A *Botrytis cinerea* aspartic protease targets grape pathogenesis-related proteins and facilitates their removal from wine

Nicholas Ian Warnock

A thesis submitted for the degree of
Doctor of Philosophy
at
Flinders University of South Australia

June, 2014

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Abstract

Botrytis cinerea is a devastating plant pathogen whose broad host specificity allows it to infect many economically important crops, including grapes. Many enzymes produced by *B. cinerea* have been implicated in pathogenesis however a specific role for secreted aspartic proteases has not yet been ascertained. *B. cinerea* contains at least 14 genes encoding aspartic proteases (*Bcap1-14*), some of which are highly expressed during the early stages of pathogenesis. One of these proteases, BcAP8, represents 23% of the total secreted protein and 71% of secreted proteolytic activity. Strains in which these *Bcap* genes have been knocked out do not demonstrate an altered virulence phenotype. Nor are they more sensitive to grape pathogenesis-related (PR) proteins, a major defence response raise by the host plant. This is despite evidence that *B. cinerea* aspartic proteases can hydrolyse PR proteins.

In an effort to understand its physiological role, BcAP8 was expressed in *Pichia pastoris* and purified to homogeneity. It was produced as an inactive zymogen containing a glycosylated inhibitory prodomain. At pH 5 and below, this prodomain was removed through autocatalytic cleavage and the enzyme activated. Its maximal activity was at pH 3.5, correlating with the conditions of ripe fruit. Kinetic characterisation with a synthetic peptide substrate revealed that BcAP8 has a similar K_M and k_{cat} to several other fungal aspartic proteases, all of which differ to pepsin, a model gastric aspartic protease.

BcAP8 was demonstrated to be capable of hydrolysing grape chitinase and thaumatin-like (TL) protein, PR proteins which are inherently resistant to proteases including pepsin. This novel activity was investigated by digesting a purified grape TL-protein and determining the sites of cleavage. A substrate specificity was revealed for this enzyme which partially differs from that of pepsin. Whilst both enzymes cleave between hydrophobic residues, BcAP8 appears to have broader substrate specificity and be less prone to inhibition by unfavourable amino acid residues. This may in part explain its ability to digest the normally resistant PR protein of the host.

In order to ascertain the specific features of this specificity which allow BcAP8 to degrade proteolysis-resistant PR proteins, TL protein cleavage sites were mapped to a model three-dimensional structure of the protein and assessed for their likely

exposure to proteases. Accessible bonds which are cleaved by BcAP8 but not by pepsin are proposed as critical points for destabilising the tertiary structure of the TL protein, exposing further cleavage sites which allow its full degradation. This analysis suggests that it is the ability of BcAP8 to cleave bonds containing glutamine, proline, glycine and lysine which confers its ability to hydrolyse PR proteins.

Grape PR proteins persist through the winemaking process and are present in white wine. Despite their stability throughout fermentation, they are unstable in the wine and can aggregate to form an unappealing visible haze. In commercial wine production, these proteins are removed through the cation exchange activity of bentonite added to the wine. Bentonite removes PR proteins efficiently but can remove wine aroma compounds and retain some of the volume of treated wine, drawbacks which represent significant economic burdens. The novel ability of BcAP8 to hydrolyse these PR proteins presents an alternative method to remove these proteins before they form haze.

Previous studies have pasteurised grape juice to remove PR proteins, a process which can be improved by the addition of proteolytic enzymes. The potential of BcAP8 to enhance this method was investigated, revealing that BcAP8 is not sufficiently thermostable to survive pasteurisation, precipitating before it is able to have any further effect on PR proteins. Despite this, inhibition of aspartic protease activity during pasteurisation suggests the presence of a grape aspartic protease which remains active in this process and may represent another research target.

During fermentation of grape juice, BcAP8 is active against TL proteins and chitinases in a dose-dependent manner. Addition of BcAP8 to a concentration of 12.5 $mg.L^{-1}$ was sufficient to remove all PR proteins visible by SDS-PAGE with Coomassie staining, with dosages as low as 0.5 $mg.L^{-1}$ also significantly lowering the concentration of these proteins. Despite the removal of the intact forms of these proteins, wine stability tests demonstrated that their proteolysis products form more haze per mass of protein. However, on a positive note, bentonite appears to remove these proteolysis products more efficiently than it does intact PR proteins, suggesting that BcAP8 treatment during fermentation may reduce the quantity of bentonite required to stabilise wine, thus conferring potential economic and sensory gains to the final product.

Declaration

I, Nicholas Ian Warnock, certify that this thesis does not incorporate, without acknowledgment, any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Nicholas Ian Warnock.

Acknowledgements

Whilst this thesis bears only my name, there are many others who deserve credit, and my vast gratitude, and without whom this thesis would never have been finished.

Firstly, I would like to thank Dr Peter Anderson, my patient supervisor whose obvious passion for science at all levels has helped to inspire and sustain my own. Peter's ability to see through the complexity of experimental data and relate everything back to physiological function is a sharp tool, and one which I hope I have begun to develop for myself.

There are many past and current members of the lab who have been amazing during my studies, especially Pradeep, My-my, Hayden and Imran. I would particularly like to acknowledge Emma who has been a tireless sounding board for ideas! You have all helped me enjoy my time at uni, especially when the work has been tough.

My family has supported me in so many ways over the years, understanding all of the issues which come with being a postgraduate student! I appreciate everything you have done for me, especially the last minute meals, your willingness to listen and your patience when I haven't been around. Thank you Mum and Dad; Nat, Tali and Isabelle; Kate and Andrew; Marlene and Charlie; Michaela and William.

I also want to say hi to my friends outside of uni - I haven't done that enough lately I but know that you are still there for me. I look forward to seeing you all more often, and continuing friendships which have endured many more years than has my study.

Bianca, you have been with me through every step of this journey. You have inspired me to a great love of science, and kept my feet on the ground with your practicality. My gratitude runs far deeper than can ever be conveyed in writing, which is why it is so exciting to leave writing behind and finally focus on the many dreams we have together. I love you and am blessed to have you by my side.