PROTEASES OF MAIZE ROOTS

by

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A thesis submitted in fulfilment of the
requirements for the degree of
Doctor of Philosophy

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April 1979
Awarded October 1979

PREFACE

Some of the results described in this thesis have been published as follows:

1. "Characterization of peptidase enzymes in the maize root"
   J.D. Shannon and W. Wallace
   Proc. Aust. Biochem. Soc. 11, 65 (1978)

ACKNOWLEDGEMENTS

I would like to express my deep thanks to Dr. W. Wallace for his invaluable, cheerful guidance and encouragement throughout this project. I would also like to thank Professor D.J.D. Nicholas for his valuable advice, particularly during the preparation of this thesis and Mr. R. Batt for his skilled technical assistance and cheerful company. Dr. R.G. Nicholls and Mr. J. Rhozinski are thanked for profitable discussions on chromatographic procedures and Mrs. M. Clarke is thanked for her skilful typing of this thesis.

I gratefully acknowledge financial support from a University Research Grant Scholarship.

DECLARATION

I hereby declare that the work presented in this thesis has been performed by myself and has not been submitted to any University for the award of any degree.

JOHN DAVID SHANNON

NOMENCLATURE AND ABBREVIATIONS

The major enzymes mentioned in this thesis are listed below with their numbers and systematic names as recommended by the Commission on Biochemical Nomenclature (1972).

Trivial Name	*E.C. Name and Number*
Alcohol dehydrogenase	Alcohol : NAD^+ oxidoreductase E.C. 1.1.1.1
Aldolase	D-Fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase E.C. 4.1.2.13
Argininosuccinate lyase	L-Argininosuccinate arginine-lyase E.C. 4.3.2.1
Aspartate aminotransferase	L-Aspartate : 2-oxoglutarate aminotransferase E.C. 2.6.1.1
Bromelain	E.C. 3.4.22.4
Carboxypeptidase A	Peptidyl-L-amino-acid hydrolase E.C. 3.4.12.2
Carboxypeptidase B	Peptidyl-L-lysine (L-arginine) hydrolase E.C. 3.4.12.3
Carboxypeptidase C	Peptidyl-L-amino-acid (L-proline) hydrolase E.C. 3.4.12.1
Cathepsin A	E.C. 3.4.12A.1
Cathepsin B	E.C. 3.4.22.1
Cathepsin D	E.C. 3.4.23.5
Chymotrypsin	E.C. 3.4.21.1
Elastase	E.C. 3.4.21.11
Ficin	E.C. 3.4.22.3

Trivial Name	*E.C. Name and Number*
Fructose 1,6-bisphosphatase	D-Fructose-1,6-bisphosphate 1-phosphohydrolase E.C. 3.1.3.11
Glutamate dehydrogenase	L-Glutamate : NAD^+ oxidoreductase (deaminating) E.C. 1.4.1.2
Hydroxymethylglutaryl-CoA reductase (NADPH)	Mevalonate : $NADP^+$ oxidoreductase (CoA-acylating) E.C. 1.1.1.34
Invertase	β-D-Fructofuranoside fructohydrolase E.C. 3.2.1.26
Lactate dehydrogenase	L-Lactate : NAD^+ oxidoreductase E.C. 1.1.1.27
Nitrate reductase (NADH)	NADH : nitrate oxidoreductase E.C. 1.6.6.1
Nitrate reductase (NAD(P)H)	NAD(P)H : nitrate oxidoreductase E.C. 1.6.6.2
Nitrate reductase (NADPH)	NADPH : nitrate oxidoreductase E.C. 1.6.6.3
Nitrite reductase	Nitric-oxide : (acceptor) oxido-reductase E.C. 1.7.99.3
Ornithine decarboxylase	L-Ornithine carboxy-lyase E.C. 4.1.1.17
Ornithine transaminase	L-Ornithine : 2-oxo-acid aminotransferase E.C. 2.6.1.13
Papain	E.C. 3.4.22.2
Pepsin	E.C. 3.4.23.1
Phaseolus proteinase	E.C. 3.4.21.13
Phenylalanine ammonia lyase	L-Phenylalanine ammonia-lyase E.C. 4.3.1.5
Phospho *enol* pyruvate carboxykinase (GTP)	GTP : oxaloacetate carboxy-lyase (transphosphorylating) E.C. 4.1.1.32

Trivial Name	*E.C. Name and Number*
Phosphoglucomutase	α-D-Glucose-1,6-bisphosphate : α-D-Glucose-1-phosphate phosphotransferase E.C. 2.7.5.1
Pyruvate kinase	ATP : pyruvate 2-*O*-phosphotransferase E.C. 2.7.1.40
Ribulose 1,5-bisphosphate carboxylase	3-Phospho-D-glycerate carboxy-lyase (dimerizing) E.C. 4.1.1.39
RNA polymerase	Nucleosidetriphosphate : RNA nucleotidyltransferase E.C. 2.7.7.6
Subtilisin	E.C. 3.4.21.14
Thrombin	E.C. 3.4.21.5
Trypsin	E.C. 3.4.21.4
Tryptophan oxygenase	L-Tryptophan : oxygen 2,3-oxido-reductase (decyclizing) E.C. 1.13.11.11
Tryptophan synthase	L-Serine hydro-lyase (adding indole) E.C. 4.2.1.20
Tyrosine aminotransferase	L-Tyrosine : 2-oxoglutarate aminotransferase E.C. 2.6.1.5
Xanthine oxidase	Xanthine : oxygen oxidoreductase E.C. 1.2.3.2
Yeast proteinase A	E.C. 3.4.23.8
Yeast proteinase B	E.C. 3.4.22.9

The plants mentioned in this thesis are listed below with their full binomial Latin names as recommended in Instructions to Authors for the Biochemical Journal [Biochem. J. (1973) 169, 2-27].

Trivial Name	*Binomial Latin Name*
barley	*Hordeum vulgare* L.
cotton	*Gossypium hirsutum* L.
French bean	*Phaseolus vulgaris* L.
maize	*Zea mays* L.
mung bean	*Phaseolus aureus* Roxb.
mustard	*Sinapis alba* L.
oat	*Avena sativa* L.
orange	*Citrus sinensis* (L.) Pers.
pea	*Pisum sativum* L.
potato	*Solanum tuberosum* L.
rice	*Oryza sativa* L.
Scot's pine	*Pinus sylvestris* L.
sorghum	*Sorghum bicolor* (L.) Moench
soybean	*Glycine max* (L.) Merr.
sunflower	*Helianthus annuus* L.
tomato	*Lycopersicon esculentum* Mill.
watermelon	*Citrullus lanatus* (Thunb.) Mansf.
wheat	*Triticum aestivum* L.
yeast	*Saccharomyces cerevisiae* Mayen ex Hansen

The abbreviations for chemicals and symbols in general follow the Instructions to Authors for the Biochemical Journal (Biochem. J. 169, 2-27 (1978)

Chemicals

ATEE	N-Acetyl-L-tyrosine ethyl ester
N-Ac-Ala-Ala-Ala-methyl ester	N-Acetyl-L-alanyl-L-alanyl-L-alanine methyl ester
BAEE	α-*N*-Benzoyl-L-arginine ethyl ester
Cbz-Glu-Tyr	*N*-Carbobenzoxy-L-glutamyl-L-tyrosine
Cbz-Phe-Ala	*N*-Carbobenzoxy-L-phenylalanyl-L-alanine
DAN	Diazoacetyl-DL-norleucine methyl ester
DFP	Diisopropyl fluorophosphate
DMSO	Dimethyl sulphoxide
DTT	DL-Dithiothreitol
NEM	*N*-ethylmaleimide
pCMB	*p*-Chloromercuribenzoic acid
pHMB	*p*-Hydroxymercuribenzoic acid
PMSF	Phenylmethylsulphonyl fluoride
SDS	Sodium dodecyl sulphate
SH	Sulphydryl
TAME	*p*-Tosyl-L-arginine methyl ester
TLCK	*p*-Toluenesulphonyllysinechloromethyl ketone
TPCK	*p*-Toluenesulphonylphenylalaninechloromethyl ketone

Symbols and Units

Δ A	change in absorbance
^{o}C	degrees Celsius
d	day(s)
µE	microeinstein
fr	fresh
g	gram
mg	milligram
µg	microgram
h	hour(s)
K_m	Michaelis constant
ℓ	litre
ml	millilitre
m	metre
cm	centimetre
mm	millimetre
M	molar
mM	millimolar
µmol	micromole
min	minute
%	per cent
ppt.	precipitate
sec	second
v/v	volume per volume
wt	weight
w/v	weight per volume

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SUMMARY

SUMMARY

1 This thesis reports an investigation on some properties of proteinases, carboxypeptidases and an aminopeptidase in maize roots, their distribution in maize and wheat tissues and involvement in the inactivation of nitrate reductase. The main steps in the purification procedure are shown in Figure 1.

2 The proteases were found in the supernatant fraction after centrifuging crude extracts at 95 000 *g* for 1 h. Treatments to remove polyphenols from crude extracts had no beneficial effect on enzyme activity or stability. All the enzymes were precipitated between 35-70% $(NH_4)_2SO_4$ saturation and after subsequent treatment at pH 4.4 they were recovered in the supernatant fraction and other than aminopeptidase, there was 3-4 fold purification of the enzymes.

3 The proteases were separated into two fractions, A and B by elution from a CM-cellulose column with a pH gradient (5-6) containing 130 mM NaCl.

4 Protease A degraded azocasein, haemoglobin, inactivated nitrate reductase and had carboxypeptidase activity. Most of the carboxypeptidase activity (carboxypeptidase A) was separated from the other components on a DEAE-Sephadex column. The remaining carboxypeptidase activity (carboxypeptidase A') was separated on a Sephadex G-100 column from an enzyme which degraded azocasein and haemoglobin and inactivated nitrate reductase (proteinase A).

5 Protease B, containing haemoglobin degrading activity (proteinase B), carboxypeptidase activity (carboxypeptidases BI and BII) and a nitrate

reductase inactivator was resolved on a Biogel A-0.5m column.

6 Proteinase A, which has a molecular weight of 53 000, degraded azocasein and haemoglobin with maximum activity at pH 9.0 and 4.0 respectively. The pH optimum for proteinase A with casein or root protein as substrate was 6.0. Azocasein degrading activity, haemoglobin degrading activity and nitrate reductase inactivating activity could not be separated by a range of techniques namely, ion exchange procedures, gel filtration, hydroxylapatite columns and affinity chromatography. Proteinase A was inhibited by PMSF and DFP and appears to be a serine proteinase.

7 Proteinase B degraded haemoglobin with maximum activity at pH 4.0. It was inhibited by PMSF (41%) and pCMB (70%) and may be an SH-proteinase.

8. Carboxypeptidases A and A', both with a molecular weight of 97 000, had a pH optimum of about 5.0 when Cbz-Phe-Ala was the substrate. Both enzymes were inhibited by PMSF and DFP and appear to have a serine residue at the active site; carboxypeptidase A' was also inhibited by pCMB.

9 Carboxypeptidases BI and BII which were sensitive to both PMSF and pCMB had a pH optimum of about 5.0.

10 The nitrate reductase inactivating activity associated with protease B appears not to be a protease and was inhibited by 1,10-phenanthroline but not by PMSF.

11 Azocasein degrading and nitrate reductase inactivating activities were found mainly in maize roots. The highest activities for carboxypeptidase and aminopeptidase were recorded in leaves of maize and wheat.

12 Proteinase, carboxypeptidase and nitrate reductase inactivating activity increased with age in maize roots while the soluble protein content decreased. The enzymes associated with protease A increased with age while those associated with protease B declined.

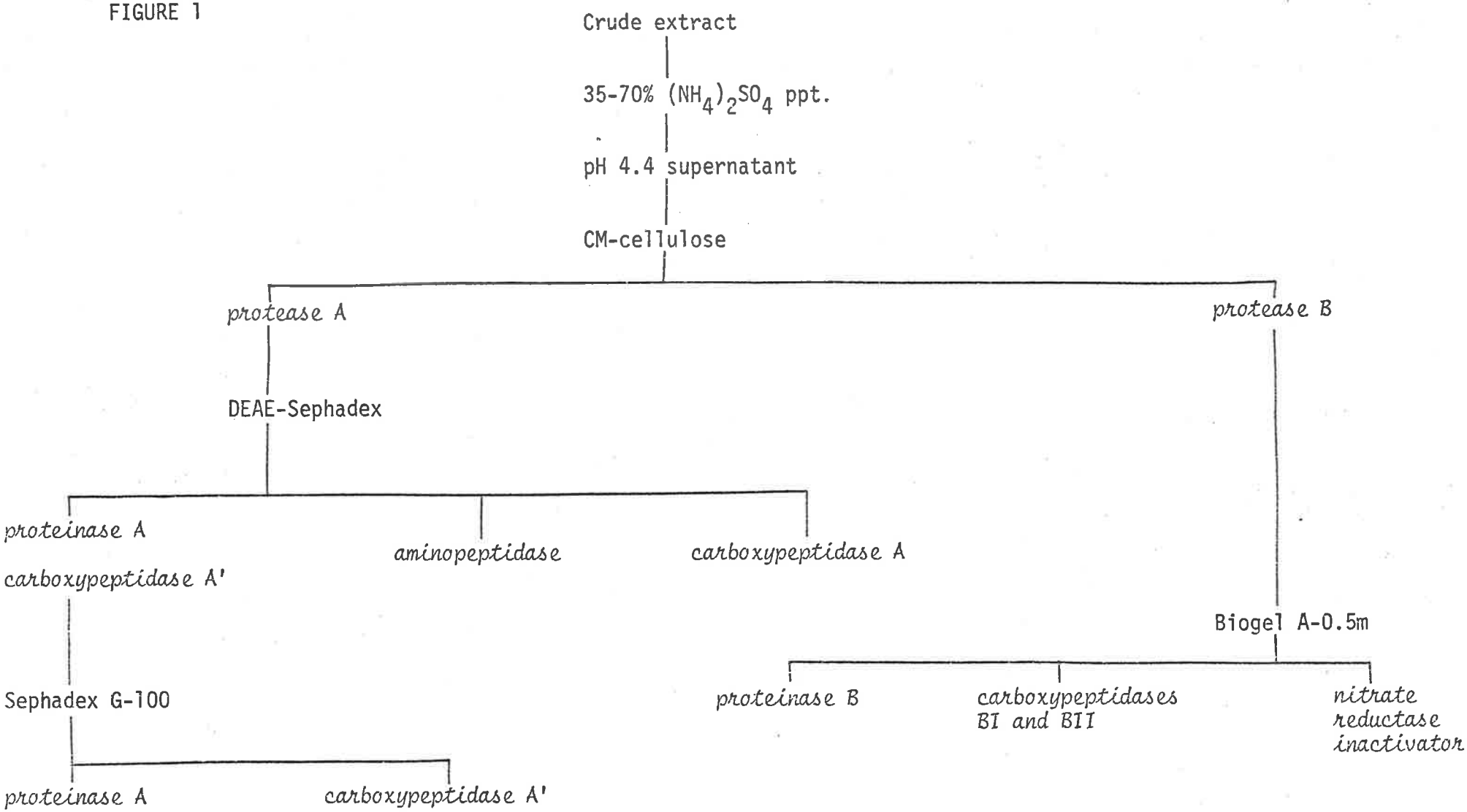
FIGURE 1

Flow diagram of the preparation of proteases from maize roots

An abbreviated scheme for the preparation of proteases from maize roots is shown. Full details are given in Sections 3.1-3.5. The enzymes shown in the Figure are described briefly below.

Protease A	A mixture of proteinase A and carboxypeptidases A and A'.
Proteinase A	Degrades azocasein and haemoglobin at pH 9.0 and 4.0 respectively; inactivates nitrate reductase. PMSF sensitive.
Carboxypeptidases A and A'	Hydrolyse Cbz-Phe-Ala at pH 5.0. PMSF sensitive (A' also inhibited by pCMB).
Protease B	A mixture of carboxypeptidases BI and BII, proteinase B and nitrate reductase inactivator.
Proteinase B	Degrades haemoglobin at pH 4.0. Inhibited by pCMB and PMSF.
Carboxypeptidases BI and BII	Hydrolyse Cbz-Phe-Ala at pH 5.0. Inhibited by pCMB and PMSF.
Aminopeptidase	Hydrolyses leucine-*p*-nitroanilide at pH 6.7; acid labile.
Nitrate reductase inactivator	Inactivates nitrate reductase. Inhibited by 1,10-phenanthroline but not PMSF or casein.

FIGURE 1


Crude extract
35-70% $(NH_4)_2SO_4$ ppt.
pH 4.4 supernatant
CM-cellulose
protease A
protease B
DEAE-Sephadex
proteinase A
carboxypeptidase A'
aminopeptidase
carboxypeptidase A
Biogel A-0.5m
Sephadex G-100
proteinase A
carboxypeptidase A'
proteinase B
carboxypeptidases BI and BII
nitrate reductase inactivator

1. INTRODUCTION

1. INTRODUCTION

1.1 Review of literature on proteases

1.1.1 Classification of proteases

The classificaton system for proteases presented in this thesis is based on that recommended by the Commission on Biochemical Nomenclature (1972). Proteases, more correctly referred to as peptide hydrolases, are divided into two groups according to the position of the peptide bond hydrolysed, namely peptidases, formerly exopeptidases, and proteinases, formerly endopeptidases. Work to be described in this thesis on peptidases will be restricted to the aminopeptidases (EC 3.4.11) which hydrolyse single amino acids from the N terminus of peptide chains and the carboxypeptidases (EC 3.4.12) which hydrolyse single amino acids from the C terminus.

The proteinases (endopeptidases) are further classified according to their catalytic mechanism based on studies on their active site and pH optima. This classification is as follows:

EC 3.4.21 serine proteinases - a serine and a histidine residue at the active site

EC 3.4.22 SH-proteinases - a cysteine residue at the active site

EC 3.4.23 acid proteinases - a pH optimum below 5 because of the involvement of an acidic residue in the catalytic mechanism

EC 3.4.24 metalloproteinases - require a metal ion for their catalytic action

The active site inhibitors most commonly used to classify proteinases are listed in Table 1. However, care must be taken in

TABLE 1

Reagents used to characterise active sites of proteases

Reagent	Probable site of action	Reference
p-chloromercuribenzoate (pCMB)	Cys, His	Kuhn *et al*(1974)
$HgCl_2$	Cys, His	Kuhn *et al*(1974)
di-isopropylfluorophosphate (DFP)	Ser	Mounter *et al* (1963)
phenylmethylsulphonylfluoride (PMSF)	Ser	Fold and Fahrney (1964)
p-toluenesulphonyllysinechloromethyl ketone (TLCK)	His	Shaw *et al* (1965)
p-toluenesulphonylphenylalanine-chloromethyl ketone (TPCK)	His	Schoellman and Shaw (1963)
ethylenediaminetetra-acetic acid (EDTA)	metal ions	Martell (1959)
1,10-phenanthroline	Fe^{2+}, Zn^{2+} Cu^{2+}	Martell and Calvin (1952)
diazoacetyl-D,L-norleucine methyl ester	Asp	Lunblad and Stein (1969); Bayliss *et al* (1969)

interpreting the effects of these inhibitors. PMSF, which reacts specifically with a single reactive serine residue in chymotrypsin even when used in twenty fold excess (Gold and Fahrney, 1964), also reacts with sulphydryl groups of the active site of the SH-proteinase papain (Whitaker and Perez-Villaseñor, 1968). Means and Feeney (1971) consider that mercurials have a far greater affinity for SH groups than for other groups but suggest that insufficient attention has been given to the possibility of reaction with residues other than cysteine. Kuhn *et al* (1974) list the probable site of action of mercurials as cysteine or histidine residues. If mercurials react with histidine residues then they may inhibit serine proteinases, which have a histidine residue at their active site, as well as SH-proteinases. Proteinase B of yeast which is inhibited by DFP and pCMB (Hata *et al*, 1967) has been classified as an SH-proteinase (EC 3.4.22.9). It is difficult however to understand why it was not classified as a serine proteinase as the reaction with DFP is generally considered to be a diagnostic test for a serine proteinase (Kraut, 1977). Possibly some proteinases require a free SH group, which is not part of the catalytic mechanism, to bind substrates or to maintain an active conformation. TLCK (*p*-toluenesulphonyllysine-chloromethyl ketone) and TPCK (*p*-toluenesulphonylphenylalaninechloro-methyl ketone) are inhibitors which have been designed to resemble substrates of trypsin and chymotrypsin respectively but possess a chloromethyl group which alkylates the reactive histidine when these molecules bind to the enzyme (Means and Feeney, 1971). However they have also been shown to react with SH groups at the active site of the proteinase papain (Whitaker and Perez-Villaseñor, 1968). Furthermore, because of the binding specificity of these reagents, they do not react with all serine proteinases (Shaw, 1970). Means and Feeney (1971) conclude that dye sensitised photochemical oxidation of imidazole groups is a more reliable procedure for checking for histidine residues at the

active sites of enzymes.

By using the inhibitor DAN (diazoacetyl-D,L-norleucine methyl ester) which reacts specifically with one aspartic acid residue in the active site of pepsin (Lundblad and Stein, 1969; Bayliss *et al*, 1969), it is possible to determine whether an acid proteinase is similar to pepsin. There are other proteinases with pH optima below 5 which are not inhibited by DAN but are affected by N-bromosuccinimide (Garg and Virupaksha, 1970; Oda and Murao, 1974). N-bromosuccinimide is widely used to cleave peptide bonds adjacent to tryptophan residues but it oxidises cysteine more rapidly (Means and Feeney, 1971). Thus a second group of 'acid proteinases', although not always inhibited by pCMB, may have an essential SH group at their active site; a good example is the enzyme from germinating maize which is inhibited by pCMB but not by DAN (Abe *et al*, 1977).

1.1.2 Plant proteinases

Studies on plant proteinases have mainly concerned those involved in the mobilisation of seed reserves during germination. Those of germinating seeds generally appear to be SH-proteinases (Abe *et al*, 1977; Baumgartner and Chrispeels, 1977; Wittenbach, 1978) and thus differ from proteinases of vegetative tissues which generally appear to be either serine or acid proteinases as described below. The enzymes of germinating seeds normally have acidic pH optima (Abe *et al*, 1977; Baumgartner and Chrispeels, 1977; Wittenbach, 1978). From time course studies on the utilisation of seed reserve proteins and on the appearance of proteinases and their specificity, it has been concluded that proteinases present in seedlings mobilise protein reserves (Preston and Kruger, 1976; Baumgartner and Chrispeels, 1977; Fujimaki *et al*, 1977). It appears that these enzymes are located within protein bodies which are thought to coalesce to form the central vacuole after depletion of the reserve protein

(Ashton, 1976). The function and properties of proteinases of germinating seeds have been extensively reviewed (Ryan, 1973; Mayer and Shain, 1974; Ashton, 1976).

Most of the proteinases in leaf tissue are inhibited by DFP or PMSF and pCMB or $HgCl_2$ (Table 2). As mentioned previously (Section 1.1.1), these enzymes should be classified as serine proteinases. Soybean trypsin inhibitor does not affect leaf proteinases although it appears that the enzymes of oat leaves and shoots contain an essential histidine residue, as does trypsin, because they are inhibited by rose bengal sensitised photochemical oxidation or TLCK (Drivdahl and Thimann, 1978; Pike and Briggs, 1972; Means and Feeney, 1971). The serine proteinases display a range of pH optima (Table 2). However the pH optimum of a proteinase may depend on the substrate used for a proteinase from mung bean seedlings has a pH optimum of 4.6 on azocoll but 5.7 on gelatin (Chrispeels and Boulter, 1975); similarly a proteinase from wheat seedlings has pH optima of 4.2 and 5.2 with haemoglobin and casein respectively (Wittenbach, 1978). Molecular weights of the serine proteinases of higher plants do not exceed 100 000 (Table 2).

Acid proteinases have been characterised in wheat leaves (Frith *et al*, 1978a,b,c). Of the six proteases isolated, three have been shown to be proteinases by determining the ratio of α-amino N to total N in the products released from haemoglobin (Frith *et al*, 1978a); they have a pH optimum of 4.5 when haemoglobin is the substrate (Frith *et al*, 1978a). One of these proteinases was inhibited by DAN and therefore positively identified as a pepsin like acid proteinase (Frith *et al*, 1978b). PMSF partially (53%) inhibited this enzyme and an acid proteinase from *Plasmodium berghei* was also inhibited by this reagent (Levy and Chou, 1974). Such inhibition may be due to the presence of a serine residue in the active site as has been shown for pepsin (Tang, 1977).

TABLE 2

Properties of proteinases of vegetative tissues of higher plants

Tissue	Molecular weight	Inhibition by			pH optimum	Reference
		DFP or PMSF	pCMB or $HgCl_2$	EDTA		
Agave americana leaves	55 000				8.0	du Toit (1976)
Phaseolus vulgaris leaves	100 000	+	+		9.0	Racusen and Foote (1970)
Zea mays leaves			+	-	6.4	Klein and Harpaz (1965)
Zea mays roots and shoots		+	+		5.4	Feller *et al* (1978)
Avena sativa shoots	62 000	+	+	-	6.4	Pike and Briggs (1972)
Avena sativa	76 000	+	-	-	4.2	Drivdahl and Thimann
leaves	76 000	+	-	-	6.6	(1977, 1978)
Glycine max root cells in culture		-	+	-	6.0	Shargool (1975)
Triticum aestivum leaves	89 000	+	-	-	4.5	Frith *et al* (1978a,c)

The presence of acid proteinase activity in wheat leaves of all ages has been interpreted to mean that they function in normal protein turnover (Frith *et al*, 1975) and an increase in activity in older tissues also indicates a role for them in protein degradation during senescence. Dalling *et al* (1976) found a significant correlation between proteinase activity in wheat leaves and the rate of nitrogen translocation from it. In the same tissue, ribulose-1,5-bisphosphate carboxylase is degraded by the acid proteinases (Peoples and Dalling, 1978) and in barley leaves loss of ribulose-1,5-bisphosphate carboxylase activity is correlated with an increase in proteolytic activity against azocasein (Peterson and Huffaker, 1975). PMSF, which inhibits two proteinases found in oat leaves (Drivdahl and Thimann, 1978), retards senescence of the leaves (Thimann *et al*, 1972) suggesting a role for these enzymes in this process.

Proteinases appear to be located in the central vacuole (Matile, 1968; Nishimura *et al*, 1978); thus these enzymes are located in an acidic compartment of the cell as are the proteolytic lysosomal cathepsins of animal cells.

Commonly proteolytic activity in plants is determined with non-physiological substrates, usually casein and haemoglobin, the latter being used at acidic pH. Ribulose-1,5-bisphosphate carboxylase has been described above as a substrate for endogenous proteinases and Racusen and Foote (1970) have shown that the enzyme of bean leaf degrades denatured soluble leaf protein. While proteolytic inactivation of phytochrome (Pike and Briggs, 1972) and some enzymes described below has been shown, the extent of the associated proteolysis has not been established.

A nitrate reductase inactivating enzyme has been described in maize roots (Wallace, 1973). This enzyme appears to be a serine proteinase and is similar to yeast proteinase B (Wallace, 1978a; Section 1.1.4).

Specificity studies show that this enzyme inactivates the NADH-cytochrome c reductase component of plant and fungal nitrate reductases but not the bacterial enzyme (Wallace, 1975). The molybdenum containing enzyme xanthine oxidase from buttermilk was not inactivated although this enzyme can complement the nitrate reductase mutant nit-1 from *Neurospora* (Ketchum *et al*, 1970). Tryptophan synthase and fructose-1,6-bisphosphatase from yeast, which are also sensitive to yeast proteinase B (Saheki and Holzer, 1974; Molano and Gancedo, 1974) are the only other enzymes known to be inactivated by the maize root proteinase (Wallace, 1978b). An increase in the activity of the inactivating enzyme occurs with root age (Wallace, 1978a).

The *in vivo* significance of proteolytic systems for inactivating nitrate reductase has been questioned by Tischler *et al* (1978). These workers found no evidence that a previously described protease in cotton cotyledons (Purvis *et al*, 1976) resulted in a loss of nitrate reductase activity *in vitro* because neither PMSF treatment nor a pH of 7.5 in the extract of cotton cotyledons which would inhibit serine and acid proteinases respectively stabilised nitrate reductase. The inhibitors NEM and iodoacetamide were also ineffective, but as these inhibitors do not always inhibit proteinases sensitive to pCMB or $HgCl_2$ (Hata *et al*, 1967a; Racusen and Foote, 1970; Pike and Briggs, 1972; Burger, 1973) and the absence of proteolytic activity was not checked directly, evidence against proteolytic inactivation of nitrate reductase in extracts of cotton cotyledons is inconclusive.

There are now reports of nitrate reductase inactivating proteins which are not proteinases. One such inhibitory molecule in the rice plant, first described by Kadam *et al* (1974), has been extensively studied by Yamaya and Ohira (1976, 1977, 1978). Its molecular weight has been estimated to be 200 000 and it was sensitive to

1,10-phenanthroline, Na_2EDTA and pCMB, suggesting the involvement of a metal ion and SH groups (Yamaya and Ohira, 1977). It also inactivates xanthine oxidase of buttermilk but not rice nitrite reductase, glutamate dehydrogenase or the NADH-cytochrome c reductase component of nitrate reductase (Yamaya and Ohira, 1977). Recently a protein inhibitor of nitrate reductase has been isolated from soybean leaves (Jolly and Tolbert, 1978). It has a molecular weight of 31 000 and again it did not appear to be a protease. Evidence was presented that the inhibitor was inactivated in light suggesting that it may have a role in the light-dark modulation of nitrate reductase activity.

Macromolecular inhibitors have been identified for a range of other plant enzymes including potato invertase (Pressey, 1967), sunflower phenylalanine ammonia lyase (Creasy, 1976), maize alcohol dehydrogenase (Ho and Scandalios, 1975) and *Lemna* aldolase (Sarawek and Davies, 1977). In these cases it was shown that a proteolytic action was not involved. Proteases may be involved in the inactivation of argininosuccinate lyase in cultured soybean cells (Shargool, 1975), potato phosphoglucomutase (Kahl and Gaul, 1975) and phosphoenolpyruvate carboxykinase from *Mesembryanthemum crystallinum* (Winter and Greenway, 1978).

1.1.3 Peptidases of higher plants and fungi

The carboxypeptidases isolated from a range of vegetative tissues of higher plants, germinating seeds, yeast and *Neurospora crassa* are similar in their properties (Table 3). They are inhibited by PMSF or DFP and therefore are likely to contain a serine residue in the active site (Section 1.1.1). An essential free SH group is also suggested in some cases because either pCMB or $HgCl_2$ inhibit. In yeast carboxypeptidase Y, pHMB prevents DFP from reacting with the enzyme, suggesting that the SH group is close to the serine residue (Hayashi *et al*, 1973b). As there

TABLE 3

Properties of carboxypeptidases of higher plants, yeast and Neurospora

Tissue	Molecular weight	Inhibition by			Esterase activity	pH optimum	Reference
		DFP or PMSF	pCMB or $HgCl_2$	EDTA			
Hordeum vulgare seedlings	90 000	+	+	-		5.2	Visuri *et al* (1969)
Phaseolus vulgaris leaves	120 000	+		-		5.6	Wells, 1965, 1968; Shaw and Wells, 1972
Gossypium hirsutum	85 000	+			+	5.8	Ihle and Dure, 1972a,b
Zea mays all vegetative tissues		+	-			4.0-5.4	Feller *et al* (1978)
Phaseolus aureus seedlings		+	-			5.0	Chrispeels and Boulter (1975)
Neurospora crassa	42 000	+	+	-	+	5.7	Siepen *et al* (1975)
Citrus sinensis leaves	175 000	+		-	+	5.3	Sprössler *et al* (1971)

(Cont.)

Table 3 (Cont.)

Tissue	Molecular weight	Inhibition by			Esterase activity	pH optimum	Reference
		DFP or PMSF	pCMB or $HgCl_2$	EDTA			
Ananas comosus stem		+	+	-	+	5.2	Doi *et al* (1974b)
Pinus sylvestris seedlings		+				4.5	Salmia and Mikola (1976a)
Lyropersicon esculentum fruit		+	+	-		5.0-5.5	Matoba and Doi (1974b)
Citrullus lanatus sarcocarp	89 000 110 000	+ +	+ +	- -	+ +	5.0-5.5 5.0-5.5	Matoba and Doi (1974a)
Triticum aestivum seedlings	55 000 61 000	+ +		- -		4.2	Preston and Kruger (1976)
Saccharomyces cerevisiae	62 000	+	+	-	+	6.0	Kuhn *et al* (1974); Hata *et al* (1967b)

is evidence for a reactive histidine residue (Hayashi *et al*, 1975), it has been concluded that the active site contains a charge relay system in which a histidine residue, aided by a neighbouring aspartic acid residue, transfers a proton to or from its reactive serine residue during peptide bond hydrolysis (Kraut, 1977). This mechanism of action is similar to that of the well known serine proteinases trypsin, chymotrypsin and subtilisin (Kraut, 1977) but the amino acid sequence in the active site of the yeast enzyme (Shaw and Wells, 1972; Hayashi *et al*, 1973b) has been shown to be different from those of the proteinases (Hartley *et al*, 1965; Smith *et al*, 1966). Metal ions and chelating agents have no effect on plant carboxypeptidases (Table 3) and it has been concluded that they do not have a metal component. Plant carboxypeptidases thus differ from the zinc dependent animal carboxypeptidases A and B (Pétra, 1970; Folk, 1970), but resemble lysosomal carboxypeptidases A and C (Barrett and Heath, 1977) including pig kidney cathepsin A (Doi *et al*, 1974a). While ~~yeast~~ carboxypeptidase Y from yeast is similar to carboxypeptidases from higher plants (Table 3), two other yeast carboxypeptidases have been described which are inhibited by Na_2EDTA but not DFP (Félix and Brouillet, 1966; Wolf and Weiser, 1977).

Molecular weights of carboxypeptidases range from 61 000 to 120 000 (estimated by gel filtration) (Table 3), hence they are larger than pancreatic carboxypeptidases A and B which are 34 000 (Pétra, 1970; Folk, 1970) but generally smaller than pig kidney cathepsin A, whose two forms have molecular weights of 100 000 and 500 000 (Doi *et al*, 1974a). Yeast carboxypeptidase Y has been shown to contain 15-20% hexose (Hata *et al*, 1967b; Hayashi *et al*, 1973a; Kuhn *et al*, 1974).

Carbobenzoxy dipeptides are normally used as substrates for carboxypeptidases and a wide range are hydrolysed, albeit at different rates (Carey and Wells, 1972; Hayashi *et al*, 1972; Preston and

Kruger, 1977). Only a small number of peptide bonds resist hydrolysis (Hayashi *et al*, 1970; Visuri *et al*, 1969). Some studies on purified carboxypeptidases have shown that these enzymes can degrade polypeptides from the C terminal end (Hayashi and Hata, 1972; Siepen *et al*, 1975; Preston and Kruger, 1977). Several carboxypeptidases are known to have esterase activity (Table 3) and others amidase activity (Hayashi and Hata, 1972; Matoba and Doi, 1975). Failure to find either activity may be due to an inappropriate choice of substrate. For peptidase activity, the pH optimum is generally around 5.0 which is also the range for maximum stability. Apparent optimal temperatures for carboxypeptidases, which may be determined by their lability at higher temperatures (Dixon and Webb, 1964), are often around 50°C.

Although carboxypeptidase activity rises during germination, this increase does not coincide with the mobilisation of protein reserves suggesting that carboxypeptidases are not involved in this process (Chrispeels and Boulter, 1975; Salmia and Mikola, 1976b). In maize plants activity increased during leaf maturation but decreased during senescence indicating that carboxypeptidases are not involved in protein degradation in older tissues (Feller *et al*, 1977, 1978). Yeast carboxypeptidase activity stays constant during sporulation when considerable protein degradation occurs (Betz and Weiser, 1976b) although there is an increase in activity during starvation for glucose or readily available nitrogen (Hansen *et al*, 1977, Lenney *et al*, 1974). While it may be considered that carboxypeptidases degrade peptides released from proteins by proteinases, the combined action of a proteinase and a carboxypeptidase appears to cause little more release of α-amino groups than the added effects of the two enzymes acting separately (Baumgartner and Chrispeels, 1977). Thus an essential role for carboxypeptidases in protein degradation has not yet been clearly demonstrated.

Carboxypeptidase has been found in the vacuoles of maize root tips (Zuber and Matile, 1968), castor bean endosperm (Nishimura and Beevers, 1978) and yeast (Lenney *et al*, 1974; Hasilik *et al*, 1974). In germinating mung bean seedlings, most of the carboxypeptidase activity is found in protein bodies (Harris and Chrispeels, 1975) which are believed to coalesce to form the central vacuole after depletion of the storage proteins (Ashton, 1976).

Aminopeptidases have been less well studied than other plant proteases. Examination of their properties (Table 4) shows that generally they are inhibited by pCMB or $HgCl_2$ and thus appear to have an essential SH group at their active site. As those of higher plants are generally not inhibited by Na_2EDTA they appear not to have a metal constituent and the lack of inhibition by PMSF and DFP suggests that there is no active site serine residue (Section 1.1.1). However metal dependent (Masuda *et al*, 1975; Siepen *et al*, 1975; Tomomatsu *et al*, 1973) and DFP sensitive (Caldwell and Sparrow, 1976) aminopeptidases have been reported. Inhibition of the enzyme from *Agave americana* by diethyl pyrocarbonate was taken to indicate the presence of an essential histidine residue at the active site (du Toit and Schabort, 1978). In contrast, animal aminopeptidases are metal dependent (De Lange and Smith, 1971).

Common substrates for aminopeptidases are amino acid derivatives of *p*-nitroaniline or β-napthylamine; each enzyme shows selectivity towards the amino acid derivatives which it hydrolyses. Normally pH optima for these reactions are around 7.0. Higher plant aminopeptidases have molecular weights of less than 100 000 (Table 5).

It has been suggested that aminopeptidases may contribute to the mobilisation of reserve protein in the endosperm of pine seeds (Salmia and Mikola, 1976b). However other studies have assigned a minor role to aminopeptidases in protein turnover in maize leaves and pea seeds

TABLE 4

Properties of aminopeptidases of higher plants, yeast and Neurospora

Tissue	Molecular weight	Inhibition by			Esterase activity	pH optimum	Reference
		DFP or PMSF	pCMB or $HgCl_2$	EDTA			
Agave americana leaves	86 000			-		7.2-7.5	du Toit *et al* (1978) du Toit and Schabort (1973)
Zea mays leaves		-	-	-		6.5-7.0	Feller *et al* (1977)
Hordeum vulgare seeds	65 000	-	+	-		7.2	Kolehmainen and Mikola (1971)
Pisum sativum	58 000	-	+	-		7.0	Elleman (1974)
cotyledons	74 000	-	+	-		7.0	
Saccharomyces cerevisiae	200 000	-	+	+		7.6	Masuda *et al* (1975)
Neurospora	85 000	-	+	+		7.25	Siepen *et al* (1975)
crassa	78 000	-	+	+		8.6	

(Feller *et al*, 1977, 1978; Tomomatsu *et al*, 1978).

1.1.4 Proteinases from yeast and *Neurospora*

Observations of the instability of tryptophan synthase in yeast extracts led to the identification of a macromolecular inactivating factor (Manney, 1968). Two inactivases were subsequently isolated (Katsunuma *et al*, 1972) and one was shown to have proteolytic activity (Schött and Holzer, 1974). When the two inactivases were compared with previously described yeast proteinases A and B (Hata *et al*, 1967a,b), they were found to be virtually identical (Saheki and Holzer, 1974). Accordingly it has been concluded that this inactivation of trytophan synthase in yeast extracts is due to proteolytic action.

Proteinase A of yeast appears to be an acid proteinase and proteinase B, while it has been classified as an SH-proteinase (Section 1.1.1), appears to also have a serine residue at its active site (Hayashi *et al*, 1967a). The molecular weights of proteinases A and B are 44 000 and 32 000 respectively (Magni *et al*, 1978; Lenney and Dalbec, 1969) which are in the same range as those of proteinases from higher plants (Section 1.1.2). Some early determinations of molecular weights of these proteinases were confounded by the formation of a complex of equimolar amounts of proteinases A and B in which both enzymes retained their activity (Hinze *et al*, 1976). The physiological significance of this complex is unknown.

Studies on the specificity of the two proteinases show that proteinase B inactivates a wide range of enzymes *in vitro* while proteinase A inactivates only some of the enzymes inactivated by proteinase B (Jusic *et al*, 1976). Some enzymes are not inactivated by either proteinase. During sporulation of yeast, activities of several enzymes fell while at the same time activities of the proteinases rose

(Betz and Weiser, 1976a,b). The enzymes whose activities decline are susceptible to the proteinases *in vitro*, but not all enzymes so inactivated lose activity during sporulation in yeast, suggesting that there is some mechanism other than the specificity of the proteinases responsible for the selective inactivation of enzymes. A role for proteinase A in protein degradation during yeast sporulation is suggested by the inability of a mutant lacking proteinase A to sporulate (Betz and Hartwell, 1976); it is not clear whether proteinase B is also essential for sporulation (Wolf and Ehman, 1978). While the yeast proteinases inactivate only the apo form of ornithine transaminase, as do the group specific proteinases of rat tissues (Afting *et al*, 1972; Section 1.1.5), they are not restricted in their action to any one group of enzymes.

Proteinases in yeast are located in the central acidic vacuole (Hasilik *et al*, 1974; Lenney *et al*, 1974) and thus separated from the enzymes they attack which occur in the cytosol. (The role of lysosomes and vacuoles in protein degradation is discussed in Section 1.2.3.) Compartmentation of the proteinases also separates them from their specific inhibitors (Section 1.1.6). Five proteases have been isolated from *Neurospora crassa* (Siepen *et al*, 1975; Table 5). The properties of the acid and alkaline proteinases are similar to those of yeast proteinases A and B respectively. Although it has been classified as an 'alkaline proteinase' because it has a pH optimum of 9.1, the *Neurospora* alkaline proteinase also has a pH optimum at 6.2. It was shown earlier (Yu *et al*, 1973) that tryptophan synthase was inactivated by a protease which has similar properties to those of the alkaline proteinase. It was also shown that the *Neurospora* tryptophan synthase was inactivated by yeast proteinase B (Tsai *et al*, 1973). Recent studies indicate that two proteases from *Neurospora crassa* can inactivate nitrate reductase (Sorger *et al*, 1978). One of these, referred to as inactivator II, which

TABLE 5

Properties of proteinases of Saccharomyces cerevisiae and Neurospora crassa

Enzyme	Molecular weight	Inhibition by			pH optimum	Reference
		DFP or PMSF	pCMB or $HgCl_2$	EDTA		
Saccharomyces						
proteinase A	44 000	-	-	-	3	Hata *et al* (1967a)
proteinase B	32 000	+	+	-	9	Magni *et al* (1978) Lenney and Dalbec (1969)
Neurospora						
acid proteinase	34 000	-	-	-	2.5	Siepen *et al* (1975)
alkaline proteinase	24 000	+	+	-	6.2, 9.1	

is inhibited by PMSF as is the alkaline proteinase described above, is repressed by ammonia and derepressed by nitrogen starvation. The other protease, referred to as inactivator I, is Na_2EDTA sensitive and is invariably present. Inhibitors for the *Neurospora* proteinases and inactivators were also described by Yu *et al* (1976) and Sorger *et al* (1978).

1.1.5 Group specific proteinases of rat tissues

Rat tissues contain three groups of proteinases which inactivate only the apo forms of either pyridoxal phosphate, NAD or FAD dependent enzymes (Katunuma *et al*, 1971a,b, 1972) and are induced by deficiencies of vitamin B_6, niacin and vitamin B_2 respectively. These mitochondrial group specific proteinases, which appear to have serine at the active site and have alkaline pH optima (Katunuma *et al*, 1975), differ from the lysosomal cathepsins B and D which are SH- and acid proteinases respectively (Barrett and Heath, 1977). When compared with the well known serine proteinases trypsin, chymotrypsin and elastase, they were inhibited by soybean trypsin inhibitor and the chymotrypsin inhibitor chymostatin but not by the elastase inhibitor elastatinal (Katunuma *et al*, 1975); the chymotrypsin substrate ATEE was hydrolysed but not the trypsin substrate TAME or the elastase substrate N-Ac-Ala-Ala-Ala-methyl ester. Analysis of the amino acid sequence of the rat intestine group specific proteinase shows approximately 30% sequence identity with trypsin, chymotrypsin and elastase and a common evolutionary origin for all these serine proteinases has been suggested (Sanada *et al*, 1978).

The proteinases acting on pyridoxal phosphate dependent enzymes inactivate their substrates at differing rates and do not inactivate the pyridoxal phosphate dependent enzymes aspartate aminotransferase or tyrosine aminotransferase (Katunuma *et al*, 1975). As only pyridoxal phosphate dependent enzymes are inactivated by the group specific

proteinases, it appears that only these enzymes in their apo forms have accessible sites containing aromatic amino acid residues, as the intestinal and muscle group specific proteinases mainly cleave peptide bonds on the carboxyl side of these residues (Kobayashi and Katunuma, 1978; Kobayashi *et al*, 1978). The inactivation of ornithine transaminase results from the hydrolysis of only a few peptide bonds (Kominami *et al*, 1975). As the peptides produced by the limited cleavage of ornithine transaminase do not accumulate *in vivo* (Kominami and Katunuma, 1976), it appears that attack by a group specific proteinase is the rate limiting step for the inactivation of some enzymes. From the limited data available, it is not possible to draw conclusions about the species specificity of the group specific proteinases although it has been claimed that they lack species specificity (Afting *et al*, 1972).

1.1.6 Proteinase inhibitors and their function

Proteinase inhibitors found in plant tissues, principally in seeds and germinating tissues, are small, heat stable proteins which usually do not affect endogenous proteinases (Richardson, 1976; Ryan, 1973). It has been proposed that they protect plants against microbial and insect attack and against degradation in the digestive tract of mammals (Richardson, 1976; Weiel and Harper, 1976; Mosolov *et al*, 1976). Ryan (1978) has described a system for the production of trypsin inhibitor in tomato leaves in response to tissue damage and suggested that this system is a defence mechanism against insects. In those seeds where inhibitors are active against endogenous proteinases, they do not appear to regulate proteinase activity. In lettuce, inhibitor activity is lost before proteinase activity reaches a maximum (Shain and Mayer, 1968) while in mung bean cotyledons, inhibitor activity decreases more rapidly than proteinase activity increases (Baumgartner and Chrispeels, 1976).

Few inhibitors are known in non-seed tissues. Wallace (1978a) has shown that acid treatment of maize root extracts increases proteinase activity and concluded that a proteinase inhibitor was present. In yeast, small, heat stable proteins which specifically inhibit yeast proteinases and carboxypeptidase have been characterised (Betz *et al*, 1974; Matern *et al*, 1974a; Saheki *et al*, 1974). These inhibitors were located in the cytoplasm (Lenney *et al*, 1974; Matern *et al*, 1974b) while the enzymes they inhibit are found in the vacuole (Hasilik *et al*, 1974). As the inhibitors turn over slowly (Betz, 1975) and rise and fall in concert with protease activity (Lenney *et al*, 1974; Betz, 1975), they do not appear to regulate proteolytic activity *in vivo*. Possibly the inhibitors protect the cytoplasm against the action of proteases which are being transported to the vacuole, but it is now known that carboxypeptidase Y is transported as a zymogen (Hasilik and Tanner, 1978). Another suggested function of the inhibitors is protection of the cytoplasm against proteolytic attack in case of rupture of the vacuole (Betz, 1975).

1.2 Protein degradation

1.2.1 Function

Degradation of protein releases amino acids for the synthesis of new proteins or provision of energy through oxidation of their carbon skeletons and it has an important role during animal starvation (Goldberg and St. John, 1976). In seeds, degradation of reserve proteins provides amino acids for new tissues during germination (Ashton, 1976). When plant tissues suffer water stress (Arad and Richmond, 1976) or mineral deficiency (Trewavas, 1972), proteins are degraded, possibly to provide energy.

Numerous experiments have shown that proteins with abnormalities resulting either from genetic mutations or from incorporation of amino

acid analogues are degraded more rapidly than their normal counterparts (Goldberg and St. John, 1976). It has therefore been suggested that proteins with abnormalities resulting from errors in translation, post-translational damage such as oxidation or from conformational changes are rapidly removed from the cell (Ballard, 1977). There is no direct evidence to support this hypothesis but the finding that some proteins have half-lives of minutes (Poole and Wibo, 1973) may indirectly substantiate this idea.

A number of enzymes, such as phosphoenolpyruvate carboxykinase (Hopgood *et al*, 1973), hydroxymethylglutaryl CoA reductase (Higgins *et al*, 1973) and nitrate reductase (Oaks *et al*, 1972) are rapidly synthesised and degraded so that alterations in the rate of synthesis or degradation can lead to rapid changes of the activity of these enzymes (Schimke and Doyle, 1970; Huffaker and Peterson, 1974). Rapidly turned over enzymes, such as those mentioned above which initiate the reactions of gluconeogenesis, cholesterol synthesis and nitrate assimilation, are often the first enzymes of metabolic pathways (Goldberg and St. John, 1976) and therefore protein degradation can rapidly and irreversibly inactivate a metabolic pathway. Inactivation of unnecessary enzymes by degradation rather than by some reversible process such as (de)phosphorylation prevents the accumulation of non-functional proteins in the cell.

1.2.2 Regulation

Protein degradation in plants and animals changes during growth and in response to external factors. In animals, starvation and tissue differentiation result in altered patterns of protein degradation (Goldberg and St. John, 1976). Increases in protein degradation in plants during germination, senescence and stress are described elsewhere

(Sections 1.1.2, 1.2.1). Hormones may control protein degradation in both plants and animals (Tavares and Kende, 1970; Ballard, 1977).

Several properties of proteins can be correlated with their rates of degradation, particularly in animal tissues and a general observation (Goldberg and St. John, 1976) is that a protein like tyrosine animotransferase with a half-life of hours is inactivated rapidly *in vitro* by trypsin while an enzyme with a half-life of days, like arginase, is not so affected (Bond, 1971).

Large proteins are degraded more rapidly than small ones e.g. RNA polymerase I, monomeric molecular weight 83 000, has a half-life of 1.3 h while isozyme 5 of lactate dehydrogenase, monomeric molecular weight 18 000, has a half-life of 144 h (Dice and Goldberg, 1975). Possible reasons for the decreased metabolic stability of large proteins are: an increased number of sites sensitive to degradative attack, decreased conformational stability and an increased probability of errors in the amino acid sequence (Goldberg and Dice, 1974). An apparent correlation between relative turnover rates of soluble proteins of pea stem and their sizes has been observed (Dice *et al*, 1973).

Acidic proteins like ornithine decarboxylase, with an isoelectric point of 4.1 and a half-life of 0.2 h, turn over rapidly whereas basic proteins like cytochrome c, with an isoelectric point of 9.2 and a half-life of 150 h, turn over slowly (Dice and Goldberg, 1975). There appears to be a similar relationship between the isoelectric points of proteins and their half-lives in mustard cotyledons (Acton and Gupta, 1978). Calculation of partial correlation coefficients shows that the relationships of size and charge to turnover rate are independent of each other (Dice and Goldberg, 1975). Possibly the effect of charge is explained by the findings that short lived proteins are generally inactivated

rapidly at low pH (Bond, 1976) possibly because of precipitation of their uncharged forms. The ready precipitation of short lived proteins (Bohley *et al*, 1976) is consistent with this suggestion.

Proteins with high affinities for hydrophobic surfaces and solvents are more rapidly degraded than other proteins (Bohley and Riemann, 1977); a related observation is that short lived proteins are preferentially adsorbed on membranes (Dean, 1975a). This data may be explained by a model of enzyme inactivation on membranes (Section 1.2.3). By examining the rates of loss of radioactivity of electrophoretically separated proteins, it has been found that glycoproteins are degraded more rapidly than non-glycoproteins (Gurd and Evans, 1976; Mathews *et al*, 1976; Kaplan and Moskowitz, 1975). This relationship is also claimed to be independent of the effects of a protein's charge and size on its turnover rate (Dice *et al*, 1978).

Binding of ligands can influence the stability of proteins (Goldberg and Dice, 1974). In pyridoxal phosphate dependent enzymes, the co-factor protects them against proteolytic attack (Katunuma *et al*, 1975) while administration of tryptophan *in vivo* decreases the rate of degradation of tryptophan oxygenase (Schimke *et al*, 1965). On the other hand, binding of actin to aldolase *in vitro* increases its susceptibility to proteolysis (Dedman *et al*, 1975).

Phosphorylatable rat liver proteins are more resistant to proteolysis when phosphorylated than when dephosphorylated (Ekman *et al*, 1978). However, phosphorylation of histones does not appear to affect their rate of degradation (Heinrich *et al*, 1976) although these proteins, which are tightly bound to DNA, may not be exposed to the same degradative processes as other cellular proteins.

Thermal stabilities and half-lives of proteins appear to be correlated (McLendon and Radany, 1978); thus pyruvate kinase has a half-

life of 30 h and a transition temperature of 42°C on its 'melting curve' while ribonuclease has a half-life of 250 h and a transition temperature of 63°C. It is suggested that the half-life of a protein is dependent on the proportion of the protein molecules which are unfolded, which is related to its thermal stability, for proteins are more susceptible to proteolysis in their unfolded state (McLendon, 1977). Unfolding of proteins may also increase their susceptibility towards other inactivating reactions (Ballard, 1977). If the thermal stability of a protein is dependent on its charge, size and content of hydrophobic amino acid residues, these properties may influence the turnover rate by determining the amount of unfolded protein present. It has also been shown that binding of a substrate to an enzyme reduces the amount of unfolded enzyme (Tanford, 1968) thus explaining the stabilising effect of substrates mentioned above. In addition, Ballard (1977) considers that because amino acid analogues markedly reduce the half-life of proteins, the conformation of a protein has a crucial role in determining the rate of degradation.

1.2.3 Mechanism

In this section mechanisms for protein degradation are described, particularly evidence that lysosomes and their proteinases are responsible for the degradation of proteins.

Lysosomes of animal cells contain many hydrolytic enzymes (Barrett and Heath, 1977) and investigations *in vitro* confirm that lysosomal proteases are capable of the complete degradation of proteins (Huisman *et al*, 1974). Plant and yeast vacuoles also contain hydrolytic enzymes and Matile (1975, 1976) has presented strong evidence that vacuoles are analogous to animal cell lysosomes. Vacuoles of yeast (Wiemken and Nurse, 1973) and liver lysosomes (Ward and Mortimore, 1978) contain

amino acid pools; in the latter case the amino acids are derived from protein degradation. These observations are consistent with protein hydrolysis in lysosomes. More direct evidence that proteinases degrade proteins *in vivo* has come from inhibitor studies. Pepstatin and leupeptin, inhibitors of the lysosomal proteinases cathepsin D and cathepsin B respectively, inhibit protein degradation in rat liver and muscle respectively (Dean, 1975b; Libby and Goldberg, 1978). In senescing oat leaves, DFP and PMSF, inhibitors of serine proteinases (Section 1.1.1), retard the loss of protein (Thimann *et al*, 1972) and it has been shown that oat leaves contain proteinases sensitive to these reagents (Drivdahl and Thimann, 1978). Recently it has been shown that vacuoles from castor bean endosperm can degrade protein (Nishimura and Beevers, 1979).

While there is strong evidence that lysosomes are involved in the hydrolysis of proteins, the processes by which degradation is initiated are less well understood. Ballard (1977) has proposed two mechanisms for protein degradation, namely autophagy and a membrane located inactivation process which leads to subsequent degradation. Autophagy is a process in which cellular organelles are digested as complete units within lysosomes; this process has been observed in animal (Dean and Barrett, 1976) and plant cells (Matile, 1975). Digestion of complete organelles could explain the lack of effect of size on rates of protein degradation observed in muscle and liver of diabetic rats (Dice *et al*, 1978) and in some membranes (Tweto and Doyle, 1976). Short lived enzymes are thought to undergo inactivation on cellular membranes, particularly the plasma membrane (Ballard, 1977). In this model (Ballard and Hopgood, 1976), the first step of the degradative pathway is for a protein to assume an unstable conformation, react with a thiol exchange enzyme or undergo some other process which causes loss of functional activity and leads to the formation of a complex of enzyme with a membrane. It has been

suggested (Ballard, 1977) that the structural features of proteins which are related to rapid turnover (Section 1.2.2) result in unstable conformations or a tendency to react with membranes. The subsequent steps in this pathway are poorly understood. Uptake of proteins by isolated lysosomes has been reported (Hayashi *et al*, 1973) and lysosomal uptake of intracellular proteins during protein degradation has been inferred (Auricchio *et al*, 1972; Haider and Segal, 1972). On the other hand, proteases in extra-lysosomal membranes (Tökés and Chambers, 1975) may digest intracellular proteins. Random autophagy is believed to be too slow to account for the rapid degradation of many proteins (Ballard, 1977). Other proteolytic systems which have been identified are a soluble system active at neutral pH in reticulocytes (Etlinger and Goldberg, 1977), mitochondrial group specific proteinases (Section 1.1.5) and a cytoplasmic protease in muscle (Reville *et al*, 1976). The relationship of these systems to the autophagic and membrane located protein degradative paths and to lysosomes is poorly understood.

There are numerous observations (Goldberg and St. John, 1976) that protein synthesis and an energy supply are required for protein degradation by intact cells but the mechanisms involved are still unclear (Ballard, 1977).

1.3 Aims of the study

The objectives of the current study were to determine the number and evaluate the properties of proteases in maize roots and their relation to the inactivation of nitrate reductase.

The following topics were investigated:

1. Separation of these enzymes using a range of chromatographic procedures.

2. Some of the properties of the proteases including their mode of action on nitrate reductase.

3. The distribution of proteases in various tissues of maize and wheat plants including the effects of age.

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Preparation of solutions and buffers

Double glass-distilled water was used to prepare all aqueous solutions. The pH of all buffers were adjusted at room temperature (15-25°C) using a Pye Model 291 pH meter standardised at pH 7 before use. The pH of Tris-HCl buffers used at 37°C was adjusted at room temperature and a correction for the change of pH with temperature was made by reference to Sigma Technical Bulletin 106 B.

PMSF and 1,10-phenanthroline and TPCK were dissolved in isopropyl alcohol and methanol respectively to give solutions of 20 mM inhibitor. The alcoholic solutions were diluted to 4 mM with water before mixing with enzyme samples. DFP solutions were prepared in ethylene glycol to reduce hydrolysis. The concentration of pCMB in aqueous solution was determined spectrophotometrically by measuring A_{232} and using a molar extinction coefficient of 1.69×10^4 $M^{-1}cm^{-1}$ as described by Means and Feeney (1971).

2.2 Biological materials

2.2.1 Plants

Maize (*Zea mays* L., hybrid variety DSC1) was supplied by De Kalb Shand Seed Co., Tamworth, N.S.W., Australia. Wheat (*Triticum aestivum* L. variety Warigal) was obtained from the Department of Agronomy, Waite Agricultural Research Institute, Glen Osmond, S.A., Australia.

2.2.2 Enzymes

Alcohol dehydrogenase (from yeast), hexokinase (Type IV from yeast) and carboxypeptidase A (Type II from bovine pancreas) were supplied by

Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). Trypsin (from pancreas) was supplied by BDH (Poole, England). Carbonic anhydrase (from bovine erythrocytes) was supplied by Boehringer Mannheim (Mannheim, West Germany).

2.3 Chemicals and other materials

2.3.1 Substrate and marker proteins

Azocasein, haemoglobin (Type II), bovine serum albumin, α-chymotrypsinogen (Type II from bovine pancreas), soybean trypsin inhibitor, ovalbumin and myoglobin were supplied by Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). Cytochrome c was supplied by Boehringer Mannheim (Mannheim, West Germany). Pepsin (2 times crystallised) was supplied by Nutritional Biochemicals (Cleveland, Ohio, U.S.A.).

Root protein substrate was prepared by the method of Storey and Beevers (1977). Maize seedlings were grown for 3.5 d on 1% (w/v) agar containing 5 mM NO_3^- as described in Section 2.4 and the primary roots (0-20 mm) harvested. The tissue (9 g) was extracted in a cold mortar and pestle with 20 ml of the following medium: 0.1M Tris-HCl, pH 8.0, 1 M NaCl, 0.1% (v/v) SDS, 5 mM mercaptoethanol, 1 mM Na_2EDTA. The extract was centrifuged at 1 000 *g* for 10 min at 2°C. To the supernatant was added 66 ml of ethanol at -15°C. After mixing for 1 h at 5°C, the mixture was allowed to stand overnight at -15°C, then centrifuged at 8 000 *g* for 10 min at 2°C. The pellet was washed successively with 100 ml each of 95% ethanol, 100% ethanol, ethanol-ether (3:1, v/v) and 5% (w/v) trichloroacetic acid. The pellet was recovered from each washing by centrifugation at 8 000 *g* for 10 min at 2°C. The final pellet was resuspended in 10 ml of 0.5 M NaOH, 0.5 M NaCl, 0.01% SDS and dialysed for 65 h against 2 changes of 10 mM NaOH, 0.001% (v/v) SDS.

2.3.2 Chromatographic materials

CM-cellulose was supplied by Whatman Biochemicals Ltd. (Springfield Mill, Kent, England). DEAE-Sephadex, Sephadex G-100, Sephadex G-25 and Sepharose 4B, CNBr activated were supplied by Pharmacia Fine Chemicals (Uppsala, Sweden). Biogel A-0.5m and Biogel HT (used for hydroxylapatite chromatography of protease B) were supplied by Bio-Rad Laboratories (Richmond, California). Amberlite XAD-4, IRA 400 and IR-120 were supplied by Rohm and Haas Co. (Philadelphia, Pennsylvania). Hypatite C (used for hydroxylapatite chromatography of protease A) was supplied by Clarkson Chemical Co. (Williamsport, Pennsylvania, U.S.A.).

2.3.3 Other chemicals

L-acetyl-L-phenylalanyl-L-diiodotyrosine, ATEE, ATP, BAEE, Cbz-Glu-Tyr, Cbz-Phe-Ala, Congo red elastin, cysteine, DAN, DFP, DTT, NADH, leucine-*p*-nitroanilide, 1,10-phenanthroline, PMSF, protamine sulphate, TAME, TLCK, TPCK and Tris were supplied by Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). NEM and pCMB were supplied by Calbiochem (San Diego, California, U.S.A.). Blue dextran was supplied by Pharmacia Fine Chemicals (Uppsala, Sweden). Acetaldehyde, glycerol, mercapto-ethanol and ninhydrin were supplied by May and Baker Ltd. (Dagenham, England). Alanine, cresol red, Folin and Ciocalteu's Reagent and N-(1-napthyl) ethylenediamine dihydrochloride were supplied by B.D.H. Ltd. (Poole, England). Polyclar AT was supplied by Edgar I. Noble Co. (Abbotsford, Vic., Australia) and washed as described by Loomis and Battaile (1966). Ammonium sulphate (Ultra-pure) was supplied by Mann Research Laboratories (New York, N.Y., U.S.A.).

All other chemicals were of the highest purity available.

2.3.4 Other materials

Coarse filter paper (V 70) used for germination of wheat was supplied by Industrial Equipment (A/sia) Pty. Ltd. (Paddington, N.S.W., Australia). Acid washed sand used during the preparation of cell free extracts was supplied by BDH (Poole, England). Miracloth was supplied by Chicopee Mills Inc. (Milltown, New Jersey, U.S.A.). Dialysis tubing (Visking tubing) was supplied by Scientific Instrument Centre Ltd. (London, England).

2.4 Growth of plants

Maize seeds were surface sterilised by washing briefly with 25% (v/v) ethanol, 0.05% (w/v) $HgCl_2$ and then four rinses of sterile distilled water. The seeds were planted on sterile agar, 1% (w/v) containing 0.1 strength Hoagland's solution and germinated in the dark for four days at 27°C and 70% relative humidity. They were then supported on a black perspex plate on a black water bath 0.32m x 0.25m and 0.13m deep so that the primary root was immersed in 0.1 strength Hoagland's solution containing 1.5 mM KNO_3 which was aerated. The plants were illuminated at an intensity of 350 $\mu E\ m^{-2}\ s^{-1}$ for 16 h/day at 21°C and were kept in darkness for 8 h at 17°C. When the seedlings were grown for more than 7 days, the nutrient solution was changed on the seventh day.

In one study where maize and wheat plants were grown in sand, 0.1 strength Hoagland's solution containing 1.5 mM KNO_3 and then 0.5 strength Hoagland's solution containing 10 mM NO_3^- was given. Growth conditions were as described for the liquid cultures. Four day wheat seedlings were germinated on coarse filter paper moistened with water under the initial incubation conditions described for maize above.

2.5 Enzyme techniques

2.5.1 Preparation of cell free extracts

After harvesting, the roots were rinsed with cold distilled water, dried with paper and weighed. They were homogenised with either a cold mortar and pestle or a cold food blender (14 d maize roots). The extraction medium was 50 mM sodium phosphate pH 7 containing 0.5 mM Na_2EDTA, 1 mM cysteine and 1% (w/v) Polyclar AT used in the ratio 2 ml/g tissue unless otherwise stated. The extract was filtered through 2 layers of Miracloth and then centrifuged at 95 000 *g* for 1 h at 2°C or in some cases at 18 000 *g* for 15 min at 2°C; in both cases, the supernatant fraction was retained.

2.5.2 Ammonium sulphate fractionation

Solid $(NH_4)_2SO_4$ was added to the solution with stirring at 0-4°C; the quantities of salt used were determined from the table given by Dawson *et al* (1969). After stirring for 30 min, the solution was centrifuged at 8 000 *g* for 10 min at 2°C. If the supernatant was further fractionated, the pH was readjusted to 7 with 0.1 M KOH and additional $(NH_4)_2SO_4$ was added to compensate for the dilution before proceeding with further fractionation. $(NH_4)_2SO_4$ ppts. were resuspended in about 0.1 of the starting volume using 20 mM sodium phosphate pH 7.0.

2.5.3 pH treatment

A dialysed $(NH_4)_2SO_4$ ppt. was adjusted to pH 4.4 with 50 mM HCl at 2°C, diluted to 1.5 times the initial volume with cold water and centrifuged at 8 000 *g* for 10 min at 2°C and the supernatant fraction retained.

2.5.4 Enzyme assays

2.5.4.1 Azocasein degradation

0.2 ml of enzyme sample, 0.3 ml of 10 mg/ml azocasein, 0.5 ml of 0.2 M Tris-HCl pH 8.5 were incubated at 37°C for 2 h. The reaction was stopped by addition of 2 ml of Hagihara protein precipitant (0.3 M acetic acid, 0.1 M trichloroacetic acid, 0.2 M sodium acetate) (Hagihara, 1954); after standing on ice for 10 min, the tubes were centrifuged at 8 000 *g* for 10 min at 2°C. To the supernatant was added 0.2 ml of 10 M NaOH and A_{440} was measured. The non-enzymic increase in A_{440} during incubation was about 0.01.

2.5.4.2 Haemoglobin degradation

(a) Standard procedure

0.1 ml of enzyme was incubated with 0.05 ml of 10 mg/ml haemoglobin and 0.1 ml of 0.25 M sodium acetate pH 4.0 for 2 h at 37°C. The reaction was stopped with 0.25 ml of 0.7 M $HClO_4$; after standing on ice for 10 min, the tubes were centrifuged at 8 000 *g* for 10 min at 2°C. 0.1 ml of the supernatant was taken for determination of free α-amino N (Section 2.7.6). The reaction was calibrated by addition of alanine; 1 A_{570} unit is equivalent to 1.25 μmol α-amino N. In some assays, the volume of the assay was doubled and for such reactions 1 A_{570} unit is equivalent to 2.75 μmol α-amino N. No non-enzymic hydrolysis of haemoglobin was observed.

(b) Modified procedure

In experiments where protease B was eluted from Biogel A-0.5m columns (Section 3.6), a modified assay was used. 0.2 ml of enzyme was incubated with 0.1 ml of 5 mg/ml haemoglobin in 0.25 M sodium acetate pH 4.0 at 37°C for up to 4 h. The reaction was stopped with

0.1 ml of 0.7 M $HClO_4$ and after standing on ice for 10 min, the tubes were centrifuged at 8 000 *g* for 10 min at 2°C. To 0.3 ml of the supernatant was added 2 ml of ninhydrin reagent containing 10.5 µl of 5 M NaOH to neutralise the $HClO_4$. The ninhydrin reaction was performed as described in Section 2.7.4 and 1 A_{570} unit is equivalent to 0.45 µmol of α-amino N.

2.5.4.3 Carboxypeptidase

0.2 ml of enzyme was incubated with 0.6 ml of 1 mg/ml Cbz-Phe-Ala in 0.1 M sodium acetate pH 5.2 at 37°C for 30 min, 1 h or 2 h depending on the activity of the sample. Reactions were terminated by addition of 2 ml of ninhydrin reagent and alanine released was determined as described in Section 2.7.6. 1 A_{570} unit is equivalent to 0.45 µmol of alanine. The substrate was dissolved in the buffer at room temperature with stirring for several hours.

2.5.4.4 Nitrate reductase inactivation

A sample of partially purified nitrate reductase from maize scutella, prepared as described in Wallace (1973), was supplied by Dr. W. Wallace. This preparation contained approximately 2 mg protein/ml and had an activity of about 3.0 µmol NO_2^- produced/h/ml. In this thesis, 1 unit of nitrate reductase is defined as the amount of enzyme that produces 1 nmol NO_2^-/h.

Nitrate reductase inactivation was determined by incubating 0.025 ml of nitrate reductase sample (approximately 75 units and 0.05 mg protein) with the inactivating sample and 25 mM potassium phosphate in a total volume of 0.4 ml at a final pH of about 7. After incubating for 1.5 h at 25°C, the following nitrate reductase assay reagents were added: 0.2 ml of 0.1 M Tris-HCl pH 7.5, 0.1 ml of 0.1 M KNO_3 and 0.1 ml of 2 mM NADH. After a further incubation for 0.5 h at 25°C, the

nitrate reductase reaction was stopped and excess NADH oxidised by adding 0.1 ml of 1 M acetaldehyde and 0.1 ml of 0.3 mg/ml alcohol dehydrogenase (14 units). After the reaction tubes had stood at room temperature for 5 min, 1 ml each of 1% sulphanilamide in 1 M HCl and 0.01% N-(1-napthyl) ethylenediamine dihydrochloride were added to enable nitrite to be determined. After 30 min, A_{540} was determined; an absorbance of 1 is equivalent to 60 nmol NO_2^-. Nitrate reductase inactivating activity was expressed as units of nitrate reductase lost. There was no loss of nitrate reductase activity in the absence of an inactivating species (Wallace, 1978b).

2.5.4.5 Aminopeptidase

The assay for aminopeptidase is based on that of Chrispeels and Boulter (1975). 0.1 ml of enzyme was incubated with 2 ml of 2 mM leucine *p*-nitroanilide in 50 mM potassium phosphate pH 6.7 containing 2% (v/v) DMSO for 30 min at 37°C and then A_{410} was read. An absorbance of 1 at 410 nm is equivalent to 0.34 µmol *p*-nitroaniline released.

2.5.4.6 Hexokinase

The assay procedure is essentially that of Darrow and Colowick (1962). Each assay contained 1.9 ml of 0.001% (w/v) cresol red in 0.3% (w/v) $MgCl_2$, 4.65 mg ATP in 0.2 ml of 0.1 M NaOH, 0.4 ml of 0.1 M sodium glycylglycine pH 9.0 and 0.5 ml of enzyme sample. The reaction mixture was incubated at 25°C in a 1 cm glass cuvette in a Varian model 635 spectrophotometer and the reaction was started by addition of enzyme. Reaction rates were determined by measuring the change in A_{560}.

2.5.4.7 Esterase

Each assay contained 0.5 ml of 2 mM ester substrate,

0.4 ml of 50 mM Tris-Cl pH 8.0 containing 10 mM $MgCl_2$. After incubating the reagents for 3 min in a 1 cm quartz cuvette (volume 1 ml) at 37°C in the spectrophotometer the reaction was started by addition of enzyme (0.1 ml). A cuvette containing the assay mixture but with 0.1 ml of the Tris-Cl buffer instead of enzyme was used as the reference for absorbance measurements which were made for 5 min. To determine wavelengths at which absorbance changes were measured, trypsin was added to the reaction mixture and incubated for 10 min to ensure complete hydrolysis of the substrate and the wavelength at which maximum or minimum absorbance occurred was noted.

2.5.4.8 Elastase

1 mg of Congo red elastin was dispersed in 0.2 ml of either 50 mM sodium acetate pH 4.0 or 50 mM Tris-Cl pH 8.5 using a trace of Tween 80 to lower surface tension of the buffer. The reaction was started by addition of 0.1 ml of enzyme. After incubating 2 h at 37°C in a shaking water bath, the tubes were centrifuged at 8 000 *g* for 10 min. A_{495} was estimated and compared to a reaction in which water was added instead of enzyme.

2.5.5 Determination of Michaelis-Menten constant

Double reciprocal plots of the data shown in Figures 19 and 23 were used to estimate K_m as described by Lineweaver and Burk (1934).

2.5.6 Determination of molecular weights

Molecular weights of enzymes were estimated from their elution volumes from gel filtration columns as detailed by Andrews (1965).

2.5.7 Dialysis

Solutions were dialysed by placing in dialysis tubing treated as

described in Section 2.7.5. The dialysis tubing was placed in one litre of dialysis buffer at 4°C and stirred. Normally dialysis was performed overnight with two changes of dialysis buffer (1 ℓ); the volume of the dialysing buffer was always at least 20 times that of the solution being dialysed.

2.5.8 Concentration of enzymes against Sephadex G-25

An enzyme solution was placed in a dialysis sac which was covered with Sephadex G-25 (coarse). After 2-3 h, the sac was covered with fresh gel. Normally this procedure was performed overnight and was always performed at 0-4°C.

2.6 Preparation and elution of chromatographic columns

2.6.1 Cation exchange chromatography

20 g of CM-cellulose were precycled by treatment with 300 ml of 0.5 M NaOH and 0.5 M HCl in the following sequence: NaOH 30 min; HCl 10 min; HCl 30 min; NaOH 10 min; NaOH 30 min; HCl 10 min; HCl 30 min. The resin was then washed with water and fines were removed as described in the Whatman laboratory manual using n = 1.3. The resin was stored in 0.03% (v/v) toluene. A slurry (1.5 of the settled volume of the resin) was poured into a glass column and the flow started immediately. If more resin was added, the addition was made before all the resin had completely settled. Columns were washed overnight with 500 ml of 20 mM sodium acetate pH 5.0 or other equilibrating buffer.

2.6.2 Anion exchange chromatography

DEAE-Sephadex was swollen for 24 h at room temperature in 20 mM sodium phosphate or other equilibrating buffer. A slurry

(1.5 x settled volume of the gel) was poured into a column and allowed to settle before washing overnight with 500 ml of equilibrating buffer with a pressure head of 2cm/cm gel height.

2.6.3 Gel filtration

Sephadex G-100 and Biogel A-0.5m were allowed to swell in equilibration buffer for 24 h. A slurry of 1.5 times the settled volume of gel was poured into the column, running the slurry down the side of the column, and left to settle. If further gel was added, this was done before all the gel had completely settled. When all the gel had settled, the column was washed with one or two column volumes of equilibrating buffer. When not in use, the columns were equilibrated with 0.02% sodium azide.

2.6.4 Hydroxylapatite chromatography

A slurry of hydroxylapatite (Hypatite C for protease A, Biogel HT for protease B) was poured into a glass column, packed under pressure and washed with several column volumes of buffer before use.

2.6.5 Affinity chromatography

Phenylalanine was coupled to CNBr-activated Sepharose essentially as described by Preston (1978). 3 g of gel was swollen in 1 mM HCl and washed with 600 ml of this acid. Then the gel was washed with 80 ml of 0.1 M sodium bicarbonate pH 9.0 and mixed with 0.4% (w/v) phenylalanine for 24 h at 4°C in a rotating mixer. The gel was then poured into a column, washed with water and then equilibrated with 25 mM sodium acetate pH 4.0.

Haemoglobin was coupled by a similar procedure but using 25 mg/ml haemoglobin.

2.6.6 Amberlite XAD-4

Amberlite XAD-4 was sieved to select beads with a diameter of 0.5-1 mm which were washed several times with methanol then water. A slurry of beads was then poured into a glass column and equilibrated with 50 mM sodium phosphate pH 7.0 containing 0.5 mM Na_2EDTA.

2.6.7 Operation of chromatographic columns

Glass tubes were fitted at both ends with rubber bungs with glass tubing through them and the lower bung was covered with two layers of nylon netting. The eluting buffer for gel filtration columns was supplied from a Mariotte flask using a head of 0.3 m for the Sephadex G-100 column and 1 m for the Biogel A-0.5m column. For other columns, the eluting buffer was pumped on to the column using an LKB peristaltic pump. Fractions were collected on an ISCO model 1200 fraction collector and the absorbance of the eluate was measured with an ISCO type 6 optical unit and model UA-5 absorbance monitor.

2.7 General techniques

2.7.1 Absorbance spectra and measurements

Absorption spectra were recorded on either a Pye SP 800 or Varian model 635 spectrophotometer using 1 cm quartz cuvettes. Absorbance measurements in the range 400-600 nm were made on a Turner spectrophotometer fitted with a glass flow through cell. Measurements at other wavelengths were made on a Shimadzu QV-50 spectrophotometer.

2.7.2 Centrifugation

Centrifugation at forces less than 30 000 *g* was performed on a Sorvall RC-2B centrifuge at 2°C using a SS-34 rotor. A Beckman L2-65B

centrifuge with a type 35 rotor was used for centrifugation at 95 000 *g* at 2°C.

2.7.3 Protein determination

Protein was determined essentially by the method of Lowry *et al* (1951). To 1 ml of protein sample was added 1 ml of 20% (w/v) trichloroacetic acid. After standing on ice for 15 min, the tubes were centrifuged at 3 000 r.p.m. on a MSE bench centrifuge for 15 min. To the pellet was added 0.8 ml of 2% (w/v) Na_2CO_3 in 0.1 M NaOH with mixing. After 30 min at room temperature, 4 ml of freshly prepared alkaline copper sulphate containing sodium potassium tartrate (50 ml of 2% (w/v) Na_2CO_3 in 0.1 M NaOH; 1 ml of 0.5% (w/v) $CuSO_4 \cdot 5H_2O$; 1 ml of 1% (w/v) sodium potassium tartrate) was added, the tubes were incubated for 20 min at 37°C and 0.5 ml of diluted (1:1 with water) Folin and Ciocalteu's reagent was added. A_{730} was measured after 30 min. Bovine serum albumin was used as a standard.

2.7.4 Determination of amino acids and soluble α-amino N

The procedure is based on that of Lee and Takahashi (1966). Each tube contained 1.9 ml of the following mixture: 14 ml of 0.5 M sodium citrate pH 5.5; 0.1 g ninhydrin; 24 ml glycerol. After addition of the sample, the contents of the tube were mixed with a Vortex mixer and the tube was placed in a boiling water bath for 12 min. After cooling the tube, A_{570} was read. Alanine was used to calibrate the reaction.

Sodium citrate buffer was prepared by dissolving 44 g of tri-sodium citrate in water, boiling for 10 min, cooling, neutralising to pH 5.5 with concentrated hydrochloric acid, and making up to 300 ml with water.

Glycerol was purified with Amberlite in exchange resins as suggested by Jolly and Tolbert (1978). IR-120 and IRA-400 ion exchange

resins were prepared as described by the makers in 'Amberlite Exchange Resins - Laboratory Guide' and equal amounts of each resin packed into a column $9cm^2$ x 47cm.

The glycerol was pumped through the column at the rate of 120 ml/h.

2.7.5 Preparation of dialysis tubing

Dialysis tubing was boiled for 10 min in 2% (w/v) Na_2CO_3 and 1.1 g/l Na_2EDTA. It was then boiled twice in separate lots of distilled water and stored in 50% (v/v) ethanol.

3. RESULTS

3. RESULTS

3.1 Extraction of proteases from maize roots

3.1.1 Use of high speed centrifugation

A crude extract of maize roots centrifuged at 95 000 *g* for 1 h had higher specific activities for azocasein degrading, haemoglobin degrading and carboxypeptidase activities than an extract centrifuged at 15 000 *g* for 15 min (Table 6). As little activity is sedimented, the enzymes appear to be in the soluble fraction of the extract.

3.1.2 Interference by phenolic compounds

Extracts of maize roots become brown on storage and show strong absorption at 260 nm (Figure 2). It therefore seemed likely that they contained phenolic compounds because oxidation of these compounds results in browning of plant extracts (Loomis, 1969) and most of the absorption at 260 nm by plant extracts is due to phenolic compounds rather than nucleic acids (Loomis, 1974). Polyclar AT, an insoluble polyvinylpyrrolidone used to protect proteins against phenolic compounds, may release soluble vinylpyrrolidone, which absorbs at 260 nm (Figure 3) during homogenisation (Loomis, 1974). However this compound appears to make only a small contribution to the absorbance of the extract; A_{260} of a maize root extract prepared with 1% (w/v) Polyclar AT, 28.5; A_{260} of 10% (w/v) Polyclar AT homogenate, 0.2; A_{260} of 1% (w/v) soluble polyvinylpyrrolidone, 0.73 (Figure 3).

Phenolic compounds often have deleterious effects on enzymes (Loomis, 1974) and in addition to Polyclar AT, the following agents have been used to protect proteins against these compounds: cysteine (Anderson, 1968), metabisulphite (Anderson and Rowan, 1967), protamine sulphate (Loomis, 1974). These reagents were added in turn to the

TABLE 6

Effect of high speed centrifugation on the purification and recovery of proteases from maize roots

7 day old maize roots were homogenised in a cold mortar and pestle with 2 ml/g fr wt of 50 mM potassium phosphate buffer pH 7.0 containing 0.5 mM Na_2EDTA, 5 mM cysteine, 1% (w/v) Polyclar AT at 4°C, filtered through two layers of Miracloth and then centrifuged as indicated at 2°C. Enzyme activities and the protein contents of each supernatant fraction were determined as described in Sections 2.5.4.1, 2.5.4.2, 2.5.4.3 and 2.7.3.

	Fraction	Protein (mg/ml)	Azocasein degrading activity ΔA_{440}/mg protein/h)	Haemoglobin degrading activity (µmol α-amino N released/mg protein/h)	Carboxypeptidase activity (µmol alanine released/mg protein/h)
I	18 000 *g* (15 min) supernatant of extract	0.67	2	4.4	8.9
II	95 000 *g* (60 min) supernatant of I	0.52	2.5 (100)*	5.1 (89)*	10 (87)*

* figures in parentheses denote percentage recovery of enzyme activity relative to fraction I

FIGURE 2

Spectrum of a maize root extract

An extract of 7 day old maize roots was prepared as described in Table 6 and centrifuged at 18 000 *g* for 15 min at 2°C. The spectrum of the supernatant fraction, diluted 15 fold, was recorded in a 1 cm quartz cuvette in a Unicam SP 800 recording spectrophotometer.

Fig 2

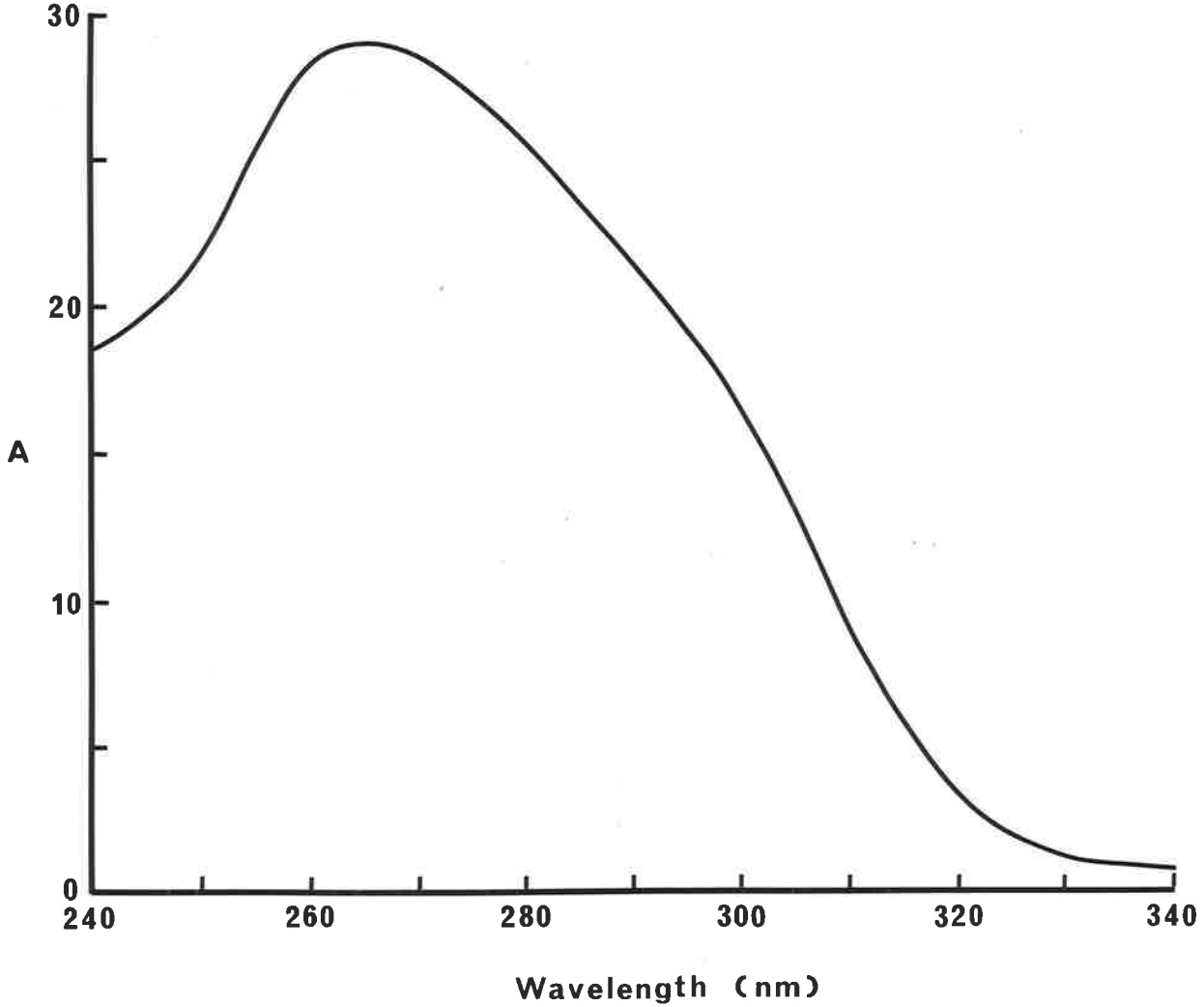


FIGURE 3

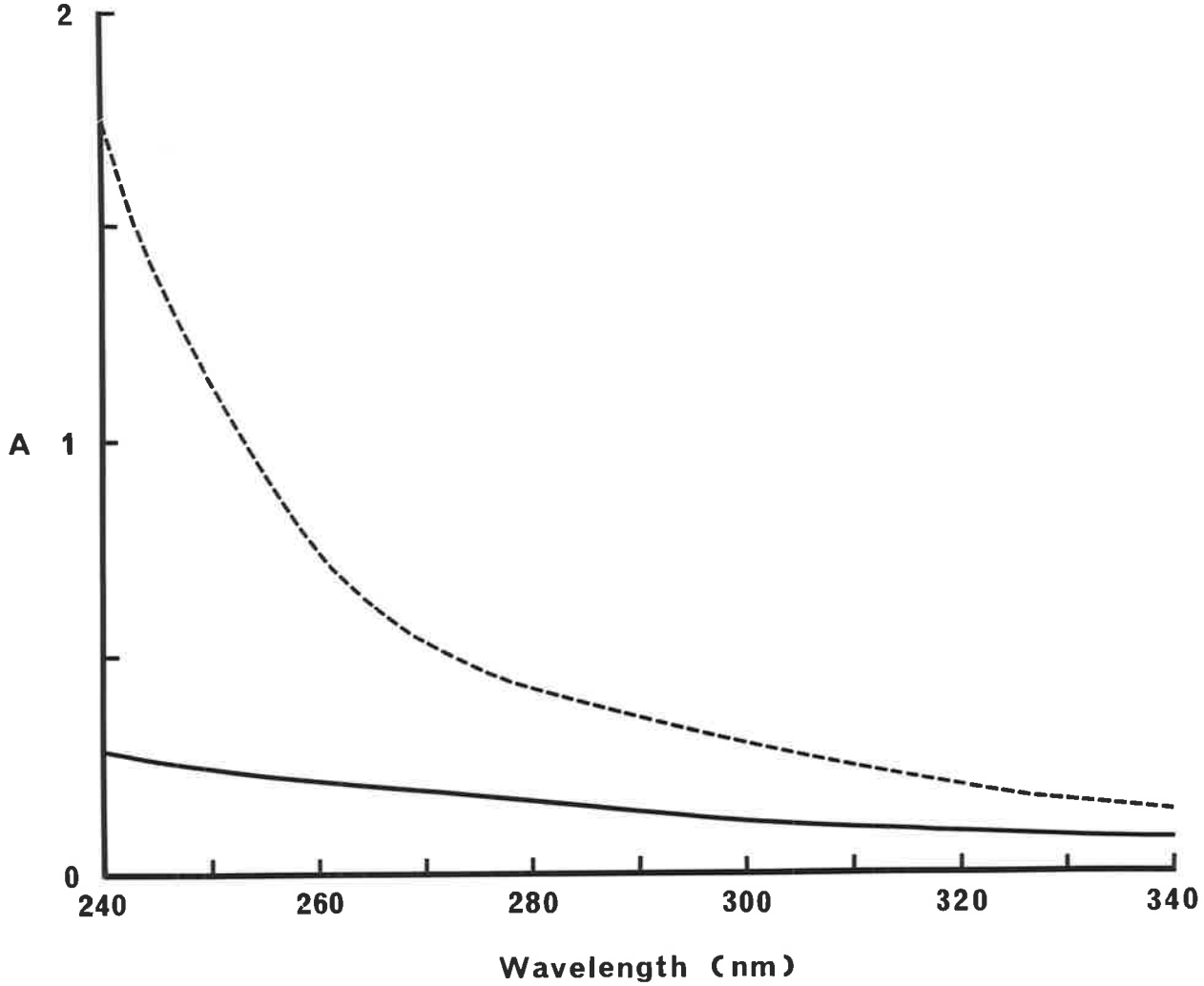
Spectra of polyvinylpyrrolidone and material released by homogenisation of Polyclar AT

A 10% (w/v) suspension of Polyclar AT in 50 mM sodium phosphate pH 7.0 containing 0.5 mM Na_2EDTA was homogenised for a few minutes in a cold mortar and pestle. After centrifuging at 18 000 *g* for 15 min at 2°C, the spectrum of the supernatant fraction was recorded in a 1 cm quartz cell using a Pye SP 800 spectrophotometer. Soluble polyvinylpyrrolidone was dissolved in water, 1% (w/v), and its spectrum recorded as described above.

supernatant of homogenised Polyclar AT

soluble polyvinylpyrrolidone

Fig 3



extracting buffer (50 mM sodium phosphate pH 7.0 containing 0.5 mM Na_2EDTA) and their effects on UV absorbance and protease activity determined (Table 7). None of the treatments significantly increased the levels of proteases detected or resulted in a marked reduction in UV absorbance compared with extracting medium A while treatment E significantly reduced the amount of carboxypeptidase activity in the extract. Treatment A was chosen because it avoided the addition of protein to the extract (treatment D) and did not involve large amounts of Polyclar AT (treatment C) which reduced the volume of extract recovered.

The polystyrene resin Amberlite XAD-4 has been used for the removal of phenolic compounds from plant extracts (Loomis, 1974). When an 18 000 *g* supernatant of an extract of maize roots was passed through an Amberlite XAD-4 column a considerable amount of UV absorbing material was removed (A_{260} was reduced from 36 to 11). This treatment also eliminated material absorbing equally at 260 and 280 nm which was present in the elution profile of a CM-cellulose column loaded with a partially purified extract of maize roots (Figure 7). In addition there was less browning, as indicated by A_{400} values, in the $(NH_4)_2SO_4$ ppt. of an XAD-4 treated extract than in a similar ppt. of an untreated extract (Table 8). (No absorbance peaks were found in the spectra of the two $(NH_4)_2SO_4$ ppts. listed in Table 8 in the range 400-800 nm so that the absorbance at 400 nm could be taken as an index of browning.)

Treatment with the polystyrene resin had no beneficial effect on the amounts of protease activity or on their stability assessed after 4 days of storage (Table 8). The metabisulphite used in the preparation of extracts treated with XAD-4 may also reduce browning by restricting phenolic oxidation (Anderson and Rowan, 1967) although this reagent did not reduce UV absorption (Table 7).

TABLE 7

Effect of reagents which protect against phenolic compounds on the activity of proteases from maize roots

7 day old maize roots were extracted with 2 ml/g fr wt of 50 mM sodium phosphate pH 7.0 containing 0.5 mM Na_2EDTA with the additives shown in a cold mortar and pestle. The extracts were centrifuged at 18 000 *g* for 15 min at 2°C and the supernatant fractions were dialysed overnight against two changes of 20 mM sodium phosphate pH 7.0 at 4°C. The dialysed supernatant fractions were analysed for protease activities as described in Sections 2.5.4.1, 2.5.4.2 and 2.5.4.3. UV spectra were recorded on a Pye SP 800 spectrophotometer using a 1 cm quartz cuvette and the maximum absorbance (in the range 250-300 nm) and the corresponding wavelength are shown.

	Reagents added to the extraction buffer	Maximum absorbance	Wave-length (nm)	Azocasein degrading activity (ΔA_{440}/ml/h)	Haemoglobin degrading activity (µmol α-amino N released/ml/h)	Carboxypeptidase activity (µmol alanine released/ml/h)
A	5 mM cysteine, 1% (w/v) Polyclar AT	1.83	267	1.3	2.5	6.3
B	10 mM sodium metabisulphite, 1% (w/v) Polyclar AT	1.74	260	1.2	2.4	5.5
C	5 mM cysteine, 10% (w/v) Polyclar AT	1.46	265	1.3	2.5	6.7
D	5 mM cysteine, 1% (w/v) Polyclar AT, 0.2% (w/v) protamine sulphate	1.86	267	1.4	2.6	7.1

(Cont.)

TABLE 7 (Cont.)

	Reagents added to the extraction buffer	Maximum absorbance	Wave-length (nm)	Azocasein degrading activity (ΔA_{440}/ml/h)	Haemoglobin degrading activity (µmol α-amino N released/ml/h)	Carboxypeptidase activity (µmol alanine released/ml/h)
E	10 mM sodium metabisulphite, 10% (w/v) Polyclar AT, 0.2% (w/v) protamine sulphate	1.29	262	1.1	2.2	1.8

TABLE 8

Effect of Amberlite XAD-4 treatment of extracts of maize roots on protease activity

7 day old maize roots were homogenised in a cold mortar and pestle with 2 ml/g fr wt of 50 mM sodium phosphate buffer pH 7.0 containing 0.5 mM Na_2EDTA and 1% (w/v) Polyclar AT. For treatment A, the extraction medium also contained 5 mM cysteine and the extract was centrifuged at 95 000 *g* for 1 h at 2°C and the supernatant fraction was retained. For treatment B, the extraction medium also contained 10 mM sodium metabisulphite and the extract was centrifuged at 18 000 *g* for 1 min at 2°C; the supernatant fraction was passed through an Amberlite XAD-4 column (1.77cm² x 10cm) equilibrated with 50 mM sodium phosphate buffer pH 7.0 containing 0.5 mM Na_2EDTA and 10 mM sodium metabisulphite at the rate of 65 ml/h. The eluate was centrifuged at 95 000 *g* for 1 h. 0-90% $(NH_4)_2SO_4$ ppts. were prepared from the 95 000 *g* supernatant fractions from treatments A and B by adding the salt (60.3 g/100 ml) with stirring at 4°C. After 30 min, the solutions were centrifuged at 8 000 *g* for 10 min and the supernatant fractions decanted. The precipitates were resuspended in 20 mM sodium phosphate buffer (0.1 of original volume), dialysed against the same buffer overnight (two changes) at 4°C and analysed for protease activity as described in Sections 2.5.4.1, 2.5.4.2 and 2.5.4.3.

	A_{400}	Azocasein degrading activity (ΔA_{440}/ml/h)	Haemoglobin degrading activity (µmol α-amino N released/ml/h)	Carboxypeptidase activity (µmol alanine released/ml/h)
0-90% $(NH_4)_2SO_4$ ppt. of untreated extract (treatment A)	1	3.1 (2.9)*	6.1 (5.9)*	15
0-90% $(NH_4)_2SO_4$ ppt. of Amberlite treated extract (treatment B) (figures corrected for dilution on Amberlite column)	0.69	3.2 (3.1)*	6.1 (6.2)*	15

* figures in parentheses are enzyme activities determined after storage for 4 days at 4°C

3.1.3 Effect of salt, detergent and high pH

As enzymes may adhere to cell wall fragments (Newcombe, 1963) and ribosomes (Peterman, 1964) the use of detergent, high salt concentrations and high pH was explored to determine whether additional enzyme activity could be extracted. Addition of either 0.2 M NaCl or 0.1% (w/v) sodium deoxycholate to 50 mM potassium phosphate pH 7.0 did not result in an increased extraction of the azocasein degrading activity and neither did extraction at pH 8.0 or 9.0 (Table 9). It will be shown in Sections 3.9.3 and 3.10.3 that some of the proteases are more labile at pH 8.0 than at pH 6.0.

3.2 Partial purification of proteases from maize roots

3.2.1 Ammonium sulphate fractionation

Between 70 and 90% of the protease activities in an extract of maize roots were precipitated between 35 and 70% saturation of $(NH_4)_2SO_4$ (Table 10) resulting in a 1.5 fold purification (Table 11). This step also concentrated the proteases.

3.2.2 pH treatment

When the pH of the dialysed 35-70% $(NH_4)_2SO_4$ ppt. prepared as described in Table 8 was lowered to pH 4.4, 75% of the protein was precipitated (Table 11). All the azocasein degrading activity was recovered and recovery of the haemoglobin degrading activity was 83%, carboxypeptidase activity 82% and aminopeptidase activity 37%. Specific activities increased about 4, 3, 4 and 1.5 fold respectively (values relative to the 35-70% $(NH_4)_2SO_4$ ppt.). Losses of aminopeptidase activity during this step are probably due to the lability of such enzymes at pH 4.4 (Kolemainen and Mikola, 1971).

TABLE 9

Effects of salt, detergent and pH on the extraction of azocasein degrading activity from maize roots

Four day old maize roots were homogenised in a cold mortar and pestle with the buffers shown (2 ml/g fr wt) and the extracts were centrifuged at 18 000 *g* for 15 min at 2°C. The supernatant fractions were assayed for azocasein degrading activity as described in Section 2.5.4.1 except that the reactions were performed at pH 6.0 and stopped with 1 M $HClO_4$.

Extracting buffer (each 50 mM)	Azocasein degrading activity (ΔA_{440}/ml/h)
potassium phosphate pH 7.0	0.17
potassium phosphate pH 7.0 + 0.2 M NaCl	0.18
potassium phosphate pH 7.0 + 0.1% (w/v) sodium deoxycholate	0.17
Tris-HCl pH 7.0	0.26
Tris-HCl pH 8.0	0.20
Tris-HCl pH 9.0	0.21

TABLE 10

Ammonium sulphate fractionation of an extract of maize roots

A 95 000 *g* supernatant fraction was prepared from 75 g of 7 day old maize roots as described for treatment A in Table 8. Ammonium sulphate fractionation was performed by the procedure described in Table 8 and after each precipitation step, the pH of the supernatant fraction was adjusted back to the initial pH of 7.0 with 0.1 M KOH. The $(NH_4)_2SO_4$ ppts. shown were resuspended in 20 mM sodium phosphate pH 7.0, dialysed overnight against two changes of the same buffer at 4°C and analysed for protease activity and protein content as described in Sections 2.5.4.1, 2.5.4.2, 2.5.4.3, 2.5.4.5 and 2.7.3.

% saturation with $(NH_4)_2SO_4$	Protein (mg)	Azocasein degrading activity (ΔA_{440}/h)	Haemoglobin degrading activity (µmol α-amino N released/h)	Carboxypeptidase activity (µmol alanine released/h)	Aminopeptidase activity (µmol *p*-nitroaniline released/h)
Crude extract	121	210	510	1 000	530
0 - 35	11	7	40	37	2
35 - 50	25	65	150	220	50
50 - 60	23	87	130	180	300
60 - 70	11	10	72	360	120
70 - 90	12	1.3	4	170	10

TABLE 11

Partial purification of proteases from maize roots by ammonium sulphate fractionation and pH treatment

A 35-70% $(NH_4)_2SO_4$ ppt. was prepared from the extract of 75 g of 7 day old maize roots as described in Table 10. The precipitate was resuspended in 20 mM sodium phosphate pH 7.0 and dialysed overnight against two changes of the same buffer at 4°C. It was then adjusted to pH 4.4 with 50 mM HCl, diluted to 1.5 of the original volume with water and centrifuged at 8 000 *g* for 10 min at 2°C. The supernatant fraction, hereafter referred to as 'pH 4.4 supernatant', was retained. Protease activities and protein content were determined as described in Sections 2.5.4.1, 2.5.4.2, 2.5.4.3, 2.5.4.5 and 2.7.3.

Fraction	Volume (ml)	Protein (mg)	Azocasein degradation (ΔA_{440}/h)	Haemoglobin degradation (µmol α-amino N released/h)	Carboxypeptidase (µmol alanine released/h)	Aminopeptidase (µmol *p*-nitroaniline released/h)
I.Crude extract	200	121	212 (1.7)*	515 (4.2)*	1 003 (8.3)*	525 (4.3)*
II.35-70% $(NH_4)_2SO_4$ ppt. of I in 20 mM sodium phosphate pH 7.0	34	58	159 (2.7)*	354 (6.1)*	761 (13)*	470 (8.1)*
III.Supernatant fraction after adjusting II to pH 4.4 and centrifuging	50	14	170 (12)*	294 (21)*	624 (45)*	173 (12)*

* figures in parentheses denote specific activity (units/mg protein)

3.3 Cation exchange chromatography of proteases from maize roots

A protease like species which degrades azocasein and inactivates nitrate reductase has been isolated from maize roots (Wallace, 1974, 1978a). This enzyme was eluted from a CM-cellulose column equilibrated in 10 mM sodium acetate pH 5.0 by 50 mM NaCl dissolved in the equilibrating buffer. In the current study, the supernatant fraction of a 35-70% $(NH_4)_2SO_4$ ppt. resuspended in 20 mM sodium phosphate pH 7.0 and treated at pH 4.4 (hereafter referred to as 'pH 4.4 supernatant') (Fraction III, Table 11) was eluted from a similar column with a gradient of 0 - 0.4 M NaCl. All the proteases investigated were eluted at 150 mM NaCl but they were not resolved (Figure 4). A CM-cellulose column loaded with pH 4.4 supernatant (Fraction III, Table 11) was then eluted with 150 mM NaCl in 20 mM sodium acetate pH 5.0 resulting in some resolution of the proteases. By lowering the NaCl concentration in the eluting buffer to 130 mM, the carboxypeptidase was separated into two fractions, both of which contained haemoglobin degrading activity (Figure 5). Azocasein degrading activity was eluted in a plateau like fraction containing haemoglobin degrading activity but aminopeptidase activity was not clearly resolved from the other protease activities. In an attempt to increase the resolution of the proteases shown in Figure 5, the effect of varying the pH of the column equilibrating buffer was investigated.

A column eluted at pH 4.5 with 150 mM NaCl gave no improvement in the resolution presented in Figure 5 while columns equilibrated at pH 5.3 and 5.5 did not adsorb all the protease activity. As some of the proteases were not adsorbed on CM-cellulose above pH 5.0, a gradient of 20 mM sodium acetate pH 5.0 to 50 mM sodium acetate pH 5.6 was tested. Carboxypeptidase activity eluted ahead of the proteinase activities which were not resolved (Figure 6). However elution with a gradient of 20 mM

FIGURE 4

Elution of proteases from maize roots from CM-cellulose by a salt gradient

A 35-70% $(NH_4)_2SO_4$ ppt., prepared as described in Table 10, was adjusted to pH 4.4 with 50 mM HCl, diluted to 1.5 times the original volume, centrifuged 10 min at 8 000 *g* at 2°C and the supernatant fraction (hereafter referred to as 'pH 4.4 supernatant') was taken. 5 ml of pH 4.4 supernatant, containing 3 mg protein were applied to a CM-cellulose column ($0.79cm^2$ x 5cm) equilibrated in 20 mM sodium acetate pH 5.0. The column, washed with 18 ml of the equilibrating buffer, was then eluted with a 90 ml gradient of 0 - 0.4 M NaCl in 20 mM sodium acetate pH 5.0 at the rate of 30 ml/h. A_{280} of the column effluent was monitored and 2 ml fractions were analysed for proteinase and carboxypeptidase activities as described in Sections 2.5.4.1, 2.5.4.2 and 2.5.4.3.

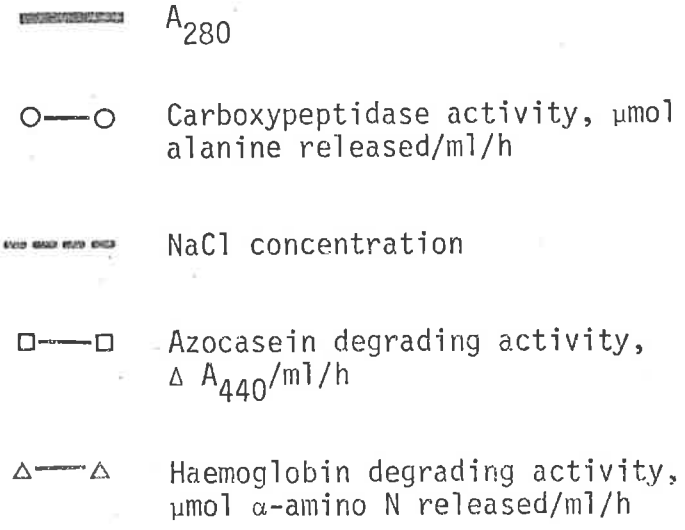
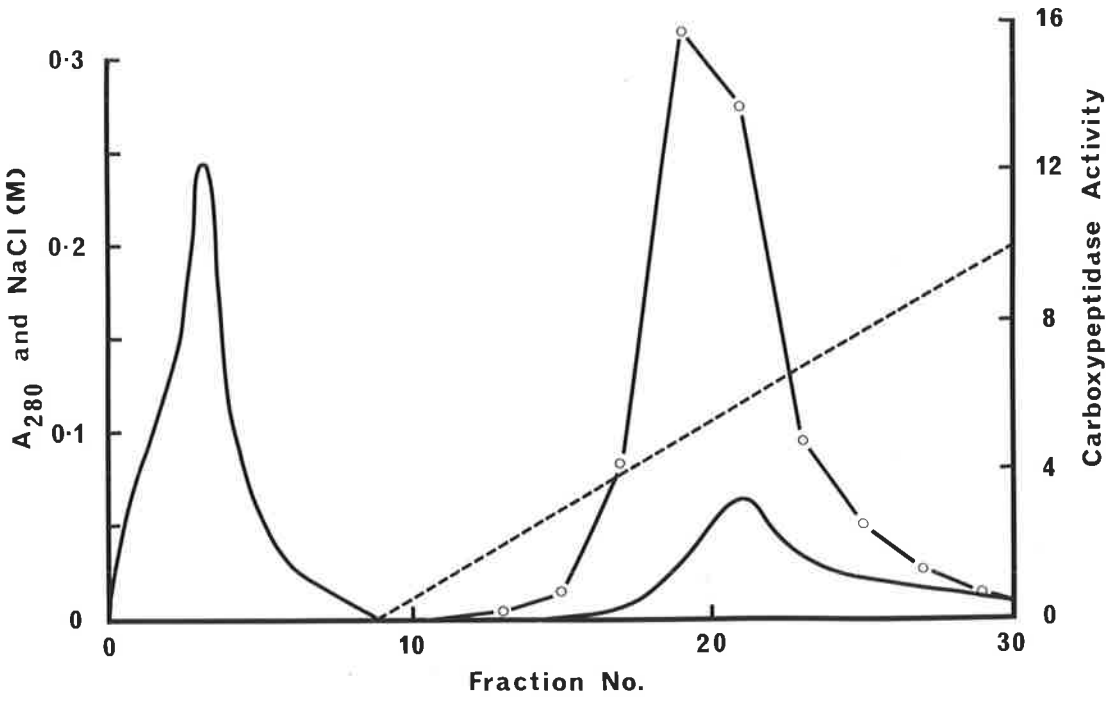


Fig 4


0·3
0·2
0·1
0
A280 and NaCl (M)
16
12
8
4
0
Carboxypeptidase Activity
0
10
20
30
Fraction No.

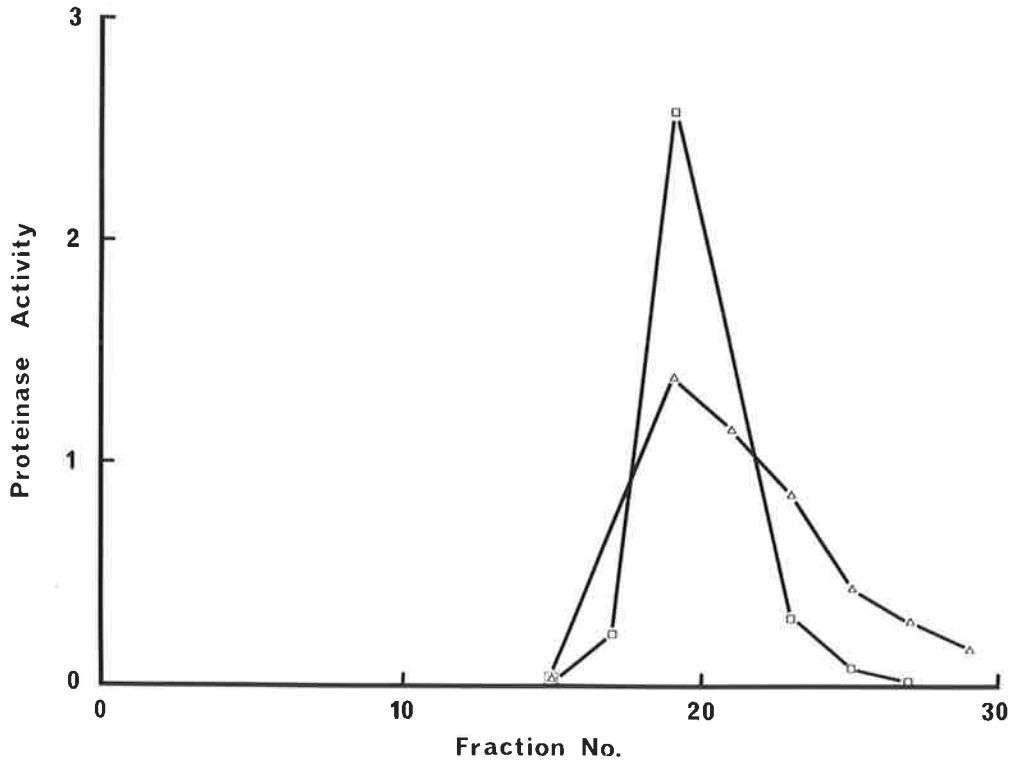

3
2
1
0
Proteinase Activity
0
10
20
30
Fraction No.

FIGURE 5

Batch elution of proteases from maize roots from CM-cellulose with 130 mM NaCl

21.5 ml of pH 4.4 supernatant (Fraction III, Table 11) containing 12 mg protein were prepared from 39 g of 7 day old maize roots as described in Table 11. This fraction was applied to a CM-cellulose column ($2cm^2$ x 10cm) equilibrated with 20 mM sodium acetate pH 5.0. UV absorbing material, similar to that shown in Figure 4, was eluted from the column with 66 ml of equilibrating buffer; no protease activity was associated with this material. The column was then eluted with 130 mM NaCl in equilibrating buffer at the rate of 10 ml/h. A_{280} of the column effluent was monitored and 3.3 ml fractions were analysed for proteinase, carboxypeptidase and aminopeptidase activities as described in Sections 2.5.4.1, 2.5.4.2, 2.5.4.3 and 2.5.4.5.

A_{280}

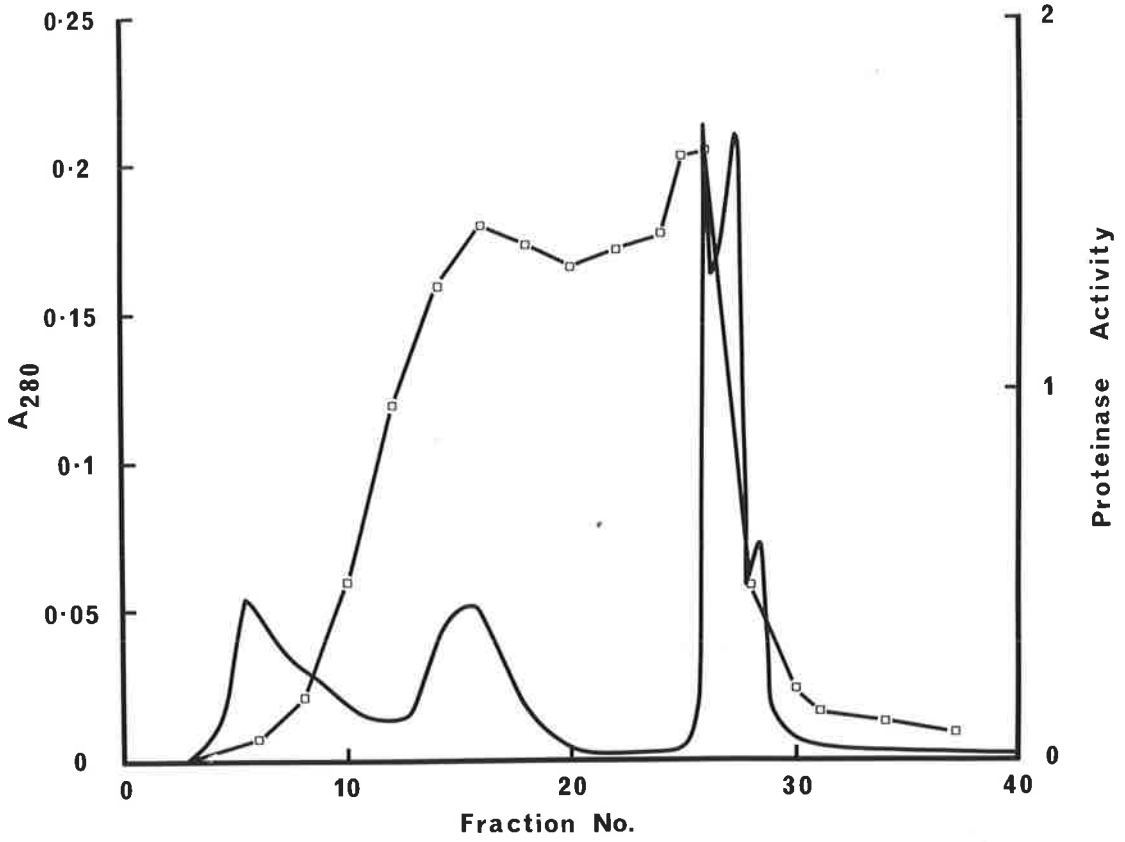
Azocasein degrading activity, ΔA_{440}/ml/h

Haemoglobin degrading activity, µmol α-amino N released/ml/h

Carboxypeptidase activity, µmol alanine released/ml/h

Aminopeptidase activity, µmol *p*-nitroaniline released/ml/h

Fig 5


0·25
0·2
0·15
0·1
0·05
0
A_{280}
2
1
0
Proteinase Activity
0
10
20
30
40
Fraction No.

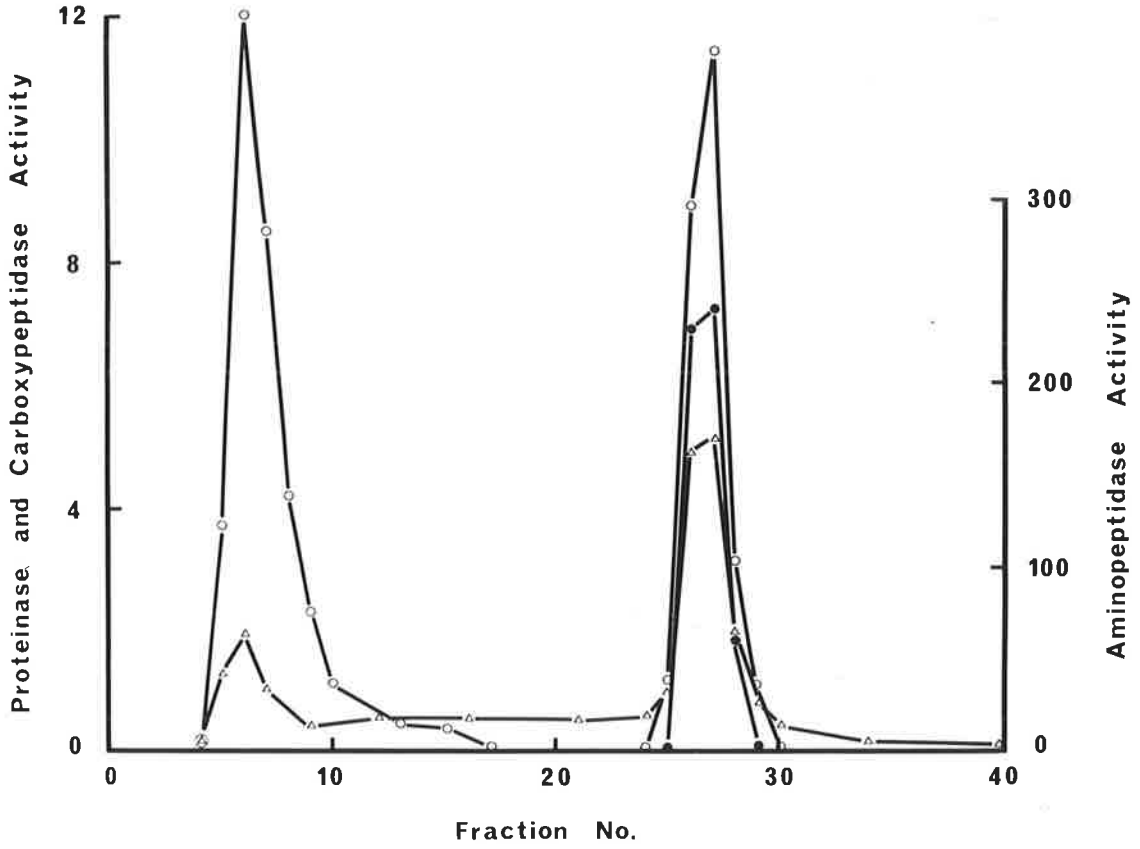
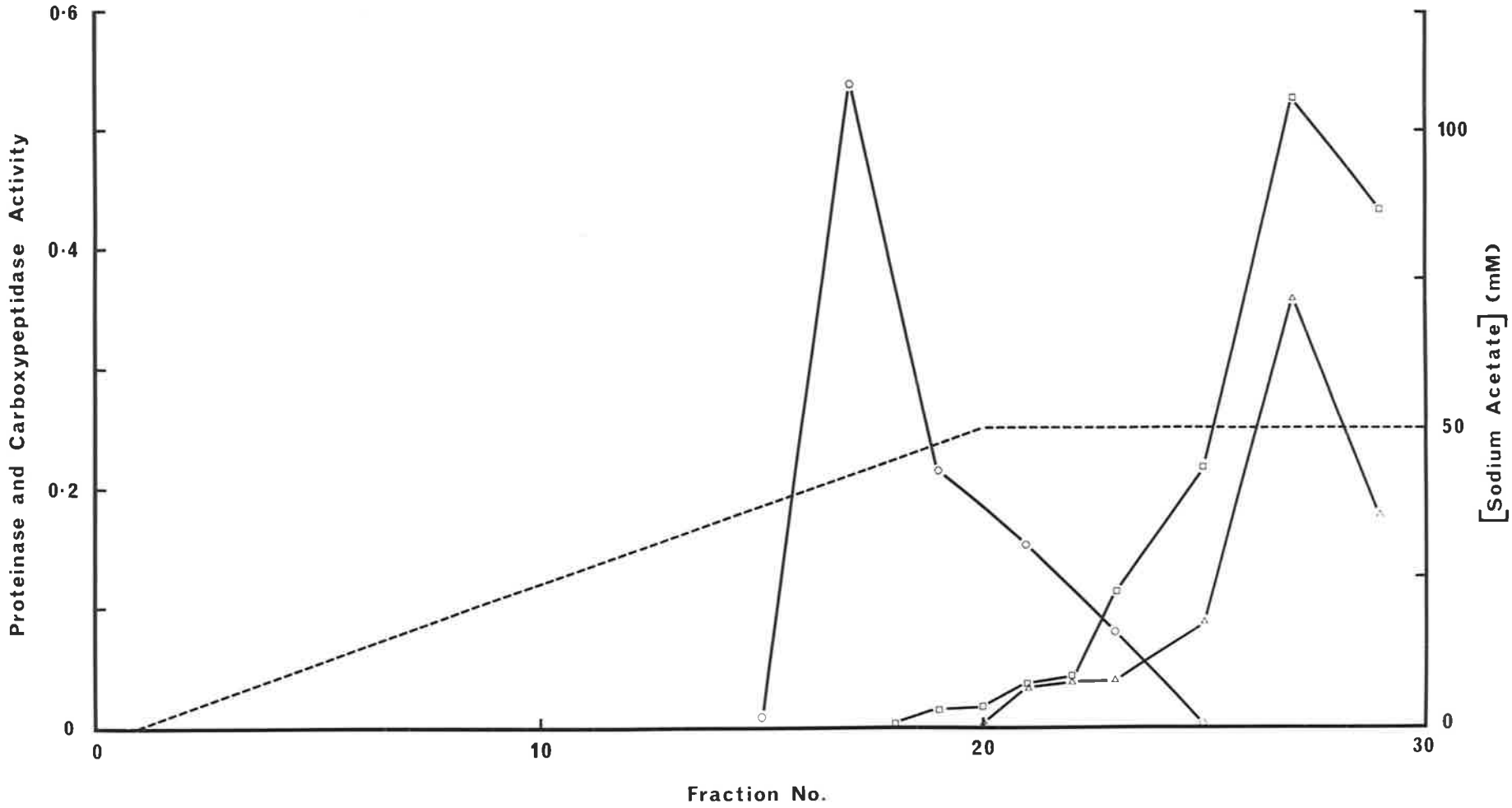

12
8
4
0
Proteinase and Carboxypeptidase Activity
300
200
100
0
Aminopeptidase Activity
0
10
20
30
40
Fraction No.

FIGURE 6

Elution of proteases from maize roots from CM-cellulose by a gradient of pH and ionic strength

5.4 ml of pH 4.4 supernatant (Fraction III, Table 11) containing 3 mg protein were loaded on a CM-cellulose column ($0.79cm^2$ x 4.5cm) equilibrated with 20 mM sodium acetate pH 5.0. After washing the column with equilibrating buffer, it was eluted at the rate of 14 ml/h with a 40 ml gradient of 20 mM sodium acetate pH 5.0 to 50 mM sodium acetate pH 5.6. After completion of the gradient at fraction 21, the column was eluted with 50 mM sodium acetate pH 5.6. 2 ml fractions were analysed for proteinase and carboxypeptidase activities as described in Sections 2.5.4.1, 2.5.4.2 and 2.5.4.3. No absorbance at 280 nm was detected on a scale of 0 - 0.3.

Fig 6


0·6
0·4
0·2
0
Proteinase and Carboxypeptidase Activity
100
50
0
[Sodium Acetate] (mM)
0
10
20
30
Fraction No.

sodium acetate pH 5.0 to 6.0 containing 130 mM NaCl resolved the proteases into two fractions (Figure 7). Approximately 30% of the gradient (125 ml) was used to elute the proteases. The first fraction, protease A, eluted at one column volume, contained 84% of the carboxypeptidase activity, all of the azocasein degrading and aminopeptidase activities, 65% of the haemoglobin degrading activity and 2% of the loaded protein. The enzymes were purified 20-34 fold with good recovery apart from aminopeptidase which is acid labile (Section 3.2.2). The second fraction, protease B, eluted after 4-5 column volumes, contained only haemoglobin degrading and carboxypeptidase activities and 23% of the loaded protein. The specific activities of these enzymes are shown in Table 12. Assays for carboxypeptidase and haemoglobin degrading activities in fractions prior to the CM-cellulose column do not distinguish the relative contributions of proteases A and B so that the recoveries and specific activities shown are not absolute values. A third fraction of UV absorbing material, eluted at fraction 11 in Figure 7, did not correspond to any peak of protease activity and was removed by Amberlite XAD-4 treatment of the crude extract (Section 1.1.2). The column equilibrating buffer (20 mM sodium acetate pH 5.0) eluted a large amount of UV absorbing material (absorbance at both 280 nm and 260 nm of about 0.4) which contained very little proteolytic activity and approximately 35% of the loaded protein.

When proteases A and B were rechromatographed on columns identical to those used to separate them, both fractions eluted in the same positions as on the original column (Figure 8) suggesting that the two fractions are not related. This experiment also showed that it is essential to use long columns $2cm^2$ x 13.3cm as used in Figure 7 because the shorter columns used in Figure 8 resulted in some overlap of proteases A and B. This overlap accounts for the appearance of a small amount of a protease A

TABLE 12

Partial purification of proteases from maize roots on a CM-cellulose column

41 ml of pH 4.4 supernatant (Fraction III, Table 11), containing 14 mg protein, was chromatographed on the CM-cellulose column shown in Figure 7. Protease activities and protein content of each fraction were determined as described in Sections 2.5.4.1, 2.5.4.2, 2.5.4.3, 2.5.4.5 and 2.7.3.

Fraction	Volume (ml)	Protein (mg)	Azocasein degradation (ΔA_{440}/h)	Haemoglobin degradation (µmol α-amino N released/h)	Carboxypeptidase (µmol alanine released/h)	Aminopeptidase (µmol *p*-nitroaniline released/h)
pH 4.4 supernatant (Fraction III, Table 11)	41	14	170 (12.2)*	294 (21)*	624 (45)*	173 (12.4)*
Protease A	49	0.25†	154 (297)*	103 (419)*	376 (1 530)*	6.7 (27)*
Protease B	16	2.6	0	55 (21)*	71 (27)*	0

* figures in parentheses denote specific activity (units/mg protein)

† estimated from A_{280}

FIGURE 7

Elution of proteases from maize roots from CM-cellulose by a pH gradient and salt

40 ml of pH 4.4 supernatant (Fraction III, Table 11) containing 11 mg protein were prepared from 75 g of 7 day maize roots as described in Table 11. This fraction was loaded on to a CM-cellulose column ($2cm^2$ x 13.3cm) equilibrated with 20 mM sodium acetate pH 5.0. After the column had been washed with 90 ml of equilibrating buffer, it was eluted with a 400 ml gradient of 20 mM sodium acetate pH 5.0 to 6.0 containing 130 mM NaCl at the rate of 10 ml/h. At fraction 31, the pH of the buffer entering the column was 5.2. A_{280} of the column effluent was monitored and 6.3 ml fractions were analysed for proteinase, carboxypeptidase and aminopeptidase activities as described in Sections 2.5.4.1, 2.5.4.2, 2.5.4.3 and 2.5.4.5. Fractions 19-25 were pooled for protease A and fractions 34-37 were pooled for protease B.

——— A_{280}

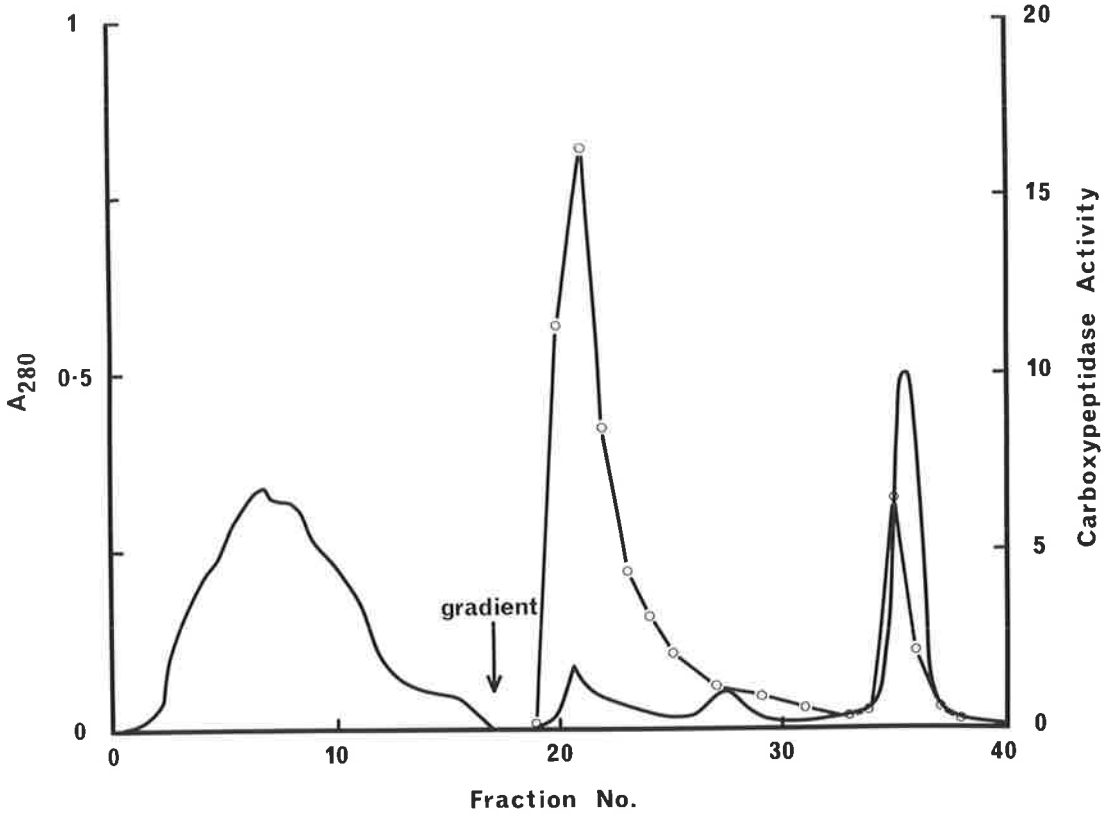
○——○ Carboxypeptidase activity, µmol alanine released/ml/h

□——□ Azocasein degrading activity, ΔA_{440}/ml/h

△——△ Haemoglobin degrading activity, µmol α-amino N released/ml/h

●——● Aminopeptidase activity, µmol *p*-nitroaniline released/ml/h

Fig 7


1
0·5
0
A_{280}
20
15
10
5
0
Carboxypeptidase Activity
gradient
0
10
20
30
40
Fraction No.

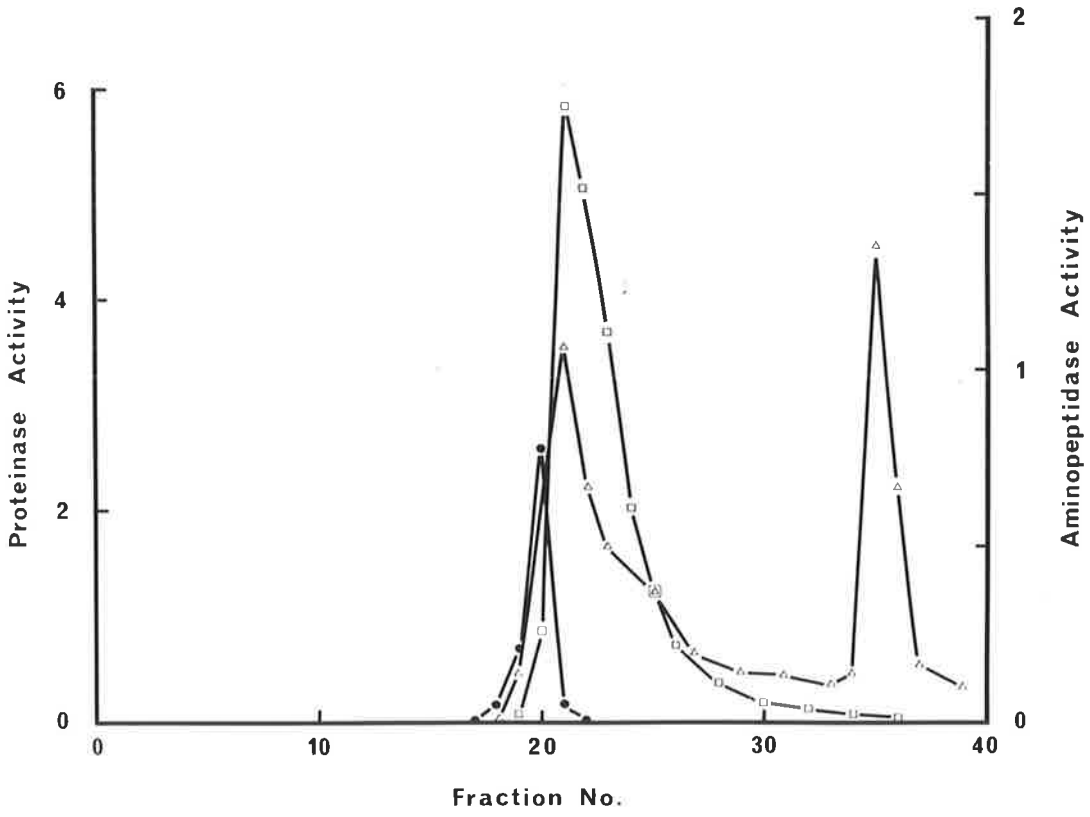

6
4
2
0
Proteinase Activity
2
1
0
Aminopeptidase Activity
0
10
20
30
40
Fraction No.

FIGURE 8

Rechromatography of proteases A and B from maize roots on CM-cellulose

A 27 ml of pH 4.4 supernatant (Fraction III, Table 11) containing 26 mg protein were prepared from 69 g of 7 day maize roots as described in Table 11. This fraction was loaded on a CM-cellulose column (1.32cm^2 x 6.8cm) equilibrated with 20 mM sodium acetate pH 5.0. After the column had been washed with 41 ml of equilibrating buffer, it was eluted with a 170 ml gradient of 20 mM sodium acetate pH 5.0 to 6.0 containing 130 mM NaCl at the rate of 10.5 ml/h. A_{280} of the column effluent was monitored and 4.6 ml fractions were analysed for proteinase and carboxypeptidase activities as described in Sections 2.5.4.1, 2.5.4.2 and 2.5.4.3. The fractions indicated by bars were pooled for rechromatography.

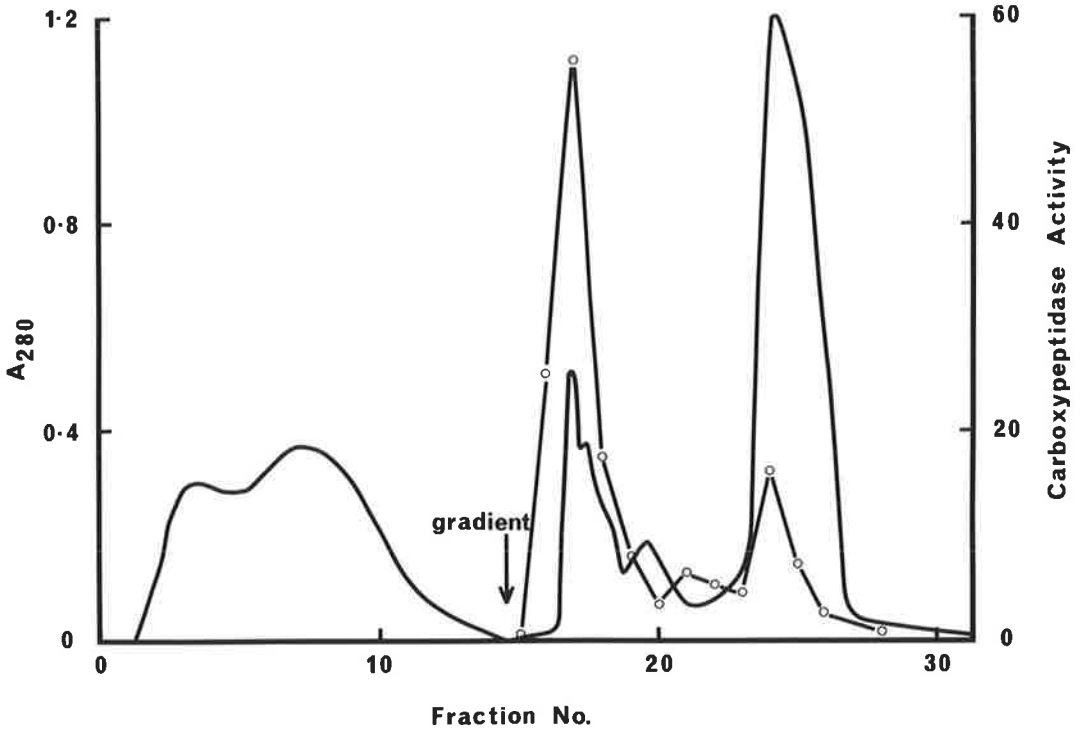
——— A_{280}

O——O Carboxypeptidase activity, µmol alanine released/ml/h

□——□ Azocasein degrading activity, Δ A_{440}/ml/h

△——△ Haemoglobin degrading activity, µmol α-amino N released/ml/h

Fig 8A


1·2
0·8
0·4
0
A_{280}
60
40
20
0
Carboxypeptidase Activity
gradient
0
10
20
30
Fraction No.

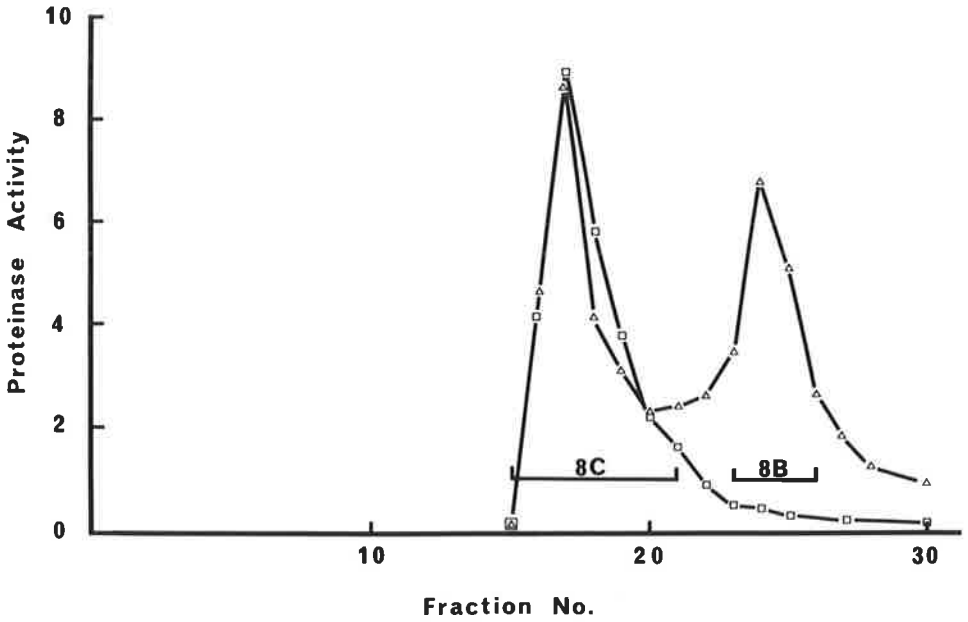

10
8
6
4
2
0
Proteinase Activity
8C
8B
10
20
30
Fraction No.

FIGURE 8

B Fractions 24-26 (protease B) from the column shown in A were dialysed against two changes of 20 mM sodium acetate pH 5.0 overnight at 4°C and loaded on to a CM-cellulose column (1.32cm^2 x 6.1cm) equilibrated in 20 mM sodium acetate pH 5.0. Elution conditions were as described in A except that the volume of the gradient was 160 ml and the fraction size was 4.1 ml.

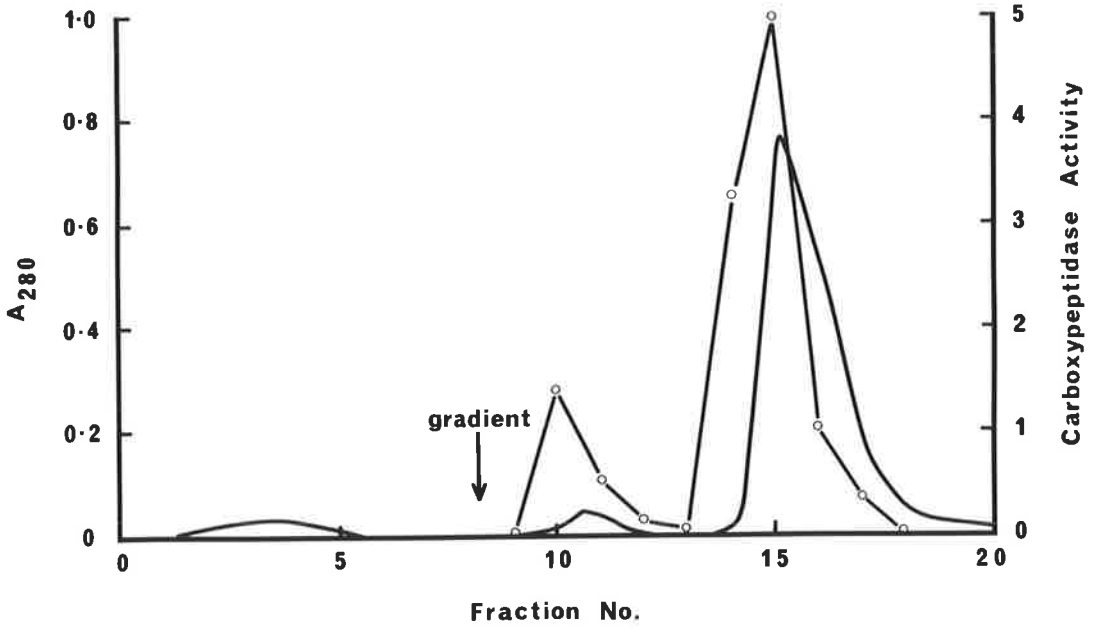
A_{280}

Carboxypeptidase activity, µmol alanine released/ml/h

Azocasein degrading activity, ΔA_{440}/ml/h

Haemoglobin degrading activity, µmol α-amino N released/ml/h

Fig 8B


1·0
0·8
0·6
0·4
0·2
0
A_{280}
gradient
5
4
3
2
1
0
Carboxypeptidase Activity
0
5
10
15
20
Fraction No.

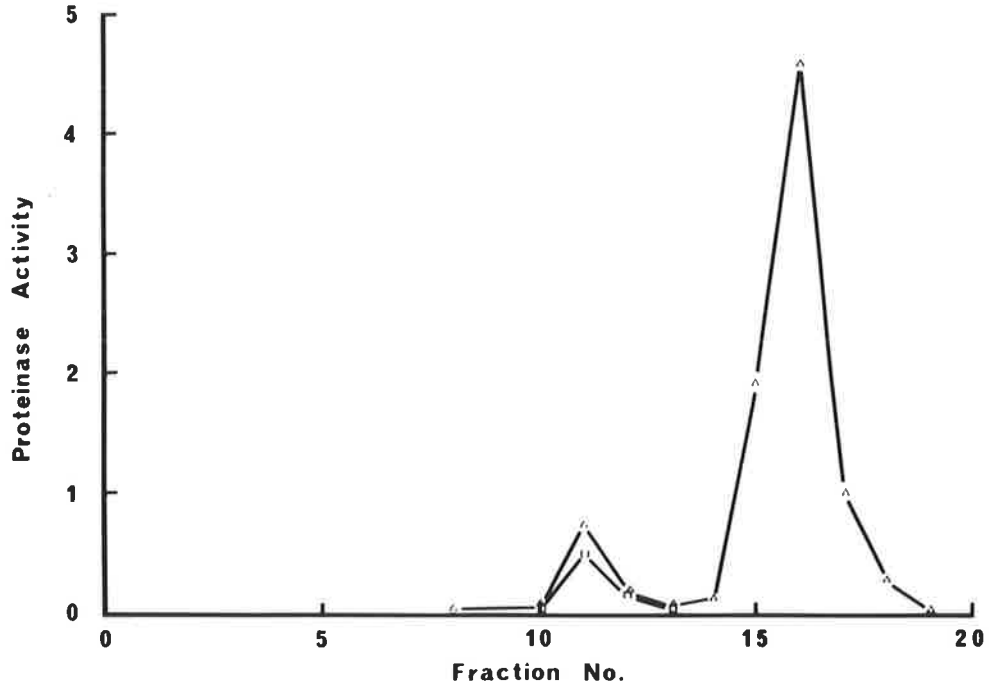

5
4
3
2
1
0
Proteinase Activity
0
5
10
15
20
Fraction No.

FIGURE 8

C Fractions 16-21 (protease A) from the column shown in A were dialysed overnight against two changes of 20 mM sodium acetate pH 5.0 at 4°C and loaded on to a CM-cellulose column (1.32cm^2 x 5.1cm) equilibrated in 20 mM sodium acetate pH 5.0. Elution conditions were as described in A except that the volume of the gradient was 160 ml and the fraction size was 4.1 ml.

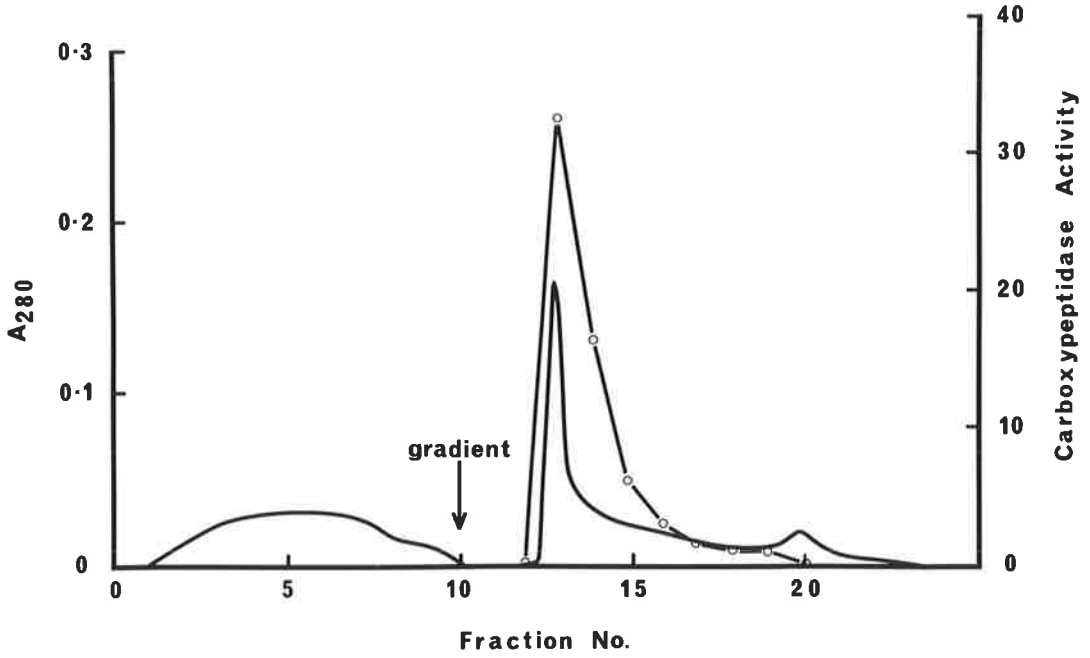
A_{280}

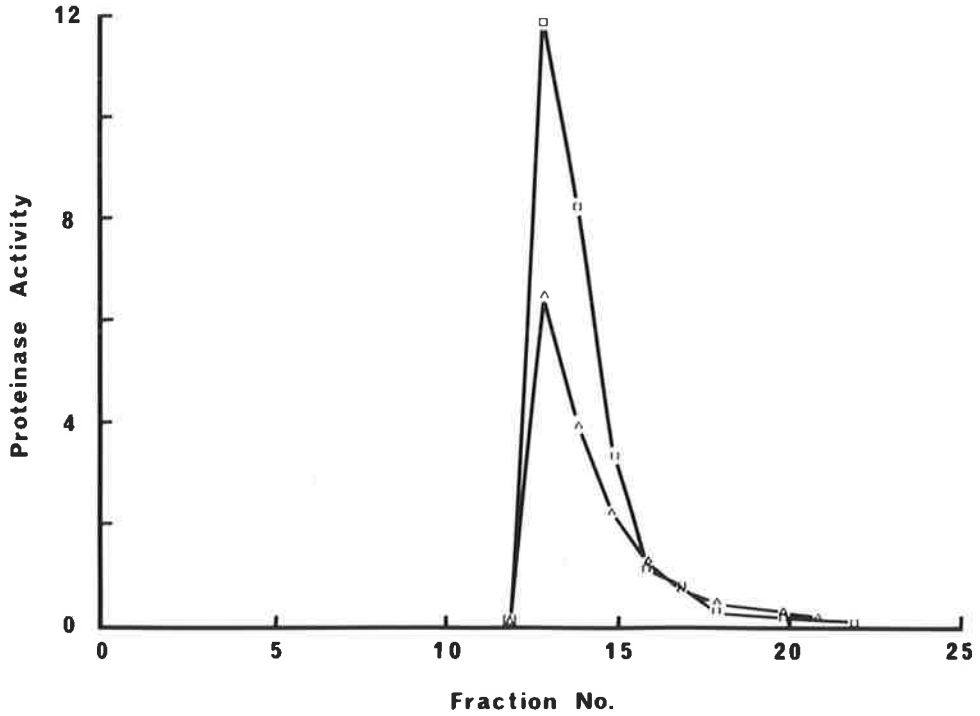
Carboxypeptidase activity, µmol alanine released/ml/h

Azocasein degrading activity, ΔA_{440}/ml/h

Haemoglobin degrading activity, µmol α-amino N released/ml/h

Fig 8C


0·3
0·2
0·1
0
A_{280}
gradient
40
30
20
10
0
Carboxypeptidase Activity
0
5
10
15
20
Fraction No.


12
8
4
0
Proteinase Activity
0
5
10
15
20
25
Fraction No.

like species on rechromatography of protease B (Figure 8B). Attempts to resolve azocasein degrading activity from haemoglobin degrading activity (protease A) and carboxypeptidase activity from haemoglobin degrading activity (protease B) by chromatography on longer CM-cellulose columns and eluting with salt concentrations less than 130 mM NaCl were unsuccessful.

The column shown in Figure 7 was used to prepare proteases A and B for subsequent studies.

3.4 Anion exchange chromatography of proteases from maize roots

Protease A from the CM-cellulose column shown in Figure 7 was further resolved on a DEAE-Sephadex column by elution with a gradient of 0 - 0.4 M NaCl in 20 mM sodium phosphate pH 6.0 (Figure 9). Most of the carboxypeptidase activity was separated from the proteinase activity, although some carboxypeptidase activity, subsequently separated on Sephadex G-100, was associated with the proteinase activity. The proteinase fraction which degrades azocasein and haemoglobin and inactivates nitrate reductase has been named proteinase A. The major carboxypeptidase fraction has been designated carboxypeptidase A while the minor fraction associated with proteinase A has been designated carboxypeptidase A'. While there was some variation in different preparations, generally carboxypeptidase A had the higher activity. Aminopeptidase activity was eluted just behind proteinase A.

All protease activities eluted at their original positions on rechromatography. When the column was eluted at pH 6.0 as described above, recovery of protease activities was 75-100%. When the column was equilibrated with 20 mM Tris-HCl pH 7.8 and eluted with a salt gradient in that buffer, the recovery of carboxypeptidase was only 8%, probably due to the instability of this enzyme at pH 7.8 (Figure 25) while 74% of the azocasein degrading activity was recovered. It was also found that on DEAE-Sephadex columns, nitrate reductase inactivating activity eluted

FIGURE 9

Fractionation of protease A from maize roots on DEAE-Sephadex

Protease A from the CM-cellulose column shown in Figure 7 was dialysed overnight against two changes of 20 mM sodium phosphate pH 6.0 at 4°C and applied to a DEAE-Sephadex column ($2cm^2$ x 4.1cm) equilibrated with 20 mM sodium phosphate pH 6.0. After the column had been washed with 56 ml of equilibrating buffer, it was eluted with a 140 ml gradient of 0 - 0.4 M NaCl in equilibrating buffer at the rate of 9 ml/h. A_{280} of the column effluent was monitored and 2.8 ml fractions were analysed for proteinase, carboxypeptidase and amino-peptidase activities as described in Sections 2.5.4.1, 2.5.4.2, 2.5.4.3 and 2.5.4.5.

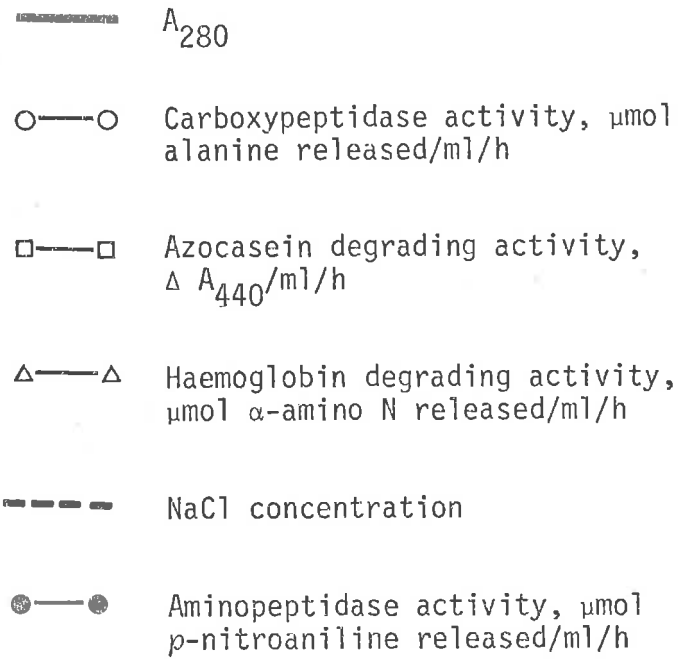
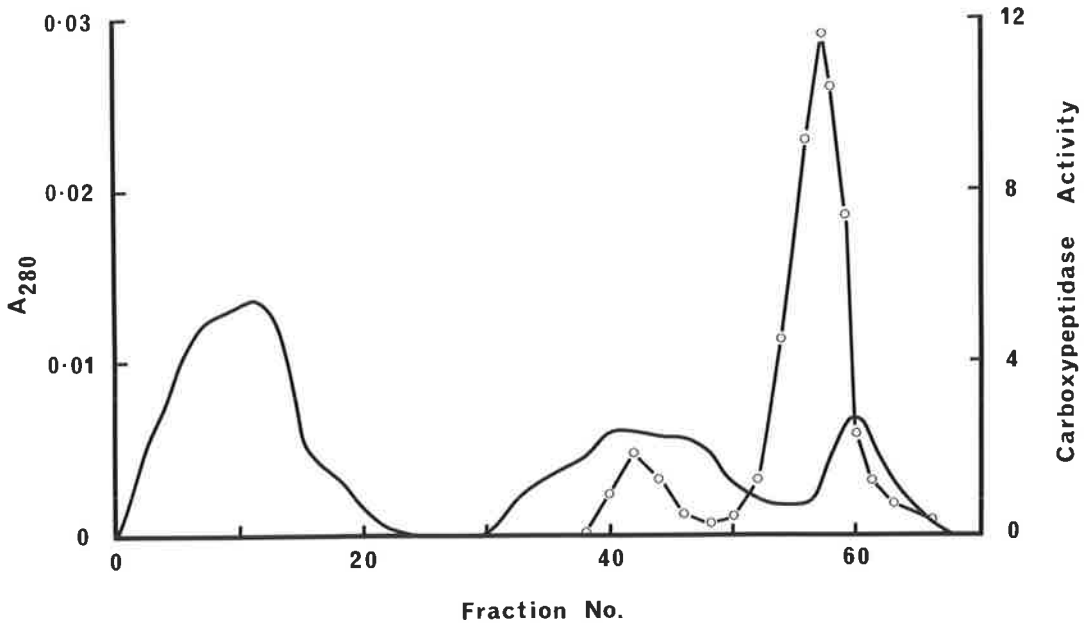
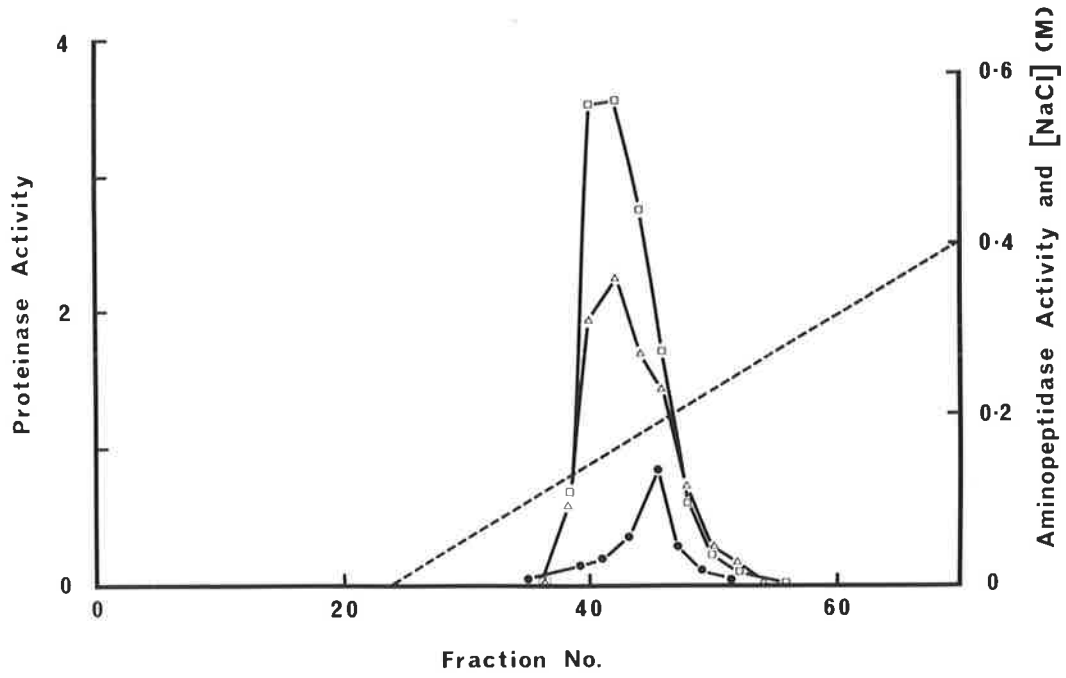


Fig 9


0·03
0·02
0·01
0
A_{280}
12
8
4
0
Carboxypeptidase Activity
0
20
40
60
Fraction No.


4
2
0
Proteinase Activity
0·6
0·4
0·2
0
Aminopeptidase Activity and [NaCl] (M)
0
20
40
60
Fraction No.

with the azocasein and haemoglobin degrading activities. Carboxypeptidase A was purified 15 fold by chromatography on DEAE-Sephadex while there was only a two fold increase in specific activity of proteinase A (Table 13).

Attempts to use a DEAE-Sephadex column to further resolve protease B which was isolated on a CM-cellulose column (Figure 7) were unsuccessful. Both the carboxypeptidase and haemoglobin degrading activities were dispersed throughout the gradient and poorly separated.

3.5 Gel filtration of proteases from maize roots

When the proteinase fraction from the DEAE-Sephadex column shown in Figure 9 was applied to a Sephadex G-100 column, the associated carboxypeptidase A' eluted ahead of proteinase A. Azocasein and haemoglobin degrading activities were not resolved and showed an identical elution pattern to that of the nitrate reductase inactivating activity (Figure 10). Carboxypeptidase A (Figure 9) had the same elution profile on Sephadex G-100 as carboxypeptidase A'. Over the range 0 - 0.1 A_{280}, no absorbing material was detected during elution of this column.

After calibration of the column with Blue dextran (completely excluded), hexokinase (102 000), bovine serum albumin (69 000) and cytochrome c (12 400) as shown in Figure 11, molecular weights for the protease species were determined as follows: carboxypeptidase A and A', 97 000; proteinase A (azocasein and haemoglobin degrading and nitrate reductase inactivating activities) 52 000. The omission of salt from the eluting buffer did not result in any appreciable effect on the resolution.

No aminopeptidase activity was detected in the experiment shown in Figure 10, probably due to the low level of activity (0.92 µmol *p*-nitroaniline released/h) applied to the column. When a sample of 35-70%

TABLE 13

Partial purification of protease A from maize roots on a DEAE-Sephadex column

A sample of protease A was prepared from 213 g of 14 day old maize roots and isolated on a CM-cellulose column as shown in Figure 7. The protease A sample was then chromatographed on a DEAE-Sephadex column as shown in Figure 9. Fractions corresponding to carboxypeptidase A and proteinase A (Section 3.4) were pooled. Protease activities and protein content were determined as described in Sections 2.5.4.1, 2.5.4.2, 2.5.4.3, 2.5.4.4 and 2.7.3.

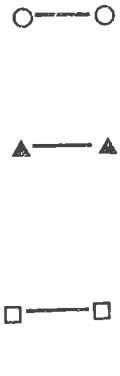
Fraction	Volume (ml)	Protein (µg)	Azocasein degradation (ΔA_{440}/h)	Haemoglobin degradation (µmol α-amino N released/h)	Carboxypeptidase (µmol alanine released/h)	Nitrate reductase inactivation (units nitrate reductase inactivated/h x 10^{-3})
Protease A from CM-cellulose	9.6	800	144 (180)*	45 (56)*	301 (376)*	109 (136)*
Proteinase A and carboxy-peptidase A'	26	410	161 (393)*	52 (127)*	50 (125)*	140 (342)*
Carboxypeptidase A	48	40	0	0	220 (5 500)*	0

* figures in parentheses denote specific activities (units/mg protein)

FIGURE 10

Gel filtration of protease A from maize roots on Sephadex G-100

Fractions 38-52 from the DEAE-Sephadex column shown in Figure 9 were concentrated to 2 ml by precipitation with 90% saturation $(NH_4)_2SO_4$ as described in Table 8 and resuspended in 2 ml of 20 mM sodium phosphate pH 6.0 containing 0.25 M NaCl. The enzyme solution was applied to a Sephadex G-100 column ($1.43 cm^2$ x 1.42m, 203ml) equilibrated with 20 mM sodium phosphate pH 6.0 containing 0.25 M NaCl. 2.8 ml fractions were collected at the rate of 4.2 ml/h and analysed for proteinase, carboxypeptidase and nitrate reductase inactivating activities as described in Sections 2.5.4.1, 2.5.4.2, 2.5.4.3 and 2.5.4.4. No absorbance was detected on a scale of 0 - 0.1 A_{280}.



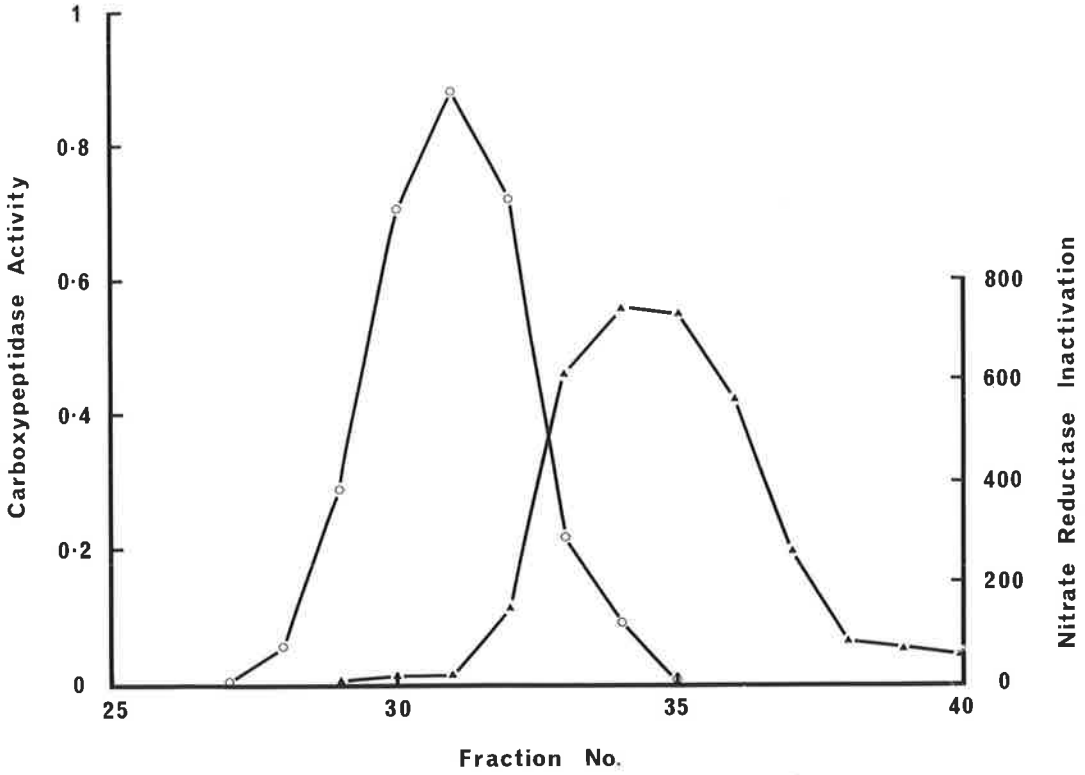
○——○ Carboxypeptidase activity, µmol alanine released/ml/h

▲——▲ Nitrate reductase inactivating activity, units nitrate reductase inactivated/ml/h

□——□ Azocasein degrading activity, ΔA_{440}/ml/h

△——△ Haemoglobin degrading activity, µmol α-amino N released/ml/h

Fig 10


Carboxypeptidase Activity
1
0·8
0·6
0·4
0·2
0
800
600
400
200
0
Nitrate Reductase Inactivation
25
30
35
40
Fraction No.

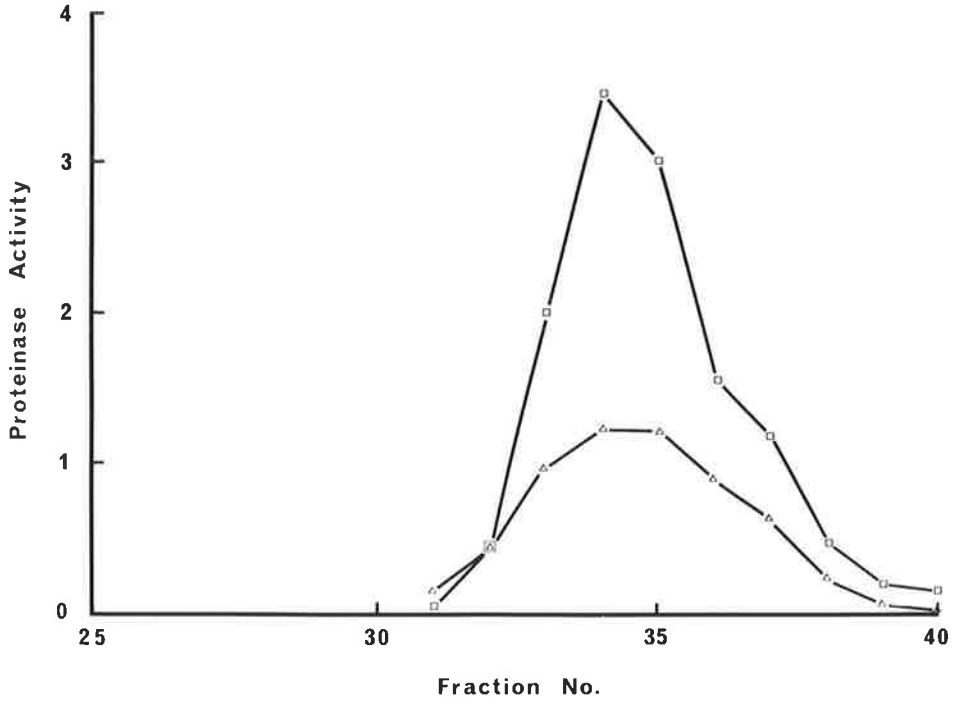
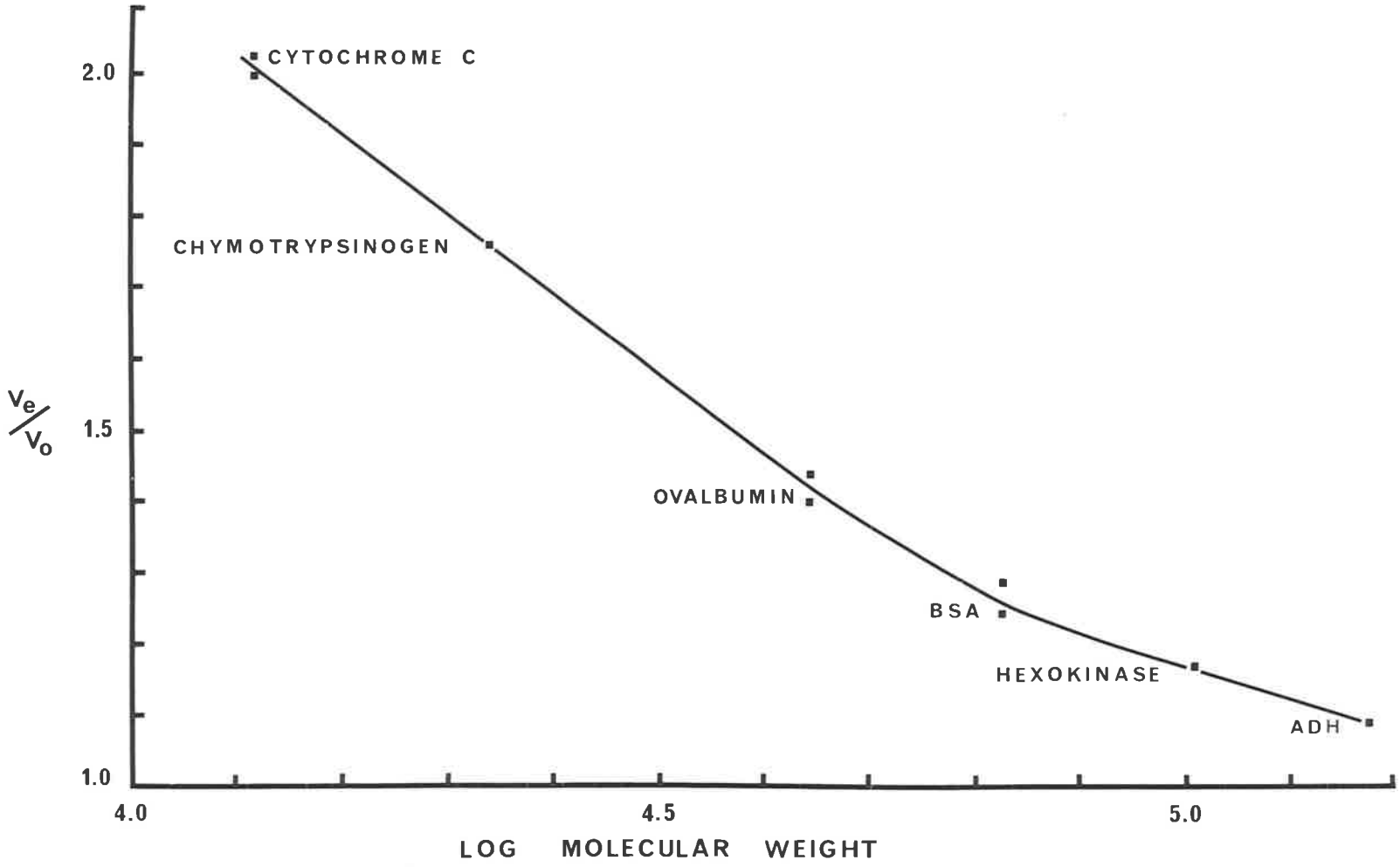

Proteinase Activity
4
3
2
1
0
25
30
35
40
Fraction No.

FIGURE 11

Calibration of a Sephadex G-100 column

Blue dextran and the proteins shown (1 - 2 mg each) were applied singly or in pairs differing markedly in molecular weight to a Sephadex G-100 column ($1.43cm^2$ x 1.42m, 203ml) equilibrated with 20 mM sodium phosphate pH 6.0 containing 0.25 M NaCl. 2.8 ml fractions were collected at the rate of 4.2 ml/h. The proteins and Blue dextran were detected in the column effluent by their absorbance at 280 nm except hexokinase which was assayed as described in Section 2.5.4.6.

Fig 11


CYTOCHROME C
CHYMOTRYPSINOGEN
OVALBUMIN
BSA
HEXOKINASE
ADH
2.0
1.5
1.0
Ve/Vo
4.0
4.5
5.0
LOG MOLECULAR WEIGHT

$(NH_4)_2SO_4$ ppt. (Fraction II, Table 11) was applied to the Sephadex G-100 column, aminopeptidase activity was detected in the eluate and the enzyme was estimated to have a molecular weight of 62 000.

Attempts to further characterise protease B from the CM-cellulose column shown in Figure 7 on a Sephadex G-100 column were unsuccessful because of losses of haemoglobin degrading and carboxypeptidase activities. When a Biogel A-0.5m column was used with 1 mM cysteine in the equilibrating buffer (20 mM sodium phosphate pH 6.0 containing 0.1 M NaCl) to stabilise the haemoglobin degrading activity (Section 3.10.4), some resolution of the protease B components was achieved (Figure 12). Carboxypeptidase activity was eluted in two fractions designated BI (first eluted) and BII (second eluted). Haemoglobin degrading activity was eluted in a broad fraction with maximal activity just after carboxypeptidase BII and nitrate reductase inactivating activity was eluted in a fraction between the two carboxypeptidases. A low molecular weight fraction which inactivates nitrate reductase shown in Figure 12 was also observed by Wallace (1974) and thought to contain phenolic compounds.

The following proteins were used to calibrate the column: alcohol dehydrogenase (150 000), bovine serum albumin (69 000), ovalbumin (44 000), pepsin (35 000), carbonic anhydrase (31 000), pancreatic carboxypeptidase A (34 000), α-chymotrypsinogen (25 700), soybean trypsin inhibitor (21 500) and myoglobin (17 000). However proteins of molecular weights below 35 000, particularly the protease carboxypeptidase A and the protease precursor α-chymotrypsinogen were retarded. All the components of protease B appear to have molecular weights below 44 000 but in view of the retardation of standard proteins, no estimates of the molecular weights of protease B components have been made.

FIGURE 12

Gel filtration of protease B from maize roots on Biogel A-0.5m

2.8 ml of protease B containing 0.63 mg protein were dialysed for 12 h against two changes of 20 mM sodium phosphate pH 6.0 containing 1 mM cysteine and 0.1 M NaCl at 4°C. The sample was applied to a Biogel A-0.5m column (1.86cm^2 x 105cm, 195ml) equilibrated with 20 mM sodium phosphate pH 6.0 containing 1 mM cysteine and 0.1 M NaCl and eluted at the rate of 23 ml/h. A_{280} of the column effluent was monitored and 3 ml fractions were analysed for carboxypeptidase and nitrate reductase inactivating activities as described in Sections 2.5.4.3 and 2.5.4.4. Haemoglobin degrading activity was determined by the modified, more sensitive procedure described in Section 2.5.4.2b.

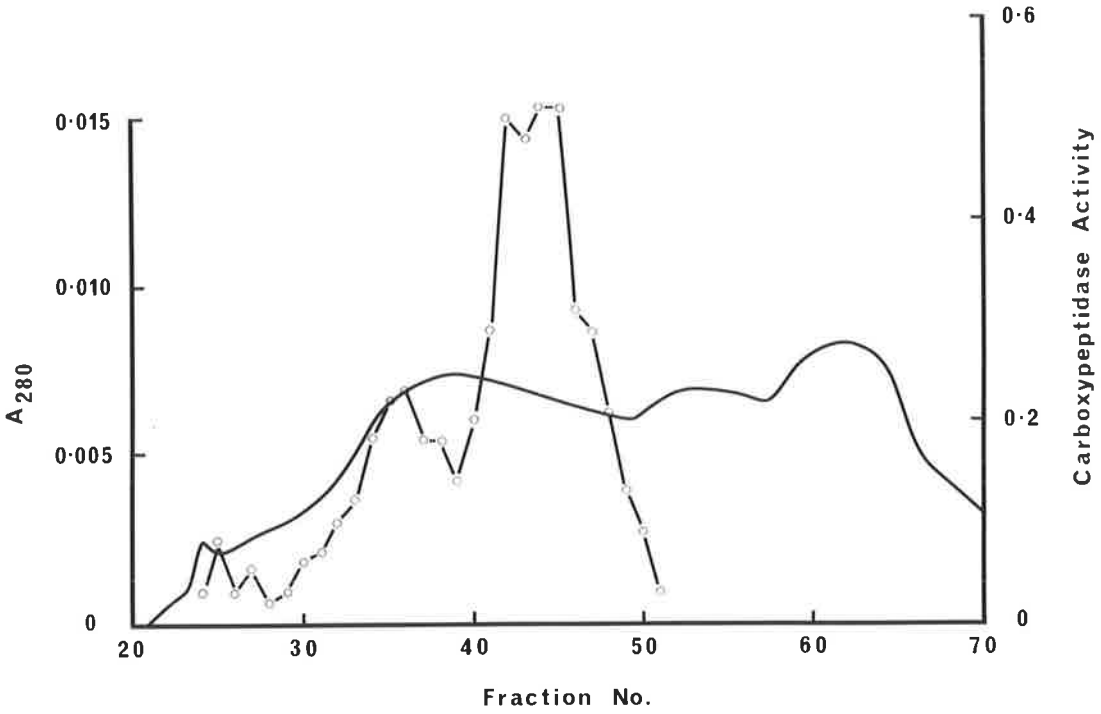
—— A_{280}

○——○ Carboxypeptidase activity, µmol alanine released/ml/h

△——△ Haemoglobin degrading activity, µmol α-amino N released/ml/h

▲——▲ Nitrate reductase inactivating activity, units nitrate reductase inactivated/ml/h

Fig 12


0·015
0·010
0·005
0
A280
0·6
0·4
0·2
0
Carboxypeptidase Activity
20
30
40
50
60
70
Fraction No.

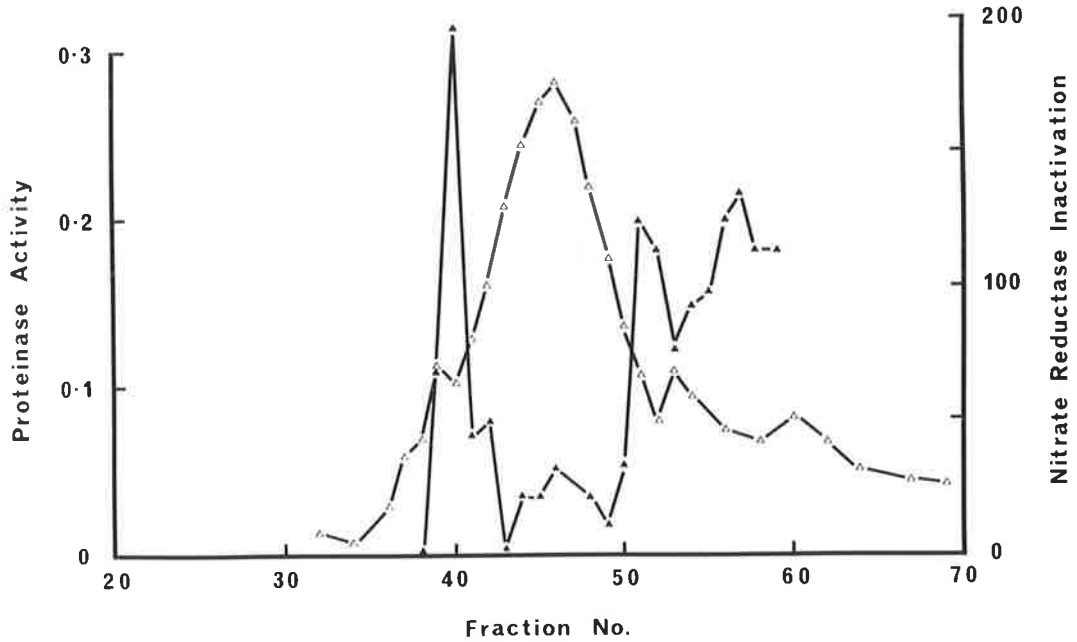
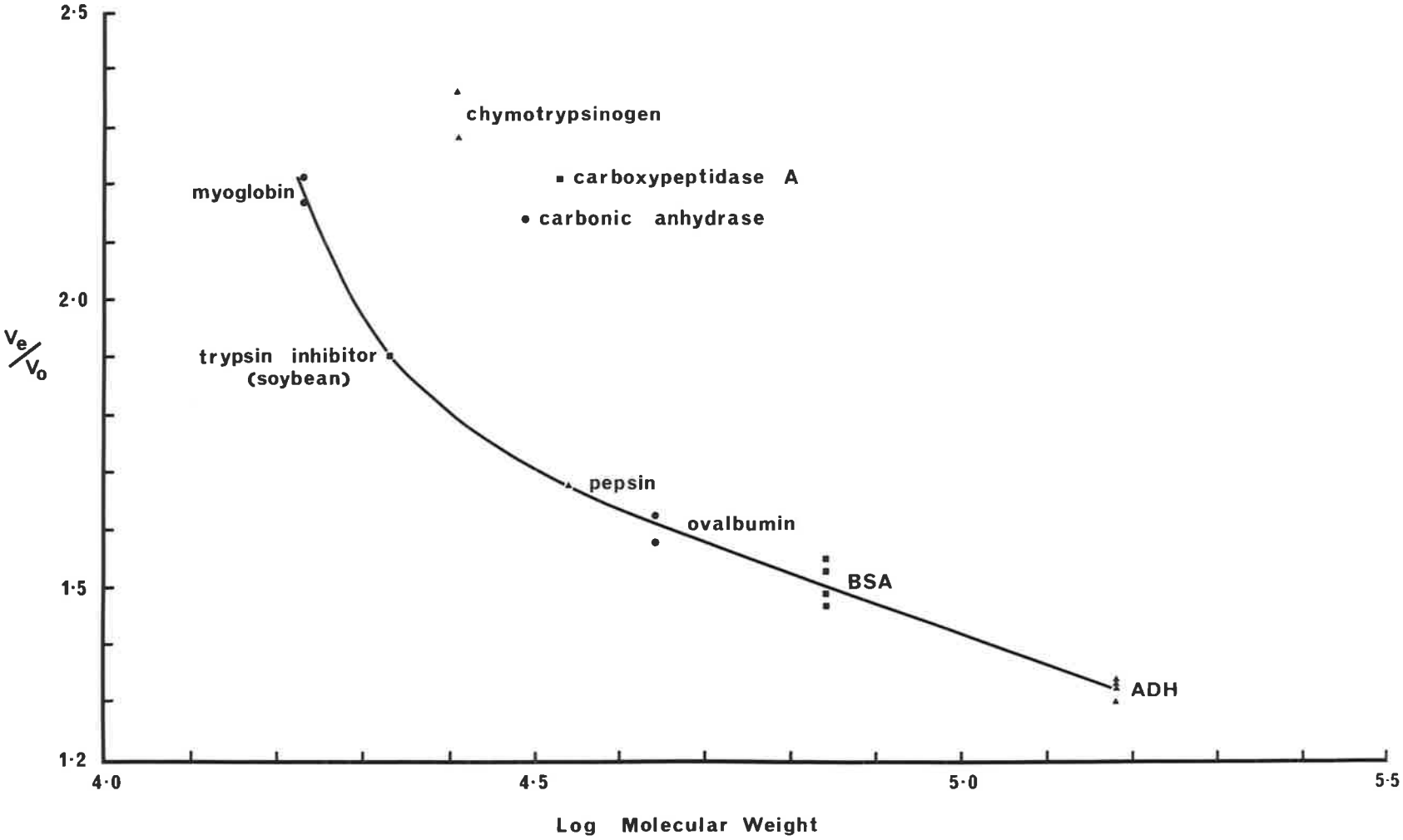

0·3
0·2
0·1
0
Proteinase Activity
200
100
0
Nitrate Reductase Inactivation
20
30
40
50
60
70
Fraction No.

FIGURE 13

Calibration of a Biogel A-0.5m column

Blue dextran (0.5 mg) and the proteins shown (2 mg each) were applied in pairs differing markedly in molecular weight to a Biogel A-0.5m column (1.86cm^2 x 105cm, 195ml) equilibrated with 20 mM sodium phosphate pH 6.0 containing 0.1 M NaCl. 3 ml fractions were collected at the rate of 23 ml/h. The proteins and Blue dextran were detected in the column effluent by their absorbance at 280 nm.

Fig 13


2·5
2·0
1·5
1·2
V_e/V_o
chymotrypsinogen
carboxypeptidase A
myoglobin
carbonic anhydrase
trypsin inhibitor
(soybean)
pepsin
ovalbumin
BSA
ADH
4·0
4·5
5·0
5·5
Log Molecular Weight

3.6 Hydroxylapatite chromatography of proteases from maize roots

Hydroxylapatite chromatography has been used successfully to separate yeast proteinases A and B (Saheki and Holzer, 1974) and it was tested for its possible use with the maize root proteases. A sample of pH 4.4 supernatant (Fraction III, Table 11) was applied to a hydroxylapatite column equilibrated with 5 mM potassium phosphate pH 7.0 and eluted with a gradient of 5 to 300 mM potassium phosphate pH 7.0. Some separation of carboxypeptidase activity from the proteinase activities occurred but the latter were not resolved (Figure 14). Use of a more shallow gradient on a similar column did not achieve any resolution of the proteinases.

Protease B from a CM-celluiose column (Figure 7) was loaded on to a hydroxylapatite column and eluted with a gradient of 10 - 400 mM sodium phosphate over 16 column volumes. The haemoglobin degrading and carboxypeptidase activities were not separated and only about 25% of both protease activities was recovered.

3.7 Affinity chromatography of proteases from maize roots

Affinity chromatography uses the substrate specificity of an enzyme as the basis for separation (Lowe and Dean, 1974). To separate the haemoglobin and azocasein degrading activities of maize root, a haemoglobin affinity column, which had been successfully used to isolate other plant proteinases (Frith *et al*, 1978c; Drivdahl and Thimann, 1977; Burger, 1973) was tested. In the current study, haemoglobin was coupled to CNBr activated Sepharose supplied by Pharmacia. When a protease sample was either applied to a haemoglobin-Sepharose column or mixed with the same material for up to 12 h, no significant binding to the haemoglobin ligand occurred. The effect of coupling Sepharose to haemoglobin in solutions of high (18 mg/ml Sepharose) and low (1.4 mg/ml Sepharose) concentration was tested; in association with the latter, a partial prehydrolysis of

FIGURE 14

Elution of proteases from maize roots from a hydroxylapatite column

5 ml of pH 4.4 supernatant (Fraction III, Table 11) containing 2.8 mg protein were dialysed overnight against two changes of 5 mM potassium phosphate pH 7.0 at 4°C. The sample was loaded on to a hydroxylapatite column (0.79cm^2 x 8.5cm) equilibrated with 5 mM potassium phosphate pH 7.0. The column was washed with 20 ml of equilibrating buffer and eluted at the rate of 8 ml/h with a gradient of 5 to 300 mM potassium phosphate pH 7.0 starting at fraction 11. A_{280} of the column effluent was monitored and 2.8 ml fractions were analysed for proteinase and carboxypeptidase activities as described in Sections 2.5.4.1, 2.5.4.2 and 2.5.4.3.

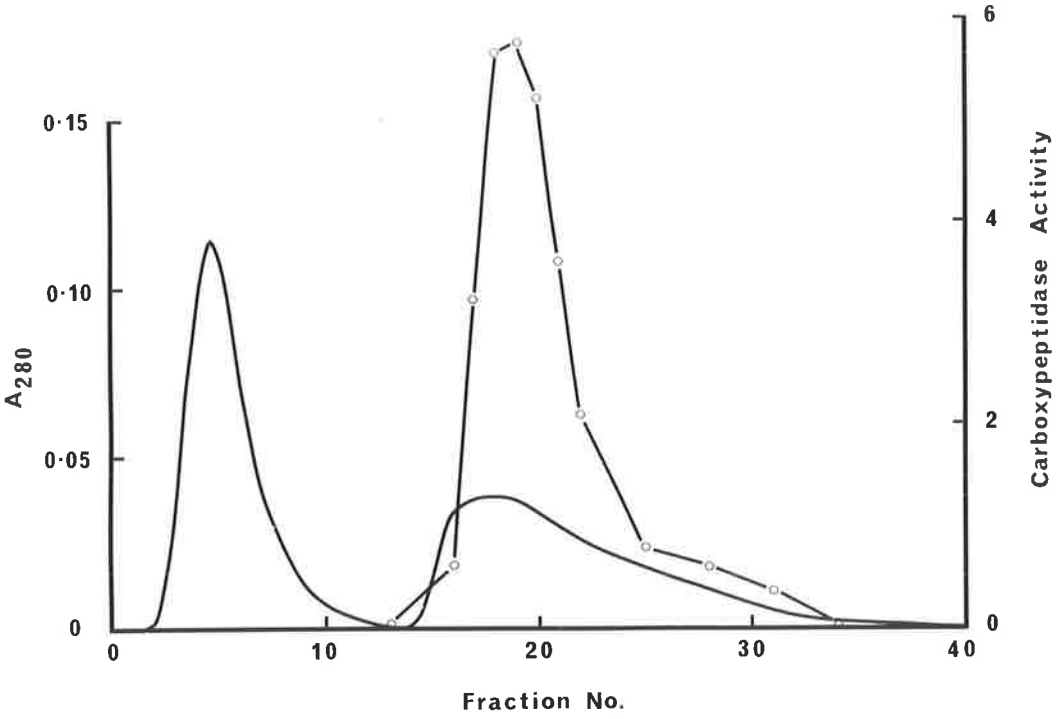
A_{280}

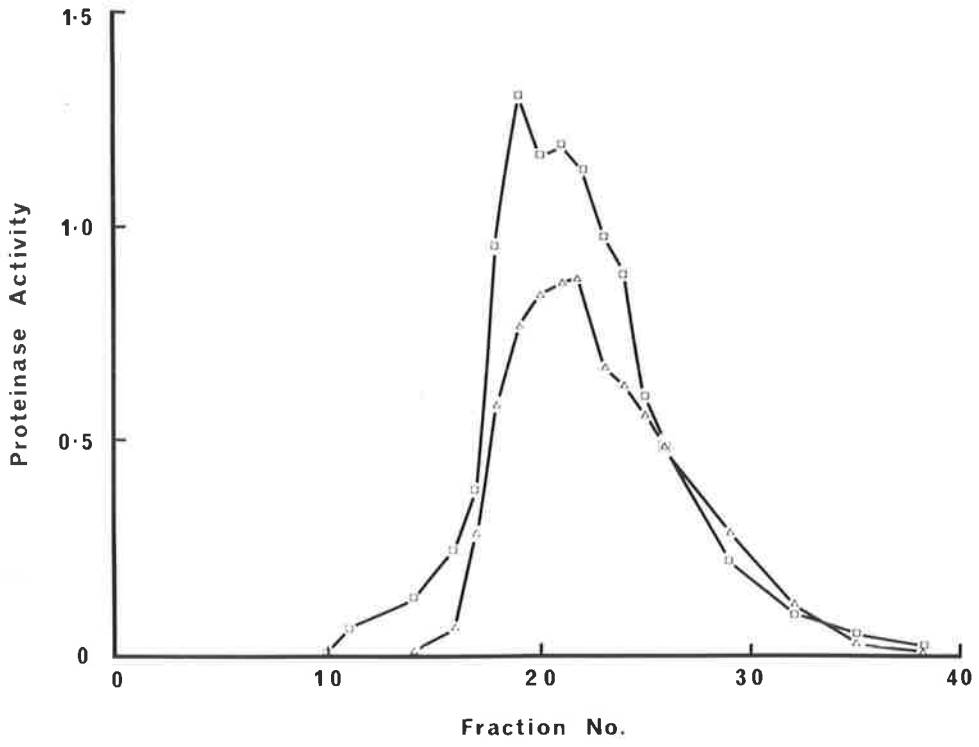
Carboxypeptidase activity, µmol alanine released/ml/h

Azocasein degrading activity, ΔA_{440}/ml/h

Haemoglobin degrading activity, µmol α-amino N released/ml/h

Fig 14


0·15
0·10
0·05
0
A_{280}
6
4
2
0
Carboxypeptidase Activity
0
10
20
30
40
Fraction No.


1·5
1·0
0·5
0
Proteinase Activity
0
10
20
30
40
Fraction No.

the CNBr activated Sepharose was performed as recommended by Pharmacia in an attempt to reduce the number of coupling sites for haemoglobin. The equilibrating buffer was usually 50 mM sodium acetate pH 4.0 but 50 mM sodium acetate pH 5.5 was also tested. One attempt to bind maize endosperm proteases to haemoglobin-Sepharose was also unsuccessful.

Preston (1978) has isolated a haemoglobin degrading enzyme from extracts of germinating wheat with a phenylalanine affinity column. A wheat seedling extract was prepared as described by Preston and Kruger (1976) and used to prepare a 35-70% $(NH_4)_2SO_4$ ppt. which was dialysed against 25 mM sodium acetate pH 4.0. When a 2 ml sample of this fraction containing 9 mg protein was applied to a phenylalanine affinity column prepared as described by Preston (1978), approximately two thirds of both the haemoglobin degrading and carboxypeptidase activities appeared to be adsorbed to the column (Figure 15). A gradient of 25 - 300 mM sodium acetate pH 4.0 eluted the haemoglobin degrading activity ahead of the carboxypeptidase activity but the two activities showed some overlap (Figure 15); one third of the total amount of each activity loaded on to the column was eluted. A similar result was obtained when 1 ml of dialysed 35-70% $(NH_4)_2SO_4$ ppt. of the wheat extract was loaded on to the column. Since the proportions of activity not adsorbed to the column were similar when either 1 or 2 ml of 35-70% $(NH_4)_2SO_4$ ppt. were loaded on to the column, it is unlikely that the column was overloaded by the 2 ml sample. Since the $(NH_4)_2SO_4$ ppt. was brown, it is possible that phenolic compounds attached to the proteins (Section 3.1.2) prevented their adsorption on to the aromatic phenylalanine ligand.

The phenylalanine affinity column was tested for its ability to resolve the components of maize root protease A. All the activity applied to the column was adsorbed; elution of the column with a gradient of 25 to 300 mM sodium acetate pH 4.0 gave two fractions of proteinase activity,

FIGURE 15

Elution of proteases from wheat seedlings from a phenylalanine affinity column

Wheat seedlings germinated for 4 days were freeze dried and then extracted in a cold mortar and pestle with 5 ml/g fr wt of 25 mM sodium acetate pH 4.0. The extract was centrifuged for 15 min at 18 000 *g* at 2°C and a 35-70% $(NH_4)_2SO_4$ ppt. prepared from the supernatant fraction as described in Section 2.5.2. The precipitate was dialysed overnight against two changes of 25 mM sodium acetate pH 4.0. 2 ml, containing 9 mg protein, were loaded on to a phenylalanine affinity column ($1.54cm^2$ x 4.2cm) equilibrated with 25 mM sodium acetate pH 4.0; preparation of the column is described in Section 2.6.5. After the column had been washed with 45 ml of equilibration buffer, it was eluted with a 120 ml gradient of 25 - 300 mM sodium acetate pH 4.0 at the rate of 10 ml/h. A_{280} of the column effluent was monitored and 3 ml fractions were analysed for proteinase and carboxypeptidase activities as described in Sections 2.5.4.1, 2.5.4.2 and 2.5.4.3.

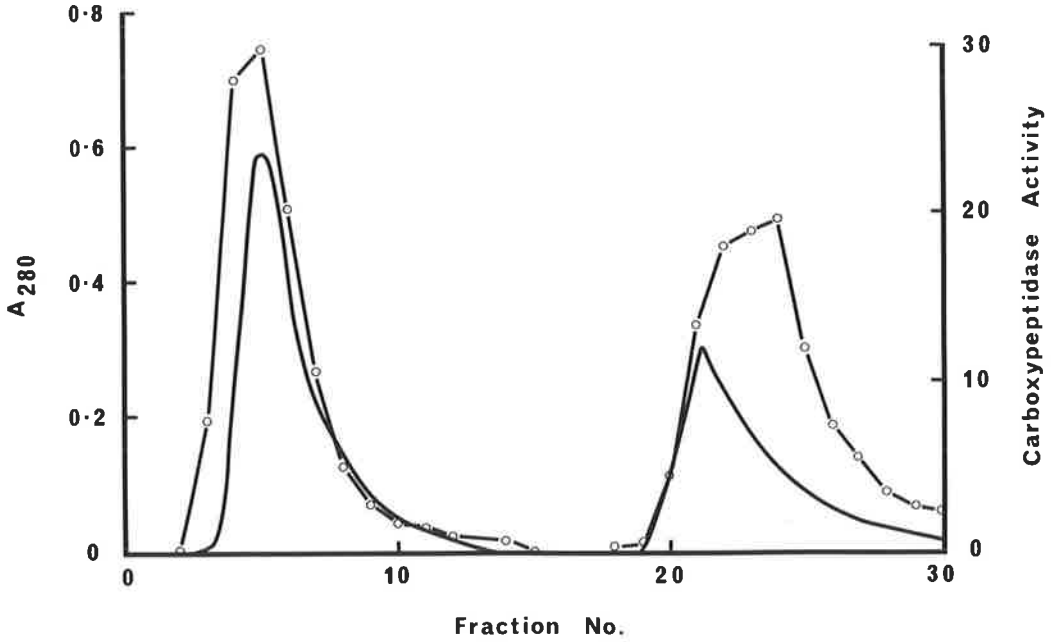
A_{280}

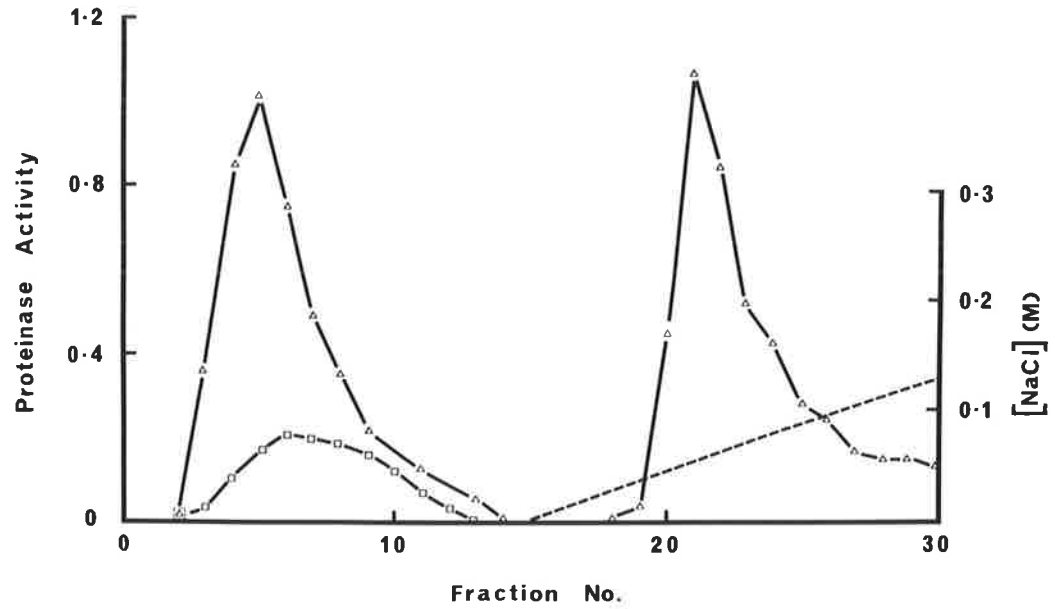
Carboxypeptidase activity, µmol alanine released/ml/h

Azocasein degrading activity, ΔA_{440}/ml/h

Haemoglobin degrading activity, µmol α-amino N released/ml/h

Fig 15


0·8
0·6
0·4
0·2
0
A_{280}
30
20
10
0
Carboxypeptidase Activity
0
10
20
30
Fraction No.


1·2
0·8
0·4
0
Proteinase Activity
0·3
0·2
0·1
[NaCl] (M)
0
10
20
30
Fraction No.

both containing carboxypeptidase activity (Figure 16). The ratios of azocasein degrading, haemoglobin degrading and nitrate reductase inactivating activities were approximately the same in both fractions; however, most of the carboxypeptidase activity was associated with the first proteinase fraction while the greater portion of the proteinase activity was in the second fraction. An apparent slight difference in the elution profiles of the azocasein and haemoglobin degrading activities observed in Figure 16 was not reproducible. Recoveries of activities were: azocasein degrading activity, 92%; haemoglobin degrading activity, 100%; carboxypeptidase, 64%. When the second proteinase fraction from the affinity column was rechromatographed on the same column, it was eluted at the same position as shown in Figure 16. As this column did not separate the azocasein degrading, haemoglobin degrading and nitrate reductase inactivating activities of proteinase A, its use was not further investigated.

3.8 Properties of proteinase A from maize roots

3.8.1 Introduction

Proteinase A refers to the endopeptidase component of the protease A fraction initially isolated on a CM-cellulose column (Figure 7). It was separated from associated carboxypeptidase activity by chromatography on DEAE-Sephadex (Figure 9) and gel filtration (Figure 10). Proteinase A degrades azocasein and haemoglobin and inactivates nitrate reductase. It has a molecular weight of 52 000 (Section 3.5).

3.8.2 pH optimum of proteinase A

The activity of proteinase A on azocasein increases with pH reaching a maximum at approximately 9.0 (Figure 17A). Trypsin, which has a pH optimum of 7.6 when acting on casein (Hata *et al*, 1967b), also has an

FIGURE 16

Elution of protease A from maize roots from a phenylalanine affinity column

3 ml of protease A containing 0.19 mg of protein were dialysed overnight against two changes of 25 mM sodium acetate pH 4.0 at 4°C. It was then loaded on the phenylalanine affinity column described in Figure 15 and eluted under the same conditions except that the rate of elution was 12 ml/h. A_{280} of the column effluent was monitored and 3 ml fractions were analysed for proteinase, carboxypeptidase and nitrate reductase inactivating activities as described in Sections 2.5.4.1, 2.5.4.2, 2.5.4.3 and 2.5.4.4.

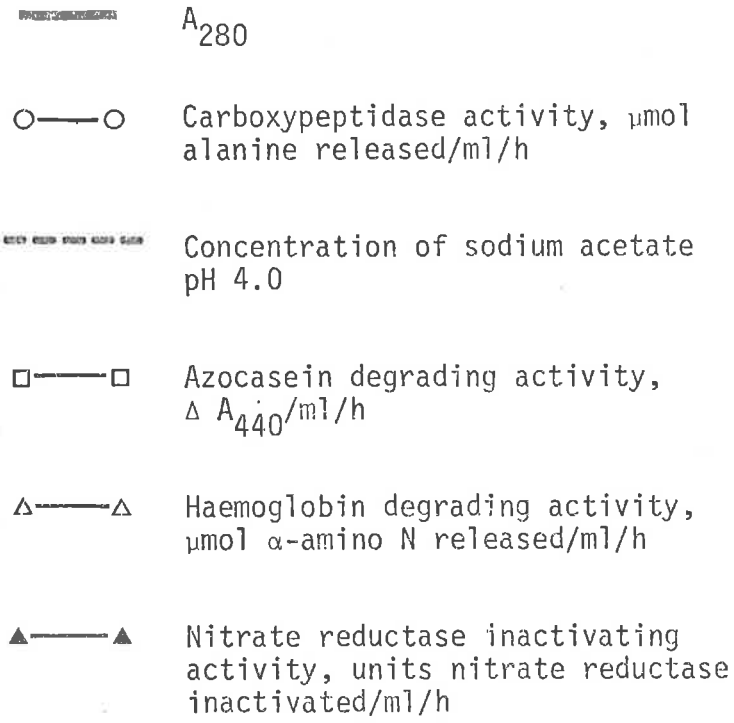
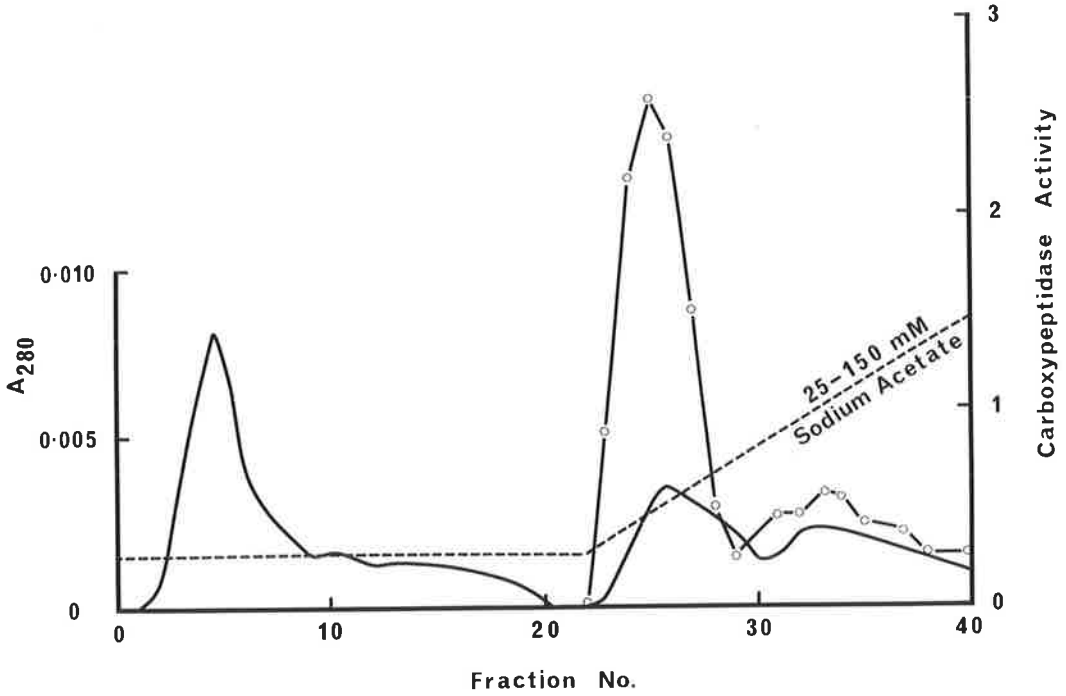


Fig 16


0·010
0·005
0
A_{280}
25–150 mM
Sodium Acetate
3
2
1
0
Carboxypeptidase Activity
0
10
20
30
40
Fraction No.

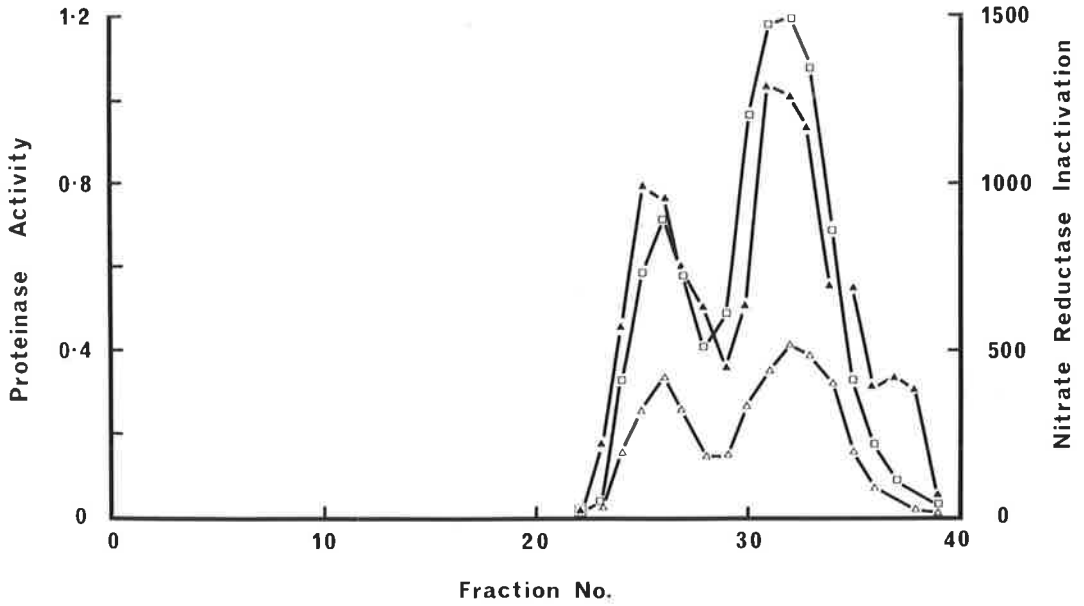

1·2
0·8
0·4
0
Proteinase Activity
1500
1000
500
0
Nitrate Reductase Inactivation
0
10
20
30
40
Fraction No.

FIGURE 17

Effect of pH on the digestion of azocasein by proteinase A from maize roots and by trypsin

A Samples of protease A (0.63 mg protein/ml) were incubated with 0.3 ml of 10 mg/ml azocasein and 0.5 ml of either sodium acetate, sodium phosphate, Tris-HCl or glycine-NaOH (each 0.2 M) at the pH values shown in a total volume of 1 ml for 2 h at 37°C. The remainder of the azocasein degradation assay procedure was then performed as described in Section 2.5.4.1. 0.2 ml of 20 mM sodium acetate pH 5.0 was incubated under the same conditions to determine non-enzymic hydrolysis of azocasein.

B 3.8 µg of trypsin was assayed as described in A.

□——□ sodium acetate

○——○ sodium phosphate

△——△ Tris-HCl

●——● glycine-NaOH

Fig 17A

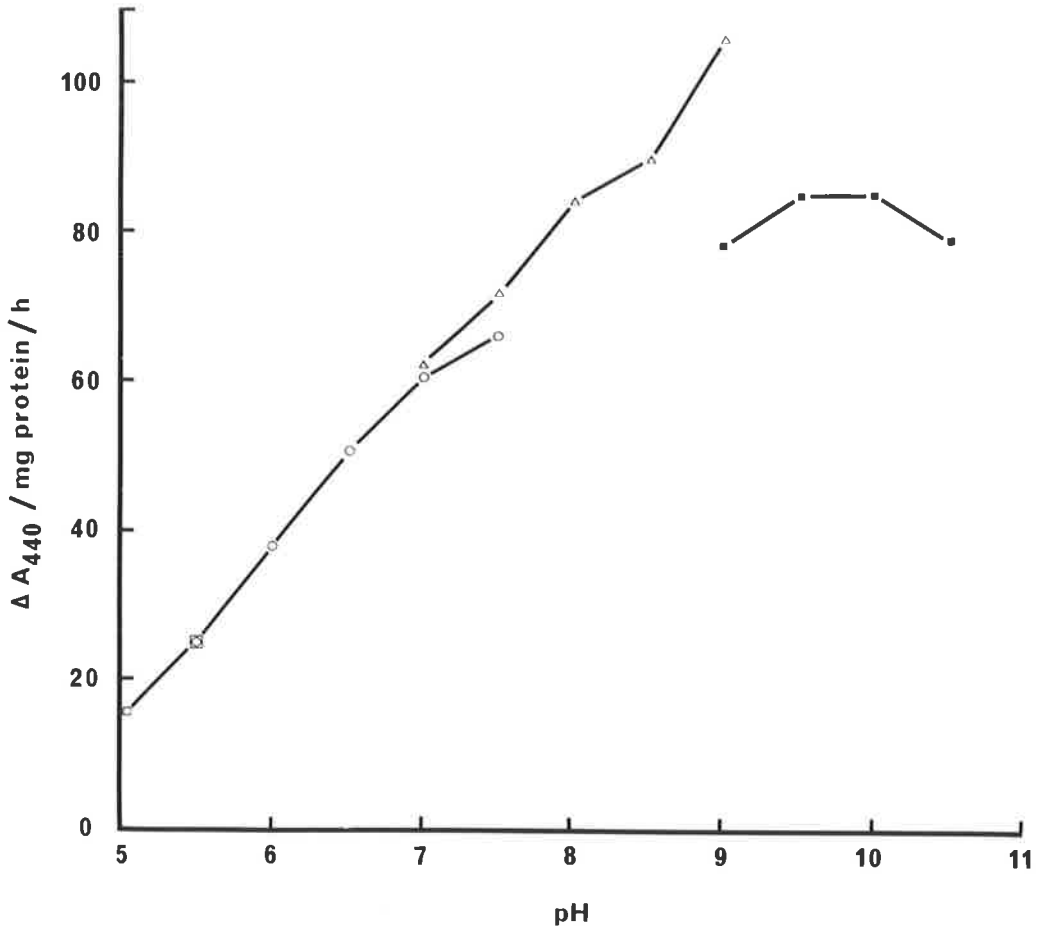
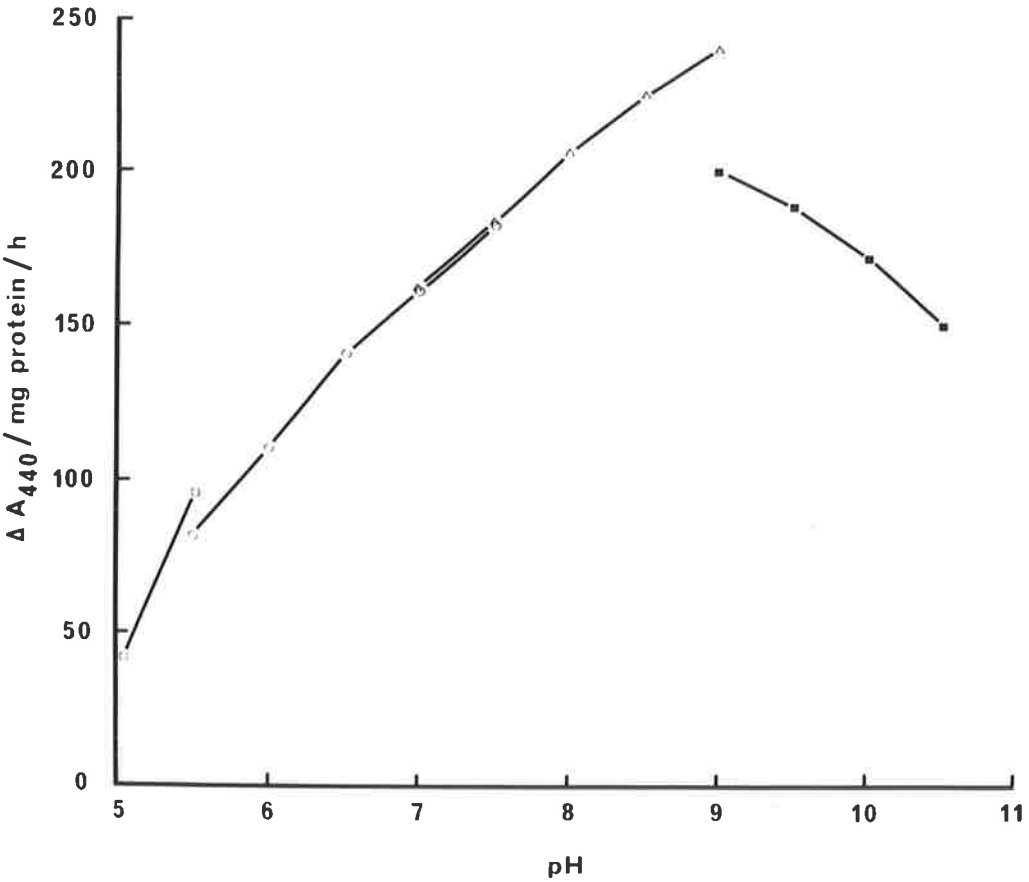


Fig 17B



apparent pH optimum of 9.0 when acting on azocasein (Figure 17B) suggesting that the apparent pH optimum of proteinase A on azocasein is due to the effect of pH on the substrate. When proteinase A acts on unsubstituted casein, the apparent pH optimum is 6.0 (Figure 18A) but there is considerable activity at pH 8.0. The apparent pH optimum of proteinase A acting on haemoglobin is 4.0 (Figure 18B). Alkaline denaturation of haemoglobin, as described by Schlamowitz and Peterson (1959), did not alter the apparent pH optimum of proteinase A acting on this substrate. Acid treatment of the haemoglobin below pH 2.0 did not alter its susceptibility to hydrolysis by proteinase A. When succinate or citrate buffer was used in the assay, there was only 75% and 44% respectively of the activity obtained with acetate buffer. The pH optimum of proteinase A acting on maize root protein is 6.0 (Figure 18C); the low activity observed may be due to the use of 0.085 mg of protein per assay whereas 0.5 mg of haemoglobin was used to assay haemoglobin degradation. Higher levels of maize root protein were shown to increase the activity of proteinase A.

3.8.3 Substrate concentration and specificity

Proteinase A exhibited normal Michaelis-Menten kinetics with both azocasein and haemoglobin and its activity on root protein increased with substrate concentration. The graphs of the relationship between concentration of azocasein and haemoglobin and enzyme activity are rectangular hyperbolae (Figure 19) with some inhibition of activity at the highest level of azocasein. From double reciprocal plots of the data, values of K_m for azocasein and haemoglobin were estimated to be 1.8 mg/ml and 5.6 mg/ml (0.08 mM) respectively.

In Table 14, it is shown that proteinase A degrades the common protein substrates azocasein, casein and haemoglobin as well as maize root protein and zein which is a storage protein of maize. In addition, nitrate reductase was inactivated by proteinase A. The following substrates were

TABLE 14

Substrate specificity of proteinase A from maize roots

Activity of proteinase A against the substrates shown was determined as described in Sections 2.5.4.1 (azocasein), 2.5.4.2 (haemoglobin, casein, zein, maize root protein), 2.5.4.4 (nitrate reductase inactivation), 2.5.4.8 (Congo red elastin), 2.5.4.3 (acetylphenylalanyl-diiodotyrosine) and 2.5.4.7 (TAME, ATEE, BAEE). Zein (10 mg/ml) was dissolved in 1 M NaOH at 50°C for 2 h then dialysed against water overnight and there was 0.085 mg of root protein present in the assay on this substrate. Activities are expressed relative to azocasein degradation.

Substrate	Degraded by	Assay pH	Activity
Azocasein	many proteinases (Peterson and Huffaker, 1975; Starkey and Barrett, 1976; Molano and Gancedo, 1974)	8.5	$\Delta A_{440} = 1$
Haemoglobin	many proteinases (Abe *et al*, 1977; Drivdahl and Thimann, 1977; Frith *et al*, 1978a)	4.0	0.53 µmol α-amino N released
Casein	many proteinases (Wells, 1968; du Toit, 1976; Feller *et al*, 1973	6.0	0.3 µmol α-amino N released
Zein	maize endosperm proteinase (Harvey and Oaks, 1974)	4.0	0.1 µmol α-amino N released
Maize root* protein		7.0	0.12 µmol α-amino N
Nitrate reductase	maize root inactivating enzyme (Wallace, 1974)	7.0	1.1×10^3 units nitrate inactivated reductase

(Cont.)

TABLE 14 (Cont.)

Substrate	Degraded by	Assay pH	Activity
Congo red elastin	elastase (Naughton and Sanger, 1961)	4.0 and 8.0	none detected
acetylphenyl-alanyl-diiodotyrosine	pepsin (Ryle and Hamilton, 1966)	5.2	none detected
TAME	trypsin, thrombin (Hummel, 1959) subtilisin (Ottesen and Svendsen, 1970)	8.0	none detected†
ATEE	chymotrypsin, trypsin (Hummel, 1959)	8.0	none detected†
BAEE	trypsin (Walsh and Wilcox, 1970) ficin (Hammond and Gutfreund, 1959) bromelain (Inagami and Murachi, 1963)	8.0	none detected

* preparation described in Section 2.3.1

† carboxypeptidases A and A' also present

FIGURE 18

Effect of pH on the digestion of casein, haemoglobin and maize root protein by proteinase A from maize roots

A 0.1 ml aliquots of protease A (6.3 µg protein) were incubated with 0.05 ml of 10 mg/ml casein and 0.1 ml of either sodium acetate or sodium phosphate (each 0.25 M) for 2 h at 37°C. The remainder of the assay was performed as for the haemoglobin degradation assay described in Section 2.5.4.2. Due to the precipitation of casein, activity was not determined below pH 5.0.

□——□ Sodium acetate

○——○ Sodium phosphate

B Activity of proteinase A on haemoglobin was determined as described for casein above using sodium citrate and sodium acetate buffers (each 0.25 M).

●——● Sodium citrate

□——□ Sodium acetate

○——○ Sodium phosphate

Fig 18A

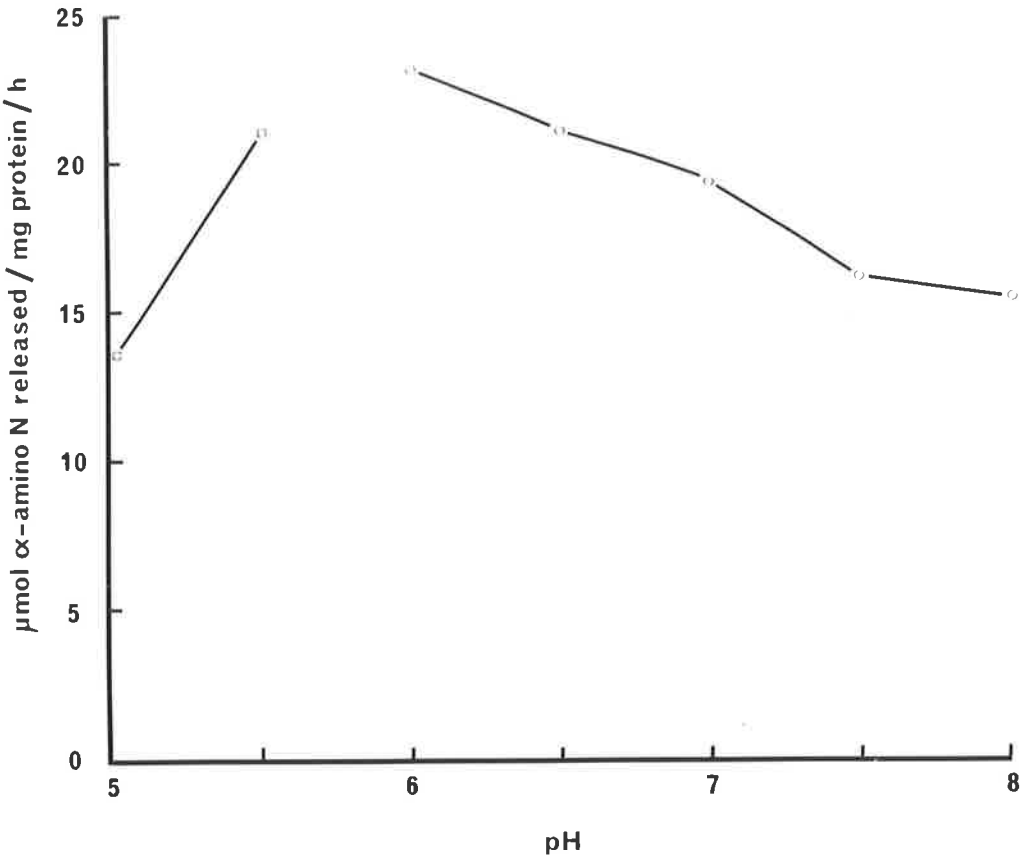


Fig 18B

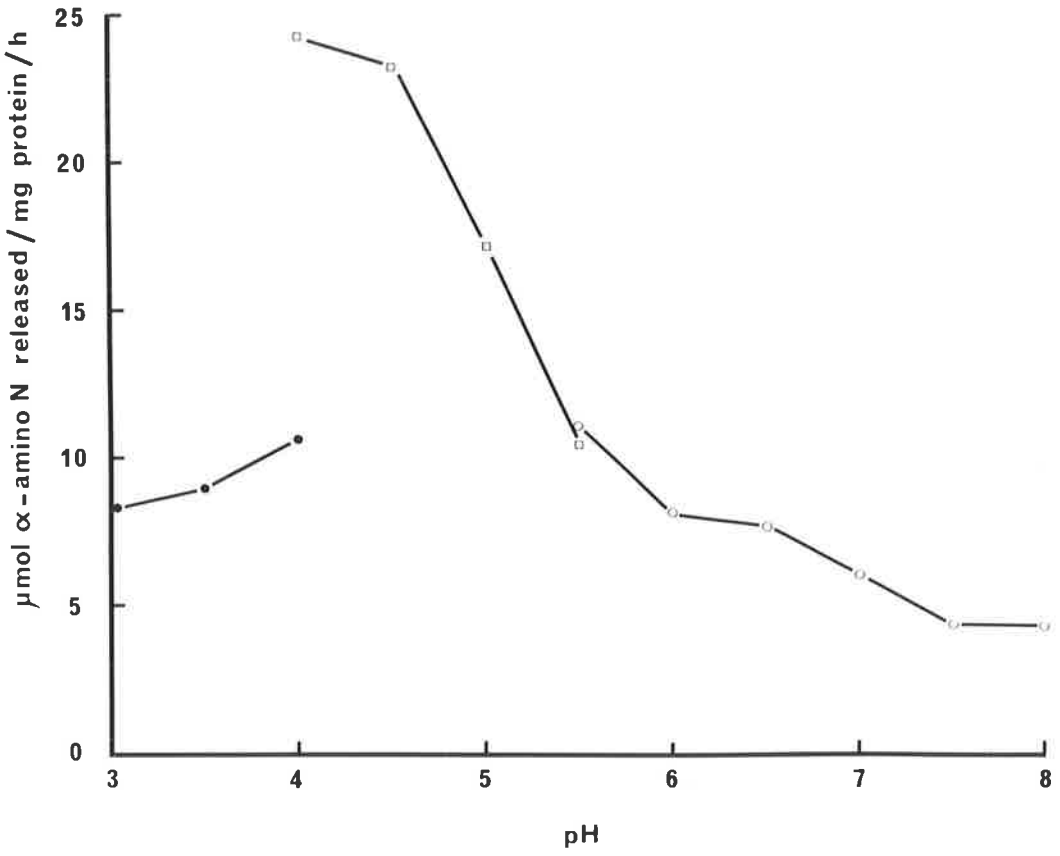


FIGURE 18

C 0.1 ml aliquots of proteinase A (1.6 µg protein) purified on a DEAE-Sephadex column (Figure 9) were incubated for 2 h at 37°C with 0.05 ml of 1.7 mg/ml root protein prepared as described in Section 2.3.1 and 0.1 ml of acetate-phosphate-borate buffer (each 50 mM and present as the sodium salt). The remainder of the assay procedure was performed as described in Section 2.5.4.2 for haemoglobin degradation. (In parts A and B of this figure a sample of protease A purified on CM-cellulose (63 µg/ml) was used rather than proteinase A from DEAE-Sephadex.)

Fig 18C

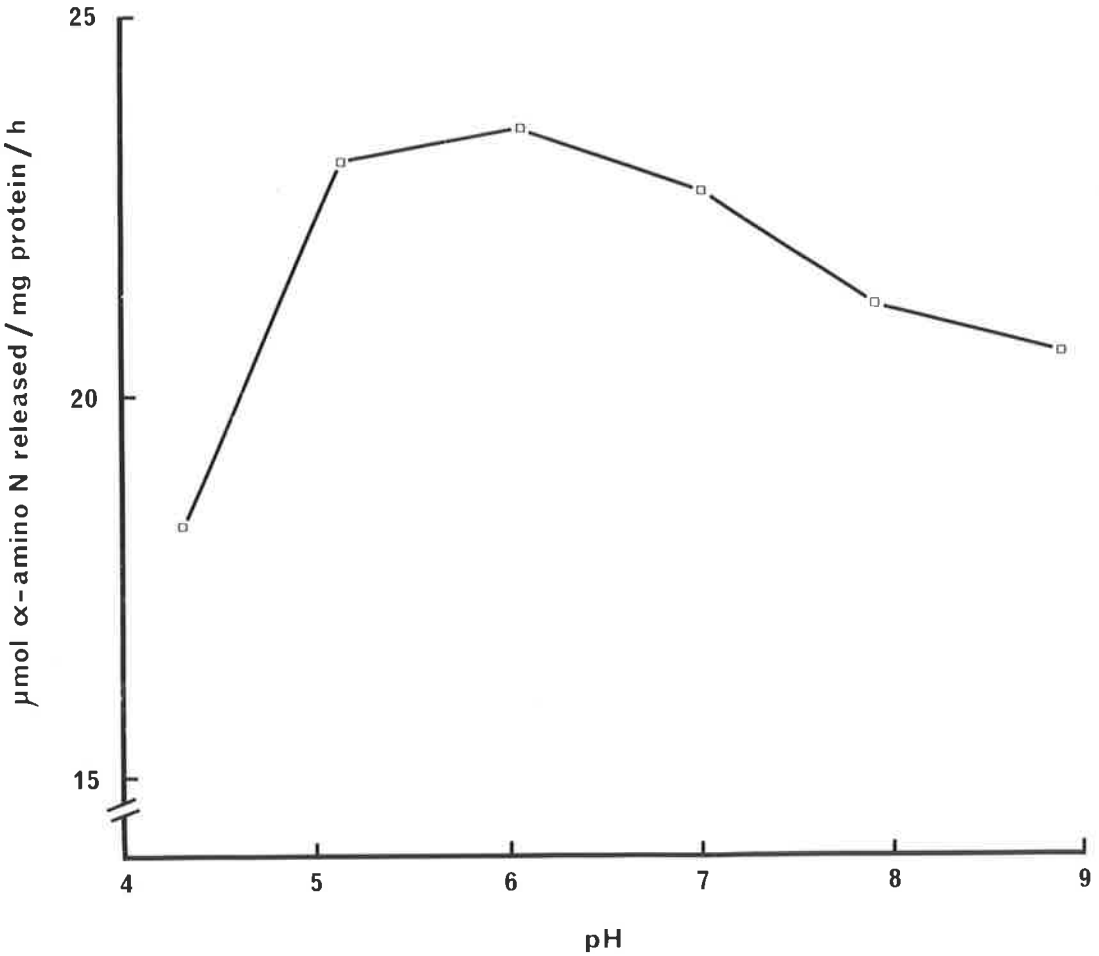


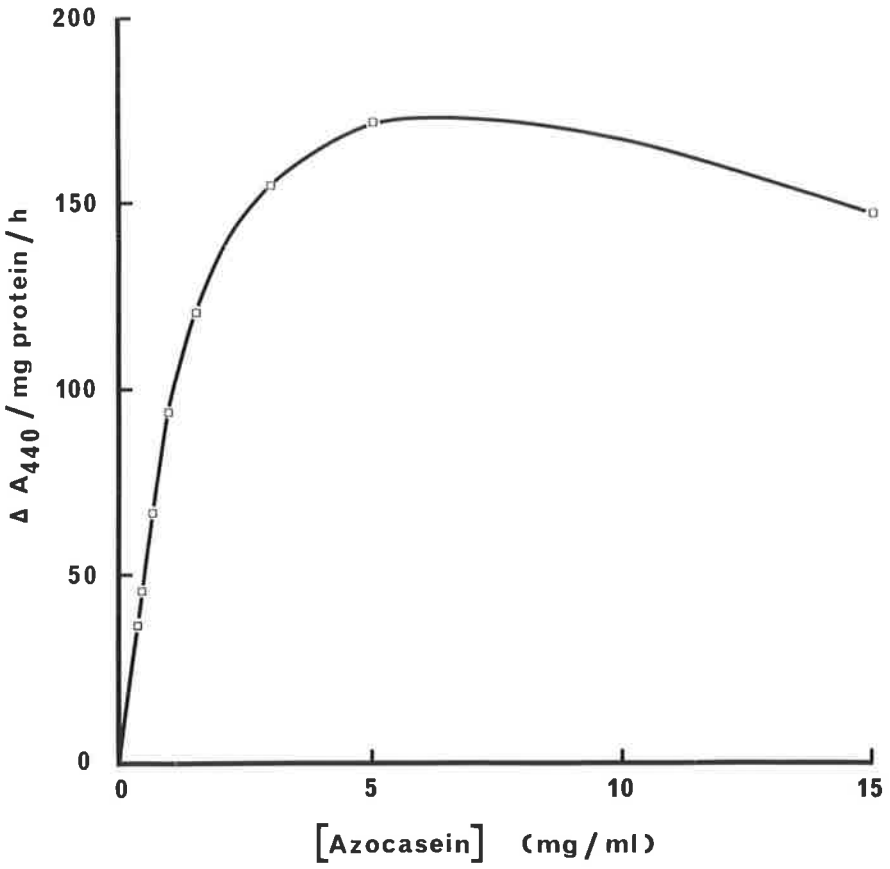
FIGURE 19

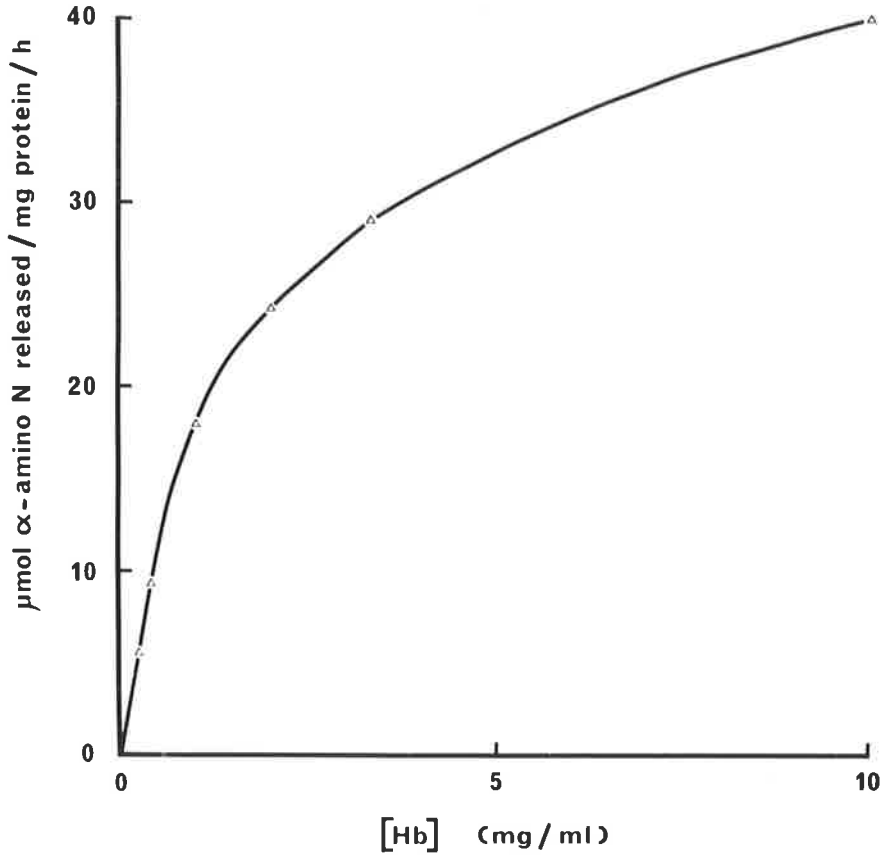
Effect of substrate concentration on the activity of proteinase A from maize roots

0.1 ml aliquots of protease A (6.3 µg protein) were assayed with azocasein as substrate at the concentrations shown as described in Section 2.5.4.1.

0.1 ml aliquots of protease A (6.3 µg protein) were assayed with haemoglobin as substrate at the concentrations shown as described in Section 2.5.4.2.

Fig 19


200
150
100
50
0
Δ A440 / mg protein / h
0
5
10
15
[Azocasein] (mg / ml)


40
30
20
10
0
µmol α-amino N released / mg protein / h
0
5
10
[Hb] (mg / ml)

not hydrolysed by proteinase A: Congo red elastin which is a substrate for the serine proteinase elastase; acetylphenylalanyl-diiodotyrosine, a substrate for pepsin; TAME, an ester substrate of the serine proteinases trypsin, thrombin and subtilisin; ATEE, an ester substrate for the serine proteinases chymotrypsin and trypsin; BAEE, an ester substrate for the SH-proteinases ficin and bromelain (Table 14).

3.8.4 Inhibitors of proteinase A

Active sites of proteases operative in the neutral pH range may contain SH groups, serine residues or require metal ions for activity (Section 1.1). The strong inhibition of proteinase A by PMSF implies that it may have a serine residue at its active site (Table 15). NEM, pCMB and $HgCl_2$ did not markedly inhibit proteinase A, apart from the inhibition of haemoglobin degradation by $HgCl_2$, suggesting that free SH groups are not essential for the action of this enzyme. TLCK and TPCK, which alkylate histidine residues at the active site of trypsin and chymotrypsin respectively (Shaw *et al*, 1965; Schoellman and Shaw, 1963) did not inhibit, nor did the trypsin inhibitor from soybeans. Mercaptoethanol, which affects the activity of some proteinases sensitive to mercurials (Pike and Briggs, 1972; Drivdahl and Thimann, 1977) also had no effect on proteinase A.

The azocasein degrading, haemoglobin degrading and nitrate reductase inactivating activities were affected similarly by the inhibitors tested (Table 15) apart from the effect of $HgCl_2$ on haemoglobin degradation. Similarity between the azocasein and haemoglobin degrading activities is further shown in Figure 20 where different levels of inhibitor were tested. Both activities were very sensitive to PMSF and insensitive to pCMB. Haemoglobin degrading activity was more sensitive to $HgCl_2$ than was the azocasein degrading activity. PMSF inhibited azocasein degradation

TABLE 15

Action of inhibitors on proteinase A from maize roots

Samples of protease A (0.65 mg protein/ml) were incubated for 20 min at 37°C with the inhibitors shown. Aliquots (0.1 ml) were withdrawn and assayed for azocasein degrading, haemoglobin degrading and nitrate reductase inactivating activities as described in Sections 2.5.4.1, 2.5.4.2 and 2.5.4.4. When the effect of pCMB on nitrate reductase inactivating activity was tested, 1 mM cysteine was included in the assay mixture for nitrate reductase.

Initial activities were: ΔA_{440}/ml/h = 1.65 (azocasein degradation); 1.07 µmol α-amino N released/ml/h (haemoglobin degradation); 8 740 units of nitrate reductase inactivated/ml/h (nitrate reductase inactivation).

Inhibitor	Concentration of inhibitor during pre-incubation	Enzyme activities expressed as a percentage of initial activity		
		Azocasein degrading activity	Haemoglobin degrading activity	Nitrate reductase inactivating activity
PMSF (in 5% (v/v) isopropyl alcohol)	1 mM	3	3	16
DFP* (in 25% (v/v) ethylene glycol)	1 mM	62	65	-
DFP* (in 25% (v/v) ethylene glycol)	0.5 mM	-	-	0
1,10-phenanthroline (in 5% isopropyl alcohol)	1 mM	105	71	96
isopropyl alcohol	5% (v/v)	102	89	-
TLCK	1 mM	86	86	109
TPCK (in 5% (v/v) methanol)	1 mM	110	84	-
methanol	5% (v/v)	102	94	-
soybean trypsin inhibitor	0.25% (w/v)	126	111	-
$HgCl_2$	1 mM	74	26	-
pCMB	0.1 mM	92	89	100
NEM	1 mM	92	89	-
mercaptoethanol	2 mM	90	104	-

* one experiment only; results expressed relative to enzyme incubated with 25% (v/v) ethylene glycol

FIGURE 20

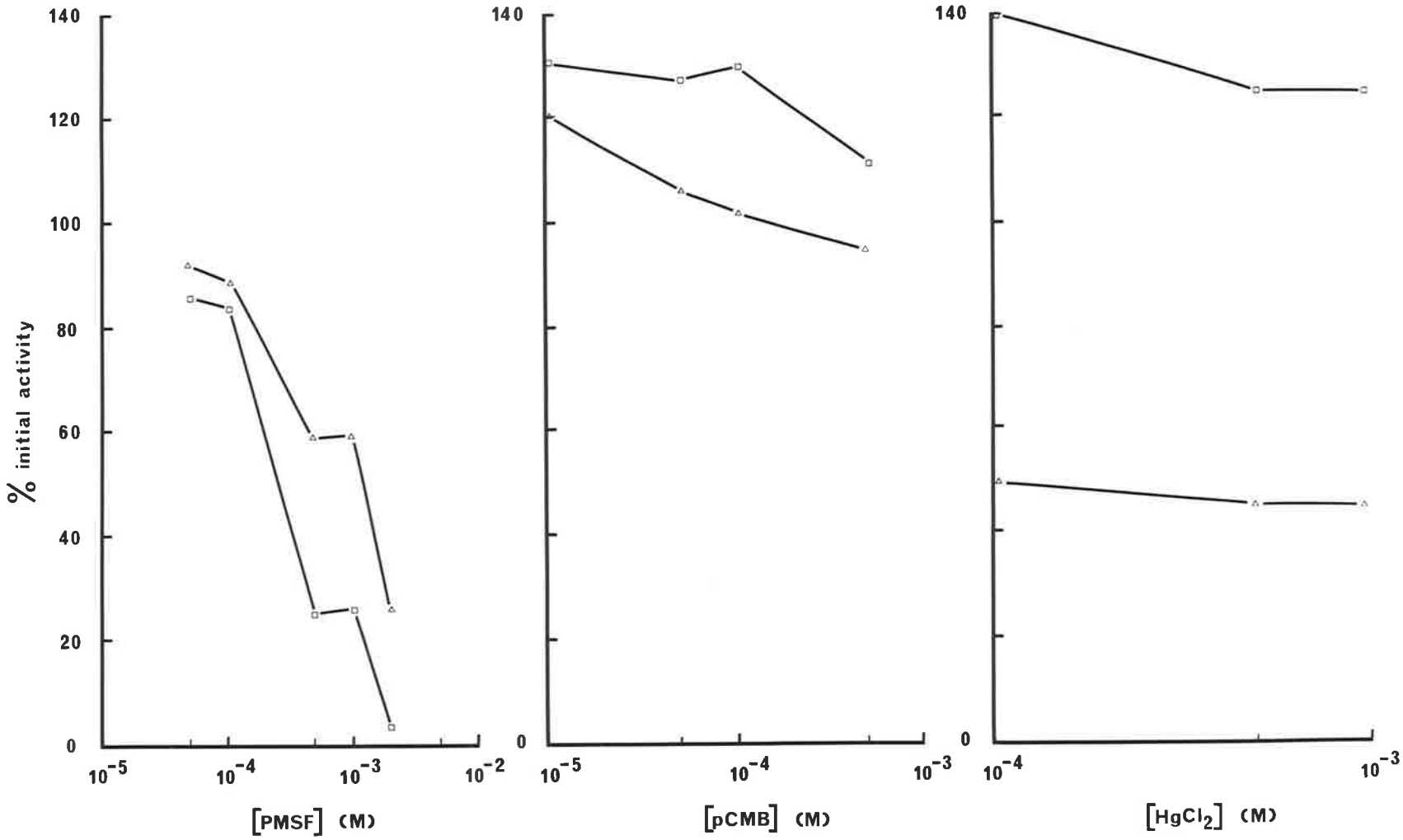
Influence of different concentrations of PMSF, pCMB and $HgCl_2$ on the activity of proteinase A from maize roots

Protease A samples (0.61 mg protein/ml) were preincubated for 20 min at 37°C with inhibitor at the concentration shown and then assayed for azocasein and haemoglobin degrading activities as described in Sections 2.5.4.1 and 2.5.4.2. Initial activities were ΔA_{440}/ml/h = 4.6 (azocasein degrading activity and 3.9 µmol α-amino N released/ml/h respectively. The concentrations of inhibitor are plotted on a logarithmic scale.

□——□ Azocasein degrading activity

△——△ Haemoglobin degrading activity

Fig 20


% initial activity
140
120
100
80
60
40
20
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
[PMSF] (M)
140
0
10^{-5}
10^{-4}
10^{-3}
[pCMB] (M)
140
0
10^{-4}
10^{-3}
$[HgCl_2]$ (M)

by 98% in the presence of 10 mM cysteine suggesting that PMSF does not react with SH groups of proteinase A.

3.8.5 Stability

No loss of azocasein degrading activity occurred during incubation for 2 h at 37°C at either pH 4.1, 6.0 or 7.8. The haemoglobin degrading activity was stable at pH 4.1 and 6.0 but lost 15% of its original activity per hour at pH 7.8 (Figure 21). Over a 16 day storage period, the azocasein degrading activity was more stable than the haemoglobin degrading activity, retaining 100% and 92% of the original activity at 4°C and -15°C respectively while the corresponding values for the haemoglobin degrading activity were 60% and 69%.

3.9 Properties of carboxypeptidase A and A' from maize roots

3.9.1 Introduction

Carboxypeptidase A refers to the carboxypeptidase activity of the protease A fraction separated on a CM-cellulose column (Figure 7). It was separated from proteinase activity on a DEAE-Sephadex column (Figure 9) although a minor carboxypeptidase species, designated A', remained associated with the proteinase activity. All the carboxypeptidase activity can be separated from proteinase activity by gel filtration (Figure 10). The molecular weight of the carboxypeptidases was found to be 97 000. Several properties of carboxypeptidase A have been determined with a protease A sample of which carboxypeptidase A' is only a minor component; the properties of the latter enzyme generally appear to be similar to those of carboxypeptidase A.

3.9.2 pH optimum

Both carboxypeptidase A and A' have a broad pH optimum around 5.0

FIGURE 21

Stability of proteinase A in relation to pH

Samples of protease A (0.63 mg protein/ml) in 5 mM sodium phosphate pH 6.0 were mixed with either sodium acetate pH 4.0, sodium phosphate pH 6.0 or *di*-sodium hydrogen phosphate (each 0.12 M) (3 volumes enzyme : 1 volume buffer); final pH values were 4.1, 6.0 and 7.8 respectively. After incubation at 37°C for the times shown, aliquots (0.2 and 0.1 ml) were withdrawn and assayed for azocasein and haemoglobin degrading activities respectively as described in Sections 2.5.4.1 and 2.5.4.2.

Azocasein degrading activity, upper panel

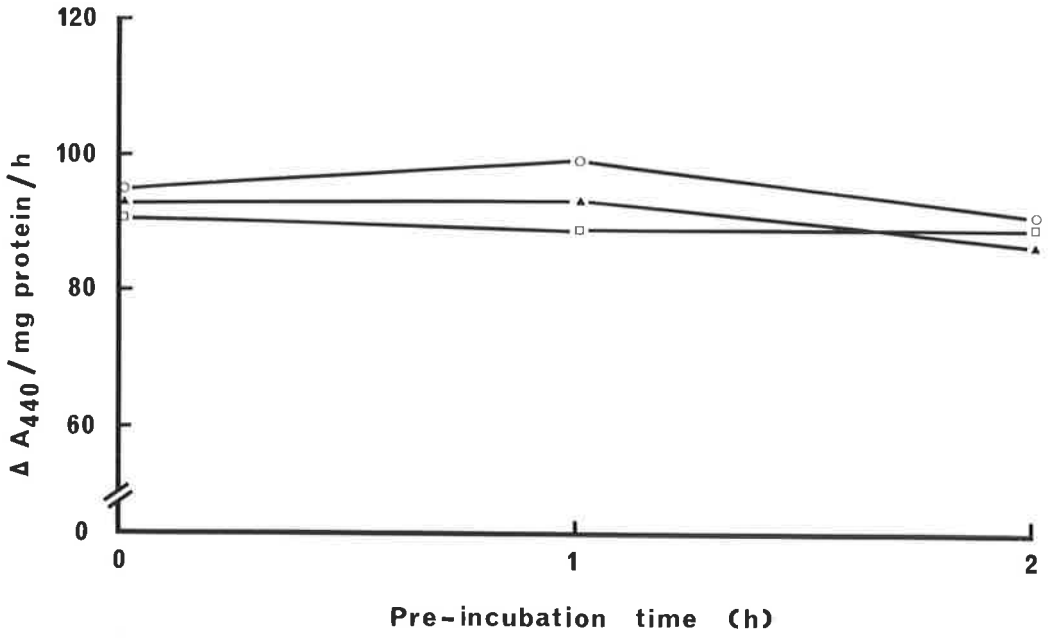
Haemoglobin degrading activity, lower panel

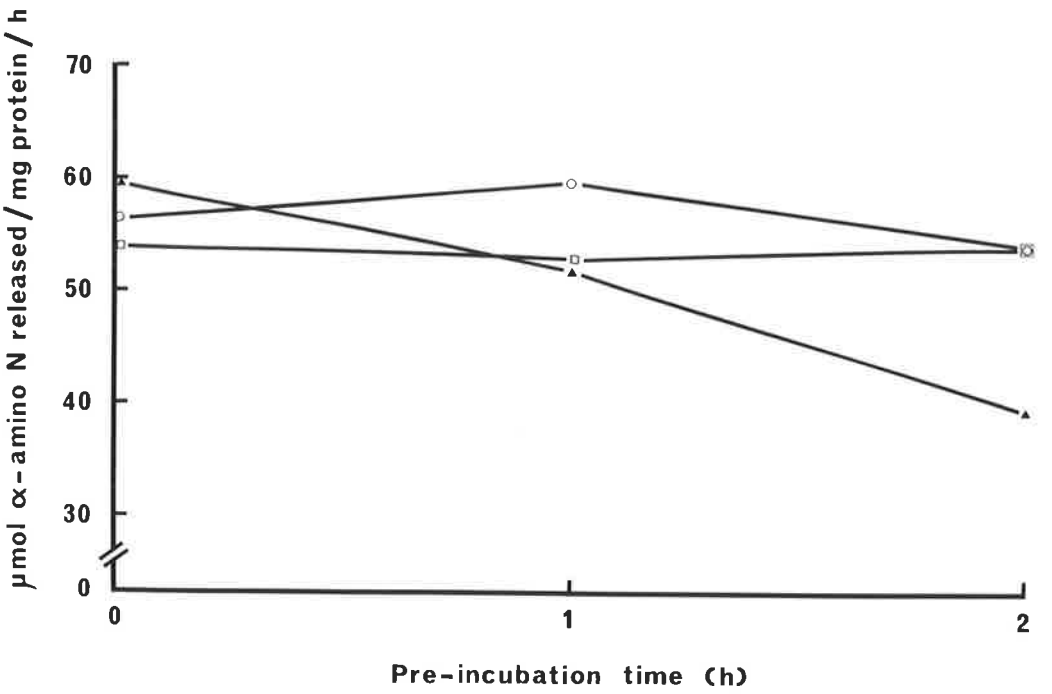
□——□ Sodium acetate (final pH 4.1)

○——○ Sodium phosphate (final pH 6.0)

▲——▲ *di*-Sodium hydrogen phosphate (final pH 7.8)

Fig 21


120
100
80
60
0
Δ A440 / mg protein / h
0
1
2
Pre-incubation time (h)


70
60
50
40
30
0
μmol α-amino N released / mg protein / h
0
1
2
Pre-incubation time (h)

when Tricine-acetate buffer was used in the assay (Figure 22). A similar result was obtained when carboxypeptidase A was assayed in sodium acetate buffer.

3.9.3 Substrate concentration and specificity

When carboxypeptidase A was assayed with different levels of substrate, the resultant activities fitted a hyperbola predicted by Michaelis-Menten kinetics (Figure 23). From a double reciprocal plot of activity and substrate concentration, the K_m of carboxypeptidase A for Cbz-Phe-Ala at pH 5.2 and 37°C was found to be 5 mM.

Cbz-Glu-Tyr, a substrate for the carboxypeptidases of yeast and *Aspergillus* (Hayashi and Hata, 1972; Ichishima, 1972), was degraded at 5% of the rate of Cbz-Phe-Ala. Acetylphenylalanyl-diiodotyrosine, a pepsin substrate, was not affected neither were the esters TAME and ATEE (Table 14). BAEE, a substrate of ficin, bromelain and trypsin, was hydrolysed by carboxypeptidase A at a rate of 0.13 µmol BAEE/µmol alanine released from Cbz-Phe-Ala. Carboxypeptidase A' hydrolysed BAEE at a rate of no more than 0.04 µmol BAEE/µmol alanine released from Cbz-Phe-Ala. Thus carboxypeptidase A hydrolysed BAEE more rapidly than the A' fraction.

3.9.4 Action of inhibitors

Carboxypeptidase A was inhibited by PMSF and DFP (Table 16) suggesting the presence of a serine residue at its active site. The chelating agent 1,10-phenanthroline did not inhibit indicating that neither Fe^{2+}, Zn^{2+} or Cu^{2+} are required for activity of this enzyme. $HgCl_2$ and pCMB were only slightly inhibitory (20%) to carboxypeptidase A.

Increasing the concentration of PMSF from 1 mM to 5 mM gave little further inhibition (Figure 24). Use of preincubation times longer than 15 min or use of a higher pH (7.4 instead of 5.2) during preincubation

TABLE 16

Action of inhibitors on carboxypeptidase A from maize roots

Aliquots of diluted protease A (1.1 µmol alanine released/ml/h; 6.3 µg protein/ml) were incubated with the inhibitors shown for 20 min at 37°C and then carboxypeptidase activity was determined as described in Section 2.5.4.3. A 20% reduction in the colour yield of the ninhydrin reaction by $HgCl_2$ and pCMB has been corrected for by reference to an alanine standard.

Inhibitor	Inhibitor concentration during pre-incubation	% activity remaining
PMSF (in 5% (v/v) isopropyl alcohol)	1 mM	30*
DFP (in 25% (v/v) ethylene glycol)	1 mM	25†
1,10-phenanthroline (in 5% (v/v) isopropyl alcohol)	1 mM	100*
isopropyl alcohol	5% (v/v)	113
pCMB	0.5 mM	78
$HgCl_2$	0.5 mM	80
NEM	1 mM	100

* compared to enzyme incubated with equivalent amount isopropyl alcohol only

† compared to enzyme incubated with 25% (v/v) ethylene glycol

FIGURE 22

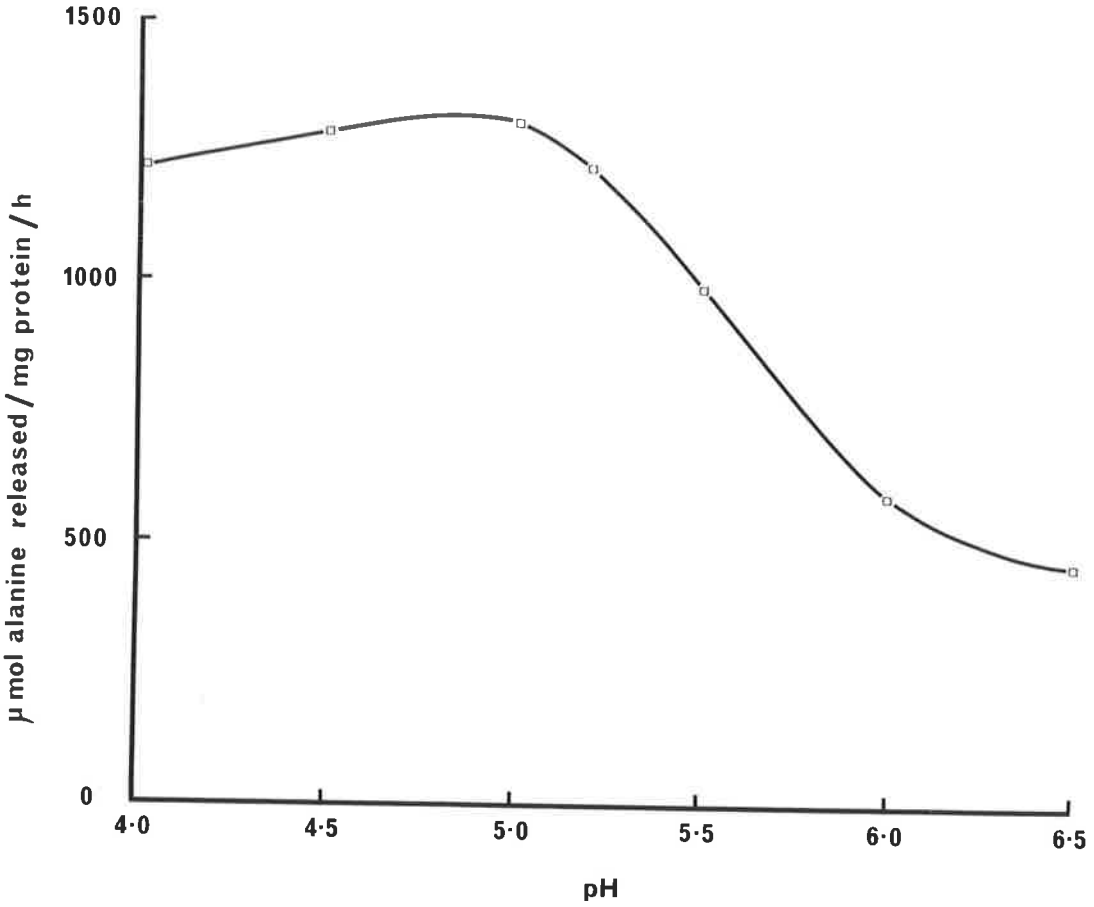
pH optimum of carboxypeptidases A and A' from maize roots

Samples of carboxypeptidases A and A' were prepared from a DEAE-Sephadex column (Figure 9). Each assay contained 0.2 ml of enzyme sample in 20 mM sodium phosphate pH 6.0 and 0.6 ml of 0.1 M Tricine-acetate at the pH values shown containing 1 mg/ml Cbz-Phe-Ala and 2% (v/v) DMSO. Activity was determined as described in Section 2.5.4.3.

Carboxypeptidase A, upper panel

Carboxypeptidase A', lower panel

Fig 22


1500
1000
500
0
µmol alanine released / mg protein / h
4·0
4·5
5·0
5·5
6·0
6·5
pH

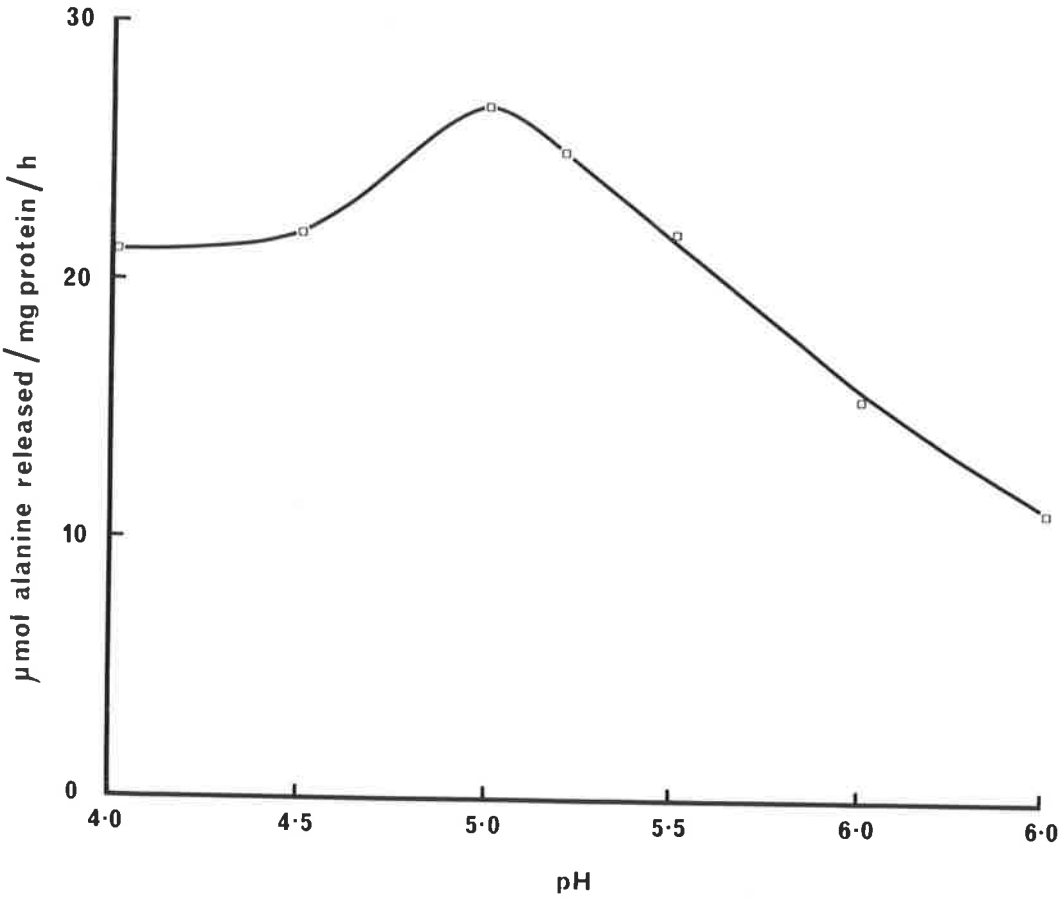

30
20
10
0
µmol alanine released / mg protein / h
4·0
4·5
5·0
5·5
6·0
6·0
pH

FIGURE 23

Effect of substrate concentration on activity of carboxypeptidase A from maize roots

Samples of protease A (0.15 mg protein/ml) were assayed for carboxypeptidase activity by incubating with 0.6 ml of 0.1 M sodium acetate pH 5.2 containing Cbz-Phe-Ala at the final concentrations shown for 30 min at 37°C as described in Section 2.5.4.3.

Fig 23

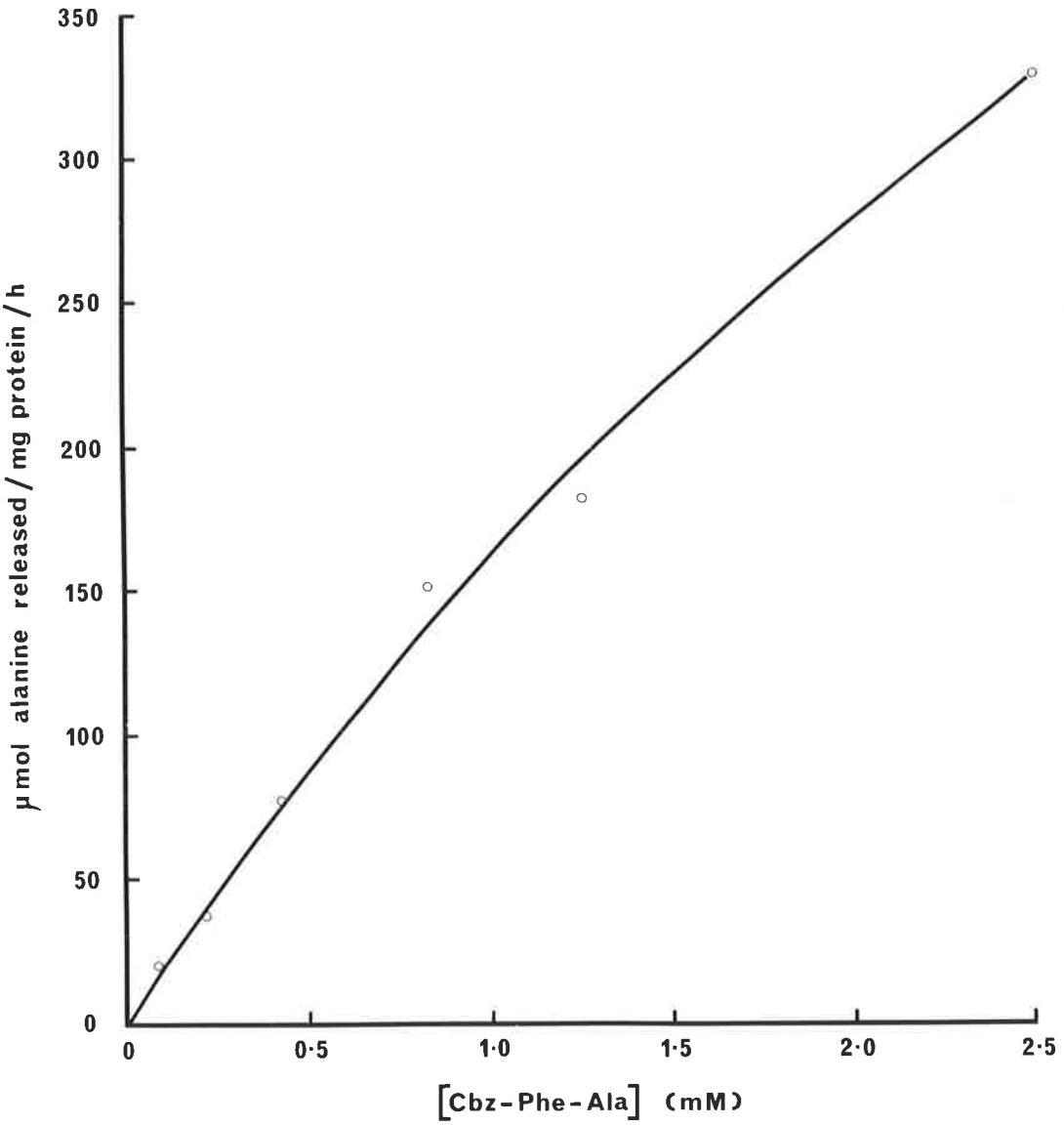
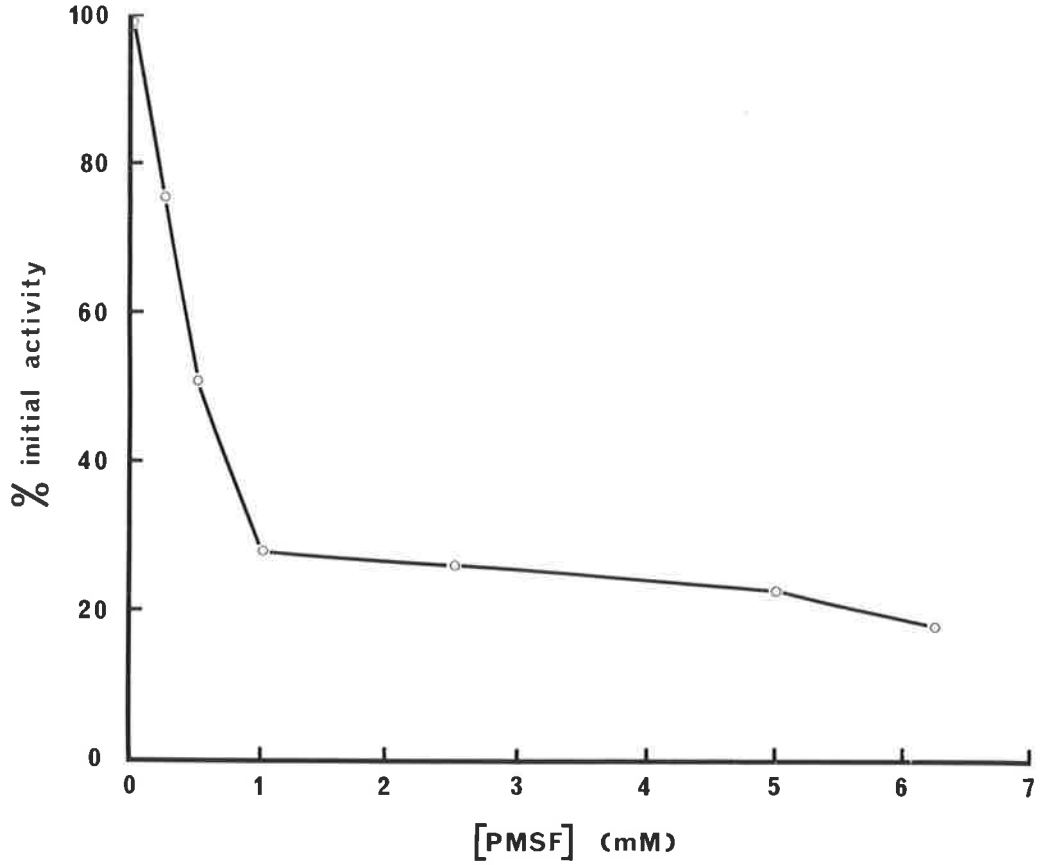

350
300
250
200
150
100
50
0
µmol alanine released / mg protein / h
0
0·5
1·0
1·5
2·0
2·5
[Cbz-Phe-Ala] (mM)

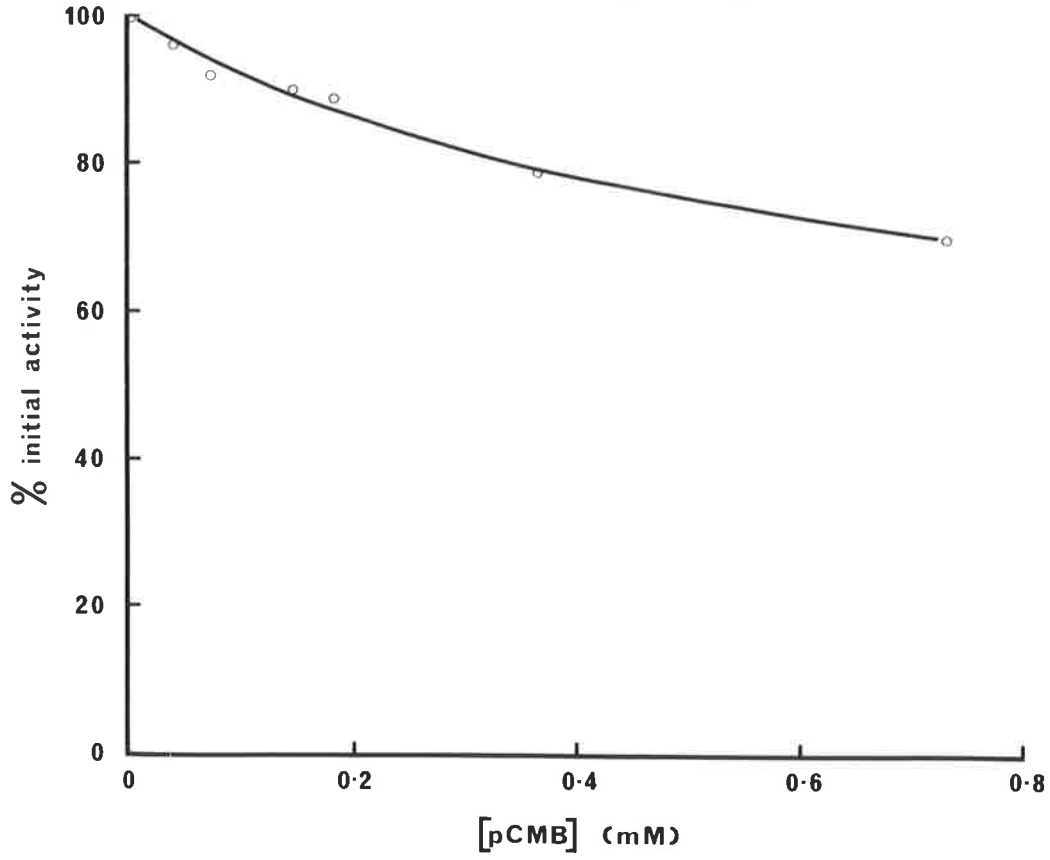
FIGURE 24

Effect of different concentrations of PMSF and pCMB on the activity of carboxypeptidase A from maize roots

Aliquots of protease A (8 µg protein) with an initial activity of 14 µmol alanine released/mg protein/h were incubated with either PMSF in isopropyl alcohol or pCMB at the concentrations shown for 20 min at 37°C and then assayed for carboxypeptidase activity as described in Section 2.5.4.3. Activity with PMSF is expressed relative to enzyme samples incubated with isopropyl alcohol only and the diminution in colour yield of the ninhydrin reaction due to the presence of pCMB was corrected for by reference to an alanine standard.

Fig 24


100
80
60
40
20
0
% initial activity
0
1
2
3
4
5
6
7
[PMSF] (mM)


100
80
60
40
20
0
% initial activity
0
0·2
0·4
0·6
0·8
[pCMB] (mM)

did not increase the degree of inhibition. As shown in Figure 24, carboxypeptidase A was relatively insensitive to pCMB at concentrations which are normally inhibitory to SH-proteinases. Preincubation times longer than 15 min did not increase the inhibition by pCMB.

Carboxypeptidase A', the minor species resolved on DEAE-Sephadex was inhibited 100% and 90% by PMSF and $HgCl_2$ respectively while the corresponding figures for carboxypeptidase A were 70% and 20% (Table 17).

3.9.5 Stability

Carboxypeptidase A was stable at pH 6.0 but labile at pH 4.1 and 7.8 when incubated at 37°C (Figure 25). However there was no marked loss of carboxypeptidase activity at pH 4.4 at 4°C. The lability at alkaline pH may account for losses of activity during chromatography at pH 7.8 on DEAE-Sephadex (Section 3.4). Carboxypeptidase A was less stable than proteinase A (Section 3.8.4).

3.10 Properties of protease B from maize roots

3.10.1 Introduction

Protease B was isolated by chromatography on CM-cellulose (Figure 7) and contains a haemoglobin degrading species (proteinase B), a nitrate reductase inactivating activity and two carboxypeptidases BI and BII (collectively referred to as carboxypeptidase B) which were separated by gel filtration (Figure 12). Due to the relatively low activity of protease B and its lack of stability (Section 3.10.4), this fraction has not been extensively studied. Studies on protease B have been conducted on the fraction isolated from a CM-cellulose column (Figure 7) as the components of protease B have not been completely resolved.

TABLE 17

Action of PMSF, pCMB and $HgCl_2$ on carboxypeptidases A and A' from maize roots

Samples of carboxypeptidases A (1 µg protein/ml) and A' (16 µg protein/ml) were prepared on a DEAE-Sephadex column as described in Figure 9. Aliquots were incubated with the inhibitors shown for 20 min at 37°C. In experiment I, 5% (v/v) isopropyl alcohol was present in the pre-incubation mixture and the activity with PMSF was compared to that of enzyme incubated with isopropyl alcohol only. In experiment II, a 20% diminution in the colour yield of the ninhydrin reaction due to the presence of pCMB and $HgCl_2$ has been corrected for by reference to an alanine standard. Initial activities of carboxypeptidase A were 1.24 µmol alanine released/ml/h (experiment I) and 1.87 µmol alanine released/ml/h (experiment II); those of carboxypeptidase A' were 0.48 µmol alanine released/ml/h (experiment I) and 0.73 µmol alanine released/ml/h (experiment II).

Experiment	Inhibitor and concentration during pre-incubation		% of initial activity A	A'
I	PMSF	1 mM	30	0
II	pCMB	0.5 mM	80	21
	$HgCl_2$	0.5 mM	80	10

FIGURE 25

Stability of carboxypeptidase A from maize roots at different pH values

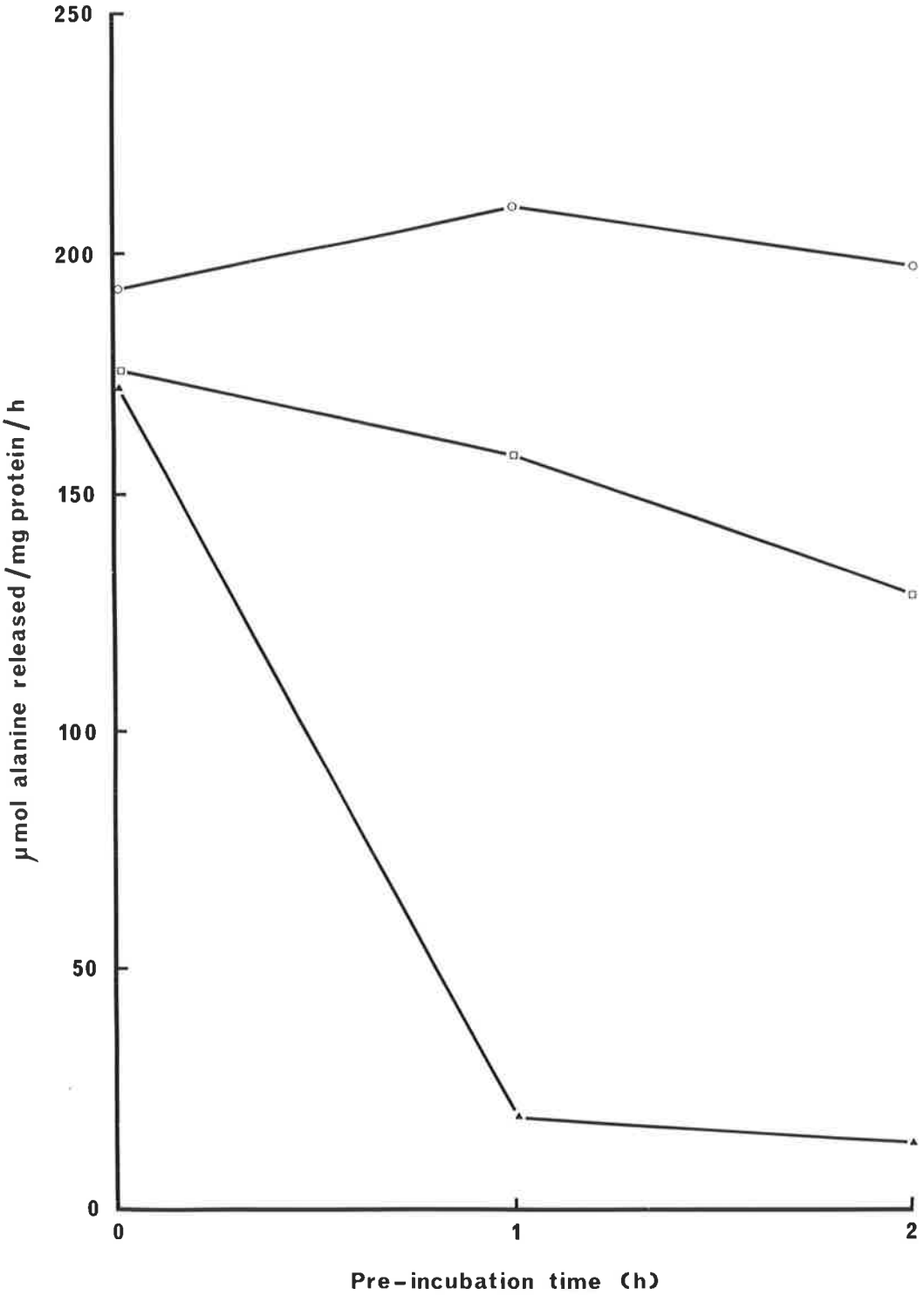
Samples of protease A (0.63 mg protein/ml) in 5 mM sodium phosphate pH 6.0 were mixed with either sodium acetate pH 4.0, sodium phosphate pH 6.0 or *di*-sodium hydrogen phosphate (each 0.12 M) (3 volumes enzyme : 1 volume buffer); final pH were 4.1, 6.0 and 7.8 respectively. After incubation at 37°C for the times shown, aliquots were withdrawn and assayed for carboxypeptidase activity as described in Section 2.5.4.3.

□——□ Sodium acetate (final pH 4.1)

○——○ Sodium phosphate (final pH 6.0)

▲——▲ *di*-Sodium hydrogen phosphate (final pH 7.8)

Fig 25


250
200
150
100
50
0
µmol alanine released /mg protein / h
0
1
2
Pre-incubation time (h)

3.10.2 pH optimum

Proteinase B acting on haemoglobin shows a pH optimum of 4.0 (Figure 26) with 80% more activity with acetate than citrate buffer. Carboxypeptidase B appears to have a pH optimum of about 5.0 as for carboxypeptidase A.

3.10.3 Action of inhibitors

Both haemoglobin degrading activity and carboxypeptidase activity are inhibited by PMSF, DFP, $HgCl_2$ and pCMB with the carboxypeptidase activity being more sensitive to PMSF and the haemoglobin degrading activity being more sensitive to $HgCl_2$ and pCMB (Table 18, Figure 27). Inhibition of haemoglobin degrading activity by pCMB increased with concentration up to 0.5 mM while the inhibition of carboxypeptidase activity was not increased by concentrations of pCMB above 0.05 mM (Figure 27). However 0.1 mM $HgCl_2$ and 1 mM $HgCl_2$ were almost equally inhibitory (Figure 27). Both pCMB and $HgCl_2$ inhibited haemoglobin degradation about 75% at a concentration of 0.5 mM. The chelating agent 1,10-phenanthroline had no effect on carboxypeptidase activity and inhibited haemoglobin degrading activity only 19% (Table 18). However the nitrate reductase inactivating activity of protease B was sensitive to 1,10-phenanthroline and Na_2EDTA but insensitive to PMSF. The nitrate reductase inactivating activity of a protease B fraction was not inhibited by casein but was heat labile. In contrast, the nitrate reductase inactivating activity of protease A could not be distinguished from proteinase activity and was inhibited by casein. DTT, which affects the activity of some mercurial sensitive proteinases (Pike and Briggs, 1972; Wittenbach, 1978) gave some inhibition (56%) as did TLCK (41%) which may react with SH groups as well as histidine residues (Whitaker and Perez-Villaseñor, 1968).

TABLE 18

Action of inhibitors on protease B from maize roots

Samples of protease B (0.16 mg/ml) were incubated with the inhibitors shown for 20 min at 37°C and then assayed for haemoglobin degrading, carboxypeptidase and nitrate reductase inactivating activities as described in Sections 2.5.4.2, 2.5.4.3 and 2.5.4.4. Initial activities were: 21 µmol α-amino N released/mg protein/h (haemoglobin degradation), 27.2 µmol alanine released/mg protein/h (carboxypeptidase) and 1.4 x 10^3 units nitrate reductase inactivated/mg protein/h (nitrate reductase inactivation). Diminution of the colour yield of the ninhydrin reaction in the presence of $HgCl_2$ and pCMB has been corrected for by reference to an alanine standard.

Inhibitor	Concentration of inhibitor during pre-incubation	Enzyme activities expressed as a percentage of initial activity		
		Haemoglobin degrading activity	Carboxypeptidase activity	Nitrate reductase inactivating activity
PMSF (in 5% (v/v) isopropyl alcohol)	1 mM	59*	41*	91*
DFP (in 25% (v/v) ethylene glycol)	1 mM	42†	42†	-
1,10-phenanthroline (in 5% (v/v) isopropyl alcohol)	1 mM	81	100	27
Isopropyl alcohol	5% (v/v)	95	86	-
$HgCl_2$	1 mM	24	50	-
pCMB	0.1 mM	30	51	-

(Cont.)

TABLE 18 (Cont.)

Inhibitor	Concentration of inhibitor during pre-incubation	Enzyme activities expressed as a percentage of initial activity		
		Haemoglobin degrading activity	Carboxypeptidase activity	Nitrate reductase inactivating activity
NEM	1 mM	95	100	-
dithiothreitol	2 mM	44	-	-
mercaptoethanol	2 mM	117	-	-
TLCK	1 mM	59	-	128

* relative to enzyme incubated with 5% (v/v) isopropyl alcohol

† relative to enzyme incubated with 25% (v/v) ethylene glycol

FIGURE 26

pH optimum of proteinase B from maize roots

Protease B (0.227 mg/ml) was assayed for haemoglobin degrading activity as described in Section 2.5.4.2 using either sodium citrate or sodium acetate buffers (each 0.25 M) at the pH values shown.

□——□ Sodium acetate

●——● Sodium citrate

Fig 26

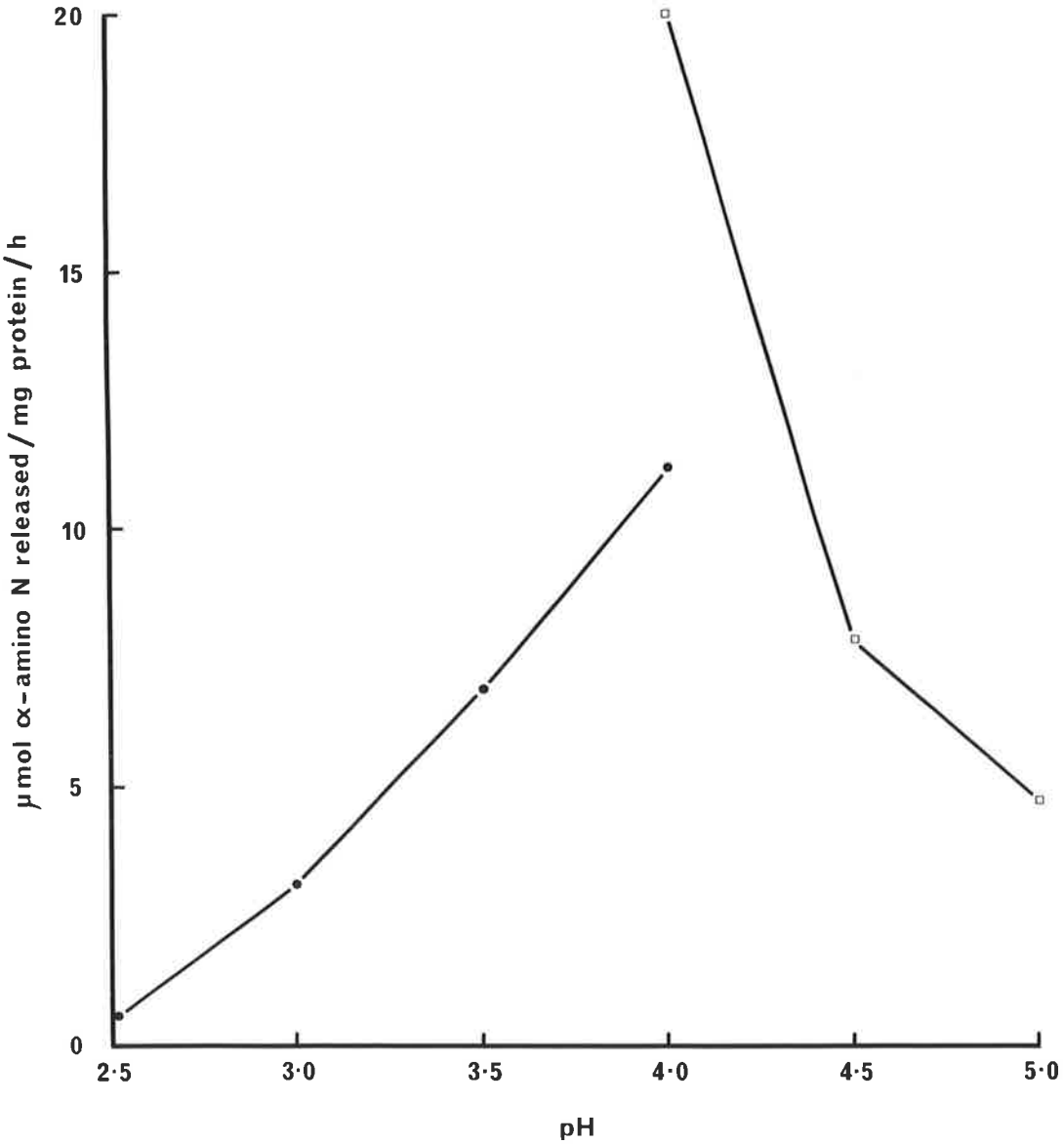

20
15
10
5
0
μmol α-amino N released / mg protein / h
2·5
3·0
3·5
4·0
4·5
5·0
pH

FIGURE 27

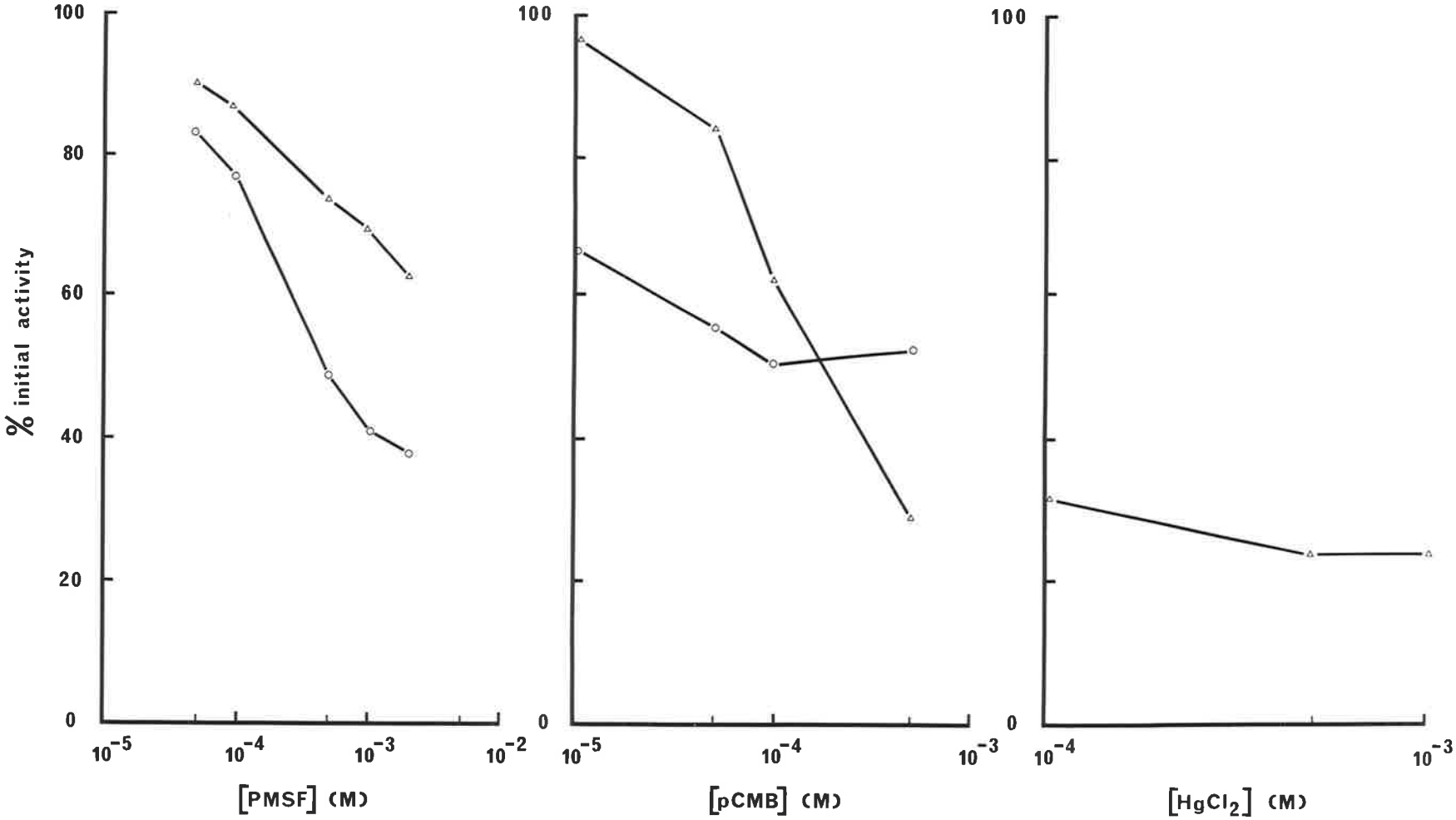
Effect of different concentrations of PMSF, pCMB and $HgCl_2$ on the activity of protease B from maize roots

Samples of protease B (0.227 mg/ml) were incubated with the concentrations of inhibitor shown for 20 min at 37°C and then assayed for haemoglobin degrading and carboxypeptidase activities as described in Sections 2.5.4.2 and 2.5.4.3. Results are expressed relative to the activities of enzyme samples incubated without inhibitor which were 17 µmol α-amino N released/mg protein/h (haemoglobin degradation) and 28 µmol alanine released/mg protein/h (carboxypeptidase activity). Inhibitor concentrations are plotted on a logarithmic scale.

△——△ Haemoglobin degrading activity

○——○ Carboxypeptidase activity

Fig 27


100
80
60
40
20
0
% initial activity
10^{-5}
10^{-4}
10^{-3}
10^{-2}
[PMSF] (M)
100
0
10^{-5}
10^{-4}
10^{-3}
[pCMB] (M)
100
0
10^{-4}
10^{-3}
[$HgCl_2$] (M)

3.10.4 Stability

The haemoglobin degrading component of protease B lost at least 30% of its activity over 2 h when incubated at 37°C at pH 4.1, 6.0 or 7.8 (Figure 28).

When stored for 16 days, 61% of the original haemoglobin degrading activity was lost at 4°C and 44% at -15°C. Dilution and gel filtration of protease B resulted in losses of both haemoglobin degrading and carboxypeptidase activities (Section 3.5).

Several agents were tested as stabilisers of protease B (Table 19). Neither sucrose nor glycerol had any effect on either the haemoglobin degrading or carboxypeptidase activity and BSA did not stabilise the former activity. (BSA was not tested on carboxypeptidase because of the reaction of BSA with ninhydrin reagent in the standard assay.) Cysteine enhanced the activities of both carboxypeptidase and the haemoglobin degrading species at a concentration of 1 mM (Figure 29). Neither mercaptoethanol nor dithiothreitol produced any marked stimulation of either activity.

3.10.5 Solubility

When a protease B sample (Figure 7) in 20 mM sodium acetate pH 5.0 containing 130 mM NaCl was concentrated 5 fold with Sephadex G-25, a precipitate containing the three enzyme activities studied was formed. Nitrate reductase inactivating activity was more soluble than either the proteinase or carboxypeptidase (Table 20). After concentration of protease B by Sephadex G-25, the recoveries of enzyme activities were as follows: proteinase 42%; carboxypeptidase 65%; nitrate reductase inactivating activity 54% (Table 20).

To redissolve the precipitated proteins, aliquots of the precipitate

TABLE 19

Effect of stabilising agents on protease B from maize roots

Samples of protease B (0.169 mg/ml) in 20 mM sodium acetate pH 5.0 containing 130 mM NaCl were incubated with the compounds listed for 2 h at 37°C. Aliquots were withdrawn and assayed for haemoglobin degrading and carboxypeptidase activities as described in Sections 2.5.4.2 and 2.5.4.3. Initial activities were 7.2 µmol α-amino N released/mg protein/h and 18 µmol alanine released/mg protein/h respectively.

Reagent and final concentration during pre-incubation	% of initial activity	
	Haemoglobin degrading activity	Carboxypeptidase activity
25% (w/v) sucrose	92	98
5% (w/v) bovine serum albumin	61	-
25% (v/v) glycerol	24	84

TABLE 20

Solubility of protease B from maize roots

15 ml of protease B (0.225 mg protein/ml) in 20 mM sodium acetate pH 5.0 containing 130 mM NaCl was placed in dialysis tubing and concentrated with Sephadex G-25 to 3 ml as described in Section 2.5.8. The concentrated solution was centrifuged at 8 000 *g* for 10 min at 2°C. The pellet, resuspended in 1 ml of 20 mM sodium phosphate pH 6.0 containing 0.1 M NaCl and 1 mM cysteine, was assayed for haemoglobin degrading, carboxypeptidase and nitrate reductase inactivating activities as described in Sections 2.5.4.2, 2.5.4.3 and 2.5.4.4. Total amounts of activity prior to concentration were 22 µmol α- amino N released/h (haemoglobin degradation), 109 µmol alanine released/h (carboxypeptidase) and 27 800 units nitrate reductase inactivated/h (nitrate reductase inactivation).

Activity	Activity recovered in soluble fraction	Activity recovered in pellet
Haemoglobin degradation (µmol α-amino N released/h	5.25	3.86
Carboxypeptidase (µmol alanine released/h)	32.1	39.6
Nitrate reductase inactivation (units nitrate reductase inactivated/h)	14 112	904

FIGURE 28

Stability of protease B from maize roots at different pH values

A Samples of protease B (0.227 mg/ml) in 5 mM sodium phosphate pH 6.0 were mixed with either sodium acetate pH 4.0, sodium phosphate pH 6.0 or *di*-sodium hydrogen phosphate (each 0.12 M) (3 volumes enzyme : 1 volume buffer); final pH values were 4.1, 6.0 and 7.8 respectively. After incubation at 37°C for the times shown, aliquots of 0.1 ml were assayed for haemoglobin degrading activity as described in Section 2.5.4.2.

B Experimental details are as described in A and 0.05 ml aliquots were assayed for carboxypeptidase activity as described in Section 2.5.4.3.

□——□ Sodium acetate (final pH 4.1)

○——○ Sodium phosphate (final pH 6.0)

▲——▲ *di*-Sodium hydrogen phosphate (final pH 7.8)

Fig 28A

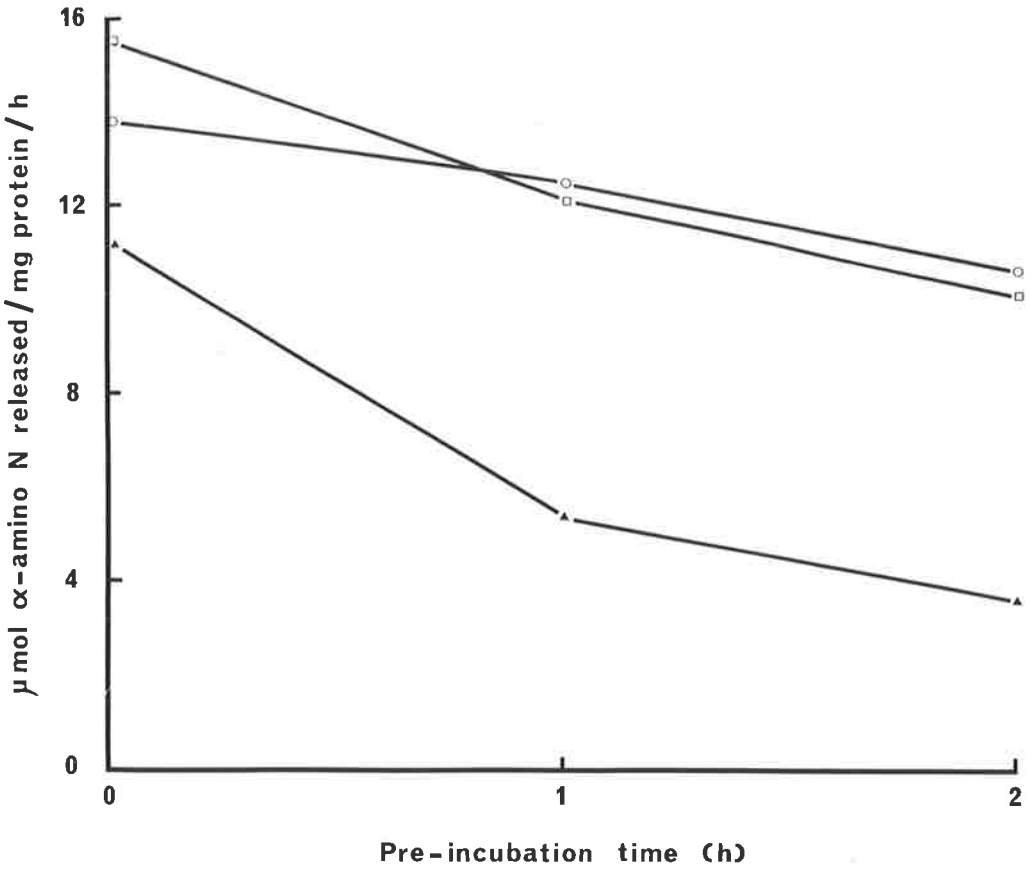


Fig 28B

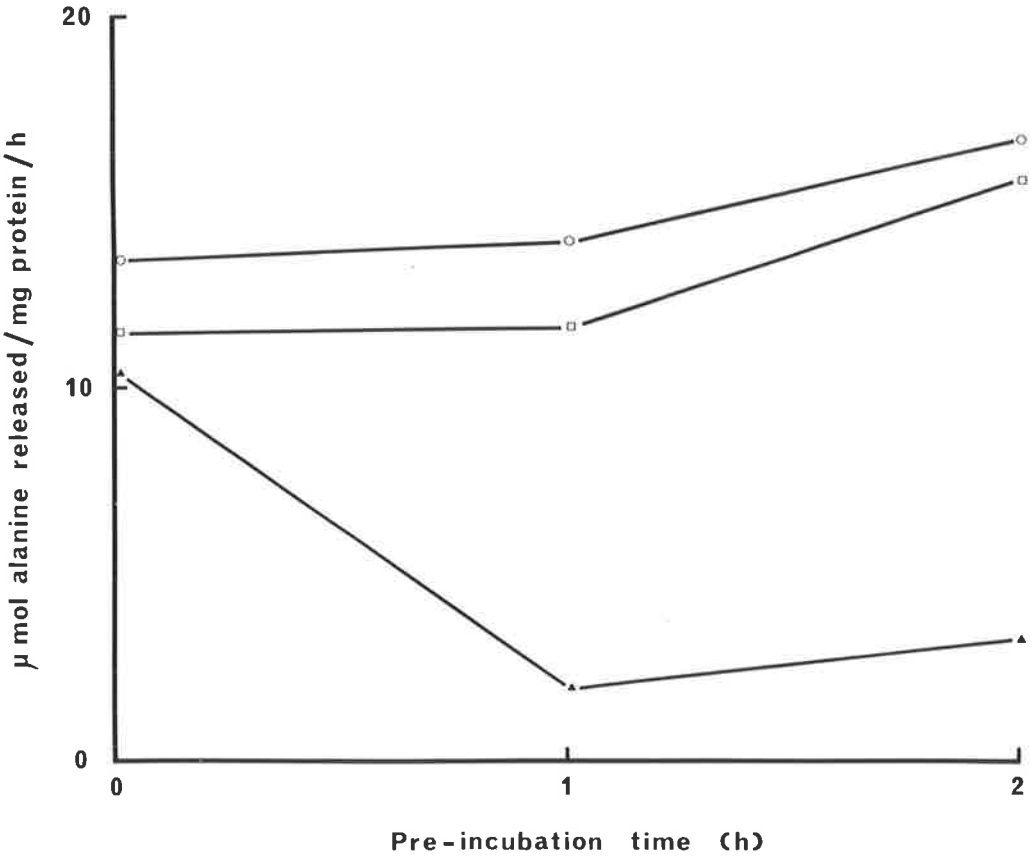


FIGURE 29

Effects of sulphydryl reagents on the activity of protease B from maize roots

A Samples of protease B (0.225 mg/ml) were incubated with either cysteine, mercaptoethanol or dithiothreitol (3 volumes enzyme : 1 volume of sulphydryl reagent) at the final concentrations shown for 2 h at 37°C. 0.1 ml aliquots were then withdrawn and assayed for haemoglobin degrading activity as described in Section 2.5.4.2. Initial activity was 7.3 µmol α-amino N released/mg protein/h and the enzyme incubated with water did not change in activity during incubation.

B Experimental details are described in A. 0.05 ml aliquots were assayed for carboxypeptidase activity as described in Section 2.5.4.3. Initial activity was 19.2 µmol alanine released/mg protein/h and the sample incubated with water increased in activity by 13% during incubation.

○——○ Cysteine

△——△ Mercaptoethanol

□——□ Dithiothreitol

Fig 29A

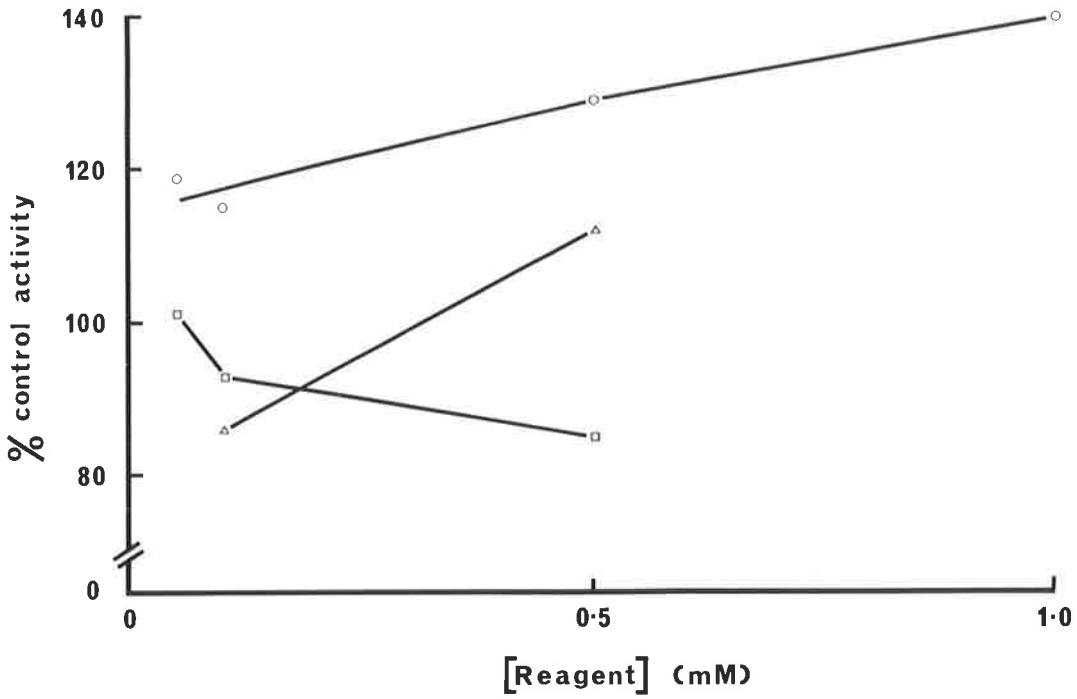
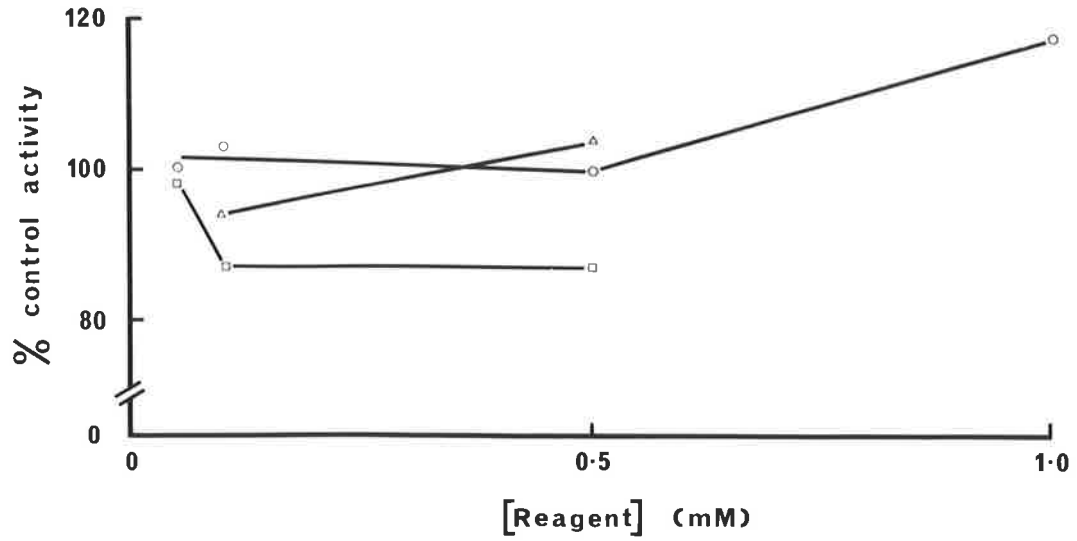


Fig 29B



formed during concentration with Sephadex G-25 were dialysed against either 20 mM sodium acetate pH 4.0, 20 mM sodium acetate pH 5.0 containing 0.6 M NaCl or 20 mM sodium phosphate pH 7.0. The highest recovery of both the carboxypeptidase and proteinase activities was in the buffer at pH 4.0 and both enzymes were most soluble in this buffer (Table 21). As the solubility of both the carboxypeptidase and the proteinase in the buffer at pH 5.0 containing salt was intermediate between their solubilities at pH 5.0 and 7.0, it appears that salt does not markedly affect the solubility of protease B.

3.11 Comparison of protease activities in leaf and root tissues of maize and wheat

In maize and wheat seedlings, the most significant finding was that high levels of azocasein degrading activity and nitrate reductase inactivating activity were restricted to primary and adventitious roots of maize (Table 22). Relatively low levels of both activities were found in leaf and root tissues and only a trace of azocasein degrading activity was found in maize leaves. There was less variation in the distribution of haemoglobin degrading activity, the highest levels being found in wheat leaves and maize roots. Because the numerical ratio of azocasein degrading activity to haemoglobin degrading activity of proteinase A from maize roots is about 2:1 (Table 14), approximately half of the latter activity of maize roots may be attributed to the action of the azocasein degrading species. High levels of exopeptidase activity were found in leaves of both wheat and maize; wheat leaves had the highest carboxypeptidase activity while maize leaves had the highest amino-peptidase activity. The data in Table 22 show that the pattern of protease activities in the primary and adventitious roots of the maize seedlings was virtually identical.

TABLE 21

Redissolving precipitated protease B

The resuspended pellet of protease B described in Table 20 was divided into 0.25 ml aliquots which were dialysed against the buffers shown for 24 h at 4°C. The dialysis tubes were rinsed with 0.1 ml of dialysis buffer after removal of the dialysis residue. The contents of the dialysis tubes (including rinsing solutions) were centrifuged at 8 000 *g* for 10 min at 2°C. The pellets thus formed were resuspended in 0.25 ml of 20 mM sodium acetate pH 5.0. Both the supernatant and pellet fractions in each case were assayed for haemoglobin degrading and carboxypeptidase activities as described in Sections 2.5.4.2 and 2.5.4.3.

Dialysing buffer	Fraction	Carboxypeptidase activity in each fraction*	Carboxypeptidase activity in each buffer* (total)	Proteinase activity in each buffer†	Proteinase activity in each buffer† (total)
20 mM sodium acetate pH 4.0	pellet	0.29		0.021	
	supernatant	6.1		0.54	
			6.4		0.56
20 mM sodium acetate pH 5.0 containing 0.6 M NaCl	pellet	1.63		0.11	
	supernatant	3.9		0.43	
			5.5		0.54
20 mM sodium phosphate pH 7.0	pellet	0.84		0.032	
	supernatant	3.0		0.30	
			3.8		0.33

* µmol alanine released/h

† µmol α-amino N released/h

TABLE 22

Distribution of protease activities in leaves and roots of maize and wheat and the effects of some inhibitors

Wheat and maize seedlings were grown in sand for 18 days as described in Section 2.4. Extracts were prepared in 50 mM sodium phosphate pH 7.0 containing 0.5 mM Na_2EDTA, 5 mM cysteine and 1% (w/v) Polyclar AT as described in Section 2.5.1, centrifuged at 18 000 *g* for 15 min and the supernatant fractions were retained. 35-70% $(NH_4)_2SO_4$ ppts., prepared as described in Section 2.5.2, were resuspended in and dialysed against 20 mM sodium phosphate pH 7.0. pH 4.4 supernatants were prepared from the dialysed 35-70% $(NH_4)_2SO_4$ ppts. as described in Section 2.5.3. Aminopeptidase, which is labile at pH 4.4 (Section 3.2.2), was estimated in the 35-70% $(NH_4)_2SO_4$ ppts. while all other determinations were made on the pH 4.4 supernatant fractions.

Activity (per g fr wt/h)	Wheat leaves	Wheat roots	Maize leaves	Maize adventitious roots	Maize primary roots
Aminopeptidase (µmol *p*-nitroaniline released)	6.2	1.3	12	4.3	4.1
Carboxypeptidase (µmol alanine released)	16	2.7	11	3.0	3.6
Azocasein degradation (ΔA_{440})	0.18	0.27	0.04	1.5	1.9
Haemoglobin degradation (µmol α-amino N released)	1.1	0.60	0.53	1.3	1.3

TABLE 22 (Cont.)

Activity (per g fr wt/h)	Wheat leaves	Wheat roots	Maize leaves	Maize adventitious roots	Maize primary roots
Nitrate reductase inactivation (units nitrate reductase inactivated)	87	224	173	1 581	1 527
% inhibition of nitrate reductase inactivation by 1 mM PMSF	0	0	22	100	96
% inhibition of nitrate reductase inactivation by 1 mM 1,10-phenanthroline	0	0	39	0	0
protein (mg/g fr wt)	0.83	0.12	0.34	0.12	0.10

The nitrate reductase inactivating activity of maize roots was inhibited by PMSF. Inactivation of nitrate reductase by proteases from other tissues was either insensitive to PMSF (wheat roots and leaves) or inhibited only 22% (maize leaves). Nitrate reductase inactivating activity from maize leaves was also only partially inhibited (39%) by 1,10-phenanthroline.

When the data for primary roots are compared with those for endosperms of maize seedlings of the same age, azocasein degrading activity was again predominantly found in the roots (Table 23). The endosperm sample contained nitrate reductase inactivating activity but it was insensitive to PMSF. Both tissues have similar levels of carboxypeptidase and haemoglobin degrading activities. As discussed above, the azocasein degrading species may be responsible for about one third of the haemoglobin degrading activity of maize roots.

3.12 Influence of age on protease activities and soluble protein content of maize roots

In Table 24 it can be seen that the protein content of crude extracts of primary maize roots decreased 50 fold between 7 and 14 days. Between 4 and 14 days there was a 21 fold increase in the specific activity of the azocasein degrading species and a 45 fold increase in that of the nitrate reductase inactivating species. The specific activity for azocasein degradation rose 4 fold between the 4th and 7th days while the corresponding increases for haemoglobin degradation and carboxypeptidase were 2 and 1.6 fold respectively. The specific activity of the carboxypeptidase increased almost 4 fold between the 7th and 14th days while there was an increase of 1.9 fold in the specific activity of the haemoglobin degrading species. Part of the latter increase may be due to the action of the azocasein degrading species on haemoglobin.

TABLE 23

Distribution of protease activities in maize roots and endosperms

Primary roots of 4 day maize seedlings grown on 1% (w/v) agar containing 5 mM KNO_3 in 0.1 strength Hoagland's solution were extracted with 50 mM sodium phosphate pH 7.0 containing 0.5 mM Na_2EDTA and 5 mM cysteine. The extract was centrifuged at 27 000 *g* for 15 min at 2°C and a 40-65% saturation $(NH_4)_2SO_4$ ppt. was prepared from the supernatant fraction as described in Section 2.5.2. The $(NH_4)_2SO_4$ ppt. was resuspended in 20 mM sodium phosphate pH 7.0 and dialysed overnight against two changes of the same buffer at 4°C. A pH 4.4 supernatant was prepared from the dialysed $(NH_4)_2SO_4$ ppt. as described in Section 2.5.3.

Endosperms of 4 day maize seedlings grown on 1% (w/v) agar containing 10 mM KNO_3 and 5 mM $Ca(NO_3)_2$ in 0.1 strength Hoagland's solution were extracted with 0.2 M sodium acetate pH 4.0. A 35-70% $(NH_4)_2SO_4$ ppt. was prepared as described above for maize roots.

The pH 4.4 supernatant prepared from roots and the 35-70% $(NH_4)_2SO_4$ ppt. prepared from endosperms were analysed for protease activities as described in Sections 2.5.4.1, 2.5.4.2, 2.5.4.3 and 2.5.4.4.

Activity (per g fr wt/h)	Roots	Endosperms
Azocasein degradation (ΔA_{440})	1.5	0.01
Haemoglobin degradation (µmol α-amino N released)	2.2	2.1
Nitrate reductase inactivation (units nitrate reductase inactivated)	760	110
Carboxypeptidase (µmol alanine released)	3	1.6

TABLE 24

Influence of age on protease and soluble protein contents of maize seedlings

Maize seedlings were grown for 4 days on 1% (w/v) agar containing 0.1 strength Hoagland's solution and thereafter with the primary root immersed in 0.1 strength Hoagland's solution as described in Section 2.4. Extracts were prepared as described in Section 2.5.1 and centrifuged at 18 000 *g* for 15 min (4 day extracts) or at 95 000 *g* for 1 h (7 and 14 day extracts). The supernatants were dialysed overnight against two changes of 20 mM sodium phosphate pH 7.0 and analysed for protease activities and protein content as described in Sections 2.5.4.1, 2.5.4.2, 2.5.4.3, 2.5.4.4 and 2.7.3.

	4 day	7 day	14 day
Protein (mg/g fr wt)	2.2	1.8	0.36
Azocasein degradation (Δ A_{440}/mg protein/h)	0.46	1.7	9.8
Haemoglobin degradation (µmol α-amino N released/mg protein/h)	2.1	4.2	7.8
Carboxypeptidase (µmol alanine released/mg protein/h)	5.1	8.3	31
Nitrate reductase inactivation (units nitrate reductase inactivated/mg protein/h)	203	-	9 100

The effects of age on the components of protease A and B were investigated after the protease fractions had been separated on a CM-cellulose column (Figure 7). Azocasein degrading, carboxypeptidase and nitrate reductase inactivating activities of protease A increased by 1.4, 1.3 and 6.2 fold between the 7th and 14th days (Table 25). In contrast, the total activities of proteinase B and carboxypeptidase B decreased 2 and 1.2 fold respectively over the same period although the nitrate reductase inactivating activity of this fraction doubled.

TABLE 25

Influence of age on the protease activities of proteases A and B from maize roots

Primary roots of 400 maize seedlings were harvested at either 7 days (63 g fr wt) or 14 days (213 g fr wt). Proteases A and B were prepared as described in Sections 3.1 to 3.3. Protease activities and protein content of the two fractions were determined as described in Sections 2.5.4.1, 2.5.4.2, 2.5.4.3, 2.5.4.4 and 2.7.3.

Protease A				
Age of plants (days)	Protein (mg)	Azocasein degradation (ΔA_{440}/h)	Carboxypeptidase (µmol alanine) released/h)	Nitrate reductase inactivation ($\times 10^{-3}$ units nitrate reductase inactivated)
7	1.2	154	376	39
14	2.3	222	502	241
Protease B				
Age of plants (days)	Protein (mg)	Haemoglobin degradation (µmol α-amino N released/h)	Carboxypeptidase (µmol alanine released/h)	Nitrate reductase inactivation ($\times 10^{-3}$ units nitrate reductase inactivated)
7	2.6	55	71	3.6
14	4.3	28	59	7.0

4. DISCUSSION

4. DISCUSSION

4.1 Appraisal of techniques used

The work described in this thesis embodies the first detailed account of the separation and characterisation of the proteases in plant roots.

The assay of proteinases has been based on their action on haemoglobin and azocasein as substrates. Haemoglobin, although it is not a physiological substrate, has been shown to be degraded by proteinases from a range of plants at acidic pH values (Abe *et al*, 1977; Drivdahl and Thimann, 1977; Frith *et al*, 1978a). Zein, a storage protein from maize, was degraded by a proteinase from maize roots in this study but it was not used routinely as a substrate because of its insolubility in aqueous solution. Casein is also a substrate for many plant proteinases (Wells 1968; du Toit, 1976, Feller *et al*, 1978) and its derivative azocasein has been used in a convenient assay procedure for a range of proteinases (Molano and Gancedo, 1974; Peterson and Huffaker, 1975; Starkey and Barrett, 1976; Thomas, 1973) at neutral and alkaline pH. It was found in the present study that the assay of degradation products of haemoglobin by the ninhydrin reagent as modified by Lee and Takahashi (1966) resulted in higher absorbance values than measurements of A_{280}. The reason is that the ninhydrin procedure detects a wide range of amino acids and peptides whereas the UV absorption method only detects aromatic amino acids and peptides containing such residues. Further, absorbance measurements at 280 nm may be spuriously high due to the presence of nucleotides released from nucleic acids (Marrink and Gruber, 1966) as well as phenolic compounds and trichloroacetic acid, the latter is commonly used to precipitate proteins.

Since the assays used in this study for proteinase and carboxypeptidase

activities are relatively non-specific, it is likely that the majority of proteinases and carboxypeptidases in maize roots have been included in the current study. In contrast, other studies with plant carboxypeptidases (Visuri *et al*, 1969) and proteinases (du Toit, 1976) have tended to concentrate on only one or two enzymes.

The most effective separation and purification technique for proteases from maize roots in the present study was found to be ion exchange chromatography. With CM-cellulose columns, marked changes in the elution profiles of the proteases resulted from minor changes in the pH and salt content of the eluting buffer.

4.2 Proteinase A

Proteinase A from maize roots described in this thesis appears to be a serine proteinase with a molecular weight of about 53 000. It has been assayed by its action in degrading azocasein and haemoglobin and in inactivating nitrate reductase. It was also shown to degrade zein, a storage protein of maize as well as soluble protein from maize roots.

No resolution of the azocasein degrading, haemoglobin degrading and nitrate reductase inactivating activities was achieved with a range of ion exchange procedures, gel filtration, hydroxylapatite chromatography or affinity chromatography on a phenylalanine substituted Sepharose column. The lack of resolution during the ion exchange studies is considered to be very significant since resolution of oxidised and reduced forms of cytochrome c was achieved using procedures similar to those employed in the current study (Dixon and Thompson, 1968) and it was suggested that proteins differing by one charge are separable with ion exchange chromatography.

Since proteinase A degraded haemoglobin, it would be expected to

bind to haemoglobin coupled to Sepharose. Thus it should be possible to isolate proteinase A by means of a haemoglobin affinity column, as used to isolate proteinases from wheat leaves (Frith *et al*, 1978c), germinating barley (Burger, 1973) and oat leaves (Drivdahl and Thimann, 1977). However no binding of proteinase A from maize roots to a haemoglobin affinity column was observed. Preston (1978) has shown that proteinases bind to a phenylalanine substituted Sepharose column and such a column was used in this study to bind proteinase A and carboxypeptidases A and A' from maize roots. The proteinase was separated into two fractions but both had similar ratios for activities on azocasein and haemoglobin and for nitrate reductase inactivation. Possibly an interaction between the carboxypeptidases and proteinase resulted in the appearance of two proteinase fractions.

Thus it has been concluded that proteinase A degrades azocasein and haemoglobin and also inactivates nitrate reductase as these three activities were not separated. Furthermore, all three activities were affected similarly by inhibitors although haemoglobin degradation was more sensitive to $HgCl_2$ than azocasein degradation. The inhibition of haemoglobin degradation by $HgCl_2$ could result from a reaction of the inhibitor with haemoglobin rendering it less susceptible to proteolysis. It was also observed that haemoglobin degrading activity was less stable than the azocasein degrading activity after storage at either 4°C or -15°C as well as during incubation at 37°C at pH 7.8 and during the purification procedure. A proteinase from *Lupinus angustifolius* showed selective loss of activity against gliadin during purification and this was attributed to changes in the carbohydrate content of the enzyme (Shepard and Moore, 1978). This suggestion is supported by observations that some rate constants of yeast carboxypeptidase Y appear to be altered by the carbohydrate content of the enzyme (Margolis *et al*, 1978).

Inhibition of proteinase A from maize roots by PMSF and DFP suggests that this enzyme is a serine proteinase. Since 1 mM PMSF inhibited azocasein degradation in the presence of 10 mM cysteine, it is unlikely that this inhibitor reacted with free SH groups as it did with the SH-proteinase papain (Whitaker and Perez-Villaseñor, 1968). Proteinases from bean leaves (Racusen and Foote, 1970), oat leaves (Drivdahl and Thimann, 1978), *Neurospora crassa* (Siepen *et al*, 1975) and yeast (Hata *et al*, 1967a) were also inhibited by DFP or PMSF. While proteinase A described herein and the enzymes from oat leaves (Drivdahl and Thimann, 1978) were insensitive to mercurials, the enzymes from bean leaves (Racusen and Foote, 1970), *Neurospora* (Siepen *et al*, 1975) and yeast (Hata *et al*, 1967a) were inhibited by these compounds. Thus maize root proteinase A does not appear to contain any free SH groups and it was not inhibited by TLCK or TPCK which are inhibitors of the serine proteinases trypsin and chymotrypsin respectively. Since these inhibitors were designed as substrate analogues (Means and Feeney, 1971), proteinase A probably has a different substrate specificity to either trypsin or chymotrypsin.

Proteinase A had a pH optimum of 6.0 when acting on root protein and casein but the pH optima for degradation of haemoglobin and azocasein were 4.0 and 9.0 respectively. Thus it appears that pH alters the susceptibility of proteins to degradation. Since proteins are more susceptible to proteolysis in their unfolded state (McLendon, 1977; Rupley and Scherega, 1963), possibly alterations in pH changed the degree of unfolding of the protein substrates used in this study. When azocasein was the substrate, the apparent pH optimum of both proteinase A of maize roots and trypsin was 9.0, while equivalent values on unsubstituted casein were 6.0 and 7.6 (Hata *et al*, 1967b) respectively. Thus it appears that sulphanilamide alters the susceptibility of casein to

hydrolysis, possibly by facilitating unfolding of the protein at high pH. In the current study, treatment of haemoglobin at pH 1.5 did not alter its susceptibility to degradation at pH 4.0 and treatment at pH 12.0 did not result in a change in the pH optimum of 4.0. A possible explanation could be that the treatments used were not sufficiently vigorous to ensure complete unfolding of the protein or that unfolding was reversible on changing the pH. Other workers have observed that the pH optima of proteinases from wheat seedlings (Wittenbach, 1978) and mung bean seedlings (Chrispeels and Boulter, 1975) depended on the substrate used.

The molecular weight of 53 000 estimated for proteinase A in this study differs from the molecular weight of 44 000 suggested for it by Wallace (1974) when it was then described as the nitrate reductase inactivating enzyme. The Sephadex G-100 column used in the present study yields more accurate estimates of molecular weights around 50 000 (Andrews, 1965) than the Sephadex G-200 column used in the earlier study (Wallace, 1974).

Proteinase A from maize roots has similar properties to yeast proteinase B (Hata *et al*, 1967a) and the alkaline proteinase of *Neurospora crassa* (Siepen *et al*, 1975). All three enzymes were sensitive to PMSF. The maize root enzyme was relatively insensitive to mercurials while the enzymes of yeast and *Neurospora* were inhibited. Proteinase A from maize roots had a pH optimum of 6.0 when casein was the substrate while proteinase B from yeast had a pH optimum of 9.0 for this substrate (Hata *et al*, 1967a). The alkaline proteinase of *Neurospora* had two pH optima at 6.2 and 9.1 when acting on casein (Siepen *et al*, 1975). Maximal stability of the yeast enzyme however was at pH 6.0 (Hata *et al*, 1967a) while the maize root enzyme was stable between pH 4.0 and 8.0. Molecular weights of the three enzymes differ: 53 000 for maize; 32 000 for yeast

(Lenney and Dalbec, 1969); 24 000 for *Neurospora* (Siepen *et al*, 1975).

The K_m of proteinase A described in this thesis when acting on haemoglobin was 0.08 mM. An acid proteinase from sorghum had apparent K_m values of 0.008 mM and 0.002 mM for albumin and N,N-dimethylalbumin respectively (Garg and Virupaksha, 1970) while the K_m of proteinases from germinating barley seeds for N,N-dimethylhaemoglobin was 0.009 mM (Burger, 1973).

4.3 Proteinase B

It is difficult to classify proteinase B of maize roots. Inhibition by pCMB and $HgCl_2$ suggests that it is an SH-proteinase. However inhibition by DFP and PMSF also suggests that a serine residue is present in the active site although the degree of inhibition (41% and 58% respectively) was less than that observed for proteinase A from maize roots. It is possible that PMSF reacted with SH groups of proteinase B described herein as it does in papain (Whitaker and Perez-Villaseñor, 1968) and that impurities in DFP also reacted with SH groups (Gould and Liener, 1965). Since proteinase B has a pH optimum of 4.0, it may be a pepsin like acid proteinase which is inhibited by DAN (Lundblad and Stein, 1969). However this inhibitor requires Cu^{2+} for a rapid, specific reaction with aspartic acid residues (Lundblad and Stein, 1969). When proteinase B was incubated with Cu^{2+} and DAN under conditions similar to those used by Frith *et al* (1978b), the Cu^{2+} inhibited the enzyme. Therefore it was not possible to determine whether DAN inhibits proteinase B.

Preston and Kruger (1976) isolated two carboxypeptidases from wheat seedlings which also degraded haemoglobin. Proteinase B of maize roots which degraded haemoglobin did not appear to be a carboxypeptidase because haemoglobin degrading activity and carboxypeptidase activity had different elution profiles on a Biogel A-0.5m column. Dissimilar elution profiles

of the two activities were also observed with DEAE-Sephadex columns and the proteinase was more sensitive than the carboxypeptidase to mercurials, glycerol and possibly PMSF.

While proteinase A and carboxypeptidase A' were easily separated by gel filtration, some difficulty was experienced with gel filtration of protease B components. At pH 6.0 in the presence of 0.1 M NaCl, proteinase B activity was resolved from carboxypeptidase and the associated nitrate reductase inactivating species. However since some marker proteins, particularly α-chymotrypsinogen and pancreatic carboxypeptidase A, were retarded on the Biogel column under these conditions, there is considerable doubt about the validity of the molecular weight estimates for protease B components obtained from this column. At a pH of 4.5, which favoured the solubility of the protease B components, all proteins tested were adsorbed to the column even in the presence of 0.1 M NaCl. Thus contrary to the claims of the manufacturer, Biogel A-0.5m can only be used satisfactorily for gel filtration near neutral pH. Lin and Castell (1978) have also recently reported that agarose gels should be used at neutral pH for gel filtration.

The procedures used to resolve the protease A components of maize roots on DEAE-Sephadex were found to be unsatisfactory for protease B because both proteinase and carboxypeptidase activities were dispersed throughout the gradient. Adsorption of the proteins on the resin may have concentrated the enzymes sufficiently to result in precipitation as was the case when protease B was concentrated against Sephadex G-25. The subsequent elution of the column with a salt gradient may have solubilised the enzymes as well as desorbing them from the resin. The lack of resolution may also have been due to the presence of many forms of proteinase B and carboxypeptidase B which differ only slightly in the number of negative charges available for interaction with the resin.

Differences in the number of negative charges carried by a protein may be associated with the attachment of different amounts of acid sugars as described for cathepsins of animal cells (Dean, 1975b). Neuraminidase has been used to remove such sugars to reduce differences in charge (Dean, 1975b) but this enzyme cleaves bonds adjacent to sialic acid residues which have not been found in plants (Jennings, 1978). As it was not known which carbohydrate residues might be present, no attempt was made to remove carbohydrates which may have been present in protease B. Different degrees of amidation, as in ficin (Kramer and Whitaker, 1969b), may also result in differences in the charge carried by subspecies of a protein.

Autolysis may result in the formation of subspecies of a proteinase which differ in size and charge, as observed in a proteinase from oat shoots (Pike and Briggs, 1972). However subspecies of proteinase B differing in positive charge or size were not detected by chromatography on either CM-cellulose or Biogel A-0.5m respectively. It was claimed that autolysis did not occur when proteinases from fig (Kramer and Whitaker, 1969a) and barley (Burger, 1973) were fractionated. Six species which degrade haemoglobin at acidic pH have been isolated from wheat leaves by chromatography on DEAE-~~Sephadex~~ Cellulose and there was no evidence that autolysis occurred (Frith *et al*, 1978a).

4.4 Carboxypeptidases A, A' and B

Carboxypeptidase A and a minor related species A' described in this thesis have the characteristics of typical plant carboxypeptidases as both are inhibited by PMSF, have a pH optimum of about 5.0 and a molecular weight of about 97 000. Separation of the two enzymes on DEAE-Sephadex, different sensitivities to pCMB and $HgCl_2$ and different rates of hydrolysis of BAEE suggest that these two carboxypeptidases are separate species.

Carboxypeptidases A and A' of maize root may be compared with the carboxypeptidases from bean leaves and yeast (Wells, 1965; Hata *et al*, 1967b). The presence of a serine residue in the active site of the latter two DFP sensitive enzymes has been confirmed by isolation of amino acid sequences containing a serine residue which has reacted with labelled DFP (Hayashi *et al*, 1973b; Shaw and Wells, 1972). Lysosomal carboxypeptidases A and C from animal tissues are also inhibited by DFP (Barrett and Heath, 1977) while pancreatic carboxypeptidases A and B require a Zn^{2+} ion for activity (Pétra, 1970;Folk, 1970).

Carboxypeptidase A from maize roots was not affected by pCMB or $HgCl_2$ unlike most plant carboxypeptidases (Table 3). However carboxypeptidase A' was sensitive to mercurials. Possibly the A enzyme either lacks free SH groups or such groups are inaccessible to the SH inhibitory reagents tested.

The molecular weight of 97 000 for both carboxypeptidases A and A' reported in this thesis falls within the range reported for other plant carboxypeptidases determined by gel filtration (Table 3).

The substrate Cbz-Phe-Ala was hydrolysed about twenty times faster than Cbz-Glu-Tyr by carboxypeptidase A from maize roots. The former peptide was also the best substrate for carboxypeptidases of wheat seedlings, barley seedlings and watermelon sarcocarp (Preston and Kruger, 1977; Visuri *et al*, 1969; Matoba and Doi, 1975). However Cbz-Glu-Tyr was used as a peptide substrate for the carboxypeptidases of yeast and *Aspergillus* (Hayashi and Hata, 1972; Ichishima, 1972).

Carboxypeptidase A from maize roots has esterase activity against BAEE which was also hydrolysed by carboxypeptidases of cottonseed and yeast (Ihle and Dure, 1972a; Doi *et al*, 1967). In work reported in this thesis, carboxypeptidase A' had little or no activity towards BAEE and

neither the A nor A' enzyme hydrolysed the esters TAME or ATEE. Possibly carboxypeptidase A' hydrolyses some other ester since most carboxypeptidases from plants have esterase activity (Table 3).

The K_m of carboxypeptidase A from maize roots utilising Cbz-Phe-Ala was 5 mM which is higher than those reported for carboxypeptidases from *Aspergillus* (Ichishima, 1972), watermelon sarcocarp (Matoba and Doi, 1975), wheat seedlings (Preston and Kruger, 1977) and bean leaves (Carey and Wells, 1972) which are in the range 0.5 - 4 mM. However a carboxypeptidase from barley seedlings had a K_m of 6.7 mM when acting on Cbz-Phe-Ala (Visuri *et al*, 1969).

The lability of carboxypeptidase A above pH 6.0 reported in this thesis is similar to the behaviour of the carboxypeptidases of barley seedlings (Visuri *et al*, 1969) and watermelon sarcocarp (Matoba and Doi, 1974a).

Differences between carboxypeptidases A and A' of maize roots in their chromatographic behaviour, sensitivity to mercurials and action on BAEE may be explained by the two enzymes having different amino acid sequences or carbohydrate contents (Margolis *et al*, 1978). If the two enzymes contain different amounts of carbohydrate, they will differ in molecular weight but differences of the order of several thousand are not detected by gel filtration on Sephadex G-100. Since carboxypeptidase A' was eluted from DEAE-Sephadex by a lower salt concentration than carboxypeptidase A, it appears to have less negative charges which interact with DEAE-Sephadex. Possibly A' has fewer acidic residues than A or else the carbohydrate present in the A' enzyme alters the interaction of negative amino acid residues with the resin by shielding effects or changing the shape of the molecule (Margolis *et al*, 1978). Differences between the A and A' enzymes in their sensitivity to mercurial agents

and in their rates of hydrolysis of BAEE suggest that the active sites of these two enzymes are different. It has recently been shown that the carbohydrate content of yeast carboxypeptidase Y alters some of its kinetic properties (Margolis *et al*, 1978). If carboxypeptidases A and A' differ in their carbohydrate contents, variations in the relative amounts of the two enzymes in different root extracts may be due to small changes in growth conditions of the maize seedlings which lead to changes in the amount of carbohydrate attached to the carboxypeptidases. It has been shown that differences in carbohydrate content of carboxypeptidase Y of yeast occur in different batches of the same strain (Margolis *et al*, 1978).

Carboxypeptidases BI and BII appear to be typical plant carboxypeptidases since both PMSF and SH group reagents were inhibitory. The pH optimum near 5.0 is typical of plant carboxypeptidases (Section 1.1.3). When isolated on Biogel A-0.5m, the molecular weights of the BI and BII enzymes described herein appeared to be less than 40 000, but as discussed earlier (Section 4.3), these estimates are unreliable. The limited studies performed did not indicate any major differences between the BI and BII enzymes apart from their size. These two enzymes resemble carboxypeptidases A and A' described in this thesis in their sensitivity to PMSF and in their pH optimum near 5.0 but resemble only carboxypeptidase A' in their sensitivity to mercurials.

4.5 Nitrate reductase inactivation

It has been shown in this thesis that the nitrate reductase inactivating activity of maize root described by Wallace (1974) is due to proteinase A. In *Neurospora crassa*, two proteolytic species which inactivate nitrate reductase have recently been described (Sorger *et al*, 1978) and one of these resembles maize root proteinase A in its sensitivity to PMSF.

Relatively low levels of proteinase A were found in other parts of the maize or wheat seedlings compared to that of maize root. Nitrate reductase inactivating activity observed in tissues other than maize roots may be due to the action of other proteases or to the non-proteolytic type of inactivators described in rice seedlings (Yamaya and Ohira, 1976) and soybean leaves (Jolly and Tolbert, 1978). A similar inactivator of nitrate reductase was also identified in the current study in the protease B fraction of maize roots. This inactivator resembles that of rice seedlings (Yamaya and Ohira, 1977) being insensitive to PMSF and casein but inhibited by metal chelating agents. As nitrate reductase has not yet been crystallised from higher plants because of its lability, it has not yet been possible to determine whether proteinase A from maize roots cleaves only a restricted number of bonds as do the group specific proteinases of rat tissues (Kobayashi and Katanuma, 1978; Kobayashi *et al*, 1978). The nitrate reductase inactivating enzyme, which now appears equivalent to proteinase A described herein, was shown previously not to inactivate nitrite reductase, isocitrate lyase, invertase or glutamate dehydrogenase from maize (Wallace, 1978b) but inactivated tryptophan synthase and fructose-1,6-bisphosphatase from yeast (Wallace, 1978a). Thus it appears to have some specificity in its action on proteins.

The sensitivity of nitrate reductase to proteolytic attack *in vitro* may be correlated with its relatively high turnover rate (half-life in maize roots 2-3 h, Oaks *et al*, 1972) because enzymes with short half-lives *in vivo* are usually more susceptible to proteolytic inactivation *in vitro* (Bond, 1971). In addition, large, acidic proteins generally have short half-lives (Section 1.2.2) so that the lability of nitrate reductase may be correlated with its molecular weight of about 200 000 and isoelectric point below 5 (Notton *et al*, 1972). Thus the susceptibility of nitrate reductase to inactivation by proteinase A

may be due to some structural feature which facilitates attack by the proteinase. Nitrate reductase was also inactivated by yeast proteinase B which has similarities to maize root proteinase A (Wallace, 1978a). However nitrate reductase was not affected by proteinase B from maize roots and appeared less susceptible to attack by proteinases from leaves and roots of wheat and from leaves and endosperms of maize than by proteinase A from maize roots. Thus nitrate reductase may be affected by a limited number of proteinases.

It is likely that nitrate reductase is located in the cytoplasm of maize roots (Dalling *et al*, 1972) while proteinase A is probably found in the vacuole (as discussed later). It has been proposed that enzymes in the cytoplasm are inactivated by some non-proteolytic agent before they are degraded by proteases in the lysosome or vacuole (Section 1.2.3). Nitrate reductase may be inactivated in the cytoplasm by the nitrate reductase inactivator associated with protease B described in this thesis. Inactive nitrate reductase could then be transported into the vacuole where it is degraded by proteinase A. It will however be necessary to determine the subcellular location of proteinases and other nitrate reductase inactivators before this hypothesis can be verified.

4.6 Distribution of proteases in plants and effect of age

It would appear from this study on the maize root and the data in the literature that there is only a limited number of proteinases in each organism. Two proteinases which have been named A and B have been identified in the current study with maize roots and similarly two proteinases have been characterised in both yeast and *Neurospora crassa* (Hata *et al*, 1967a, Siepen *et al*, 1975). As discussed previously, proteinase A of maize roots is similar to proteinase B of yeast and the alkaline proteinase of *Neurospora* while proteinase B of maize is similar

to proteinase A of yeast and the acid proteinase of *Neurospora* in having an acid pH optimum when acting on haemoglobin. Bean leaves contain two proteinases (Wells, 1968), one of which has been identified as a serine type (Shaw and Wells, 1967) and two proteinases have also been isolated from oat leaves (Drivdahl and Thimann, 1977). Six fractions degrading haemoglobin, of which at least three are proteinases, have been isolated from wheat leaves (Frith *et al*, 1978a) but the relationship between them has not yet been fully determined. In maize tissues, Feller *et al* (1978) have shown that proteinase activity with casein as substrate was partially inhibited by PMSF and pHMB. The effects of these inhibitors in the study of Feller *et al* (1978) are consistent with the action of proteinases A and B described herein which are sensitive to PMSF and pCMB respectively. In a study of endopeptidases of maize, Melville and Scandalios (1972) found that only one enzyme hydrolysed BANA (α-N-benzoyl DL-arginine β-napthylamide HCl). This enzyme resembled proteinase B described herein in that its activity declined with plant age.

Cathepsin B, an SH-proteinase, and cathepsin D, an acid proteinase, are found in many animal tissues (Barrett and Heath, 1977) while other cathepsins appear restricted to polymorphonuclear leukocytes and bone marrow. Thus it appears that eukaryote cells generally contain only two intracellular proteinases.

Four carboxypeptidase species have been identified in maize roots. As discussed earlier, carboxypeptidases A and A' may differ only in their carbohydrate contents. In both wheat seedlings (Preston and Kruger, 1976) and watermelon (Matoba and Doi, 1974a), two carboxypeptidases have been isolated which differ only in size. Possibly more carboxypeptidases would have been identified by other workers had a greater range of separation techniques been used.

The proteases described in this thesis were found in the soluble fraction of maize roots and this location is consistent with a vacuolar location *in vivo* since vacuoles would be ruptured under the extraction conditions used. Moreover the solubility and stability of the proteases (apart from aminopeptidase) at pH 4.4 is also in agreement with the suggestion that they occur in vacuoles. Matile (1968) and Zuber and Matile (1968) have shown that proteinase and carboxypeptidase activities of root tip cells of maize are found in the vacuole. As described in Section 1.1.2, plant proteinases appear to be located in the central vacuole. Proteases of yeast, which are similar to those of maize roots as discussed previously, are found in the central vacuole (Lenney *et al*, 1974).

The increase in the total activities of proteinase A and carboxypeptidase A per root with age suggests that these enzymes are being continuously synthesized. In contrast, levels of protease B and carboxypeptidase B per root declined with age. The activity of an acid proteinase from maize endosperm which resembles proteinase B described herein declined (per g fr wt) from the 7th to the 14th day (Harvey and Oaks, 1974). Feller *et al* (1978) reported that between 4 and 7 days the activities (per g fr wt) of carboxypeptidase and casein degradation measured at pH 7.5 in maize roots increased but there was no change in casein degradation assayed at pH 5.4. The results reported in this thesis are in general agreement with those of Feller *et al* (1978). Low levels of soluble protein in older roots could have resulted from an increase in protein degradation which may be due to the action of protease A described herein. Decreases in the levels of proteinase B and carboxypeptidase B per plant with age suggest that these enzymes are important in younger tissues.

Higher activity (per g fr wt) for proteinase in roots rather than

in leaves of maize seedlings has also been reported by Feller *et al* (1978). In work reported in this thesis, in 18 day old wheat seedlings the specific activities for the degradation of both haemoglobin and azocasein were higher in roots than in leaves. A higher specific activity for acid proteinase in roots than leaves of wheat seedlings was also reported by Frith *et al* (1975). In potato plants, protease activity (per g fr wt) was higher in roots than leaves (Santarius and Belitz, 1978).

The high activity (per g fr wt) for the degradation of azocasein in maize roots is noteworthy since low levels were found in maize endosperms and leaves as well as in wheat leaves and roots. However the highest activities (per g fr wt) of carboxypeptidase and aminopeptidase were located in leaves of maize and wheat. Feller *et al* (1978) also observed higher levels of aminopeptidase in maize shoots than roots while levels of carboxypeptidase in the two tissues were the same. The significance of this distribution of peptidase activities is unclear for it is generally thought that peptidases have a secondary role in protein degradation (Section 1.1.3).

No major differences were found in this study between the types of proteases found in the primary and adventitious roots of maize seedlings. Thus senescence of the primary roots did not appear to influence protease activities in the primary roots examined in this thesis.

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