Molecular and Biochemical Characterisation of Esterases from *Oenococcus oeni* and Their Potential For Application In Wine

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Abstract

This study examined the complex array of ester synthesis and hydrolysis activities in whole cells by cloning, heterologous expression, partial purification, and biochemical characterisation of EstA2, EstB28 and EstCOo8 esterase proteins from *O. oeni* alongside EstC34, an esterase from *Lactobacillus hillgardii*. With a view to applying these esterases to wine, enzyme function under frequently encountered harsh physicochemical conditions in wine was examined. EstB28, EstCOo8 and EstC34 showed highest activity with *p*NP-acetate (C_2) and *p*NP-butanoate (C_4). The highest activity of EstA2 was observed with *p*NP-hexanoate (C_6). All enzymes retained at least some activity under conditions relevant to winemaking. However, only EstB28 and EstA2 showed an increase in activity above 10% ethanol. EstB28 and EstA2 also had a higher relative activity to EstCOo8 and EstC34. Once characterisation of these esterases showed that they should retain at least partial activity under wine-like conditions, EstA2 and EstB28 were assayed for activity in two separate wine samples by SPME-GCMS. Studies were also conducted to determine activity on natural substrates. Synthesis and hydrolysis of ethyl esters with acyl chain lengths of between C_2-C_8 was measured by SPME-GCMS. All four esterases where found to have the ability to synthesise and hydrolyse ethyl esters under optimal conditions (pH 5.0).

Observed strain-specific differences in whole cell ester hydrolysis were also investigated. Firstly wine MLF trials were conducted in Chardonnay and Cabernet Sauvignon wines to determine if these strain specific differences could be translated into hydrolysis ability in wine. Eight strains of LAB were initially used in this study and strains with the lowest activity against *p*NP-linked ester substrates (Ooeni2, Ooeni3, Ooeni6, Lac34 and Lac40) were compared to the strains with the highest activity (Ooeni8, Ooeni12 and Ooeni28).

In an effort to better understand the *O. oeni* strain specific differences in esterase activity real-time qPCR was carried out on an independent set of samples (n = 30) using strain Ooeni28 („high“ activity against *p*NP-linked ester substrates). Expression of esterase genes was measured in the presence of known esterase substrates. Both *estA2* and *estA7* showed an increase in expression in the presence of ethanol and butyric acid, whereas *estB28* and *estC* expression increased in the presence of ethyl butyrate. However, when the same experiment was repeated with Ooeni2 („low“ activity) there was little change in the expression of the characterised esterase genes. Further investigation is required to determine if this is response is related to the phenotype of „low“ activity.

Finally esterase genes were also sequenced from strains demonstrating „low“ and „high“ esterase hydrolysis activity to establish if there are any differences in predicted protein sequences amongst these strains. All strains sequenced had a homologue of EstA2, EstA7 and EstB28. Based on EstA2 and EstB28 sequences the strains with „high“ whole cell activity can be separated from strains with „low“ whole cell activity through single nucleotide polymorphisms (SNP).

This study will improve the understanding of the functioning of LAB esterases in wine conditions and the reason for strain specific differences in activity, with a view towards modulating and controlling the impact of LAB in ester profile modifications during the MLF.

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Thesis Structure and Overview

Overview

The aim of this study was to characterise esterase enzymes from wine LAB and to investigate the range of these enzymes and how they might fit into the context of whole cell activity. The possibility of exploiting these enzymes to alter the aroma profile of wine was also investigated. Six papers have been either published or submitted for publication during the course of this research. Therefore this thesis is submitted as a combination of publications or manuscript. In addition, a conventional thesis chapter is included on the nucleotide sequencing of *O. oeni* strains and their comparison with previously observed esterase activities.

The first chapter paper is a published literature review outlining the importance of esters and esterase activity in wine in both LAB and *Saccharomyces* spp. Following on from this, Chapters 2 through 4 report the cloning and characterisation of four esterase enzymes from LAB; three from *O. oeni* and one from *Lactobacillius hilgardii*. Chapter 4 also includes comparison of the activities of two purified esterase enzymes in wine.

Chapters 5 – 7 describe efforts to identify the basis for strain specific differences in esterase activity. Thus Chapter 5 reports the ability of esterase enzymes to both hydrolyse and synthesise esters in an aqueous environment, and compares strain specific differences in wine to hydrolysis seen against natural substrates, as previously observed for these strains. Following on from this Chapter 6 reports use of *in silico* methods (translation of the nucleotide sequences into the predicted protein sequence and analysis of single nucleotide polymorphisms (SNPs)) to further understand strain specific differences. The presence of SNPs and their potential to affect protein function was then compared with each strains‟ hydrolysis activity. Finally Chapter 7 describes initial experiments using

real-time qPCR to investigate esterase gene expression in the presence of natural substrates.

Introduction

Lactic acid bacteria (LAB), particularly *Oenococcus oeni* are important in winemaking as they carry out malolactic fermentation (MLF) and deacidify wine. *O. oeni* is the species most commonly used to carry out MLF in wine. It is acidophilic and indigenous to wine and similar environments. Aside from impacts on acidity, LAB can metabolise precursors present in wine during fermentation, and as a consequence can alter the chemical composition of the wine. Aroma compounds such as esters and the quantities in which they are present can play an important role in determining the quality of wine. A significant proportion of esters in wine are formed during the primary fermentation by yeast; after this, LAB have been shown to contribute by increasing and decreasing the ester concentration during MLF.

As a group, esters are a quantitatively significant constituent of beverages such as wine and most are present in wine at concentrations around the threshold value. This implies that minor concentration changes might have a dramatic effect on wine aroma and flavour. For this reason, an understanding of the hydrolysis and synthesis of esters in winemaking and how these may be manipulated is essential. Ester hydrolysis and synthesis can be catalysed by esterases and many LAB produce esterases (EC 3.1.1.1) with high activity towards water-soluble short-chain esters. While extensive research has been carried out on the enzymes responsible for ester formation by wine strains of the yeast *Saccharomyces cerevisiae*, esterase activity for wine related LAB is not well documented.

While there are now three strains of *O. oeni* with published genome sequences (CP000411.1, AAUV00000000, ACSE00000000.1) information on the function of

esterase genes and their potential contribution to food and beverage aroma is still limited. Previous studies have highlighted the potential of wine associated lactic acid bacteria (LAB) as a source of novel esterase enzymes for use as additives in winemaking (Matthews et al. 2004). Prior to this study there were no reports of characterisation of esterases from wine associated LAB. Characterisation of such enzymes may not only have practical implications for processes using LAB but is also of fundamental interest. This is reviewed in the following paper (Sumby et al. 2010, Chapter 1) which summarises the research directed at defining ester changes during fermentation in wine by both *Saccharomyces* spp. and LAB. It also describes what is known about the enzymes and the mechanism responsible for ester synthesis and hydrolysis.

Most characterisation of esterases in LAB has focused on dairy isolates (Fenster et al. 2000; Fernandez et al. 2000; Fenster et al. 2003a; b; Choi et al. 2004). Parallel work in a wine context has been limited despite general acceptance of the importance of esters in wine. The characterisation of esterase EstB28 from *O. oeni* wine strain Ooeni28 (Chapter 2) was the first report of the characterisation of such enzymes at the biochemical or genetic level in this organism. Until recently, most evidence that wine LAB possess esterase activity came from wine volatile profiling studies which investigated the changes in concentration of individual esters during MLF (Maicas et al. 1999; Delaquis et al. 2000; Ugliano and Moio 2005). Such changes in ester concentration were strain specific and had the potential to greatly affect the final aroma of wine. This study provides information on the esterase activity of wine LAB, by firstly characterising four enzymes responsible for this activity and then subsequently analysing the reasons for strain specific differences.