# Identification of Mitogenic Factors in Bovine Whey

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by

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**FIGURE TITLES HAVE BEEN ABBREVIATED*

# ABBREVIATIONS

| | |
|---|---|
| **$\alpha_2$-M** | alpha two-macroglobulin |
| **$A_{214\ nm}$** | absorbance at 214 nm |
| **$A_{280\ nm}$** | absorbance at 280 nm |
| **A431** | human epidermoid carcinoma cell line |
| **AG2804** | simian virus 40-transformed human lung fibroblasts |
| **BALB/c 3T3** | mouse BALB/c 3T3 embryo fibroblasts |
| **BHK-21** | baby hamster kidney fibroblasts |
| **BSA** | bovine serum albumin |
| **CSIRO** | Commonwealth Scientific and Industrial Research Organisation |
| **DMEM** | Dulbecco's modified minimal essential Eagles Medium |
| **DNA** | deoxyribonucleic acid |
| **EGF** | epidermal growth factor |
| **EGFR** | EGF receptor |
| **FBS** | fetal bovine serum |
| **FGF** | fibroblast growth factor |
| **FGFR** | fibroblast growth factor receptor |
| **FPLC** | fast protein liquid chromatography |
| **h** | hour |
| **HB-EGF** | heparin binding growth factor |
| **HBSS** | HEPES-buffered saline |
| **HER/ erbB** | human EGF receptor/ EGF receptor |
| **HFBA** | heptafluorobutyric acid |

| | |
|---|---|
| **HPLC** | high pressure liquid chromatography |
| **HSPG** | heparan sulphate proteoglycans |
| **IEC-6** | rat small intestinal epithelial cells |
| **IGF** | insulin-like growth factor |
| **IgG** | immunoglobulin G |
| **L6** | rat L6 skeletal muscle myoblasts |
| **LAP** | latency associated peptide |
| **LTBP** | latent TGF-β binding protein |
| **MDCK** | canine kidney epithelial cells |
| **min** | minute |
| **Mr** | molecular weight |
| **Mv1Lu** | mink lung epithelial cells |
| **PAGE** | polyacrylamide gel electrophoresis |
| **PDGF** | platelet-derived growth factor |
| **PDGF-Rα** | platelet derived growth factor receptor alpha |
| **PDGF-Rβ** | platelet derived growth factor receptor beta |
| **RRA** | radioreceptor assay |
| **SDS** | sodium dodecyl sulphate |
| **SPARC** | secreted protein, acidic and rich in cysteine |
| **TFA** | trifluoroacetic acid |
| **TGF-β** | transforming growth factor-beta |
| **TGF-α** | transforming growth factor alpha |
| **Tris** | tris (hydroxymethyl) aminomethane |

This list excludes nomenclature of amino acids, chemical elements and SI units.

# ABSTRACT

Bovine milk contains factors that can support the growth of cells in culture. However, milk growth factors are at very low concentrations and not easily purified and investigated. Cation-exchange chromatography of cheese whey was shown by Francis *et al.* (1995) to produce a mixture of whey proteins with enriched growth factor activity (termed whey extract). In the current thesis, growth factors and binding proteins of bovine milk that have not been extensively investigated are done so using whey extract as the starting material.

Initially, whey extract was shown to contain more than one type of growth factor and to support the *in-vitro* growth of mesodermal-derived cells (such as BALB/c 3T3 cells) but inhibit epithelial cell growth. Gel-filtration experiments showed that mitogens and epithelial cell growth inhibitors present in whey extract are attached to high molecular weight whey proteins and are released from such associations under acid conditions.

Platelet-derived growth factor (PDGF) was identified and measured in acid gel-filtration fractions of whey extract. There was only a small amount of PDGF found in whey extract and this was predominantly the PDGF-BB isoform. Importantly, PDGF did not account for the majority of the BALB/c 3T3 bioactivity of whey extract. PDGF was associated with a high molecular weight whey protein that conferred latency on this factor and was released from this association under acid conditions. The PDGF binding protein was not identified, but appeared not to be related to known PDGF binding proteins.

A small amount of fibroblast growth factor (FGF)-1 and FGF-2 was detected in bovine whey extract and is the first report of FGF in bovine milk. However, when FGF

was removed from whey extract there was no significant effect on its BALB/c 3T3 cell bioactivity.

The epithelial cell growth inhibitory activity found in whey extract was identified as transforming growth factor-beta (TGF-β). The bulk of TGF-β in bovine whey and whey extract was latent and could be activated by acid. Over 85% of this activity shown to be TGF-β2. An 80 kDa latent TGF-β complex present in whey extract was identified and is the first description of this size complex in a biological fluid. TGF-β was a significant contributor to the BALB/C 3T3 bioactivity of acid treated whey extract. However, neither TGF-β or any other growth factor known to be present in bovine milk could account for all the BALB/C 3T3 bioactivity of whey extract. Epidermal growth factor (EGF) has previously not been purified from bovine milk. In the current thesis, an EGF-like molecule was identified in bovine whey extract and may be a major contributor to the BALB/c 3T3 bioactivity of whey extract. Heparin affinity chromatography showed that the EGF-activity of whey extract was a betacellulin-like molecule.

The results of this thesis show bovine whey extract contain small amounts of active growth factors and large amounts of latent mitogens that can be activated by acid treatment. It is also concluded that in addition to the known levels of IGF, the major growth factors in bovine whey are TGF-β and to a lesser extent PDGF. FGF appears to be a minor bovine whey growth factor. Preliminary results show an EGF-like molecule may be a major BALB/c 3T3 cell mitogen of whey extract.

# STATEMENT

This thesis contains no material that has been accepted for the award of any other degree or diploma at any university or other tertiary institution, and to the best of my knowledge, it contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I give consent to this copy of my thesis when deposited in the University library, being available for loan and photocopying.

Mary-Louise Rogers

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# PUBLICATIONS FROM THESIS

## Refereed Journal Articles

Rogers M-L, Belford DA, Francis GL, Ballard FJ. (1995) Identification of fibroblast growth factors in bovine cheese whey *Journal of Dairy Research* **62** (3) 501-507

Belford DA, Rogers M-L, Regester GO, Francis GL, Liepe IJ, Priebe IK, Ballard FJ. (1995) Milk-derived Growth factors as Serum Supplements for the Growth of Fibroblast and Epithelial Cells *In Vitro Cell. Dev. Biol.* **31** (10) 752-760

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## Abstracts

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## Patent

Belford DA, Rogers M-L, Francis GL, Regester GO, Smithers GW, Ballard FJ. (1994) Improved Cell Growth Promoting Preparations from Milk and Milk by-products *Patent Specification AU95/00237*Australian Industrial Property Organisation, Woden, A.C.T., Australia.

# CHAPTER One:

# Introduction and Literature Review

## 1.1 Introduction

Since the late 1970's, bovine milk has been found to contain factors that induce the growth of cells in culture. These growth factors need to be identified before developing hypotheses on how milk affects neonatal and mammary development. In the past, the volume of milk required to purify even nano molar amounts of mitogens was considered not practical to consider seriously. It has since been found found that cation-exchange chromatography of bovine cheese whey has the capacity to produce a highly concentrated fraction of whey protein containing cell-growth promoting activity (Francis *et al.* 1995). In this current study, the cation-exchange fraction of bovine cheese whey was used to evaluate and characterise growth factor activity of milk. Since commencing this project, numerous studies have focused on further analysing the presence and properties of growth factors in bovine whey. This chapter will primarily encompass a review of literature up to 1992, however, where appropriate, more recent references have been included.

## 1.2 Cell-growth Promoting Agents in Bovine Milk

Klagsbrun was the first to publish that bovine colostrum contains factors that stimulate DNA synthesis and cellular division in mammalian cells (Klagsbrun and Neuman 1979). Tissue culture media supplemented with as little as 0.4% (v/v) colostrum was shown to induce the growth of BALB/c 3T3 fibroblasts. Significantly, bovine milk obtained 60 hours after birth contained only 1% of the growth factor activity of colostrum. Steimer *et al.* (1981) then showed that mature bovine milk can

support the growth of many types of cells *in-vitro* if supplemented with factors that promote cell attachment. Since bovine serum contains cell attachment factors, it was found that supplementing bovine milk with as little as 0.5% serum produces a typical cell growth response seen equivalent to 10% serum (Sereni and Berserga 1981; Pakkanen *et al.* 1992; Derouiche *et al.* 1990; Ramirez *et al.* 1990).

It is known that various factors are present in whole milk that may promote cell growth. These include growth factors, bioactive peptides, lactoferrin, hormones, vitamins, and trace elements (Shing and Klagsbrun 1984; Brantl *et al.* 1979; Jahnke and Lazarus 1984; Masson *et al.* 1971; Schams *et al.* 1984, Schams *et al.* 1986; Blanc 1981). Of all these agents in milk, only growth factors and bioactive peptides can produce significant cell growth *in-vitro*. Lactoferrin, vitamins and trace elements are present in bovine milk at relatively low concentrations and by themselves do not significantly promote cell growth in tissue culture media. (Wattanbe *et al.* 1984; Lampreave *et al.* 1990; Blanc 1981; Steimer *et al.* 1981). Hormones such as growth hormone, thyroid hormones and prolactin are present in bovine milk but are not known to produce notable cell growth *in-vitro* (Forsyth 1986; Schams *et al.* 1984, Schams *et al.* 1986; Ronge and Blum 1988; Collier *et al.* 1993). On the other hand, insulin, peptide growth factors and bioactive casein fragments isolated from bovine milk are understood to promote the growth of cells *in-vitro* (Hadsell *et al.* 1990; Ruan *et al.* 1992; Cox and Burk 1991; Shing and Klagsbrun 1987; Brantl *et al.* 1979; Jahnke and Lazarus 1984; Nagaune *et al.* 1989; Mati *et al.* 1993; Wilson *et al.* 1989; Antila *et al.* 1991). The work submitted for this thesis investigates peptide mitogens found in bovine whey.

## 1.3 Polypeptide Growth factors

Polypeptide growth factors can be defined as proteins that regulate a variety of cellular events including cell division, differentiation, and gene transcription. As such, growth factors may contribute to the growth of cells supplemented with milk fractions *in-vitro*. Eight families of polypeptide growth factors have been isolated and characterised from mammals. These are the epidermal growth factor family, the platelet-derived growth factors, insulin-like growth factors, fibroblast growth factors, transforming growth factor-betas, nerve growth factors, interleukins, and hemopoietic growth factors (Bradshaw and Cavanaugh 1990).

To date, bovine milk has been shown to contain only three types of growth factors. These are the insulin-like growth factors, transforming growth factor-betas and platelet-derived growth factor-like molecules. Milk growth factors that have been most widely investigated (see **Table 1.2**) are insulin-like growth factors and transforming growth factor-beta (Malvern *et al.* 1987; Francis *et al.* 1988; Cox and Burk 1991; Jin *et al.* 1991). A platelet-derived growth factor-like molecule has been purified from bovine colostrum but not identified in mature milk (Shing and Klagsbrun 1984, *op cit* 1987; Shing *et al.* 1987). Although epidermal growth factor has not been purified from bovine milk, Read *et al.* (1985) showed that pasteurised cow milk contained a small amount of epidermal growth factor-like activity, whereas Yagi *et al.* (1986) reported a much higher concentration. However, Iacopetta *et al.* (1992) reported that use of highly specific EGF assays show there is little if any epidermal growth factor in cow milk.

Nerve growth factor (NGF) has been isolated from mouse, human and rat milk but not from bovine milk (Wright and Gaull 1985; Siminowski *et al.* 1986; Murphy *et al.*

1977; Hirata and Orth 1979). Although NGF has been identified in both neuronal and non-neuronal tissues of the cow but does not significantly induce the growth of cells other than those of neuronal origin (Meier *et al.* 1986; Aloe *et al.* 1982; Greene and Shooter 1980; Chao 1990). Haemopoietic factors such as tissue necrotising factor-alpha (TNF-α), and colony stimulating factors in addition to the interleukins have been isolated from human milk (Mushtaha *et al.* 1989; Rudolff *et al.* 1992; Sinha and Yunis 1983; Gilmore *et al.* 1994; Soder 1987; Munoz *et al.* 1990; Saito *et al.* 1991; Palkowetz *et al.* 1994). Although these factors are particularly important in the cellular immune system, there is no suggestion in the literature that their presence in milk would promote cell growth (Palkowetz *et al.* 1994; Saito *et al.* 1991; Rudolff *et al.* 1992). NGF, haemopoietic factors and the interleukins will not be addressed in this thesis.

### 1.3.1 *Insulin-like growth factors of bovine milk*

The only growth factor found in bovine milk that has been extensively investigated is insulin-like growth factor (IGF). Bovine milk has been shown to contain the two types of IGF found in mammals. Both IGF-I and IGF-II have been purified and characterised from bovine milk and whey (Malvern *et al.* 1987; Francis *et al.* 1988). Although minor constituents of milk protein, they are potent mitogens, anabolic agents, and differentiation factors for a range of cells *in-vitro*, where IGF-I and IGF-II have similar biologic activity (Rechler and Nissley 1990). They are 7.5 kDa peptides that contain three disulphide bonds and have identical amino acids in 62% of their sequence. In addition, the amino acid sequence of the IGFs is highly conserved across species (Zangger *et al.* 1987). In this respect, the sequence of bovine and human IGF-I is identical and the sequence of bovine IGF-II is 94% identical to the human molecule (Honegger and Humbel 1986).

In raw cows milk, the average concentration of IGF-I is 4.3 ng/ml and IGF-II is 1.0 ng/ml (Collier *et al.* 1991; Vega *et al.* 1991). A truncated IGF-I lacking the first three N-terminal residues des (1-3) IGF-I has also been isolated from bovine colostrum (Francis *et al.* 1988). This truncated IGF-I was initially discovered by Sara *et al.* (1986) in human fetal and adult brain extracts. It has also been isolated from the porcine uterus and human platelet lysates, but is absent in adult serum (Ogasawara *et al.* 1989; Karey *et al.* 1989). Bovine colostrum des (1-3) IGF-I comprises approximately 50% of the IGF-I fraction (Francis *et al.* 1986). Although the exact concentration of des-IGF-I is unknown in mature milk, it has been found to account for no more than 3% of IGF-I concentration (Shimamoto *et al.* 1992).

A very important control of the biologic activity of IGF in milk is its binding proteins (IGFBPs). The majority of IGF in milk, blood, and other extracellular fluids is complexed to binding proteins. At least six IGFBPs have been identified, and four of them (IGFBP-1-4) are found in serum in significant concentrations (Ballard *et al.* 1989; Langford and Miell 1993). Greater than 90% of circulating IGF is found in a complex comprising IGF attached to the 45-53 kDa glycosylated IGFBP-3 that is bound to a second acid-labile sub-unit of 84-85 kDa (Baxter *et al.* 1989). The remainder of the circulating IGFs are bound to IGFBP-1, or -2,-4,-5 or -6 in 25-40 kDa complexes (Langford and Miell 1993). The attachment of IGF to IGFBPs has been shown to prolong the half life of IGF, prevent binding to its receptors and enhance the activity of IGF in some cultured cell lines (Richler and Nissley 1990). In bovine milk, a 45 kDa IGFBP that could be detached from IGF-I under acid conditions was detected by Campbell and Baumrucker (1989), who suggested that it was IGFBP-3 devoid of the 84-85 kDa acid labile subunit. This was confirmed by a study showing IGFBP-3 that lacks the acid labile subunit, and IGFBP-2 are present in bovine milk (Vega *et al.* 1991).

Other lower molecular weightIGF-binding proteins also appear to be present in bovine milk but they have not been identified (Ronge and Blum 1989; McGrath *et al.* 1991; Skaar *et al.* 1991; Vega *et al.* 1991).

Bovine colostrum des (1-3) IGF-I is five to ten times more potent than IGF-I in *in-vitro* assays (Ballard *et al.* 1987; Francis *et al.* 1988). This has been shown to be the result of an increased availability to bind receptors because of reduced affinity for several of the binding proteins (Ross *et al.* 1989; Ballard *et al.* 1989). Site directed mutagenesis and biological assays showed that if the third amino acid residue of IGF-I (glutamate) is missing (as found in des (1-3) IGF-I), there is reduced binding with IGFBP-2 and increased potency *in-vitro* (Bagley *et al.* 1989; Carlsson-Skwirut *et al.* 1989). This was confirmed by *in-vitro* studies that compared the potency of IGF-I to des (1-3) IGF-I in the presence of IGFBPs. When IGFBPs were added to a cell line that did not secrete IGFBPs into the medium, the actions of IGF-I but not des (1-3) IGF-I were inhibited (Ross *et al.* 1989). Other *in-vitro* and *in-vivo* studies have verified that the lack of binding to IGFBPs by des (1-3) IGF-I makes it more potent than native IGF (McGrath *et al.* 1991; King *et al.* 1992; Gillespie *et al.* 1990).

Binding of IGF to its receptors mediates its functions at a cellular level. In the bovine mammary gland, IGF receptors are localised to the epithelial cells (Dehoff *et al.* 1988; Collier *et al.* 1989; Hadsell *et al.* 1990). There are two types of receptors that bind IGF with significant affinity and the insulin receptor which binds the IGFs with lower affinity. The type I IGF receptor shares 40% structural homology with the insulin receptor. This receptor binds IGF-I with a higher affinity than IGF-II, and exhibits a lower affinity for insulin (Ullrich *et al.* 1986). The type II IGF receptor is the 274 kDa IGF-II/Mannose-6-phosphate receptor (MacDonald *et al.* 1988). It binds IGF-II and proteins that contain a mannose-6-phosphate recognition site, but has lower affinity for

IGF-I and does not bind insulin (Kiess *et al.* 1988). Both IGF-I and IGF-II bind to the insulin receptor, but with only 1% of the affinity of insulin (Czech 1989). The type I IGF receptor has been suggested to mediate the mitogenic effects of IGF since antibodies directed against this receptor block both IGF-I and IGF-II stimulated DNA synthesis in cultured cells (Flier *et al.* 1986; Conover *et al.* 1986). The role of the type II IGF receptor is still unclear. It has been proposed that the type II receptor sequesters IGF-II into the interior of cells and targets lysosomal enzymes to the lysosome (Oka *et al.* 1985; Braukle *et al.* 1987; Pfeffer 1988). Interestingly, a soluble protein that is composed of the extracellular domain of the type II IGF receptor has been found in fetal serum (White *et al.* 1982; Causin *et al.* 1988; Hey *et al.* 1987; Gelato *et al.* 1988; Czech *et al.* 1990). This serum receptor carries as much as 20-40% of circulating IGF-II in fetal monkeys and sheep (Gelato *et al.* 1988; Hey *et al.* 1987). However, in the rat, the soluble type II receptor binds only 1-2% of fetal serum IGF-II, whereas the binding proteins are more significant carriers of IGF-II (White *et al.* 1982).

IGF found in bovine mammary secretions has been suggested to originate from either the mammary gland or maternal serum. *De-novo* synthesis of IGF has been demonstrated in stromal fibroblasts but not epithelial cells of the bovine mammary gland. Hauser *et al.* (1990) showed that IGF-I mRNA is present in mammary tissue which supported the hypothesis of local synthesis. It was then demonstrated that IGF-I is produced by bovine mammary explants but not isolated acini *in-vitro*, so that the source of IGF-I is mammary stromal fibroblasts (Campbell *et al.* 1991). However, it was noted that IGF production by stromal cells is lower than that required for colostrum, but may contribute to IGF in mature milk. The second origin proposed for IGF in bovine milk is from maternal serum. Prosser *et al.* (1990, Prosser *et al* 1992; Prosser and Fleet 1992) demonstrated that $^{125}$I-IGF-I or $^{125}$I-IGF-II infused into lactating goats

mammary gland artery is transported from the serum into milk. IGF-I was transported from the serum to goat milk though the epithelial cell layer, but IGF-II entered milk via intracellular means. It has also been found that there are greater numbers of IGF receptors on bovine mammary epithelial cells during pregnancy and a dramatic increase in IGF binding to mammary tissue at the onset of lactation (Hadsell *et al.* 1990; Dehoff *et al.* 1988; Collier *et al.* 1989). This has led to the suggestion that epithelial receptors actively sequester IGF from maternal serum in early lactation. It is important to note that in general the appearance of IGF are accompanied by specific IGFBPs. In this respect, most IGFs in the milk are bound to IGFBPs which are synthesised and secreted by bovine mammary secretory epithelial cells. It has been demonstrated that IGFBPs are localised to the bovine mammary epithelial cells *in-vivo*, secreted by these cells *in-vitro* and up-regulated by IGFs (Collier *et al.* 1993; McGrath *et al.* 1991). Indeed, IGFBPs are secreted in prepartum and colostral secretions of the bovine mammary gland at sufficient concentration to bind free IGF entering milk (Campbell *et al.* 1991).

The concentration of IGF in bovine milk has been found to be related to stage of lactation and the number of lactations that cows have had. IGF-I concentration is high in colostrum, and within two days after parturition declines below that found in serum. A gradual decline in IGF-I concentration then continues through the remainder of lactation (Ronge and Blum 1988; Ronge *et al.* 1988; Vega *et al.* 1991; Collier *et al.* 1991). Cows also exhibit lower concentrations of IGF-I in first lactations compared with subsequent lactations (Campbell and Baumrucker 1989). Collier *et al.* (1991), showed that although older cows have a higher concentration of IGF-I in their milk than animals that are undergoing their first lactation, no correlation existed between milk yield and IGF-I concentration. It was suggested that the higher concentration in older

animals is related to body size, numbers of type I IGF receptors and rate of uptake or metabolism of IGF-I in the mammary gland.

Although serum growth hormone stimulates IGF-I production in the liver, there is no evidence that it directly controls milk IGF produced in bovine lactation. It was initially found that treatment of lactating cows with growth hormone increases IGF-I levels in milk, and causes a dramatic rise in intracellular IGF-I in mammary epithelial cells (Glimm *et al.* 1988; Prosser *et al.* 1989). In addition, growth hormone increases IGFBP production by mammary epithelial cells (Byatt *et al.* 1992). However, Ronge and Blum (1988), showed that serum growth hormone increases naturally after parturition and reaches a maximum concentration at peak milk production, at a time when milk IGF levels are low. Subsequently, Hadsell *et al.* (1993) showed growth hormone treatment increased serum IGF levels in cows at all stages of lactation, but had no effect on mammary prepartum IGF-I and produced only a modest increase in colostral IGF-I. It was also found that des (1-3) IGF-I concentration is not increased in lactating cows treated with growth hormone (Shimamato *et al.* 1992). Current evidence therefore suggests that the level of IGF secreted into bovine milk is controlled locally in the mammary gland, rather than by serum growth hormone. Examples of local control are the change in IGF receptor population on mammary epithelial cells and IGFBP levels in mammary tissue that may modulate the level of IGF secreted into colostrum (Hadsell *et al.* 1990, Hadsell *et al.* 1993).

Bovine milk IGF has been suggested to be important in mammary gland development and in the maturing neonatal gut. Collier *et al.* (1993) showed that IGF-I increased bovine mammary epithelial cell DNA synthesis *in-vitro* and infusion of IGF-I into late pregnant cows increased mammary growth. Since 50% of IGF-I in bovine colostrum is des (1-3) IGF-I, it has been suggested that this factor is an important regulator of

mammary growth and differentiation (Ruan *et al.* 1992). Recently, mice that over-express des (1-3) IGF-I in the mammary gland have been produced by Hadsell *et al.* (1996). Mammary tissue from these mice showed incomplete mammary involution, ductile hypertrophy, and loss of secretory lobules associated with increased deposition of collagen. There are several factors that suggest bovine milk IGF is resistant to digestion and may retain bioactivity within the neonatal gut. The first is that IGFs are stable to acid conditions and not altered by transient heat used in pasteurising bovine milk (Collier *et al.* 1991). Secondly, at birth neonatal calves are monogastric and have low gastic acid and intestinal enzyme activity. In addition, it has been postulated that milk IGFs may be protected from degradation by the presence of IGFBPs and protease inhibitors such as colostrum-specific trypsin inhibitor (Lindberg 1979). A role for bovine milk IGF-I in intestinal cell proliferation was suggested by Corps and Brown (1987) after showing that both IGF-I and bovine milk induce rat intestinal epithelial cell growth. A subsequent study by Baumrucker and Blum (1993) showed that calves fed a milk replacement that contained IGF had more ilieal and jejunal proliferation than without IGF. (Further research is necessary to determine all the roles of bovine milk IGF in neonatal growth and development).

### 1.3.2 *Other growth factors of bovine milk*

Transforming growth factor-beta (TGF-β) and platelet-derived growth factor (PDGF) have been purified from bovine mammary secretions (Cox and Burk 1991; Jin *et al.* 1991; Shing and Klagsbrun 1984, *op cit* 1987). Other growth factors such as fibroblast growth factors and members of the epidermal growth factor family have been identified in bovine tissue or fluids but not in milk (Gospodarowicz 1975; Thomas *et al.* 1980; Zurfluh *et al.* 1990).

Shing and Klagsbrun (1984, *op cit* 1987; Shing *et al.* 1987), showed that bovine milk contained different growth factors than human milk. Under their experimental conditions, bovine mammary secretions (unlike human milk), did not contain a high concentration of epidermal growth factor (EGF). Instead, they found a PDGF-like molecule was the most predominant factor in bovine colostrum. Interestingly, the methods used by Shing and Klagsbrun (1987) to isolate PDGF are now known to preclude the isolation of TGF-β in that whey was separated from casein by acid-treating colostrum and then heating it for 30 minutes at 100°C. Brown *et al.* (1990) has since shown that native and recombinant TGF-β is denatured at tempearatures above 70°C at incubation times of more than 10 minutes. More recently, Tokuyama and Tokuyama (1989) identified a TGF-β-like substance in bovine colostrum and Cox and Burk (1991) formally characterised TGF-β in bovine milk.

#### 1.3.2.1 *Transforming growth factor-betas*

Transforming growth factor-betas (TGF-β) have been identified in bovine milk but their form and function in mammary secretions have not been described (Cox and Burk 1991; Jin *et al.* 1991; Tokuyama and Tokuyama 1989, Tokuyama and Tokuyama 1993). TGF-β was originally described as a substance present in transformed cells or tumours that promoted anchorage-independent growth of normal rat kidney (NRK) fibroblasts in the presence of EGF (DeLarco and Todaro 1978; Roberts *et al.* 1981). It has since been found that TGF-βs are present in almost all mammalian cells and abundant in platelets and bone (Assoian *et al.* 1983; Seyedin *et al.* 1987; Danielpour *et al.* 1989; Flanders *et al.* 1989). Ongoing research has revealed that TGF-β modulates the growth and function of many cell types, regulates extracellular matrix production and is an immuno-

suppressive agent (Roberts and Sporn 1993). The description of TGF-β has therefore been modified to a growth factor/cytokine with multifunctional properties that is present in most mammalian cells (Roberts and Sporn 1993).

Five forms of TGF-β have been identified in vertebrates, and on the basis of structural homology belong to the TGF-β supergene family that also includes inhibins, activins, Mullerian inhibitory substance and bone morphogenic proteins (Mason *et al.* 1985; Ling *et al.* 1986; Cate *et al* 1986; Wang *et al.* 1988). The TGF-βs occur principally as 25 kDa homodimers, although heterodimeric forms of TGF-β have been found in low abundance (Cheifetz *et al.* 1988; Ogawa *et al.* 1992). Three distinct isoforms: TGF-β1, TGF-β2, and TGF-β3 are expressed in mammals (Derynck *et al.* 1985; Seyedin *et al.* 1987; Cheifetz *et al.* 1987; Derynck *et al.* 1988). Even though TGF-β1, TGF-β2 and TGF-β3 are encoded by different genes, there is a significant degree of homology in their amino acid sequences. The position of the nine cysteine residues are precisely conserved in all isoforms of TGF-β, which is important in disulphide links within and between the peptide chains of TGF-β (Derynck *et al.* 1985; Marquardt *et al.* 1987; Derynck *et al.* 1988). Human TGF-β1 is 71% homologous to TGF-β2 and 77% homologous to TGF-β3, while TGF-β2 is approximately 80% homologous to TGF-β3 (Derynck *et al.* 1988; ten Dijke *et al.* 1990). All the TGF-βs have similar *in-vitro*, but distinct *in-vivo* actions in mammals (Cheifetz *et al.* 1990; Joyce *et al.* 1990).

The primary structure of the TGF-βs found in mammals is highly conserved. The amino acid sequences of all mammalian TGF-β1 are identical, with the exception of murine TGF-β1, which substitutes a serine for an alanine in position 75 (Derynck *et al.* 1986; Derynck *et al.* 1988; Derynck and Rhee 1987; Van Obberghen-Schilling *et al.* 1987). The primary structure of TGF-β2 is totally conserved among human, bovine and

porcine species (Marquardt *et al.* 1987; Seyedin *et al.* 1987; Cheifetz *et al.* 1987). Human TGF-β3 is identical to that found in cattle and over 90% homologous to pig TGF-β3 (ten Dijke *et al.* 1988; Graycar *et al.* 1989; Derynck *et al.* 1988).

The concentration of TGF-β in colostrum and bovine milk is not known but, it has been revealed that TGF-β is present in the whey fraction of colostrum and milk (Tokuyama and Tokuyama 1989; Cox and Burk 1991). The data presented by Tokuyama and Tokuyama (1989) suggests that like IGF, the concentration of TGF-β in mammary secretions is related to the stage of lactation. They showed TGF-β activity is higher in bovine colostrum than milk samples taken 5 days after parturition. Research has also shown that TGF-β2 is the most predominant form of TGF-β in mammary secretions (Tokuyama and Tokuyama 1993; Jin *et al.* 1991). In colostrum, the majority of TGF-β purified was found to cross-react to antibodies specific to TGF-β2 (Tokuyama and Tokuyama 1993). However; the presence of small amounts of TGF-β1 in the purified colostral TGF-β was not excluded. In mature milk, the ratio of TGF-β1 to TGF-β2 in was found by Jin *et al* (1991) to be 15:85.

TGF-β is expressed by cells in an inactive complex from which that mature TGF-β must be released before it can exert biologic effects in its environment. Activation of latent TGF-β complexes is probably one of the most important steps in regulating the actions of this factor *in-vivo*. There are two types of latent TGF-β complexes released from producer cells, one larger than the other. The first is comprised of active TGF-β and its latency associated peptide (LAP) and is called 'the small latent complex' (Wakefield *et al.* 1989; Miyazono and Heldin 1991). The second is the LAP and the latent TGF-β binding protein (LTBP) and is termed the 'large latent complex' (Wakefield *et al.* 1988; Miyazano *et al.* 1988; Kanzaki *et al.* 1990).

Analysis of the cDNA coding for TGF-β and of the expressed protein has revealed that TGF-β1 is initially synthesised as a 390 amino acid precursor peptide or pre-pro-protein (Derynck *et al.* 1985; Gentry *et al.* 1987). Active TGF-β1 is a dimer of 112 amino acids derived from the carboxyl terminus of the precursor protein and proteolytically cleaved from the N-terminal remnant of the precursor. On its release from cells, TGF-β1 remains non-covalently associated with a 249 amino acid dimer of the N-terminal remnant of the pre-pro-protein called the pro-protein (Gentry *et al.* 1987, Gentry *et al.* 1988; Wakefield *et al.* 1988). TGF-β2 and-β3 have also been found in complexes with their pro-proteins when their precursor cDNA are transfected into mammalian cells (Madisen *et al.* 1990; Brown *et al.* 1990). Further investigation revealed that the pro-protein is required for processing and secretion of TGF-β. Gray and Mason (1990) were unable to get cells to express TGF-β when they were transfected with the cDNA of mature TGF-β1 without its pro-protein.

Research has shown that the pro-protein confers latency to mature TGF-β. The pro-protein has since been termed the 'latency associated peptide (LAP) and association of mature TGF-β with the LAP described as the 'small latent complex' (Wakefield *et al.* 1989; Miyazono and Heldin 1991). Investigations revealed that the small latent complex could not bind TGF-β receptors or specific antibodies (Pircher *et al.* 1986; Gentry *et al.* 1987, Gentry *et al.* 1988). However, TGF-β could be released from association with its pro-protein by acid, base, heat or plasmin and then bind the specific receptors and antibodies (Lawrence *et al.* 1985; Wakefield *et al.* 1988; Lyons *et al.* 1988; Lyons *et al.* 1990; Miyazono *et al. 1*993). In addition, Wakefield *et al.* (1988), were able to show that the bonds joining LAP with TGF-β are non-covalent because some re-association of TGF-β with LAP occured after transient acid treatment.

The latent TGF-β complex released from platelets and some cultured cell lines was found to be larger than the small latent complex. In addition to the LAP, a latent TGF-β1 binding protein (LTBP) of 125-200 kD was present with TGF-β1 as a 'large latent complex' (Wakefield *et al.* 1988; Miyazano *et al.* 1988; Okada *et al.* 1989; Kanzaki *et al.* 1990). LTBP is transcribed from a gene that is separate to the pre-pro-protein of TGF-β and LAP (Kanzaki *et al.* 1990; Miyazano *et al.* 1991). Olofsson *et al.* (1992) revealed that the precursors of all three isoforms of TGF-β can be expressed with LTBP in large latent complexes *in-vitro*. Importantly, research showed that LTBP is not required for TGF-β latency. LTBP was found to be attached to LAP by disulphide bonds, but not bound to mature TGF-β (Wakefield *et al.* 1988; Miyazono *et al.* 1988; Kanzaki *et al.* 1990). Furthermore, Gentry *et al.* (1987) demonstrated small latent TGF-β complexes that lack LTBP are latent. Chinese hamster ovary cells transfected with the cDNA of pre-pro-proteinTGF-β but not LTBP, were shown to secrete small TGF-β complexes that are latent. More recent research revealed that there are several types of human LTBP, two of which have been isolated and cloned. LTBP-1 is transcribed from a gene that is separate to LTBP-2, but they have similar amino acid sequences, especially the EGF-like repeats and cysteine-rich regions (Kanzaki *et al.* 1990; Moren *et al.* 1994). To date, the presence of species specific LTBPs has not been investigated.

Several roles have been proposed for the LTBP, including the intracellular processing and secretion of latent TGF-β, targeting of latent TGF-β to extracellular matrix, and cell-mediated activation of latent TGF-β (Miyazono *et al. 1*991; Taipale *et al. 1*994; Moren *et al. 1*994; Mizoi *et al. 1*993; Flaumenhaft *et al. 1*993). Studies on the biosynthesis and processing of TGF-β in cells such as human erythroleukemia cell line (HEL) revealed that the large latent complex is more efficiently excreted from cells when compared to the small latent complex (Miyazono *et al. 1*991, Miyazono *et al.*

1993). The latent TGF-β binding protein has been shown to regulate the targeting of TGF-β to extracellular matrix (Taipale *et al.* 1994; Moren *et al.* 1994; Mizoi *et al.* 1993). It is also required for the cell-mediated activation of TGF-β by macrophages and co-cultures of endothelial and smooth muscle cells (Nunes *et al.* 1995; Flaumenhaft *et al.* 1993b).

Small latent complexes are the predominant form secreted by chinese hamster ovary (CHO) or COS cells transfected with precursor cDNA coding for TGF-β1 or TGF-β2 (Gentry *et al.* 1987; Madisen *et al.* 1990). They are also found in tissue culture medium conditioned by bone organ cultures and osteoblast cell lines, HT1080 fibrosarcoma cells, BSC 40 monkey kidney cells and human glioblastoma cells (Dallas *et al.* 1994; Bonewald *et al.* 1991; Wakefield *et al.* 1988; Lioubin *et al.* 1991; Olofsson *et al.* 1992). Large latent complexes are predominant in platelets (Wakefield *et al.* 1988; Miyazono *et al.* 1988; Okada *et al.* 1989) They are also secreted by human skin fibroblasts, HEL cell lines, human glioblastoma cell lines, and cultures of bovine smooth muscle and endothelial cells (Kanzaki *et al.* 1990; Moren *et al.* 1994; Miyazono *et al.* 1991; Olofsson *et al.* 1992; Flaumenhaft *et al.* 1993b). Platelets and several cell lines were found to produce multiple latent forms (Lioubin *et al. 1*991; Olofsson *et al. 1*992; Grainger *et al.* 1995).

There has been no information on the type of latent TGF-β present in bovine milk, or the proportion of active to latent TGF-β in bovine mammary secretions. Cox and Burk (1991), showed that acid treatment of whole milk results in an increase of TGF-β detected when compared to untreated milk. Since acid treatment is known to release TGF-β from its interaction with LAP, it was assumed that there was a significant amount of latent TGF-β in milk (Pircher *et al.* 1984; Lawrence *et al.* 1985; Brown *et al.* 1990). There is no data on the latency of TGF-β in bovine colostrum. However,

Tokuyama and Tokuyama (1989) suggested it was active because high levels of TGF-β were detected in colostrum compared to TGF-β in serum which was known to be latent (Tokuyama and Tokuyama 1989). It is now known that the mild acid used by Tokuyama and Tokuyama (1989) to separate whey from casein could release active TGF-β from LAP (Pircher *et al.* 1984; Lawrence *et al.* 1985). A subsequent study of by Saito *et al.* (1993) reported the proportions of latent to active TGF-β in human mammary secretions. Whey was separated from casein by centrifugation and TGF-β activity was then measured before and after acid activation. In this study, latent TGF-β accounted for approximately 50% of colostral TGF-β and 80% of mature TGF-β in human milk (Saito *et al.* 1993). To date, their is no information on the size of latent TGF-β in mammalian milk, or if LTBPs are present.

Most cells have TGF-β receptors and three different glycoslated receptors have been identified (type I, II and III). Although none of the bovine TGF-β receptors have been cloned or sequenced, TGF-β binds to all types of mammalian cells which suggests there is high homology between bovine and other mammals receptors (Frolik *et al.* 1984; Massague *et al.* 1992). Indeed, when human, rat, pig and mouse receptors for TGF-β were cloned and sequenced their primary structures were found to be very similar (Lin *et al.* 1992; Franzen *et al.* 1993; Ebner *et al.* 1993). All TGF-β isoforms can bind to TGF-β receptors, however, their receptor affinities are different. TGF-β1 binds the type I receptor equally as well as TGF-β2, but TGF β3 binds the type I receptor with less affinity than the other isoforms. TGF-β1 binds to the type II receptor with much greater affinity than TGF-β2 which in turn binds type II stronger than TGF-β3. Type III TGF receptor binds all TGF-β species with the same affinity (Massague 1992). The type I and II receptors are transmembrane serine-theonine kinases of 55 and 80 kDa

respectively (Franzen *et al.* 1993; Lin *et al.* 1992). They have been suggested to mediate the cell growth inhibitory and stimulatory effects of TGF-β by serine-threonine phosphorylation (Wrana *et al.* 1992). The type III receptor is a membrane bound proteoglycan of 250-350 kDa called betaglycan (Lopez-Casillas *et al.* 1991; Wang *et al.* 1991). It does not transduce TGF-β signals but may function to concentrate TGF-β on the cell surface and present TGF-β to the type I and II receptors (Lopez-Casillas *et al.* 1991; Lopez-Casillas *et al.* 1993). Interestingly, plasmin has been shown to cleave TGF-β from betaglycan found on fibroblast cells in-vitro (Lamarre *et al.* 1994). This suggests that similar to the latent complexes, TGF-β bound to betaglycan may associate with the extracellular matrix and act as an intracellular store for TGF-β (Fukushima *et al.* 1993; Lamarre *et al.* 1994; Taipale *et al.* 1994).

In serum and some cell culture media, active TGF-β binds to proteins that result in its inactivation. These include decorin and α2-macroglobulin (Yamaguchi *et al. 1*990; Huang *et al. 1*988). Decorin is a small proteoglycan that has been shown to be expressed on some cells in-vitro and binds active TGF-β1 (Yamaguchi *et al. 1*990). Importantly, it neutralised the growth stimulatory activity of TGF-β *in-vitro*. Plasma TGF-β is predominantly found in covalent associations with $\alpha_2$-macroglobulin (α2-M), a 720 kDa tetramer which exhibits differential affinity for TGF-β depending on its activation status and the isoform of TGF-β (Danielpour and Sporn 1990; Webb *et al. 1*994, Webb *et al* 1995).

All the available evidence supports the hypothesis that the main role for α2-M is to bind excess serum TGF-β and present it to organs for turnover or serum clearance. Wakefield *et al.* (1987, Wakefield *et al* 1988) showed that active TGF-β can associate with α2-M and be released from the complex under strong acid. However, acid

denatured the α2-M tetramer in such a way that prevented any reforming of the TGF-β-α2-M complex, or any further activity of α2-M. This is the opposite to that observed for the latent TGF-β complexes, where some TGF-β reassociates with the latency associated peptide (LAP) following transient acidification and reneutralisation (Wakefield *et al.* 1988). Other agents such as heat, transient alkalinity, and plasmin that activate TGF-β have not been reported to activate the TGF-β-α2-M complex (Wakefield *et al.* 1988; Brown *et al.* 1990). This led to the suggestion that unlike the interaction of TGF-β with LAP which is non-covalent and reversible, TGF-β binds to α2-M covalently and irreversibly. Further research showed that a specific conformation of α2-M binds to active TGF-β in the serum and more readily binds hepatic α2-M receptors (Huang *et al.* 1988; Lemarre *et al.* 1991) This TGF-β-α2-M complex can not interact with TGF-β receptors but is cleared from the circulation by α2-M receptors in the liver (Wakefeild *et al.* 1988; Wakefield *et al* 1990; Lemarre *et al.* 1991; Miyazono *et al. 1*993). However, McCaffrey *et al.* (1989, McCaffrey *et al* 1992, McCaffrey *et al* 1994) has proposed that some of the TGF-β binds non covalently to α2-M in such a way that results in TGF-β being available to cells. Data produced, showed a minor portion of TGF-β-α2-M complexes are formed by bonds that can be dissociated by heparin. The biological significance of this release of TGF-β from the TGF-β-α2-M complex is unknown.

TGF-β in bovine milk has been suggested to originate from mammary gland epithelial cells, maternal plasma, or milk cells. Expression of all three TGF-β isoforms have been detected in the bovine mammary gland (Maier *et al. 1*991). TGF-β1 and TGF-β2 were expressed most strongly by alveolar epithelial cells which secrete milk protein in the lactating bovine mammary gland (Maier *et al. 1*991). No TGF-β

expression was detected in cytospin preparations of bovine milk-derived cells (Maier *et al. 1*991). This suggests that maternal cells such as leucocytes and macrophages that are transferred to the newborn during suckling and are known to contain TGF-β are not an important source of TGF-β. Interestingly, the literature appears to show their is variation in the origin of milk TGF-β across species. In contrast to cow milk, Letterio *et al.* (*1*994) reported TGF-β1 in cytospin preparations of mouse milk derived cells. Like the cow, expression of TGF-β isoforms in the mouse mammary gland was found in the alveolar cells during lactation but the pattern of expression for each isoform was also different to that observed in the cow (Robinson *et al. 1*991). Curiously, Palkowetz *et al.* (1994) reported that TGF-β was not present in medium conditioned by human mammary gland epithelial cells. Conversely, cultured rat mammary epithelial cells secrete latent TGF-β (Either and Van De Velde 1990). To date, there has been no animal studies on the transfer of TGF-β from maternal serum to milk. However, Letterio's *et al.* (*1*994) study on TGF-β1 null mice, demonstrated that TGF-β passes from the mother to the foetus, where it is bound by extracellular matrix. Further animal studies are required to determine the exact source of milk TGF-β for each mammal.

Proposed roles for milk-derived TGF-β in the neonate have included regulation of intestinal maturation and host defence. Receptors for TGF-β are present in neonatal tissue, suggesting roles for TGF-β during development (Heine *et al.* 1987; Flanders *et al.* 1989; Thompson *et al.* 1989). Whether milk derived TGF-β is important during neonatal gut maturation is still unclear. Their is some evidence that TGF-β is important in epithelial differentiation in the gut. TGF-β has been shown to inhibit proliferation of human and rodent intestinal epithelial cells and promote their differentiation *in-vitro* and *in-vivo* (Kurokowa *et al.* 1987; McCabe *et al.* 1993; Puolakkainen *et al. 1*994). In

addition, TGF-β mRNA is expressed in gastrointestinal epithelium, and a rat intestinal cell line was demonstrated to expresses TGF-β mRNA and secrete latent TGF-β (McCabe *et al.* 1993; Kurokowa *et al.* 1987; Koyama and Podolsky 1989). Significant roles for TGF-β in the immune system have been reported in the literature (Palladino *et al.* 1990; Fontana *et al.* 1992). It is therefore not surprising that milk TGF-β has been implicated in host defence of the neonate. TGF-β strongly inhibits the immune function of T cells, B cells, macrophages and killer cells (Ranges *et al.* 1987; Kuppner *et al.* 1989; Kehrl *et al*, 1986; Espevik *et al.* 1987; Rook *et al.* 1986). It is also involved in the class switch from IgM to IgA in the B lymphocytes (Lebman *et al.* 1989; Chen and Li 1990). Since the developing B cells are present in the lymph nodes of the gastrointestinal tract, this has led to the suggestion that milk derived TGF-β may stimulate intestinal immunoglobulin production in the neonate (Chen and Li 1990). In addition, Donnet-Hughes *et al.* (1995), showed that TGF-β2 could modulate the expression of MHC antigens in gut intestinal epithelial cells. They postulated that milk TGF-β could down regulate class II MHC antigens in the neonatal gut until weaning. Other studies have suggested that milk TGF-β may protect the neonatal gut against infections and allergic diseases (Stoek *et al.* 1989; Ishizaka *et al. 1*994).

Interestingly, orally administered TGF-β can be mainly recovered from the kidney and to a lesser extent the heart, lung and liver when given to neonatal mice (Letterio *et al.* 1994). This suggests that TGF-β may have physiological actions at sites distant from the gastrointestinal tract. Alternatively, the molecule may bind to proteins in the serum such as α2-M and be eliminated. Plasma clearance studies in adult mice have shown that intravenously administered TGF-β quickly accumulates in the lungs, kidneys and liver (Lamarre *et al.* 1991). Conversely, TGF-β bound to an α2-M conformation amassed mostly in the liver and when the α2-M receptors were blocked, TGF-β-α2-M

persisted in the circulation (Lamarre *et al.* 1991). It was suggested that free TGF-β in the serum bound to receptors located in multiple organs, but the TGF-β-α2-M was eliminated through the α2-M receptors in the liver. However, it is yet to be determined whether TGF-β present in mammary secretions is biologically accessible and in sufficient quantity to have significant roles in the neonate. Current evidence would suggest, however, that a primary role of mammary gland-derived TGF-β is as an autocrine regulator of glandular growth and milk protein production (Sudlow *et al. 1*994; Robinson *et al. 1*993; Robinson *et al. 1*991). In this regard, significant expression of TGF-β isoforms in the mouse mammary gland during pregnancy was found to decrease during lactation (Robinson *et al. 1*991).

#### 1.3.2.2 *Platelet-derived growth factor*

A platelet-derived growth factor (PDGF) like molecule has been identified in bovine mammary secretions but not sequenced (Shing and Klagsbrun 1984; *op cit* 1987; Shing *et al.* 1987). PDGF has also been purified from platelets obtained from bovine blood but was not sequenced (Narczewska *et al.* 1985). Nevertheless, receptor binding and radio-immuno assay studies have shown that the structure of PDGF is highly homologous among mammals. Human PDGF was found to bind equally well to cells from cows, pigs, monkeys and humans (Bowen-Pope and Ross 1982). PDGF-like activity cells isolated from sera of a wide range of species is able to displace iodinated human PDGF from fibroblasts *in-vitro* (Singh 1982; Singh 1987). In addition, a polyclonal antibody produced against human PDGF was found to recognise cow, rabbit, rat and pig PDGF (Raines and Ross 1985; Huang *et al.* 1983; Owen *et al.* 1984). The description of the structure of human PDGF is therefore thought to apply to the cow.

Human PDGF is a cationic glycoprotein composed of disulphide linked dimers of 16-18 kDa PDGF-A and 14-16 kDa PDGF-B chains (Antoniades 1981; Deuel *et al.* 1981; Raines and Ross 1982; Antoniades and Hunkapiller 1983). The mature A and B chains show 60% amino acid sequence similarity with perfect conservation of all eight cysteine residues (Johnsson *et al.* 1982; Betsholtz *et al.* 1986; Bonthron *et al.* 1988) Specifically, PDGF-AA, PDGF-BB and PDGF-AB are the three types of PDGF found in platelets, macrophages and cultures of normal and transformed cells of humans (Heldin *et al.* 1986; Shimokado *et al.* 1985; Hammacher *et al.* 1988; Hart *et al.* 1990).

The highly conserved binding of PDGF to its receptors has been used to define the type of PDGF found in cow and other mammals cells. The predominant isoform in platelets of the cow, chicken, dog, rat, pig and sheep was found to be PDGF-BB (Bowen-Pope *et al.* 1989). However, approximately 70% of human platelet PDGF consists of PDGF-AB, the remainder is principally PDGF-BB together with small amounts of PDGF-AA (Hammacher *et al.* 1988; Hart *et al.* 1990). The form of PDGF produced by activated macrophages of many species including the cow, and that secreted by cultured endothelial and epithelial cells is mainly PDGF-BB (Shimokado *et al.* 1985; Kartha *et al.* 1988; Bowen-Pope *et al.* 1989). Conversely, smooth muscle cells, nerve cells and fibroblasts secrete PDGF-AA *in-vitro* (Sejersen *et al.* 1986; Seifert *et al.* 1984; Noble *et al.* 1988; Nister *et al.* 1988; Paulsson *et al.* 1987; Bowen-Pope *et al.* 1989).

The major growth factor activity in bovine colostrum was purified and described by Shing and Klagsbrun (1984, *op cit* 1987; Shing *et al.* 1987) as a PDGF-like molecule. It was a 30 kDa disulphide linked dimer comprised of two identical chains and had an isoelectric point of 10. These were structural properties similar to human PDGF. However, they did not sequence the molecule nor did they determine which PDGF

chain(s) the dimer was composed of. Purified bovine colostral factor was also able to displace human PDGF from receptors on human fibroblasts in radio-receptor experiments. Even though the growth factor was not sequenced, the highly specific radio-receptor assay was enough evidence for Shing and Klagsbrun (1987) to conclude it was a PDGF-like molecule. Interestingly, Brown and Blakeley (1984) partially purified a PDGF-like molecule from goat colostrum. Although they did not purify the molecule to homogeneity nor did they sequence it, they were able to show that it had biochemical and physical similarities to human PDGF.

The mitogenic activity of the different isoforms of PDGF depends on the cell type and parallels the ability of the cells to bind the different isoforms. PDGF stimulates the proliferation of a wide range of cells and the migration of mesenchymal cells by binding to specific receptors. As previously mentioned, the interaction of PDGF with its receptors is highly conserved. $^{125}$I-human-PDGF binds with equal affinity with cells from mouse, cow, monkey and man (Bowen-Pope and Ross 1982). Two cell-associated receptors for PDGF have been characterised, one is the PDGF-Rα, the other PDGF-Rβ, and both contain tyrosine kinases (Hart *et al.* 1988; Heldin *et al.* 1988; Matsui *et al.* 1989, Matsui *et al* 1989). Soluble forms of PDGF receptors have also been identified in human plasma and cell-conditioned medium (Tiesman and Hart 1993). Receptor binding studies have shown that PDGF-Rα recognises PDGF-A and PDGF-B, whereas PDGF-Rβ binds only PDGF-B (Heldin *et al.* 1988; Hart *et al.* 1988; Seifert *et al.* 1989).

A number of models have been developed concerning the mechanism of PDGF receptor signal transduction. The most widely excepted are that receptors must dimerise for signal transduction to occur (Hammacher *et al.* 1989; Seifert *et al.* 1989; Heldin *et al.* 1989; Eriksson *et al.* 1992). In the presence of PDGF, a complex is formed, consisting of one PDGF isoforms and two receptor molecules. The three possible

receptor dimers are α/α, α/β and β/β. In the absence of PDGF, receptors are present on the cell as independent monomers or weakly associated dimers (Seifert *et al.* 1989). According to the specificities of the two receptor chains, the α/α should bind all three PDGF forms, the α/β both PDGF-AB and PDGF-BB and β/β only PDGF-BB (Seifert *et al.* 1989; Eriksson *et al.* 1992; Ueno *et al.* 1993). However, it has been shown that PDGF-AB can bind and signal through the β/β receptor with low affinity (Seifert *et al.* 1993; Abboud *et al.* 1994).

PDGF receptors have been found on cells derived from connective, muscle, and nervous tissue, but rareley on endothelial cells or epithelial cells and not on activated macrophages or megakarocytes (see Raines *et al.* 1990 for review). Most of the cell types examined have at least 5 times as many PDGF-Rβ as PDGF-Rα and no natural cell type expresses only PDGF-Rα. For example, human dermal fibroblasts do not express α-subunits, and are poorly stimulated by PDGF-AA (Seifert *et al.* 1989; Grotendorst *et al.* 1991). There appears to be little difference in the biologic activities of PDGF-AA, -AB and -BB. Rather, the type and number of receptor subunits expressed on cells reflects the responsiveness of cells to each type of PDGF.

PDGF released into the circulation is rapidly inactivated by binding to $\alpha_2$-macroglobulin ($\alpha_2$-M), and to extracellular matrix proteins such as "secreted protein, acidic and rich in cysteine" (SPARC), as well as proteoglycans (Huang *et al.* 1983, *op cit* 1984; Raines *et al.* 1984; Raines *et al.* 1992; Raines and Ross 1992). Experimental evidence shows that PDGF-AB and PDGF-BB, but not PDGF-AA bind to native and proteinase activated forms of $\alpha_2$-M non-covalently (Bonner and Orsonio-Vargas 1995). Binding to native $\alpha_2$-M appears to protect PDGF-BB and PDGF-AB from degradation and serves as an extracellular reservoir for this molecule (Bonner *et al.* 1995). On the

other hand, proteinase-activated fast $\alpha_2$-M mediates the clearance of PDGF-BB and PDGF-AB from the liver through the $\alpha_2$-M receptor/low density lipoprotein receptor-related protein system (Bonner *et al.* 1995). SPARC is an extracellular matrix protein that is up-regulated following vascular injury. PDGF-B chains have been demonstrated to bind SPARC non-covalently, in a reversible pH dependent manner (Raines *et al.* 1992). Since macrophages secrete mainly PDGF-B and mesenchymal-derived (fibroblast and smooth muscle) cells PDGF-A chains, it has been postulated that $\alpha_2$-M and SPARC allow for discrimination during tissue repair processes or during the pathogenesis of fibroproliferative diseases (Bonner *et al.* 1995; Bonner and Orsonio-Vargas 1995). In addition, purified heparin sulphate proteoglycans that are known to associate with endothelial cell membranes or matrices also bind long dimeric forms of PDGF containing basic sequences encoded by exon 6 of the A and B chains (Raines and Ross 1992). It was suggested that the larger forms of PDGF are immobilised on cell membranes or matrix and cleaved to release the active molecules.

Accumulating evidence suggests that PDGF has important roles in fibroblast proliferation, wound repair, fibrotic disease, atherosclerosis and tumour growth. *In-vitro* experiments have shown PDGF is required for the BALB/c 3T3 fibroblast cell cycle that culminates in the synthesis of new DNA and mitosis (Pledger *et al.* 1977; Vogel *et al.* 1978). The involvement of PDGF in wound repair has been demonstrated *in-vitro* and *in-vivo*. Platelets, activated macrophages, capillaries, fibroblasts and smooth muscle cells that are found at wound sites all secrete large amounts of PDGF (Kaplan *et al.* 1979; Mornex *et al.* 1986; Pierce *et al.* 1988, Pierce *et al* 1991). PDGF also induces a motility response in mesenchymal-derived cells and mononuclear cells associated with wound repair (Grotendorst *et al.* 1981; Deuel *et al.* 1982; Hammacher *et al.* 1989; Seigbahn *et al.* 1990). In addition, other events in wound healing such as fibroblast

proliferation and secretion of collagenase and fibronectin are stimulated *in-vitro* by PDGF (Seppa *et al.* 1982; Peirce *et al.* 1991; Bauer *et al.* 1985). Confirmation of the importance of PDGF in wound healing has been provided by *in-vivo* experiments. A clinical trial showed that locally applied PDGF-BB to chronic pressure ulcers accelerates wound closure (Robson *et al.* 1992). Atherosclerosis and some tumours that involve excess cellular proliferation have been proposed to be driven by abnormal expression of PDGF. In situ hybridisation experiments showed that in artherosclerotic plaques, increased expression of PDGF and the PDGF-β receptor is associated with excess endothelial and vascular smooth muscle cell proliferation respectively (Wilcox *et al.* 1990). PDGF-BB has also been identified as an autocrine growth factor for sarcomas and as a paracrine factor in malignant tumours of mammary epithelial cells (Wang *et al.* 1994; Coltrera *et al* 1995).

PDGF has been suggested to be important in the proliferation and development of maternal, embryonal and fetal tissues. It was found that PDGF chains and PDGF-R subunits are expressed in many cell types within fetal and maternal tissues (Mercola *et al.* 1988, Mercola *et al* 1990; Goustin *et al.* 1985; Bidwell *et al.* 1995). The importance of PDGF in fetal development has been supported by the physiology of transgenic mice deficient for PDGF-B or the β PDGF-receptor. Mice pups deficient for PDGF-B died shortly before or at birth from haemorrhages and/or fatal renal and cardiovascular abnormalities (Leveen *et al.* 1994). Post mortum showed that the kidneys lacked mesengial cells, the heart and major vessels were dilated, and the mice were thrombocytopenic and severely anaemic. Mice lacking the β receptor died from similar renal and red blood cell abnormalities, but did not have defects in the major vessels or heart (Soriano 1994). It was suggested that the PDGF-B chain and β receptor are essential for the development of the kidney mesangium and normal red blood cells. It

was also proposed that the maturation of major blood vessels involves the signalling of PDGF-BB or PDGF-AB through the α/α receptor but not the α/β or β/β dimers.

There has been only one study of PDGF in mammary secretions. As already mentioned Shing and Klagsbrun identified a PDGF-like molecule in colostrum and proposed that it was the major growth factor in bovine milk (Shing and Klagsbrun 1984, *op cit* 1987; Shing *et al.*1987). They found that the level of PDGF-like molecule was highest in colostrum but not detectable in mature milk. Since milk PDGF has not been extensively investigated, many more studies are needed to determine the structure and function of this factor. Firstly, it will have to be established if any PDGF is present in mature milk. Then other work could include purifying and determining the sequence of bovine milk derived bovine PDGF and if there are binding proteins that protect PDGF from inactivation. In addition, the source of bovine milk PDGF could be investigated. The role of bovine milk-derived PDGF in the new born should only be studied after determining there is sufficient quantity to be physiologically relevant.

## 1.4 Other Bioactive Peptides from Proteins Found in Bovine Milk

Peptides with various types of biological activity (other than growth factors) have been isolated from the hydrolysates of casein and whey protein. These include the bombesin-like peptides, peptide kinins, the opioid peptides, and casein fragments with mitogenic activity (Jahnke and Lazaus 1984; Antila *et al.* 1991; Wilson *et al.* 1989; Nagaune *et al.* 1989; Mati *et al.* 1993).

Gastrointestinal peptides with hormonal activities have been isolated from bovine milk. These comprise bombesin and peptide kinins. A bombesin like peptide has been

shown to be present in bovine milk (Jahnke and Lazarus 1984). This peptide acts as a hormone that effects the release of gastric acid and a wide variety of gastrointestinal peptides and hormones throughout the gut. It does not by itself induce the growth of cells in culture (Puccio and Lehy 1989; Jiang *et al.* 1989). Both bradykinin and kinin peptides have been purified from bovine milk (Wilson *et al.* 1989). These peptides are important in the contraction of muscle cells especially in the gut, but do not induce growth of cells in culture (Berseth *et al.* 1990).

Opioid peptides such as the β-casomorphins, β-lactorphin and α-lactorphin have been isolated from bovine milk. These peptides are fragments of large milk proteins such as casein, β-lactoglobulin or α-lactalbumin (Brantl *et al.* 1979; Antila *et al.* 1991). They all have effects on opioid receptors and smooth muscle cells but do not produce cell growth *in-vitro*.

Peptide fragments derived from tryptic hydrolysis of bovine β-casein or phospho-glycoproteins have been shown to stimulate DNA synthesis in BALB/c 3T3 and hybridoma cells respectively (Nagaune *et al.* 1989; Mati *et al.* 1993). The mitogen derived from β-casein hydrolysis was equivalent to the amino acid region 177-183 of bovine β-casein, but the fragment derived from phospho-glycoprotein has not been sequenced. The importance of these peptides is not known at present. Further experiments that show whether they are present when milk is incubated with the natural acid stomach contents will indicate their biological significance.

## 1.5 Lactoferrin

Lactoferrin is an iron-binding glycoprotein widely distributed in external secretions (Groves 1960; Masson *et al.* 1966; Harmon *et al.* 1976). The concentration of

lactoferrin in bovine milk is much less than found in human milk. The average concentration in mature bovine milk is 0.1 mg/ml and in human milk is 2 mg/ml (Masson and Heremans 1971). Each lactoferrin molecule binds two iron molecules. It has been proposed that lactoferrin contributes to the lack of free iron in milk which may inhibit the growth of bacteria. Indeed, bacterial species which rely on iron to transport oxygen in aerobic metabolism are affected by the lack of available iron in milk (Byers and Arveneaux 1971; Oram and Reiter 1968). Also, lactoferrin has direct cytotoxic activity against bacteria, fungi and viruses (Bullen *et al.* 1972; Reiter 1983, *op cit* 1985; Rainard 1986; Smith and Oliver 1981). Besides these protective effects it exerts immunoregulatory functions. It has roles in the regulation of myelopoiesis, iron deposition in the monocyte-macrophage system and hydroxyl radical production by neutrophils (Birgens *et al.* 1983; Birgens 1984; Oria *et al.* 1988, Oria *et al.* 1993). It acts as an inhibitor of IL-1, IL-2 and TNF-β, and represses the synthesis of IL-6 in monocytes upon stimulation with lipopolysaccharide (Zucali *et al.* 1979, Zucali *et al.* 1989; Wang *et al.* 1984; Crouch *et al.* 1992).

Another possible function of milk lactoferrin is that of a promoter of iron absorption in the neonate. Animal experiments have shown that lactoferrin-iron is as well absorbed as iron supplements (Fransson *et al.* 1983; Fransson *et al.* 1983b). The presence of lactoferrin receptors in the intestinal brush border of humans and monkeys have been reported (Cox *et al.* 1979; Davidson and Lonnerdal 1988). The binding of lactoferrin to these receptors has been suggested to be evidence for the hypothesis that receptor-mediated uptake of iron is responsible for the high bioavailability of human breast milk iron. This hypothesis holds that when milk lactoferrin is iron saturated it is protected from proteolysis during its passage through the gastrointestinal tract. After it reaches the lactoferrin receptors at the brush border it releases iron into the circulation, and the

lactoferrin subsequently becomes partly digested. This agrees with the appearance of intact lactoferrin in the faeces of breast fed infants (Prentice *et al.* 1989; Davidson and Lonnerdal 1987). However, a subsequent study on the influence of lactoferrin on iron absorption from human milk in infants has been inconclusive (Davidson *et al.* 1994). The absorption of iron from human milk by breast fed infants was low and increased with the removal of lactoferrin. Further studies are needed to define the role of milk lactoferrin and its intestinal receptors in the neonate.

There are some reports that lactoferrin can stimulate the growth of cells in-vitro. (Oria *et al.* 1988; Amouric *et al.* 1984; Azuma *et al.* 1989b). Although iron is required for cell growth, there has been no evidence that lactoferrin is involved in transporting iron *in-vitro*. This is in contrast to transferrin which is the iron-binding protein present in plasma. Transferrin supplies iron to cells by binding a specific receptor followed by endocytosis during which iron is retained in the cell and the receptor is recycled (Dautey-Varsat 1986). No such iron-transport function has been established for lactoferrin i*n-vitro*. Although lactoferrin receptors have been described on lymphocytes, monocytes and macrophages (Mazurier *et al.* 1989; Birgens *et al.* 1983; Birgens 1984) a study by Oria *et al.* (1988) showed that iron uptake from lactoferrin by monocytes was poor. Nevertheless, despite its inability to act as an iron-transport protein it can stimulate cell growth *in-vitro*. Lactoferrin purified from milk has been shown to promote the growth of bovine epithelial cells, human cancer cell lines, L6 myoblasts, BALB/c 3T3 cells and rat crypt cells (Rejman *et al.* 1992, Rejman *et al.* 1992b; Hashizume *et al.* 1983; Amouric *et al.* 1984; Byatt *et al.* 1990; Azuma *et al.* 1989b; Nichols *et al.* 1987). However, since lactoferrin binds non-specifically to a number of milk proteins, the possibility that growth promoting agents were co-purified with lactoferrin may need to be investigated (Wattanbe *et al.* 1984; Lampreave *et al.* 1990).

The anti-bacterial, cytotoxic and immune effects of milk lactoferrin have suggested roles for this protein in breast fed infants. However, bovine milk contains a relatively small amount of lactoferrin in comparison to human milk.

## 1.6 Isolation of Bioactive Proteins from Bovine Milk

The starting point for detailed analysis of milk growth factors is a method to separate the cell-growth promoting activity from other components of milk. Mitogenic factors in bovine milk are a minor proportion of total milk protein (see **Table 1.1**). They are also contained in the whey fraction of milk, but not the casein component (Brown and Blakeley 1983; Klagsbrun 1980; Shing and Klagsbrun 1984). Importantly, the cell-growth promoting activity of mature milk can be recovered from whey produced as an industrial residue; specifically, whey produced as a by-product of cheese manufacture or from acid or enzyme-induced precipitation of milk casein (Klagsbrun 1979; Damerdji *et al.* 1988; Derouiche *et al.* 1990; Pakkanen *et al.* 1992; Legrand *et al.* 1993; Capiaumont *et al.* 1994). The use of large scale cheese whey fractions to study milk growth factors is economically advantageous. Indeed, cheese whey is considered a waste product and world wide production of cheese whey is in the order of 130 million tonnes per year. Current use in animal feed and food manufacturing is far less than cheese whey created (Zall 1992).

There are various methods that could be used to produce fractions of whey that contain the growth factors of milk. To date, large scale membrane exclusion techniques and cation-exchange chromatography have been used to produce mitogenic fractions from cheese whey (Damerdji *et al.* 1988; Derouiche *et al.* 1989; Legrand *et al.* 1993;

Francis *et al.* 1995). Mitogenic cheese whey concentrates of molecular mass greater than 10 kDa have been obtained by ultafiltration procedures (Damerdji *et al.* 1988; Derouiche *et al.* 1989; Legrand *et al.* 1993). This method concentrates whey protein but does not separate the low percentage of protein comprising growth factors from the major whey proteins listed in **Table 1.1**. Indeed, a 10-30 kDa concentrate of whey protein which contained the majority of whey mitogens was shown to contain up to 85% of total whey protein (Francis *et al.* 1995). Specifically, the major whey proteins β-lactoglobulin (18 kDa) and α-lactalbumin (14 kDa) were still present in this concentrate. This means that the growth factors are still a very minor component of the total protein present in this whey fraction. Conversely, cation-exchange chromatography of cheese whey has been utilised to produce a whey fraction that has no major whey proteins whilst retaining high concentrations of mitogens. It was known that β-lactoglobulin and α-lactalbumin have different isoelectric points than most growth factors (see **Table 1.1 and Table 1.2**). Francis *et al.* (1995), showed that cation-exchange chromatography of cheese whey could eliminate the majority of whey protein, while retaining the whey growth factors. Over 97% of whey proteins including β-lactoglobulin, α-lactalbumin, casein fragments and serum albumin were removed by this process.

Cation-exchange chromatography of cheese whey can produce a fraction of highly concentrated growth factor activity. All the growth factor activity contained in cheese whey adheres to cation-exchange resins at neutral pH and elutes with 0.4-0.6 M NaCl (Francis *et al.* 1995). Typically, 30 to 60g of whey extract is obtained from one litre of whey, which comprises 0.5% of whey protein. Significantly less of the whey extract protein was needed than that of microfiltered whey to produce cell growth promotion. This means there has been a significant purification of the mitogenic fraction of whey while eliminating the majority of whey protein. Moreover, the non-adsorbed material

contained most of the whey protein, but no cell-growth promoting activity. In addition, stepwise removal of whey protein bound to the resin with 1.0 M NaCl after the 0.4 M NaCl elution revealed material bound between 0.4 and 1.0 M NaCl did not induce cell-growth. The major protein in this fraction was lactoferrin, which in previous reports had been shown to stimulate the growth of cells in culture (Hashizuma *et al.* 1983; Azuma *et al.* 1989b; Byatt *et al.* 1990). Nevertheless, poor stimulation of cell-growth in the fraction containing lactoferrin suggested the earlier reported activity of lactoferrin may be due to co-purified growth factors.

Before assigning biologic functions to milk growth factors, their form and concentration has first to be established. There is little information on fibroblast growth factors (FGF), platelet-derived growth factors (PDGF), transforming growth factor-betas (TGF-β) and epidermal growth factors (EGF) in bovine milk (Shing and Klagsbrun 1987; Cox and Burk 1991; Jin *et al.* 1991; Read *et al.* 1985; Yagi *et al.* 1986). It is known however, that they are at very low concentrations in milk. Large amounts of a cation exchange fraction that contains concentrated growth factor activity could therefore be used to simplify the search and characterisation of milk growth factors. The results presented in **Chapters 3 to 7** represent experimental work on growth factors in bovine whey conducted between February 1992 and November 1995. More recent information from literature that relates to my thesis will be included in the appropriate chapters.

**Table 1.1 Classification, properties and distribution of bovine milk proteins (Adapted from Eigel *et al.* 1984)**

| *Class of milk protein* | | *Mol. weight* | *Isoelectric point* | *Amount in milk* | *% of whey protein* |
|---|---|---|---|---|---|
| | | **kDa** | **pI** | **g/l** | **%** |
| **Caseins** | | | | | |
| | αs1 | 22-23 | 4.2-4.8 | 12-15 | 0 |
| | αs2 | 25 | 4.8-5.1 | 3-4 | 0 |
| | β | 23-24 | 4.8-5.1 | 9-11 | 0 |
| | κ | 19 | 4.1-5.8 | 2-4 | 0 |
| **MFGM proteins** | | | | | |
| | zone A to D | 30-200 | | 0.4 | 0 |
| **Whey proteins** | | | | | |
| | Soluble casein and MFGM fragments | 4-80 | 4.7-5.8 | 1-3 | 10-20[1] |
| **Major** | | | | | |
| | β-Lactoglobulin | 18 | 5.1-5.5 | 2-5 | 55-70 |
| | α-Lactalbumin | 14 | 4.2-4.5 | 0.6-1.7 | 15-25 |
| | Serum albumin | 66 | 5.1 | 0.4 | 5 |
| | Immunoglobulins | 153-1000 | 5.5-8.3 | 0.7 | 10 |
| **Minor** | | | | | |
| | Lactoferrin/ Transferrin | 75-80 | 8.8 | 0.02-0.1 | 0.3 |
| | β2-Microglobulin | 46 | 10 | 0.006 | 0.01 |
| | Growth Factors | 5-30 | 5.0-10 | NR | NR |
| **Enzymes** | | | | | |
| | Lysosyme | 15 | 9.5 | 0.0001 | 0.001 |
| | Lipases | 60-96 | 4.0-5.0 | 0.002 | 0 |
| | Proteinases | 83 | various | 0.3-trace | 0 |
| | Phosphatases | 40-190 | 7.0-8.0 | 0.02 | 0 |
| | Lactoperoxidase | 78 | 8.5 | 0.1 | 1.5 |
| | Ribonuclease | 13.6 | 8.5 | 0.02 | 0.3 |
| **Total** | | | | **29-37** | **100** |

[1]This includes peptides derived from proteolysis of β-casein and glycomacropeptides derived from hydrolysis of κ-casein. MFGM: milk fat globule membrane proteins.
NR: not recorded

**Table 1.2 Characteristics of growth factors found in bovine milk and colostrum**

| | *Isoform* | *Mol. weight* | *Isoelectric point* | *Amount* |
|---|---|---|---|---|
| | | **kDa** | **pI** | **ng/ml** |
| **Platelet-derived growth factor (PDGF)** | | | | |
| | PDGF-like molecules [1] | 30 | 10 | NR [A] |
| **Transforming growth factor-β (TGF-β)** | | | | |
| | TGF-β1 | 25 | NR | NR [B] |
| | TGF-β2 | 25 | NR | NR [B] |
| **Epidermal growth factor (EGF)** | | | | |
| | EGF | 6-200 | NR | 2-324 [C] |
| **Insulin-like growth factor (IGF)** | | | | |
| | IGF-I | 7.6 | 8.8 | 4.3 [D] |
| | des (1-3) IGF-I | 7.0 | 8.0 | 3-50% of IGF-I [E] |
| | IGF-II | 7.5 | 6.7 | 1.0 [F] |

[1] Refers to growth factors detected in bovine colostrum only. NR: not recorded.
[A] Shing and Klagsbrun (1987)
[B] Jin *et al.* (1991); Cox and Burk (1991)
[C] Read *et al.* (1985); Iacopetta *et al.* (1992)
[D] Collier *et al.* (1991)
[E] Francis *et al.* (1986); Shimamoto *et al.* (1991)
[F] Vega *et al.* (1991)

# CHAPTER Two:

# General Materials and Methods

## 2.1 Materials

### 2.1.1 *Recombinant growth factors*

Platelet-derived growth factor-AA and -BB (PDGF-AA, PDGF-BB), acidic and basic fibroblast growth factors (aFGF/FGF-1, bFGF/FGF-2) and transforming growth factor-β1 (TGF-β1) were purchased from Austral Biologicals (San Ramon, California, USA). Insulin-like growth factor-I and II (IGF-I, IGF-II) were obtained from GroPep (Adelaide, South Australia, Australia) and epidermal growth factor (EGF) was from Chiron Mimotopes Peptide Systems (Rosebank, Victoria, Australia).

### 2.1.2 *Cells*

The following cell lines were purchased from the American Type Culture Collection, (Camden, New Jersey, USA): Mouse BALB/c 3T3 embryo fibroblasts (CCL 163), rat L6 skeletal muscle myoblasts (CRL 1458), rat small intestine epithelial cells (IEC-6; CRL 1592), baby hamster kidney cells (BHK-21, ATCC CCL 10), mink lung epithelial cells (Mv1Lu, CCL64) and the human epidermoid carcinoma cell line A431 (CRL 1555). Canine kidney epithelial cells (MDCK) were donated by Arthur Webster Pty Ltd (Castlle Hill, New South Wales, Australia). Human diploid skin fibroblasts were provided by the Women's and Children's Hospital, (North Adelaide, South Australia, Australia). A Simian virus 40-transformed human lung fibroblast, AG2804, was obtained from the Institute of Medical Research (Camden, New Jersey, USA).

### 2.1.3 *Antibodies*

Anti-human PDGF IgG raised in goats that recognises human, bovine and porcine PDGF and neutralises their activity in cell culture was obtained from Upstate Biotechnology Incorporated (Lake Placid, New York, USA). Anti-goat/sheep IgG conjugated to alkaline phosphatase and anti-goat/sheep IgG was bought from Silenus Laboratories (Hawthorn, Melbourne, Australia) Polyclonal anti-bovine FGF-2 was purchased from Sigma Aldrich Pty Ltd (Castle Hill, New South Wales, Australia) and anti-FGF-1 was a gift from Dr I Hendry, John Curtin School of Medical Research (Australian National University, ACT, Australia). The FGF antibodies were raised in rabbits and anti rabbit IgG conjugated to alkaline phosphatase was bought from Sigma Aldrich Pty Ltd (Castle Hill, New South Wales, Australia). Monoclonal mouse anti-TGF-β was from Genzyme Corporation (Cambridge, Massachusetts, USA). A control monoclonal antibody of anti-alkaline phosphatase was bought from Silenus Laboratories, (Hawthorn, Melbourne, Australia).

### 2.1.4 Reagents

Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, Utah, USA), or Cytosystems (Castle Hill, New South Wales, Australia). Dulbecco's Modified Eagle's Medium, fungizone and trypsin were obtained from Flow Laboratories (Irvine, Strathclyde, Great Britain). Radio-immunoassay grade bovine serum albumin, heparin (sodium salt), nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, streptomycin and penicillin were from Sigma Aldrich Pty Ltd (Castle Hill, New South Wales, Australia). $^{125}I$ and rainbow™ molecular weight markers were obtained from

Amersham Australia. (North Ryde, New South Wales, Australia). Analytical grade acetonitrile and propanol were purchased from Waters Corporation Australia (Rydalmere, New South Wales, Australia). Trifluoroacetic acid was obtained from BDH Chemicals Australia Pty Ltd (Kilsyth, Victoria, Australia) and heptafluorobutyric acid was from Beckman Pty Ltd (Gladesville, New South Wales, Australia).

## 2.2 Methods

### 2.2.1 *Production of whey extract*

Cation-exchange chromatography was used to obtain a fraction of cheese whey (whey extract) as previously described by Francis (*et al.* 1995) (**Figure 2.1**). Pasteurised whey obtained as an end product of cheese manufacture (pH 5.6) was passed through a 0.8 μm ceramic filter to remove solids (Membralox, Bajet, France) (**A**). The ultrafiltrate was adjusted to pH 6.5 and applied to a Pharmacia BPG 575 cm diameter column filled with 3000 ml of S-Sepharose Fast Flow cation-exchange resin (AMRAD Pharmacia Biotech, North Ryde, New South Wales, Australia). The column had previously been equilibrated with 50 mM sodium citrate buffer, pH 6.5 (**B**). After washing the column with equilibration buffer, the adsorbed material was eluted with 0.5 M NaCl made in the same buffer (**C**). Salt was then removed from the eluted material by diafiltering the extract against de-ionised water. This was done with a 3 kDa excluding membrane (Sartorius, Gottingen, Niedersachsen, Germany) in an Amicon DC-10 ultrafiltration unit (Amicon, Danvers, Massachusetts, USA). The material was then concentrated (**D**) using the same device and then freeze-dried (**E**). Protein content of the whey extract was measured as described in **2.2.5** and was consistently greater than

**Figure 2.1: Isolation of whey extract from bovine cheese whey**

*Pasteurised Cheddar Cheese Whey*

**A** Ultrafiltration: 0.8 μm membrane then adjusting whey to pH 6.5

**B** Cation-exchange: whey loaded onto S-Sepharose Fast Flow media

**C** Protein eluted with 0.5 M NaCl

**D** Eluate diafiltered and concentrated: 3 kDa membrane

**E** Desalted protein concentrate freeze-dried

98% of the dried weight. The freeze-dried cation-exchange fraction is termed '**whey extract**'.

### 2.2.2 *Cell culture*

All cells used were cultured in Dulbecco's modified minimal essential Eagle's medium (DMEM) containing 60 μg/ml penicillin, 100 μg/ml streptomycin, 500 μg/ml fungizone and 10% (v/v) FBS in an atmosphere of 5% (v/v) $CO_2$ at 37° C. Stock cultures were maintained in Corning 75 $cm^2$ flasks (Corning Australia, Alexandria, New South Wales, Australia) and routinely passaged after suspension in 0.125% (v/v) trypsin/0.5 mM EDTA made in Dulbecco's phosphate buffered saline to detach cells. The 96-well plates used for cell growth assays were purchased from Nunc Pty Ltd (Roskilde, Denmark). All culture media and material used in culture (except fungizone) were routinely passed though 0.22 μm GV filters on sterile glass filtering devices under suction or through those attached to syringes (Waters-Millipore, Rydalmere, New South Wales, Australia).

### 2.2.3 *Cell growth assay*

The ability of whey extract fractions or recombinant growth factors to induce the growth of mammalian cells was determined by a 96-well plate dye binding assay modified from Oliver (1989). Cells were subcultured in DMEM containing 10% (v/v) FBS and 100 μl containing 10-20 x $10^3$ cells added to each of the 96 wells in Nunc tissue culture plates (Nunc Pty Ltd, Roskilde, Denmark). The plates were incubated overnight at 37° C to facilitate cell attachment and then wells were washed in DMEM for 2-4 hours to remove residual FBS. Dilutions of whey extract or growth factors were added to wells in a final

volume of 100 µl. DMEM alone and dilutions of FBS were also included on all 96-well plates as standard references of cell growth. Plates were incubated for 48 h at 37° C and then wells were washed with 0.15 M NaCl three times before fixing cells with 100 µl of methanol/well for 30 min. Each well was then stained with 100 µl of 1% (w/v) methylene blue made in 0.01 M disodium tetraborate, pH 8.5, and the culture plates incubated for 30 min at room temperature. Wells were then washed with 0.01 M disodium tetraborate, pH 8.5, before adding 100µl of 1:1 (v/v) ethanol: 0.1 M HCl. The cell mass was then quantified by determining the absorbance of methylene blue at 655 nm in each well using an automated plate reader (Biorad model 450). Data was expressed as a percentage of the response observed in 10% (v/v) FBS, with the growth in DMEM alone subtracted. The results were graphed using Sigma Plot and analysed using one-way ANOVA (Jandel Scientific, San Rafael, California, USA).

### 2.2.4 *Gel-filtration*

Gel-filtration of dried whey extract was carried out using a Pharmacia Superdex 75 HR 35/600 (3.5 x 60 cm) size exclusion column coupled to a fast protein liquid chromatography (FPLC) system (AMRAD Pharmacia Biotech). A running buffer of 50 mM $NaH_2PO4$, 150 mM NaCl and 10% (v/v) acetonitrile, pH 7.4, was used for neutral gel-filtration. Acid gel-filtration was undertaken in a buffer of 150 mM NaCl, 1 M acetic acid and 10% (v/v) acetonitrile, pH 2.0. The Superdex column was equilibrated in the relevant running buffer and loaded with 15 ml of a 25 mg/ml solution of filter sterilised whey extract. Protein was then eluted from the column at a flow rate of 22 cm/h and five minute fractions of 17.5 ml collected. The Superdex 75 HR 35/600 column was calibrated under neutral conditions using protein standards. Anomalous

behaviour of standards in acid prevented calibration of the gel-filtration column under acid conditions. The molecular weight standards used were IgG (150 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) cytochrome c (12.4 kDa) (Sigma Aldrich Pty Ltd Castle Hill, New South Wales, Australia.) and human insulin (7 kDa) (Humulin, Melbourne, Victoria, Australia).

### 2.2.5 *Protein measurement*

Protein was measured by with reference to bovine serum albumin (Sigma Aldrich Pty Ltd Castle Hill, New South Wales, Australia) by the method of Lowry *et al.* (1951) or by a bichinoninic acid-binding assay as described in the manufactures instructions (Pierce, Rockford, Illinios, USA).

# CHAPTER Three:

# Cell-growth Promotion by Whey Extract

## 3.1 Introduction

Specific growth factors have been suggested to control the progression of normal cells through a cell cycle. The interval between consecutive mitosis that encompasses DNA synthesis (S phase) and mitosis (M) has been termed the mammalian cell cycle. $G_1$ is the gap between mitosis and the onset of DNA synthesis, and $G_2$ between S phase and mitosis. The parameters of the BALB/c 3T3 fibroblast cell cycle have been well defined, and research has shown which growth factors are necessary for fibroblasts to traverse a cycle. BALB/c 3T3 fibroblasts that have become growth arrested by high cell density or serum starvation enter a quiescent phase designated $G_0$, and do not enter $G_1$. Stiles and colleagues demonstrated that BALB/c 3T3 cells in $G_0$ proceed through the cell cycle when provided with a sequence of platelet rich and platelet poor fractions of serum (Stiles *et al.* 1979; Smith and Stiles 1981). $G_0$ cells were described as being made 'competent' to proliferate by transient exposure to PDGF released from platelets and then enter early $G_1$. Competent BALB/c 3T3 cells then 'progress' through a cell cycle when supplied with platelet-poor plasma components such as EGF and IGF-I. Competence factors did not cause progression, nor progression factors competence. Further research has shown that EGF manifests its function at early $G_1$ and IGF-I at the $G_1$ /S boundary or a few hours before the onset of DNA synthesis (Leof *et al.* 1983; Campsi and Pardee 1984). In addition, FGF and bombesin have been described as competence factors for endothelial and Swiss 3T3 embryo fibroblasts respectively (Bouche *et al.* 1987; Rozengurt and Sinnett-Smith 1983).

The coordinated control of cell proliferation by growth factors observed in BALB/c 3T3 cells *in-vitro,* is not found in many other types of mammalian cells. The length of

$G_1$ can often be heterogenous, and not all cells show a competence response (Chen and Rabinovitch 1989; Mulder and Childress-Fields 1990). Also, cells derived from the epithelial layer like MDCK cells, respond to different growth factors than those from the mesenchyme such as BALB/c 3T3 cells. However, the presence of a variety of growth factor receptors on mammalian cells suggests that cell growth and development *in-vitro* and *in-vivo* are determined by combinations of growth factors. An example of this is that TGF-β may induce BALB/c 3T3 cells to grow in the presence of FGF by increasing the production of proteoglycans in the extracellular matrix which bind FGF (Nugent and Edelman 1992). In addition, TGF-β may also act indirectly through the induction of PDGF expression. Research showed that binding of TGF-β to its type I and II receptors on human skin fibroblasts and L6 myoblasts, induced the expression of PDGF which then stimulated the same cells to proliferate (Soma and Grotendorst 1989; Segarini 1990; Blachowski *et al.* 1993) In contrast, most epithelial cells lack PDGF receptors, so that TGF-β may not act as an indirect mitogen in these cells (Raines *et al.* 1990). Indeed, epithelial cells such as MDCK or IEC-6 cells are potently inhibited by TGF-β (Kurokowa *et al.* 1987; Barnard *et al.* 1989; Danielpour *et al.* 1989). Epithelial cells do, however, grow in the presence of EGF or FGF (Malo and Menard 1982; Thompson *et al.* 1987).

The specific response of mammalian cells lines to growth factors can be used to suggest the types of mitogens present in complex biological fluids such as bovine whey. This requires that cell lines be grown in media supplemented with whey and the response compared to that achieved by growth factors. To date, insulin-like growth factors (IGF) and transforming growth factor-beta (TGF-β) are the only mitogens that have been isolated and purified from mature bovine milk whey (Malvern *et al.* 1987; Cox and Burk 1991). There is no literature to suggest only IGF and TGF-β determine

the growth of mammalian cells in bovine milk (Hadsell *et al.* 1990; Ruan *et al.* 1992; Cox and Burk 1991; Steimer and Klagsbrun 1981; Steimer *et al.* 1981, Shing and Klagsbrun 1987). Other as yet unspecified mitogens may be responsible for the observed growth of cells in mature bovine milk. Platelet-derived growth factor activity has been identified by Shing and Klagsbrun (1984, *op cit* 1987; Shing *et al.* 1987) in bovine colostrum, but not in mature milk. Shing and Klagsbrun (1984) also suggested EGF was absent from bovine milk, but both Read *et al.* (1985) and Yagi *et al.* (1986) claimed to have measured detectable levels of the molecule. Conversely, Iacopetta *et al.* (1992) found there was little if any EGF in bovine milk. Fibroblast growth factors and a member of the epidermal growth factor family called transforming growth factor-alpha (TGF-α), have been identified in bovine tissue and fluids but not milk (Gospodarowicz 1975; Thomas *et al.* 1980; Kobrin *et al.* 1987; Zurfluh *et al.* 1990).

The aim of the current study was to use a bovine whey fraction in mammalian cell growth studies to suggest the types of growth factors present in bovine milk. Cation-exchange chromatography has been shown to recover the mitogenic activity of cheese whey (Francis *et al.* 1995). This fraction (termed whey extract), will be used to characterise the mitogens of mature bovine milk. Initially, the growth of BALB/c 3T3 cells in media supplemented with bovine whey will be contrasted with that achieved with whey extract. This will be followed by observing the growth of mesenchymal-derived and epithelial cells in whey extract. The growth of cells in media containing a recombinant growth factor will then be compared to the level of stimulation induced by whey extract. Growth factors that will be used include recombinant PDGF, TGF-β, FGF, EGF and IGF. Neutral and acid gel-filtration will then be used to separate the growth factor activity of whey extract. This study will show if whey extract can be used to isolate mitogens that have not yet been described in bovine milk.

## 3.2 Materials and Methods

Pasteurised bovine cheese whey was passed though a 0.2 μm syringe filter (Amicon, Danvers, Massachusetts, USA) before use in the cell growth assay. The production of whey extract was carried out as described in **2.2.1**. One litre of whey yeilded 60 mg freeze-dried whey extract. Gel-filtration of whey extract under neutral and acid conditions was done using a Superdex 75 HR 35/600 column as outlined in **2.2.4**.

### 3.2.1 *Promotion of cell growth by whey and whey extract*

The ability of whey extract to promote the growth of BALB/c 3T3 fibroblasts was compared to that produced by dilutions of microfiltered whey using the cell growth assay described in **2.2.3**. Cells were subcultured in 96-well tissue culture plates (20 x $10^3$/100 μl well) in DMEM containing 10% (v/v) FBS, and allowed to attach overnight. Cheese whey was microfiltered and the protein content measured as described in **2.2.5**. Whey was serially diluted in DMEM and the maximum amount of whey added to 100μl wells was 20μl. Whey extract was dissolved in DMEM at a concentration of 25 mg/ml and also serially diluted. Wells were washed for 2 hr in DMEM before the addition of the indicated dilutions of whey or whey extract in a final volume of 100 μl and incubated for a further 48 h. DMEM and DMEM containing 10% (v/v) FBS were also included on each plate and all treatments were assayed in triplicate. The growth of L6 myoblasts, human skin fibroblasts and BALB/c 3T3 cells (10-20 x $10^5$/96-well plate) in whey extract alone was also compared using the same method. Increase in cell mass was quantified by the methylene blue binding assay as indicated in **2.2.3**. Data are

expressed as the percentage of cell mass response observed in 10% (v/v) FBS, with the growth in DMEM subtracted.

The ability of whey extract to promote the growth of epithelial cells was also examined using the cell growth assay described in **2.2.3**. MDCK, and IEC-6 cells were subcultured in 96-well tissue culture plates (20 x $10^3$/100 µl well) in DMEM containing 10% (v/v) FBS and allowed to attach overnight. Whey extract was dissolved in DMEM at a concentration of 25 mg/ml or in DMEM supplemented with 1% (v/v) FBS and then serially diluted in the same media. Wells were washed for 2 hr in DMEM before the addition of the indicated dilutions of whey extract in a final volume of 100 µl and incubated for a further 48 h. DMEM and DMEM containing 10% (v/v) FBS were also included on each plate and all treatments were assayed in triplicate. Cell mass was quantified by the dye binding assay as indicated in **2.2.3** and data expressed as percentage of response observed in 10% (v/v) FBS, with the growth in DMEM subtracted.

### 3.2.2 *Cell growth in whey extract compared to recombinant growth factors*

The response of L6 myoblasts, BALB/c 3T3 fibroblasts and human skin fibroblasts to PDGF-AA, PDGF-BB, TGF-β1, EGF, aFGF/FGF-1, bFGF/FGF-2, IGF-I and IGF-II in comparison to whey extract were determined by the 96-well dye binding assay. Whey extract and FBS were diluted in DMEM; recombinant growth factors were diluted in DMEM containing 0.1% (wt/v) bovine serum albumin (BSA). The cells (10-20 x $10^5$/96-well plate) were grown overnight in 100 µl of DMEM/10% (v/v) FBS as indicated in **2.2.3** and then washed in DMEM for 2-4 h to remove residual serum. Dilutions of whey extract or recombinant growth factors were then added to wells in

final volume of 100 µl. DMEM/0.1% (wt/v) BSA and DMEM containing 10% (v/v) FBS were also included on each plate. Cell mass was then quantified after 48 h by the methylene blue binding described in **2.2.3**. Data are expressed as the percentage of the response observed in 10% (v/v) FBS, with the growth in DMEM containing 0.1% (wt/v) BSA subtracted.

### 3.2.3 *Cell growth in the presence of gel-filtration fractions*

The growth of L6 myoblasts, BALB/c 3T3 cells and human skin fibroblasts in response to neutral and acid gel-filtration fractions of whey extract was determined using the 96-well plate methylene blue binding assay. Samples (375 mg) of whey extract were subject to neutral and acid gel-filtration as described in **2.2.4** and 100 µl of each 17.5 ml fraction was freeze-dried with 10 µl of 10 mg/ml BSA and then dissolved in 1 ml of DMEM. L6 myoblasts, BALB/c 3T3 cells and human skin fibroblasts were subcultured in 96-well plates (10-20 x $10^5$/plate) as described in **2.2.3** and allowed to attach overnight in DMEM containing 10% (v/v) FBS. After washing the cells in DMEM for 2-4 h, 100 µl of each dissolved fraction was added to wells in triplicate. DMEM and dilutions of FBS were also incorporated onto each plate. After a 48 h incubation period, cell mass was quantified by methylene blue binding as indicated in **2.2.3**. Data is shown as the percentage of cell growth observed in 10% (v/v) FBS, with the response to DMEM alone subtracted.

### 3.2.4 *Epithelial cell growth inhibition produced by gel-filtration fractions*

Neutral and acid gel-filtration fractions were tested for their ability to inhibit the growth of MDCK and IEC-6 epithelial cells by modifying the 96-well plate cell growth

assay. Cells were allowed to attach overnight (20 x $10^5$/96-well plates) in DMEM containing 10% (v/v) FBS. 100 µl of neutral and acid gel-filtration fractions were freeze-dried with 10 µl of 10 mg/ml BSA and made up in 1 ml of DMEM that was supplemented with 5% (v/v) FBS. Cells were then washed in DMEM for 2-4 h before 100 µl of each dissolved fraction containing 5% (v/v) FBS was added to three wells of each plate. Standard references of DMEM containing 5% (v/v) FBS and DMEM alone were included on each plate. Cell mass was then quantified after a further 48 h incubation by the dye-binding assay as indicated in **2.2.3**. Data are expressed as a percentage of growth in DMEM containing 5% (v/v) FBS in the absence of gel-filtration fractions, with the growth in DMEM alone subtracted.

## 3.3 Results

### 3.3.1 *Cell growth in response to whey and whey extract*

The ability of whey and whey extract to promote the growth of BALB/c 3T3 cells were examined by the 96-well plate dye-binding assay. Cells were allowed to attach overnight in the presence of 10% (v/v) FBS before the addition of whey or whey extract. The amount of whey that could be added to cells growing in tissue culture plates is around 20% of volume or 80 µg of whey protein per 100 µl well. In contrast to this, powdered whey extract was dissolved in tissue culture media at concentrations of 25 mg/ml and the maximum amount added to 100 µl wells was 500 µg. **Figure 3.1** shows there was very little BALB/c 3T3 cell growth produced by whey in comparison to whey extract. Whey extract at 0.5 mg/ml produced cell growth that was nearly three times greater than bovine cheese whey at the same protein concentration. Furthermore, the maximum BALB/c 3T3 cell growth induced by whey was only 20% of that produced by

10% fetal calf serum. In comparison, **Figure 3.1** shows that whey extract could produce maximum cell growth 1.25 fold greater that that achieved with 10% FBS.

The growth of L6 myoblasts, and human skin fibroblast in tissue culture media supplemented with whey extract was also examined (**Figure 3.2**). All cell lines grew in media supplemented with whey extract in a dose dependent manner and achieved growth greater than that seen in 10% (v/v) FBS. Human skin fibroblasts were particularly responsive; as little as 250 μg/ml induced a 48 h growth response equivalent to 10% (v/v) FBS.

The growth of IEC-6 and MDCK epithelial cells in media supplemented with whey extract is shown in **Figure 3.3**. The growth of MDCK and IEC-6 cells in DMEM supplemented with 5 mg/ml whey extract was depressed to the level seen in DMEM alone. However, 0.2 to 1.2 mg/ml of whey extract induced IEC-6 cell growth to reach a maximal cell density equivalent to 50% of the response to 10% (v/v) FBS (**Figure 3.3 A**). MDCK cell density was highest at 0.1 mg/ml of whey extract and only reached the same level of growth as 20% of that observed with 10% (v/v) FBS (**Figure 3.3 B**).

The ability of whey extract to supplement epithelial cell-growth in a low concentration of serum was then examined by diluting whey extract in DMEM supplemented with 1% (v/v) FBS (**Figure 3.3**). The inclusion of 1% (v/v) FBS in the dilution series of whey extract enhanced IEC-6 and MDCK cell growth in an additive manner. IEC-6 and MDCK cell growth was inhibited the most at the highest concentration (5 mg/ml) of whey extract (**Figure 3.3 A, B**). Significantly, the response of IEC-6 cells to 1.2 to 2.5 mg/ml whey extract in 1% (v/v) FBS was greater than that obtained with 10% (v/v) FBS (**Figure 3.3 A**). The maximal growth of MDCK cells supplemented with 1% (v/v) FBS was reached with low concentrations of whey extract and was nearly equivalent to that obtained with 10% (v/v) FBS (**Figure 3.3 B**).

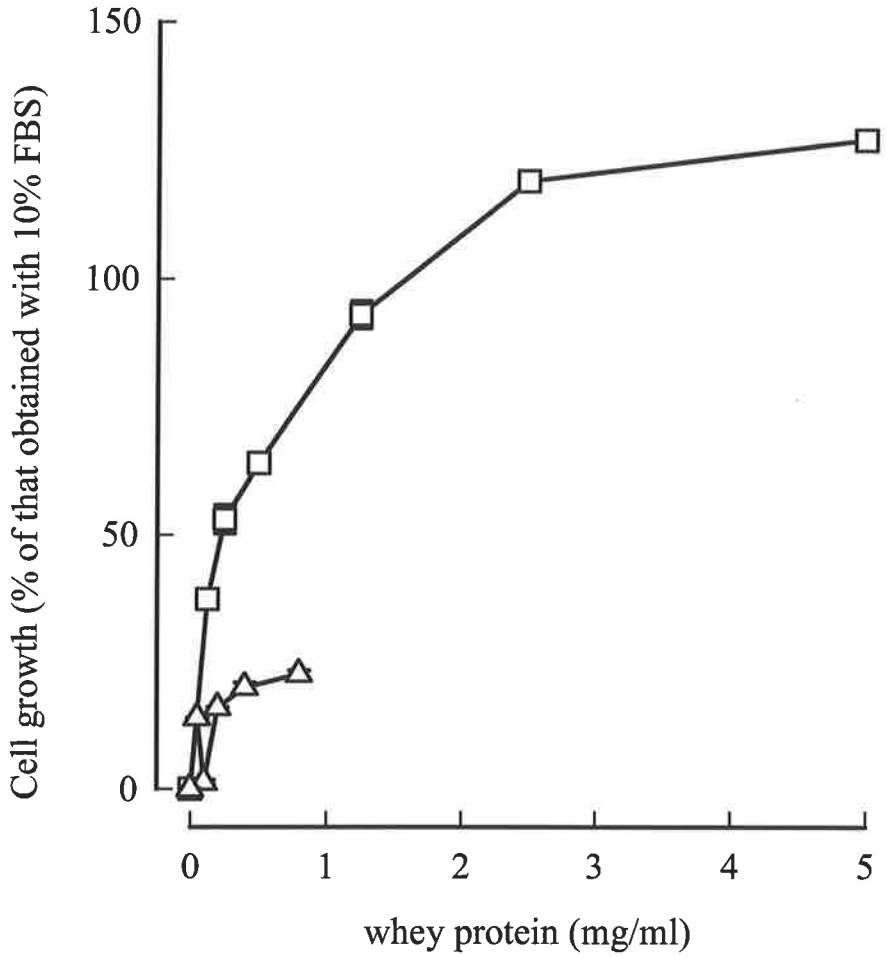



**Figure 3.1** Growth of BALB/c 3T3 cells in response to whey and whey extract. BALB/c 3T3 fibroblasts were subcultured in DMEM containing 10% FBS using 96-well plates, and allowed to attach overnight. Wells were then washed in DMEM to remove residual FBS before the addition of the indicated concentrations of whey (Δ) or whey extract (□) diluted in DMEM. After a 48-h incubation period, cell growth was quantified using an automated methylene blue dye-binding assay. Results are expressed as a percentage of the response to a 10% FBS standard incorporated onto each plate (mean ± S.E.M of triplicate measurements). The response to DMEM alone has been subtracted.

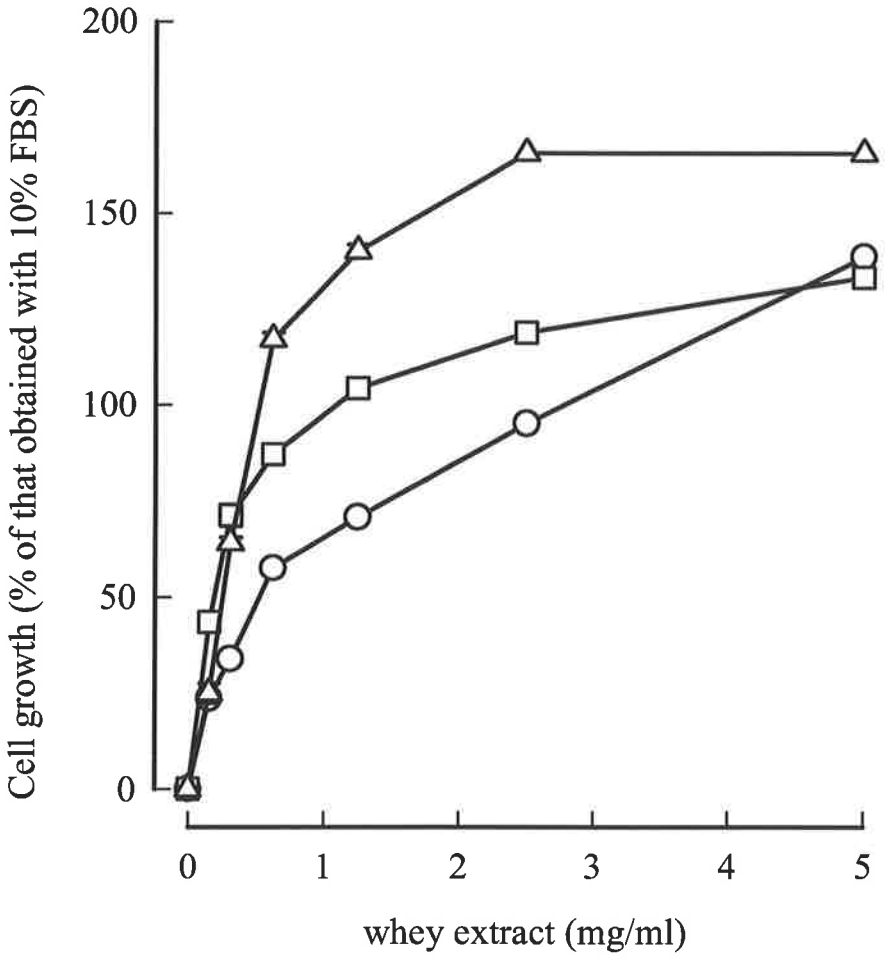



**Figure 3.2** Growth of cells in response to whey extract. L6 myoblasts (○), BALB/c 3T3 fibroblasts (□), and human skin fibroblasts (Δ) were subcultured in DMEM containing 10% FBS using 96-well plates, and allowed to attach overnight. Wells were then washed in DMEM to remove residual FBS before the addition of the indicated concentrations of whey extract diluted in DMEM. After a 48-h incubation period, cell growth was quantified using an automated methylene blue dye-binding assay. Results are expressed as a percentage of the response to a 10% FBS standard incorporated onto each plate (mean ± S.E.M of triplicate measurements). The response to DMEM alone has been subtracted.

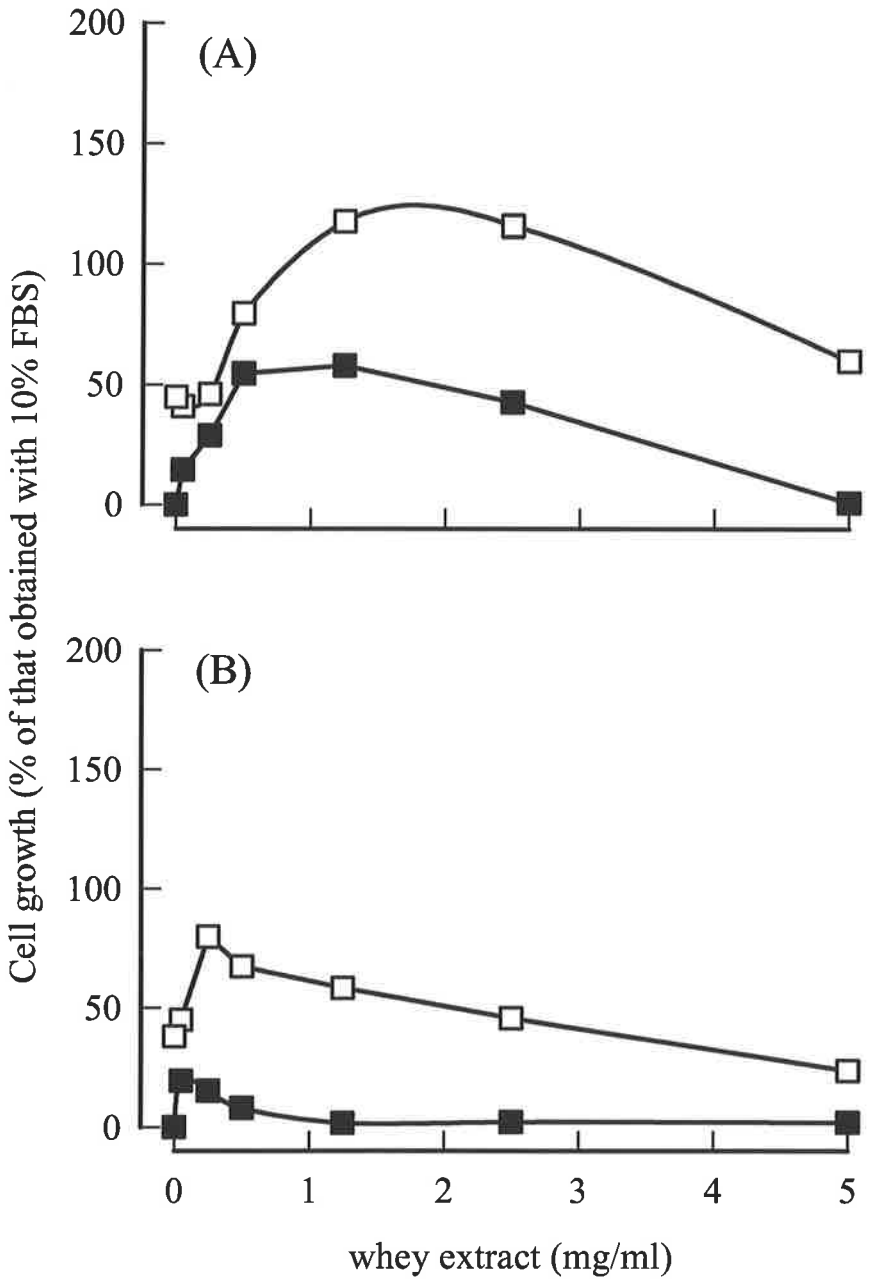



**Figure 3.3** Growth of epithelial cells in response to whey extract alone (■), or whey extract in the presence of 1% FBS (□). IEC-6 (A) and MDCK (B) cells were subcultured into 96-well plates, and allowed to attach overnight in DMEM/10% FBS. Wells were then washed in DMEM to remove residual FBS, before the addition of the indicated concentrations of whey extract diluted in DMEM or DMEM supplemented with 1% FBS. After a 48-h incubation period, cell density was determined using an automated methylene blue dye-binding assay. Results are expressed as a percentage of the response to a 10% FBS standard incorporated onto each plate (mean ± S.E.M of triplicate determinations). The response to DMEM alone has been subtracted.

### 3.3.2 *Cell growth by whey extract compared with pure growth factors*

The cell growth produced by whey extract was compared to that generated by recombinant human PDGF-AA, PDGF-BB, TGF-β1, FGF-1, FGF-2, IGF-I and IGF-II using 96-well plate dye-binding assays. A series of dilutions of whey extract and each growth factor were added to 96-well plates of L6 myoblasts, BALB/c 3T3 fibroblasts and human skin fibroblasts. In terms of maximal cell density attained for each cell line, whey extract was more potent than any pure growth factor tested or 10% (v/v) FBS (**Figures 3.4, 3.5, 3.6, 3.7, 3.8**).

PDGF-AA and PDGF-BB were the most potent growth factors for BALB/c 3T3 and human skin fibroblasts (**Figure 3.4 D, F**). PDGF-BB was slightly more potent than PDGF-AA on BALB/c 3T3 cells, especially at 0.5 to 50 ng/ml. In contrast, both factors were equipotent on human skin fibroblasts and 100 ng/ml produced maximal cell density. PDGF-BB at concentrations of 6-100 ng/ml exerted a growth response on L6 myoblasts equal to half of that seen with 10% (v/v) FBS (**Figure 3.4 B**). However, PDGF-AA was inactive on L6 myoblasts.

TGF-β1 induced the growth of BALB/c 3T3 and human skin fibroblasts to nearly 50% of the response observed with 10% (v/v) FBS at concentrations of 12-50 ng/ml and 1-3 ng/ml respectively. Maximal L6 myoblast cell density produced by TGF-β1 was less than 25% of the 10% (v/v) FBS response and required 50 ng/ml of TGF-β1 (**Figure 3.5 B, D, F**).

EGF concentrations of 6 to 12 ng/ml stimulated the growth of BALB/c 3T3 cells to nearly 50% of that seen with 10% (v/v) FBS (**Figure 3.6 D**). In contrast, L6 myoblasts

and human skin fibroblasts did not grow much above that seen DMEM in media supplemented with EGF (**Figure 3.6 B, F**).

Over 100 ng/ml of FGF increased the cell density of BALB/c 3T3 and human skin fibroblasts to that seen with 25-50% of the response to 10% (v/v) FBS (**Figure 3.7 D, F**). FGF-2 was more potent than FGF-1 on BALB/c 3T3 fibroblasts (**Figure 3.7 D**). The maximum L6 myoblast cells were induced to grow in FGF was less than 25% of the cell growth seen with 10% FBS. This was with 50 ng/ml of FGF (**Figure 3.7 B**).

IGF-I produced maximum L6 myoblast and human skin fibroblast cell growth of approximately 40% of that in 10% (v/v) FBS at 100 and 12 ng/ml respectively (**Figure 3.8 B, F**). IGF-I and IGF-II induced similar growth of L6 myoblasts and skin fibroblasts (**Figure 3.8 B, F**). BALB/c 3T3 cells did not grow much above that observed in DMEM in media supplemented with either isoform of IGF (**Figure 3.8 D**).

### 3.3.3 *Bioactivity of gel-filtration fractions of whey extract*

The bioactivity of whey extract was further investigated by gel-filtration. Samples of whey extract were subject to both neutral and acid Superdex 35/600 chromatography. The ability of the recovered fractions to induce the growth of L6 myoblast, BALB/c 3T3 and human skin fibroblasts, and inhibit epithelial cell-growth was measured using the 96-well plate dye binding assay.

Neutral gel-filtration of whey extract showed that the most potent fractions for L6 myoblast, BALB/c 3T3 and human skin fibroblast cells were near the 150 kDa standard (**Figures 3.9 A, 3.10 A, 3.11 A**). In contrast, fractions recovered from acid gel-filtration that most increased cell density had little detectable protein and chromatographed below the 45 kDa standard (**Figures 3.9 B, 3.10 B, 3.11 B**). Indeed, a larger number of acid than neutral fractions induced cell growth in all three cell lines. Under acid conditions,

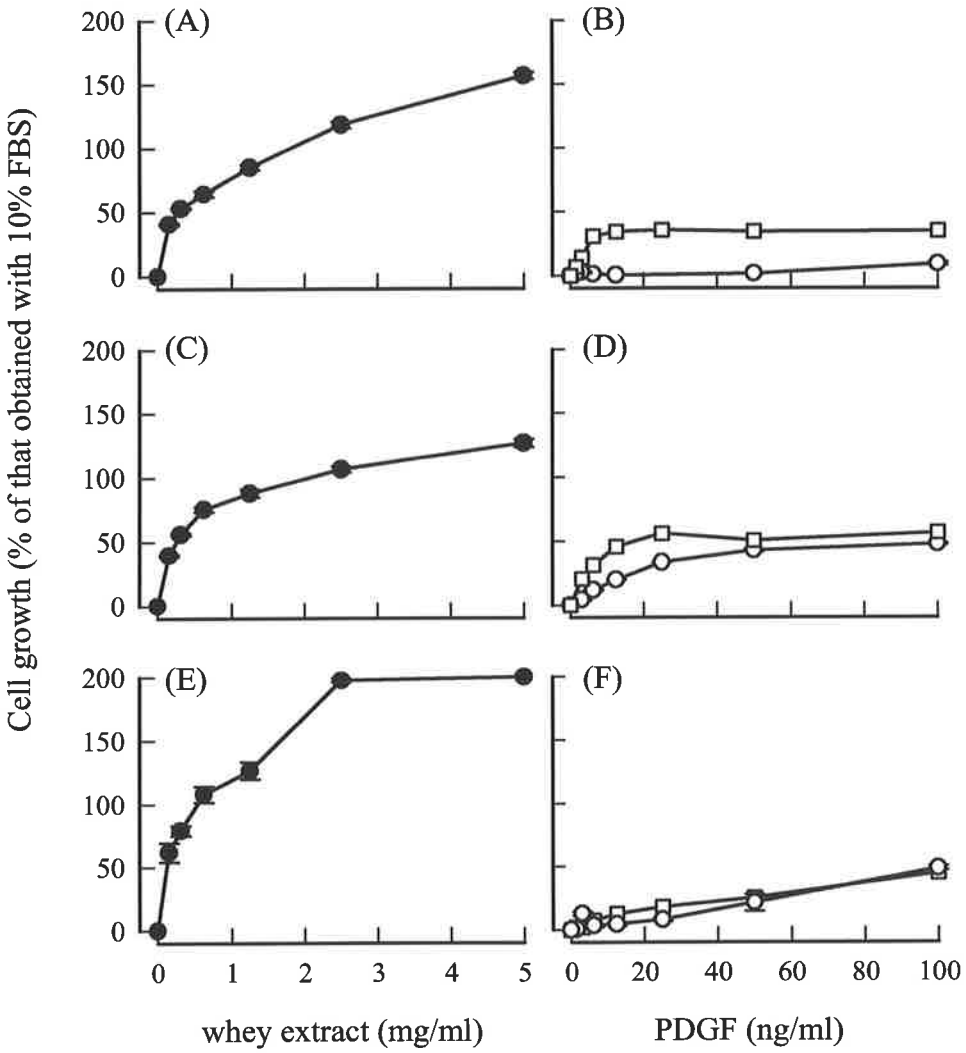



**Figure 3.4** Growth of cells in response to whey extract (●) compared to PDGF-BB (□) or PDGF-AA (○). L6 myoblasts (A,B), BALB/c 3T3 fibroblasts (C,D) and human skin fibroblasts (E,F) were subcultured in 96-well plates in DMEM/10% FBS and allowed to attach overnight. After washing cells for two hours in DMEM, a dilution series of PDGF-BB, PDGF-AA, and whey extract were added to the 96-well plates, which were then incubated for 48 h. Cell growth was then measured using an automated methylene dye-binding assay (**2.2.3**). Results are expressed as a percentage of the response to a 10% FBS standard incorporated onto each plate (mean ± S.E.M of three measurements). The response to DMEM alone has been subtracted.

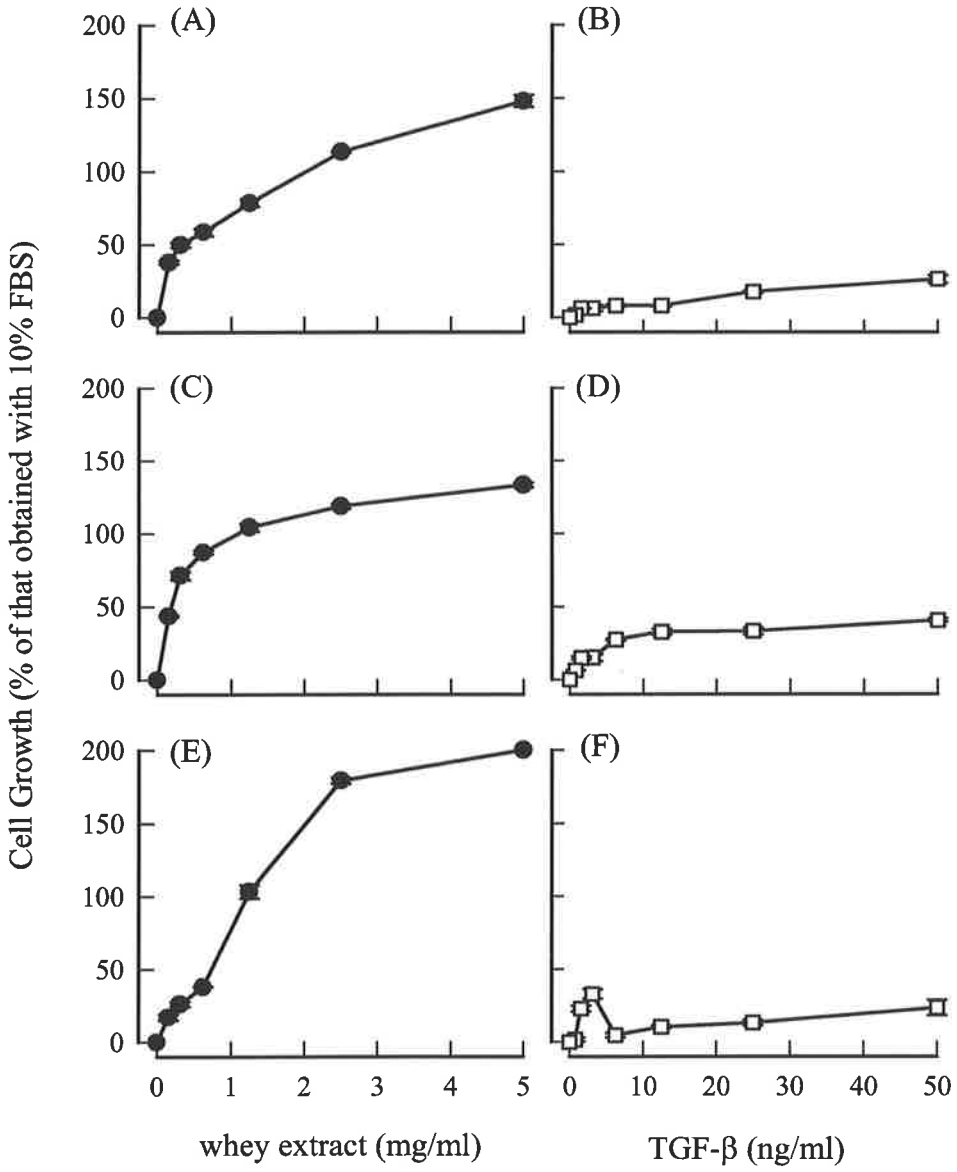



**Figure 3.5** Growth of cells in response to whey extract (●) compared to TGF-β1 (□). Experimental details are as for **Figure 3.4**. Cells are: L6 myoblasts (A,B), BALB/c 3T3 fibroblasts (C,D), and human skin fibroblasts (E,F). Results are the mean ± S.E.M of three measurements and expressed as a percentage of the response to a 10% FBS standard. The response to DMEM alone has been subtracted from all data.

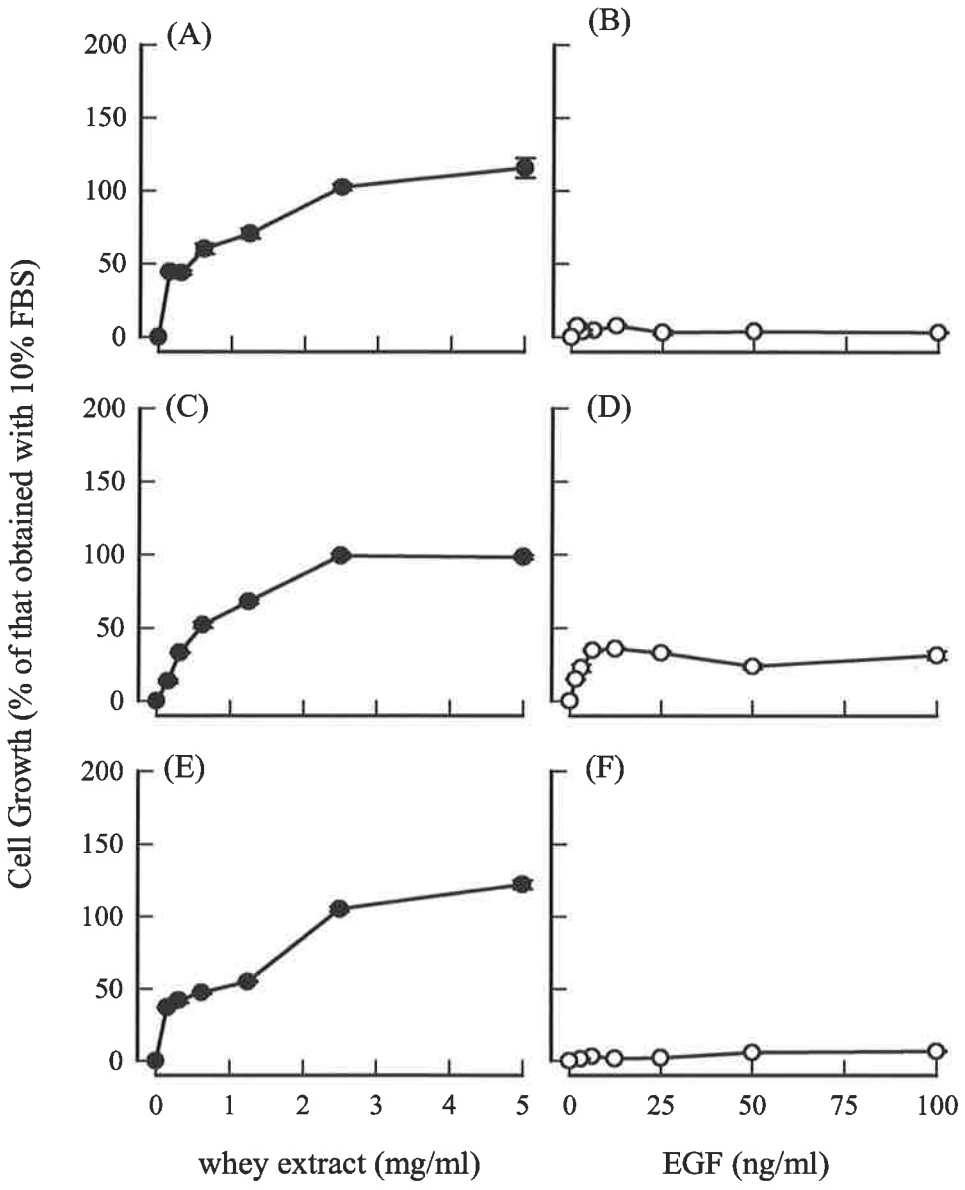



**Figure 3.6** Growth of cells in response to whey extract (●) compared to EGF (○). Experimental details are as for **Figure 3.4**. Cells are: L6 myoblasts (A,B), BALB/c 3T3 fibroblasts (C,D), and human skin fibroblasts (E,F). Results are the mean ± S.E.M of three measurements and expressed as a percentage of the response to a 10% FBS standard. The response to DMEM alone has been subtracted from all data.

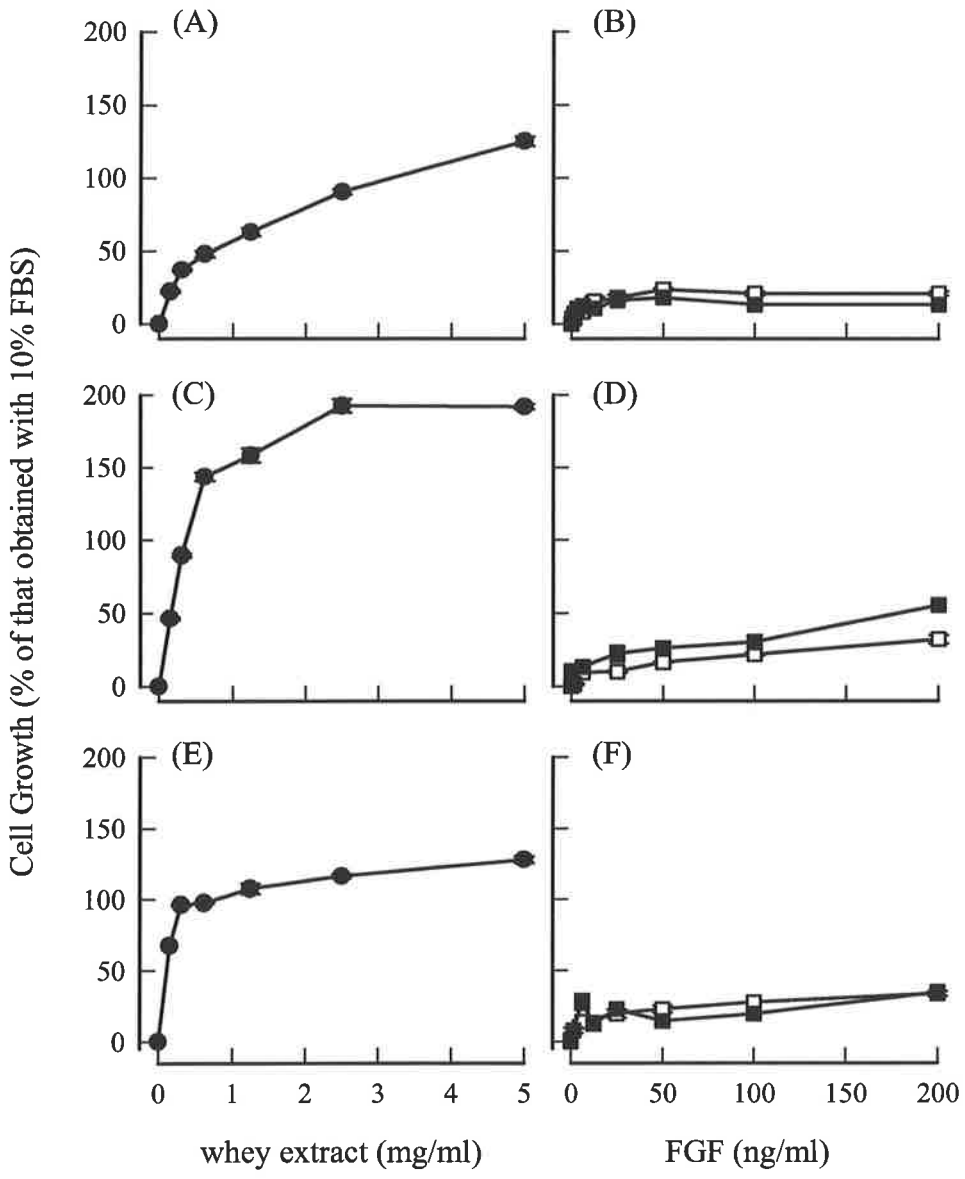



**Figure 3.7** Growth of cells in response to whey extract (●) compared to FGF-1 (□) and FGF-2 (■). Experimental details are as for **Figure 3.4**. Cells are: L6 myoblasts (A,B), BALB/c 3T3 fibroblasts (C,D), and human skin fibroblasts (E,F). Results are the mean ± S.E.M of three measurements and expressed as a percentage of the response to a 10% FBS standard. The response to DMEM alone has been subtracted from all data.

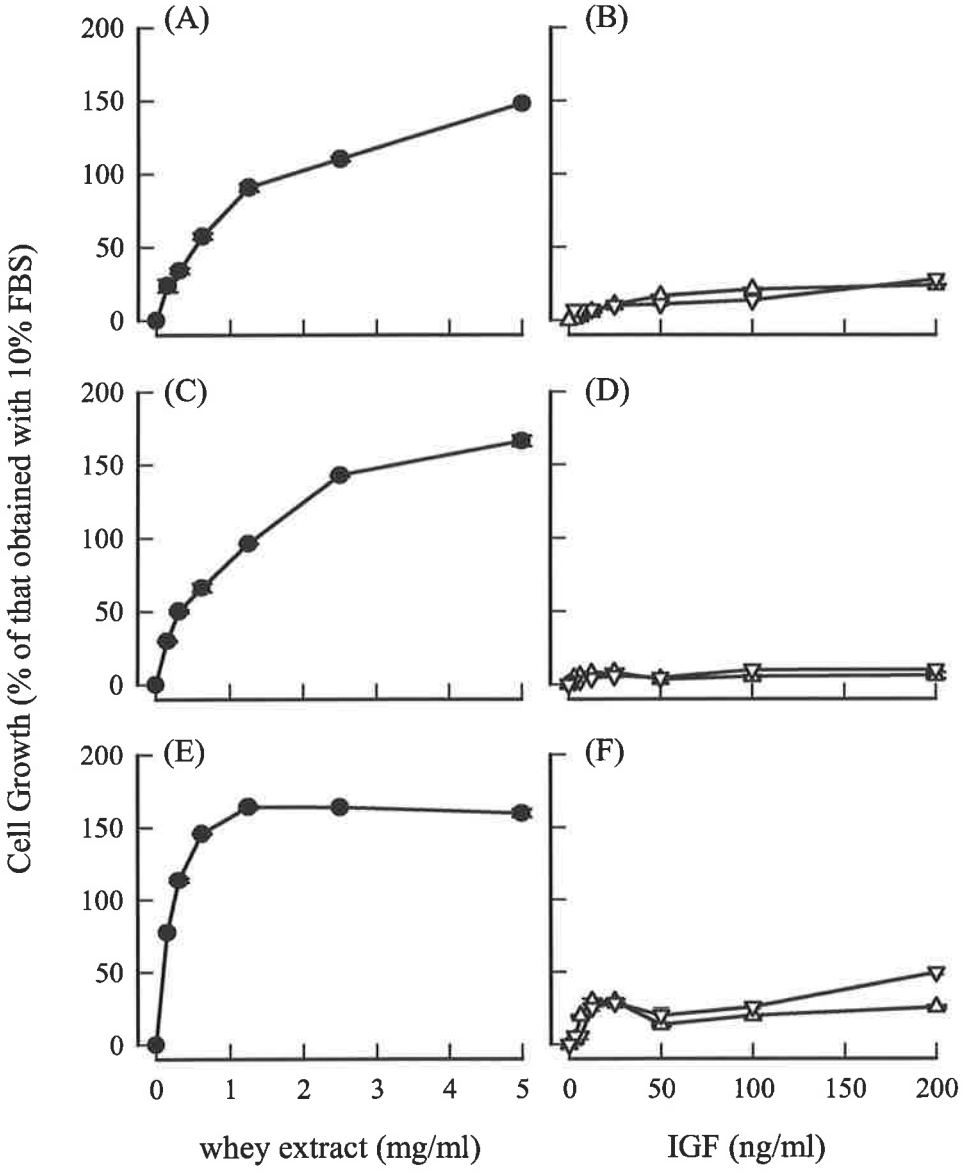



**Figure 3.8** Growth of cells in response to whey extract (●) compared to IGF-I (Δ) and IGF-II (∇). Experimental details are as for **Figure 3.4**. Cells are: L6 myoblasts (A,B), BALB/c 3T3 fibroblasts (C,D), and human skin fibroblasts (E,F). Results are the mean ± S.E.M of three measurements and expressed as a percentage of the response to a 10% FBS standard. The response to DMEM alone has been subtracted from all data.

the major peak of bioactivity for L6 myoblasts and human skin fibroblasts chromatographed near the 7 kDa standard. A smaller group of acid fractions that produced growth in the same cells were eluted between the 45 and 12.4 kDa standards (**Figure 3.9 B, Figure 3.11 B**). In comparison, bioactivity for BALB/c 3T3 cell growth, above 60% of that seen with 10% (v/v) FBS, eluted near the 29 kDa standard (**Figure 3.10 B**). A small group of fractions also produced BALB/c 3T3 cell growth and chromatographed below the 12.4 kDa standard.

The nature of the inhibitory activity for MDCK and IEC-6 cells observed in **Figure 3.3** was also investigated by gel-filtration. Portions of fractions from neutral and acid gel-filtration of whey extract were tested on MDCK and IEC-6 cells in the presence of 5% (v/v) FBS by a cell growth inhibition assay. Some fractions recovered from neutral gel-filtration of whey extract that chromatographed below the 12.4 kDa standard slightly inhibited the growth of MDCK and IEC-6 in 5% (v/v) FBS (**Figure 3.12 A and Figure 3.13 A**). In contrast, there was a distinct group of acid gel-filtration fractions that inhibited epithelial cell growth. They eluted between the 29 and 12.4 kDa standards and inhibited MDCK and IEC-6 cell growth to 20% of that seen in 5% (v/v) FBS alone (**Figure 3.12 B and Figure 3.13 B**).

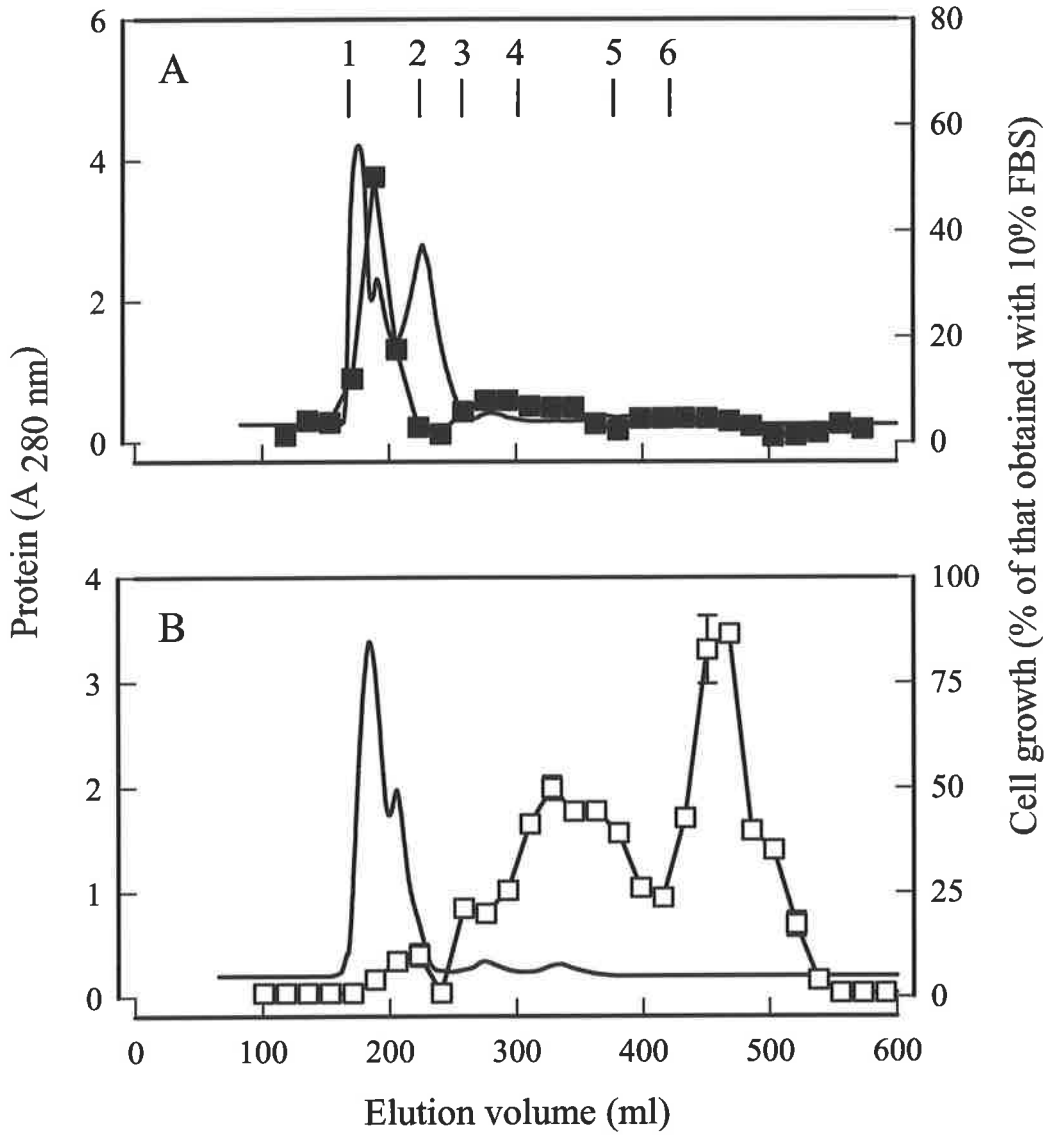



**Figure 3.9** Growth of L6 myoblasts produced by fractions recovered from neutral and acid Superdex 75 gel-filtration of whey extract. A 375 mg sample of whey extract was chromatographed under neutral conditions (**A**), using a running buffer of 50 mM $NaH_2PO_4$, 150 mM NaCl and 10% (v/v) acetonitrile, pH 7.4, at 22 cm/h. Acid gel-filtration of a 375 mg sample of whey extract (**B**) was undertaken in 150 mM NaCl, 1 M acetic acid and 10% (v/v) acetonitrile, pH 2.0, at 22 cm/h . Five minute fractions (36) of 17.5 ml were collected. Portions of neutral (■) and acid (□) fractions were tested for their ability to increase L6 myoblast density using a 96-well plate dye binding assay. Results are expressed as a percent of the response to a 10% FBS standard incorporated onto each plate (mean ± S.E.M of three measurements). The response to DMEM alone has been subtracted. The column was standardised under neutral conditions with IgG (1; 150 kDa), BSA (2; 67 kDa), ovalbumin (3; 45 kDa), carbonic anhydrase (4; 29 kDa), cytochrome C (5; 12.4 kDa) and insulin (6; 7 kDa).

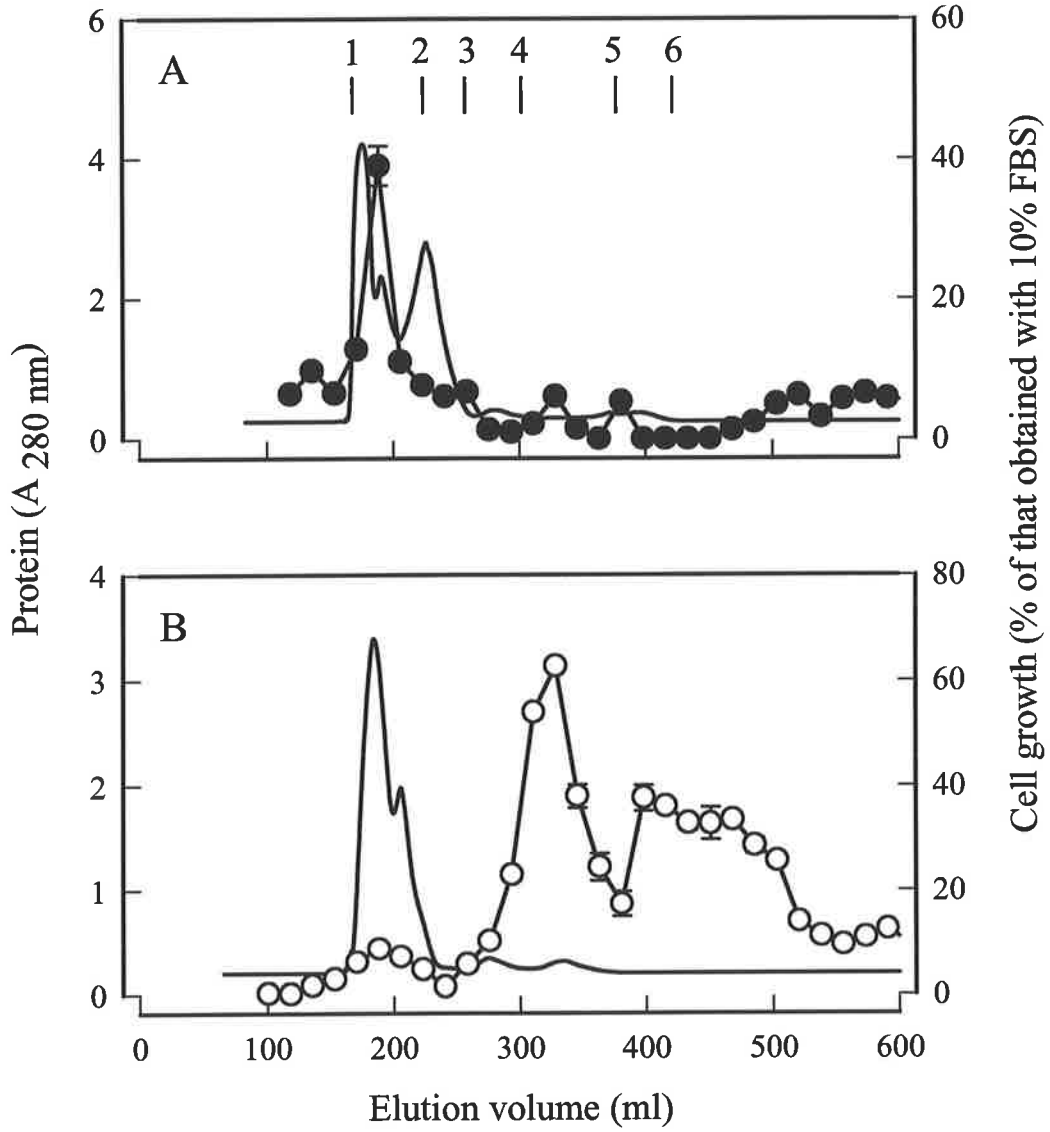



**Figure 3.10** The ability of fractions obtained from neutral (A) and acid (B) Superdex 75 gel-filtration of whey extract to induce BALB/c 3T3 cell growth. Experimental procedures that include measuring the growth of BALB/c 3T3 cells in neutral (●) and acid (○) fractions were carried out as for **Figure 3.9**. Results are the mean ± S.E.M of triplicate determinations. The response to DMEM alone has been subtracted. The column was standardised under neutral conditions with IgG (1; 150 kDa), BSA (2; 67 kDa), ovalbumin (3; 45 kDa), carbonic anhydrase (4; 29 kDa), cytochrome C (5; 12.4 kDa) and insulin (6; 7 kDa).

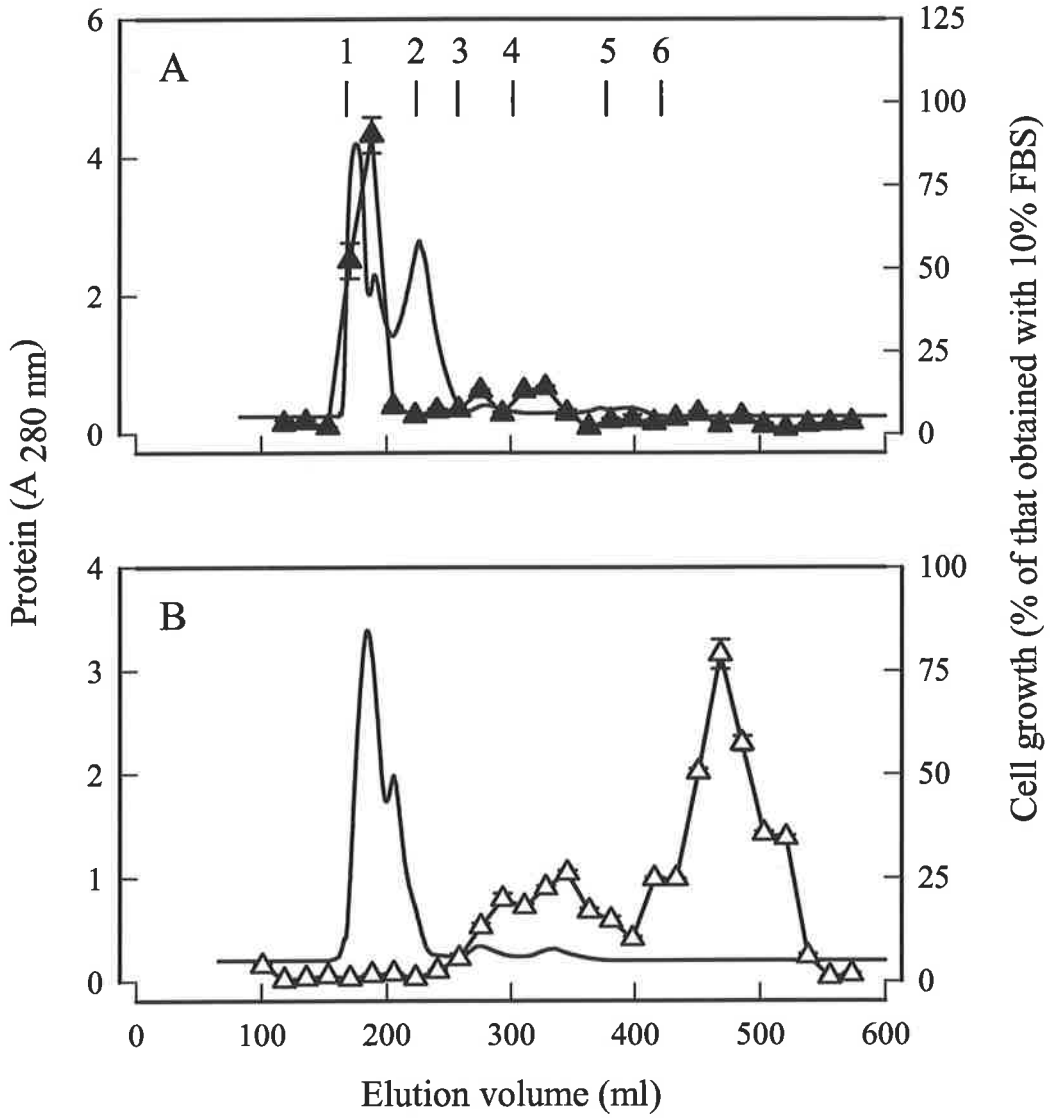



**Figure 3.11** Growth of human skin fibroblasts produced by fractions obtained from neutral (**A**) and acid (**B**) Superdex 75 gel-filtration of whey extract. Experimental procedures that include measuring the growth of human skin fibroblasts in neutral (▲) and acid (Δ) fractions were carried out as for **Figure 3.9**. Results are the mean ± S.E.M of triplicate determinations and the response to DMEM alone has been subtracted. The column was standardised under neutral conditions with IgG (1; 150 kDa), BSA (2; 67 kDa), ovalbumin (3; 45 kDa), carbonic anhydrase (4; 29 kDa), cytochrome C (5; 12.4 kDa) and insulin (6; 7 kDa).

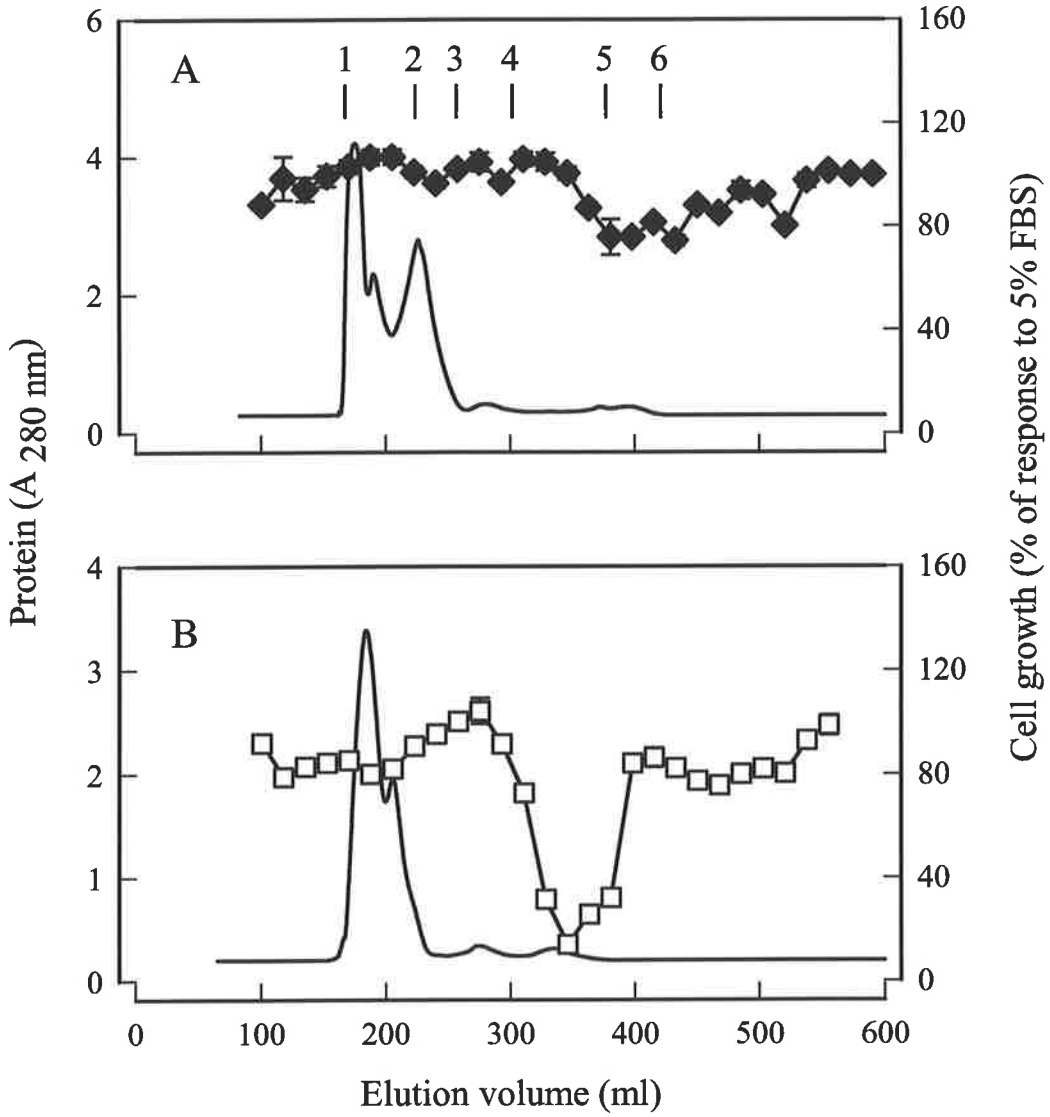



**Figure 3.12** Inhibition of MDCK cell growth produced by fractions obtained from neutral (**A**) and acid (**B**) Superdex 75 gel-filtration of whey extract. A 375 mg sample of whey extract was chromatographed under neutral and acid conditions as described in **Figure 3.9**. Portions of neutral (◆) and acid (□) fractions were then tested for their ability to decrease MDCK density in the presence of 5% FBS. Cell growth was quantified by the 96-well plate dye-binding assay and expressed as a percent of the 5% FBS response, each point representing the mean ± S.E.M of triplicate cultures. The response to DMEM alone has been subtracted. The column was standardised under neutral conditions with IgG (1; 150 kDa), BSA (2; 67 kDa), ovalbumin (3; 45 kDa), carbonic anhydrase (4; 29 kDa), cytochrome C (5; 12.4 kDa) and insulin (6; 7 kDa).

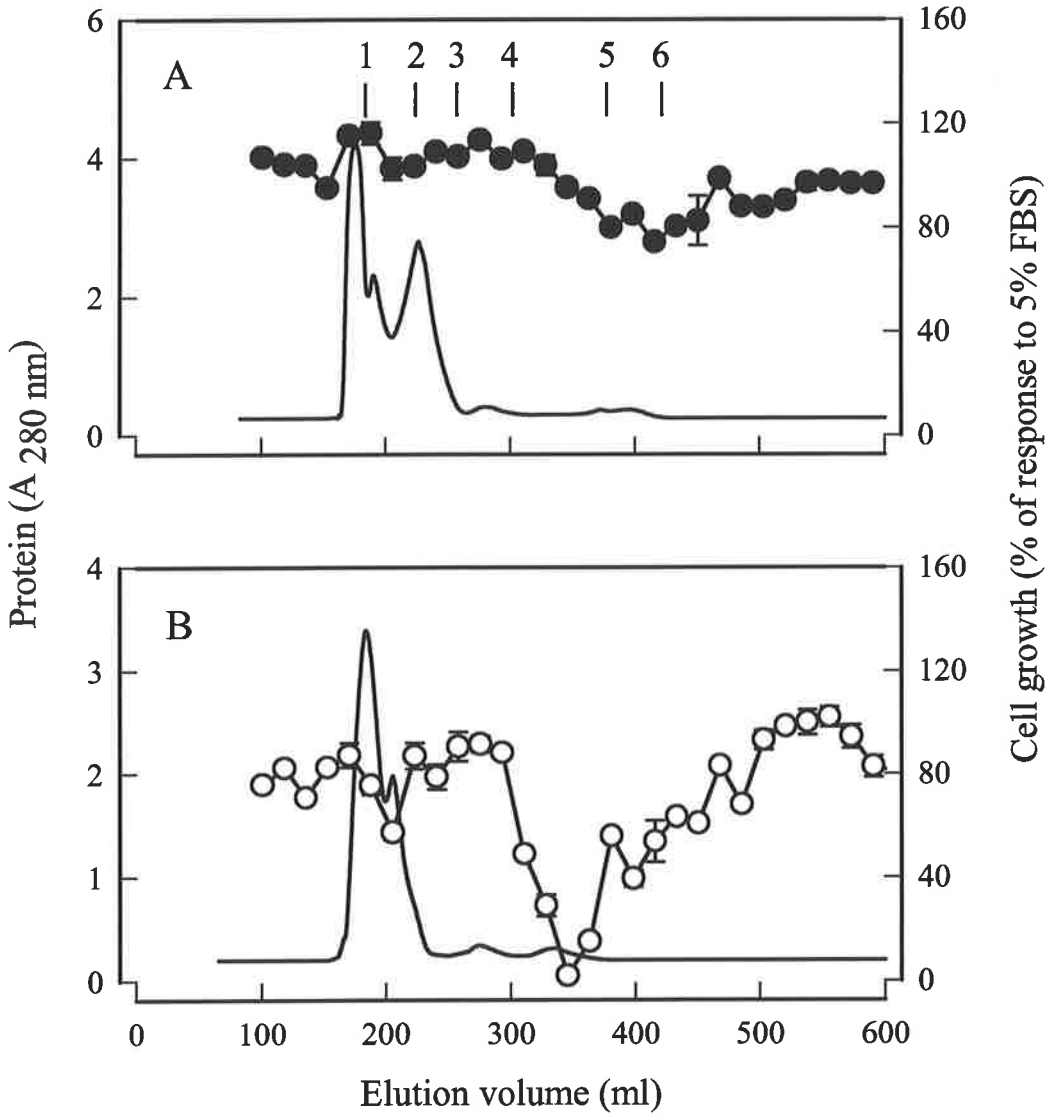



**Figure 3.13** Inhibition of IEC-6 cell growth produced by fractions obtained from neutral (**A**) and acid (**B**) Superdex 75 gel-filtration of whey extract. A 375 mg sample of whey extract was chromatographed under neutral and acid conditions as described in **Figure 3.9**. Portions of neutral (■) and acid (○) fractions were then tested for their ability to decrease IEC-6 growth in the presence of 5% FBS. Cell growth was quantified by the 96-well plate dye-binding assay and expressed as a percent of the 5% FBS response, each point representing the mean ± S.E.M of triplicate determinations. The response to DMEM alone has been subtracted. The column was standardised under neutral conditions with IgG (1; 150 kDa), BSA (2; 67 kDa), ovalbumin (3; 45 kDa), carbonic anhydrase (4; 29 kDa), cytochrome C (5; 12.4 kDa) and insulin (6; 7 kDa).

## 3.4 Discussion

The results presented in this chapter show that whey extract contains mitogens that induce the growth of mesenchymal derived cells and inhibit the growth of epithelial cells. Previous research had suggested that mature bovine milk contains very little mitogenic activity (Klagsbrun and Neuman 1979; Shing and Klagsbrun 1984). It was also found that bovine milk, acid whey or cheese whey could support a small amount of growth in cells that were pre-treated with attachment factors (Steimer *et al.* 1981; Derouiche *et al.* 1990; Legrand *et al.* 1993). Although this earlier work suggested that mature bovine milk contains very little mitogenic activity, subsequent research revealed it contained at least two classes of growth factors. These are the insulin-like growth factors (IGFs) and transforming growth factor-betas (TGF-βs; Ronge and Blum 1988; Collier *et al.* 1991; Cox and Burk 1991). In this current chapter, a cation-exchange fraction of bovine cheese whey (whey extract) was used to investigate the cell growth promoting activity of bovine milk. Cation-exchange chromatography had been shown to exclude most major whey proteins because of their more acidic isoelectric points than growth factors (Francis *et al.* 1995). The results presented in this chapter show that whey extract contains several types of growth factor activity, not all of which can be explained by the IGFs and TGF-βs.

The cell growth studies presented in this chapter are from a dye-binding procedure that quantified change in mass and number of cells grown in 96-well plates (Oliver *et al.* 1989). The cells were grown overnight in the presence of 10% serum to promote cell attachment and then washed for two hours in serum free medium before exposure to dilutions of test materials. **Figure 3.1** shows whey extract is a more potent growth

promoter of BALB/c 3T3 cells than cheese whey. This could be explained by the cation exchange process enriching the growth factor component already present in bovine cheese whey. On average, cheese whey contains between 4 and 6 mg/ml protein, it contains only traces of mitogens; with most of the whey proteins being large globular proteins such as β-lactoglobulin, α-lactalbumin, immunoglobulins and bovine serum albumin (Eigel *et al.* 1984; Whitney 1984). Typically, 30 to 60 mg of protein termed 'whey extract' is obtained from cation-exchange chromatography of one litre of whey, which comprises only 0.5% of total whey protein. Most of the major whey proteins are eliminated during the process, and fluid is removed when the final product is freeze-dried. Whey extract can then be made up to 25 mg/ml in tissue culture media and added to cells at concentrations up to 5 mg/ml. In contrast, the amount of mitogenic protein that can be added to 100 µl wells when microfiltered whey is tested on cells is limited to 20 µl/100 µl well or 0.8-1.2 mg/ml. Indeed, earlier studies found that whey only supports the growth of attached cells when it comprises 10-20% of tissue culture media (Steimer *et al.* 1981; Damerdji *et al.* 1988; Derouiche *et al.* 1990). By eliminating most of the non-mitogenic proteins and fluid, whey extract serves as a useful starting point to characterise the growth factor activity of whey.

The maximal cell density of L6 myoblasts, BALB/c 3T3 and human skin fibroblasts achieved with whey extract exceeded that observed in 10% fetal bovine serum (**Figure 3.1**, **3.2**). Whey extract also induced significantly higher growth than any single recombinant growth factor tested, including PDGF, TGF-β1, EGF, FGF and IGF (**Figures 3.4**, **3.5**, **3.6**, **3.7 3.8**). This is evidence for the proposal that the cation-exchange process used to isolate whey extract did not purify only one class of growth factor activity. Instead, the results suggest several classes of factors are present in whey extract and they act additively or synergistically. A number of investigators have shown

that cells require more than one growth factor for sustained growth. Initial research on BALB/c 3T3 fibroblasts demonstrated that both competence and progression factors are required for the cells to traverse a cell cycle. PDGF is an example of a competence factor and EGF a progression factor (Stiles *et al.* 1979; Smith and Stiles 1981). However, the assay used in the present study could not distinguish between competence and progression factors because the BALB/c 3T3 cells were not quiescent when the factors were added to cells. Indeed, BALB/c 3T3 cells responded to the competence factors PDGF and FGF as well as the progression factors IGF and EGF (**Figures 3.4, 3.7, 3.8, 3.6**).

Importantly, research has revealed that both fibroblast and myoblast cells used in the current study do respond both additively and synergistically to mixtures of growth factors. Both fibroblast and myoblast cell lines have receptors for a variety of growth factors including the IGFs, PDGFs, TGF-βs, FGFs and EGF (Rechler and Nissley 1990; Raines *et al.* 1990; Segarini 1990; Carpenter and Wahl 1990). Research has also shown that IGF-I together with PDGF and/or EGF can induce additive growth to the same level as serum in BALB/c 3T3 cells and human skin fibroblasts (Stiles *et al.* 1979; Scher *et al.* 1979; Leof *et al.* 1982; Phillips and Cristofalo 1988; Chen and Rabinovitch 1989). An example of the synergistic properties of growth factors is shown in the action of TGF-β enhancing the growth promoting action of FGF and PDGF in cell culture. TGF-β induce BALB/c 3T3 cells to grow in the presence of FGF by increasing the production of proteoglycans in the extracellular matrix which bind FGF (Nugent and Edelman 1993; Soma and Grotendorst 1989; Segarini 1990; Blachowski *et al.* 1993). It could be argued that since enhanced cell growth can be produced by mixtures of growth factors, the response of attached cells to whey extract could be explained by more than one mitogen.

Although a source of growth factor activity for L6 myoblasts, BALB/c 3T3 cells and human skin fibroblasts, whey extract primarily inhibited the growth of epithelial cells (**Figure 3.3**). IEC-6 cells grew more than MDCK epithelial cells in whey extract. However, MDCK cells were stimulated to grow at very low concentrations of whey extract. The addition of 1% FBS to the culture media containing a dilution series of whey extract produced an additive response in the IEC-6 and MDCK cells (**Figure 3.3**). Similar additive responses have been reported after addition of low amounts of FBS to fibroblasts and hybridomas growing in whey protein fractions (Damerdji *et al.* 1988; Ramirez *et al.* 1990; Legrand *et al.* 1993).

The cell growth inhibition shown in **Figure 3.3** does not support earlier research on epithelial cell growth in bovine colostrum and mature milk (Steimer *et al.* 1981; Corps and Brown 1987). In these reports, colostrum and to a lesser extent mature milk induced the growth of attached epithelial cells such as MDCK and rat intestinal epithelial cells. Corps and Brown (1987) also showed that IGF-I or EGF but not PDGF promoted the growth of intestinal epithelial cells that were also responsive to bovine colostrum. However, there was no direct evidence that IGF and EGF were responsible for epithelial cell growth observed in the presence of colostrum. IGF binding proteins which inhibit the action of IGF are found in bovine milk and EGF is said to be absent from bovine milk (Skaar *et al.* 1991; Vega *et al.* 1991; Iacopetta *et al.* 1992). Importantly, whey extract may also contain epithelial cell growth promoting agents, albeit at lower levels than epithelial cell growth inhibitors. This is shown in the response of IEC-6 cells (**Figure 3.3 A**) and to a lesser extent MDCK cells (**Figure 3.3 B**) to a dilution series of whey extract. The strong inhibitory action of whey extract on epithelial cells could be explained by the growth factor profile of whey extract being different from that found in colostral and mature whey. It is known that bovine

colostrum contains higher concentrations of growth factors than mature bovine milk. Indeed, the concentration of milk mitogens which include epithelial cell growth inhibitors decrease over the lactation period as the volume of mammary secretions increases (Ronge and Blum 1988; Collier *et al.* 1991; Tokuyama and Tokuyama 1989; Cox and Burk 1991). The results presented in this chapter do not necessarily suggest that whey extract contains significantly different epithelial cell mitogens and inhibitors than mature milk. Rather, the cation exchange process used to obtain whey extract may have enriched the epithelial cell inhibitors of whey more than growth promoters.

The inhibition of MDCK and IEC-6 cells by whey extract could be explained by TGF-β-like activity. This factor has been shown to potently inhibit the growth of MDCK and IEC-6 cells used in the present study (Kurokowa *et al.* 1987; Barnard *et al.* 1989; Danielpour *et al.* 1989). Moreover, TGF-β has been identified and purified from both bovine colostrum and milk (Tokuyama and Tokuyama 1989; Tokuyama and Tokuyama 1993; Cox and Burk 1991; Jin *et al.* 1991). In addition, TGF-β released from cells is normally in a latent form which is also suggested by the current chromatography experiments of whey extract (**Figure 3.12 and Figure 3.13**). Most of the TGF-β secreted by cells and found in platelets and bone is inactive. It can be activated and released from its association with a 'latency associated peptide' by *in-vitro* exposure to acid (Pircher *et al.* 1984; Lawrence *et al.* 1985; Brown *et al.* 1990). In the current experiments, acid treatment of whey extract during acid size-exclusion increased the ability of the whey fraction to inhibit the growth of epithelial cells. Curiously, the inhibitory activity chromatographed with an apparent molecular weight below that predicted for the active 25 kDa TGF-β dimer (**Figure 3.12 B and Figure 3.13 B**). However, the hydrophobic nature of the active 25 kDa TGF-β homodimer has been alluded to by Jin *et al.* (1991), and hydrophobic binding of peptides to gel-filtration

resins has been reported (O'Callaghan *et al. 1*995). Although the current data suggests the epithelial cell inhibitory activity of whey extract is TGF-β, further experiments are needed to confirm this.

The gel-filtration experiments also revealed that whey extract contains L6 myoblast, BALB/c 3T3 and human skin fibroblast growth promoting activities that can be separated from whey large proteins by acid treatment (**Figures 3.9**, **3.10**, **3.11**). Only two to three fractions recovered from neutral gel-filtration of whey extract induced cell growth. These fractions chromatographed between the 67 and 150 kDa standards, where the bulk of the whey extract protein eluted. In contrast, over five times as many fractions obtained from acid gel-filtration of whey extract increased cell density. These fractions eluted near the 6-29 kDa standards, where absorbance at 280 nm revealed there was little detectable whey extract protein. This suggests that the smaller mitogens found in whey extract associate with larger proteins at neutral pH, and acid treatment prevents such aggregates forming. The large proteins that attach to the mitogens at neutral pH could be their growth factor binding proteins and/or other whey proteins. In tissues and the circulation many growth factors such as IGF, PDGF and TGF-β are associated with large binding proteins (Baxter *et al.* 1989; Bonner *et al.* 1995a; Raines *et al.* 1992; Wakefield *et al.* 1988; Brown *et al.* 1990). The growth factor-binding proteins complexes can-not bind to receptors and so are inactive. Transient acid treatment can release the bonds between the growth factors and binding proteins so that the mitogens regain their bioactivity. This may explain the increased number of mitogenic fractions produced by acid compared to neutral gel-filtration of whey extract. However, since whey extract is still active without acid treatment and elutes with the major whey proteins at neutral pH, (**Figures 3.1**, **3.2**, **3.9**, **3.10**, **3.11**), some of the mitogens may be in transient physical associations with large proteins. Indeed, under equilibrium

conditions it would be expected that some of the growth factors will be bound to the large proteins and some will be free. Indeed, aggregation of whey proteins have been reported. For example, the 78 kDa lactoperoxidase, the most abundant protein in whey extract forms physical associations with other whey proteins such as lysosyme, RNAase and immunoglobulins (Frances *et al.* 1995; Hulea *et al.* 1989).

The presence of IGF in whey extract is suggested by the response of L6 myoblasts to pure IGF and to acid gel-filtration fractions of whey extract (**Figure 3.8 B and Figure 3.9 B**). L6 myoblasts responded to purified IGF-I and IGF-II above that seen in DMEM alone (**Figure 3.8 B**). Although this response was small, it agrees with previous research that established pure IGF preparations are mitogenic for L6 cells (Richman *et al.* 1980; Ballard *et al.* 1986). However, other factors such as PDGF-BB, TGF-β1 and FGF are also known to be weakly mitogenic for L6 myoblasts, which is also confirmed by **Figures 3.4, 3.5, 3.7** (Blachowski *et al.* 1993; Segarini 1990; Linkhart *et al.* 1980). The presence of IGF binding proteins (IGFBPs) are also suggested by the shift in L6 bioactivity from the high (**Figure 3.9 A**) to low (**Figure 3.9 B**) molecular weight after neutral and acid gel-filtration of whey extract receptively. It is well established that some complexes of 7 kDa IGFs with acid labile IGFBPs are disrupted by gel-filtration under acidic conditions (Zapf *et al.* 1975; Moses *et al.* 1976; Rechler and Nissley 1990). Previous research has also established that bovine milk contains IGF-I, IGF-II and IGFBP-2 and 3 (Ronge and Blum 1988; Vega *et al.* 1991; McGrath *et al.* 1991; Skaar *et al.* 1991). However, under equilibrium conditions some IGF may be present in whey extract unattached to IGFBPs and therefore be bioactive and others bound to IGFBPs. This is seen by the fact that whey extract induces L6 cell growth at neutral pH (**Figure 3.8 A**). In addition, after neutral gel-filtration of whey extract, only the high molecular weight fractions induced L6 growth (**Figure 3.9 A**). Significantly, IGF by itself cannot

account for the bioactivity of whey extract. Saturating concentrations (100 ng/ml) of recombinant IGF did not produce the same level of L6 growth as whey extract (**Figure 3.8 A,B**) or the 6-7 kDa peak of acid gel-filtration fractions of whey extract (**Figure 3.9 A**).

Two peaks of BALB/c 3T3 activity were recovered from acid size exclusion chromatography of whey extract (**Figure 3.10 B**). BALB/c 3T3 cells also responded to pure preparations of PDGF, TGF-β1, EGF and FGF (**Figures 3.4, 3.5, 3.6, 3.7**). The peak of mitogenic activity eluting between the 45 and 12.4 kDa standards may contain growth factors such as 30 kDa PDGF, the 25 kDa TGF-β and the 16-25 kDa FGF-1. Indeed, Shing and Klagsbrun (1984, *op cit* 1987) purified an acid and heat stable 30 kDa PDGF-like factor from bovine colostrum that induced BALB/c 3T3 cell growth. TGF-β has been purified from bovine milk and low concentrations of TGF-β have been shown to be mitogenic for fibroblasts, whereas high concentrations can be inhibitory (Jin *et al.* 1991; Roberts *et al.* 1985; Benzakour *et al.* 1992). FGF-1 and FGF-2 are heparin binding proteins that have been traditionally thought to be absent from physiological fluids such as serum and milk, but, Hill *et al.* (1994) demonstrated soluble FGF is in maternal serum. In addition, Sandowski *et al.* (1993), found heparin binding factors in bovine prepartum mammary secretions. However, the instability of FGF-2 in acid suggests only FGF-1 could be recovered from acid gel-filtration of whey extract (Thomas *et al.* 1980; Thomas *et al.* 1984). The second group of acid gel-filtration fractions that induced high BALB/c 3T3 cell density chromatographed between the 12.4 and 6 kDa standards (**Figure 3.10 B**), and may contain the 7 kDa IGF's or 6 kDa EGF. However, the BALB/c 3T3 cell growth activity found in these fractions is greater than that of saturating concentrations of IGF and EGF (**Figure 3.8 D, 3.6 D**). In addition, only trace amounts of the 6 kDa EGF have been detected in bovine milk (Iacopetta *et al.*

1992). Further studies are required to determine what milk growth factors contribute to the peak of low molecular fractions which induce BALB/c3T3 cell growth.

Maximal growth of human skin fibroblasts in whey extract was 100-200% of that seen in 10% FBS (**Figure 3.1-3.8**). PDGF, TGF-β1, FGF and IGF were also mitogenic for human skin fibroblasts (**Figures 3.4 F, 3.5 F, 3.7 F, 3.8 F**). Human skin fibroblasts are known to contain receptors for each of these growth factors and for EGF (Heldin *et al.* 1988; Roberts *et al.* 1985; Bouche *et al.* 1987; Clemmons and Van Wyk 1985; Conover *et al.* 1986; Xu *et al.* 1984; Phillips and Cristofalo 1988). Acid gel-filtration separated whey extract into two groups of fractions that induced the growth of human skin fibroblasts (**Figure 3.11 B**). These peaks of mitogenic activity were similar to those which promoted L6 cell density (**Figure 3.9 B**), with peak bioactivity eluting at 6-7 kDa (**Figure 3.11 B**). This is in the region where IGF-I (7 kDa) and EGF (6 kDa) would be expected to elute. Research has previously shown that IGF-I and EGF are required for serum free growth of human skin fibroblasts. It has also revealed that IGF-I together with EGF can induce the same level of cell growth as serum in human skin fibroblasts (Phillips and Cristofalo 1988; Chen and Rabinovitch 1989). Pure IGF or EGF alone did not induce the level of cell growth produced by the 6-7 kDa peak of acid gel-filtration fractions of whey extract (**Figure 3.8 F**, **Figure 3.6 F**, **Figure 3.11 B**), although combination experiments were not undertaken in the current study. The presence of undefined mitogens or synergistic actions of growth factors in the peak of fractions eluting at 6 kDa after acid gel-filtration of whey extract must be considered.

Whey extract has potential for defining the mitogens of bovine whey. Although it comprises 2 % of total whey protein, at concentrations up to 5 mg/ml it is more potent than 10% (v/v) FBS or any pure growth factor on L6 myoblasts, BALB/c 3T3 and human skin fibroblasts. Gel-filtration experiments confirmed that more than one type of

mitogen is present in whey extract. They also showed whey extract mitogens can be associated with high molecular weight whey proteins, either in acid-labile complexes or in physical associations with whey proteins such as lactoperoxidase. In addition, a group of fractions obtained from acid gel-filtration of whey extract potently inhibited the growth of epithelial cells. Saturating concentrations of recombinant growth factors did not produce the level of L6 myoblast, BALB/c 3T3 and skin fibroblast growth seen in the peak of bioactivity eluting near the 6 kDa standard. A second peak of bioactive fractions recovered from acid gel-filtration of whey extract (eluting between the 29 and 12 kDa standards) induced BALB/c 3T3 cell growth greater than that seen with pure growth factors. In the following chapters whey extract will be used to investigate both mesenchymal and epithelial cell growth factors of bovine milk.

# CHAPTER Four:

# Platelet-Derived Growth Factor in Bovine Whey

## 4.1 Introduction

Shing and Klagsbrun (1984, *op cit* 1987) suggested platelet derived growth factor (PDGF) is the principal BALB/c 3T3 cell growth promoting activity in bovine colostrum. They first found BALB/c 3T3 cells could be stimulated to grow by media supplemented with colostrum but not mature milk (Klagsbrun and Neuman 1979; Shing and Klagsbrun 1984). Subsequently, they investigated the growth factor activity in bovine colostrum alone (Shing and Klagsbrun 1987). The major BALB/c 3T3 growth promoting activity in bovine colostrum was purified and found to have a molecular weight of 30 kDa and isoelectric point (pI) of 10. It was also shown to be heat stable and composed of two polypeptide chains linked by disulphide bonds (Shing and Klagsbrun 1987; Shing *et al.* 1987). The BALB/c 3T3 cell growth promoting activity, dimmeric structure, pI and heat stability of the purified colostrum-derived growth factor, suggested it was related to human PDGF. Further, purified bovine colostral factor displaced human $^{125}$I-PDGF from receptors on human fibroblasts in radio-receptor experiments, which was enough evidence for Shing and Klagsbrun (1987) to conclude it was a PDGF-like molecule. Brown and Blakeley (1984) also partially purified a PDGF-like molecule from goat colostrum and were also able to show that it had biochemical and physical similarities to human PDGF.

Although PDGF has not been extensively characterised in milk, it is one of the best characterised mitogens of connective tissue cells such as fibroblasts and smooth muscle cells (Kohler and Lipton 1974; Ross *et al.* 1974; Rutherford and Ross 1976; Busch *et al.* 1976). Other cellular responses to PDGF include chemotaxis, differentiation, and modulation of functions such as contraction and the production of extracellular matrix

components (Grotendorst *et al.* 1981; Seigbahn *et al.* 1990; Raff 1989; Berk *et al.* 1986; Gullberg *et al.* 1990; Owen *et al.* 1982; Majack *et al.* 1985).

Human PDGFs has been well characterised and found to be cationic glycoprotein composed of the 16-18 kDa PDGF-A and/or 14-16 kDa PDGF-B chains linked by disulphide bonds (Heldin *et al.* 1979; Antoniades 1981; Deuel *et al.* 1981; Antoniades and Hunkapiller 1983). The three forms are PDGF-AA, PDGF-BB and PDGF-AB (Hart *et al.* 1990). Active PDGF is stable to heat or acid and contains a high number of basic residues that give it a pI of 9.8-10 (Deuel *et al.* 1981). Two cell-associated receptors for PDGF have been characterised, one is the PDGF-Rα, the other PDGF-Rβ, and they must dimmerise before signal transduction occurs (Hart *et al.* 1988; Heldin *et al.* 1988; Nister *et al.* 1988; Hammacher *et al.* 1989; Seifert *et al.* 1989; Heldin *et al.* 1989; Eriksson *et al.* 1992). The α/α binds all three PDGF forms, the α/β both PDGF-AB and PDGF-BB and β/β only PDGF-BB (Seifert *et al.* 1989; Eriksson *et al.* 1992; Ueno *et al.* 1993).

The amino acid sequence of bovine PDGF is not known; although a 28-31 kDa PDGF-like molecule, composed of 14 and 16 kDa chains has been purified from bovine platelets it was not sequenced (Narczewska *et al.* 1985). However, receptor and immunological studies revealed bovine PDGF shares a high degree of homology with its human counterpart. Human PDGF was found to bind equally well to cells from cows, and humans (Bowen-Pope and Ross 1982). PDGF-like activity from bovine sera was also able to displace iodinated human PDGF from fibroblasts *in-vitro* (Singh *et al.* 1982, Singh 1987). In addition, a polyclonal antibody produced against human PDGF was found to recognise bovine PDGF-like activity (Raines and Ross 1985; Huang *et al.* 1983). The highly conserved binding has been used to identify PDGF-like activity in many bovine tissues, cells and in colostrum. The predominant isoform in bovine

platelets was found to be PDGF-BB (Bowen-Pope *et al.* 1989). This contrasts with human platelets, where approximately 70% consists of PDGF-AB, the remainder is principally PDGF-BB together with small amounts of PDGF-AA (Hart *et al.* 1989). The form of PDGF produced by activated macrophages of many species including the cow, and that secreted by cultured endothelial and epithelial cells is mainly PDGF-BB. Conversely, smooth muscle cells, nerve cells and fibroblasts secrete PDGF-AA *in-vitro* (Claesson-Welsh *et al.* 1988; Paulsson *et al.* 1987; Seifert *et al.* 1984; Noble *et al.* 1988; Shimokado *et al.* 1986). Shing and Klagsbrun (1987), showed that a purified bovine colostral factor displaced human PDGF from receptors on human fibroblasts. However, there is no data on which type of human PDGF was used in the experiments, or on the isoforms present in bovine colostrum.

In tissues and body fluids such as milk, growth factors can bind to protective proteins that prevent degradation and serve as a reservoir of the molecule. In **Chapter 3**, it was demonstrated that the bioactivity of whey extract can be separated from high molecular weight proteins by acid gel-filtration. PDGF is known to bind to $\alpha_2$-macroglobulin ($\alpha_2$-M), and extracellular matrix proteins such as 'secreted protein acidic and rich in cysteine' (SPARC), as well as proteoglycans (Huang *et al.* 1983, *op cit* 1984; Raines *et al.* 1984; Raines *et al.* 1992; Raines and Ross 1992). In blood or tissues, it appears that the ability of PDGF to bind these proteins depends on the PDGF isoform present. PDGF-AA is rapidly degraded and does not bind protective proteins in the circulation, although it can bind proteoglycans on cell membranes and matrices (Huang *et al.* 1983, *op cit* 1984; Raines *et al.* 1984; Raines *et al.* 1992). In the circulation fast $\alpha_2$-M bind PDGF-BB or PDGF-AB covalently and destroys their bioactivity and the complexes are rapidly excreted through the liver (Bonner *et al.* 1995; Bonner and Osornio-Vargas 1995; Raines *et al.* 1992). However, during tissue repair processes PDGF-BB and

PDGF-AB bind native $\alpha_2$-M or SPARC non-covalently and appear to be protected from degradation (Bonner *et al.* 1995; Bonner and Osornio-Vargas 1995). Although there is little literature on milk PDGF it would be expected that to protect it from rapid degradation it would be attached to similar protective proteins.

There are no studies on PDGF in mature bovine milk. Shing and Klagsbrun (1984) found that the level of PDGF-like activity was high in colostrum but declined as lactation progressed till it was undetectable in mature milk. However, as already seen in **Chapter 3**, the mitogens of mature milk are enriched in whey extract. As little as 2.0 mg of whey extract induced BALB/c 3T3 cells to grow in excess of that seen in 10% (v/v) fetal bovine serum (**Figure 3.1**). The most potent recombinant growth factors for BALB/c 3T3 cells were found to be PDGF (**Figure 3.4**). Significantly, BALB/c 3T3 cells were potently stimulated by a group of fractions recovered from acid gel-filtration of whey extract that eluted at the molecular weight region consistent with the presence of PDGF (**Figure 3.10**). In this current study, acid gel-filtration fractions recovered from whey extract will be used to determine if PDGF is present in bovine whey. These fractions will be tested for their ability to displace $^{125}$I-human-PDGF-AA from BALB/c 3T3 cell monolayers and the presence of PDGF confirmed by immunoblotting. The contribution PDGF makes to the ability of BALB/c 3T3 cells to grow in whey extract will be then tested by a neutralising antibody directed against PDGF-AB, PDGF-AA and PDGF-BB.

## 4.2 Materials and Methods.

The production of whey extract was carried out as previously described in section **2.2.1**. One litre of whey yielded 60 mg freeze-dried whey extract Acid gel-filtration of dried whey extract was undertaken using a Superdex 75 HR 35/600 column as described in **2.2.4**.

### 4.2.1 *BALB/c 3T3 cell growth in gel-filtration fractions of whey extract*

The growth of, BALB/c 3T3 cells in response to acid gel-filtration fractions of whey extract was determined using the 96-well plate methylene blue binding assay. A 375 mg sample of whey extract was subject to acid gel-filtration as described in **2.2.4** and 100 µl of each 17.5 ml fraction was freeze-dried with 10 µl of 10 mg/ml BSA and then dissolved in 1 ml of DMEM. BALB/c 3T3 cells were subcultured in 96-well plates as described in **2.2.3**.

### 4.2.2 *PDGF radioreceptor assay*

Radioreceptor assays (RRA) as described by Singh (1987) were used to detect PDGF-like molecules in acid gel-filtration fraction of whey extract. $^{125}$I-h-PDGF-AA (specific activity 50 µCi/µg) was prepared using the chloramine-T method (Van Obberghen-Schilling and Pouyssegur 1983) and separated from free $^{125}$I by G-50 Sephadex gel-filtration. BALB/c 3T3 cells were subcultured in Costar 24-well plates (Costar, Cambridge, Massachusetts, USA) and grown to confluence in DMEM containing 10% (v/v) FBS. The monolayers of cells were then washed twice in HEPES-

buffered saline (0.1 M HEPES, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, and 8 mM glucose, pH 7.6) containing 0.5% (wt/v) bovine serum albumin (BSA), and incubated at 4°C for 4 h. Portions (1 ml) of each fraction obtained from acid gel-filtration of whey extract were dried under vacuum and then made up in 1 ml of HEPES without saline, pH 7.6 that contained 0.5% (wt/v) BSA. PDGF-AA and PDGF-BB were diluted in HEPES-buffered saline containing 0.5% (wt/v) BSA. The cells were then incubated for 12 h at 4°C in HEPES-buffered saline containing $^{125}$I-h-PDGF-AA ($1.5 \times 10^4$ cpm) and the dissolved acid gel-filtration fractions or growth factor standards in a final volume of 0.5 ml. Monolayers were harvested by washing three times with Hanks' balanced salts solutions (HBSS) at 4°C (1.26 mM $CaCl_2$, 5.36 mM KCl, 0.44 mM $KH_2PO_4$, 0.82 mM $MgSO_4$, 136.9 mM NaCl, 0.033 mM $Na_2HPO_4$, 5.55 mM glucose, 0.01 g/l phenol-red, pH 7.3). This was followed by dissolution in 1 ml of 0.5 M NaOH containing 0.1% (v/v) Triton X-100.

Radioactivity was counted in a gamma counter, and the ability of fractions or PDGF to displace $^{125}$I-h-PDGF-AA from receptors expressed as a percentage of binding in HEPES-buffered saline. Standard curves for $^{125}$I-h-PDGF-AA displacement were obtained by using increasing amounts of either PDGF-AA or PDGF-BB. Portions (1.5 ml) of acid gel-filtration fractions of whey extract that displaced $^{125}$I-h-PDGF-AA were pooled, freeze-dried and made up and diluted in HEPES containing 0.5% (wt/v) BSA. PDGF-like activity recovered from acid gel-filtration of whey extract was then estimated by comparing the ability of dilutions of pooled activity to displace $^{125}$I-h-PDGF-AA to that of PDGF-AA and PDGF-BB using the standard curves.

Data was fitted to a four parameter logistic dose response equation using Tablecurve (Jandel Scientific, San Rafael, California, USA). Non-specific binding in HEPES-buffered saline containing excess PDGF (500 ng/ml PDGF-AA; 800 ng/ml of PDGF-

BB) was consistently 10-15% of the total binding. For each plate, the total amount of $^{125}$I-h-PDGF-AA bound to monolayers in HEPES-buffered saline was around 20% of the 1.5 x $10^4$ cpm added. The protein concentration of the pooled fraction was determined as shown in **2.2.5**.

### 4.2.3 *PDGF immunoblots*

Evidence that fractions obtained by acid gel-filtration of dried whey extract contain PDGF was provided by immunoblotting. The salt contained in each fraction was removed before electrophoresis, by dialysing against 10 mM acetic acid for 24 h at 4°C in Spectra/por #3 dialysis tubing (2.9 cm diameter, 3.5 kDa cut off, Spectrum, Houstin, Texas, USA). Portions of the desalted fractions (2.5 ml) were vacuum dried and subject to 12.5% SDS-PAGE under reducing and non-reducing conditions (Laemmli 1970). Recombinant human PDGF-AA and PDGF-BB (500 ng/lane) were included as positive controls and rainbow molecular weight makers utilised for estimating molecular weight. Protein was transferred from the gels to nitrocellulose sheets (Schleicher and Scheuell Pty Ltd, Dassel, Germany) using a semi-dry Novablot system (AMRAD Pharmacia Biotech, North Ryde). The nitrocellulose sheets were then incubated in wash buffer (0.2 M NaCl, 0.1 % (wt/v) BSA, 0.1% (wt/v) polyethylene Glycol, 50 mM Tris, pH 7.4) containing 0.3% (wt/v) BSA to block non-specific binding of antibodies. Nitrocellulose sheets were then incubated at 4°C overnight with polyclonal anti-human PDGF-AB (10 μg/ml of wash buffer) that had been raised in goats and recognises human, bovine and porcine PDGF-AA, -BB and -AB (Upstate Biotechnology Inc New York, New York, USA). The non-bound antibody was removed by soaking the nitrocellulose sheets in fresh wash buffer with frequent changes over 1 h. The nitrocellulose sheets were then incubated in a second antibody of anti-goat/sheep IgG conjugated to alkaline

phosphatase (Silenus Laboratories, Hawthorn, Australia) that was diluted in wash buffer (1:500) for 1 h. After removing non-bound antibody by frequent changes of wash buffer over 1.5 h, the immuno-reactive bands were developed with a 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium substrate (Sigma-Aldrich Pty Ltd).

### 4.2.4 *Immuno-neutralisation of PDGF induced BALB/c 3T3 cell growth*

To determine the contribution PDGF makes to the ability of whey extract to induce the growth of BALB/c 3T3 cells, whey extract was incubated with an anti-human PDGF IgG (Upstate Biotechnology Inc New York, New York, USA) which neutralises PDGF activity. Preliminary experiments showed 25 μg/ml of this antibody neutralised BALB/c 3T3 fibroblasts growth produced by 25 and 10 ng/ml PDGF-BB. A control antibody of the same subclass (goat/sheep IgG) was also tested on dilutions of PDGF-BB in the same assay (Silenus Laboratories, Hawthorn, Melbourne, Australia).

Whey extract was made to 25 mg/ml in sterile water and a portion (10 ml) acidified to pH 2 with 25 μl of concentrated HCl. Both neutral and acidified whey extract were then sterile filtered and made to 1 mg/ml with DMEM containing 0.1% (wt/v) BSA. BALB/c 3T3 cells were subcultured into 96-well plates as described in **2.2.3**. Dilutions of whey extract and recombinant PDGF were then added to plates with and without 25 μg/ml of anti-PDGF-AB. After 48 h cell mass was then quantified by the methylene blue binding assay (**2.2.3**) and data expressed as the percentage of growth response observed in 10% (v/v) FBS, with the growth in DMEM alone subtracted. The data obtained from neutralising BALB/c 3T3 cell growth activity were also examined by analysis of variance. An F-test was used to compare the results obtained from treatments and when this showed a significant difference, means were subject to the

standardised student's t-test. When there was only two treatments, they were compared using a simple student's t-test (Miller *et al.* 1990).

## 4.3 Results

### 4.3.1 *Detection of PDGF-like activity by radioreceptor assays*

In **Chapter 3**, the results presented in **Figure 3.10**, showed that acid gel-filtration of whey extract separates BALB/c 3T3 bioactivity from the bulk of whey extract proteins. This BALB/c 3T3 bioactivity was now screened for PDGF activity by radioreceptor assays. A sample of whey extract (375 mg) was subject to acid gel-filtration (**2.2.4**) and portions of the recovered fractions tested for ability to displace $^{125}$I-h-PDGF-AA from BALB/c 3T3 monolayers (**4.2.2**). The same set of fractions were also tested for ability to induce BALB/c 3T3 cell growth by the 96-well plate dye binding assay (**4.2.1**). **Figure 4.1** shows the PDGF and BALB/c 3T3 cell activity of fractions eluting from acid gel-filtration of whey extract. A single peak of fractions that displaced $^{125}$I-h-PDGF-AA from BALB/c 3T3 monolayers was detected and chromatographed between the 45 and 29 kDa standards. However, the major group of fractions that produced BALB/c 3T3 cell growth activity eluted after the 29 kDa standard, in agreement with **Figure 3.10 B**.

### 4.3.2 *Identification of PDGF by immunoblotting*

Confirmation that acid gel-filtration fractions of whey extract that displaced $^{125}$I-h-PDGF-AA from BALB/c 3T3 monolayers contained PDGF, was provided by

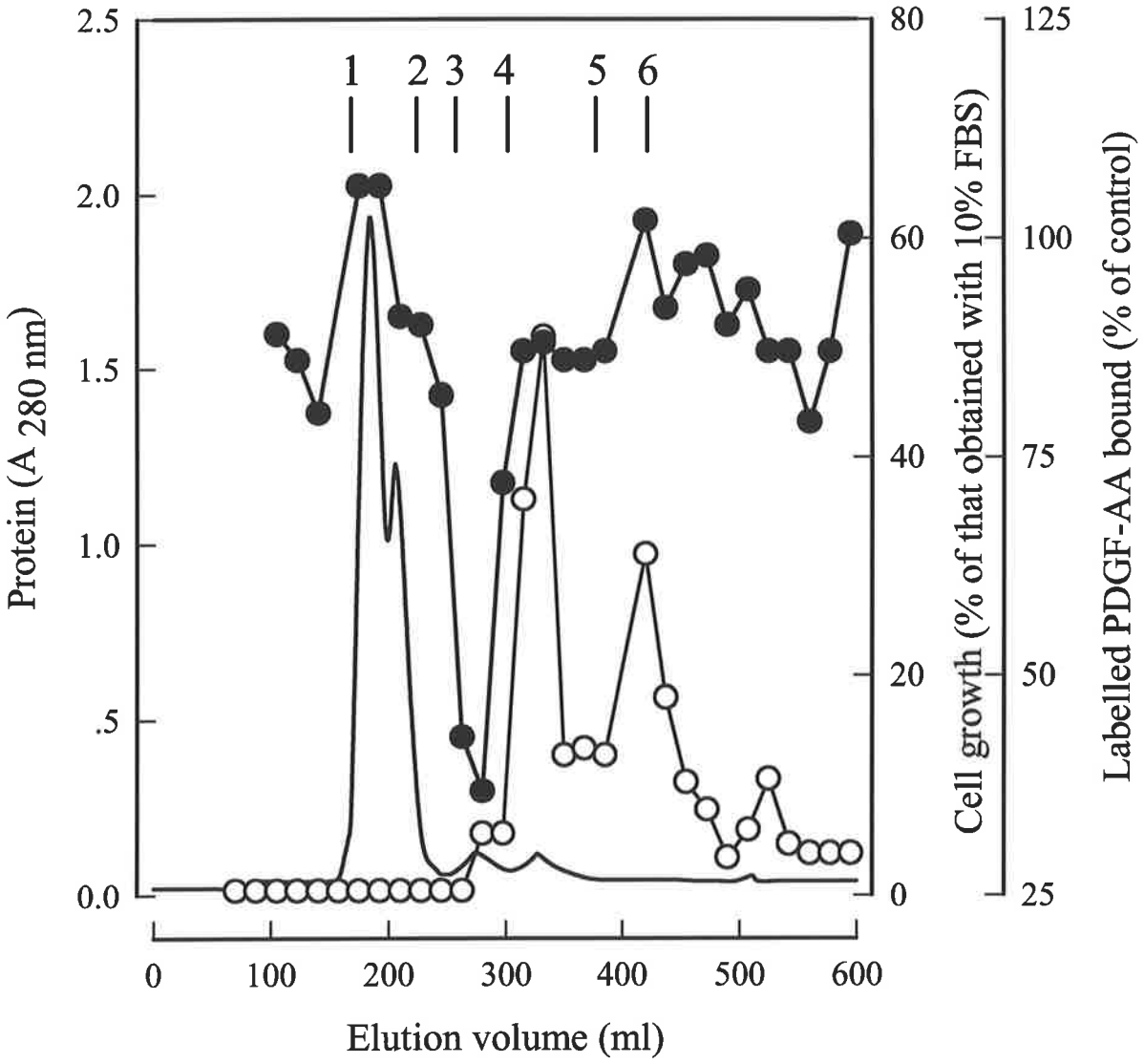



**Figure 4.1** PDGF and BALB/c 3T3 activity recovered from acid gel-filtration fractions of whey extract. A 375 mg sample of whey extract was chromatographed under acid conditions as described in **2.2.4** on a Superdex 75-35/600 column at 22 cm/h. Five minute fractions of 17.5 ml were collected. Portions (1 ml) of fractions were tested for PDGF activity by measuring their ability to displace $^{125}$I-h-PDGF-AA from BALB/c 3T3 cells (●) as described in **4.2.2**. Results are expressed as a percentage of the total binding of $^{125}$I-h-PDGF-AA to cells. A portion of each acid gel-filtration fraction (100 µl) was also tested for ability to induce BALB/c 3T3 growth (○) outlined in **2.2.3**. Cell growth data is expressed as a % of the response to a 10% FBS standard incorporated onto each plate and the response to DMEM alone has been subtracted. Values are the means of triplicate determinations. The column was standardised under neutral conditions with IgG (1; 150 kDa), BSA (2; 67 kDa), ovalbumin (3; 45 kDa), carbonic anhydrase (4; 29 kDa), cytochrome C (5; 12.4 kDa) and insulin (6; 7 kDa).

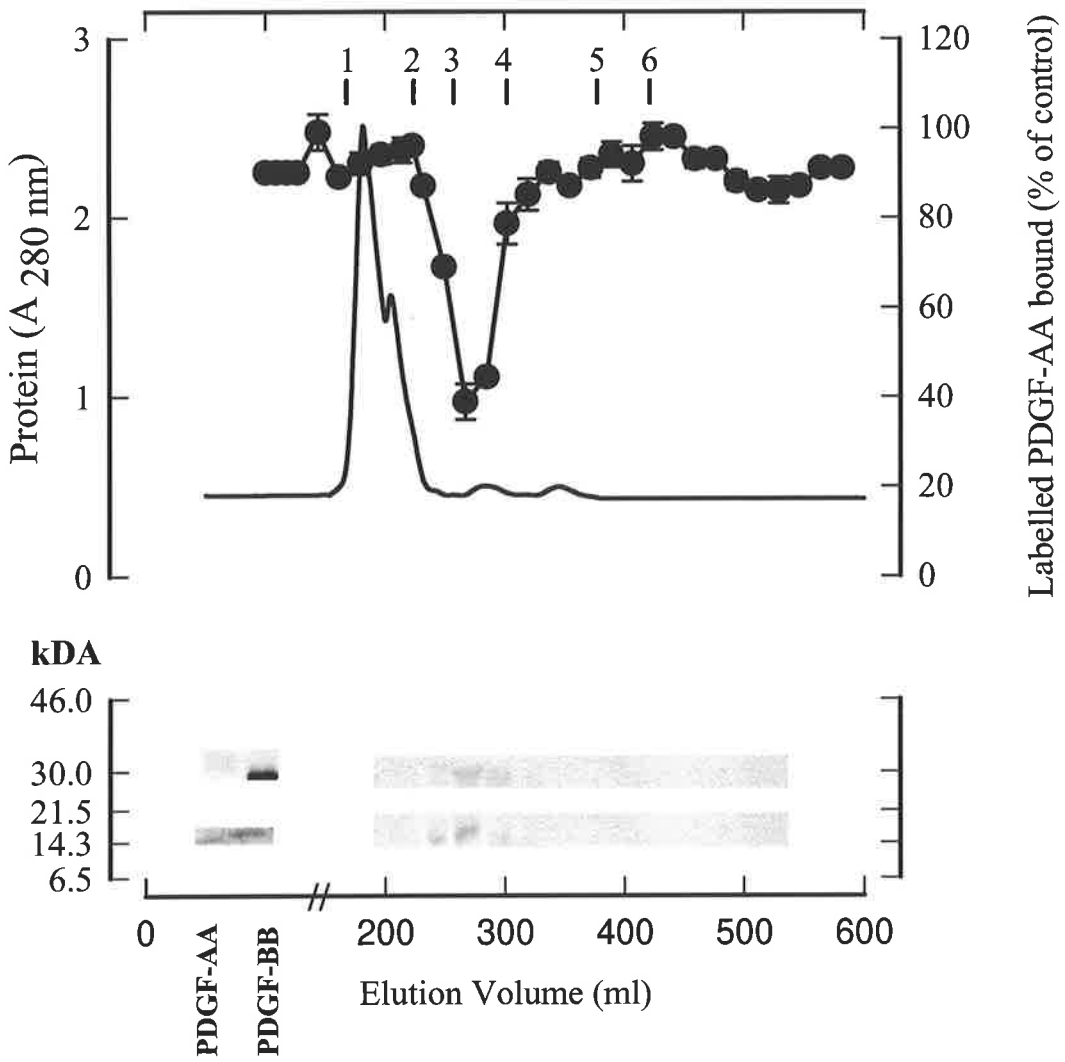



**Figure 4.2** PDGF activity in acid whey extract detected by both PDGF radioreceptor assay (RRA) and immunoblotting. A 375 mg sample of whey extract was subject to acid gel-filtration (pH 2.0) as described in **Figure 4.1**, and PDGF activity measured by the RRA (●) described in **4.2.2**. Results are expressed as a percentage of the total binding of $^{125}$I-h-PDGF-AA to cells (mean ± S.E.M of three measurements). The column was standardised as for **Figure 4.1** (IgG (1; 150 kDa), BSA (2; 67 kDa), ovalbumin (3; 45 kDa), carbonic anhydrase (4; 29 kDa), cytochrome C (5; 12.4 kDa) and insulin (6; 7 kDa)). The fractions were de-salted and portions (2.5 ml) were freeze dried and subject to SDS-PAGE under reducing and non-reducing conditions and then immunoblotted and exposed to anti PDGF-AB as described in **4.2.3**. PDGF-AA and PDGF-BB (500 ng) were included on immunoblots as positive controls; the positions of the molecular weight standards on the immunoblots are indicated (ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5), lysosyme (14.3) and aprotinin (6.5 kDa)).

immunoblotting. A 375 mg sample of whey extract was subject to acid gel-filtration and portions of the acid fractions were dialysed against 10 mM acetic acid to remove salt and vaccum dried. Portions of the desalted fractions and PDGF-AA and PDGF-BB were then subjected to non-reducing and reducing SDS-PAGE. Recombinant human PDGF-AA and PDGF-BB (500 ng) were also included as controls. The protein from gels was then transferred to nitocellulose and probed with 10 μg/ml of anti-PDGF-AB which recognises all PDGF isoforms. The desalted fractions were also tested for PDGF activity by the radioreceptor assay. Under non-reducing conditions, broad bands migrating at 30 kDa were detected in protein from fractions that had also competed for $^{125}$I-h-PDGF-AA in the radioreceptor assay (**Figure 4.2**). Recombinant human PDGF-AA and PDGF-BB migrated to 32 and 30 kDa respectively. Reducing conditions revealed a band at 15-16 kDa for the fractions containing PDGF-like activity and bands at 14 kDa for PDGF-AA and 16 kDa PDGF-BB respectively.

### 4.3.3 *Quantifying PDGF activity of whey extract*

To quantify the amount of PDGF recovered from acid gel-filtration of whey extract, PDGF activity that eluted in two fractions between 262 and 297 ml (**Figure 4.2**) was pooled, diluted and ability to displace $^{125}$I-h-PDGF-AA compared to PDGF-AA and PDGF-BB. **Figure 4.3** shows that binding of $^{125}$I-h-PDGF-AA to BALB/c 3T3 monolayers was competed for by dilutions of pooled PDGF activity and both PDGF-AA and PDGF-BB. The slope of the dose response curve obtained from displacement of $^{125}$I-h-PDGF-AA by dilutions of pooled PDGF-like activity was identical to PDGF-BB, but not PDGF-AA. $^{125}$I-h-PDGF-AA was competed for by the pooled column fractions with a 50% reduction in binding at 0.5 mg/ml and PDGF-AA and PDGF-BB competed

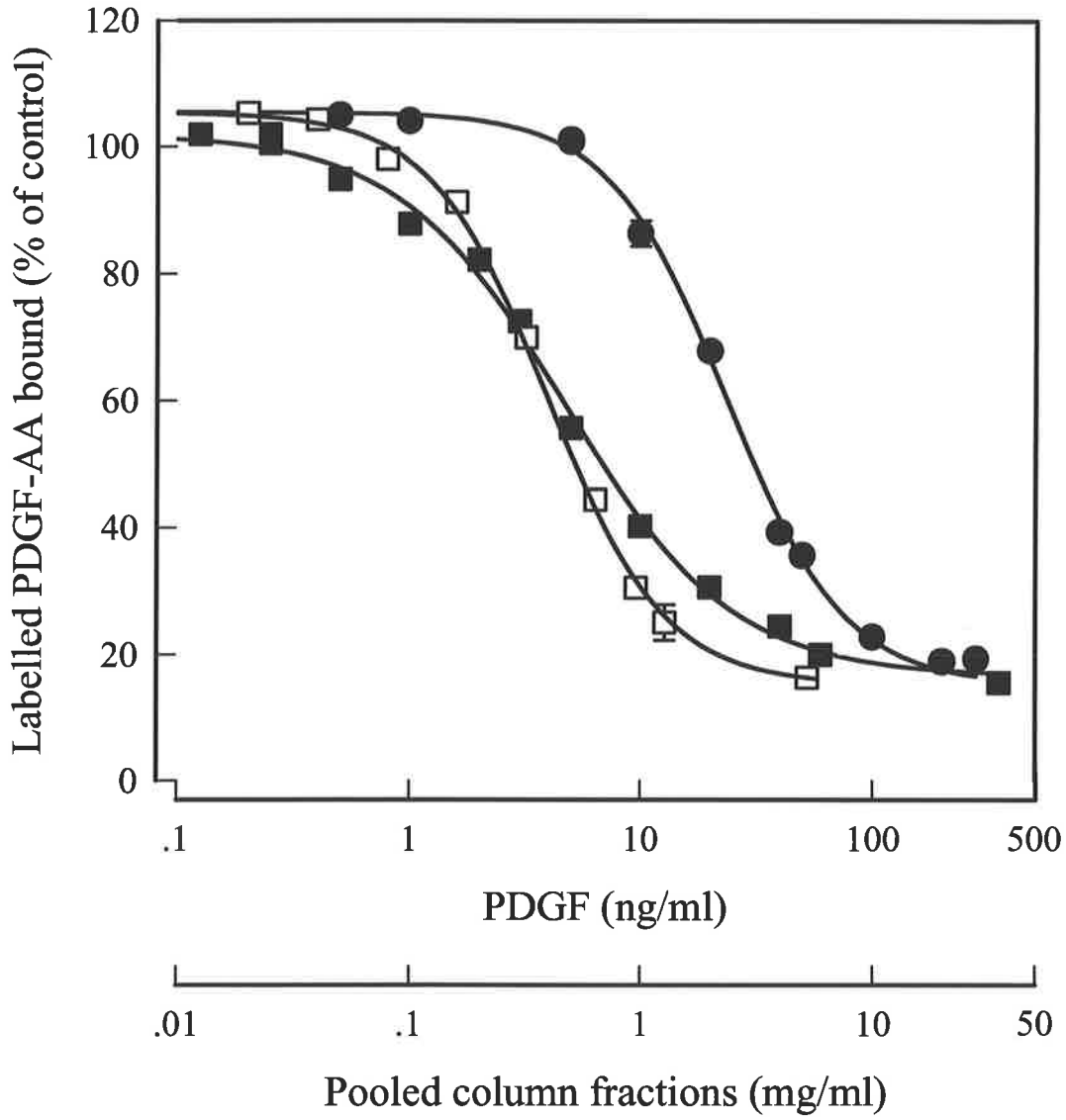



**Figure 4.3** Quantification of PDGF in pooled acid gel-filtration fractions of whey extract. Two acid Superdex 35/600 fractions of whey extract eluting between 262 and 297 ml that had PDGF-like activity (**Figure 4.2**) were pooled. The PDGF-like activity was serially diluted and tested for their ability to displace $^{125}$I-h-PDGF-AA from BALB/c 3T3 cell monolayers (□) as indicated in **4.3.3**. The amount of PDGF was estimated by reference to standard curves for dilutions of PDGF-AA (■) and PDGF-BB (●). Results (mean ± S.E.M of three measurements) are expressed as a percentage of the total binding of $^{125}$I-h-PDGF-AA to cells. Protein in the pooled fractions was estimated by comparison to bovine serum albumin by the method of Lowery *et al.* (1951) and shown in **2.2.5**.

with half maximal inhibition at 7 and 31 ng/ml respectively. A total of 0.36 μg of PDGF-AA and 1.9 μg of PDGF-BB equivalents were recovered from loading 375 mg of whey extract onto the gel-filtration column. These results and a series of three similar experiments showed 1.1 ± 0.1 ng of PDGF-AA or 4.8 ± 0.4 ng of PDGF-BB like molecules were present per mg of whey extract loaded onto the Superdex column (mean ± S.E.M, n=4).

### 4.3.4 *Contribution of PDGF to the growth factor component of whey extract*

The contribution of PDGF to the BALB/c 3T3 stimulator activity of whey extract was investigated by immuno-neutralising PDGF. Since PDGF-activity was recovered from acid gel-filtration of whey extract, the ability of immuno-neutralising PDGF to inhibit BALB/c 3T3 growth induced by acid-treated whey extract was also studied. Both acidified and non-acidified whey extract (1.0 mg/ml) were incubated with an antibody that neutralises PDGF, and their ability to stimulate BALB/c 3T3 cell growth measured by the 96-well plate dye binding assay. **Figure 4.4** shows that 25 μg/ml of anti-PDGF-AB neutralised 25 ng/ml of PDGF-BB activity but the control antibody (anti sheep/goat IgG) had little effect on BALB/c 3T3 cell growth. There was no significant difference in the BALB/c 3T3 cell growth produced by 1 mg/ml neutral whey extract before and after treatment with 25 μg/ml of anti-PDGF-AB. However, there was a small but significant difference in the BALB/c 3T3 response to 1 mg/ml acid-treated whey extract after it was incubated with 25 μg/ml of anti-PDGF.

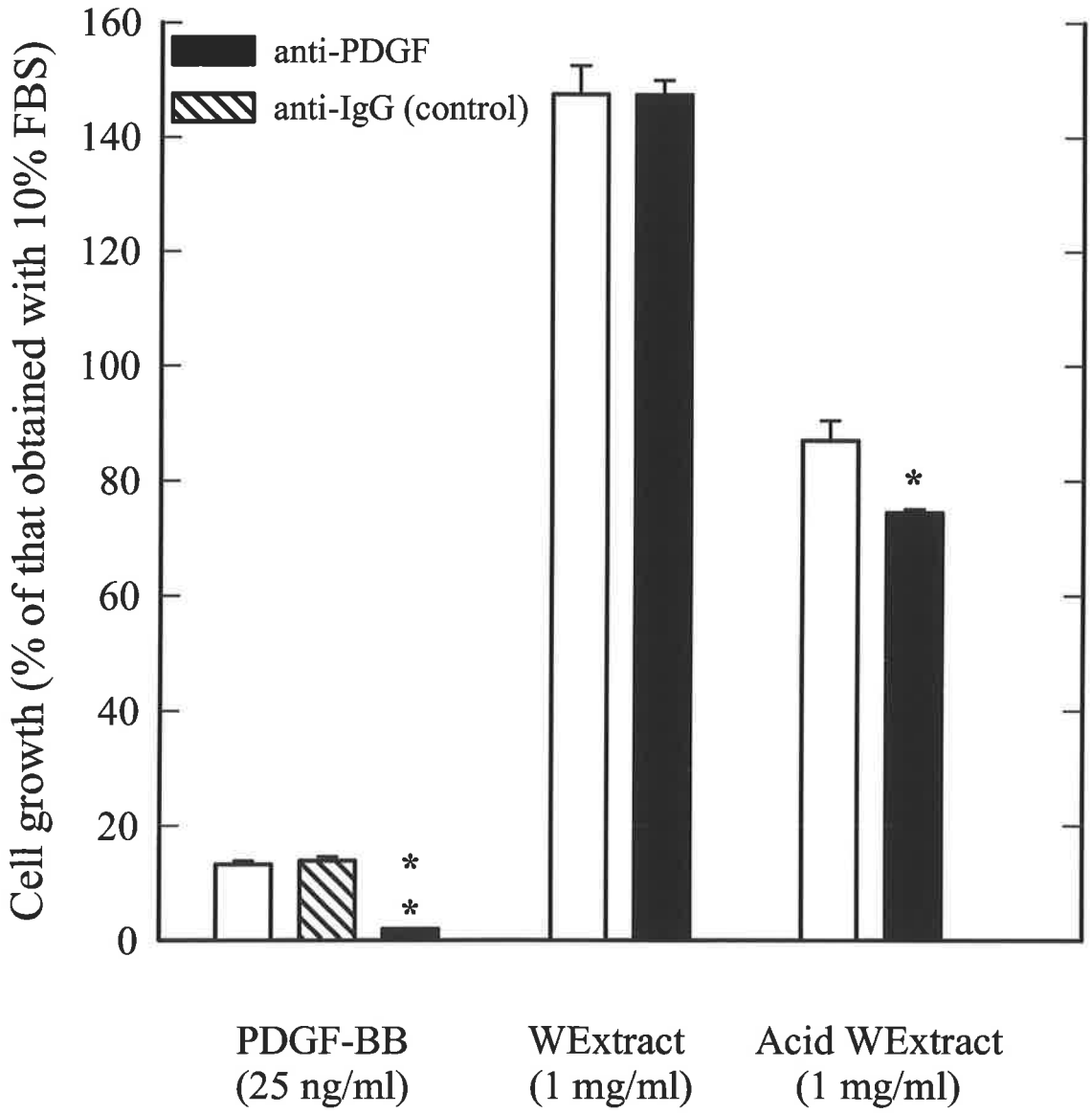



**Figure 4.4** The effect of anti-PDGF on the BALB/c 3T3 cell growth response to neutral and acid-treated whey extract. Whey extract was made to 25 mg/ml and a portion acidified to pH 2 with HCl and this and the untreated material diluted to 1 mg/ml in DMEM containing 0.1% (wt/v) BSA and tested on BALB/c 3T3 cells in 96-well plates as described in **4.2.4**. Samples (100 µl) of neutral (**WExtract**) and acid whey extract (**Acid WExtract**) in addition to 25 ng/ml PDGF-BB were assayed with and without 25 µg/ml of anti-PDGF-AB at the time of culture. The PDGF-BB standard (25 ng/ml) was also tested with a control antibody of 25 µg/ml of anti-goat/sheep IgG. After 48-h, the increase in cell mass was quantified by the methylene blue assay described in **2.2.3**. Results are expressed as a percentage of the response to a FBS standard incorporated onto each plate (mean ± S.E.M of three measurements). The response to DMEM alone has been subtracted from the results. **$p<0.05$ anti-PDGF-AB added to PDGF-BB vs no antibody; *$p<0.05$ vs no antibody.

## 4.4 Discussion

The results of this study show that bovine whey extract contains a platelet-derived growth factor (PDGF). Previous research had established that a PDGF-like molecule is a major mitogen of bovine and goat colostrum and induces BALB/c 3T3 cell growth (Shing and Klagsbrun 1984, *op cit* 1987; Brown and Blakeley 1984). The low level of BALB/c 3T3 cell growth factor activity in mature bovine milk compared to colostrum has to date, led to the assumption that PDGF is found only in colostrum (Shing and Klagsbrun 1984, *op cit* 1987; Shing *et al.* 1987). However, the highly concentrated mitogens in a cation-exchange fraction of bovine cheese whey (whey extract) has provided an ideal material for determining if PDGF is present in mature bovine milk. Homology in PDGF ligand-receptor interactions across species supplied a means to detect PDGF in whey extract by a radioreceptor assay (Singh *et al.* 1982; Bowen-Pope and Ross 1982; Bowen-Pope *et al.* 1989). It is apparent from **Figure 4.1** that a group of acid gel-filtration fractions of whey extract displaced human $^{125}$I-PDGF-AA from BALB/c 3T3 monolayers. The presence of PDGF in these acid gel-filtration fractions of whey extract was confirmed by immunoblotting with anti-PDGF-AB (**Figure 4.2**). In addition, the shift in immuno-reactive bands from 30 kDa under non-reducing to 15-16 kDa with reducing conditions (**Figure 4.2**) is consistent with previous observations that PDGF is a disulphide linked dimer (Raines and Ross 1982; Antoniades and Hunkapiller 1983). To date, this is the first report of PDGF-like activity in mature bovine milk.

Measurement of PDGF activity in whey extract by radioreceptor assays demonstrates that bovine milk contain only small amounts of PDGF. According to the features of PDGF receptor binding, $^{125}$I-hPDGF-AA binds the $\alpha/\alpha$ receptor dimer and all three

PDGF isoforms present in whey extract displace it in radioreceptor assays (Seifert *et al.* 1989; Eriksson *et al.* 1992; Ueno *et al.* 1993). As seen by **Figure 4.3**, the slope of the PDGF-like activity in whey extract is similar to human PDGF-BB, but not PDGF-AA. This could indicate that the measurement of PDGF-BB in bovine whey is more accurate than PDGF-AA, or more likely, that PDGF-BB is the predominant isoform in whey. In this regard, there were 4.8 ng of PDGF-BB-like molecules present per mg of whey extract, which translates to 0.14 ng of PDGF-BB equivalents present per ml of bovine whey. Previous radioreceptor assays has demonstrated that the predominant isoform in bovine platelets, bovine macrophages and most bovine cells is PDGF-BB (Shimokado *et al.* 1985; Kartha *et al.* 1988; Bowen-Pope *et al.* 1989; Seifert *et al.* 1984).

The data presented in this chapter indicate that PDGF does not contribute to the BALB/c 3T3 cell growth promoting activity of neutral whey extract but does to acid-treated whey extract. Radioreceptor assays that tested PDGF activity in neutral gel-filtration fractions of whey extract were inconclusive due to interference from major whey proteins (data not shown). However, antibodies that neutralised the BALB/c 3T3 cell growth promoting activity of PDGF had little effect on the potency of neutral whey extract (**Figure 4.4**). The same antibody had a small but significant effect on the BALB/c 3T3 cell activity of acid-treated whey extract (**Figure 4.4**). These results are expected as PDGF activity was recovered from whey extract after it was subject to acid gel-filtration (**Figure 4.1** and **Figure 4.2**). Indeed, previous research has shown that human PDGF found in plasma and the extracellular matrix is bound to and inactivated by binding proteins (Raines *et al.* 1984; Raines *et al.* 1992; Raines and Ross 1992). PDGF activity may be inactivated by high molecular weight proteins in whey extract before it is treated with acid.

The acid gel-filtration experiments show that whey extract fractions containing PDGF activity do not contain the most BALB/cT3 bioactivity. PDGF activity eluted between the 45 and 29 kDa standards, whereas the major peak of BALB/c 3T3 activity eluted near to the 29 kDa standard (**Figure 4.1**). Previous literature has shown that PDGF in bovine colostrum accounts for most of its bioactivity in BALB/c 3T3 cells. Shing and Klagsbrun (1984, *op cit* 1987), showed that the peak of BALB/c 3T3 activity recovered from acid gel-filtration of colostrum is primarily due to PDGF. Brown and Blakeley (1984), also found that goat colostrum contained a high concentration of a PDGF-like molecule. The results reported here argue that PDGF does contribute to the BALB/c 3T3 cell activity of acid-treated whey extract but is not the major mitogen in this fraction. Other mitogens eluting with the major peak of BALB/c 3T3 cell activity at 29 kDa may also include such factors as FGF and TGF-β. Indeed, research has shown that both FGF and TGF-β induce fibroblast growth and may be present in fluids such as milk (Gospodarowicz 1974; Roberts *et al.* 1985; Hill *et al.* 1994; Cox and Burk 1991). Since bFGF is unstable in acid, only aFGF could contribute to the 29 kDa peak of BALB/c 3T3 activity shown in **Figure 4.1** (Gospodarowicz 1975; Thomas *et al.* 1984). In **Chapter 3**, **Figures 3.3**, **3.12**, **3.13** show whey extract contains TGF-β-like activity in the form of epithelial cell growth inhibition. In addition, **Figure 4.1**, show fractions of whey extract that contained the highest level of TGF-β-like activity co-eluted with high BALB/c 3T3 cell bioactivity. Further work is needed to clarify whether FGF and TGF-β are major contributors to the BALB/c 3T3 cell growth of acid-treated whey extract.

Previous research revealed that growth factors can act synergistically or additively to induce high levels of BALB/c 3T3 cell growth. It could be argued that the peak of fractions from acid gel-filtration of whey extract that induce BALB/c 3T3 cell growth

may contain PDGF, FGF, TGF-β and other un-identified factors. The synergistic or additive effect of these factors, even in small concentration could be responsible for this growth (Soma and Grotendorst 1989; Segarini 1990; Nugent and Edelman 1992; Blachowski *et al.* 1993).

Although the association of binding proteins to milk-derived PDGF has not been described, the plasma PDGF binding protein $\alpha_2$-macroglobulin ($\alpha_2$-M) has been identified in both bovine colostrum and mature milk (Perez *et al.* 1989; Rantamaki and Muller 1992). Also, two PDGF binding proteins of 150 and 40 kDa that have less affinity for PDGF have been identified in serum but have not been characterised (Raines *et al.* 1984). It has been proposed that $\alpha_2$-M enters mammary secretions from the circulation or is locally produced in the mammary gland (Perez *et al.* 1989). Indeed, $\alpha_2$-M concentrations in colostrum expressed as percent of serum levels resembled those reported for albumin and therefore suggested that $\alpha_2$-M is passively transferred into milk from serum. Local production of $\alpha_2$-M by mammary epithelial cells or macrophages, or leucocytes entering the secretory cells cannot be excluded as almost all cell types secrete $\alpha_2$-M *in-vitro* (Mosher *et al.* 1977; White *et al.* 1980; Hovi *et al.* 1977). Notably, the 725 kDa $\alpha_2$-macroglobulin ($\alpha_2$-M) has been shown to be the most potent binding protein for plasma derived PDGF (Huang *et al.* 1983; Bowen-Pope *et al.* 1984; Raines *et al.* 1984; Bonner *et al.* 1992). PDGF-BB and PDGF-AB bind to native and proteinase activated $\alpha_2$-M but, PDGF-AA does not bind $\alpha_2$-M (Bonner and Osornio-Vargas 1995). This previous data on $\alpha_2$-M in plasma suggests that if this factor was present in whey extract it would bind bovine whey derived PDGF-BB. Indeed, PDGF-BB is the predominant form of PDGF in whey extract (**Figure 4.3**). Also, other reports show the concentration of $\alpha_2$-M in bovine whey (4.5 μg/ml) may be sufficient to

bind the low concentration of PDGF-BB detected in whey extract (Perez *et al.* 1989; Bonner and Osornio-Vargas 1995).

Further chromatography is required to detect the presence and form of $\alpha_2$-M in bovine whey extract. The Superdex 35/600 gel-filtration column used in the current experiments does not have the capacity to separate proteins greater than 150 kDa, so that binding proteins such as the 725 kDa $\alpha_2$-M will be eluted in the void volume. This will also happen when the pH of 2.0 used in the current gel-filtration experiments denatures the 725 kDa $\alpha_2$-M tetramer into subunits of 440 kDa (Raines *et al.* 1984; Bonner *et al.* 1992). Future experiments could involve assaying neutral and Superose 6 chromatography fractions of whey extract for PDGF and $\alpha_2$-M. This is needed to determine if native and/or proteinase activated $\alpha_2$-M are major PDGF binding proteins in milk.

In addition to plasma derived PDGF binding proteins, extracellular proteins have been demonstrated to bind and inactivate PDGF (Raines and Ross 1992; Raines *et al.* 1992). PDGF-B dimers, but not PDGF-AA, binds to the extracellular glycoprotein SPARC (or "secreted protein, acidic and rich in cysteine") in a reversible pH dependent non-covalent manner (Raines *et al.* 1992). It has been demonstrated that SPARC is expressed in low levels in platelets and normal adult tissues, but is up-regulated along with PDGF-B chain expression following vascular injury. Also, purified heparin sulphate proteoglycans that are known to be associated with endothelial cell membrane or matrices have been shown to bind long dimeric forms of PDGF A and B chains containing basic sequences (Raines and Ross 1992). Although both SPARC and heparin-like molecules have been detected in platelets and serum, there is no evidence that these extracellular proteins are present in milk (Snow *et al.* 1987; Raines *et al.* 1992).

The role of PDGF in bovine milk and its origin is not known at present. However, the high concentration of PDGF detected in colostrum (200 ng/ml) compared to bovine whey extract measured in the current study suggests it is important in the colostral phase (Shing and Klagsbrun 1987). Importantly, the validity of using whey extract to measure PDGF in bovine milk is strong considering both the characteristics of PDGF and cheese whey. PDGF is a cationic protein with a pI of 10 and would be expected to bind the cation-exchange resin in the production of whey extract from cheese whey (Deuel *et al.* 1981). Further, the pH of approximateley 5.6 used to produce bovine cheese whey helps prevent degradation of protein by encouraging the association of PDGF with protective binding proteins (Whitney 1988).

Production of transgenic mice deficient in either PDGF-B or the PDGF-Rβ has demonstrated the importance of PDGF for fetal development. Deficient mice showed renal, cardiovascular and haematological abnormalities and died before or at birth (Leveen *et al.* 1994; Soriano 1994). However, experiments which involve calves receiving colostrum deficient in PDGF are required to demonstrate the importance of this growth factor for neonatal development. Although transfer of PDGF to mammary secretions from secretory epithelial cells has not been demonstrated, Coltrera *et al.* (1995) have shown that PDGF-BB produced by human mammary epithelial cells is strongly correlated with mesenchymal proliferation. These findings suggest that bovine milk derived PDGF may be important for mammary development. Other possible sources of milk PDGF include macrophages that secrete mainly B chain isomers. Indeed, serum derived macrophages are the most predominant cell type found in bovine milk (Shimokado *et al.* 1985; Saad and Ostensson 1990). Since PDGF can be released from binding proteins in acid conditions, this suggests that milk derived PDGF will be active in the neonatal gut (Bonner *et al.* 1992; Raines *et al.* 1992). Further research is

needed to establish if PDGF and protein decline in parallel over bovine lactation and physiological relevance of PDGF in mature milk. The current data indicates that unlike colostral whey, mature whey does not contain high levels of PDGF.

# CHAPTER Five:

# Heparin-binding Factors in Bovine Whey

## 5.1 Introduction

The capacity of crude preparations of tissue or physiological fluids to stimulate fibroblast growth can be partly explained by growth factors. Those mitogens capable of inducing fibroblast growth include the platelet-derived growth factors (PDGFs), the transforming growth factors (TGFs), epidermal growth factor, and the fibroblast growth factors (FGFs) (Stiles *et al.* 1979; Roberts *et al.* 1985; Anzano *et al.* 1983; Leoff *et al.* 1983; Gospodarowicz 1974). Importantly, very few of these factors have been identified in bovine milk and nor have the milk mitogens that support the growth of fibroblasts when media is supplemented with bovine milk. The results presented in **Chapter 3** and **Chapter 4** of this document have demonstrated that whey extract can be used to survey the growth factors in mature milk. Although comprising only 2% of whey protein, at concentrations up to 5 mg/ml whey extract is more potent than 10% FBS on BALB/c 3T3 cells (**Figure 3.1 and 3.2**). Whey extract was also used to identify PDGF-like molecules in mature milk (**Chapter 4**). To date, there is no literature on fibroblast growth factors (FGFs) in milk, although they have been found in bovine tissue and other fluids (Gospodarowicz 1975; Thomas *et al.* 1980). Notablly, they have been identified in mammary tissue and in pre-partum mammary secretions (Barraclough *et al.* 1990; Ke *et al.* 1993; Sandowski *et al.* 1993).

Basic fibroblast growth factor (FGF-2/bFGF) and acidic fibroblast growth factor (FGF-1/aFGF) were initially characterised by their ability to promote the growth of cultured fibroblasts (Gospodarowicz 1974; *op cit* 1975; Thomas *et al.* 1984; Bohlen 1984). However, since 1985 more than seven additional members of the FGF family have been identified by their sequence homology to FGF-1 or FGF-2, rather than their

ability to promote fibroblast growth (Baird and Klagsbrun 1991). The most fully characterised FGFs are FGF-1 and FGF-2. The amino acid sequence of bovine and human FGF-1 differs in only 11 amino acids, whereas FGF-2 found in cows and humans are identical (Esch *et al.* 1985; Abraham *et al.* 1986; Gimenez-Gallego *et al.* 1985; Gimenez-Gallego *et al.* 1986). FGFs bind at high affinity with tyrosine kinase-containing FGF receptors that includes at least four members designated FGFR-1 to FGFR-4 (Lee *et al.* 1989; Dionne *et al.* 1990; Keegan *et al.* 1991; Partanen *et al.* 1991; Givol and Yayon 1992). Splicing variants of these receptors have also been found and they have altered affinities for members of the FGF family (Werner *et al.* 1992; Shi *et al.* 1993). FGFs also bind at low affinity with the heparan sulfate chain of cell surface proteoglycans (HSPGs) (Moscatelli *et al.* 1987 a; Keifer *et al.* 1990; Yayon *et al.* 1991). Heparan sulfate proteoglycans are obligate partners in binding of FGF-1 or FGF-2 to FGFRs and for induction of mitogenesis (Rapraeger *et al.* 1991; Ornitz and Leder 1991; Ornitz *et al.* 1992; Rapraeger Reiland and 1993). This has led to the suggestion that a ternary complex of FGF, FGFR and HSPGs is required for FGF binding and biological response (Bellot *et al.* 1991; Yayon *et al.* 1991; Kan *et al.* 1993; Savona *et al.* 1991).

FGF-1 and FGF-2 are potent modulators of cell proliferation and differentiation and are important regulators of angiogenesis and wound repair (Klagsbrun 1989; Gospodarowicz 1990;. Gospodarowicz 1990b; Baird and Bohlen 1990). As such their presence in milk would add to the list of factors present in milk that can influence cell growth in mammary glands and in the neonatal gut. One method of isolating FGFs makes use of their ability to bind heparin. Indeed, heparin-affinity chromatography has been widely used to purify and characterise FGF from a wide variety of tissues of mesodermal and ectodermal origin (Gospodarowicz *et al.* 1987; Klagsbrun 1989; Baird and Bohlen 1990). Indeed, two heparin-binding growth factors of 19 and 6 kDa were

recovered from bovine pre-partum (milk-like) secretions but no direct evidence for FGF-1 or FGF-2 in milk has yet been obtained (Sandowski *et al.* 1993),.

In this chapter, heparin-affinity chromatography is used to isolate FGF factors from whey extract. Elutions from heparin-affinity chromatography of a whey extract will be tested on BALB/c-3T3 fibroblasts in cell growth assays. The presence of FGF-1 and FGF-2 will then tested in the heparin-binding fractions by immunoblotting and radioreceptor assays. The amount of FGF in whey extract will be estimated; its contribution to the mitogenic activity of whey extract will be examined before and after the heparin-binding factors are removed.

## 5.2 Materials and Methods

The production of whey extract from bovine cheese whey was carried out as previously described in section **2.2.1**. One litre of whey yielded 30 mg freeze-dried whey extract. Materials are described in **Chapter 2** unless otherwise stated.

### 5.2.1 *Heparin-Sepharose chromatography*

Lobb *et al.* (1986) showed that FGF-1 and FGF-2 bind and elute from heparin-affinity columns using stepwise concentrations of salt and a flow rate of 14 cm/h. This research showed only heparin-binding proteins bind to affinity columns and FGF-1 elutes in 1.2 M NaCl and FGF-2 in 2.0 M NaCl. In the current procedure, chromatography was performed using heparin-Sepharose CL-6B in a (2.5 cm radius) Bio-rad column (AMRAD Pharmacia Biotech, North Ryde, New South Wales,

Australia). Heparin-Sepharose was packed into the column as described by the manufacturer, with a final column volume of 98 ml.

Initially, the heparin-affinity column was pre-equilibrated with 0.6 M NaCl in 10 mM Tris-HCl, pH 7.0 and 1 g of whey extract was dissolved in the same buffer at a concentration of 25 mg/ml and loaded onto the column. The column was then attached to an FPLC system (AMRAD Pharmacia Biotech), washed with 0.6 M NaCl in 10 mM Tris-HCl, pH 7 and eluted stepwise with 2 column volumes of 0.8 M, 1.2 and 2.0 M NaCl in 10 mM Tris-HCl, pH 7 at 14 cm/h. The recovered protein from each step was desalted by dialysing against 0.1 M $NH_4HC0_3$ using Specta/Por # 3 cellulose membranes that exclude molecules less than 3.5 kDa (Spectrum, Houston, Texas, USA).

To maximise recovery of protein, NaCl was replaced in heparin-affinity elution buffers by the volatile $NH_4HC0_3$ so that the eluted protein does not require desalting. After equilibrating with 0.6 M NaCl in 10 mM Tris-HCl, pH 7.0, the heparin-affinity column was loaded with 1.0 g of whey extract dissolved in equilibration buffer and the column washed in of 0.1 M $NH_4HC0_3$, pH 7.0. The protein was then eluted stepwise from the heparin-Sepharose at 14 cm/h (4 ml/min) with 200 ml of 0.8, 1.2, and 2.0 M $NH_4HC0_3$, pH 7.0. Under these conditions, FGF-1 is eluted first in the 1.2 M eluate and then FGF-2 in the 2.0 M fraction (Lobb *et al.* 1986). Eight ml fractions were collected, and the peak of protein recovered at each $NH_4HC0_3$ concentration pooled. The 0.8, 1.2 and 2.0 M $NH_4HC0_3$ pools were then sub-divided and freeze-dried. Protein was determined using the method described in **2.2.5** with bovine serum albumin as reference (RIA grade; Sigma Aldrich Pty Ltd, Castle Hill, New South Wales, Australia).

Whey extract that had not bound to heparin-Sepharose during affinity chromatography was desalted and freeze-dried. This pool included whey extract passing though the column during loading and that washed off with equilibration buffer and 0.1

M $NH_4HC0_3$. The material was desalted by diafiltering against deionised water in an Amicon CH-2 ultrafiltration unit with a 3 kDa excluding membrane (Amicon, Danvers, Massachusetts, USA; Sartorius, Gottingen, Niedersachsen, Germany). It was concentrated using the same device and then freeze-dried and is termed 'non heparin-binding whey extract'.

### 5.2.2 *Cell-growth assays*

The growth of BALB/c 3T3 cells in response to heparin-binding fractions of whey extract was determined using the 96-well plate methylene blue binding assay described in **2.2.3**. One g of whey extract was subject to heparin-Sepharose chromatography as outlined in **5.2.1**. A dried portion of the protein recovered from the column at each $NH_4HC0_3$ concentration was dissolved in DMEM containing 0.1% bovine serum albumin (BSA) and then serially diluted in the same media. Dilutions of recombinant FGF-1/aFGF and FGF-2/bFGF were included on each plate. Samples of whey extract before and after removing the heparin-binding factors were also dissolved in DMEM and diluted in the same cell media. BALB/c 3T3 cells were subcultured in 96-well plates as described in **2.2.3** and allowed to attach overnight in DMEM containing 10% (v/v) FBS. After washing the cells in DMEM for 2-4 h, 100 µl of the diluted samples were added to wells in triplicate. DMEM and dilutions of FBS were also incorporated onto each plate. After a 48 h incubation period, cell mass was quantified by methylene blue binding as indicated in **2.2.3**. Data is presented as the percentage of cell growth observed in 10% (v/v) FBS, with the response in DMEM alone subtracted.

### 5.2.3 *Radioreceptor assays*

Radioreceptor assays (RRA's) to detect FGF-like molecules in heparin-affinity fractions of whey extract were performed using BHK-21 cells (Neufeld and Gospodarowicz 1985; Moscatelli 1987). $^{125}$I-h-FGF-2 with a specific activity of 54 μCi/μg was prepared using a modification of the chloramine-T method (Van Obberghen-Schilling and Pouyssegur 1983; Gargosky *et al.* 1990). It was then separated from free $^{125}$I by G-50 Sephadex gel-filtration. BHK-21 cells were subcultured in 24-well plates (Costar, Cambridge, Massachusetts, USA) and grown to confluence in DMEM containing 10% (v/v) FBS. The monolayers of cells were then washed twice in HEPES-buffered saline (HBSS; 0.1 M HEPES, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, and 8 mM glucose, pH 7.6) containing 0.5% (wt/v) bovine serum albumin (BSA), and incubated at 4°C for 4 h. Portions of the dried 1.2 and 2.0 M $NH_4HC0_3$ elutions from heparin-affinity chromatography of whey extract were made up in HBSS with 0.5% (wt/v) BSA. They were then serially diluted in the same buffer as were recombinant FGF-1 and FGF-2. The cells were incubated for 12 h in HEPES-buffered saline containing diluted samples and $^{125}$I-FGF-2 ($1.5 \times 10^4$ cpm) in a final volume of 0.5 ml.

Research has indicated that FGFs may bind both their high affinity receptors and heparan sulphate proteoglycans. Moscatelli (1987), showed that binding of FGF to heparan sulphate proteoglycans is sensitive to high salt concentrations. To ensure the current assay is only measuring FGFs bound to the high affinity transmembrane receptor, the monolayers were washed with a high salt buffer. Monolayers were first harvested by washing twice with Hanks' balanced salts solutions (1.26 mM $CaCl_2$, 5.36 mM KCl, 0.44 mM $KH_2PO_4$, 0.82 mM $MgSO_4$, 136.9 mM NaCl, 0.033 mM $Na_2HPO_4$,

5.55 mM glucose, 0.01 g/l phenol-red, pH 7.3) at 4°C. The cells were then washed once with Hanks' containing 2.0 M NaCl at 4°C. The monolayer were then dissolved by adding 1 ml of 0.5 M NaOH containing 0.1% (v/v) Triton X-100 to wells. Radioactivity was counted in a gamma counter, and the ability of samples to displace $^{125}$I-h-FGF-2 from receptors expressed as a percentage of binding in HEPES-buffered saline. FGF-like activity recovered from heparin-affinity fractions of whey extract was then estimated by comparing the ability of dilutions of pooled activity to displace $^{125}$I-h-FGF-2 to that of FGF-2 and FGF-1 using the standard curves. Data was fitted to a four parameter logistic dose response equation using Tablecurve (Jandel Scientific, San Rafael, California, USA). Non-specific binding in HEPES-buffered saline containing excess FGF (400 ng/ml FGF-2; 400 ng/ml of FGF-1) was consistently 10-15% of the total binding. For each plate, the total amount of $^{125}$I-h-FGF-2 bound to monolayers in HEPES-buffered saline was around 20% of the 1.5 x $10^4$ cpm added. The protein concentration of the pooled fractions were determined as shown in **2.2.5**.

### 5.2.4 *FGF immunoblots*

Immunoblotting was used to confirm the presence of FGF in heparin-binding fractions of whey extract (Wadzinski *et al.* 1987). Samples from the 1.2 and 2.0 M $NH_4HC0_3$ elutions were dissolved in 100 µl SDS-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (5% (wt/v) SDS, 100 mM Tris, and 5% (wt/v) bromophenol blue, pH 8.0) containing the reducing agent dithiothreitol (1.5%) (Sigma Aldrich Pty Ltd, Castle Hill, New South Wales, Australia). Portions of recombinant aFGF/FGF-1 and bFGF/FGF-2 were also dissolved in reducing SDS-PAGE buffer in addition to a sample of rainbow molecular weight markers. The samples were then heated for 5 minutes at 95°C, and loaded onto 15 % polyacrylamide gels and subject to SDS-PAGE. The

amount of protein loaded was 9 μg of the 1.2 M peak, 2.7 μg of the 2.0 M peak and 220 ng of FGF-1 and FGF-2. On each gel there were also 5μg of each protein present in the rainbow molecular weight markers. The protein from the gels was then electroblotted onto nitrocellulose (Schleicher and Schuell Pty Ltd, Dassel D-3354, Germany) sheets. This was done using a semi-dry Novablot system as described by the manufacturer (AMRAD Pharmacia Biotech, North Ryde, New South Wales, Australia). The nitrocellulose sheets were incubated in wash buffer (0.2 M NaCl, 0.1 % (wt/v) BSA, 0.1% (wt/v) polyethylene Glycol, 50 mM Tris, pH 7.4) containing 0.3% (wt/v) BSA to block non-specific binding of antibodies. Nitrocellulose sheets were then incubated at 4°C overnight with either polyclonal anti-bovine FGF-1 (1:100; Sigma Aldrich Pty Ltd, Castle Hill, New South Wales, Australia) or anti-bovine FGF-2 (1:1000; I Hendry, Australian National University, ACT, Australia). The non-bound antibody was then removed by soaking the nitrocellulose sheets in fresh wash buffer (4 x 15 min). The nitrocellulose sheets were then incubated in a second antibody of anti-rabbit IgG conjugated to alkaline phosphatase (Silenus Laboratories, Hawthorn, Australia) that was diluted in wash buffer (1:500) for 1 h. After removing non-bound antibody by frequent changes of wash buffer over 1.5 h, the immuno-reactive bands were developed with a 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium substrate (Sigma-Aldrich Pty Ltd, Castle Hill, New South Wales, Australia).

### 5.2.5 *Polyacrylamide gel electrophoresis*

The FGF-activity recovered from heparin-affinity chromatography of whey extract was subject to reducing SDS-polyacrylamide gel-electrophoresis (SDS-PAGE). Samples of the 0.8, 1.2 and 2.0 M $NH_4HC0_3$ elutions from heparin-affinity of whey extract (**5.2.1**) and FGF-1 and FGF-2 were analysed by 8-25% SDS-PAGE with a

Pharmacia Phast System. The samples were all dissolved in reducing SDS-PAGE buffer and heated (as described in **5.2.4**). Four µl of each sample and Phast-gel molecular weight markers was loaded on to a 8-25% polyacrylamide preformed slab gel. They were then subject to PAGE and stained with Coomassie blue according to the manufacturers instructions (AMRAD Pharmacia Biotech, North Ryde, New South Wales, Australia).

## 5.3 Results.

### 5.3.1 *Heparin-affinity chromatography.*

As a first step in isolating heparin-binding growth factors, classical heparin-affinity chromatography was used on whey extract. Whey extract was loaded onto a heparin-Sepharose column in the presence of 0.6 M NaCl in 10 mM Tris, pH 7.0, and eluted stepwise with 0.8, 1.2 and 2.0 M NaCl in 10 mM Tris, pH 7 at 14 cm/h. However, only a small amount of protein was recovered, which was barely detectable after the samples were desalted (data not shown). Use of the volatile salt $NH_4HC0_3$ in the elution buffer allowed immediate freeze-drying of the recovered fractions and resulted in detectable protein. Subsequently, 1 g of whey extract was loaded onto a heparin-Sepharose column and eluted with 0.8, 1.2 and 2.0 M $NH_4HC0_3$ at 14 cm/h (**Figure 5.1**). This yielded 2.2, 0.48 and 0.22 mg of protein respectively.

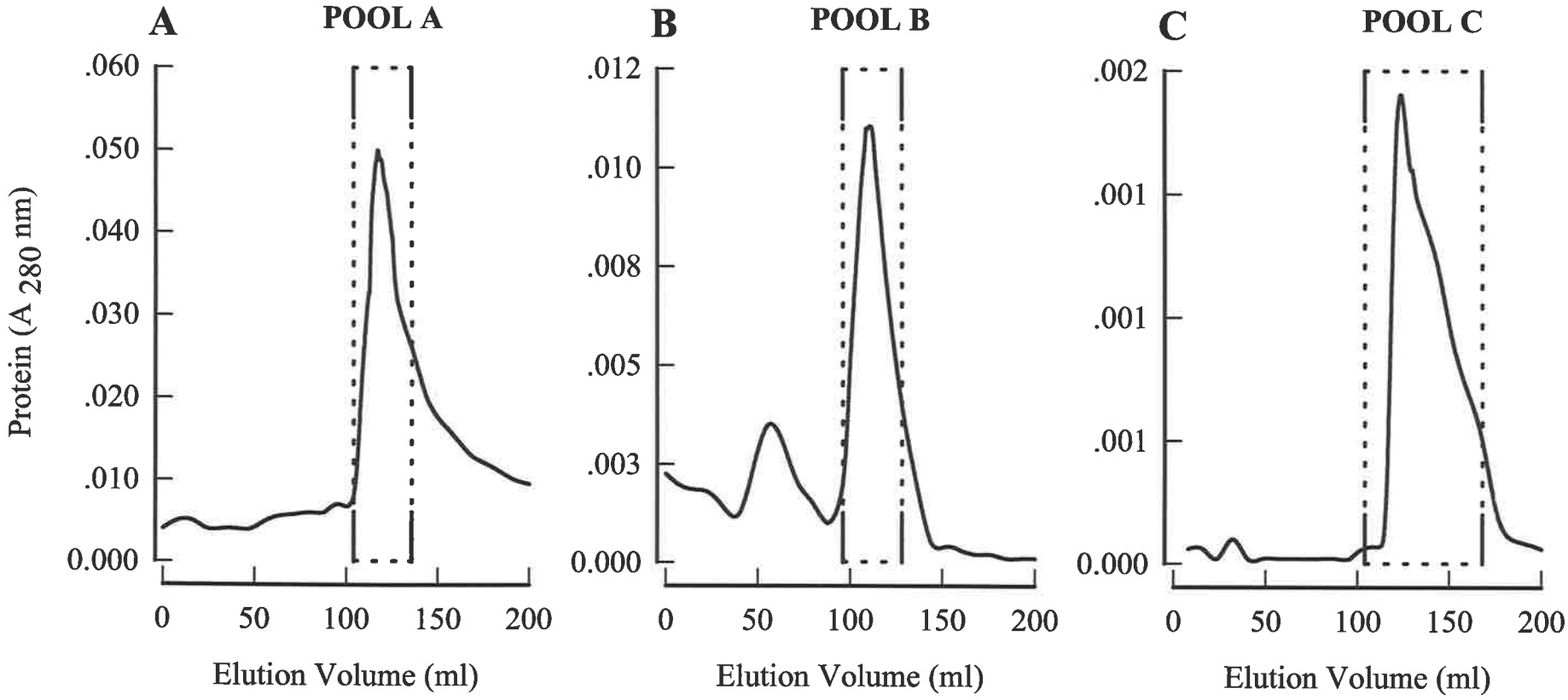



**Figure 5.1** FPLC profile from heparin-Sepharose chromatography of 1.0 g of whey extract. A heparin-Sepharose column was equilibrated with 0.6 M NaCl in 10 mM Tris-HCl, as described in **5.2.1**. One gram of whey extract was dissolved in the equilibration buffer at a concentration of 25 mg/ml, loaded on to the column, washed with the same buffer and then coupled to an FPLC system. The column was then washed with 0.1 M $NH_4HC0_3$ and then stepwise eluted with 0.8 (**A**), 1.2 (**B**), and 2.0 M (**C**) $NH_4HC0_3$ at 14 cm/h. Fractions (of 8 ml) were collected, and each peak of absorbance pooled and freeze dried (**Pool A**, **Pool B**, **Pool C**). The total protein recoveries were 1.2 mg, 0.48 mg and 0.22 mg respectively.

### 5.3.2 *Cell-growth activity of heparin-binding whey fractions*

Dilutions of the protein recovered from heparin-affinity chromatography of whey extract were tested for their ability to induce BALB/c-3T3 cell growth using the dye binding assay (**2.2.3**). Cells were allowed to attach overnight in the presence of 10% (v/v) FBS before the addition of dilutions of pooled protein collected at each $NH_4HC0_3$ concentration used in heparin-affinity chromatography of whey extract. Dilutions of recombinant aFGF/FGF-1 and bFGF/FGF-2 were included each plate. As shown in **Figure 5.2 A** and **B**, the protein recovered in all fractions and FGF induced the growth of BALB/c-3T3 cells in a dose-dependant fashion. Maximal responses to both the 1.2 and 2 M fractions were achieved at between 10-20 μg/ml of protein. This was nearly ten times less than the 0.8 M fraction.

### 5.3.3 *Verification of FGF activity by radioreceptor assay*

Evidence that the heparin-binding fractions of whey extract contained FGF-1 and FGF-2 was provided by radioreceptor assay using BHK-21 cells. Samples of the 1.2 and 2.0 M $NH_4HC0_3$ elutions recovered from heparin-Sepharose chromatography of whey extract were tested for their ability to displace $^{125}I$ labelled FGF-2 from BHK-21 cells (**Figure 5.3**). Dilutions of both the 1.2 and 2.0 M eluates were effective in competing with labelled FGF-2 for receptor sites on cell monolayers (**Figure 5.3 A** and **B**). Comparison to a FGF-2 standard (**Figure 5.3 A**) indicated 3 ng of FGF-2/μg of protein was found in the 2.0 M $NH_4HC0_3$ fraction. This indicated that 660 ng of FGF-2 was present in the 220 μg of protein recovered in the 2.0 M fraction. Since 30 mg of

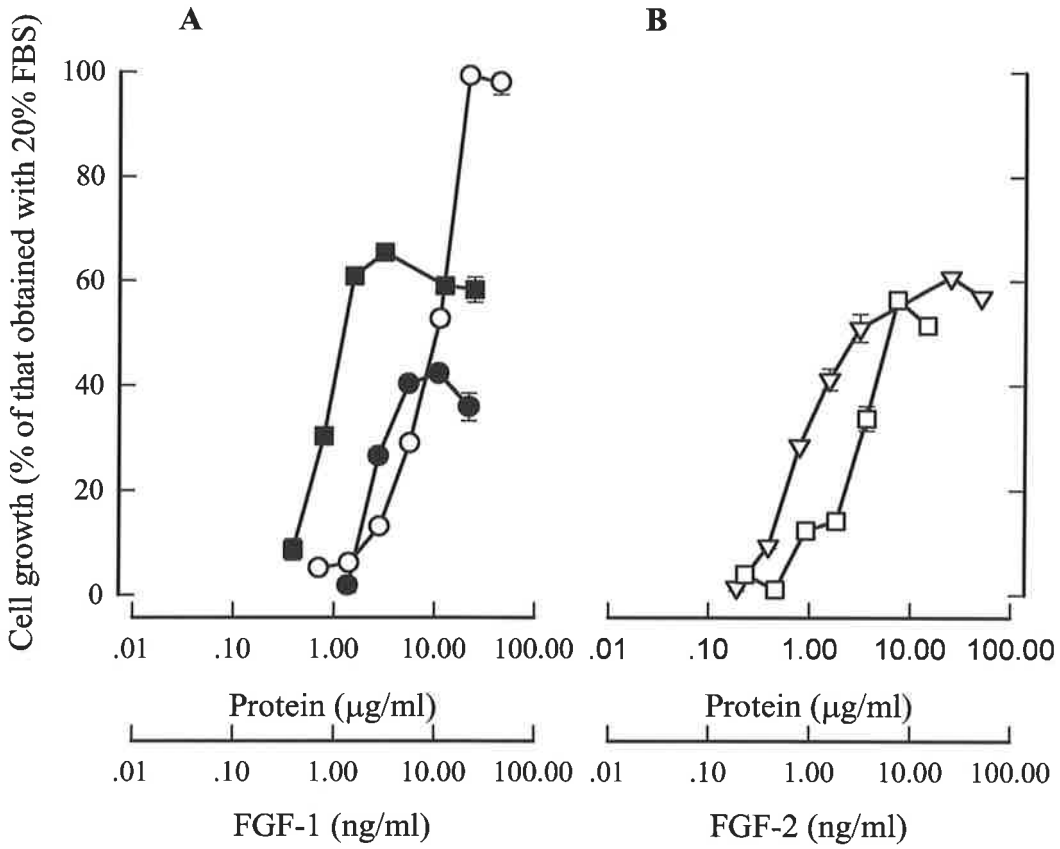



**Figure 5.2** Growth of BALB/c 3T3 cells in response to fractions obtained from heparin-affinity chromatography of whey extract. Whey extract was subject to heparin-Sepharose chromatography as described in **Figure 5.1** and **Pool A**, **Pool B** and **Pool C** collected from the 0.8 M, 1.2 M and the 2.0 M $NH_4HC0_3$ elutions respectively. A dilution series of the 0.8 M elution (●), the 1.2 M elution (○) and FGF-1 (■) were tested for their ability to induce BALB/c 3T3 cell growth (**A**) in 96-well plates as described in **5.2.2**. Using the same method, dilutions of the 2.0 M fraction (□) and FGF-2 (▽) were also tested on BALB/c 3T3 cells (**B**). Data are expressed (mean ± S.E.M of three measurements) as a percentage of the response to a 20% FBS standard incorporated on to each plate, with the response to DMEM alone subtracted. The protein in each elution has been estimated by comparison to bovine serum albumin by the method described in **2.2.5**.

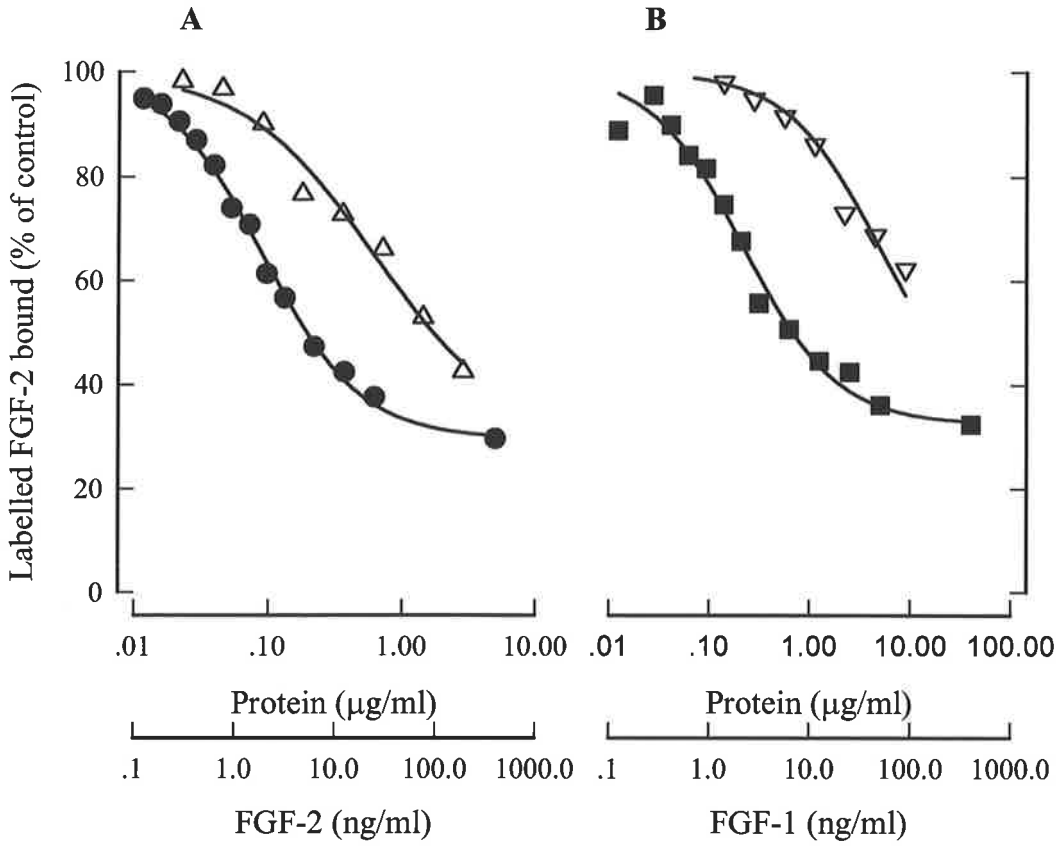



**Figure 5.3** Radioreceptor assays of heparin-affinity fractions of whey extract. Whey extract was subject to heparin-Sepharose chromatography as described in **5.2.1** and **Figure 5.1** A portion of the pooled fractions recovered from both the (**A**) 2.0 M (Δ) and (**B**) the 1.2 M (∇) $NH_4HC0_3$ elutions were serially diluted and tested for their ability to displace $^{125}$I-FGF-2 from BHK-21 cells as described in **5.2.3**. This was compared to indicated concentrations of unlabelled FGF-2 (●) and FGF-1 (■). Data are expressed (mean of two measurements) as the percentage bound in the absence of unlabelled peptide. Non-specific binding (10-20%) has not been subtracted. Protein in each elution has been estimated by comparison to bovine serum albumin by the method described in **2.2.5**.

whey extract protein was recovered from each litre of whey, this indicates the presence of 19.8 ρg of FGF-2/ml of whey. Using the same assay with reference to a recombinant FGF-1 standard (**Figure 5.3 B**), 0.4 ng of FGF-1/μg protein was detected in the 1.2 M eluate. This corresponds to 192 ng of FGF-1 in the 480 μg recovered from the 1.2 M elution, corresponding to 5.8 ρg of FGF-1/ml of whey.

### 5.3.4 *Detection of FGF by immunoblotting*

Further confirmation that whey-derived heparin-binding fractions contains FGF was provided by immunoblotting. Portions of the 1.2 M (9 μg) and 2.0 M (2.7 μg) $NH_4HC0_3$ eluates recovered from heparin-affinity chromatography of whey extract were subject to SDS-PAGE. They were then electroblotted onto nitrocellulose and probed with anti-bovine FGF-1 or anti-bovine FGF-2. The presence of FGF-1 in the protein eluting at 1.2 M $NH_4HC0_3$ was confirmed by the detection of a single broad immunoreactive band at 15 kDa (**Figure 5.4 A, lane 1**). This protein migrated slightly further than recombinant human FGF-1 (1-154) (**Figure 5.4 A, lane 2**). Two bands at 14 and 17 kDa were detected in the 2.0 M eluate when probed with an antibody raised against a synthetic peptide corresponding to the first 24 amino acids of bovine FGF-2 (**Figure 5.4 B lane 3**). The higher molecular mass band migrated to the same position as the recombinant FGF-2 (1-146) standard (**Figure 5.4 B lane 4**). There was no evidence of immunoreactive FGF-1 or FGF-2 in the 0.8 M $NH_4HC0_3$ fraction or of cross-reactivity between anti-bovine FGF-1 and FGF-2 or anti-bovine FGF-2 and FGF-1 (results not shown).

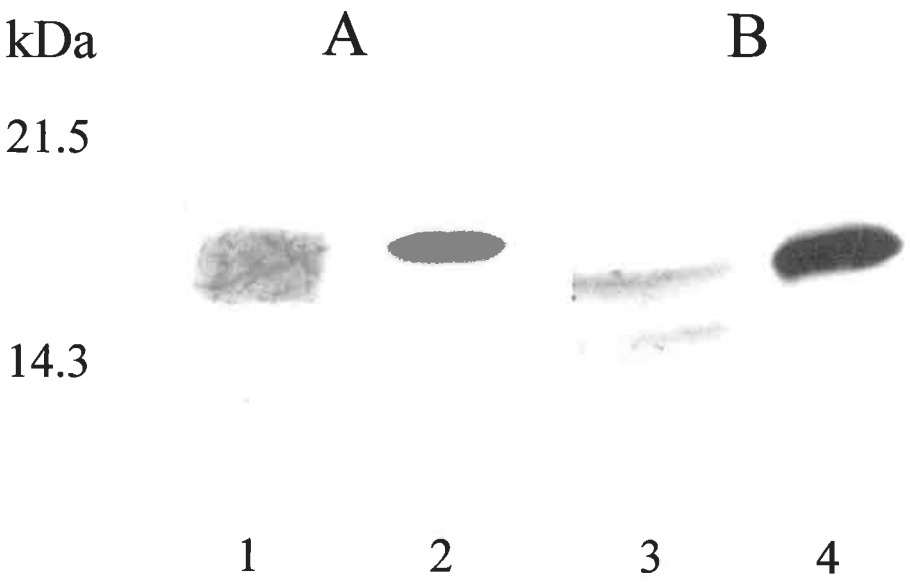



**Figure 5.4** Immunoblots of fractions from heparin-Sepharose chromatography of whey extract probed with **A**: anti-bovine FGF-1 and **B:** anti-bovine FGF-2. Whey extract was applied to a heparin-affinity column and eluted from with 0.8, 1.2 and 2.0 M $NH_4HC0_3$ as described in **5.3.1**. Portions of the 1.2 M elution (**lane 1**; 9 μg) and the 2.0 M elution (**lane 3**; 2.7 μg) recovered from heparin-affinity chromatography of whey extract were subject to SDS-PAGE and then electroblotted onto nitrocellulose and then probed with either anti-bovine FGF-1 or anti-bovine FGF-2 as described in **5.2.4**. 220 ng of recombinant FGF-1 (**lane 2**) and FGF-2 (**lane 4**) were also included on all gels in addition to rainbow molecular weight markers (5 μg of each protein). The positions of the rainbow molecular weight markers of 21.5 kDa (trypsin inhibitor) and 14.3 kDa (lysosyme) are shown above. There was no cross-reactivity between anti-bovine FGF-1 and FGF-2 or anti-bovine FGF-2 and FGF-1 (data not shown).

### 5.3.5 *SDS-PAGE of heparin-binding whey fractions*

The purity of the FGF-like activity in heparin-affinity fractions of whey extract was examined by SDS 8-25% PAGE. The amount of protein loaded was 3 μg of the 0.8 M peak, 2 μg of the 1.2 M peak, 0.55 μg of the 2.0 M peak and 200 ng of FGF-1 and FGF-2. **Figure 5.5** shows photographs of the reducing gel. In comparison to the 1.2 and 2.0 M eluates, the 0.8 M fraction resolved into numerous bands of protein. The 1.2 M eluate separated into at least four proteins, one of which migrated near ~15 kDa (**Figure 5.5 A**). In the 2.0 M elution only two protein bands were detected and these were of relatively low molecular weight (**Figure 5.5 B**). However, the low purity of each fraction means that FGF could not be visible in either the 1.2 or 2.0 M elutions.

### 5.3.6 *Cell-growth activity of heparin-binding whey fractions*

The ability of whey extract to promote BALB/c-3T3 cell growth before and after the removal of heparin-binding factors was examined by the 96-well plate dye-binding assay (**2.2.3**). The whey extract that did not bind the heparin-Sepharose was pooled, desalted and freeze-dried as described in **5.2.1**. This 'non heparin-binding whey extract' and the original whey extract was dissolved in DMEM at 25 mg/ml, serially diluted in the same media and tested for ability to promote BALB/c-3T3 cell growth. **Figure 5.6** shows that the ability of whey extract to induce BALB/c 3T3 cell growth was not significantly affected by the removal of heparin-binding factors.

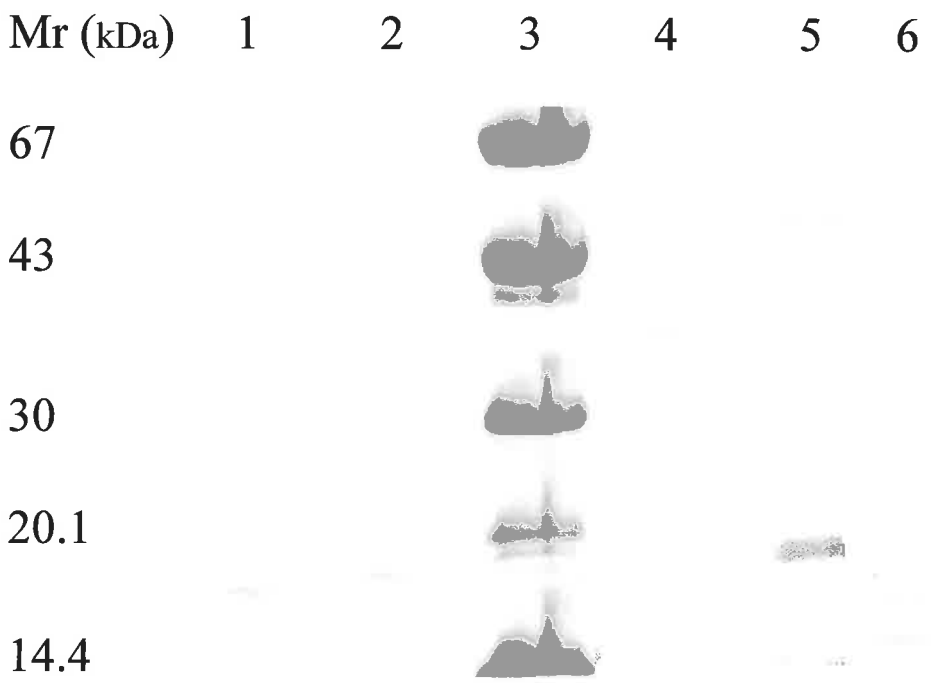



**Figure 5.5** SDS-PAGE of fractions from heparin-Sepharose chromatography of whey extract. Whey extract was applied to a heparin-affinity column and eluted with 0.8, 1.2 and 2.0 M $NH_4HC0_3$ as described in **5.3.1** A sample of the 0.8 M elution (3 μg; **lane 5**), the 1.2 M elution (2 μg; **lane 4**) and the 2.0 M elution (0.55 μg; **lane 6**) was subject to SDS-PAGE under reducing conditions on a 8-25% Polyacrylamide preformed slab gel and stained with Coomassie blue (**5.2.5**). Portions (200 ng) of recombinant FGF-2 (**lane 1**) and FGF-1 (**lane 2**) were also included on the gels in addition to molecular weight markers (3 μg; **lane 3**). The positions (**Mr** in **kDa**) of the molecular weight markers are also shown (bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4kDa)).

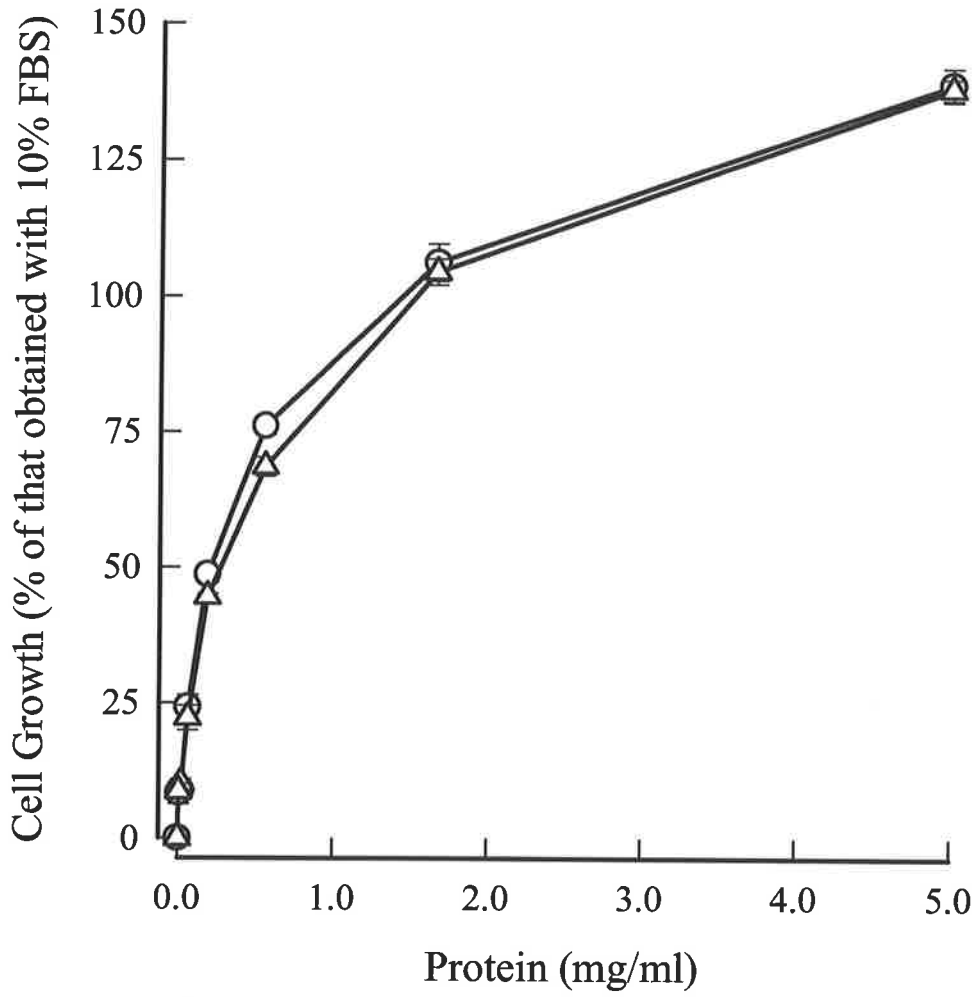



**Figure 5.6** Growth of BALB/c 3T3 cells in response to whey extract and whey extract that has had heparin-binding factors removed. Whey extract was subject to heparin-Sepharose chromatography; (**Figure 5.1**) and the non-heparin binding material collected, de-salted and freeze-dried as described in **5.2.1** A dilution series of a portion of whey extract (O) and non heparin-binding whey extract (Δ) were tested for their ability to induce BALB/c 3T3 cell growth in 96-well plates as shown in **5.2.2**. Data are expressed (mean ± S.E.M of three measurements) as a percentage of the response to a 10% FBS standard incorporated on to each plate, with the response to DMEM alone subtracted.

## 5.4 Discussion

Although fibroblast growth factors are found in several different mammalian tissues, they have not been identified in milk or colostrum (Gospodarowicz *et al.* 1987; Klagsbrun 1989). In this chapter, heparin-Sepharose chromatography was used to identify acidic FGF/FGF-1 and basic FGF/FGF-2 in bovine whey. To achieve this result, a cation-exchange fraction was first obtained from whey that contained high cell growth activity and this was then subject to heparin-affinity chromatography. Indeed, the cation-exchange fraction (whey extract) was shown in **Chapter 3** and **Chapter 4** to induce fibroblasts to grow at a higher level than whey or even 10% (v/v) FBS (**Figure 3.1**, **3.2**). A similar cation-exchange step is included in the routine isolation of FGF-1 and FGF-2 from bovine brain (Lobb *et al.* 1986). The 1.2 M elution from heparin-affinity chromatography of whey extract contained FGF-1 and the 2.0 M fraction FGF-2, which is consistent with previous tissue-derived FGF isolation procedures (Lobb *et al.* 1986).

The small contribution of FGF proteins to the BALB/c 3T3 cell bioactivity of whey extract agrees with their reported instability in serum and their autocrine/paracrine action. As shown in **Figure 5.6**, removal of the heparin-binding fraction from whey extract made little difference to its BALB/c 3T3 growth factor activity. Moreover, only a small amount of FGF was measured in whey extract, equivalent to 18 ng of FGF-2 and 5.8 ng FGF-1 from each litre of whey (**Figure 5.3**). Since FGF-2 is unstable in the acid conditions used to separate whey from solids, this may account for its observed low frequency (Gospodarowicz 1975; Thomas *et al.* 1980). Importantly, there is little evidence that FGFs are circulating hormones (Gauthier *et al.* 1987). However, like most

growth factors, it is more likely that they are attached to binding proteins that make them inactive in serum and fluids like milk. Binding proteins related to the external domain of the FGFR-1 have been found in human and bovine blood and maternal serum (Hanneken *et al.* 1994; Hill *et al.* 1994). Interestingly, the 78 kDa lactoperoxidase that was found by Francis *et al.* (1995) to be the most abundant protein in whey extract, is known to form physical associations with other whey proteins such as lysosyme, RNAase and immunoglobulins (Hulea *et al.* 1989). It is possible it also forms physical associations with growth factors in the whey extract.

Although growth factors are a minor component of whey protein, the impurity of the FGF containing fractions (seen in **Figure 5.5**) was surprising given the reported specificity of heparin-affinity chromatography (Gospodarowicz *et al.* 1987; Klagsbrun 1989; Baird and Bohlen 1990). Furthermore, addition of an ammonium sulphate precipitation step to the isolation procedure (see Lobb *et al.* 1986) did not significantly enhance the purity of the final product (data not shown). This may reflect the diversity of protein in the whey fraction. Attempts to quantify FGF in crude whey or the cation-exchange fraction by Radioreceptor assays were also inconclusive due to interference from major whey proteins.

All three fractions recovered from the heparin-affinity column induced the growth of BALB/c-3T3 cells, which were also responsive to purified recombinant FGF. Although the bioactive constituents in the 0.8 M fraction were not identified, it may contain factors such as platelet-derived growth factor (PDGF). This mitogen has been shown to elute from a heparin-affinity column in 0.8 M salt (Raines and Ross 1982; Ferrera and Henzel 1989; Raines and Ross 1992). Identification of FGF-1 in the 1.2 M fraction by radioreceptor and immuno assays is consistent with previously reported protocols (Lobb *et al.* 1986). N-terminal heterogeneity of tissue-derived FGF-1 is well described and

would account for the lower molecular mass of the whey-derived molecule observed in immunoblots compared to the recombinant FGF-1(1-154) standard (**Figure 5.4**; Gospodarowicz *et al.* 1987; Baird and Bohlen 1990). However, other heparin-binding factors may be present in the 1.2 M fraction as the BALB/c 3T3 cell growth response to this fraction could not be explained by the presence of FGF-1 alone (**Figure 5.2 A**).

To date, FGF-2 is the only known factor that requires 2.0 M salt for elution from a heparin-affinity column (Lobb *et al.* 1986; Klagsbrun 1992). The presence of FGF-2 in the 2.0 M whey-derived eluate was confirmed by immunoblotting (**Figure 5.4**). The presence of a 14 kDa band may reflect cleavage during the relatively harsh conditions of the cheese-making process. Although multiple forms of FGF-2 have been reported, they are generally of higher molecular mass and consistent with alternative translation sites (Brigstock *et al.* 1990). The concentration of FGF-2 in the 2 M eluate would account for the observed bioactivity in the BALB/c-3T3 cell-growth assay (**Figure 5.2 B**).

The detection of FGF-1 and FGF-2 in whey adds to the list of growth factors described in milk (Brown and Blakeley 1984; Francis *et al.* 1988; Jin *et al.* 1991). It is consistent with a previous report describing heparin-binding growth factors in bovine prepartum secretions (Sandowski *et al.* 1993). The source and significance of FGF in milk are unknown; assuming no losses during the isolation procedure, the concentration of FGF-2 in milk (19.8 ng/l) is within the physiologically active range (Gospodarowicz *et al.* 1987). Earlier studies showing expression of FGF is a feature of mammary gland fibroblasts and myo-epithelial cells, rather than the epithelial cells, would argue against a mammary gland origin (Barraclough *et al.* 1990; Ke *et al.* 1993). Indeed, the presence of soluble FGF is unusual and may be derived from lysis of the cellular component of milk during cheesemaking (Lee *et al.* 1980; Brooker *et al.* 1983; Saad and Ostensson 1990; Klagsbrun 1992).

# CHAPTER Six:

# Transforming Growth Factor-Beta in Bovine Whey

## 6.1. Introduction

Preliminary findings in **Chapter 3**, **Figures 3.3**, **3.12** and **3.13** indicate that whey extract contains a large amount of epithelial cell growth inhibitory material suggesting the presence of TGF-β. This factor has been identified in bovine milk and whey and its activity studied over a lactation (Tokyuyama and Tokuyama 1989; Cox and Burk 1991). However, the concentration of TGF-β in milk and whey is not known, nor its molecular weight form and stability.

TGF-β belongs to a supergene family of polypeptides that influence cell-growth, extracellular matrix production and development (Roberts and Sporn 1990). Three isoforms (TGF-β1 2 and 3) with similar *in-vitro*, but distinct *in-vivo* actions, have been described in mammalian species (Derynck *et al.* 1985; Seyedin *et al.* 1987; Cheifetz *et al.* 1987; Derynck *et al.* 1988; Cheifetz *et al.* 1990; Joyce *et al.* 1990). The primary structure of the TGF-βs found in mammals is highly conserved. The amino acid sequences of human and bovine TGF-β1, TGF-β2 and TGF-β3 are identical (Dernyck *et al.* 1987; Van Obberghen-Schilling *et al.* 1987; Seyedin *et al.* 1987; Cheifetz *et al.* 1987; ten Dijke *et al.* 1988; Graycar *et al.* 1989). TGF-β is expressed by cells in an inactive complex from which the mature TGF-β must be released before it can exert biologic effects in its environment. Latent TGF-β complexes can be activated *in-vitro* by heat, extreme pH, dissociating agents such as urea and sodium dodecyl sulphate, and by enzymes including plasmin (Lawrence *et al.* 1985; Wakefield *et al.* 1988; Lyons *et al.* 1988; Lyons *et al.* 1990; Miyazono *et al. 1*993). Activation of latent TGF-β complexes is one of the most important steps in regulating the actions of this factor *in-vivo*. Once released *in-vivo*, the active TGF-β is bound by several proteins and

proteoglycans, including $\alpha_2$-macroglobulin, betaglycan and decorin (Huang *et al. 1*988; Lamarre *et al. 1*994; Yamaguchi *et al. 1*990).

There are two types of latent TGF-β complexes released from producer cells, one larger than the other. The first is comprised of active TGF-β and its latency associated peptide (LAP) and is called 'the small latent complex' (Wakefield *et al.* 1989; Miyazono and Heldin 1991). The second is the LAP and the latent TGF-β binding protein (LTBP) and is termed the 'large latent complex' (Wakefield *et al.* 1988; Miyazano *et al.* 1988; Kanzaki *et al.* 1990). The LTBP was found to be covalently bound to the LAP by disulphide bond(s), but not to mature TGF-β and was not required for TGF-β latency. Furthermore, it was demonstrated that the association of TGF-β with LAP is sufficient to confer latency to mature TGF-β (Pircher *et al.* 1986; Gentry *et al.* 1987, *opcit* 1988). Large latent complexes are the predominant form of TGF-β found in platelets (Wakefield *et al.* 1988; Miyazono *et al.* 1988; Okada *et al.* 1989). They are also secreted by various cell lines that include human skin fibroblasts, bovine smooth muscle and endothelial cells (Kanzaki *et al.* 1990; Moren *et al.* 1994; Miyazono *et al.* 1991; Olofsson *et al.* 1992; Flaumenhaft *et al.* 1993b). Several cell lines and platelets produce multiple latent forms of TGF-β (Lioubin *et al. 1*991; Olofsson *et al. 1*992; Grainger *et al.* 1995). Significantly, small latent complexes of TGF-β have not been recovered from *in-vivo* sources.

The aims of the research undertaken in this current chapter are to investigate the form of TGF-β found in bovine milk and determine its contribution to the growth factor activity of milk. Prior results in **Chapter 3** suggest that a large proportion of TGF-β in whey extract requires acid treatment before it is active and that this TGF-β-like activity is an important mitogen for BALB/c 3T3 cells. In the current study, the concentration of TGF-β in several batches of milk, whey and whey extract will be determined. Whey

extract will be used to investigate the nature and stability of bovine milk-derived latent TGF-β. TGF-β will then be purified from whey extract to homogeneity and its contribution to the BALB/c 3T3 bioactivity of whey extract determined.

## 6.2 Materials and Methods

Pasteurised milk and whey were supplied by the Division of Food Science and Technology, Commonwealth Scientific and Industrial Research Organisation (CSIRO; Highett, Victoria, Australia). The production of whey extract from bovine cheese whey was carried out as previously described in section **2.2.1**. One litre of whey yielded 60 mg freeze-dried whey extract. Recombinant human TGF-β1 (TGF-β1) was obtained from Austral Biologicals, (San Ramon, California, USA) and monoclonal mouse anti-TGF-β was from Genzyme Corporation (Cambridge, Massachusetts, USA). Monoclonal mouse anti-alkaline phosphatase was obtained from Silenus Laboratories (Hawthorn, Melbourne, Australia). Heparin (sodium salt) was from Sigma Aldrich Pty Ltd (Castle Hill, New South Wales, Australia).

### 6.2.1 *TGF-β bioassay*

A cell-growth inhibition assay was performed according to the method of Absher *et al.* (1991), with some modifications. Mv1Lu cells were routinely cultured in Dulbecco's modified minimal essential Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) at 37°C in 5% (v/v) $CO_2$. Cells were plated at ($20 \times 10^3$/100 µl well) in 96-well microplates in DMEM with 10% (v/v) FBS and allowed to attach overnight. After a 2 h wash in serum free DMEM, diluted milk-derived samples, whey

fractions or TGF-β1 were added in a final volume of 100 µl containing 5% (v/v) FBS and incubated for a further 48 h. Seven dilutions of each sample were routinely assayed in triplicate. Standard references of DMEM containing 5% (v/v) FBS and DMEM alone were included on each plate. Cell mass was then measured after a further 48 h incubation by the dye-binding assay as indicated in **2.2.3**. Data are expressed as the percent of growth in DMEM containing 5% (v/v) FBS in the absence of TGF-β or test samples, with the growth in DMEM alone subtracted. TGF-β was quantified by comparing Mv1Lu cell-growth inhibition in response to milk-derived samples with that of a standard TGF-β1 using Tablecurve (Jandel Scientific, San Rafael, California, USA).

### 6.2.2 *Preparation milk and whey samples*

Pasteurised milk was centrifuged at 15,000 g for 1 h at 4°C and filtered through a 10 µm filter (Waters-Millipore, Rydalmere, New South Wales, Australia) under suction to remove residual lipids. The skimmed milk was then centrifuged at 30,000g for 1.5 h at 4°C. Latent TGF-β in milk and whey was activated at room temperature by drop wise addition of 5 M HCl to pH 2.0, then neutralised with 5 M NaOH and diluted with phosphate buffered saline (PBS; 1.47 mM $KH_2PO_4$, 12.5 mM $Na_2HPO_4$, 2.68 mM KCl, 136.9 mM NaCl, pH 7.3). All whey and milk samples were routinely filtered through 0.22 µm GV syringe filters before the bioassays (Waters-Millipore, Rydalmere, New South Wales, Australia).

### 6.2.3 *Stability of latent TGF-β in whey extract*

Whey extract obtained from cation-exchange chromatography of cheese whey was

exposed to acid, alkali, or heat prior to the Mv1Lu bioassay, as described by Lawrence (1991). In addition, both crude and acid-activated cationic whey extract were assayed in the presence of heparin. All samples were routinely filtered through 0.22 μm GV syringe filters before the bioassay (Waters-Millipore).

*pH treatment*: Whey extract was dissolved in sterile water at 26 mg/ml, and 5 M HCl or NaOH added drop-wise to pH 2.0 or 11 respectively. The protein solution was left at room temperature for 10 min, neutralised with 5 M HCl or NaOH, and the final concentration of protein made up to 25 mg/ml.

*Heat treatment*: Whey extract was made up to 25 mg/ml in sterile water before boiling for 2 minutes.

*Urea treatment*: Whey extract (25 mg/ml) was made up in 10 mM $NaH_2PO_4$ buffer (pH 7.0) containing 8 M urea, and allowed to stand at room temperature for 4 hours before assaying.

*Heparin treatment*: The stability of TGF-β present in whey was further examined by adding heparin (100 μg/ml) to dilutions of both crude and acid-activated whey extract immediately prior to the Mv1Lu bioassay.

### 6.2.4 *Gel-filtration*

Gel-filtration of whey extract was carried out using a Pharmacia Superose 6 HR 10/30 (1.0 x 30 cm) or Superose 12 HR 10/30 column (1.0 x 30 cm) coupled to an FPLC system (AMRAD Pharmacia Biotech, North Ryde, New South Wales, Australia). Neutral chromatography (pH 7.4) was carried out in a running buffer of 50 mM $NaH_2PO4$, 150 mM NaCl and 10% (v/v) acetonitrile. Acid gel-filtration (pH 2.0) was undertaken in a buffer containing 150 mM NaCl, 1 M acetic acid and 10% (v/v) acetonitrile. Whey extract was dissolved in the relevant running buffer at 25 mg/ml,

sterilised through a 0.22 μm GV syringe filter (Waters-Millipore) and 200 μl applied to the Superose column. The column was then eluted at a flow rate of 23 cm/hour (0.3 ml/min) and 30 fractions of 0.9 ml were collected and tested for TGF-β activity both before and after acid activation.

Fractions recovered from neutral Superose gel-filtration of whey extract that were found to contain latent Mv1Lu cell-growth inhibitory activity were then pooled and acid activated. This was by drop-wise addition of 5 M HCl to pH 2.0 and re-neutralising with 5 M NaOH after 10 minutes. The pooled activated fractions were then filter sterilised and a portion (200 μl) re-chromatographed as described for neutral size exclusion chromatography. Portions of collected fractions were tested for TGF-β activity.

Columns were calibrated under neutral conditions using α2-macroglobulin (720 kDa, Boeringer Mannheim Australia Pty Ltd, Castle Hill, NSW, Australia), thyroglobulin (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa) (AMRAD Pharmacia Biotech, North Ryde, New South Wales, Australia). The void volume (Vo) and total column volume (Vt) was determined with blue dextran (2,000 kDa AMRAD Pharmacia Biotech, North Ryde, New South Wales, Australia) and acetone respectively (BDH Laboratory Supplies, Kilsyth, Victoria, Australia).

### 6.2.5 *Purification of TGF-β*

TGF-β was purified from bovine cheese whey using a five-step chromatographic procedure (**Figure 6.1**). Each stage was followed by testing the ability of the fractions to inhibit the growth of Mv1Lu cells.

***A*** Whey extract was prepared from bovine cheese whey as described in **2.2.1**.

***B*** A portion of whey extract was then subject to acid gel-filtration using two Amicon Moduline columns (15 x 60 cm) operating in series, each filled with 10 l of Amicon Cellufine Media (Amicon Corp, Danvers, Massachusetts, USA), controlled by an FPLC system. The columns were pre-equilibrated with a running buffer of 150 mM NaCl, 10 mM HCl (pH 2.0). Whey extract (66g) was dissolved at 25 mg/ml, applied to the column, and eluted in the same running buffer at 23 cm/h (67 ml/min). A low molecular weight pool (less than 65 kDa by SDS PAGE), was collected and neutralised with 5 M NaOH and diafiltered against $H_2O$. This was done using an Amicon CH-2 ultrafiltration unit with a 3 kDa excluding membrane (Amicon, Danvers, Massachusetts, USA; Sartorius, Gottingen, Niedersachsen, Germany). The material was concentrated in the same device and then freeze-dried.

***C*** A second acid gel-filtration step was then performed using a Pharmacia Superdex 75 HR 35/600 size exclusion column (3.5 x 60 cm), equilibrated in a running buffer of 150 mM NaCl, 1 M acetic acid and 10 % (v/v) acetonitrile, pH 2.0 (AMRAD Pharmacia Biotech, North Ryde, New South Wales, Australia). 250 mg of the low molecular weight fraction from **Step *B*** was dissolved in the running buffer at 25 mg/ml, pumped on to the column, and eluted at 22 cm/h using an FPLC system. Forty 17.5 ml fractions were collected.

***D*** Fractions from ***C*** that contained TGF-β activity were pooled and then subject to reverse-phase HPLC on a Waters Deltapak C4 (25 x100 mm) column (Waters-Millipore, Rydalmere, New South Wales, Australia) equilibrated in 0.1% (v/v) TFA, pH 2.0. The pool of TGF-β from ***C*** was injected onto the C4 column and eluted with a linear gradient of 0-80 % (v/v) acetonitrile over 1 hour. Thirty fractions of 10 ml were collected.

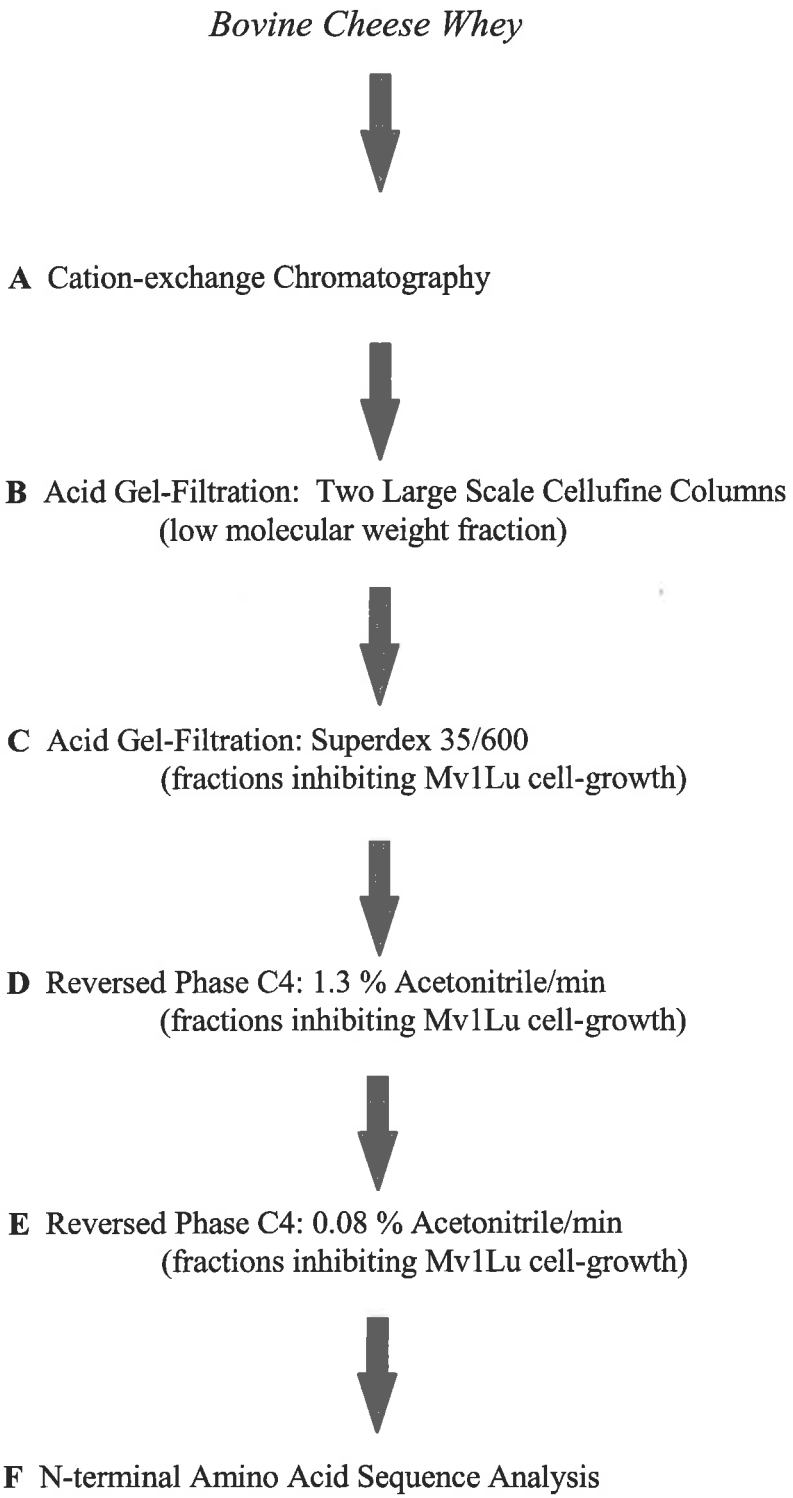
***E*** TGF-β containing fractions were then further diluted in 0.1% (v/v) TFA, re-applied to the C4 column, and eluted with a gradient of 0.08 % (v/v) acetonitrile per minute over three hours. Fractions of 10 ml (90) were collected.

***F*** A portion of the pooled TGF-β activity after ***E*** was freeze-dried and then subject to N-terminal sequence analysis as described in **6.2.7**.

### 6.2.6 *SDS-polyacrylamide gel electrophoresis*

The TGFβ-activity of whey extract was subject to SDS-polyacrylamide gel-electrophoresis (SDS-PAGE). Samples of the purified activity from **Figure 6.1** ***E*** were lyophilised and dissolved in either SDS-PAGE buffer or reducing SDS-PAGE buffer and subject to non-reducing or reducing electrophoresis respectively. PAGE was undertaken on a 8-25% polyacrylamide preformed slab gel using a Pharmacia Phast System (AMRAD Pharmacia Biotech, North Ryde, New South Wales, Australia). The SDS-PAGE buffer contained 5% (wt/v) SDS, 100 mM Tris, and 5% (wt/v) bromophenol blue, pH 8.0; the reducing buffer also contained dithiothreitol (1.5%) (Sigma Aldrich Pty Ltd, Castle Hill, New South Wales, Australia). Phast-gel molecular weight standards (AMRAD Pharmacia Biotech) and a TGF-β1 standard (Austral Biologicals, San Ramon, California, USA) was also made up in both non-reducing and reducing sample buffer. All samples were heated for 5 minutes at 95°C, and 4 µl of each sample were loaded on to 8-25% polyacrylamide preformed gels and subject to SDS-PAGE according to the manufacturers instructions (AMRAD Pharmacia Biotech, North Ryde, New South Wales, Australia). The gels were then silver stained (Merril *et al.* 1984).

**Figure 6.1: Purification of TGF-β from bovine cheese whey**



### 6.2.7 *Sequence analysis*

The amino acid sequence was analysed by Edman degradation with an automated gas phase sequencer (Hewlett-Packard G1000A, Hewlett-Packard Company Corvallis, Oregon, U.S.A; (Hunkapiller *et al. 1*983)).

### 6.2.8 *Protein measurements*

Protein concentrations were determined with reference to bovine serum albumin (Sigma Aldrich Pty Ltd, Castle Hill, New South Wales, Australia) by the method of Lowry *et al.* (1951) or by a bichinoninic acid-binding assay as described in the manufactures instructions (Pierce, Rockford, Illinios, USA).

### 6.2.9 *Neutralising TGF-β bioactivity*

A TGF-β antibody was used to neutralise the Mv1Lu cell growth inhibitory activity in whey. The antibody was a monoclonal antibody that recognises TGF-β1, -β2 and -β3 and can neutralise their activity in Mv1Lu cells (Genzyme Corp., Cambridge, Massachusetts, USA; Dasch *et al.* 1989). Initially, anti-TGF-β was tested on a recombinant human TGF-β1 standard (Austral Biologicals, San Ramon, California, USA) in the Mv1Lu bioassay in 96-well plates described in **6.2.1**. In a preliminary experiment, several concentrations of anti-TGF-β were tested on diluted TGF-β1 standard and after 48 h incubation, the cell mass was quantified as indicated in **6.2.1** Six μg/ml of anti-TGF-β was found to block 2-3 ng/ml of TGF-β activity in Mv1Lu cells.

The bioactivity of whey fractions and the TGF-β1 standard in Mv1Lu assays were then subject to 6 μg/ml of neutralising anti-TGF-β. Mv1Lu cells were subcultured in DMEM/10% (v/v) FBS in 96-well plates as described in **6.2.1** and washed for 2 h in DMEM. The whey fractions and the TGF-β standard were diluted in DMEM/5% (v/v) FBS and added to wells in the presence and absence of anti-TGF-β (6 μg/ml) in a final volume of 100 μl. Control wells consisted of a control antibody (anti-alkaline phosphatase) with and without TGF-β1, DMEM alone, DMEM/6 μg/ml anti-TGF-β, and DMEM/5% (v/v) FBS. All treatments were assayed in triplicate and after 48 h incubation, the increase in cell mass in the 96-well plates was quantified by the methylene blue binding assay described in **6.2.1**. Data are expressed as the percentage of cell mass response observed in 5% (v/v) FBS, with the growth in DMEM alone subtracted. TGF-β concentration was quantified by comparing Mv1Lu cell-growth inhibition in response to whey-derived samples with that of standard TGF-β1, using Tablecurve (Jandel Scientific, San Rafael, California, USA).

The ability of anti-TGF-β to neutralise the BALB/c 3T3 cell growth activity in whey fractions was examined using the cell growth assay described in **2.2.3**. Initially, anti-TGF-β was tested on recombinant human TGF-β1 standard (Austral Biologicals, San Ramon, California, USA) in the 96-well plate BALB/c 3T3 cell growth assays described in **2.2.3**. Ten μg/ml was found to neutralise the bioactivity of 5 ng/ml of recombinant TGF-β1. Whey fractions and a TGF-β standard were then subject to anti-TGF-β (10 μg/ml) in the cell bioassay. The samples were diluted in DMEM/0.1% (wt/v) BSA and added to wells in the presence and absence of anti-TGF-β (10 μg/ml) in a final volume of 100 μl. The assays also incorporated a control antibody (anti-alkaline phosphatase) with and without TGF-β1, DMEM/0.1% (wt/v) BSA alone, DMEM/0.1% (wt/v) BSA

with 10 μg/ml anti-TGF-β, and DMEM containing 10% (v/v) FBS. PDGF-BB (10 μg/ml) was also incubated with anti-TGF-β to show the specificity of the antibody. All treatments were assayed in triplicate and after 48 h, the increase in cell mass in the 96-well plates was quantified by the methylene blue binding assay as indicated in **2.2.3**. Data are expressed as the percentage of cell mass response observed in 10% (v/v) FBS with the growth in DMEM/0.1% (wt/v) BSA subtracted.

The data obtained from neutralising Mv1Lu cell bioactivity in whey and neutralising BALB/c 3T3 cell growth activity in whey were each examined by analysis of variance. An F-test was used to compare the results obtained from treatments and when this showed a significant difference, means were subject to the standardised student's t-test. When there was only two treatments, they were compared using a simple student's t-test (Miller *et al.* 1990).

## 6.3 Results

### 6.3.1 *Latent TGF-β like activity in bovine milk and whey*

The concentration of TGF-β in milk was determined by comparing Mv1Lu cell growth inhibition in the presence of defatted bovine milk samples to a TGF-β1 standard (**Table 6.1**). Transient acidification, a process that results in precipitation of the casein component of milk, was found to increase the Mv1Lu cell-growth inhibitory activity in the acid whey fraction (**Table 6.1**). Accordingly, commercial whey was then evaluated as a source of TGF-β. The concentration of bioactive TGF-β in whey was

increased 12-13 fold after transient acidification (**Table 6.1** and **Figure 6.2**). Their appears to be no notable difference between the concentration of acid-activated TGF-β in skim milk whey and commercial cheese whey. However, the cell-growth inhibition curve in response to dilutions of non-acidified raw milk was not parallel to that produced by the TGF-β1 standard (data not shown), indicating interference in the assay by components present in milk. In contrast, the Mv1Lu cell-growth inhibition curve produced in response to acid-activated whey is parallel to the TGF-β1 standard (**Figure 6.2**).

**Table 6.1 TGF-β activity in bovine milk and cheese whey**

| | **Bovine skim milk (n=5)** | | **Bovine cheese whey (n=9)** | |
|---|---|---|---|---|
| **TGF-β** | ***no acid*** | ***acidified*** | ***no acid*** | ***acidified*** |
| ng/ml | 1.8 ± 0.3 | 4.3 ± 0.9 | 0.3 ± 0.08 | 3.7 ± 0.7 |
| ng/mg (protein) | 0.1 ± 0.01 | 0.7 ± 0.05 | 0.05 ± 0.01 | 0.7 ± 0.04 |

Samples of milk and cheese whey were tested for TGF-β activity before and after transient acidification using the Mv1Lu cell growth inhibition assay (**6.2.1**). For each experiment, seven dilutions of the sample were assayed in triplicate and the mean TGF-β concentration determined. Values represent the mean ± S.E.M of the indicated number of experiments.

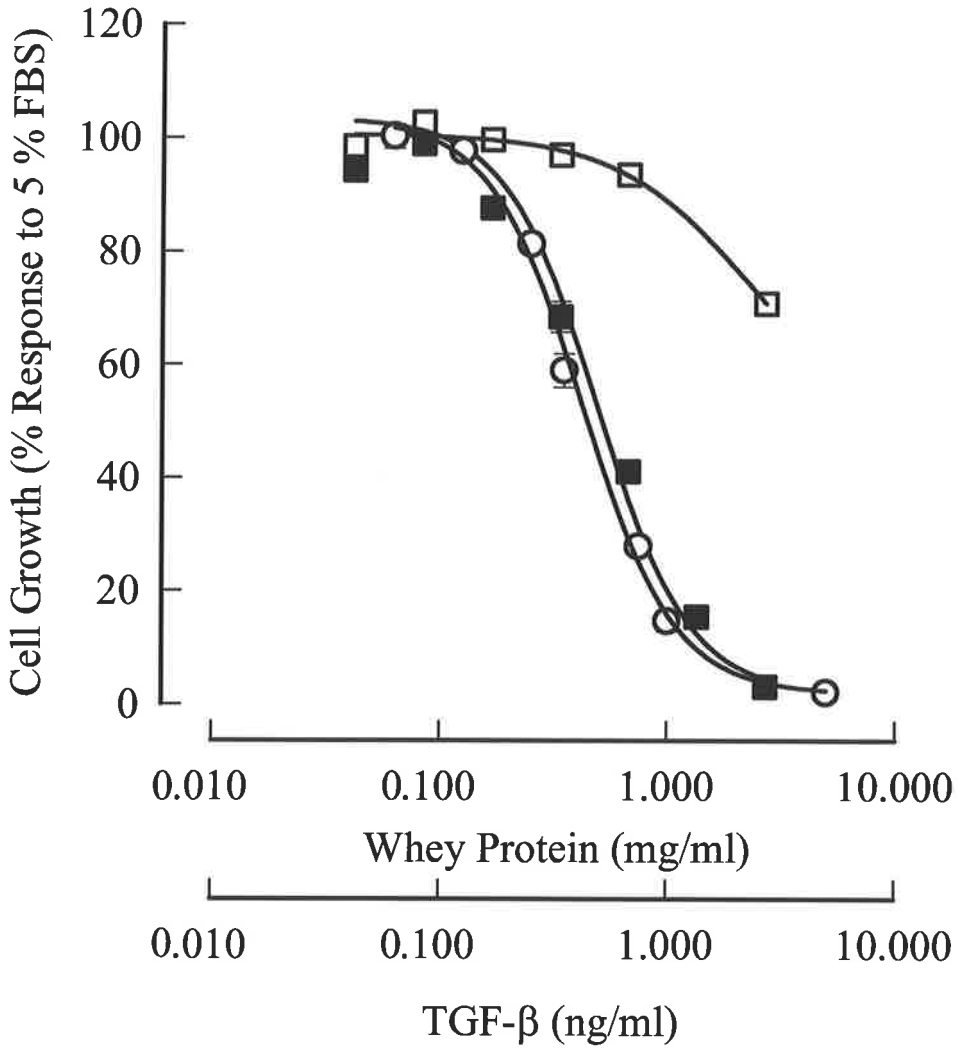



**Figure 6.2** Inhibition of Mv1Lu cell growth by untreated (□) and acidified (■) bovine cheese whey in comparison to TGF-β1 (O). Crude and acidified whey, and a TGF-β1 standard were diluted in PBS and assayed in Mv1Lu cells in the presence of 5% FBS as described in **6.2.1**. The response is expressed as a percentage of cell growth in 5% FBS, each point representing the mean ± S.E.M. of triplicate cultures. The growth response to DMEM alone has been subtracted.

### *6.3.2 Latent TGF-β in whey extract*

The results in **6.3.1** show that the concentration of TGF-β in milk or whey is too low to permit further investigation of its stability and form. However, as indicated in **Chapter 3**, the cation-exchange fraction of commercial cheese whey (whey extract) contains concentrated latent TGF-β activity. Indeed, **Figures 3.3**, **3.12** and **3.13** indicate that whey extract contains a large amount of epithelial cell growth inhibitory activity after it was acid-treated, suggesting the presence of TGF-β. In a series of 4 separate experiments, 2.5 ± 0.2 ng of total TGF-β/ml whey bound to the cation-exchange resin and was eluted with 0.5 M NaCl (whey extract), compared to 0.05 ± 0.03 ng total TGF-β/ml in the column flow through. Whey extract was used in all further experiments on TGF-β in bovine whey.

### 6.3.3 *Stability of latent TGF-β*

Stability of whey-derived latent TGF-β was examined by exposing solutions of whey extract to acid, alkali, 8 M urea and heat. **Table 6.2** shows that latent TGF-β activity in the Mv1Lu cell-growth inhibition bioassay was increased by all treatments, although acidification resulted in the most consistent activation. In a separate series of experiments, addition of heparin (100 μg/ml) to dilutions of the neutral and acidified whey extract increased TGF-β activity by 1.9 fold and 1.6 fold respectively (**Table 6.3**). Notably, the activity of dilutions of a TGF-β1 standard were also increased 1.7 fold by the inclusion of heparin in the tissue culture medium (data not shown).

**Table 6.2 Stability of whey-derived latent TGF-β**

| Treatment | TGF-β (ng/mg) |
|---|---|
| None (n=9) | 0.8 ± 0.1 |
| Acid (n=9) | 42.2 ± 6.8 |
| Alkali (n=3) | 26.0 ± 6.0 |
| Heat (n=2) | 9.07 ± 3.0 |
| Urea (n=3) | 52.0 ± 17.0 |

Latent TGF-β in whey was concentrated by cation exchange chromatography as described in section **2.2.1**. One litre of whey yielded 60 mg freeze-dried whey extract. Whey extract was resuspended in the relevant buffer (see **6.2.3**) before exposure to acid (pH 2.0), alkali (pH 11), urea (8 M), or heat (100° C). TGF-β activity was measured by comparing the ability of the treated fractions to inhibit growth of Mv1Lu cells to TGF-β1 standard (**6.2.1**). For each experiment, 7 dilutions of each treatment group were assayed in triplicate and the mean TGF-β activity determined. Values are the mean ± S.E.M of the indicated number of experiments.

**Table 6.3 Effect of heparin on whey-derived TGF-β activity**

| | TGF-β (ng/mg) | |
|---|---|---|
| | ***no heparin*** | ***with heparin*** |
| Neutral whey extract | 0.15 ± 0.03 | 0.28 ± 0.05 |
| Acid-activated whey extract | 55.8 ± 7.6 | 85.6 ± 18.6 |

Crude and acid-activated whey extract were prepared as described in **6.2.3** and assayed in the presence and absence of heparin (100 μg/ml). Seven dilutions of each treatment group were assayed in triplicate and the mean TGF-β activity determined (**6.2.1**). Values are the mean ± S.E.M of six experiments.

### 6.3.4 *Fractionation of latent TGF-β by gel-filtration*

The nature of the latent TGF-β complex in whey was further investigated by gel-filtration chromatography. Samples of whey extract were subject to both neutral and acid Superose 6 and 12 size exclusion chromatography and TGF-β activity in the recovered fractions measured using the Mv1Lu bioassay. Where gel-filtration was undertaken under neutral conditions, the fractions were assayed both before and after acidification. **Figure 6.3 A** shows the elution profile and TGF-β activity of the cationic whey extract after neutral Superose 6 chromatography. TGF-β activity in untreated fractions was less than 5% of the activity observed after transiently acidifying portions of these fractions prior to bioassay. The major peak of activity eluted with an apparent molecular weight of 80 kDa. A smaller peak of TGF-β activity was detected at an elution volume corresponding to 600 kDa. Acid Superose 6 gel-filtration of the cationic whey extract produced the bioactivity profile shown in **Figure 6.3 B**. TGF-β activity was detected in the low molecular mass region, consistent with the release of the latency associated peptide (LAP), and the appearance of the active molecule. No activity was retained in either high molecular weight complex. Retention of active TGF-β on the Superose column was greater than expected for the 25 kDa TGF-β homodimer.

To confirm the molecular weight of the latent whey-derived TGF-β complex, and to exclude the possibility that the low molecular weight activity shown in **Figure 6.3 B** represented retention of a high molecular mass complex under acidic conditions, the elution volume of acid activated high molecular weight TGF-β activity was accurately determined under neutral conditions using a Superose 12 column (**Figure 6.4 A, B**).

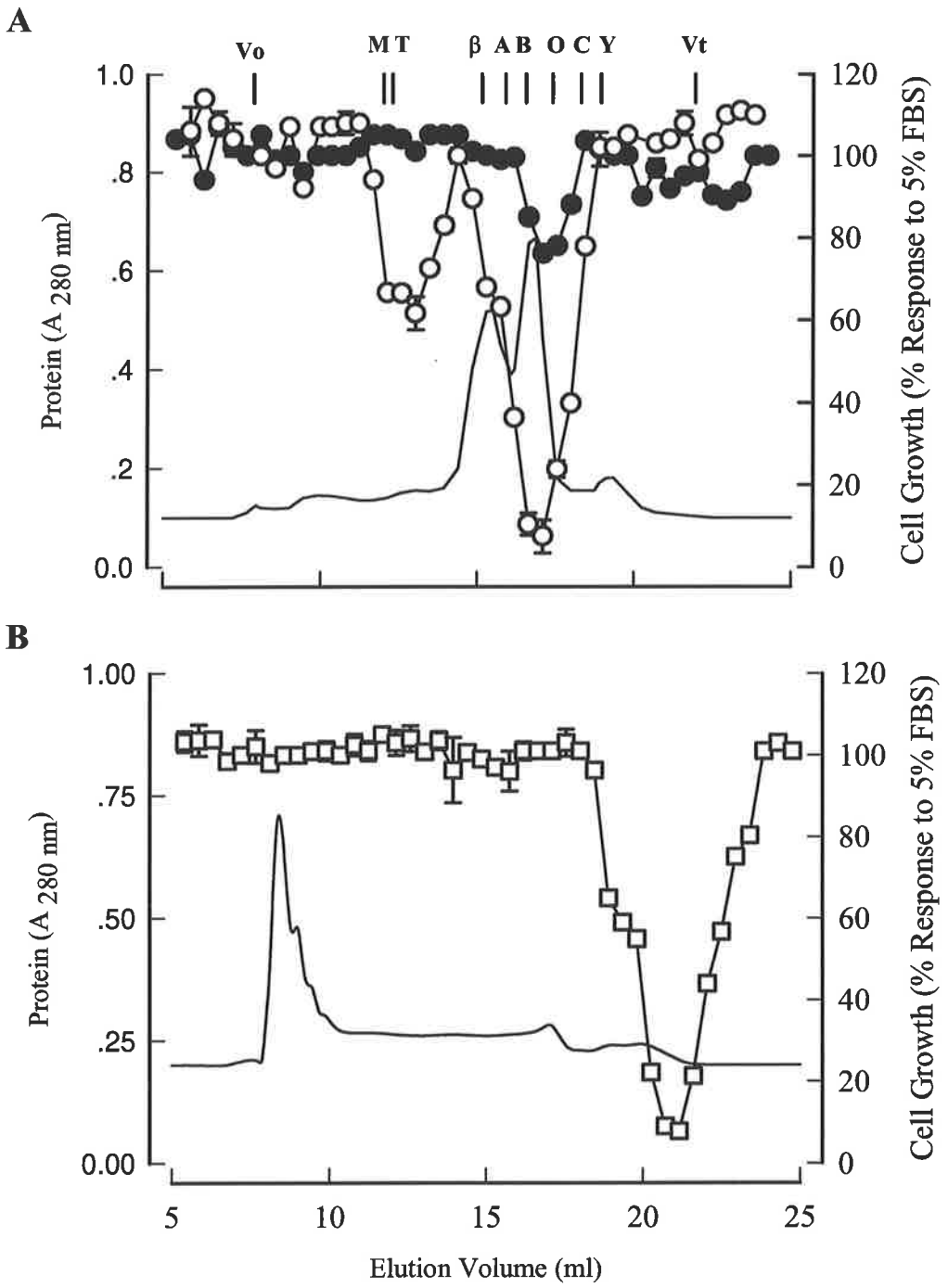



**Figure 6.3** TGF-β activity in neutral and acid Superose 6 gel-filtration fractions of whey extract. A 5 mg sample of whey extract was chromatographed under neutral (**A**) or acidic (**B**) conditions as described in **6.2.4**, and each fraction tested for inhibition of Mv1Lu cell growth in the presence of 5% FBS (**6.2.1**). Neutral fractions (**A**) were assayed both before (●) and after (O) transient acid treatment. Acid fractions (**B**) were tested without further treatment (□). Mv1Lu growth is expressed as a percentage of the 5% FBS response, each point representing the mean ± S.E.M. of triplicate determinations. The growth response to DMEM alone has been subtracted. The column was standardised under neutral conditions (α2-macroglobulin (M; 720 kDa), thyroglobulin (T; 669 kDa), β-amylase (β; 200 kDa), alcohol dehydrogenase (A; 150 kDa), bovine serum albumin (B; 67 kDa), ovalbumin (O; 45 kDa), carbonic anhydrase (C; 29 kDa) and cytochrome c (Y;12.4 kDa)). The void volume (Vo) and Vt were determined using blue dextran (2000 kDa) and acetone respectively.

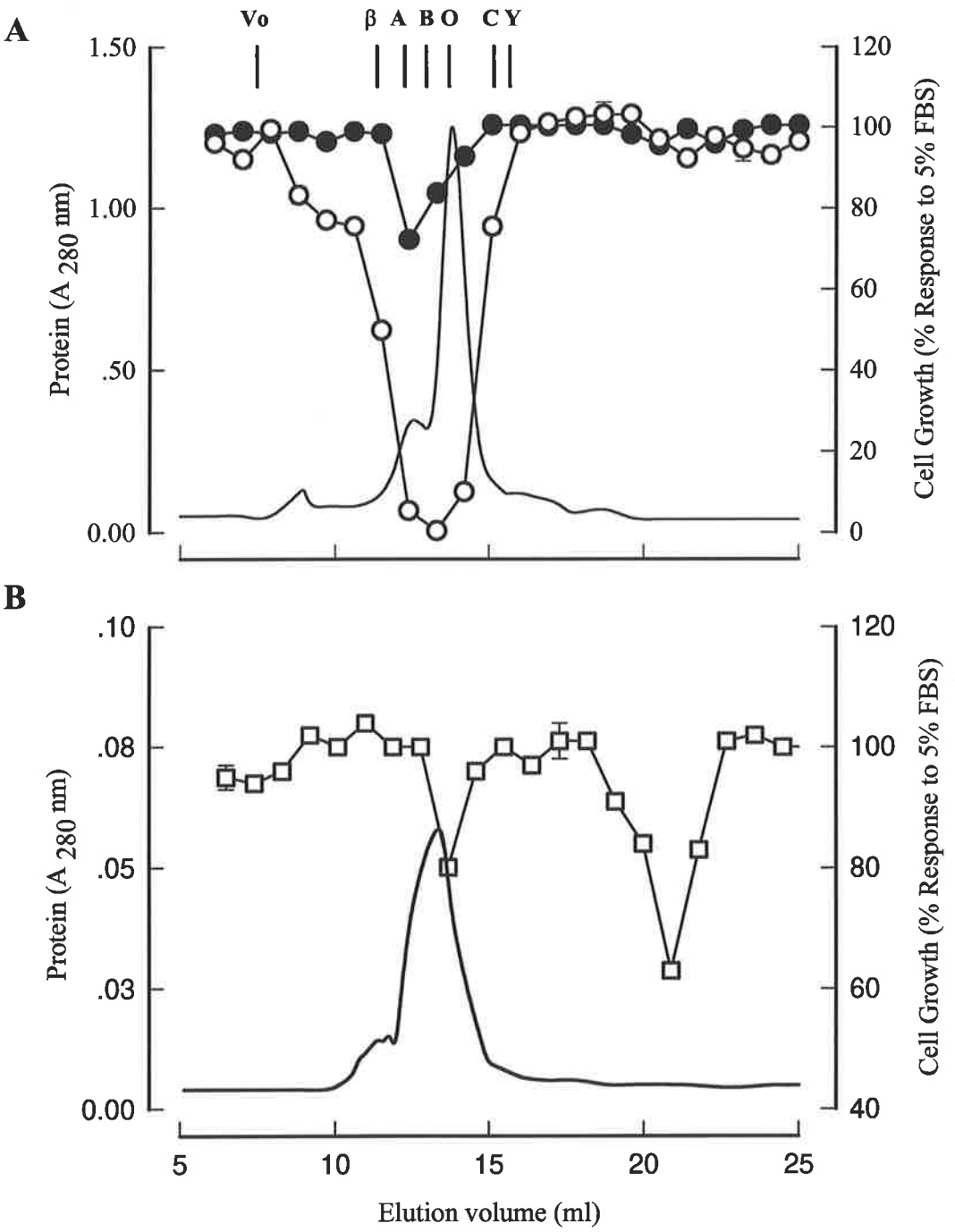



**Figure 6.4**. TGF-β activity in Superose 12 gel-filtration fractions of neutral and acid treated whey extract. (**A**) A 5 mg sample of whey extract was chromatographed under neutral conditions (**6.2.4**), and each fraction tested for ability to inhibit Mv1Lu cell growth in the presence of 5% FBS (**6.2.1**). Fractions were assayed both before (●) and after (O) transient acid treatment. (**B**) A portion of the pooled fractions containing latent activity, corresponding to an elution volume of 11-13 ml in Figure **A**, was transiently acidified, neutralised and re-chromatographed under neutral conditions (□). Mv1Lu growth is expressed as a percentage of the 5% FBS response, each point representing mean ± S.E.M. of triplicate determinations. The growth response to DMEM alone has been subtracted. The column was standardised with β-amylase (β; 200 kDa), alcohol dehydrogenase (A; 150 kDa), bovine serum albumin (B; 67 kDa), ovalbumin (O; 45 kDa), carbonic anhydrase (C; 29 kDa) and cytochrome C (Y;12.4 kDa). The void volume (Vo) was determined using blue dextran (2000 kDa).

Although the Superose 12 column did not resolve the 600 kDa peak, the major peak of latent activity again eluted with an apparent molecular weight of 80 kDa (**Figure 6.4 B**). A portion of the high molecular weight TGF-β peak, corresponding to an elution volume of 12-14 ml in **Figure 6.4 A**, was then transiently acidified and re-chromatographed under neutral conditions. Under these conditions over 85 % of TGF-β activity chromatographed at a low molecular weight, corresponding to an elution volume of 20-22 ml (**Figure 6.4 B**). Again, retention on the column was greater than expected for the active 25 kDa TGF-β homodimer. Some 15% of the bioactivity was eluted in a position corresponding to an apparent molecular mass of 80 kDa, suggesting re-association of TGF-β with LAP under neutral conditions.

### 6.3.5 *Purification of TGF-β*

To establish that the Mv1Lu cell-growth inhibitory activity of bovine whey could be accounted for by TGF-β alone, the measured activity was purified by the five step procedure outlined in **Figure 6.1**. A 66 g of batch of whey extract was prepared from microfiltered cheese whey and subjected to two acid gel-filtration steps followed by reverse-phase HPLC steps. The final reverse phase HPLC step yielded two peaks of Mv1Lu growth inhibitory activity (**Figure 6.5**) which chromatographed at 30-31% and 37-38% acetonitrile.

The bulk of TGF-β activity eluted in the second peak (**Peak B**), which was identified as bovine TGF-β2 by N-terminal sequencing (ALDAAYCFRN; Cheifetz *et al.* 1987). **Table 6.4** summarises the results of the purification. Approximately 33 μg of TGF-β2-like activity was recovered from 5940 g of cheese whey and the specific activity of 616197 ng/mg indicated it was around 62% pure. The overall purification of TGF-β2

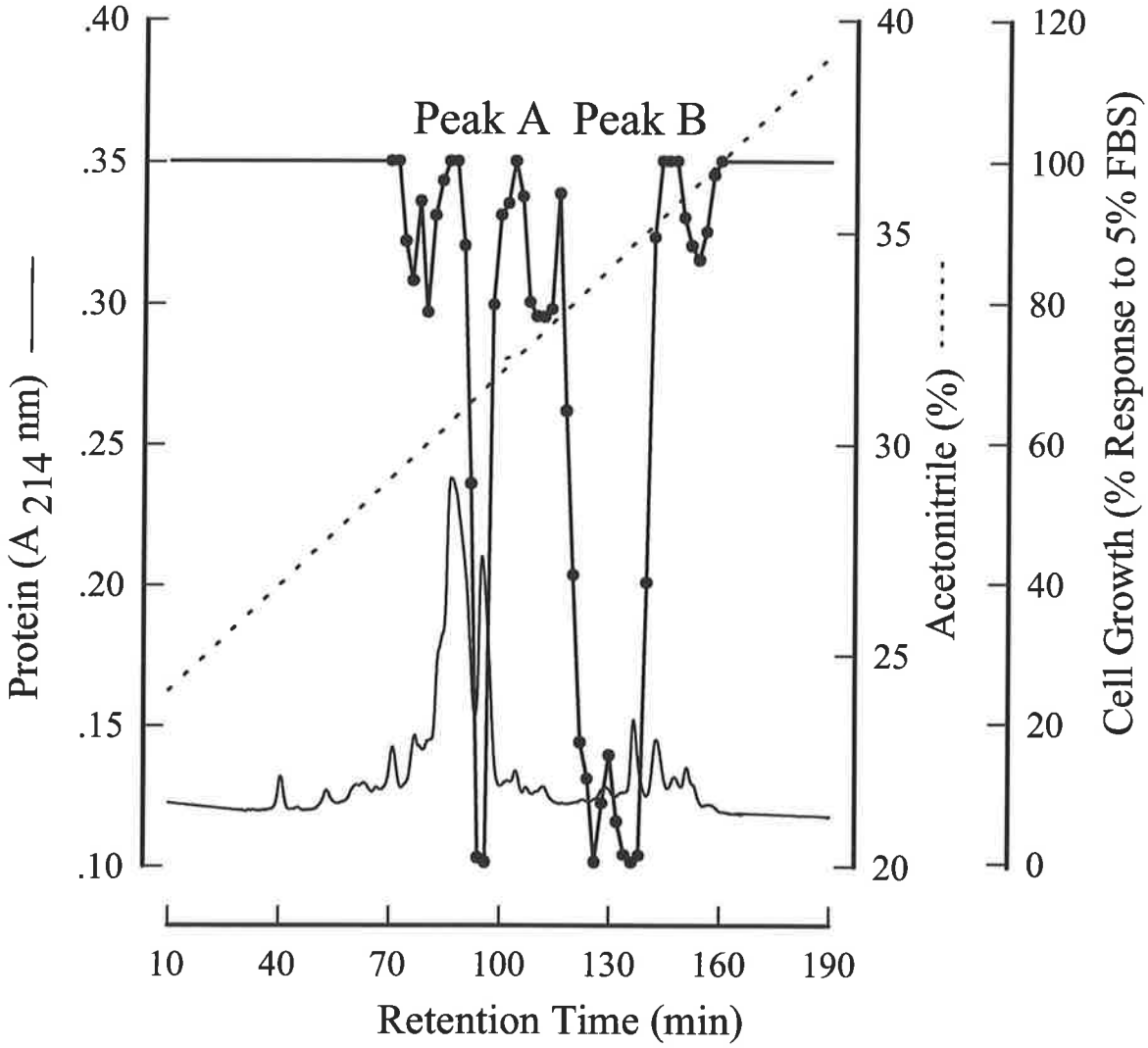



**Figure 6.5** **Step *E*** of the purification of TGF-β from bovine cheese whey **(Figure 6.1)**. Active fractions from **Step *D*** were pooled, diluted in 0.1% TFA, and pumped over a C4 reverse phase column which was eluted with a gradient of 0.08 % acetonitrile/min (---; **6.2.5**). The fractions were assayed for TGF-β activity (**6.2.1**) by measuring their ability to induce Mv1Lu cell growth in 5% FBS (●), each point representing the mean of triplicate determinations. An N-terminal sequence identical to bovine TGF-β2 was obtained from **Peak B**.

## Table 6.4 Purification of TGF-β from bovine cheese whey

| Purification Step | Protein (mg/ml) | Total Protein (mg) | Total Activity (ng) | Specific Activity (ng/mg) | Recovery (%) | Relative Purification Fold |
|---|---|---|---|---|---|---|
| **Cheese whey** | 5.4 | 5940000 | 4070000 | 0.70 | 100 | 1 |
| **Cation Exchange** | 25.0 | 66000 | 594000 | 9.0 | 15 | 13 |
| **Step *B*** | 25.0 | 7000 | 511000 | 73 | 12 | 107 |
| **Step *C*** | 3.66 | 3100 | 465360 | 151 | 11 | 222 |
| **Step *D*** | 0.13 | 130 | 408639 | 3220 | 10 | 4700 |
| **Step *E* (Peak A)** | 0.05 | 15 | 35454 | 2302 | 1 | 3385 |
| **Step E (Peak B)** | 0.0004 | 0.5 | 333144 | 616197 | 8 | 906170 |

TGF-β was isolated from bovine cheese whey as described in **6.2.5**. Mv1Lu bioactivity was measured in the fractions collected at each step (**6.2.1**) and the active fractions pooled and assayed in triplicate over 7 dilutions. TGF-β activity was then quantified by comparing the pooled fractions Mv1Lu cell-growth inhibitory activity with that of standard TGF-β1. Protein was measured by a dye binding assay (**6.2.8**) and the total and specific TGF-β activity at each step in **Figure 6.1** determined. Two peaks of activity were recovered from the final purification step (see **Figure 6.5**).

was 906170-fold with a recovery of 0.8%. Almost 4 μg of TGF-β-like activity was recovered from **Peak A** of the final HPLC step (**Figure 6.5**). Previous results reported by Jin *et al.* (1991), suggest this material may be TGF-β1. In the current experiments, this material was only 10-20% of the total TGF-β activity recovered from cheese whey and the specific activity of 2302 ng/mg indicated it was only 2.3% pure.

A portion of **Peak A** (300 ng) and **Peak B** (140ng) shown in **Figure 5** and recombinant TGF-β1 (150 ng; Austral Biologicals) were subject to reducing and non-reducing SDS-PAGE. The sample from **Peak B** that contained TGF-β2 (**Figure 6.6 A, lane 3**) resolved under non-reducing conditions at a apparent molecular weight of 25 kDa similar to that of the TGF-β1 standard (**lane 1**). When the TGF-β2 sample (**Peak B**) and the TGF-β1 standard were dissociated with dithiothreitol only single bands were visible near 14 kDa (**Figure 6.6 B, lane 3**). This indicates it was a dimmeric protein that behaved identically to recombinant TGF-β1 in gel-electrophoresis. The relatively impure sample of **Peak A** resolved into numerous bands under non-reducing conditions (**Figure 6.6 A, lane 4**), and then into two main bands at 25 kDa and 14 kDa under reducing conditions (**Figure 6.6 B, lane 4**).

### 6.3.6 *Neutralisation of TGF-β bioactivity*

To show that the Mv1Lu inhibitory activity measured in whey was accounted for by TGF-β alone, a monoclonal anti-TGF-β was used to immuno-neutralise the *in-vitro* activity of TGF-β. Preliminary experiments demonstrated that a concentration of 6 μg/ml of monoclonal anti-TGF-β was required to neutralise 2-3 ng of recombinant TGF-β1 activity in Mv1Lu cells. A control antibody of the same immunoglobulin subclass (monoclonal; anti-alkaline phosphatase Immunoglobulin $G_1$) did not affect the

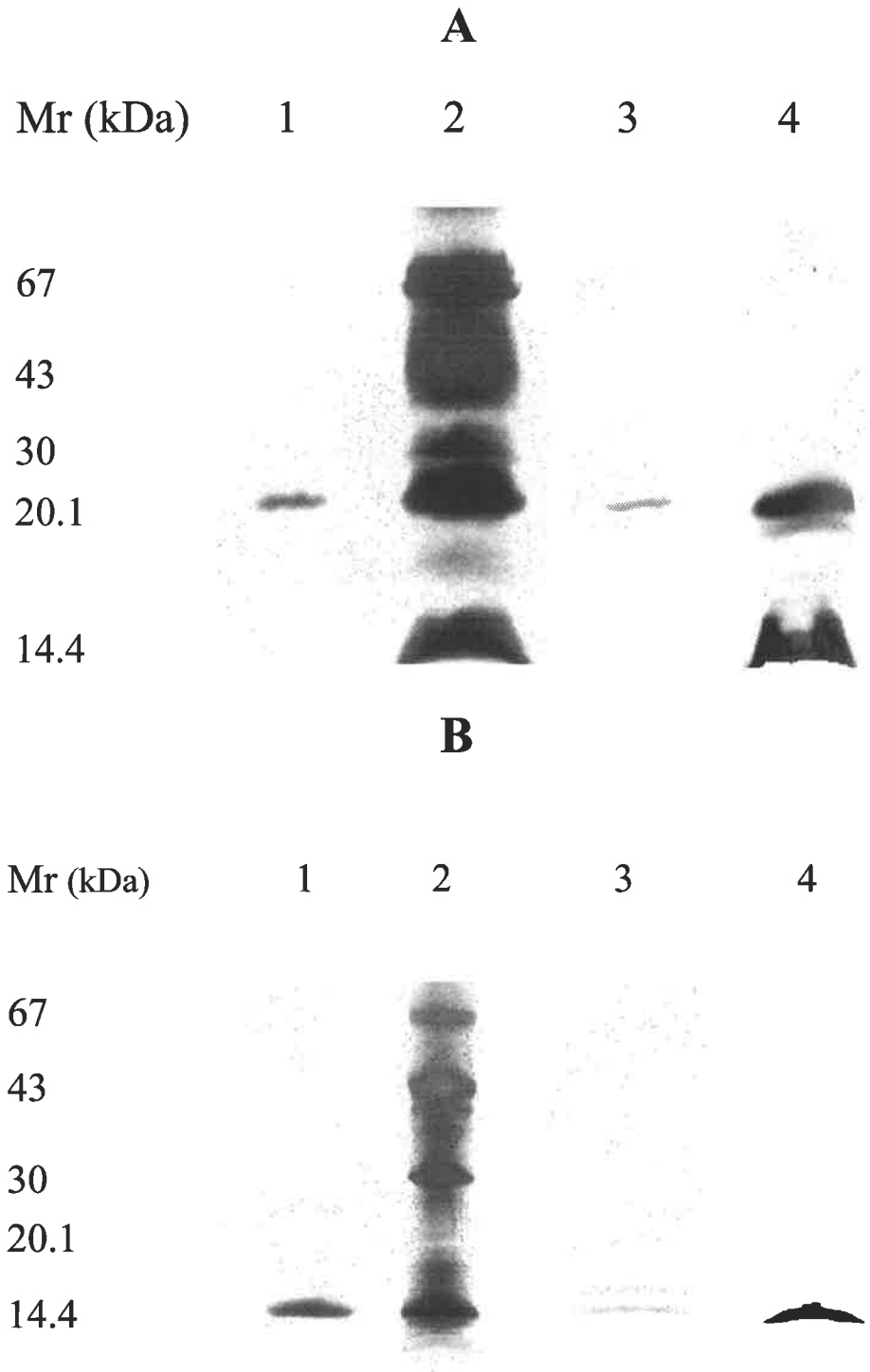



**Figure 6.6** Non-reducing and reducing SDS-PAGE of TGF-β from bovine whey. TGF-β was purified from bovine whey as described in **6.2.5,** with the final reverse phase HPLC producing two peaks of TGF-β activity (**Figure 6.5: Peak A and Peak B**). Samples of **Peak A** (300ng; **lane 4**), **Peak B** (140 ng; **lane 3**), recombinant TGF-β1 (150 ng; **lane 1**) and molecular weight markers (500 ng; **lane 2**) were subject to SDS-PAGE under non-reducing (**A**) and reducing (**B**) conditions on preformed slab gels and then silver stained (**6.2.6**; Merril *et al.* 1984). The positions of the molecular weight markers (**Mr** in **kDa**) are indicated (bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and .α-lactalbumin (14.4 kDa)).

inhibition of Mv1Lu cells by TGF-β1 (see **Figure 6.7**). The Mv1Lu inhibitory activity in acid-treated whey, whey extract and acid-treated whey extract was neutralised by inclusion of anti-TGF-β in the medium (**Figure 6.7**). Notably, acidified but not neutral whey was affected by the antibody. The MV1Lu activity of both the whey extract and acid-treated whey extract were neutralised by anti-TGF-β. Indeed, the bioactivity of whey extract at all stages of TGF-β purification was susceptible to immuno-neutralisation by this antibody (**Table 6.5**).

The ability of anti-TGF-β to neutralise TGF-β activity in BALB/c 3T3 cells was also examined. TGF-β alone (5 ng/ml) stimulated an increase in BALB/c 3T3 cell growth, which was significantly inhibited by 10 μg/ml of anti-TGF-β but not a control antibody at the same concentration. A higher concentration of TGF-β (50 ng/ml) was only partially neutralised by this level of anti-TGF-β (**Figure 6.8**). Ten μg/ml of anti-TGF-β inhibited the BALB/c 3T3 cell growth response to 5.0 mg/ml of whey extract but not 0.5 mg/ml of whey extract (**Figure 6.8**). Anti-TGF-β at the same concentration (10 μg/ml) significantly decreased the cell growth response produced by both 0.5 and 0.1 mg/ml of acid-treated whey extract. The cell growth response produced by 10 ng/ml PDGF-BB was not affected by anti-TGF-β (data not shown).

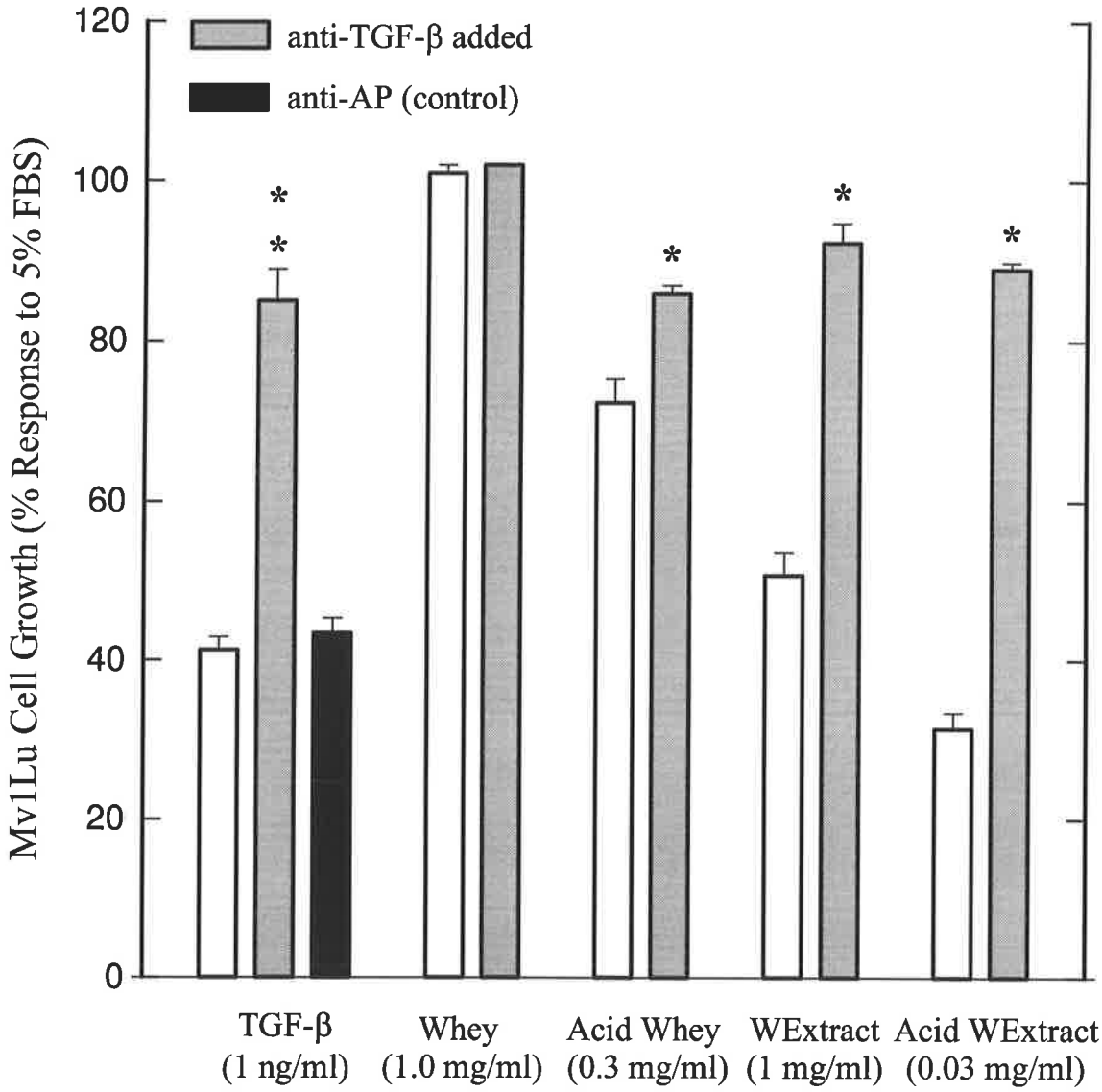



**Figure 6.7** Neutralising the Mv1Lu cell growth inhibitory activity of whey. Whey and acidified whey were prepared as described in **6.2.2**; whey extract (**WExtract**) and acidified whey extract (**Acid WExtract**) processed as shown in **6.2.3**. These and a TGF-β1 standard were diluted in DMEM/5% FBS and assayed in Mv1Lu cells in the presence and absence of 6 μg/ml of **anti-TGF-β** as described in **6.2.9**. The TGF-β1 standard was also tested with 6 μg/ml of a control monoclonal antibody (alkaline phosphatase) of the same subclass ($IgG_1$; **anti-AP**). The response is expressed as a percentage of cell growth in 5% FBS, with the response in DMEM alone subtracted (**6.2.1**). Dilutions of each treatment were assayed in triplicate and the values represent the mean ± S.E.M. of three experiments. ** $p<0.05$ anti-TGF-β added to TGF-β1 vs no antibody; * $p<0.05$ vs no antibody.

**Table 6.5 Neutralising the effect of TGF-β purified from bovine whey**

| Purification Step | Specific Activity | TGF-β Activity | |
|---|---|---|---|
| | | - aTGF-β | + aTGF-β |
| | (ng/mg) | (ng/ml) | (ng/ml) |
| **1. Cheese whey** | 0.70 | 3.8 ± 0.7 | 1.5 ± 0.6 |
| **2. Cation Exchange fraction** | 9.0 | 3.0 ± 0.4 | 0.1 ± 0.06 |
| **3. Gel-filtration (*B*)** | 73 | 5.6 ± 0.9 | 0.9 ± 0.1 |
| **4. Gel-filtration (*C*)** | 151 | 1.8 ± 0.05 | 0.2 ± 0.1 |
| **5. C4 HPLC (*D*)** | 3220 | 3.6 ± 0.2 | 0.6 ± 0.06 |
| **6. C4 HPLC (*E*)** | | | |
| **(Peak A)** | 2302 | 1.7 ± 0.1 | 0.07 ± 0.05 |
| **(Peak B)** | 616197 | 4.4 ± 0.5 | 0.4 ± 0.1 |

TGF-β was isolated from bovine cheese whey as described in **6.2.5** and the recoveries described in **Table 6.4**. Dilutions of the pool obtained from each step were incubated in the presence and absence of 6 μg/ml of neutralising antibody (**+aTGF-β**, **-aTGF-β**; **6.2.9**). TGF-β activity was measured by comparing the ability of each fraction to inhibit Mv1Lu growth to TGF-β1 (**6.2.1**, mean ± S.E.M of triplicate measurements). Two peaks of activity were recovered from the final purification step (see **Figure 6.5**). Protein was measured by a dye binding assay described in **6.2.8**.

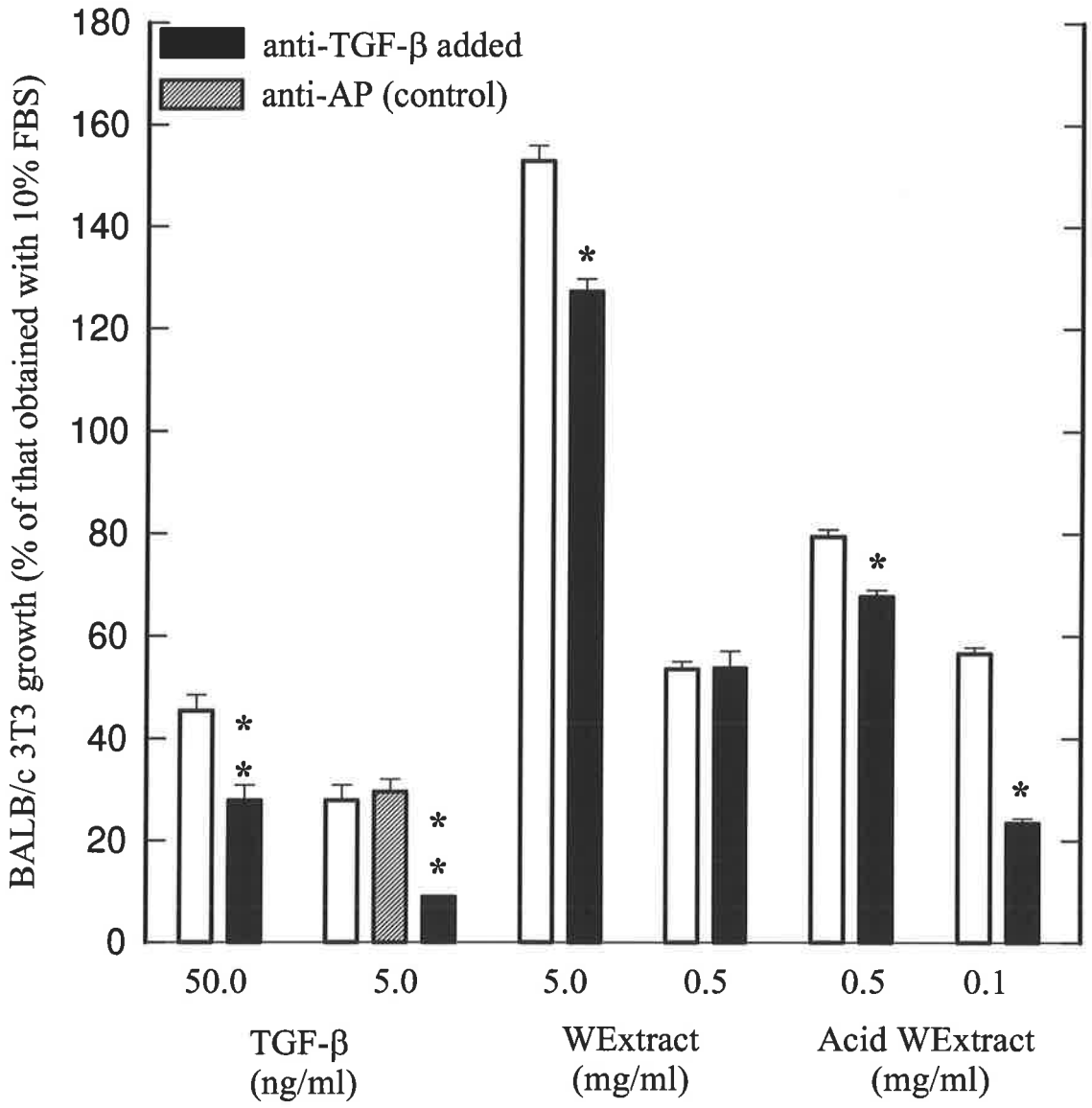



**Figure 6.8** BALB/c 3T3 cell growth response to whey extract treated with a TGF-β antibody. Whey extract (**WExtract**) was prepared as described in **2.2.1** and acidified whey extract (**Acid WExtract**) processed as shown in **6.2.3**. These samples and a TGF-β1 standard were then diluted in DMEM/0.1% BSA and assayed in BALB/c 3T3 cells as described in **6.2.9**. A monoclonal **anti-TGF-**β1,-2,-3 (10 μg/ml) was added to portions of the diluted samples at the time of culture. The TGF-β1 standard was also tested with 10 μg/ml of a control monoclonal antibody (alkaline phosphatase) of the same subclass ($IgG_1$; **anti-AP**). The response is expressed as a percentage of cell growth in 10% FBS, with the response in DMEM alone subtracted. Each treatment were assayed in triplicate and the values represent the mean ± S.E.M. of three experiments. ** $p<0.05$ anti-TGF-β added to TGF-β1 vs no antibody; * $p<0.05$ vs no antibody.

## 6.4 Discussion

In the current chapter, the research undertaken aimed to quantify and describe the active and latent TGF-β activity in bovine milk and investigate the significance of TGF-β to the mitogenic activity of milk. The concentration of total (acid-activatable) TGF-β in milk was 4.3 ± 0.8 ng/ml. Similar concentrations of total TGF-β activity were found in commercial cheese whey (3.7 ± 0.7 ng/ml), suggesting little if any loss into the curd during the cheese making process. Indeed, acid treatment of milk results in precipitation of the casein component of milk to produce an 'acid whey' (Jennes 1988; Bassette and Acosta 1988; Zall 1992). The results presented in **Chapter 3** have already shown that cation-exchange chromatography of cheese whey can produce a fraction that contains concentrated whey mitogens. When total TGF-β activity was measured throughout this process, the amount recovered in the cation-exchange fraction (whey extract) was greater than 98% of that present in the original material. Since most of the major whey proteins and other non-mitogenic peptides and sugars are removed during the cation-exchange process, there was a 60-fold increase in specific total TGF-β activity (from 0.7 ± 0.04 in whey to 42.2 ± 6.8 ng/mg in whey extract).

Further characterisation of bovine milk-derived TGF-β was therefore undertaken using whey extract. Chemical and physical treatments, including exposure to acid, alkali, urea and heat all increased the TGF-β activity of whey extract (**Table 6.2**). This is in agreement with other research on the activation of latent TGF-β *in-vitro* (Lawrence *et al.* 1985; Wakefield *et al.* 1988; Brown *et al. 1*990). However, boiling of the cationic whey extract was found to cause significant protein precipitation, which may account for the lower degree of activation compared to an earlier study using recombinant TGF-

β (Brown *et al.* 1990). Remarkably, heparin was found to increase TGF-β activity in both the TGF-β standard and whey extract. This suggests that heparin was interfering with the association of TGF-β with FBS-derived $\alpha_2$-macroglobulin in the assay, rather than dissociating TGF-β from milk-derived $\alpha_2$-macroglobulin (Danielpour and Sporn 1990; McCaffrey *et al.* 1989).

Around 90% of TGF-β activity recovered from whey was identified as TGF-β2 by purification to homogeneity and sequencing. Purified TGF-β2 from cheese whey migrated on SDS PAGE to approximately 25 kDa and when the sample was dissociated only a single band was observed (**Figure 6.6**). A second, smaller peak of TGF-β activity was recovered from the last stage of purification (**Peak A**, **Figure 6.5**, **Table 6.4**). The two peaks of TGF-β obtained from cheese whey are similar to those obtained by Jin *et al.* (1991) from bovine milk and indicate the second peak of TGF-β activity is likely to be TGF-β1. Importantly, TGF-β activity detected in both milk and whey and throughout the purification procedure was neutralised by a monoclonal antibody that recognised all mammalian isoforms of TGF-β (**Figure 6.7**, **Table 6.5**).

Neutral Superose 6 gel-filtration chromatography of the whey-derived cation-exchange fraction resolved two peaks of latent TGF-β activity. Acidification followed by chromatography under neutral conditions, or chromatography under acidic conditions, released active TGF-β from both high molecular weight latent peaks (**Figure 6.3**, **6.4**). The major peak of activity corresponded to a molecular weight of 80 kDa, suggesting that milk-derived TGF-β exists predominantly as the small latent complex, lacking the LTBP. Small latent complexes have been detected in tissue culture medium conditioned by bone organ cultures and osteoblast cell lines (Dallas *et al.* 1994; Bonewald *et al.* 1991), HT1080 fibrosarcoma cells (Wakefield *et al.* 1988), BSC 40 monkey kidney cells (Lioubin *et al.* 1991), human glioblastoma cells (Olofsson *et al.*

1992) and are the primary forms secreted by CHO or COS cells transfected with TGF-β1 or TGF-β2 precursor cDNA (Moren *et al.* 1994; Gentry *et al.* 1987; Madisen *et al.* 1990). In addition, the small latent complex constitutes 20-40% of platelet TGF-β1 and is retained in the clot after degranulation (Grainger *et al.* 1995b). However, the majority of latent TGF-β isolated from rat and human platelets and human serum comprises the large latent complex and chromatographs on a Superose 6 column with an apparent molecular weight of 400-700 kDa (Miyazono *et al.* 1988; Wakefield *et al.* 1988; Okada *et al.* 1989; O'Connor-McCourt and Wakefield 1987). The current report is the first description of a small latent-like complex in a biological fluid.

The latent TGF-β binding protein has been shown to regulate the targeting of TGF-β to extracellular matrix and is required for the cell-mediated activation of TGF-β by macrophages and co-cultures of endothelial and smooth muscle cells (Taipale *et al.* 1994; Moren *et al.* 1994; Mizoi *et al.* 1993; Nunes *et al.* 1995; Flaumenhaft *et al.* 1993b). Other authors have further suggested that latent forms of TGF-β lacking the LTBP are characteristic of secreted or soluble forms of TGF-β (Dallas *et al.* 1994; Bonewald *et al.* 1991). Whilst the data in this chapter would support this contention, the *in-vivo* mechanism of activation, and physiological role of small latent complexes remain to be elucidated.

An additional smaller peak of latent whey-derived TGF-β activity was observed at an elution volume corresponding to 600 kDa (**Figure 6.3**, **6.4**). Both α2-macroglobulin-bound TGF-β and the large latent complex elute from Superose 6 columns in the 500-700 kDa region (Bonewald *et al.* 1991; Wakefield *et al.* 1988; Miyazono *et al.* 1988). Indeed, α2-macroglobulin has been detected in bovine milk (Rantamaki and Muller 1992); and Wakefield *et al.* (1987) and Wakefield *et al.* (1988) showed that active TGF-β can associate with α2-M and be released from the complex under strong acid.

However, in Wakefield *et al*'s (1987) experiments, acid denatured the α2-M tetramer to prevent any reforming of the TGF-β-α2-M complex, or any further activity of α2-M. In contrast to the TGF-β-α2-M complex, the large latent complex consists of the LTBP covalently bonded to the LAP, not TGF-β (Wakefield *et al.* 1988; Miyazono *et al.* 1988; Kanzaki *et al.* 1990). Transient acid treatment of this large latent complex results in some TGF-β reassociating with the LAP under equilibrium conditions (Wakefield *et al.* 1988; Miyazono *et al.* 1988). Significantly, **Figure 6.4 B** shows that whey contains some acid activated TGF-β that can re-associate with a large protein and remain bioactive, suggesting that the binding protein is not α2-M. Indeed, other dissociating agents that worked for whey extract such as heat and transient alkalinity, (**Table 6.2**) are not known to activate the TGF-β-α2-M complex (Wakefield *et al.* 1988; Brown *et al.* 1990). Further studies will be required to fully describe the structure of the large latent TGF-β complex found in bovine milk.

Active TGF-β chromatographed on both the Superose 12 and Superose 6 resins with an apparent molecular weight below that predicted for the active 25 kDa dimer (**Figure 6.3**, **6.4**). An identical result was reported by Miyazono *et al.* (1988) for acid-activated platelet TGF-β after passage down a Superose 6 column. Similar chromatographic behaviour was seen on a Superdex 75 Prep grade resin (**Step *C*, 6.1**), in this case, further purification of active fractions yielded sequence pure TGF-β2 (**Figure 6.5**). The hydrophobic nature of the active 25 kDa TGF-β homodimer has also been described by other workers (Miyazono *et al.* 1988; Brown *et al.* 1990; Jin *et al.* 1991). In addition, hydrophobic binding of peptides to gel-filtration resins have been reported (O'Callaghan *et al. 1*995). Indeed, acid Superose 12 gel-filtration chromatography did not resolve whey-derived TGF-β into a discernible peak of activity unless acetonitrile was included

in the running buffer. In contrast, the elution volume of the 80 kDa latent form of whey-derived TGF-β was identical in both the presence and absence of acetonitrile.

The data presented in this chapter indicates that a high proportion of whey derived TGF-β is inactive. Over 92% of TGF-β in cheese whey and 98% in whey extract was latent and could be activated by acid treatment (**Figure 6.2**, **Table 6.1**, **6.2**). Accordingly, the contribution of TGF-β to the ability of non-acidified whey extract to induce BALB/c 3T3 cell growth appears to be minimal. A neutralising TGF-β antibody was tested on both whey extract and acid-treated whey extract. This antibody neutralises the ability of the active mature molecule of TGF-β1, TGF-β2 and TGF-β3 in BALB/c 3T3 cell culture (Genzyme Corporation; Dasch *et al.* 1990). **Figure 6.8** shows that 10 μg/ml of anti-TGF-β neutralised 5 ng/ml of TGF-β activity in BALB/c 3T3 cells but a control antibody of the same subclass had no consequence on cell growth produced by TGF-β. Importantly, anti-TGF-β only had a significant effect at the maximal concentration of neutral whey extract (5 mg/ml) that can be added to BALB/c 3T3 cells in culture (**Figure 6.8**). Lower concentrations of neutral whey extract contained little active TGF-β (**Table 6.2**), and therefore neutralising the effect of TGF-β had minimal consequence on the BALB/c 3T3 bioactivity of whey extract. Moreover, **Figure 3.5 B** shows the BALB/c 3T3 cell density produced by low amounts of TGF-β is barely above zero. Importantly, anti-TGF-β had a much greater effect on the bioactivity of whey extract after it was acidified and contained large amounts of active TGF-β (**Figure 6.8**). Indeed, 0.1 mg/ml of acid-treated extract used in the data presented in **Figure 6.8** contains 4.2 ng of TGF-β activity (**Table 6.2**) and induced BALB/c 3T3 cell growth to nearly 60% of that produced by 10% FBS. This BALB/c 3T3 cell growth activity was significantly inhibited to less than half of this value by anti-TGF-β and is within the range of TGF-β that can be neutralised by 10 μg/ml anti-TGF-β.

The current work again argues that the growth factors in whey extract only significantly induce BALB/c 3T3 cell growth when they are activated by acid treatment. As shown in **Chapters 5** and **4**, FGF and PDGF do not notably contribute to the BALB/c 3T3 cell bioactivity in neutral whey extract. **Figure 5.6** shows that removing the FGF fraction from whey extract made little difference to its BALB/c 3T3 growth factor activity. **Figure 4.4** indicates that an antibody that neutralised the BALB/c 3T3 cell growth promoting activity of PDGF had little effect on the potency of neutral whey extract. A significant component of the BALB/c 3 T3 cell growth promoting activity in neutral whey extract is uncharacterised.

TGF-β in milk could be derived from the mammary gland, maternal plasma, epithelial cells, or milk cells (Maier *et al. 1*991; Either and Van De Velde 1990; Letterio *et al. 1*994). The concentrations of TGF-β detected in bovine milk are well within the physiological range for this growth factor. Moreover, TGF-β activity has been shown to be increased in bovine colostrum compared to milk samples taken 5 days after parturition (Tokuyama and Tokuyama 1989). Proposed roles for milk-derived TGF-β in the neonate have included regulation of gut epithelial differentiation and immune function (Puolakkainen *et al. 1*994; Donnet-Hughes *et al. 1*995; Ishizaka *et al. 1*994). Letterio *et al. (*1994) also showed that orally administered TGF-β is absorbed by the gastrointestinal tract and may have roles at sites distant from the gastrointestinal tract. However, the primary role of mammary gland-derived TGF-β may be as an autocrine regulator of glandular growth and milk protein production (Sudlow *et al. 1*994; Robinson *et al. 1*993; Robinson *et al. 1*991). Indeed, significant expression of TGF-β isoforms in the mouse mammary gland during pregnancy decreases during lactation (Robinson *et al. 1*991). Thus, the presence of a small latent complex in milk may

represent an "excretory" rather than "secretory" form of TGF-β after release from a primary site of action within the mammary gland.

# CHAPTER Seven:

# Epidermal Growth Factor-Like Activity in Bovine Whey

## 7.1 Introduction

In the previous four chapters, the results have shown that the BALB/c 3T3 bioactivity produced by bovine whey can not be explained by PDGF, FGF, and TGF-β alone. Other mitogens present in whey that induce fibroblast growth could include members of the epidermal growth factor family. Epidermal growth factor (EGF) and the closely related transforming growth factor-alpha (TGF-α) have been identified in a number of mammalian milks but not in bovine milk. EGF has been found in human, mouse, porcine and rat milk (Carpenter 1980; Beardmore and Richards 1983; Jaeger *et al.* 1987; Raaberg *et al.* 1990). TGF-α has been identified in human milk and in rodent mammary tissue (Zweibel *et al.* 1986; Connolly and Rose 1988; Okada *et al.* 1991; Snedeker *et al.* 1991). EGF and TGF-α bind receptors on cells of mesenchymal origin and stimulate events associated with cell proliferation. They bind the same receptor and produce similar *in-vitro* proliferate effects (Massague 1983, *op cit* 1983b). The ability of human EGF (hEGF) to stimulate BALB/c 3T3 cell growth is illustrated in **Figure 3.6 D** in **Chapter 3**. Here, 6 to 12 ng/ml of hEGF induces growth to nearly 40% of that seen with 10% (v/v) FBS.

The most striking characteristic of the primary structure of both EGF and TGF-α is the conservation of six cysteines which form three disulphide bonds (Savage *et al.* 1973; Winkler *et al.* 1986). This pattern of six cysteines found in a region of 40 amino acids (called the EGF-like motif) has also been detected in a number of proteins. Mitogens containing this motif include amphiregulin, heregulin and neuregulins schwannoma-derived growth factor, heparin-binding EGF and betacellulin (Shoyab *et al.* 1989;

Kimura *et al.* 1990; Marchionni *et al.* 1993; Toyoda *et al.* 1995; Higashiyama *et al.* 1991; Shing *et al.* 1993; Wattanbe *et al.* 1994). To date, EGF and TGF-α are the only mitogens containing EGF-motifs that have been detected in mammary secretions.

EGF is a small molecular weight mitogen of 5000 to 6000 Da that has been purified and sequenced from the human, rat and guinea pig, mouse and horse, but not the cow (Gregory 1975; Simpson *et al.* 1985; Savage *et al.* 1972; Stewart *et al.* 1994). It is synthesised as a large precursor molecule that is anchored to the plasma membrane (Scott *et al.* 1983; Bell *et al.* 1986). Precursor EGF (prepro-EGF) comprises a hydrophobic signal sequence, the mature 53 amino acid EGF, a hydrophobic transmembrane domain, at least 7 EGF-like motifs and a cytoplasmic domain (Mroczkowski *et al.* 1989). Their are eight EGF-motifs in the precursor; in addition to the one found in the mature EGF sequence (Bell *et al.* 1986; Scott *et al.* 1983; Scott *et al.* 1985).

In humans and mice, prepro-EGFs are 130 kDa glycoproteins containing 1217 and 1207 amino acid respectively (Bell *et al.* 1986; Scott *et al.* 1985). In the submaxillary gland of the mouse and the salivary gland of humans prepro-EGFs are rapidly processed to release the mature 53 amino acid EGF (Scott *et al.* 1985; Bell *et al.* 1986; Poulsen *et al.* 1986). The mature EGF is stored in both glands in association with a 30 kDa binding protein found to be an arginine esterase (Taylor *et al.* 1974). However, in other organs such as the mammary gland and kidney, large amounts of precursor EGF is stored as membrane-bound protein and not rapidly processed (Hirata and Orth 1979; Poulsen *et al.* 1986; Scott *et al.* 1985; Breyer and Cohen 1990). Interestingly, it has been proposed that membrane bound EGF precursor is biologically active because it binds the EGF receptor and participates in cell-to-cell signalling (Raaberg *et al.* 1990; Nexo *et al.* 1990; Mroczkowski *et al.* 1989; Breyer and Cohen 1990). After the mature

EGF peptide is released from prepro-EGF in the kidney and mammary gland, it is rapidly excreted and not stored in association with binding proteins (Rall *et al.* 1985). Moreover, the release of mature peptide from prepro-EGF is facilitated by the action of species and organ-specific kallikrein-like enzymes. The action of these enzymes may account for the presence of diverse molecular weight forms of EGF (Kurobe *et al.* 1986; Tsukomo *et al.* 1987; Pesonen *et al.* 1987; Mroczkowski *et al.* 1989; Parries *et al.* 1995). Indeed, various species of prepro-EGF ranging in size from 20-165 kDa are found in body secretions, the mammary gland and kidney (Rall *et al.* 1985; Brown *et al.* 1989; Mroczkowski and Reich 1993).

TGF-α was isolated from sarcoma preparations and categorised as a member of the EGF family of peptide. This was based on its ability to bind the EGF receptor and mediate EGF-like responses (Todaro and De Larco 1976; Azano *et al.* 1983). Mature TGF-α has a molecular weight of 6000 Da and has been found in the human, rat and cow (Marquardt *et al.* 1984; Marquardt *et al.* 1983; Kobrin *et al.* 1986). This molecule is initially synthesised (like EGF) as a membrane bound precursor (Derynck *et al.* 1984). The TGF-α precursor molecule (prepro-TGF-α) is comprised of an N-terminal hydrophobic signal sequence attached to mature TGF-α and a hydrophobic transmembrane domain connected to a C-terminal cytoplasmic domain (Derynck *et al.* 1984; Lee *et al.* 1985; Teixedo and Massague 1988). It is much smaller than the EGF precursor and only the mature molecule contains the EGF-motif (Marquardt *et al.* 1983; Derynck *et al.* 1984; Marquardt *et al.* 1984). The 6 kDa mature TGF-α is released from the extracellular domain of prepro-TGF-α through proteolytic cleavage. Various sized TGF-α species have been found, which can be explained by differential cleavage of the precursor and glycosylation of the molecule (Ignotz *et al.* 1986; Teixedo and Massague 1988). Like EGF, precursor TGF-α molecules can bind EGF receptors and are

considered active factors (Teixedo and Massague 1988; Brachman *et al.* 1989; Wong *et al.* 1989).

The cellular responses to EGF or TGF-α are transmitted by a cascade of events which start with receptor binding. EGF and TGF-α bind to a tyrosine kinase receptor called the EGF-Receptor (EGFR or ErbB-1) that is expressed in most cell types (with the exception of haematopoietic cells; Carpenter and Wahl 1990). This is a 170 kDa glycoprotein consisting of a membrane bound hydrophobic transmembrane sequence, an N-terminal extracellular domain that forms the ligand binding pocket, and an intracellular tyrosine kinase domain that is regulated by ligand binding (Ullrich *et al.* 1984). The EGFR or ErbB-1 is a member of the larger family of receptors consisting the ErbB-2/HER-2, the ErbB-3/HER-3 and ErbB-4/HER-4 tyrosine kinase receptors. These were all named for their homology to the protein encoded by the avian erythroblastosis virus gene (v-*erb*β) and similarity to the Human EGF Receptor (HER) (Downward *et al.* 1984; Coussens *et al.* 1985; Plowman *et al.* 1990; Plowman *et al.* 1993). The biologic effects of EGF and TGF-α are transmitted by the EGFR/ErbB-1, whereas oncogenic proteins such as amphiregulin, heregulin and neuregulins bind other HERs/ErbBs (Shoyab *et al.* 1989; Marchionni *et al.* 1993; Toyoda *et al.* 1995; Higashiyama *et al.* 1997; Chang *et al.* 1997). Other members of the EGF family of proteins such as heparin-binding EGF (HB-EGF) and betacellulin can bind both EGFR and other HERs/ErbBs (Higashiyama *et al.* 1991; Shing *et al.* 1993; Wattanbe *et al.* 1994; Riese *et al.* 1996).

The work of Weber *et al.* (1984) and Massague *et al.* (1983b), showed that each EGF receptor binds a single EGF or TGF-α molecule. Binding of ligand to the EGFR causes a conformational change in the extracellular domain. This then enables two occupied receptors to dimmerise (Yarden and Schlessinger 1987; Zhou *et al.* 1993). Receptor

binding studies have revealed that in such dimers, EGF molecules occupy EGFRs for over 5 minutes and that this is energetically favourable (King and Cuatrecasas 1982; Yarden and Schlessinger 1987; Zhou *et al.* 1993). Receptor dimerisation also results in stimulation of intrinsic tyrosine kinase activity of the EGFRs (reviewed in Schlessinger 1988). The occupation of EGFRs by EGF or TGF-α over 6-10 hours stimulates tyrosine, serine and theonine phosphorylations. This leads to DNA synthesis, cell replication and differentiation (Carpenter and Cohen 1979; Schlessinger and Ullrich 1992). Interestingly, PDGF increases the mitogenic activity of EGF and reduces the number of high affinity EGFRs. Fibroblasts made competent by PDGF are more responsive to EGF and treatment with PDGF decreases the number of high-affinity EGFRs at physiological temperatures (Wharton *et al.* 1983; Bowen-Pope *et al.* 1983). The increase in cell growth stimulation produced by EGF with PDGF treatment has been shown to be the result of PDGF activating non-receptor kinases and reducing receptor internalisation (Bowen-Pope *et al.* 1983; Collins *et al.* 1983; Olashaw *et al.* 1986; Walker and Burgess 1991). This process may involve an increase in the time that EGFR kinases are active and result in an increased growth response to EGF. However, there is no evidence this occurs *in-vivo* or in all cell types, or that TGF-α can replace EGF in this process.

EGF has not been identified in the cow, and it's role as a milk growth factor can only be speculated from studies of EGF in other mammals and their milks. High levels of EGF are found in rat, mouse, and human mammary secretions (Jansson *et al.* 1985; Beardmore and Richards 1983; Raaberg *et al.* 1990; Connolly and Rose 1988; Read *et al.* 1985). High concentrations are also found in urine, prostatic and seminal fluids and saliva (Hirata and Orth 1979; Gregory *et al.* 1977; Gregory *et al.* 1986; Hirata *et al.* 1987). In contrast, EGF levels in blood are relatively low and EGF is present in platelet

granules, illustrating that EGF does not function as a classical hormone (Oka and Orth 1983; Savage *et al.* 1986). Importantly, the EGFs share very little sequence homology across mammals. For example, horse EGF has 55 % sequence homology to EGF from guinea-pig, and only 50% of human EGFs amino acid sequence is conserved across species (Carpenter and Wahl 1990; Stewart *et al.* 1994). This has made it difficult to isolate bovine EGF and detect its presence by using other mammalian EGF antisera in bioassays.

TGF-α was first discovered in culture media of retrovirus-transformed fibroblasts and in a variety of tumours and in embryonic tissues (DeLarco and Todaro 1978; Todaro *et al.* 1980; Twardzik 1985). It was also detected in many types of human cells. This includes pituitary, brain, and intestinal tissue in addition to skin keratinocytes and macrophages (Kobrin *et al.* 1987; Wilcox and Derynck 1988; Coffey *et al.* 1987; Barnard *et al.* 1991; Madtes *et al.* 1988). Importantly, messenger RNA for TGF-α has been detected in a number of bovine tissues, including the pituitary, kidney and mammary gland (Kobrin *et al.* 1987; Zurfluh *et al.* 1990). Unlike EGF, the similarity between the TGF-α sequences of various species is high. This has facilitated the detection of TGF-α and TGF-α mRNA in many tissues. Indeed, the predicted amino acid sequence of bovine TGF-α is 96% homologous to the human sequence (Zurfluh *et al.* 1990). However, the level of conservation between EGF and TGF-α within any one species is low: human 42%, and rat 36%. (Gregory 1975; Marquardt *et al.* 1983; Savage 1972; Marquardt *et al.* 1984).

One of the first biological activities established for EGF was that it is able to induce precocious eyelid opening in new born mice (Cohen 1962). Subsequently, comparison of human TGF-α with human or murine EGF indicated that application of either factor induced this response (Anzano *et al.* 1983; Marquardt *et al.* 1983). Both EGF and TGF-

α induce other changes in somatic development including accelerated tooth eruption, maturation of the gastrointestinal tract and liver and inhibition of gastric acid secretion (Carpenter and Wahl 1990 for review). EGF also up regulates the production of pituitary hormones and stimulates placental lactogen secretion from placental tissue; it is also implicated in wound healing (Fisher and Lakshmann 1990 for review). Importantly, both EGF and TGF-α induce cell division and differentiation in fibroblastic, myoblastic and epithelial type cells (Carpenter and Cohen 1979; Marquardt *et al.* 1984; Schreiber *et al.* 1986; Derynck 1988). However, in some *in-vivo* assays and in organ cultures, TGF-α is more potent than EGF. For example, low concentrations of TGF-α induces neo-vascularisation while EGF does not and TGF-α is a more potent stimulator of $Ca^{2+}$ release in bone organ cultures (Schreiber *et al.* 1986; Ibbotson *et al.* 1986). It has also been found that subdomain II of the EGFR selectively regulates binding of TGF-α and is therefore implicated in the observed differences in *in-vivo* potencies (Harte *et al.* 1995).

Research on EGF in mammary glands of mice and rats has found that it influences the growth of mammary tissue during pregnancy and lactation. In whole organ culture *in-vitro*, EGF has been shown to stimulate lubulo-alveolar development and epithelial cell proliferation (Vonderhaar 1987; Toneli and Sorof 1980). Immuno-histochemical experiments have revealed that EGF is predominantly associated with the proliferation of luminal cells of ductal epithelium in mouse mammary glands (Snedker *et al.* 1991). As previously mentioned, membrane-bound precursor EGF appears to be the major source of EGF found in the mammary gland and this has also been localised to the luminal border of alveolar cells (Hirata and Orth 1979b; Brown *et al.* 1989; Poulsen *et al.* 1986). The EGF receptors (EGFRs) have also been found to be localised to surrounding fibroblasts, as well as ductal epithelium (Edery *et al.* 1985). Research has

also revealed that EGF and EGFRs (in addition to estrogen and progesterone) are key regulators of ductal growth during the prepartum period (Vonderhaar 1987; Coleman *et al.* 1988). However, the effect of EGF on mammary growth gradually decreases from the onset of lactation. EGF binding to EGFR in mice mammary glands declines from high levels in gestation, to less than virgin levels during lactation (Edery *et al.* 1985). Moreover, It has been postulated that as gestation proceeds, increasing amounts of mammary gland EGF down regulate binding to EGFR to barely detectable levels during lactation (Edery *et al.* 1985; Plaut 1993).

EGF has also been suggested to be a local mediator of hormonal action in mammary milk production and to influence milk protein synthesis. The lactogenic hormones cortisol and prolactin modulate expression of mRNA transcripts of EGF in rodent mammary tissue (Vonderhaar and Nakhasi 1986; Fenton and Shefield 1991). Also, EGF concentration in mouse milk peaks during mid-lactation, at a time when prolactin receptor levels are at the highest (Beardmore and Richards 1983; Edery *et al.* 1985). Vonderhaar and Nakhasi (1986), showed that the concentration of EGF influences milk protein synthesis. They also showed that high concentration of EGF decreases the synthesis of milk protein.

Research on EGF concentration in mammary secretions during human lactation has revealed that it peaks in the pre-partum/colostral phase and then declines in parallel with milk protein production. By way of illustration, EGF content in human pre-partum secretions has been recorded at 200 ng/ml, colostrum 25-200 ng/ml and mature milk 5-50 ng/ml (Read *et al.* 1984; Read *et al.* 1985; Jansson *et al.* 1985). It is noteworthy that these changes in EGF concentration over a lactation are not conserved across species. For example, EGF concentration in colostral milk of mouse (55 ng/ml) is lower than that found in mid lactation (130-427 ng/ml; Beardmore and Richards 1983). Also some

mammals have much higher concentrations of milk EGF than others. The concentration of EGF in mature milk of mice (130-427 ng/ml) is higher than that of humans (5-50 ng/ml), whereas levels in rats are lower (3-12 ng/ml) (Beardmore and Richards 1983; Jansson *et al.* 1985; Read *et al.* 1985; Raaberg *et al.* 1990). However, it is important to note that the above authors used various assay systems that may not be directly comparable. This may also account for the range in concentration reported for each species.

Milk EGF may be derived from maternal circulation or locally produced in the mammary gland; but current evidence would seem to preclude maternal origin. The 30-1000 fold milk: plasma EGF concentration ratio coupled with low circulating levels of EGF (< 0.01 ng/ml in humans, 0.3 ng/ml in mice) indicates that mammary uptake from plasma requires an active transport mechanism. Brown *et al.* (1986), showed that the lactating goat mammary gland took up to 83% of an infused $^{125}$I-h-EGF. However, only 3% of the dose appeared in milk, which indicates that serum is not the primary source of milk EGF. Importantly, when the submaxillary or salivary glands are removed from lactating mice and rats, their is little change in milk EGF levels (Grueters *et al.* 1985). Furthermore, milk contains various molecular weight forms of active EGF. Since EGF is not a circulating hormone, but is present within platelet granules in various molecular weight forms, it is difficult to believe that it can be actively transported into the mammary gland. Rather, the high molecular weight forms of active EGF in milk and the prepro-EGF mRNA in lactating mammary tissue suggests local origin from membrane bound precursor (Rall *et al.* 1985; Raaberg *et al.* 1990; Schaudies *et al.* 1990; Mroczkowski and Reich 1993; Brown *et al.* 1989).

TGF-α is a potent effector of normal mammary development and lactation. Research has shown that TGF-α controls early development of the mammary gland. TGF-α is

associated with the proliferating cap cell layer in the mammary glands of 5 week old mice (Snedker *et al.* 1991). It also controls lubulo-alveolar development of adult mouse mammary glands. Vonderhaar (1987) showed that locally implanted TGF-α but not EGF, can replace the need for estrogen and progesterone in lobulo-alveolar development in mouse whole organ culture. Interestingly, TGF-α levels in human milk are much lower than EGF and remain constant over a lactation (Okada *et al.* 1991). Indeed, human milk TGF-α concentration is high in prepartum secretions (0-50 ng/ml) and then declines in colostrum to a level which does not change with the progression of lactation (2-7 ng/ml) (Connolly and Rose 1988; Okada *et al.* 1991).

There is little literature on how TGF-α is transported into milk. However, mRNA for TGF-α has been detected in ductal and alveolar cells in pregnant and lactating rats (Liscia *et al.* 1990). Also, Lui (*et al.* 1987), showed that ovariectomy causes a rapid decline in TGF-α mRNA expression in rat mammary glands and that estrogen induces both TGF-α mRNA and immunoreactive TGF-α in rat mammary tumour cells *in-vitro*. These results suggest local synthesis, as does the appearance of multiple molecular weight TGF-α's in human milk that have a similar size to those cleaved from membrane-spanning TGF-α precursors (Okada *et al.* 1991; Teixido and Massague. 1988).

Although the model of EGF and TGF-α's role in the mammary gland is based on rodents and humans, similar changes may occur in cows. Like mice, binding of human EGF to bovine EGFRs *in-vitro*, is high in virgin cows, increases during early pregnancy and declines during the remainder of gestation and lactation (Spitzer and Grosse 1987). This data also suggested the possibility of a bovine EGF equivalent that can bind EGFRs may exist in the cow. However, to date, EGF has not been isolated from cows, but, mRNA for TGF-α has been found in the bovine mammary gland (Zurfluh *et al.*

1990). Moreover, both human EGF and bovine TGF-α are mitogenic for mammary epithelial cells isolated from pregnant heifers (Collier *et al.* 1993). Further research is needed to establish if EGF or other members of the EGF family are present in bovine mammary glands and milk.

Previous research on the amount and characteristics of EGF in bovine milk has been inconclusive and produced variable results depending on the method used. As already mentioned, the low sequence homology of EGFs across mammals has made it difficult to isolate bovine EGF and produce its antisera. This precludes the use of anti-bovine EGF in radio-immunoassays and no other species antisera cross-reacts with bovine EGF. Shing and Klagsbrun (1984) used the cell growth response of BALB/c 3T3 cells to detect EGF in fractions of bovine colostrum. They separated growth factor activity from bovine colostrum and determined the size and charge of the peaks of fractions that induced BALB/c 3T3 cell-growth. On this basis, Shing and Klagsbrun (1984) concluded that bovine colostrum contained a spectrum of mitogens that did not include EGF. Conversely, both Read *et al.* (1985) and Yagi *et al.* (1986) measured detectable levels of EGF (2.5 and 320 ng/ml respectively) in bovine milk using radioreceptor assays. Read *et al.* (1985), used intact human lung fibroblasts (AG2804s) in their radioreceptor assays, whereas Yagi *et al.* (1986), utilised human placental membranes as a source of receptors for their assay.

Iacopetta *et al.* (1992), revisited both Read *et al.* (1985) and Yagi *et al.* (1986)'s methods to measure EGF in cow milk. Similar levels of EGF were found in bovine milk to those measured by Read *et al.* (1985), when radioreceptor assays were used with intact A431 human epidermoid carcinoma cells in place of AG2804 cells. However, using Yagi *et al.* (1986)'s methods, Iacopetta *et al.* (1992) found non-specific effects of high molecular weight milk proteins interfered with EGF receptors on human placental

membranes producing erroneous results. Further, because the amount of EGF in bovine mammary secretions measured by intact A431 radioreceptor assays was at the lower range of detection, Iacopetta *et al.* (1992), concluded that there may be even less EGF-like activity than measured.

The lack of bovine EGF antisera means that radioreceptor assays are (to date) the only method available to detect EGF in bovine milk. However, this method used by Iacopetta *et al.* (1992) and Read *et al.* (1985) can not distinguish between members of the EGF family that bind EGFRs. Both EGF and TGF-α bind the EGFR/ErbB-1, and bovine TGF-α binds with the same potency and to the same extent as human or rodent EGF in A431 radioreceptor assays (Massague 1983b; Zurfluh *et al.* 1990; Samsoondar *et al.* 1986). Also, TGF-α isolated from human milk has similar potency to EGF in such assays (Zweibel *et al.* 1986; Connolly and Rose 1988; Okada *et al.* 1991). Research has shown that in addition to TGF-α and EGF, other EGF-like peptides such as heparin-binding growth factor and betacellulin can bind the EGFR/ErbB-1 (Higashiyama *et al.* 1991; Shing *et al.* 1993; Wattanbe *et al.* 1994). However, the dose response curves of these proteins are different than EGF or TGF-α in radioreceptor assays because they bind both ErbB-1 and other ErbBs (Higashiyama *et al.* 1991; Shing *et al.* 1993; Wattanbe *et al.* 1994). This effect may not be as evident in cell lines such as A431 and AG2804 cells that over express ErbB-1 and not other ErbBs (Massague 1983b; Higashiyama *et al.* 1991; Shing *et al.* 1993; Wattanbe *et al.* 1994). Importantly, the A431 and AG2804 radioreceptor assays can only detect EGF-like molecules and can not distinguish between EGF, TGF-α, HB-EGF and betacellulin. However, heparin-affinity chromatography could be used to differentiate between some of these factors. EGF and TGF-α do not bind heparin-Sepharose (Besner *et al.* 1990; Thompson *et al.* 1994). Heparin-binding EGF (HB-EGF), amphiregulin and betacellulin all bind heparin-

Sepharose and require differing concentrations of NaCl to elute from heparin-binding columns (Higashiyama *et al.* 1991; Shing *et al.* 1993; Thompson *et al.* 1994; Wattanbe *et al.* 1994).

All the growth factors previously identified in bovine milk, including PDGF, the IGFs and TGF-β have fairly basic isoelectric points (pI) of 10, 7-9 and 8.2 respectively (Shing and Klagsbrun 1987; Collier *et al.* 1991; Francis *et al.* 1986; Vega *et al.* 1991). In the current study, it would be expected that only those growth factors with near to basic isoelectric points would be recovered from whey by cation-exchange chromatography. Indeed, growth factors that were investigated in the previous four chapters are known to have fairly basic isoelectric points. These include FGF-1, pI 5.6 (Thomas *et al.* 1984); FGF-2, pI 9.6 (Gospodarowicz 1975); PDGF, pI 10 (Deuel *et al.* 1981) and TGF-β, pI 8.2 (Assoian *et al.* 1983; Frolik *et al.* 1983). Since EGFs recovered from mammals have fairly acidic isoelectric points near 4.6, the recovery of bovine EGF from cation-exchange chromatography of whey may be low (Taylor *et al.* 1972; Shing and Klagsbrun 1984). Nevertheless, other EGF-like proteins have more basic isoelectric points and may be recovered by this process. Accordingly, TGF-α has an isoelectric point of 6.8 (Frolik and DeLarco 1987) and HB-EGF 7.8 (Higashiyama *et al.* 1992). The isoelectric point of betacellulin is not recorded but, the cation-exchange process that Shing *et al.* (1993) and Wattanbe *et al.* (1994) used to purify this factor suggests it is fairly basic, as does it's homology with TGF-α and HB-EGF.

The aim of the current study is to determine if the fraction of whey extract that induces BALB/c 3T3 cell growth contains EGF-like molecules. In the previous four chapters, the results show acid-gel filtration of whey extract produced a peak of bioactive fractions that could not be solely explained by the presence of IGF, FGF, PDGF and TGF-β. In this chapter, the EGF-like activity in fractions of whey extract

will be investigated using a radioreceptor assay with intact AG2804 lung fibroblasts. BALB/c 3T3 cell growth assays will then be used to determine if the EGF-like activity of whey extract enhances BALB/c 3T3 growth. Heparin-affinity chromatography will be utilised to determine if the EGF-like activity in whey extract is a heparin-binding EGF-like factor. The bovine EGF-like molecule will then be partially purified.

## 7.2 Materials and Methods

The production of whey extract was carried out as previously described in section **2.2.1**. Cell culture conditions and growth assays were as described in sections **2.2.2** and **2.2.3**. Whey extract and ultrafiltration fractions of whey extract were subject to acid gel-filtration as shown in **2.2.4**. This was achieved using the Pharmacia Superdex 75 HR 35/600 size exclusion column coupled to a FPLC system (AMRAD Pharmacia Biotech, North Ryde, New South Wales, Australia). The protein concentrations of samples were determined as shown in **2.2.5**.

### 7.2.1 *EGF radioreceptor assays*

Radioreceptor assays (RRA) to detect EGF were performed as described by Read (1984), with some modifications. $^{125}$I-h-EGF (specific activity 100 μCi/μg) was prepared using the chloramine-T method (Van Obberghen-Schilling and Pouyssegur 1983) and separated from free $^{125}$I by gel-filtration. Binding of EGF was determined using the epidermoid carcinoma cell line, A431 and/or the SV40-transformed human lung fibroblast line, AG2804. Cells were subcultured in Costar 24-well plates (Costar, Cambridge, Massachusetts, USA) and grown to confluence in DMEM containing 10%

(v/v) FBS. The monolayers of cells were then washed twice in HEPES-buffered saline (0.1 M HEPES, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, and 8 mM glucose, pH 7.6) containing 0.5% (wt/v) bovine serum albumin (BSA), and incubated at 4°C for 4 h. Samples of dried whey fractions were made up in HEPES without saline pH 7.6, that contained 0.5% (wt/v) of BSA. Human EGF was diluted in HEPES-buffered saline containing 0.5% BSA (wt/v). The cells were then incubated for 18 h at 4°C in HEPES-buffered saline containing $^{125}$I-h-EGF ($1.5 \times 10^4$ cpm) and either the dissolved samples of whey or h-EGF or HEPES-buffered saline, all in a final volume of 0.5 ml. The cells were then washed three times with Hanks' balanced salts solutions (HBSS; 1.26 mM $CaCl_2$, 5.36 mM KCl, 0.44 mM $KH_2PO_4$, 0.82 mM $MgSO_4$, 136.9 mM NaCl, 0.033 mM $Na_2HPO_4$, 5.55 mM glucose, 0.01 g/l phenol-red, pH 7.3) at 4°C to remove un-bound material from receptors. The monolayers were dissolved in 1 ml of 0.5 M NaOH containing 0.1% (v/v) Triton X-100. Radioactivity was counted in a gamma counter, and the ability of fractions or h-EGF to displace $^{125}$I-h-EGF from receptors expressed as a percentage of binding in HEPES-buffered saline. Total and non-specific binding were determined by using in place of test samples binding buffer and excess unlabelled EGF respectively. Data from dilution series of test samples were compared to hEGF standard curves to estimate EGF concentration using four parameter logistic dose response equations (Tablecurve; Jandel Scientific, San Rafael, California, USA).

### 7.2.2 *Ultrafiltration of whey extract*

Ultrafiltration was evaluated as method for concentrating EGF-like activity derived from the cation-exchange fraction of bovine cheese whey. Whey extract was made from cation-exchange chromatography of cheese whey as per **2.2.1** and **Figure 2.1**, except the end product was not freeze-dried (liquid whey extract). One hundred and fifteen liters

of 'liquid whey extract' derived from 2300 litres of whey was acidified to pH 2.5 with HCl and incubated at 4°C overnight. It was then concentrated to 5 liters against a 100 kDa polysulphonate membrane filter (Sartorius, Gottingen, Niedersachsen, Germany) in an Amicon DC-250 ultrafiltration unit (Amicon, Danvers, Massachusetts, USA). The concentrated whey extract was diafiltered against 20 liters of 150 mM NaCl-HCl, pH 2.5 in the same device and is subsequently referred to as 'high molecular weight fraction of whey extract' or 'retentate'. The material that had passed through the 100 kDa membrane was adjusted to pH 7.0 with NaOH and is herein after referred to as 'low molecular weight fraction of whey extract' or 'permeate'. Both the retentate and permeate were then concentrated and diafiltered against 150 mM $NH_4HCO_3$ using a 3 kDa excluding membrane (Sartorius, Gottingen, Niedersachsen, Germany) in an Amicon DC-10 ultrafiltration unit (Amicon, Danvers, Massachusetts, USA) and freeze-dried. There were 66.3 and 5.2 mg of protein recovered per litre of whey in the retentate and permeate, respectively.

### 7.2.3 *Heparin-Sepharose chromatography of permeate*

Heparin-affinity chromatography was used to determine if the EGF-like activity in the low molecular weight fraction of whey extract was a heparin-binding factor. A portion (1 ml) of EGF-like activity recovered from acid gel-filtration of low molecular weight permeate was freeze dried and made up in 1 ml of 10 mM Tris-HCl, pH 7.0. This was then applied to a 0.9 cm diameter column (Amicon, Danvers, Massachusetts, USA). This was filled with 1.1 ml of heparin-Sepharose CL6B and coupled to an FPLC system (AMRAD Pharmacia Biotech) and pre-equilibrated in 0.2 M NaCl, 10 mM Tris-HCl pH 7. After washing the column in 28 column volumes of equilibration buffer, bound protein was eluted with a 26 minute gradient of 0.2 to 1.5 M NaCl in 10

mM Tris-HCl, pH 7.0 at 14 cm/h (0.15 ml/min). Fractions of 0.45 ml were collected and portions (0.2 ml) assayed for displacement of $^{125}$I-h-EGF in the AG2804 RRA (**7.2.1**). The delay to the start of the salt gradient was determined by detecting the absorbance of 0.1% (v/v) benzyl alcohol or 2% (wt/v) phenyl red delivered by pump B of the FPLC.

### 7.2.4 *Partial purification of EGF-like activity from whey extract*

The protocol used for purification is outlined in **Figure 7.1**, with each stage followed by testing fractions for their ability to displace $^{12}$5I-h-EGF in the AG2804 radioreceptor assay (RRA; **7.2.1**).

***A*** Low molecular weight permeate was produced from liquid whey extract, as indicated in **7.2.2**.

***B*** Five hundred and eighty two-mg of permeate was subject to acid gel-filtration at 15.6 cm/h in a buffer containing 150 mM NaCl, 1 M acetic acid and 10% (v/v) acetonitrile, pH 2.0, as described in **2.2.4**. 36 fractions of 17.5 ml were collected.

***C*** The group of fractions from ***B*** containing EGF-like activity were pooled, and half applied to a Waters Deltapak reversed phase $C_4$ (25 x100 mm) HPLC column (Waters-Millipore, Rydalmere, New South Wales, Australia). This had been pre-equilibrated in 0.1% (v/v) trifluroroacetic acid (TFA), pH 2.0. The column was then washed in 10 column volumes of 0.1% (v/v) TFA and bound protein eluted. This was done at 5 ml/min using a gradient of 0 to 80% (v/v) acetonitrile in 0.08% (v/v) TFA over 80 minutes and 10 ml fractions collected. Protein was detected by monitoring at 214 nm and the same procedure repeated with the other half of the pooled EGF-like activity of low molecular weight permeate. Portions of the 10 ml $C_4$ fractions (50 µl) were freeze-

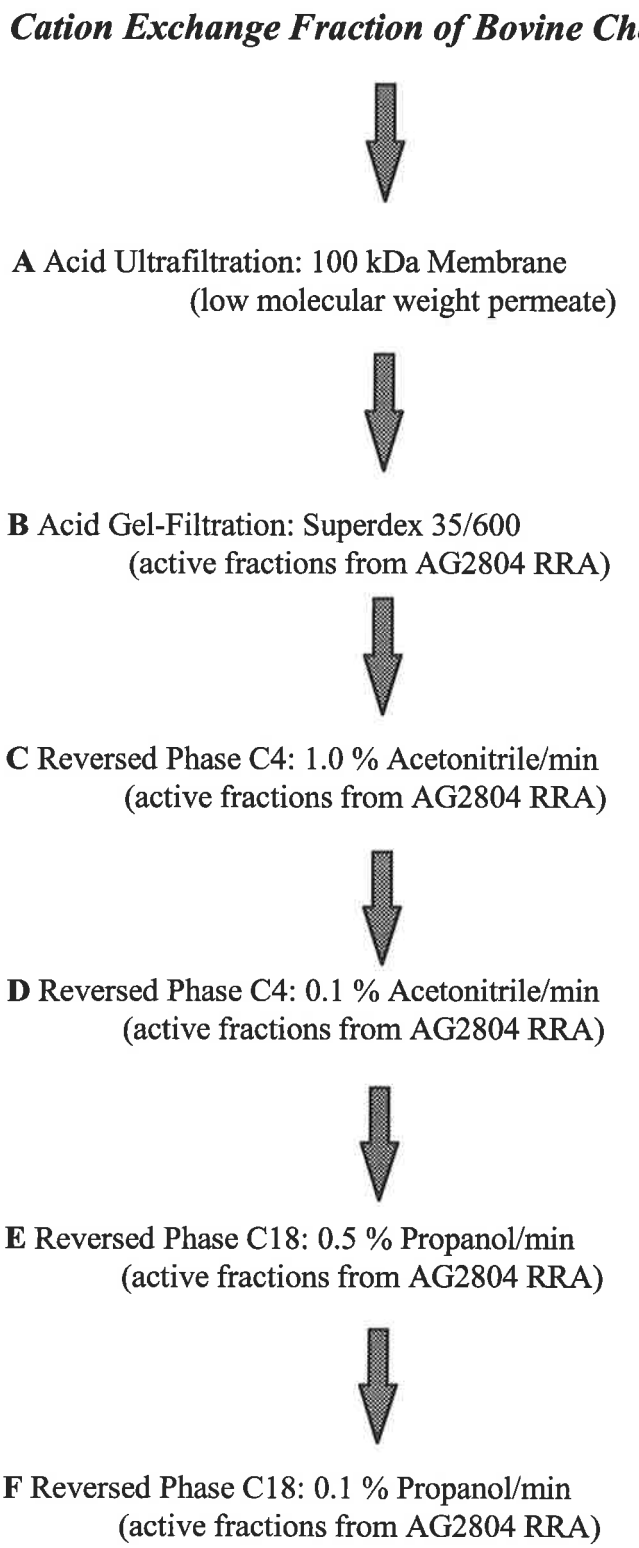
dried, made up in 2.25 ml of HEPES-buffered saline and tested for EGF activity by the AG2804 RRA.

***D*** Active fractions from ***C*** were pooled and diluted 25 times with 0.1% (v/v) TFA, and re-applied to the $C_4$ column that had been equilibrated in 0.1% (v/v) TFA. After washing the column in 10 column volumes of 0.1% (v/v) TFA, protein was eluted at 5 ml/min with a gradient of 20 to 40% (v/v) acetonitrile in 0.08% TFA (v/v) over 200 minutes and 10 ml fractions collected. Portions of fractions (100 µl) were freeze-dried, made up in 900 µl of HEPES-buffered saline and tested for displacement of $^{125}$I-h-EGF by the AG2804 RRA.

***E*** Biologically active fractions were then diluted 25 times in 0.13% (v/v) heptafluorobutyric acid (HFBA) and applied to a Nova-Pack Waters $C_{18}$ (8 mm x 100 mm) column (Waters-Millipore). This had been pre-equilibrated in 0.13% (v/v) HFBA. Protein was detected at 280 nm and eluted at 1 ml/min by a gradient of 0 to 80% (v/v) propan-1-ol in 0.13% (v/v) HFBA over 160 minutes and 2 ml fractions collected. A part of each fraction (50 µl) was freeze-dried, dissolved in 900 µl of HEPES-buffered saline and tested for EGF activity by the RRA.

***F*** Active fractions were pooled, diluted twelve times in 0.13% (v/v) HFBA and re-applied to the $C_{18}$ column. Protein was eluted at 1 ml/min by a gradient of 25 to 45% (v/v) propan-1-ol in 0.13% (v/v) HFBA over 200 min and two min fractions collected. Portions of fractions (25 µl) were freeze-dried, made up in 900 µl of HEPES-buffered saline and tested for EGF activity by the RRA.

**Figure 7.1: Partial purification of bovine EGF from whey extract**



### 7.2.5 *SDS-polyacrylamide gel electrophoresis*

The EGF-activity of whey extract was subject to reducing SDS-polyacrylamide gel-electrophoresis (SDS-PAGE). The peak of EGF-activity recovered from the last purification step (**Figure 7.1** (***F***)) was pooled and a 2 ml sample freeze dried and made up in 5 µl of reducing SDS-PAGE buffer. In addition, 1µg of freeze-dried h-EGF (Chiron Mimotopes Peptide Systems, Rosebank, Victoria, Australia) and a 3 µg portion of Phast-gel molecular weight markers (AMRAD Pharmacia Biotech) were each dissolved in 5 µl of reducing SDS-PAGE buffers. The sample buffer contained 5% (wt/v) SDS, 100 mM Tris, and 5% (wt/v) bromophenol blue, pH 8.0 and reducing conditions were achieved by adding 1.5% (wt/v) dithiothreitol. The samples were heated at at 95°C for 5 minutes, and 0.5 µl of each sample and Phast-gel molecular weight markers was loaded on to a 8-25% polyacrylamide preformed slab gel. The gel was then subject to SDS-PAGE on the Phast gel system according to the manufacturers instructions (AMRAD Pharmacia Biotech, North Ryde, New South Wales, Australia), and then silver stained (Merril *et al.* 1984).

## 7.3 Results

### 7.3.1 *EGF-like activity recovered from whey extract*

In the present study, bioactivity recovered from acid gel-filtration of whey extract was screened for EGF activity by radioreceptor assays. Initially, both the epidermoid carcinoma cell line, A431 and the SV40-transformed human lung fibroblast line,

AG2804 were used as the source of receptors in the RRA. **Figure 7.2** shows that the concentration of EGF that displaces half the total bound $^{125}$I-h-EGF from A431 monolayers was around 300 ng/ml and this assay could not detect EGF activity below 30 ng/ml. In comparison, the concentration of EGF required to displace half the total bound radioactivity from AG2804 cells was 3 ng/ml and the detection limit was 0.3 ng/ml. Non-specific binding in HEPES-buffered saline containing 1500 ng/ml of h-EGF was 2-4% in the A431 assay and 4% in the AG2804 assay. For each plate, the total amount of $^{125}$I-h-EGF bound to A431 and AG2804 monolayers in HEPES-buffered saline were consistently 55% and 25% respectively of the 1.5 x $10^4$ cpm added.

A sample of whey extract (375 mg) was subject to Superdex 35/600 chromatography under acid conditions at 22 cm/h (**2.2.4**). The recovered fractions were tested for their ability to displace $^{125}$I-h-EGF from both A431 and AG2804 monolayers. Monolayers of A431 and AG2804 cells were prepared in 24-well plates and washed as described in **7.2.1**. Portions (1 ml) of the recovered gel-filtration fractions were freeze dried and made up in 1.2 ml of HEPES without saline, pH 7.6 that contained 0.5% (wt/v) of BSA. A sample (450µl) of each dissolved fraction was added to individual wells that contained 50 µl of $^{125}$I-h-EGF (1.5 x $10^4$ cpm). Standard curves of hEGF were included on all plates. The cells were incubated for 12 h, washed, lysed and radioactivity counted and expressed as a percentage of binding in HEPES-buffered saline alone (**7.2.1**). A sample (100 µl) of each of the fractions of whey extract was also freeze dried and tested for their ability to induce BALB/c 3T3 cell growth by the 96-well plate dye binding assay described in **2.2.3**. **Figure 7.3** shows that a single peak of fractions displaced $^{125}$I-h-EGF from AG2804 monolayers and chromatographed between the 29 and 12.4 kDa standards. Importantly, the major peak of fractions that induced BALB/c 3T3 cell growth co-eluted with EGF activity detected by the AG2804 and A431 radioreceptor

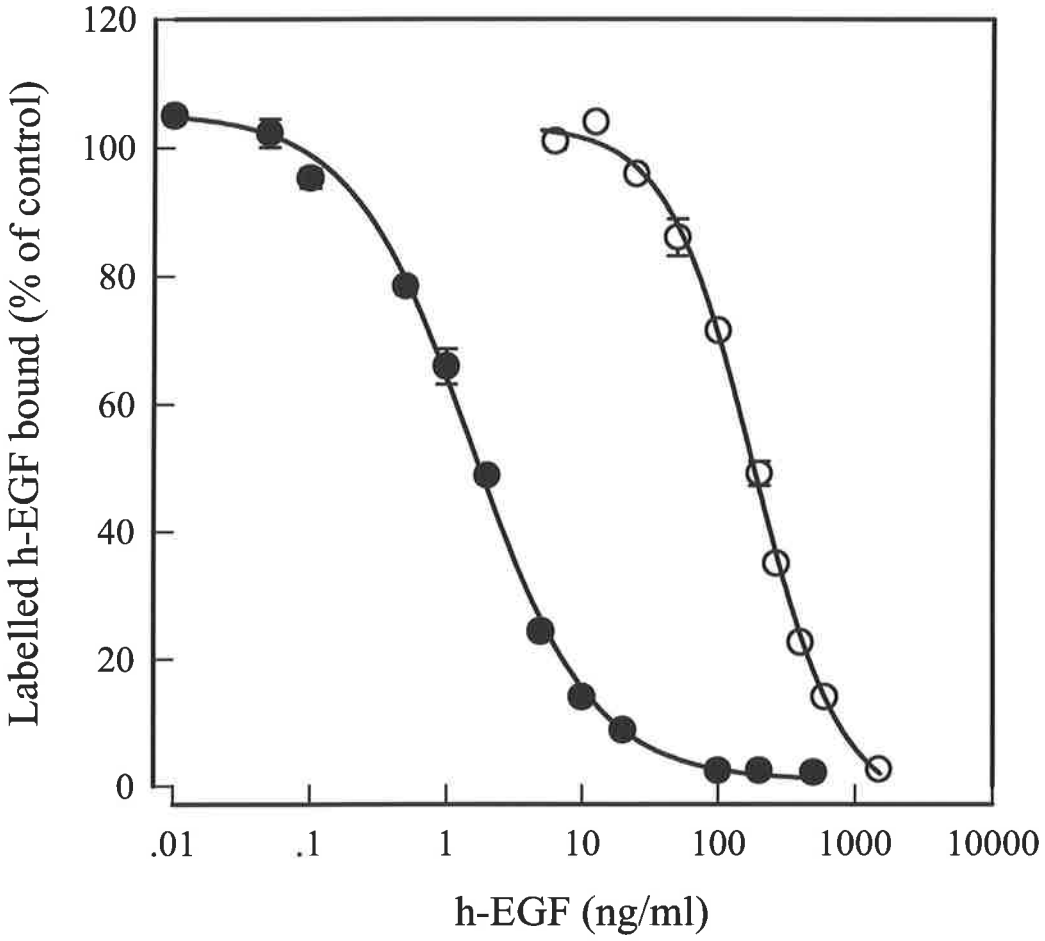



**Figure 7.2** Standard curves for EGF radioreceptor assays. Human EGF was diluted in HEPES-buffered saline that contained 0.5% (wt/v) bovine serum albumin, and its ability to displace $^{125}$I-h-EGF from A431 (O) and AG2804 (●) monolyers measured. Results (mean ± S.E.M of three measurements) are expressed as a percentage of total radioactivity bound to cells incubated in HEPES-buffered saline alone (**7.2.1**). The total amount of $^{125}$I-h-EGF bound to A431 and AG2804 cells incubated in HEPES-buffered saline was 55% and 25% respectively of the 1.5 x $10^4$ counts per minute added. Non-specific binding in the presence of 1500 ng/ml of unlabelled h-EGF was 4% of bound counts for the A431 assay and 2% for 800 ng/ml of h-EGF in the AG2804 assay.

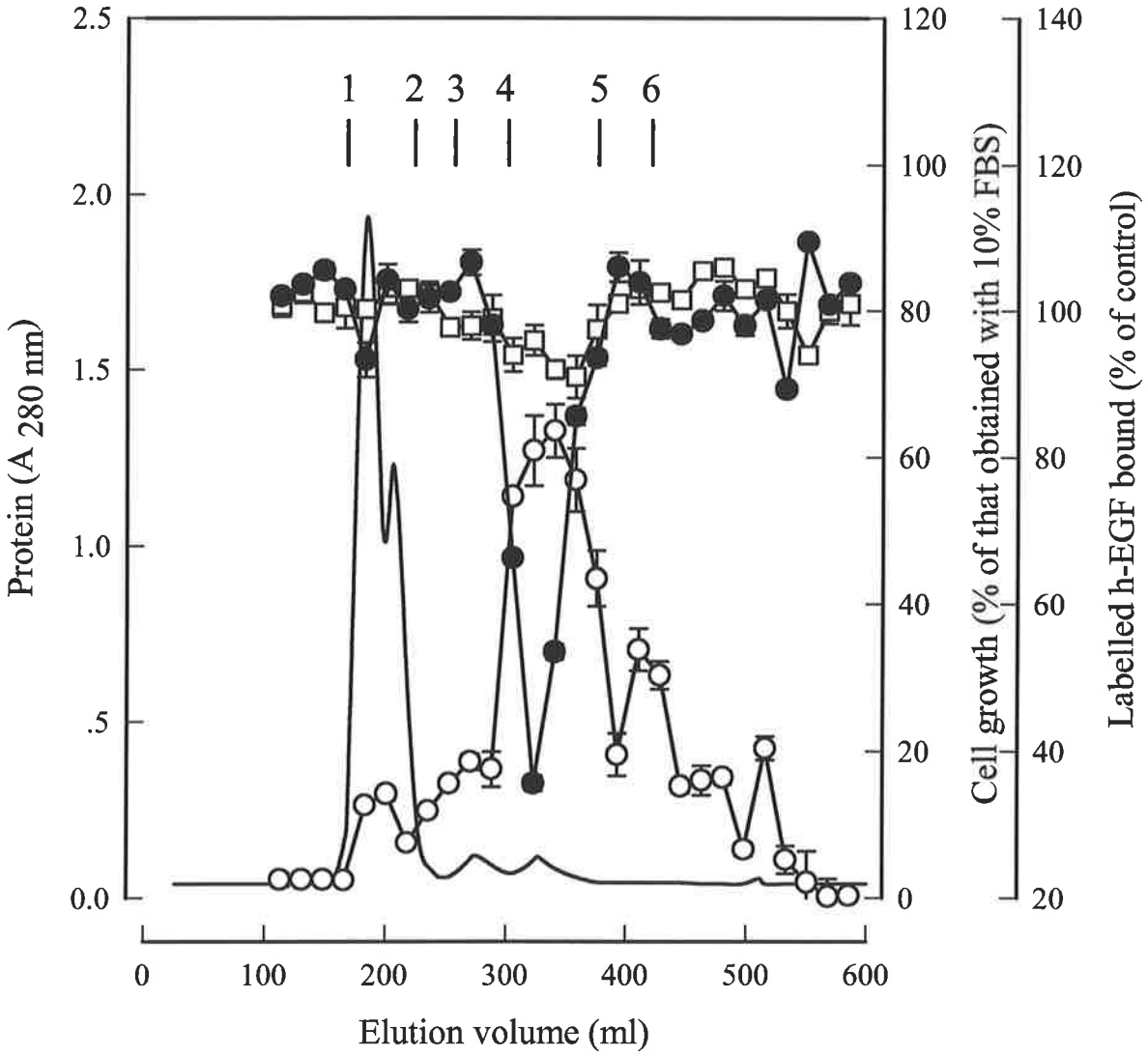



**Figure 7.3** EGF-like activity and BALB/c 3T3 bioactivity recovered from acid gel-filtration of whey extract. A 375 mg sample of whey extract was dissolved at 25 mg/ml and chromatographed at 22 cm/h on a Superdex 75-35/600 column (**2.2.4**), using a running buffer of 150 mM NaCl, 1 M acetic acid and 10% (v/v) acetonitrile, pH 2.0. Five minute fractions (17.5 ml) were collected, and 1 ml portions tested for EGF activity in the A431 (□) and AG2804 (●) radioreceptor assays (**7.2.1**). Results (mean ± s.e.m of three measurements) are expressed as a percentage of radioactivity bound to cells incubated in HEPES-buffered saline. A sample (100 µl) of each acid gel-filtration fraction was also tested for BALB/c 3T3 bioactivity (○) in the 96-well plate dye binding assay (**2.2.3**). Cell growth (mean ± S.E.M of triplicate determinations) is shown as a % of the response to a 10% FBS standard incorporated onto each plate and the response to DMEM alone has been subtracted. The column was standardised under neutral conditions with IgG1 (1; 150 kDa), BSA (2; 67 kDa), ovalbumin (3; 45 kDa), carbonic anhydrase (4; 29 kDa), cytochrome C (5; 12.4 kDa) and insulin (6; 7 kDa).

assay. Notably, there was much less displacement of $^{125}$I-h-EGF from A431 than AG2804 monolayers by the acid gel-filtration fractions. This result is expected as the A431 RRA can not detect below 30 ng/ml of EGF-like activity (**Figure 7.2**). Subsequently, displacement of $^{125}$I-h-EGF from AG2804 monolayers is the only assay used to detect EGF-activity in whey extract fractions.

EGF-like activity recovered from acid gel-filtration of whey extract, was pooled and measured by RRA. Portions (3 ml) of the four fractions from acid gel-filtration of whey extract that eluted between 305 and 375 ml (**Figure 7.3**) were pooled, freeze-dried and the pooled protein measured (**2.2.5**). This material was then made up and diluted in HEPES without saline, pH 7.6 that contained 0.5% (wt/v) of BSA. The diluted samples and a range of h-EGF concentrations were then tested in the AG2804 RRA. **Figure 7.4** shows that the slope of the dose response curve of pooled EGF-like activity of whey extract was identical to h-EGF. To demonstrate the specificity of the assay 200 ng/ml of both PDGF-AA and PDGF-BB were tested in the same AG2804 RRA. As shown in **Figure 7.4**, neither PDGF-AA nor PDGF-BB displaced $^{125}$I-h-EGF from AG2804 cell monolayers. Half maximal displacement of $^{125}$I-h-EGF from AG2804 cell monolayers occurred at 230 μg/ml of pooled whey derived EGF-like activity and 2 ng/ml of h-EGF. A total of 296 ng of EGF equivalents and 26 mg were recovered (11 ng/mg) from loading 375 mg of whey extract onto the gel-filtration column. These results and a series of two similar experiments showed 0.8 ± 0.1 ng of EGF-like molecules were present per mg of the 375 mg of whey extract loaded onto the Superdex column (mean ± S.E.M, n=3).

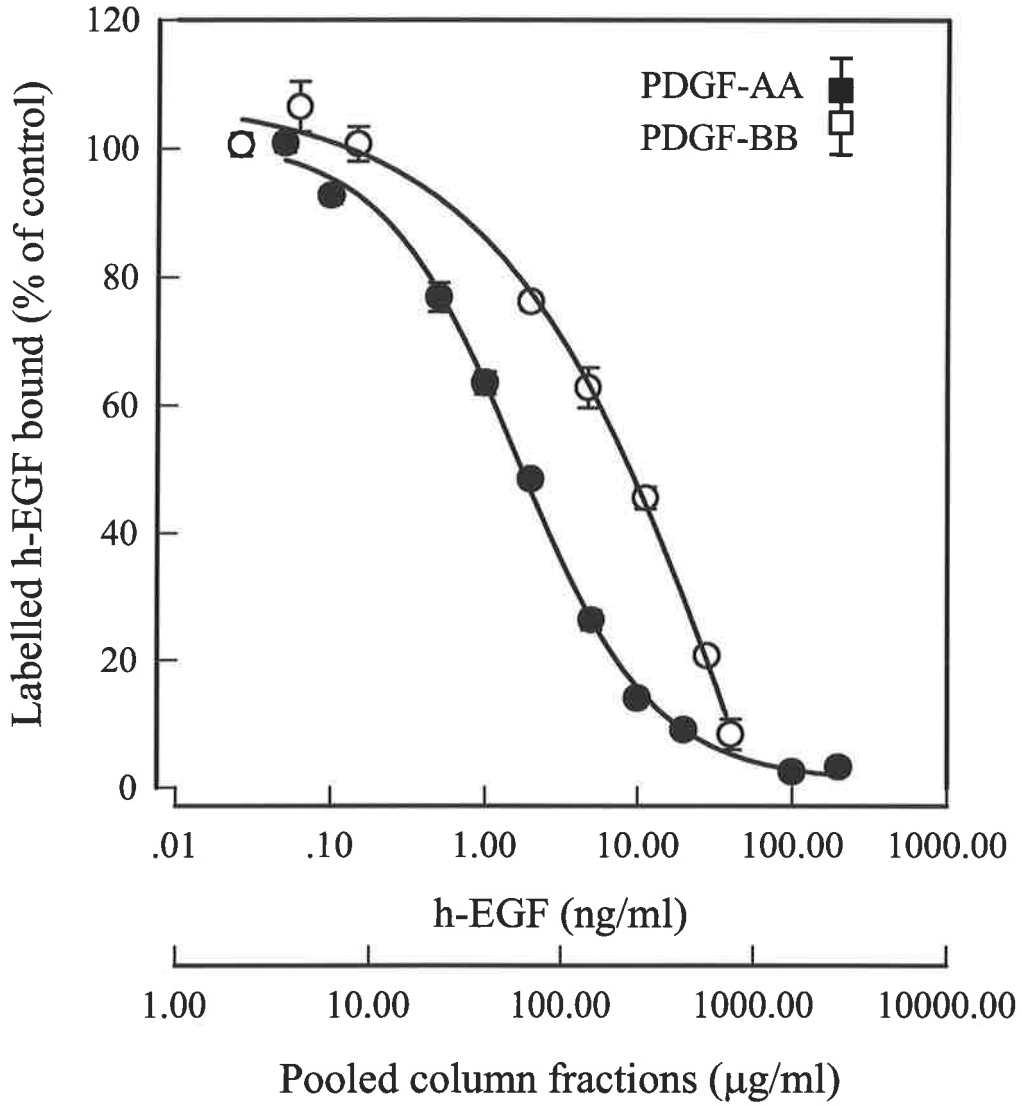



**Figure 7.4** EGF activity in pooled acid gel-filtration fractions of whey extract. EGF-like activity of whey extract eluting between 305 and 375 ml (**Figure 7.2**) was pooled, serially diluted and tested for ability to displace $^{125}$I-h-EGF from AG2804 cell monolayers (O). The amount of EGF was estimated in the pooled fraction by reference to a standard curve of h-EGF (●) included in the same assay (**7.2.1**). Results (mean ± S.E.M of three measurements) are expressed as a percentage of radioactivity bound to AG2804 cells incubated in HEPES-buffered saline alone. Protein in the pooled fractions was estimated by a bicinchoninic acid-binding assay kit with reference to bovine serum albumin (Pierce, Rockford, Illinois, USA). To demonstrate the specificity of the assay 200 ng platelet-derived growth factors (PDGF) -AA (□) and -BB (■) were included in the AG2804 RRA.

### 7.3.2 *Concentration of whey EGF-like activity by ultrafiltration*

To aid characterisation and purification of bovine milk derived EGF, a low molecular weight fraction of whey extract was produced by acid ultrafiltration. High molecular weight proteins of acidified whey extract were partitioned from low molecular weight proteins using a 100 kDa membrane and both fractions were neutralised, concentrated and freeze-dried. Initially, 115 l of liquid whey extract recovered from the cation exchange chromatography of 2300 l of cheese whey was subject to 100 kDa ultrafiltration (**7.2.2**) 11.96 g of the total protein in 115 l of liquid whey extract was recovered in the low molecular weight permeate and the rest (152.5 g) was in the high molecular weight retentate.

Portions (582.5 mg) of the permeate and retentate were then subject to acid gel-filtration (**2.2.4**). The recovered fractions were then tested in the AG2804 EGF RRA and BALB/c 3T3 cell assay. **Figures 7.5** and **7.6** show there was greater displacement of $^{125}$I-h-EGF by fractions of low molecular weight permeate compared to the corresponding fractions of high molecular weight retentate. Indeed, there were six fractions recovered from acid gel-filtration of permeate that displaced greater than 50% of bound radioactivity. Notably, the permeate fractions with the most EGF-like activity contained the greatest BALB/c 3T3 bioactivity and eluted between the 29 and 12.4 kDa standards (**Figure 7.5**). These results are comparable to that observed for acid gel-filtration fractions of whey extract (**Figure 7.3**). The fractions that eluted from acid gel-filtration of high molecular weight retentate did not displace more than 10% of $^{125}$I-h-EGF tracer from AG2804 cell monolayers (**Figure 7.6**). However, some of the retentate

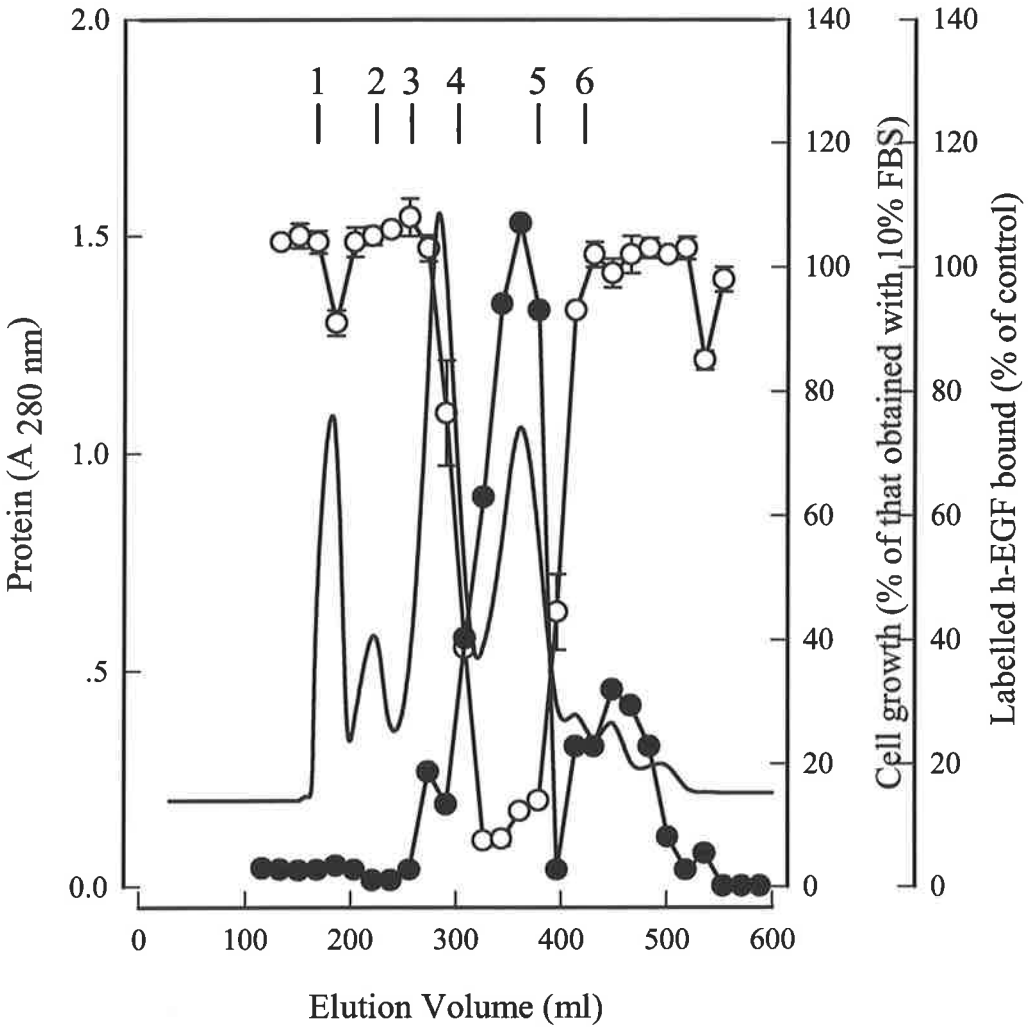



**Figure 7.5** EGF-like activity and BALB/c 3T3 bioactivity recovered from acid gel-filtration of a low molecular weight fraction of whey extract. A sample of low molecular weight (>100 kDA) permeate (582 mg) was dissolved at 25 mg/ml in a buffer of 150 mM NaCl, 1 M acetic acid and 10% (v/v) acetonitrile pH 2.0, and chromatographed on a Superdex 75-35/600 column at 15.6 cm/h (**2.2.4**), using the same buffer. Fractions of 17.5 ml were collected and 1 ml of each fraction freeze dried and tested for EGF activity in the AG2804 RRA (O) (**7.2.1**). Results (mean ± S.E.M of three measurements) are shown as a percentage of radioactivity bound to cells incubated in HEPES-buffered saline alone. A portion (40 µl) of each acid gel-filtration fraction was also tested for BALB/c 3T3 bioactivity (●) as described in **2.2.3**. Cell growth is shown as a % of the response to a 10% FBS standard incorporated onto each plate with the response to DMEM alone subtracted The column was standardised under neutral conditions with IgG1 (1; 150 kDa), BSA (2; 67 kDa), ovalbumin (3; 45 kDa), carbonic anhydrase (4; 29 kDa), cytochrome C (5; 12.4 kDa) and insulin (6; 7 kDa).

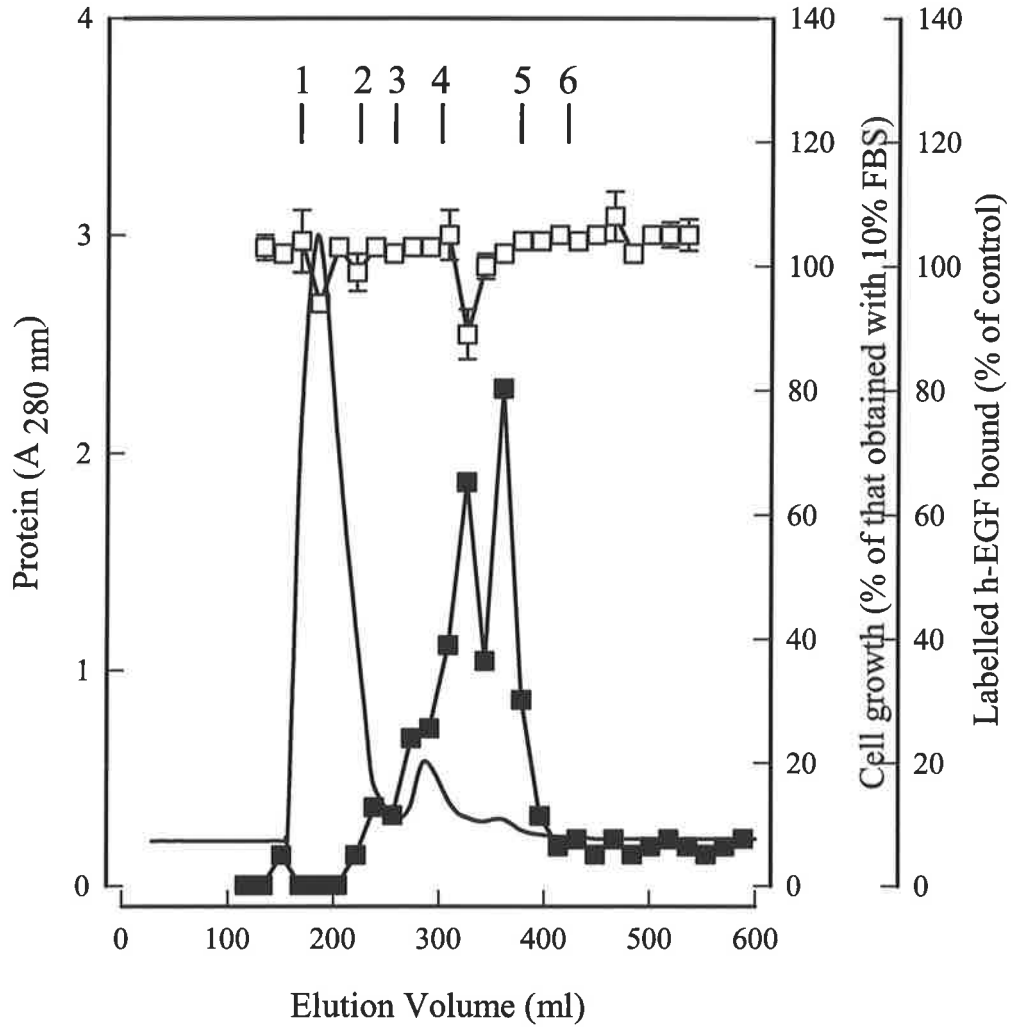



**Figure 7.6** EGF-like activity and BALB/c 3T3 bioactivity recovered from acid gel-filtration of a high molecular weight fraction of whey extract. A sample of high molecular weight (<100 kDa) retentate (572 mg) was dissolved at 25 mg/ml in a buffer of 150 mM NaCl, 1 M acetic acid and 10% (v/v) acetonitrile pH 2.0, and chromatographed at 15.6 cm/h on a Superdex 75-35/600 column at 22 cm/h (**2.2.4**), using the same buffer. fractions of 17.5 ml were collected and 1 ml portions tested for EGF activity by the AG2804 RRA **7.2.1** (□). Results (mean ± S.E.M of three measurements) are expressed as a percentage of radioactivity bound to AG2804 cells incubated in HEPES-buffered saline alone. A 40 µl sample of each fraction was also tested for BALB/c 3T3 bioactivity (■) as described in **2.2.3**, and cell growth shown as a % of the response to a 10% fetal bovine serum (FBS) standard subtracting the DMEM response. The column was standardised under neutral conditions with IgG1 (1; 150 kDa), BSA (2; 67 kDa), ovalbumin (3; 45 kDa), carbonic anhydrase (4; 29 kDa), cytochrome C (5; 12.4 kDa) and insulin (6; 7 kDa).

fractions induced BALB/c 3T3 cell growth in two small peaks that eluted between the 29 and 12.4 kDa standards (**Figure 7.6**).

The concentration of EGF-like activity recovered from acid gel-filtration of low molecular weight permeate was then measured. Portions (1 ml) of the permeate fractions that eluted between 326 and 396 ml (**Figure 7.5**) from the acid gel-filtration column were pooled, freeze dried and the pooled protein measured (**2.2.5**). The displacement of a dilution series of pooled material from $^{125}$I-h-EGF from AG2804 monolayers was then compared to dilutions of h-EGF. The dose response curves for the diluted fractions and h-EGF had similar slopes (results not shown). There was 4950 ng of EGF-like activity and 128 g protein recovered from loading 583 mg of permeate onto the gel-filtration column, giving a specific activity of 39 ng/mg recovered protein. This represents a 3-fold increase from the 11 ng/mg recovered from gel-filtration of whey extract. Since 11.96 g of permeate was produced from 165 g of whey extract, this gives a possible recovery of 102 μg of EGF-like activity from the permeate.

### 7.3.3 *Heparin-affinity of whey EGF-like activity*

The EGF-like activity recovered from acid gel-filtration of the permeate was further characterised by determining the strength it binds Heparin-Sepharose. EGF-like activity that eluted between 326 and 396 ml (**Figure 7.5**) was pooled and a portion (1 ml) freeze-dried. This was then made up in 1 ml of 10 mM Tris-HCl, pH 7.0 and loaded onto a Heparin-Sepharose column equilibrated in 0.2 M NaCl, 10 mM Tris-HCl, pH 7.0. After washing the column in the same buffer, bound protein was eluted at 14 cm/h using a 0.2 to 1.5 M gradient of NaCl. Portions (0.2 ml) of the recovered fractions were then measured for EGF-like activity by the AG2804 RRA (**7.2.1**). **Figure 7.7** shows that all

the permeate loaded on to the column bound to the heparin-Sepharose and did not elute before the salt gradient started. AG2804 radioreceptor assays indicated that EGF-like activity was recovered between 0.4 and 0.6 M NaCl and eluted with the major peak of protein.

### 7.3.4 *Partial purification of EGF-like activity of whey extract*

In an attempt to purify the EGF-like activity of whey extract, a five step procedure was used as outlined in **Figure 7.1** (***A-F***). A 582.5 mg sample of low molecular weight permeate produced from 8036 mg whey extract was subjected to acid gel-filtration followed by $C_4$ and $C_{18}$ reversed phase chromatographic steps.

Low molecular weight peremeate was subject to acid-gel filtration and EGF-like activity collected from fractions that eluted between 326 and 396 ml (see **Figure 7.5**; **Figure 7.1**(***B***)). This gel-filtration step eliminated over 75% of contaminating protein present in the low molecular weight permeate.

The recovered EGF-like activity was then subjected to $C_4$ reversed phase HPLC with a 0-80 % (v/v) acetonitrile gradient (**Figure 7.1** (***C***)). EGF-like activity was eluted at 35% (v/v) acetonitrile with a large peak of protein detected at 214 nm (**Figure 7.8 A**). This step resulted in the elimination of 60% of protein found in the pooled fractions collected from ***Step B*** and a 2 fold increase in specific activity (**Table 7.1**). The EGF-like activity was then reloaded onto the $C_4$ column and a gradient of 20 to 40% (v/v) acetonitrile used over 200 minutes (**Figure 7.1** (***D***)). A peak of EGF-like activity was eluted from the column at 23.1-24.3% (v/v) acetonitrile (**Figure 7.8 B**) This procedure eliminated approximately 99% of the protein present in ***Step C*** and increased the specific activity 80 fold from (**Table 7.1** (***C***)).

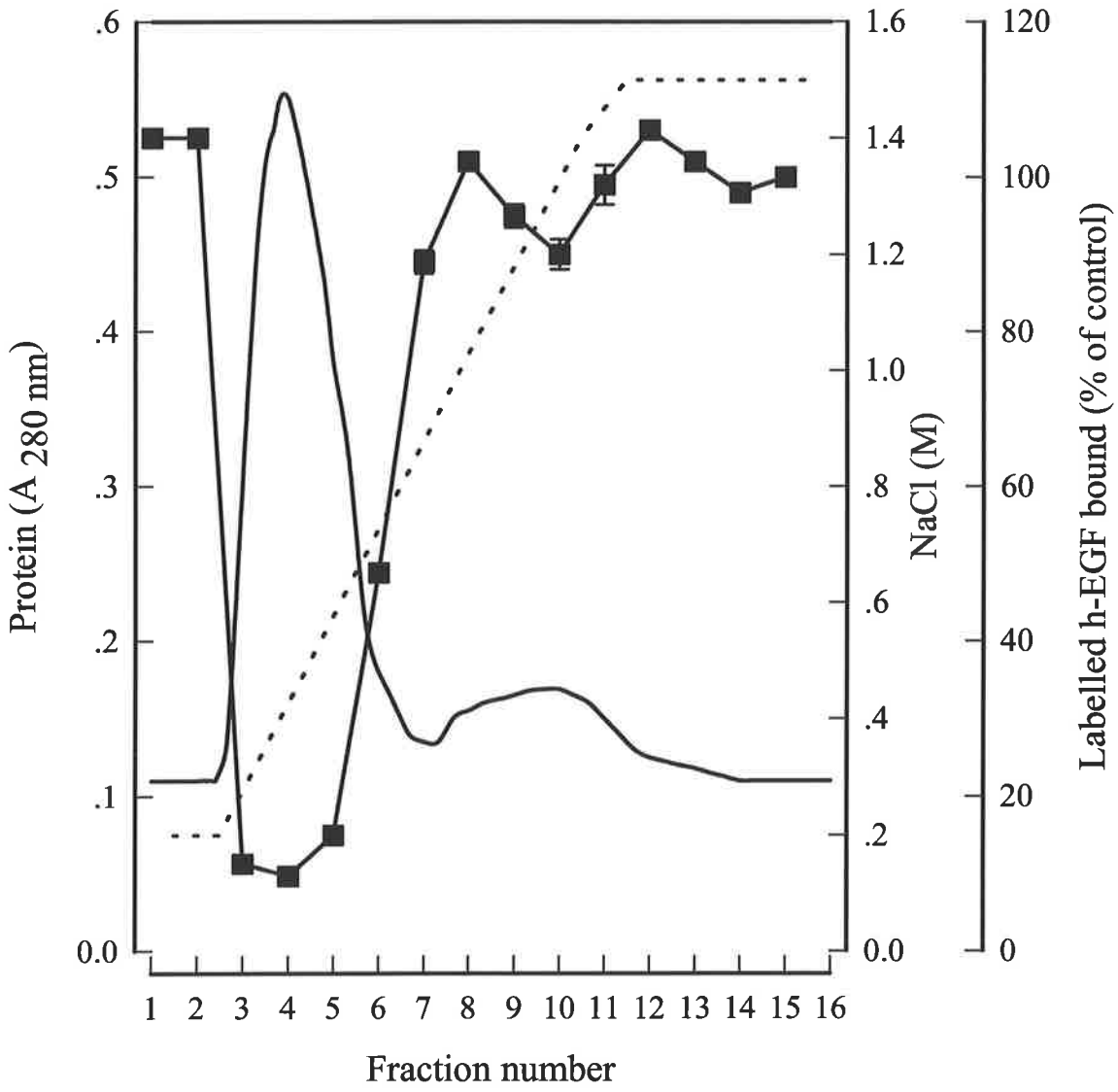



**Figure 7.7** Heparin-affinity analysis of EGF-like activity of low molecular weight whey extract. EGF-like activity of low molecular weight permeate that eluted from acid gel-filtration between 326 and 396 ml (**Figure 7.5**) was pooled, and a portion (1 ml) freeze-dried. This was then dissolved in 1 ml of 10 mM Tris-HCl, pH 7.0 and then loaded onto a 0.9 cm diameter column filled with 1.1 ml of Heparin-Sepharose equilibrated in 0.2 M NaCl, 10 mM Tris-HCl, pH 7.0. After washing the column in the same buffer, bound protein was eluted at 0.15 ml/min (14 cm/h) using a 0.2 to 1.5 M NaCl (- -) gradient over 26 minutes, and 0.45 ml fractions collected. EGF-like activity of portions (0.2 ml) of the recovered fractions were measured by the AG2804 RRA (**7.2.1**) (■). Results (mean ± S.E.M of three measurements) are expressed as a percentage of radioactivity bound to AG2804 cells incubated in HEPES-buffered saline, and non-specific binding (1% of bound counts) has not been subtracted.

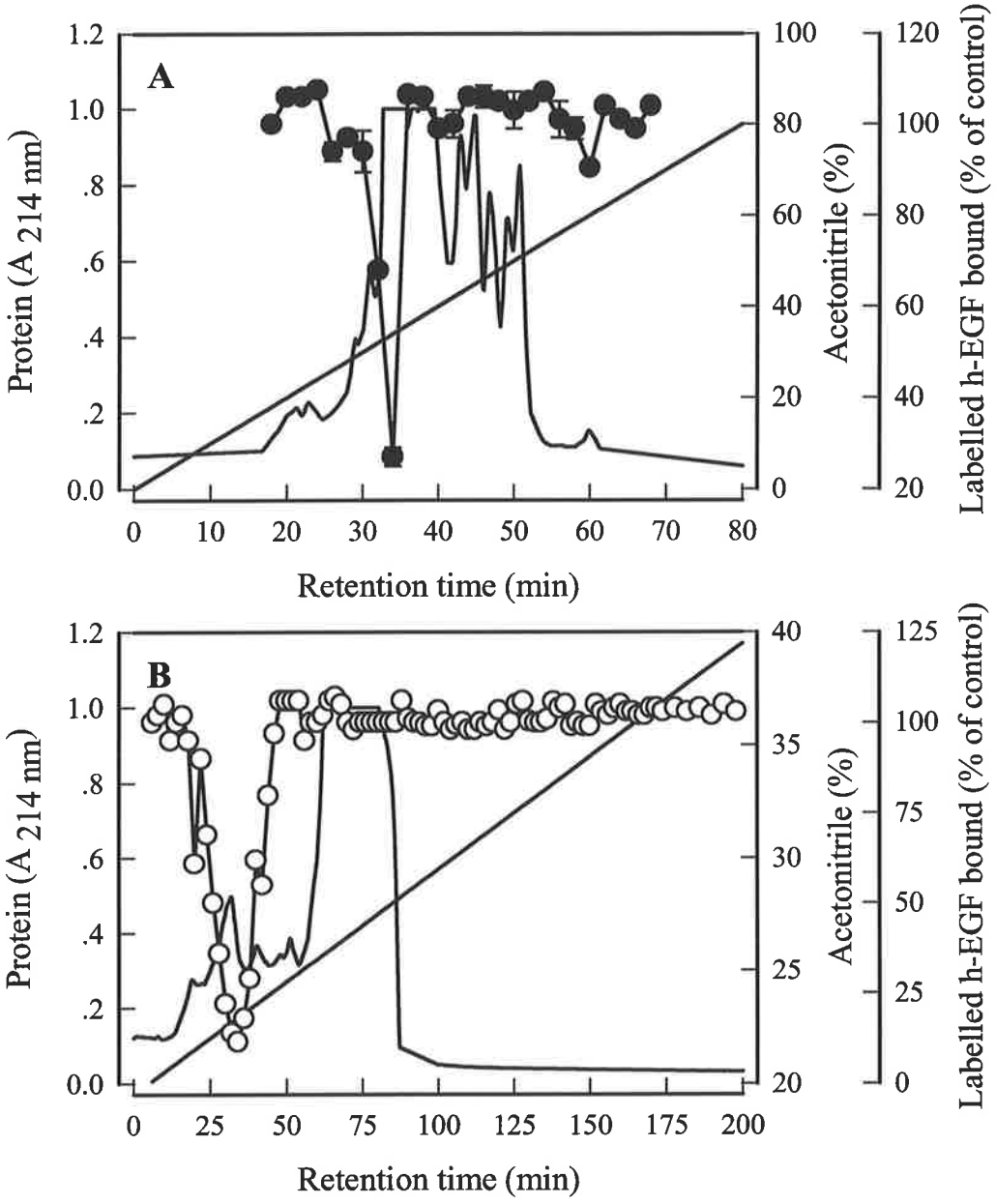



**Figure 7.8** EGF activity and initial RP-HPLC of low molecular weight permeate fraction of whey extract. Permeate was subject to acid gel-filtration (**Figure 7.1** (***B***)) and EGF-like activity pooled and loaded onto a $C_4$ (25 x100 mm) column (**A**). Bound protein was eluted at 5 ml/min with a gradient of 0-80% (v/v) acetonitrile (/) over 80 minutes, and 2 minute fractions collected (**Figure 7.1** (***C***)). EGF-like activity was measured by RRA (**7.2.1**) (●) then pooled and diluted 25 times. The EGF-like activity was then reloaded onto the $C_4$ column and protein eluted at 5 ml/min (**B**) with a gradient of 20-40% (v/v) acetonitrile (/) over 200 minutes (**Figure 7.1**(***D***)) and bioactivity measured by RRA (**7.2.1**) (○). RRA results (mean ± S.E.M of three measurements) are expressed as a percentage of radioactivity bound to AG2804 cells incubated in HEPES-buffered saline alone.

The EGF-like activity of whey extract recovered from the second $C_4$ purification (**Figure 7.8 B**) was subjected to $C_{18}$ reverse phase HPLC with a 0-80% linear gradient of propan-1-ol (**Figure 7.1** (***E***)). EGF-like activity was eluted at 33-37% (v/v) propan-1-ol in a fairly discrete peak that was separated from four other peaks of protein (**Figure 7.9 A**). This step resulted in purifying the EGF-like activity 3 fold from that found in ***Step D***. A gradient of 25 to 45% (v/v) propan-1-ol over 200 min was then used to further purify the EGF-like activity (**Figure 7.1** (***F***)). This final step resulted in EGF-like activity that eluted with 31.65 to 33.9% (v/v) propan-1-ol (**Figure 7.9 B**). The recovery from this step produced a 12 fold purification from that in ***Step E***. The whole procedure increased the EGF-like activity from 0.8 ng/mg in whey extract to 266400 ng/mg in the last $C_{18}$ reversed phase step (**Table 7.1**). It should be noted that the measurement of EGF-like equivalents by the AG2804 assay did not produce absolutely consistent results when the assays were repeated at each stage of the assay. Previous authors have reported variability in measuring bovine milk EGF-like activity by radioreceptor assays (Iacopetta *et al.* 1992).

The purity of the EGF-like activity was then examined by SDS 8-25% PAGE under reducing conditions. **Figure 7.10** shows a photograph of the gel. Although the EGF-like activity was less than 50% pure (by RRA), when 30 ng of EGF-like activity (100 ng protein) was loaded onto the gel, the resulting bands were stronger than that observed with 100 ng of h-EGF. **Figure 7.10** shows a very strong diffuse band appeared near the 20.1 kDa standard and appeared to smear to the base of the gel and another band appeared around 26 kDa. Around 1998 ng of the EGF-like activity was then subject to N-terminal sequence (**6.2.7**). However, three proteins of equal amount were present, so it was impossible to distinguish the sequence or primary and secondary proteins.

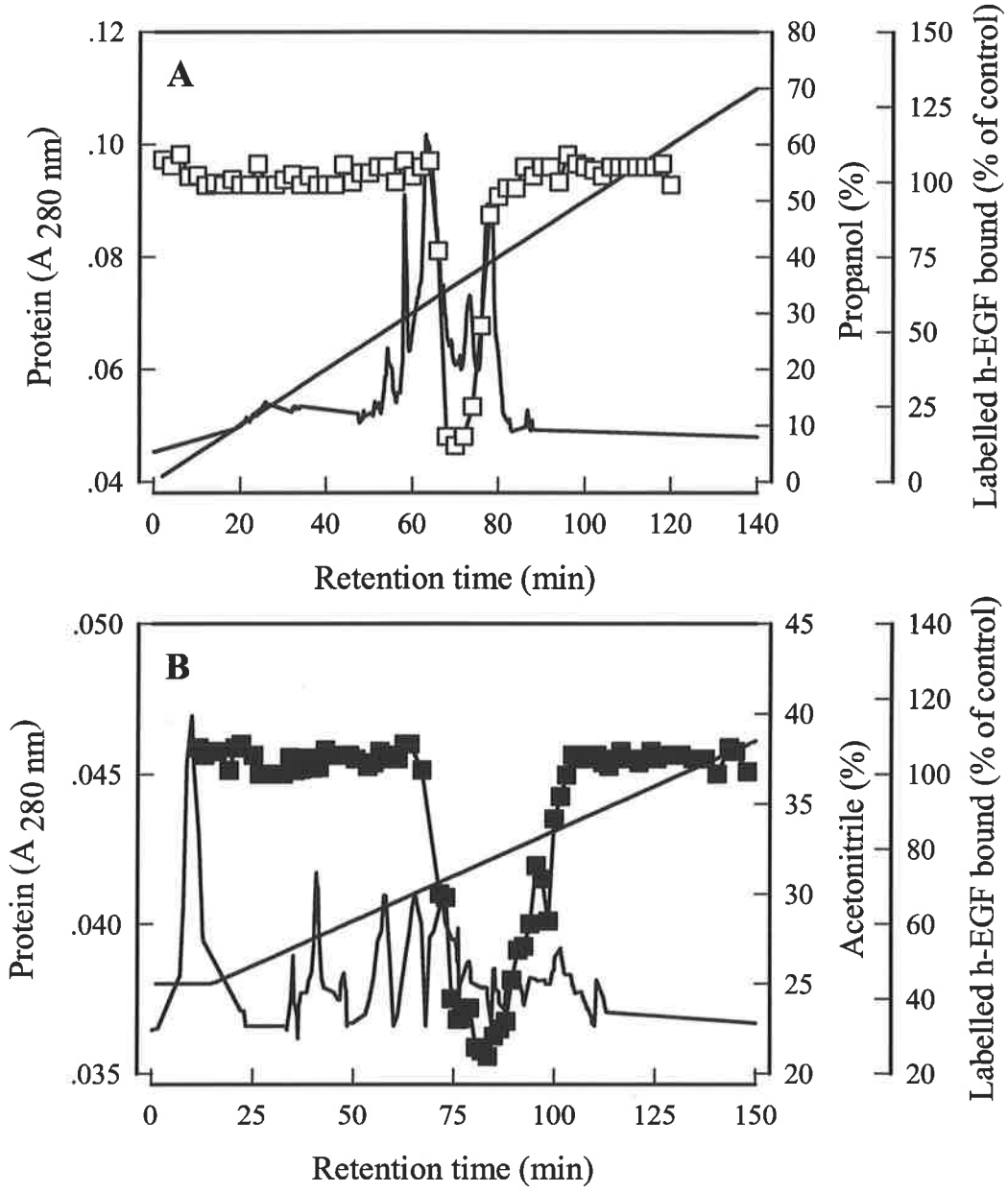



**Figure 7.9** Further purification of bovine EGF-like activity from whey extract. EGF-like activity of whey recovered from $C_4$ HPLC (**Figure 7.8 B**) was pooled, diluted 25 times in 0.13% heptaflurobutyric acid (HFBA) and loaded onto a $C_{18}$ (8 x100 mm) column (**A**). Bound protein was then eluted at 1 ml/min with a gradient of 0-80% (v/v) propan-1-ol (/) over 160 minutes and 2 minute fractions collected (**Figure 7.1** (***E***)). EGF-like activity was measured by the RRA (**7.2.1**) (□), pooled, diluted 12 times with 0.13% (v/v) HFBA and reloaded onto the $C_{18}$ column (**B**) (**Figure 7.1** (***F***)). Protein was eluted at 1 ml/min with a gradient of 25-45% (v/v) propan-1-ol (/) over 200 minutes; 2 minute fractions were collected and bioactivity measured by RRA (**7.2.1**) (■). RRA results (mean ± S.E.M of three measurements) are expressed as a percentage of radioactivity bound to AG2804 cells incubated in HEPES-buffered saline.

## Table 7.1 Purification of EGF-like activity from whey extract

| Purification | Protein | Total activity | Specific activity | Recovery | Relative purification |
|---|---|---|---|---|---|
| **step** | **mg** | **EGF ng eq** | **EGF eq/mg** | **%** | **fold** |
| ***Whey Extract*** | 8036.2 | 6429 | 0.8 | 100.0 | 1 |
| ***Ultrafiltration*** | 582.5 | 4950 | 8.5 | 76.9 | 11 |
| ***Step B*** | 127.8 | 4950 | 38.7 | 76.9 | 48 |
| ***Step C*** | 47.7 | 4015 | 84.2 | 62.4 | 105 |
| ***Step D*** | 0.57 | 3830 | 6719.3 | 59.6 | 8399 |
| ***Step E*** | 0.163 | 3724 | 22846.7 | 57.9 | 28558 |
| ***Step F**** | 0.0091 | 2424 | 266400.3 | 37.7 | 333000 |

EGF was partially purified from whey extract using the chromatographic steps described in **Figure 7.1**. The fractions obtained from each step were tested for EGF-like activity by the AG2804 radioreceptor assay (**7.2.1**). The active fractions from each procedure were pooled and recovered h-EGF equivalents (eq) then quantified by comparing the pooled fractions EGF-like activity with that of standard h-EGF. Protein was measured by a dye binding assay with reference to bovine serum albumin (**2.2.5**) and the total and specific EGF activity at each step in **Figure 7.1** determined. * protein estimated by N-terminal sequence

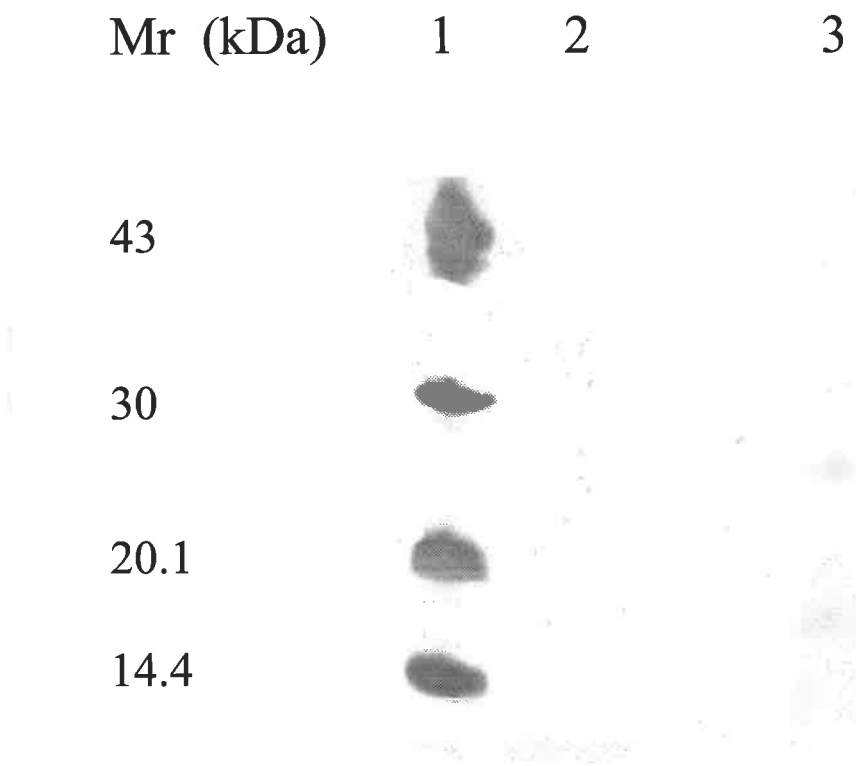



**Figure 7.10** SDS-PAGE of EGF-like activity from whey extract. EGF-like activity was purified from whey-extract as described in **7.2.4**. A sample recovered from the final purification step (**Figure 7.1** (***F***)) of EGF-like activity from whey extract (100 ng; **lane 3**) was subject to SDS-PAGE under reducing conditions on a 8-25% Polyacrylamide preformed slab gel and silver stained (**7.2.5**). Recombinant EGF (100 ng; **lane 2**) and molecular weight markers (300 ng; **lane 1**) were also subject to SDS-PAGE. The positions (**Mr** in **kDa**) of the molecular weight markers are also shown (bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa)).

## 7.4 Discussion

The results presented in the current chapter indicate that whey extract contains a protein that is a member of the EGF family of growth factors. This EGF-like protein may also be a major BALB/c 3T3 mitogen of bovine whey. Members of the EGF family that are growth factors include EGF, TGF-α, HB-EGF, amphiregulin, heregulin, and betacellulin (Savage *et al.* 1972; Marquardt *et al.* 1983; Higashiyama *et al.* 1991; Shoyab *et al.* 1989; Holmes *et al.* 1992; Wattanabe *et al.* 1994). Of these factors, EGF with an anionic pI (of 4.6) would be unlikely to bind the cation-exchange resin used to produce whey extract (Taylor *et al.* 1972). Indeed, Shing and Klagsbrun (1984) used anion-exchange chromatography to purify EGF-like activity from human milk. In the current study, it appears that a less anionic member of the EGF family of growth factors was recovered in the cation-exchange fraction of cheese whey. Future work should include surveying EGF activity in the whey fraction that does not bind the cation-exchange resin in the production of whey extract.

Radioreceptor assays (RRA) on whey extract fractions showed that whey extract contains ligands that displace labelled EGF from receptors. The RRA used in the current study can detect proteins with EGF receptor binding capacity but is unable to discern the ligand being measured. Notably, EGF, TGF-α, HB-EGF and betacellulin all compete for EGF receptors used in radioreceptor assays (Massague 1983b; Higashiyama *et al.* 1991; Wattanbe *et al.* 1994). Indeed, **Figure 7.4** shows that the dilution curve of the EGF-like activity in whey extract and human EGF have similar slopes and so appear to compete for the same receptors.

There was approximately 0.8 ng of EGF-like activity recovered per mg of acidified whey extract loaded on to the gel-filtration column. However, the amount of EGF-like activity in whey extract may not have been estimated accurately by the method available. The RRA was limited in that it is unable to discern which specific ligand is being measured and therefore if there is more than one EGF-like molecule in whey extract. In addition, although EGF ligands compete for over expressed EGFR/ErbB-1 receptors on AG2804 cells, the ligands may have slightly different affinities if they also bind other less frequently expressed ErbB receptors. For example, betacellulin binds all possible dimmeric combinations of EGFR//ErbB-1, ErbB-2/HER-2, ErbB-3/HER-3 and ErbB-4/HER-4 receptors (Wattanbe *et al.* 1994; Alimandi *et al.* 1997). Also, the bovine EGF-like molecule of whey extract may be a different size than the 6 kDa h-EGF used in the RRAs and therefore not exactly equivalent on a molar basis when comparing displacement curves. Nevertheless, although the radioreceptor assay was not an optimum procedure, it was useful for surveying EGF activity through various purification steps. More accurate assays such as radio-immuno assays could be developed when antisera to the purified bovine EGF-like molecule is available.

The gel-filtration data presented in this chapter shows that the EGF-like factor of bovine whey elutes from gel-filtration columns at a similar molecular weight range as HB-EGF, amphiregulin and betacellulin. The EGF-like activity of whey extract was eluted from acid gel-filtration between the 12.4 and 29 kDa standards (**Figure 7.3**, **7.5**). Previous gel-filtration research has shown that human EGF and TGF-α elute at approximateley 6 kDa (Gregory 1975; Marquardt *et al.* 1984). However, HB-EGF and amphiregulin elute from gel-filtration chromatography at approximately 12-14 kDa, and betacellulin between 26 and 45 kDa (Higashiyama *et al.* 1992; Shoyab *et al.* 1989; Wattanbe *et al.* 1994).

Heparin-affinity chromatography confirmed that the EGF-like molecule found in whey extract is not EGF or TGF-α. Members of the EGF family can be divided into heparin-binding and non heparin-binding molecules. EGF and TGF-α do not bind heparin and elute in the void volume from heparin-affinity columns (Besner *et al.* 1990; Thompson *et al.* 1994). Amphiregulin, heparin-binding EGF (HB-EGF) and betacellulin all bind heparin-Sepharose with differing affinities. Amphiregulin and HB-EGF require 1.0 M NaCl to elute from heparin-sepharose columns, whereas betacellulin elutes with 0.5 M NaCl (Besner *et al.* 1990; Higashiyama *et al.* 1991; Shing *et al.* 1993; Wattanbe *et al.* 1994; Thompsom *et al.* 1994). **Figure 7.7** shows that EGF-like activity of whey extract bound heparin sepharose and was eluted with 0.4 to 0.6 M NaCl. The EGF-like activity in whey extract could therefore be a betacellulin-like molecule.

The EGF-like protein was partially purified using a six-step procedure (**Figure 7.8**, **7.9**, **Table 7.1**). The final step did not produce enough protein to produce an unambiguous N-terminal sequence. In addition, there was more than one band of protein shown in the PAGE of the recovered activity from the final purification step (**Figure 7.10**). The current purification procedure should be repeated until a distinct N-terminal sequence can be obtained. The purified molecule can then be run on PAGE and pI gels and recombinant molecule manufactured. The pure molecule could then be used to produce antibody for more accurate radioimmunoassays, western blots and immuno-neutralisation experiments.

Previous research on the growth factor component of milk has shown that EGF is a predominant growth factor in human milk but not bovine milk (Shing and Klagsbrun 1984; Carpenter 1980; Iacopetta *et al.* 1992). In the earlier chapters presented for this thesis, the results have shown whey extract contains small amounts of FGF and PDGF and a significantly large amount of acid-activatable TGF-β. Neither PDGF nor TGF-β

activity isolated from acid-gel filtration fractions of whey extract eluted exactly co-incident with the fractions containing the most BALB/C 3T3 bioactivity (**Figure 3.12, Figure 4.1**). However, the EGF-like activity of whey extract co-eluted with BALB/c 3T3 bioactivity (**Figure 7.3**). The significance of this finding is that these EGF-like molecules could be a major BALB/c 3T3 mitogen of bovine milk. However, the BALB/c 3T3 cell data does not indicate which member of the EGF family is present in whey extract because all are similarly potent BALB/c 3T3 mitogens (Schreiber *et al.* 1986; Seno *et al.* 1996; Wattanbe *et al.* 1994).

The data presented in this chapter indicates that the EGF-like activity of bovine whey may require acid treatment for it to be fully active. Throughout this thesis the results have shown that mitogens in whey extract associate with large whey proteins under neutral conditions and are then separated from such proteins by acid treatment (**Figure 3.10, 4.2, 6.3, 6.4**). **Figure 3.10** shows that only two neutral gel-filtration fractions of whey extract induce a small BALB/c 3T3 cell growth response and these elute between the 67 and 150 kDa standards. Conversely, 20 acid gel-filtration fractions that elute between the 150 and 67 kDa standards induce a large amount of BALB/c 3T3 cell growth. Given that the EGF-like activity co-elutes with acid gel-filtration fractions containing high BALB/c 3T3 bioactivity (**Figure 7.3**), then some of the EGF-like activity in whey extract may be latent. Interestingly, binding proteins that dissociate with low pH have been reported in human milk, but have not been characterised (Azuma *et al.* 1989). Significantly, the results presented in this chapter do not indicate that the EGF-like activity in whey extract is similar to the high molecular weight active forms of prepro-EGF found in human milk. In most reports of EGFs in human milk, size heterogeneity has been observed and attributed to differential enzymatic cleavage of precursor EGF (Read *et al.* 1984; Mroczkowski *et al.* 1989; Brown *et al.* 1989;

Personen 1987; Mroczkowski and Reich 1993). Indeed, gel-filtration experiments on human EGF have shown that the soluble high molecular weight precusors found in milk are biologically active and do not cleave under acid conditions to release low molecular weight EGF (Read *et al.* 1984; Mroczkowski *et al.* 1989; Personen 1987; Mroczkowski and Reich 1993). More comprehensive work should be done to determine the form and size of the EGF-like molecule in bovine whey extract and if it is latent. This could include acid and neutral Superose 6 and 12 gel-filtration chromatography.

The data presented in this chapter indicates that the putative EGF-like molecules in whey extract may be a major BALB/c 3T3 mitogen of whey extract. It can not be implied from the current data that this molecule is at the same concentration in neutral whey extract as in acid-treated whey extract. Also, such data can not be translated to whole milk and whey. All the measurements and isolation of the EGF-like molecule were taken after the whey extract was acidified. To study the contribution of growth factors such as the EGF-like protein to the overall mitogenic effect of milk, whey and whey extract the actual concentration in neutral and acid-treated milk fractions must be known. This could be achieved when anti-sera and immuno-assays are available that can detect the factor in milk, whey and whey extract samples before and after they are acidified.

The significance of the EGF-like molecule is not known. Previous work has shown that members of the EGF family of mitogens have roles in mammary development and function, in addition to gut development in the suckling young (Vonderhaar 1987; Coleman 1988; Falconer 1987; Berseth 1987; Gale *et al.* 1989; Shen and Xu 1996). Speculation at to the role of the EGF-like molecule in such processes are premature and should be examined after the molecule is completely characterised.

# CHAPTER Eight:

# Conclusion

## 8.1 Summary of Thesis and Future Work

### 8.1.1 *Growth factor concentrations in whey extract*

**Table 8.1** shows the concentrations of growth factors in bovine whey extract and whey determined in the results presented for this thesis. The concentration of growth factors recorded for whey is from acid-treated whey for TGF-β; for the other factors the levels were determined from acidified whey extract, using the recovery of 30-60 mg of whey extract/litre of whey. Further work needs to be done to determine the concentration of PDGF-BB, PDGF-AA, and EGF-like molecule in neutral whey extract and confirm the concentration of these factors in neutral and acified whey. The concentration of FGF-1 in acid-treated whey extract could also be determined.

**Table 8.1 Concentration of growth factors in bovine whey extract and whey**

| Factor | Neutral Whey Extract | Acidifed Whey Extract | Acidified Whey Equivalents |
|---|---|---|---|
| | **ng/mg** | **ng/mg** | **ng/ml** |
| **PDGF-BB** | NR | 4.8 ± 0.4 (n=3) | 0.14 |
| **PDGF-AA** | NR | 1.1 ± 0.1 (n=3) | 0.033 |
| **FGF-1** | 0.19 (n=2) | NR | 0.006 |
| **FGF-2** | 0.66 (n=2) | * | 0.018 |
| **TGF-β** | 0.8 ± 0.1 (n=9) | 42.2 ± 6.8 (n=9) | 3.7 ± 0.7 (n=9) ** |
| **EGF-like molecule** | NR | 0.8 ± (n=3) | 0.05 |

NR: not Recorded; *= unstable in acid. ** measured in acid-treated whey

### 8.1.2 *Cell growth studies with whey extract*

Whey extract is a cation-exchange fraction produced from bovine cheese whey. Most of the non-mitogenic whey proteins and fluids are removed in the production of whey extract, but almost all the mitogenic activity is retained. Previous studies found whey extract more potent than cheese whey in stimulating growth of L6 rat myoblasts, BALB/c 3T3 cells and human skin fibroblasts (Francis *et al.* 1995). In **Chapter 3**, the ability of whey extract to induce cell growth was examined further and the size of the mitogenic proteins were determined by gel-filtration. Whey extract was shown to induce mesenchymal-derived cell growth greater than that achieved with 10% FBS. It also induced L6 Myoblast, BALB/c 3T3 cell and human skin fibroblast growth more than any single recombinant growth factor, including PDGF, TGF-β, EGF, FGF and IGF.

Although a source of growth factor activity for mesenchymal-derived cells, whey extract essentially inhibited epithelial cell growth. However, epithelial cell growth was increased at very low concentrations of neutral whey extract. In addition, fractions recovered from acid gel-filtration of whey extract were strongly inhibitory for epithelial cells. This suggested the presence of TGF-β and that it is mostly inactive before acid treatment. Other research has revealed that TGF-β is present in bovine milk, but its concentration and the proportion of active and latent TGF-β was not investigated (Cox and Burk 1991; Jin *et al.* 1991). The strong inhibitory action observed with whey extract on attached epithelial cells does not support earlier research that found such cells were induced to grow by colostrum and to a lesser extent mature milk (Steimer *et al.*

1981; Corps and Brown 1987). However, the cation-exchange process used to isolate whey extract may have enriched the epithelial cell growth inhibitors more than growth promoters.

Gel-filtration experiments showed that whey extract contains low molecular weight mesenchymal-derived cell growth promoting activity that can be separated from their associations with larger whey proteins by acid treatment. In contrast to neutral gel-filtration where two high molecular weight fractions induced cell growth, nearly 20 lower molecular weight acid gel-filtration fractions induced significant mesenchymal-derived cell growth. Under acid conditions, whey extract proteins with high absorbance at 280 nm chromatographed between the 67 and 150 kDa standards and did not induce cell growth. Rather, it was those fractions that eluted between the 6-29 kDa standards that contained mitogenic activity, where the absorbance at 280 nm detected minimal protein. This data suggests that most of the growth factor activity in whey extract is attached to larger whey proteins and such associations are broken under acid conditions. This agrees with previous findings that all growth factors known to be in milk can be found in associations with high molecular weight binding proteins, soluble receptors or transport proteins (Grosvenor *et al.* 1992).

The acid gel-filtration experiments on whey extract also indicated that there were groups of fractions which induced L6 and skin fibroblast growth and others that induced BALB/c 3T3 cell growth. There were two peaks of fractions recovered from acid gel-filtration of whey extract that induced both L6 myoblast and human skin fibroblast cell growth. One peak was between the 45 and 12.4 kDa standard, the other near the 7 kDa standard. The response of L6 myoblasts to whey extract fractions suggests the presence of IGF and IGF-BPs, which are known to be in bovine whey (Ronge and Blum 1988; Vega *et al.* 1991; McGrath *et al.* 1991; Skaar *et al.* 1991). The group of acid gel-

filtration fractions of whey extract that induced BALB/c 3T3 cell growth were different than those inducing L6 myoblast and skin fibroblast growth. A large group of fractions eluting near the 29 kDa standard and a smaller peak of fractions below the 12.4 kDa standard were mitogenic for BALB/c 3T3 cells. Growth factors such as PDGF, FGF, TGF-β and EGF may contribute to the peaks of mitogenic acid gel-filtration fractions and will be investigated in this thesis.

Future work could include determining the mixture of growth factors that most approximates the mesenchymal-derived cell growth factor activity of whey extract. This may include cell growth studies using quiescent cells to study the contribution of competence and progression factors to the bioactivity of whey extract. Other experiments could be undertaken to discern whether an additive or synergistic effect of pure recombinant growth factors found in whey extract can approximate the cell growth bioactivity of whey extract.

### 8.1.3 *Platelet-derived growth factor*

The results in **Chapter 3** show that BALB/c 3T3 cells were potently stimulated by acid gel-filtration fractions of bovine whey extract that eluted at the molecular weight region consistent with the presence of platelet derived growth factor (PDGF). In addition, the most potent recombinant factor for BALB/c 3T3 cells was found to be PDGF. In **Chapter 4**, the results indicate that PDGF-like activity was recovered from acid gel-filtration fractions of whey extract as shown by radioreceptor assays and immunoblotting. PDGF-BB and not PDGF-AA like molecules seemed to be the predominant isoform in whey extract. Previous work has found PDGF-BB was the predominant form in bovine platelets and many bovine cell types (Seifert *et al.* 1984;

Shimokado *et al.* 1984; Kartha *et al.* 1988; Campochiaro *et al.* 1989; Bowen-Pope *et al.* 1989).

The PDGF-like activity in acid gel-filtration fractions of whey extract was pooled and measured by radioreceptor assay. There was 4.8 ng of PDGF-BB equivalents per mg of acid-treated whey extract loaded onto the gel-filtration column. This translates to 0.14 ng of PDGF-BB per ml of acidified cheese whey. The acid gel-filtration experiments also showed that the PDGF activity of whey extract eluted earlier than the major peak of BALB/c 3T3 bioactivity. The PDGF-like activity of whey extract eluted between the 45 and 29 kDA standards, whereas the major peak of BALB/c 3T3 bioactivity eluted near the 29 kDa standard.

Previous research has shown that PDGF can associate with binding proteins like plasma derived $\alpha_2$-M and extracellular proteins such as SPARC and dissociate with low pH (Raines and Ross 1992; Raines *et al.* 1992). However, no bovine milk derived PDGF binding proteins have been reported, although $\alpha_2$-M has been detected in bovine whey (Perez *et al.* 1989; Rantamaki and Muller 1992). In addition, the 725 kDa $\alpha_2$-M would elute in the void volume from the column used for acid gel-filtration of whey extract, as will its 440 kDa subunit produced when $\alpha_2$-M is subject to low pH (Raines *et al.* 1984; Bonner *et al.* 1992). Importantly, the current results show that PDGF-like activity may be inactivated by high molecular weight proteins present in whey extract and activated by low pH.

An antibody that neutralised the BALB/c 3T3 cell growth promoting activity of PDGF, had a small effect on the BALB/c 3T3 bioactivity of acid-treated whey extract but none on neutral whey extract. Although there is only a small amount of PDGF in milk whey (0.14 ng/ml) compared to colostral whey (200 ng/ml; Shing and Klagsbrun 1987), the antibody study show that the PDGF activity in whey extract is only bioactive

for BALB/c 3T3 cells after the extract is acidified. These results also agree with the acid gel-filtration data showing that PDGF-like activity in whey extract is inactivated by associations with high molecular weight whey proteins and activated after it is acidified.

The work on PDGF in whey extract differs from Shing and Klagsbruns (1984) observation that PDGF is not found in mature bovine milk. However, the low amounts of PDGF in bovine whey extract suggest that unlike colostrum it is not a major mature milk growth factor. Future research could include purifying the PDGF-like molecule and its binding proteins to homogeneity. In addition, an assay such as a radio-immunoassay could be developed that can measure PDGF accurately in neutral whey extract, mature milk and colostrum. PDGF could then be measured in both neutral and acid gel-filtration fractions of whey extract to determine the form of PDGF in milk and the size of the binding proteins. Assays for $\alpha_2$-M could be used to indicate if this factor is a major binding protein for PDGF in bovine milk.

### 8.1.4 *Fibroblast growth factor*

Fibroblast growth factors (FGFs) are found in several different mammalian tissues but are not usually soluble factors and have not been identified in milk or colostrum (Gospodarowicz *et al.* 1987; Klagsbrun 1989; Klagsbrun 1992). In **Chapter 5**, heparin-Sepharose chromatography was used to identify acidic FGF/FGF-1 and basic FGF/FGF-2 in bovine whey extract. A classical three step procedure was used whereby whey extract was loaded on to a heparin-affinity column in a 0.6 M salt buffer, washed with same buffer and then eluted step-wise with 0.8, 1.2 and 2.0 M salt (Lobb *et al.* 1986). FGF was then identified by BHK-21 radioreceptor assays and confirmed by immunoblotting. FGF-1 was identified in the 1.2 M fraction and FGF-2 in the 2.0 M fraction. However, only a small amount of FGF was found in whey extract and this was

fairly impure. There was 0.19 ng of FGF-1 and 0.6 ng of FGF-2/mg whey extract that translated into 5.8 ρg of FGF-1 and 18 ρg of FGF-2 per ml of whey respectively. As FGF-2 is unstable in the acid conditions used to separate whey from solids, this may account for its observed low frequency (Gospodarowicz 1975; Thomas *et al.* 1980). Importantly, removal of heparin-binding factors from whey extract made little difference to its bioactivity on BALB/c 3T3 cells. Although heparin-affinity chromatography is specific, whey derived FGF was fairly impure and may reflect the diversity of whey proteins and their physical characteristics on chromatography media (Gospodarowicz *et al.* 1987; Klagsbrun 1989; Baird and Bohlen 1990).

The current work on FGF is the first description of FGF-1 and FGF-2 in bovine whey and is consistent with a previous report describing heparin-binding growth factors in bovine prepartum secretions (Sandowski *et al.* 1993). If the concentration of FGF-2 is similar in whole bovine milk (19.8 ρg/ml) it is within physiological active range (Gospodarowicz *et al.* 1987). However, the source and significance of soluble FGF in milk is unknown but may be derived from lysis of the cellular component of milk during cheesemaking (Lee *et al.* 1980; Brooker *et al.* 1983; Saad and Ostensson 1990; Klagsbrun 1992).

Future work should include purifying bovine milk FGF-1 and FGF-2 to homogeneity and developing an assay such as a radioimmuno asssay that can be used to quantify FGF in all milk fractions. The FGF binding proteins could also be investigated by producing sufficient 1.2 and 2.0 M elution protein from heparin-affinity chromatography of whey extract for gel-filtration experiments.

### 8.1.5 *Transforming growth factor-betas*

The cell growth experiments described in **Chapter 3** showed that whey extract contains a large amount of epithelial cell growth inhibitory activity suggesting the presence of transforming growth factor beta (TGF-β). The cell growth studies also indicated that a large proportion of TGF-β in whey extract requires acid treatment before it is active and that this TGF-β-like activity may be an important mitogen for BALB/c 3T3 cells. Previous research has identified TGF-β in bovine milk but its concentration in milk was not known nor the molecular form it takes, or its contribution to the growth factor activity of milk (Tokyuyama and Tokuyama 1989; Cox and Burk 1991). In **Chapter 6**, the research undertaken aimed to quantify and describe the active and latent TGF-β activity in bovine milk and investigate the significance of TGF-β to the mitogenic activity of milk.

A 96-well plate Mv1Lu cell growth inhibitory assay was developed to measure TGF-β activity in milk and whey fractions. The total amount of acid-activatable TGF-β in milk was 4.3 ± 0.8 ng/ml and similar concentrations were found in commercial cheese whey (3.7 ± 0.7 ng/ml). The amount of TGF-β recovered in whey extract and measured throughout its production was greater than 98% (2.5 ± 0.2 ng/ml) of that present in the original material. There was also a 60-fold increase in specific TGF-β activity (from 0.7 ± 0.04 in whey to 42.2 ± 6.8 ng/mg in whey extract). This suggests little loss during the cheese making process and in the cation-exchange process used to make whey extract; further characterisation of bovine milk-derived TGF-β was therefore undertaken using whey extract. Chemical and physical treatments, including exposure to acid, alkali, urea and heat all increased TGF-β bioactivity of the whey extract.

The bulk (90%) of TGF-β activity recovered from bovine whey was identified as TGF-β2 by purification to homogeneity and sequencing. A second, smaller peak of TGF-β activity was recovered from the last stage of purification. The two peaks of TGF-β obtained from cheese whey are similar to those obtained by Cox and Burk (1991) and Jin *et al.* (1991) from bovine milk and indicate the second peak of TGF-β activity is probably TGF-β1. Importantly, active TGF-β detected in both milk and whey and throughout the purification procedure was neutralised by a monoclonal antibody that recognised all mammalian isoforms of TGF-β.

Gel-filtration chromatography of the whey extract resolved two peaks of latent TGF-β activity. Previous work has revealed there are there are two types of latent TGF-β complexes. The first is comprised of active TGF-β and its latency associated peptide (LAP) called 'the small latent complex' and is sufficient to confer latency to mature TGF-β (Pircher *et al.* 1986; Gentry *et al.* 1987, *opcit* 1988; Wakefield *et al.* 1989; Miyazono and Heldin 1991). The second is the latent TGF-β binding protein (LTBP) covalently bound to the LAP termed the 'large latent complex', with the LTBP not required for latency (Wakefield *et al.* 1988; Miyazano *et al.* 1988; Kanzaki *et al.* 1990). In the current experiments, acidification followed by chromatography under neutral conditions, or chromatography under acidic conditions, released active TGF-β from both high molecular weight latent peaks. The major peak of activity corresponded to a molecular weight of 80 kDa, suggesting that milk-derived TGF-β exists predominantly as TGF-β connected to its LAP (small latent complex) and lacks the latent LTBP associated with the large latent complex. Small latent complexes have been detected from *in-vitro* sources and are the primary forms secreted by CHO or COS cells transfected with TGF-β1 or TGF-β2 precursor cDNA and constitute 20-40% of platelet TGF-β1 (Dallas *et al.* 1994; Bonewald *et al.* 1991; Wakefield *et al.* 1988; Lioubin *et al.*

1991; Olofsson *et al.* 1992; Moren *et al.* 1994; Gentry *et al.* 1987; Madisen *et al.* 1990; Grainger *et al.* 1995). In contrast, the majority of TGF-β isolated from rat and human platelets chromatographs from gel-filtration columns at an apparent molecular weight of 400-670 kDa and has been identified as the large latent complex (Miyazono *et al. 1*988; Wakefield *et al. 1*988; Okada *et al. 1*989). Some authors have further suggested that latent forms of TGF-β lacking the LTBP are characteristic of secreted or soluble forms of TGF-β (Dallas *et al. 1*994; Bonewald *et al. 1*991). Whilst the data in **Chapter 6** would support this contention, the *in-vivo* mechanism of activation, and physiological role of small latent complexes remain to be elucidated. The current report is the first description of a small latent-like complex in a biological fluid.

A smaller peak of latent bovine whey-derived TGF-β activity was found at an elution volume corresponding to 600 kDa. Previous research has shown that α2-macroglobulin-bound TGF-β found in human serum and the large latent complex elute from Superose 6 columns in the 500-700 kDa region (O'Connor-McCourt and Wakefield 1987; Bonewald *et al. 1*991; Wakefield *et al. 1*988; Miyazono *et al. 1*988). Indeed, α2-macroglobulin has been detected in bovine milk (Rantamaki and Muller 1992). In addition, Wakefield *et al.* (1987) and Wakefield *et al.* (1988) showed that active TGF-β can associate with α2-M and be released from the complex under strong acid, but, acid denatured the α2-M tetramer to prevent any reforming of the TGF-β-α2-M complex, or any further activity of α2-M. In contrast to the TGF-β-α2-M complex, the large latent complex consists of the LTBP covalently bonded to the LAP, not TGF-β (Wakefield *et al.* 1988; Miyazono *et al.* 1988; Kanzaki *et al.* 1990). Transient acid treatment of this large latent complex results in some TGF-β reassociating with the LAP under equilibrium conditions (Wakefield *et al.* 1988; Miyazono *et al.* 1988). The current experiments shows that whey contains some acid activated TGF-β that can re-

associate with a large protein and remain bioactive, suggesting that the binding protein is not α2-M. Indeed, other dissociating agents that worked for whey extract such as heat and transient alkalinity, are not known to activate the TGF-β-α2-M complex (Wakefield *et al.* 1988; Brown *et al.* 1990). Further studies will be required to fully describe the structure of the large latent TGF-β complex found in bovine milk.

The data presented in **Chapter 6** indicates that over 92% of TGF-β in bovine whey and 98% in whey extract was latent and could be activated by acid treatment. In addition, the contribution of TGF-β to the ability of non-acidified whey extract to induce BALB/c 3T3 cell growth appears to be minimal. A neutralising TGF-β antibody only had a significant effect at the maximal concentration of neutral whey extract (5 mg/ml) that can be added to BALB/c 3T3 cells in culture. Lower concentrations of neutral whey extract contained little active TGF-β and therefore neutralising TGF-β had minimal effect on the BALB/c 3T3 bioactivity of whey extract. Significantly, anti-TGF-β had a much greater effect on whey extract after it was acid activated and therefore contained larger amounts of TGF-β that can induce BALB/c 3T3 growth. The previous work in this thesis has also shown that FGF and PDGF do not notably contribute to the BALB/c 3T3 cell bioactivity in neutral whey extract. A significant component of the BALB/c 3 T3 cell growth promoting activity in neutral whey extract is uncharacterised.

Future work could include purifying and fully characterising the small and large latent complexes in milk. Since the amount of TGF-β detected in bovine milk is within the physiological range for this growth factor and latent TGF-β could be activated in the acid stomach, further experiments to assess its role could be undertaken. Firstly, experiments should be carried out to determine the site of milk derived TGF-β production and the method it is sequestered into milk. This could include more extensive analysis of its expression by Northern blotting and the use of radioactive

tracers to determine where it is produced. The amount of latent and active TGF-β in neonatal stomachs after milk meals could also be measured to find out the level of activation and show its potential as a neonatal gut factor.

### 8.1.6 *Epidermal growth factor*

Previous work undertaken in this thesis has shown that the BALB/c 3T3 bioactivity produced by bovine whey extract can not be explained by PDGF, FGF, and TGF-β alone. The objective of the work undertaken for **Chapter 7** was to determine if EGF-like molecules are present in bovine whey and if such molecules are major contributors to the BALB/c 3T3 bioactivity of whey extract. Previousally, EGF has not been convincingly identified in the cow, and it's low pI would make it unlikely to bind the cation-exchange resin used to produce whey extract (Iacopetta *et al.* 1992; Taylor *et al.* 1972). However, other less anionic members of the EGF family may be present in whey extract and the presence of such factors was examined in the work presented in **Chapter 7**. Other members of the EGF family of growth factors include TGF-α, HB-EGF, amphiregulin, heregulin, and betacellulin (Marquardt *et al.* 1993; Higashiyama *et al.* 1991; Shoyab *et al.* 1989; Holmes etal. 1992; Wattanabe *et al.* 1994).

Research has shown there is little EGF sequence homology across species or sequence conservation between the various members of the EGF family, making it impossible to produce an EGF antibody that can detect EGF-like molecules in the cow (Carpenter 1990). This has meant that antibody is not available to develop immuno assays or Western blots. However, all EGF-like molecules appear to compete for the same over expressed EGFR on cells such as A431 and AG2804 cells. Accordingly, an AG2804 radioreceptor assay was developed to scan milk fractions for EGF-like molecules. EGF-like activity was detected in acid gel-filtration fractions of whey

extract by this RRA. There was approximately 0.8 ng of EGF-like activity recovered per mg of acidified whey extract loaded on to a gel-filtration column. The EGF-like activity of whey extract was eluted from acid gel-filtration between the 12.4 and 29 kDa standards. Previous research has shown that human EGF and TGF-α elute near 6 kDa, whereas HB-EGF and amphiregulin elute from gel-filtration chromatography at approximately 12-14 kDa, and betacellulin between 26 and 45 kDa (Gregory 1975; Marquardt *et al.* 1984; Higashiyama *et al.* 1992; Shoyab *et al.* 1989; Wattanbe *et al.* 1994).

Heparin-affinity chromatography revealed that the EGF-like molecule found in whey extract is a heparin-binding member of the EGF family. Previous research has indicated that EGF and TGF-α elute in the void volume from heparin-affinity columns, whereas HB-EGF and amphiregulin require 1.0 M NaCl and betacellulin elutes with 0.5 M NaCl from heparin-affinity columns (Besner *et al.* 1990; Thompson *et al.* 1994; Higashiyama *et al.* 1991; Shing *et al.* 1993; Wattanbe *et al.* 1994). The results in **Chapter 7** showed that EGF-like activity of whey extract bound heparin-Sepharose and was eluted with 0.4 to 0.6 M NaCl. This data shows that the EGF-like activity in whey extract could be a betacellulin-like molecule. The EGF-like protein was partially purified but no clear N-terminal sequence was obtained.

Future work should include repeating the current purification procedure until a distinct N-terminal sequence can be obtained. The purified molecule can then be run on PAGE and pI gels and recombinant molecule manufactured. The pure molecule could then be used to produce antibody for more accurate radio-immunoassays, western blots and immuno-neutralisation experiments. Importantly, it can not be inferred from the data presented in **Chapter 7** that the EGF-like molecule is present at the same concentration in neutral whey extract as acid-treated whey extract. Once more accurate

assays are available, it should be determined if the EGF-like molecule is latent. This could be achieved by measuring the bovine EGF-like molecules concentration in milk and whey and whey extract before and after acid treatment. In addition, acid and neutral gel-filtration experiments could be used to show the size of the whey binding proteins that confer such latency to the EGF-like molecule. Further, it should be assessed if the EGF-like molecule is present at the same level in whole milk, whey and cheese whey and if most of it is recovered in whey extract. Also, EGF-like activity should be examined in the whey fraction that does not bind the cation-exchange resin. This will determine the recovery of the EGF-like molecule in whey extract and importantly if other more anionic members of the EGF family such as EGF are present in bovine milk.

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# AMENDMENTS TO THESIS

***Page 6, paragraph 1, line 1, sentence should read:***
In raw cow milk, the average concentration of IGF-I is 4.3 ng/ml and IGF-II is 1.0 ng/ml (Collier *et al.* 1991; Vega *et al.* 1991).

***Page 7, paragraph 1, line 1, sentence should read:***
Other lower molecular weight IGF-binding proteins also appear to be present in bovine milk but they have not been identified (Ronge and Blum 1989; McGrath *et al.* 1991; Skaar *et al.* 1991; Vega *et al.* 1991).

***Page 24, paragraph 3, line 25, sentence should read:***
However, they did not sequence the molecule nor did they determine which PDGF chain(s) comprised the dimer.

***Page 51, paragraph 2, line 10, an additional sentence should be included:***
Note that 'washing' refers only to incubation of cells in DMEM for 2h.

***Page 76, paragraph 2, line 18, sentence should read:***
The growth factor-binding proteins complexes cannot bind to receptors and so are inactive.

***Page 115 paragraph 1, line 10, sentence should be omitted:***
This was nearly ten times less than the 0.8 M fraction.

***Page 120 paragraph 1, line 6, sentence should read:***
The 1.2 M eluate separated into at least four proteins, one of which migrated near ~15 kDa (**Figure 5.5, lane 4**). In the 2.0 M elution only two protein bands were detected and these were of relatively low molecular weight (**Figure 5.5, lane 6**).

***Page 199, paragraph 2, line 20, sentence should read:***
Indeed, **Figure 7.4** shows that the dilution curve of the EGF-like activity in whey extract and human EGF have similar slopes and so appear to compete for EGF receptors with similar affinities.

***Page 206, paragraph 1, line 2, sentence should read:***
Most of the non-mitogenic whey proteins and fluids are removed in the production of whey extract, but almost all the mitogenic activity is retained (Francis *et al.* 1995).

***Page 207, paragraph 1, line 3, an additional sentence should be included:***
Indeed, whey extract may not be representative of all bovine milk growth factors.