FUNCTIONS OF A PROTEINASE SECRETED
BY THE SAP-STAINING FUNGUS OPHIOSTOMA PICEAE

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Wood Science

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August, 1995

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Department of Wood Science

The University of British Columbia
Vancouver, Canada

Date 18 September 1995
Fungal colonisation that discolours wood decreases its market value, reducing profits on Canadian lumber products. Disrupting key enzymes involved in fungal metabolism could be a way of preventing such wood-inhabiting fungi from colonising lumber. Enzyme-targeted antisapstain formulations would be expected to have a low potential for adverse environmental impact. The production of proteinases by sapstaining fungi may be key to the acquisition of nitrogen required for growth since protein is considered to be the major source of organic nitrogen in wood.

Proteolytic activity detected in wood powder and culture filtrates after growth of Ophiostoma piceae was inhibited by PMSF and EDTA. The major protein detected in culture filtrates, a proteinase with a pI of 5.6 and a molecular weight of 33 kDa, was subsequently purified by hydrophobic interaction chromatography. The proteolytic activity of the purified proteinase was determined to be optimal at pH 7 to 9 and 40°C. The N-terminal sequence of the protein showed a high degree of homology with fungal alkaline serine proteinases classified as subtilisin class II enzymes. Agreements in inhibition patterns, electrophoretic and catalytic properties suggested the secretion of the same proteinase during growth on wood. Proteinase production was associated with active growth, suggesting a role in primary retrieval of nitrogen from wood proteins. Preliminary attempts to selectively inactivate the proteinase by application of chelators or serine proteinase inhibitors on wood prior to infection were inconclusive. Further efforts were hampered by the current lack of stable, non-toxic, specific proteinase inhibitors.
The subtilisin-like serine proteinase was degraded by autoproteolysis under conditions of heating, altered pH or partial depletion of protein-bound ions by EDTA. The proteinase consisted of two major hydrolytic fragments, 19 kDa and 14 kDa, which had N-terminal sequences of Ala$^1$-Tyr$^2$-Thr$^3$-Thr$^4$-Gln$^5$-Thr$^6$-Gly$^7$-Ala$^8$-Pro$^9$- and Ser$^{170}$-Glu$^{171}$-Pro$^{172}$-Ser$^{173}$-Val$^{174}$-X$^{175}$-Thr$^{176}$-Val$^{177}$-Gly$^{178}$-Ala$^{179}$, respectively. Since the former sequence was identical to the N-terminus of the native protein, the major autoproteolytic cleavage site for a class II subtilase appeared to be the N-side of Ser$^{170}$, consistent with a similar region identified for class I subtilases.

The cleavage specificity of this subtilase was investigated on the insulin B-chain using electrospray ionisation mass spectrometry. Cleavage sites after hydrophobic, polar, and charged amino acids indicated a broad specificity. Degradation of proteins extracted from the xylem tissue of poplar was observed after incubation with the proteinase. Other proteins hydrolysed by the proteinase included gelatin, collagen, albumin, edestin, globulins and casein. This supports the conclusion that the proteinase has a broad specificity and is able to degrade physiological substrates.

A thorough understanding of the nutritional requirements of staining fungi has important implications for preventing the growth of these fungi and other economically important ophiostomatoid fungi. The approach taken in this work - identifying key physiological enzymes as a strategy for controlling sapstaining fungi - has shown that these fungi require proteinases to breakdown wood proteins into assimilable nitrogen. Therefore, these enzymes are vital components of fungal physiology and their selective inactivation may be the target for future bioprotectants.
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<td>AEBSF</td>
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<td>ASTM</td>
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<td>ATAPNA</td>
<td>N-acetyl-Ala-Ala-Ala-(p)-nitroanilide</td>
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</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulphonic acid</td>
</tr>
<tr>
<td>Met or M</td>
<td>methionine</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>tandem mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>N</td>
<td>asparagine</td>
</tr>
<tr>
<td>O.D.</td>
<td>optical density</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>paper chromatography</td>
</tr>
<tr>
<td>Phe</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulphonyl fluoride</td>
</tr>
</tbody>
</table>
PQ-8 a commercial antisapstain formulation containing DDBSA and Cu-8
Pro or P proline
PVDF polyvinylidene difluoride
Q glutamine
R arginine
rDNA ribosomal deoxyribonucleic acid
rpm revolutions per minute
RT room temperature
sAAPF succinyl-Ala-Ala-Pro-Phe-p-nitroanilide
S.D. standard deviation
SDS sodium dodecyl sulphate
Ser or S serine
TCA trichloroacetic acid
TFA trifluoroacetic acid
Thr or T threonine
TLC thin layer chromatography
TPCK tosyl phenylalanyl chloromethylketone
TPEN N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine
Tricine N-[ tris-(hydroxymethyl)-methyl]glycine
Tris tris-(hydroxymethyl)-aminoethane
Trp tryptophan
Tyr tyrosine
Val or V valine
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$</td>
<td>maximum velocity</td>
</tr>
<tr>
<td>Vol</td>
<td>volume</td>
</tr>
<tr>
<td>W</td>
<td>tryptophan</td>
</tr>
<tr>
<td>w/w</td>
<td>weight by weight</td>
</tr>
<tr>
<td>w/v</td>
<td>weight by volume</td>
</tr>
<tr>
<td>Y</td>
<td>tyrosine</td>
</tr>
</tbody>
</table>
I would like to express my appreciation to Dr. Colette Breuil for her dedication in supervising this project. I valued her enthusiasm and interest in the work, and her willingness to generously give of her time. I have also benefited enormously from all the members of the Chair of Forest Products Biotechnology group, both past and present. A special thanks to Ken Wong, Alex Yu, Mike Chester and fellow graduate students who encouraged me through the “occasional” rough patches and to my industrial mentors Paul Morris and Tony Byrne for their willingness to share their years of experience.

I am especially grateful to people who made various scientific resources available: Dr. J. Davies and Kevin Chow, Department of Microbiology and Immunology at UBC for use of the densitometer, Dr. G. Mauk and Dean Hildebrand, Department of Biochemistry and Molecular Biology at UBC for access to CD spectroscopy, Forintek Canada for use of the micro-Kjeldahl and testing facilities, and Dave Chow and Hamish Morrison, Biomedical Research Centre, UBC for introducing me to the power of LC/MS. I would also like to thank Aaron Roth, Claudia Yagodnik, Eric Yung, Dave Bradshaw, Elizabeth Molitor and Hector Gamboa for their input in the project.

Funding for this project was gratefully received through a strategic grant from Natural Sciences and Engineering Research Council of Canada, and a Graduate Research Engineering and Technology award from the Science Council of British Columbia. Industrial support was received from Forintek Canada Corp., Canada.

I extend my thanks to my parents, family and friends for their encouragement, and to Ken, who believes in my ability even when I doubt it. Finally, my faith in God has sustained and supported me through all my academic endeavours, and for that I am truly grateful.
This thesis describes the functional characterisation of a proteinase from an economically important sapstain fungus that discolours wood. The work described is part of a long-term programme towards developing environmentally acceptable control treatments for such fungi. While there is a considerable amount of information available in the literature on the composition of wood, and on the physiological and biochemical features of decay fungi, there is little available on sapstaining fungi. Effective wood protection will be impossible without a more thorough understanding of the biology of sapstaining fungi (Seifert, 1993). Chapter 1 begins with a discussion of the problem of sapstain in industry, focusing on the Canadian lumber industry. This is followed by a brief review of the fungi responsible for the discoloration, again emphasising the fungi isolated in Canada. The nitrogen availability in wood and nitrogen metabolism by fungi is also summarised. Proteinase production by wood-colonising fungi and the classification of proteinases is discussed, highlighting research on the serine proteinases. Finally the research approach and objectives are outlined.

1.1 Problem of sapstain from an industrial perspective

Canada’s forests make up about 11.6% of the world total and cover an area larger than all of western Europe (COFI, 1994). The forests and the economic, environmental and social benefits they provide are part of the foundation of the Canadian way of life. In 1993, forest products contributed $23.7 billion to the Canadian trade balance (COFI, 1994) - greater than any other
single industrial sector. Lumber constitutes more than one third of the value of forest products that are exported (Figure 1.1).

![Diagram: Total value of Production: $40.3 Billion, Total value of Exports: $26.6 Billion]

**Figure 1.1** Canada's exports of forest products in 1993 (Source: Statistics Canada)

In fact, Canada is the largest exporter of softwood lumber in the world, controlling 55.0% of the market in 1992 (COFI, 1994). British Columbia is the largest contributor to Canada's softwood lumber exports, accounting for 32.4% of world exports. Most of the lumber produced in Ontario and Québec is used in Canada rather than being exported, whereas less than 25% of the wood products from B.C. were used in Canada (COFI, 1994). Spruce, lodgepole pine and true fir make up 60% of the logs harvested in B.C., while hemlock, cedar and Douglas fir cumulatively account for 36% (Figure 1.2)(COFI, 1994). The major markets for forest products (lumber, plywood, shingles/shakes, millwork) from B.C. include the U.S.A., Japan, and the European community. In 1993, these markets received 46.8%, 21% and 4.3% of B.C. shipments respectively. Some of the
overseas markets require clear, unseasoned, defect-free lumber and pay higher prices for these premium grades.

B.C. Log harvest - 1993

Figure 1.2 Log harvests in British Columbia in 1993
(Source: Statistics Canada and B.C. Ministry of Forests)

Therefore one of the challenges facing the lumber industry in B.C., as the nation’s prime exporter, is to keep wood free of discoloration during processing of logs, storage of the lumber and transportation of logs and lumber to the market place. Although there has been a concerted effort
to export clean wood, the industry has periodically sustained significant losses in insurance claims due to the discoloration caused by sapstain fungi and moulds. Actual costs to the industry are difficult to obtain because most claims are confidential and they may not be exclusively attributed to damage by staining fungi and moulds. Upon enquiry, for the month of June in 1995, loss of value in lumber exports from B.C. was about 1 million dollars in three claims alone (Tony Byrne, Forintek Canada Corp., personal communication). Over and above monetary issues, repeat business with the same customer may be jeopardized (Smith, 1991). Stains on wood can be abiotic (e.g. iron stains), but for wood with a moisture content greater than 20% in the sapwood region, the most common stain is due to colonisation by fungi. This type of discoloration is referred to in the industry as “blue stain” or “sapstain” or simply “stain”. In the context of this thesis these terms will always refer to discoloration of the sapwood of logs or lumber due to the presence of pigmented fungal hyphae.

The damage to wood from sapstain is cosmetic, in contrast to structural damage produced by soft rot or decay fungi (Eriksson, 1981). Sapstaining fungi have little effect on the strength properties of wood (Liese, 1970), generally only causing small losses in dry weight. The only major mechanical concern is the use of stained wood for applications requiring impact toughness (Scheffer, 1973; Subramanian, 1983). This property can be reduced by up to 30% in heavily stained pine (Chapman and Scheffer, 1940). However, sapstain is of significant economic importance since the cosmetic discoloration is objectionable to buyers (Wilcox, 1973; Zabel and Morrell, 1992).
Several strategies have been used in different parts of the world to prevent losses caused by sapstain. In addition to implementation of preventative logging practices such as avoiding storage of material during high risk periods (e.g. in the warmer summer months), rapid handling of logs in warmer climates, water storage and saturating log piles by sprinkling (Findlay, 1959; Phillips and Burdeken, 1982; Dickinson, 1988), chemical treatment and kiln-drying have been used to protect timber during transportation and temporary storage (Byrne and Smith, 1987).

In 1994, approximately 3 billion board feet of softwood lumber, with an export value exceeding $2.9 billion, was treated with antisapstain chemicals in B.C. (COFI, 1995). The market for antisapstain chemicals in Canada is currently worth about $16 to $20 million annually. The most widely used chemical over the past 50 years has been sodium pentachlorophenate (PCP), however findings concerning the toxicity and environmental fate of the dioxin contaminants of PCP have now severely restricted its acceptance in Europe, Pacific Rim countries and North America. Alternative chemicals have failed to satisfy an industry that had become used to the very effective chlorinated phenols (Seifert, 1993).

The ideal antisapstain product would be effective to give a 12 month shelf life to treated lumber. To avoid toxicity to man and the environment, it should specifically target the fungi causing discoloration. Other desirable properties are that it be non-corrosive, non-leachable, water-soluble and easily handled (Zabel and Morrell, 1992). The demands on antisapstain chemicals have grown over the years. Concerns about safety with respect to the environment and the lumber treating process have become increasingly important. However, any chemicals proposed still need to be cost effective to provide an affordable end product.
In Canada increased emphasis has been placed on formulations containing environmentally friendly chemicals, such as borax, soda, quaternary ammonium compounds (e.g. didecyl-dimethylammonium chloride), and triazoles (e.g. azaconazole and hexaconazole) (Smith, 1991). There are currently seven registered active ingredients used in various formulations (Konascwich and St. Quintin, 1994), but none have all the properties of the ideal protectant as described earlier.

Kiln-drying has been used to prevent sapstain by reducing the moisture content of wood to levels where fungi are unable to grow. However, kiln-drying is only feasible in specific circumstances because it can cause drying defects and it is not suitable for lumber of large dimension. Furthermore, it is expensive and the cost cannot always be recovered by increasing lumber prices (Byrne and Smith, 1987; Rayner and Boddy, 1988). The success of this treatment is also dependent on ensuring that all the timber is adequately dried and remains so for the entire period of transit and storage. In some countries (eg. Finland) sawn softwood is treated with antisapstain chemicals even when it is kiln-dried, as an insurance against rewetting during transportation (Dickinson, 1988).

Despite a century of concern, sapstain of wood remains a serious problem for the lumber industry. Most research has concentrated on chemical protection of lumber and there is limited information available on the biology and ecology of sapstaining fungi. A basic understanding of the organisms responsible for stain and their metabolic requirements would facilitate the development of a biorational approach to preventing sapstain.
1.2 Fungi causing sapstain and other wood-colonising fungi

Discoloration of lumber is caused mostly by saprobic fungi growing in and staining the sapwood after wood is cut (Figure 1.3). Sapstain caused by pathogenic or endophytic fungi in the living tree may be less costly for the industry because infected wood can be discarded before or during processing (Seifert, 1993). Growth of saprobic fungi is more insidious because colonisation of the wood can occur at any time after the tree is felled. Sapstain then becomes evident only when the wood is exposed to conditions favourable for fungal growth.

Most staining fungi grow primarily on the nutritive substances in the parenchyma cells of the sapwood producing pigmented hyphae (Mathiesen-Käärik, 1960, Ballard et al., 1982) due to melanin formation (Zink and Fengel, 1988). Various colours occur, although the most commonly seen are bluish to bluish-black and sometimes brown (Zabel and Morrell, 1992). Artists in the 15th century recognised the unique place for stained wood in intarsia masterpieces (Blanchette et al., 1992), and stained wood is frequently used by Swedish wood workers for its artistic effects.

Others have investigated its use for producing attractive violins (Seifert, 1993).

Historically, many fungal species have been associated with stain of living trees, logs and processed lumber. However, if the problem is confined to the saprobic fungi then the fungi most often implicated are from the genera Ceratocystis and Ophiostoma and their anamorphs. Moulds and black yeasts are also important (Seifert, 1993); especially since it is not unusual to find several different fungal species growing in close proximity on a single piece of wood. Different sapstain
species predominate in different geographical areas and although some species are limited to certain timbers, most are found on a variety of timbers (Seifert, 1993).

Figure 1.3 A pine board showing the radiating pattern of discoloration characteristic of sapstaining fungi (courtesy of R. Smith, Forintek Canada Corp.)

In Canada, some work has been done on the taxonomy of staining fungi isolated from trees and lumber (Griffin, 1968), but published work on the prevalence and distribution of sapstaining fungi in Canadian wood is scant. Nevertheless, a survey conducted by Forintek Canada Corp. indicated that staining fungi were isolated from most of the economically important wood species in Canada, including those in the genera *Abies, Pinus, Picea, Larix, Pseudotsuga, Populus* and *Tsuga*.
In this survey, the fungi were categorised as Ophiostomatales e.g. *Ophiostoma* and *Ceratocystis* spp., black yeasts e.g. *Aureobasidium pullulans*, dematiaceous moulds e.g. *Cladosporium* spp., or green moulds e.g. *Penicillium* spp. One of the key conclusions from the survey was that *Ophiostoma piceae* is the most frequent sapwood inhabiting fungus on Canadian lumber, occurring on more than 50% of the samples examined (Seifert and Grylls, 1991).

Black yeasts and dematiaceous moulds appeared to be the least important categories of sapwood inhabiting fungi. However, they may be more important at different times of year. Black yeasts, such as *Aureobasidium pullulans*, are capable of causing significant sapstain, particularly of wood in service (Rayner and Boddy, 1988, Sharpe and Dickinson, 1992; Zabel and Morrell, 1992). They produce wind dispersed spores in drier environments, and slimy yeast cells that disperse in water films or within the cells of a living tree in wet conditions (Seifert and Grylls, 1991). Discoloration by dematiaceous moulds is generally caused by masses of dark spores which can be removed by planing, but penetration by pigmented hyphae is also common. Examples include *Cladosporium* and *Alternaria*, which produce dry masses of darkly pigmented conidia that are probably mostly transmitted by air currents. Species of *Alternaria* produce a dark, penetrating stain that is similar to that produced by some members of the Ophiostomatales.

The green moulds discolour wood by forming masses of pigmented asexual spores on the wood surface (Wilcox, 1973). Because these spores can usually be brushed or planed off without leaving any residual discoloration, the green moulds are not usually considered sapstain fungi (Seifert, 1993). They are generally of minor economic importance in the wood industry and they

Members of the Ophiostomatales, including species of *Ceratocystis* and *Ophiostoma*, and their anamorphs were prevalent on all woods sampled in the Forintek survey (Seifert and Grylls, 1991). Although the taxonomy of these fungi has been somewhat controversial, there appears to be general agreement that *Ceratocystis* and *Ophiostoma* represent discrete genera on the basis of differences in cell wall composition, sensitivity to cycloheximide (Przybyl and De Hoog, 1989), ascospore shape, anamorph structure (De Hoog and Scheffer, 1984) and, more recently, partial rDNA sequences (Hausner et al., 1993). In this thesis, the nomenclature for species of these two genera is consistent with that described by Seifert et al., (1993). In particular, *piceae* as an accepted species of *Ophiostoma* is in agreement with the body of literature currently available.

The Ophiostomatales are carried by bark beetles or mites that live on bark beetles (Bridges and Moser, 1983; Seifert and Grylls, 1991). Some of the associations with insects are very specific and are restricted to single species of insect and fungus; in others, one species of fungus may be associated with several insects or vice versa (Leach, 1940; Käärik, 1960). These fungi produce ascospores in dark sexual fruiting bodies known as perithecia and conidia in asexual structures known as synnemata. In both cases the spores are released in a mucilagenous matrix which is transported via insect vectors (Käärik, 1971; Dowding, 1970). These fungi invade and discolour wood during log storage and the initial stages of lumber seasoning (Zabel and Morrell, 1992). Pigmented hyphae spread primarily through the sapwood rays, although they can also spread
through the longitudinal tracheids or vessel elements (Findlay, 1959; Liese, 1970; Ballard et al., 1984). Hyphae penetrate through pits in wood cell walls, but occasionally produce fine bore holes as well. Most experimental work suggests that the bore hole formation is by mechanical means, with only a few studies implicating enzymatic processes (Wilcox, 1973).

The patterns of colonisation of untreated lumber and felled logs are quite similar in that bacteria, stain and mould fungi are isolated early on, followed by soft-rot fungi and finally decay fungi (Butcher, 1968; Clubbe, 1980; Rayner and Boddy, 1988). The establishment of decay fungi is accompanied by a second influx of moulds which utilise cellulose or the breakdown products of decay fungi (Clubbe 1980). Several studies cited by Rayner and Boddy (1988) suggest that discoloration is a necessary first stage before decay can develop. The early-colonising blue stain fungi may modify the wood substrate and make it susceptible to subsequent attack by rot fungi. Several explanations have been offered: (1) removal of some of the natural fungal inhibitors in wood, for example, *Aureobasidium pullulans* is known to degrade phenolics (Bjurman, 1988); (2) a variety of physical and chemical changes in wood (Swift, 1976); or (3) depletion of carbon sources (Garrett, 1963).

Because succession patterns are complex and variable, it is difficult to unequivocally establish exact patterns. This is due to the uncertainly of reliably isolating all the principal microorganisms involved (Zabel and Morrell, 1992). Despite this, three major points emerge from these studies: staining fungi cause aesthetic damage to wood, they are possibly involved in preconditioning lumber for decay, and Ophiostomatales are the most prevalent staining fungi on Canadian lumber.

Therefore, an *Ophiostoma* species was selected as the model organism for this research.
1.3 Wood as an environment for sapstaining fungi

Wood structure and the nutrients available in wood affect the growth and survival of fungi that colonise the wood. This section will begin with a description of the anatomical features in wood which affect fungal growth, followed by a description of the nutrients available in wood. Sapstain fungi grow in wood mainly through the natural system of passages in the wood. Natural passages are provided by axial and radial elements that provide for the distribution of water and nutrients within the xylem of the living tree. Vessels and tracheids, which function in the conduction of water as well as providing mechanical strength, provide the major axial route for mycelial development. In angiospermous wood, known as hardwoods, the vessel elements vary in quantity, size and distribution pattern, all of which can affect fungal colonisation (Rayner and Boddy, 1988). Gymnospermous wood, known as softwoods, have a more homogenous distribution of tracheid elements. In general, tracheids do not attain the dimensions possible in vessels and thus provide a less effective pathway for axial spread than vessels. An important feature of vessels and tracheids is the occurrence of pits in the lateral walls which provide the main opportunity for transverse passage between elements. The size, structure and distribution of pits is therefore a significant factor affecting the accessibility of wood to fungal hyphae.

Although pits provide a limited degree of radial access, this is insignificant in comparison with the opportunities afforded by the radially elongated elements which constitute the ray system. These elements include parenchyma cells which may contain living protoplasts, especially in functioning sapwood. These cells act as sources of nutrients because they contain assimilable substrates, and as radial passages for fungi. Rays provide a direct route for fungal colonisation originating in bark
tissues. Sapstaining fungi which colonise the ray parenchyma produce characteristically wedge-shaped columns of stain (*Figure 1.3*).

Wood is basically composed of the structural polymers cellulose, lignin and hemicellulose which make up the cell walls. Cellulose microfibrils are coated with hemicellulose and embedded in lignin to form lignocellulose (*Figure 1.4*). Lignocellulose is a complex polymer mixture, rich in carbon but poor in other essential nutrients for fungi (Carlile and Watkinson, 1994).

![Figure 1.4 Composition of wood where minor components include starch, pectin, soluble sugars, proteins and ash (after Fengel and Wegener, 1989)](image)

Compounds such as soluble sugars, starch, extractives, proteins and peptides occur in relatively small amounts (often less than 10% of the dry weight), and are found almost exclusively in living
or recently dead sapwood parenchyma (Rayner and Boddy, 1988). Besides the structural cell wall components, primary metabolites and storage compounds, wood contains a wide range of extractive material. Extractives include waxes, fats, fatty acids and alcohols, steroids, higher carbon compounds and resins. The chemical composition, distribution and quantity of extractives varies between different species. Generally they are found in larger quantities in heartwood than in the sapwood (Fengel and Wegener, 1989). They can affect the growth of fungi in three ways: as carbon sources, inhibitors or growth stimulants (Rayner and Boddy, 1988). The main components of wood ash are potassium, calcium and magnesium (Fengel and Wegener, 1989). Among the nonstructural nutrients in wood, nitrogen is thought to play the most important role (Highley and Kirk, 1979).

The carbon to nitrogen ratio of wood varies from about 350:1 to 1250:1 (Merrill and Cowling, 1966), depending on the tree species, the individual tree, the part of the tree, the location of the tree and the time of year. At 0.01 to 0.3 % of the dry weight of wood, little nitrogen is available to fungi colonising wood. These low nitrogen values are growth-limiting factors for fungi invading wood (Merrill and Cowling, 1966; Levi and Cowling, 1969).

Work by King et al. (1976) and Boutelje (1990), indicated that during the drying of lumber, the soluble nitrogen migrates to the wood surfaces, where nitrogen can accumulate to concentrations five times higher than in green wood. The nitrogen in wood can affect the competition between wood microflora and consequently the microbial activity. For example, it may block the production of certain enzymes and enhance the breakdown of available cellulose by decay fungi.
Theander et al., (1993) have also shown a good correlation between wood nitrogen, sugar contents and the growth of moulds at the surface of lumber.

Most of the nitrogen is present in organic form, primarily as proteins (Chapin and Kedrowski, 1983; Dill et al., 1984; Wetzel et al., 1989b; Langheinrich and Tischner, 1991; Sauter and van Cleve, 1990; Sauter et al., 1989). It is well known that trees store considerable amounts of nitrogenous compounds such as proteins, amino acids, and nucleic acids in the parenchyma cells of wood and bark (Laidlaw and Smith, 1965; Kramer and Kozlowski, 1979). The storage proteins are present in large quantities during winter and absent during summer (Langheinrich and Tischner, 1991). It is assumed that with budbreak, these proteins are degraded into amino acids, which are then translocated to the growing tissues of the trees. In some wood species, protein bodies in the phloem and xylem ray parenchyma have been suggested to be analogous to protein bodies of seeds (Wetzel et al., 1989a). These have been shown to be the sites for the storage of specific proteins in several hardwoods and softwoods (Sauter and van Cleve, 1990; Wetzel and Greenwood, 1991). Thus overwintering protein storage in trees may follow sequences similar to protein deposition and subsequent catabolism during seed development and germination (Wetzel et al., 1989a).

There is not a lot of information on the amino acid composition of storage proteins found in wood, bark and leaves of trees. Most of the recent work has focused on the regulatory mechanisms controlling the production of storage proteins. However, Coleman and Chen (1993) isolated and sequenced a gene encoding a 32 kDa poplar bark storage protein, and
they showed by the derived amino acid sequence that the protein is rich in serine, leucine, phenylalanine and lysine (Coleman et al., 1992).

1.4 Nitrogen metabolism in fungi

Nitrogen is found in nearly all complex macromolecules of all living cells and is a major component of proteins and nucleic acids. It is required to synthesise amino acids, the building blocks for structural proteins and enzymes. Nitrogen is an important component of the fungal protoplasm, contributing between 2 and 5% of the dry weight, depending on the environmental conditions and the age of the mycelium (Moore-Landecker, 1982). Thus, the nature and quantity of nitrogen available in the fungal environment will control the development of the fungus. Absence of available nitrogen may lead to autolysis or sporulation.

Organic nitrogen in the form of amino acids and inorganic nitrogen in the form of ammonia can be utilised by most fungi, whereas other inorganic forms (nitrates, nitrites) may not support fungal growth (Jennings, 1989). Those fungi which are able to utilise nitrates take up the nitrate ion by active transport, reduce it to the oxidation level of ammonia, and assimilate it into organic compounds. Nitrate is not utilised by Aspergillus nidulans or Neurospora crassa unless the cells are depleted of favoured nitrogen compounds namely ammonia, glutamate, or glutamine. Molecular biology studies have confirmed that the genes encoding the nitrate reductase enzyme are controlled at the level of transcription (Marzluf, 1993). Fungi that are unable to utilise nitrates, presumably because they cannot reduce the nitrate ion, may use nitrogen in the form of the ammonium ion or in the form of organic nitrogen that has the same oxidation level as the
ammonium ion. Most of the amino acids assimilated by fungi are used directly or by initial deamination.

The principal route of amino acid catabolism is oxidative deamination, but a number of other mechanisms exist. For example, asparagine may be hydrolysed to aspartic acid and ammonium, and threonine may be cleaved to glycine and acetaldehyde. The ammonia freed by the deamination reaction or the inorganic nitrogen enters into the amino acid biosynthetic pathway by combining with α-ketoglutaric acid (Moore-Landecker, 1982). Alternatively, this reaction may function in reverse, in that glutamic acid may lose the amino group as ammonia, and the carbon skeleton of the amino acid is introduced into the tricarboxylic acid (TCA) cycle as α-ketoglutaric acid, which is then oxidised (Figure I.5). Transamination may convert amino acids into other amino acids.
Fungi actively decompose proteins in nature to their component amino acids, which can be assimilated. The utilisation of exogenous proteins by fungi requires the extracellular release of enzymes and the enzymatic degradation of protein to peptides and amino acids before cellular uptake. Proteinase production in Aspergilli and *N. crassa* is regulated by carbon, nitrogen, sulphur or phosphorus catabolite repression (Cohen, 1980; North, 1982). In *Aspergillus* starvation for any one of these elements is sufficient to induce proteinase production; whereas in *N. crassa* protein must also be present. Fungal growth can also be supported with small peptides generated from protein breakdown (Jennings, 1989), since an oligopeptide transport system capable of
transporting peptides with up to five amino acid residues has been reported (Wolfinbarger, 1980). However, peptidases are widely distributed in fungi and presumably function to cleave off amino acids from peptides and proteins either in the cell or prior to uptake (Breddam, 1986).

Finally, several studies have shown that certain Basidiomycetes are able to utilise proteins as the sole source of carbon, nitrogen and sulphur (Kalisz et al., 1986, 1987). In these fungi, unlike Aspergillus and N. crassa, proteinase activity was not repressed by the presence of glucose, ammonium and sulphate in the medium.

1.5 Fungal growth and secretion of enzymes

With the exception of unicellular yeasts, fungi typically grow by means of hyphae that extend only at their apices and ramify into a mycelium (Bartnicki-Garcia, 1968). By extending at their apices, the hyphae can penetrate solid substrata such as wood, at the same time secreting the lytic enzymes which convert substrata polymers into products small enough to be taken up as nutrients (Wessels, 1993; Wood, 1985). The efficacy of fungal hyphal spreading is enhanced by the ability of the hyphae to continue apical growth in non-nutritive substrata by translocating water and nutrients from a food base (Rayner, 1991; Wessels, 1993). This ability for translocation also permits the development of aerial structures such as fruiting bodies of basidiomycetes.

Various fungal genera have the dimorphic capability of changing their growth form between mycelial and yeast, depending on the environmental conditions. Factors such as nutrient regimes, pH, temperature and chemical inducers are capable of influencing the balance between yeast and
hyphal growth (Kulkarni and Nickerson, 1981; Gow, 1994). Culture methods often manipulate many of these factors and may lead to changes in the morphological forms, particularly when using agitated liquid cultures. The predominance of yeast cells in liquid media is in contrast to the filamentous growth which occurs in wood. Proteinases are likely involved in the regulation of enzymes required for the synthesis of cell wall polymers during the mycelial form of growth. For example, in the case of chitin synthesis, the onset of activity and the life span of chitin synthetase may be regulated by proteolysis (Deshpande, 1992).

Filamentous fungi secrete a broad spectrum of enzymes, with the majority being hydrolytic enzymes that play an important role in nutrition, releasing carbon and nitrogen locked in insoluble compounds (Wood, 1985). Cytological studies carried out more than two decades ago provided circumstantial evidence that secretion of proteins by filamentous fungi was probably restricted to the tips of growing hyphae. Evidence supporting this hypothesis has been obtained by immunocytochemical methods for the secretion of glucoamylase by *Aspergillus niger* and for enzymes involved in lignin degradation by *Phanerochaete chrysosporium* (Peberdy, 1994).

The secretory pathway begins intracellularly in the lumen of the endoplasmic reticulum where post-translational processes are initiated. Vesicles carry molecules to the Golgi system, or its equivalent, where the processing continues. Finally vesicles and/or vacuoles transfer the proteins to the tip of the growing hyphae where they fuse with the plasma membrane, releasing their contents into the periplasmic space (Wessels, 1993). Enzymes are released from the surface of the plasma membrane into the periplasmic space, where they may be incorporated into the cell wall or, in many instances, may be secreted across the cell wall into the external medium.
Before proteins are secreted, they undergo several post-translational modifications: (1) proteolytic cleavage to remove the signal sequence and a propeptide sequence, if present; (2) folding process involving the formation of disulphide bonds to develop the tertiary and quaternary structures of the protein; and (3) glycosylation (Halban and Irminger, 1994). Therefore, secreted proteins undergo two important proteinase cleavage reactions. The first is the removal of the signal peptide sequence, possibly by an endoproteinase in the lumen of the endoplasmic reticulum. The second is the activation of enzymes, many of which are synthesised as inactive zymogens (Peberdy, 1994).

1.6 Proteinase enzymes

Fungi from different taxonomic classes produce extracellular and intracellular proteinases. In addition to a role in nutrition, proteinases have other functions such as degrading fungal proteins from senescent mycelium, penetrating host tissue during pathogenesis (North, 1982), and regulating cellular functions (e.g. protein turnover, translocation, sporulation, germination) (Deshpande, 1992). The ability of proteolytic enzymes to carry out selective modification of proteins by limited cleavage means that proteinases are well suited to a regulatory function (North, 1982). Proteinases or endopeptidases are the major subject of this thesis. However, a complete understanding of proteolysis should also consider the action of exopeptidases (aminopeptidases, carboxypeptidases and dipeptidases). These will be covered briefly where necessary.

In 1967, Schechter and Berger introduced a system of nomenclature to describe the interaction of proteases with their substrates. In this system, the binding site for a polypeptide substrate on a protease is envisioned as a series of subsites in the active site; each subsite interacting with one
amino acid residue of the substrate. By convention, the substrate amino acids are called P and the subsites on the protease that interact with the substrate are called S. The amino acids residues on the amino-terminal side of the scissile bond are numbered P1, P2, P3 as shown in Figure 1.6.

**Figure 1.6** The Schechter and Berger nomenclature for amino acid residues of a substrate (P1 etc.) and corresponding subsites (S1 etc.) of the active site of a protease. The arrow indicates the peptide bond to be split by the enzyme (the scissile bond or the reactive site peptide bond).

The basis for classifying proteinases is evolving. In the past proteinases were classified on the basis of the pH range over which they are active (acid, neutral or alkaline), on the basis of their ability to hydrolyse specific proteins (keratinase, elastase, collagenase etc.), and on the basis of their similarity to well characterised proteinases such as pepsin, trypsin, and chymotrypsin (North, 1982). Presently, the most satisfactory classification scheme is that proposed by Hartley (1960). This scheme is based on catalytic mechanisms and forms the basis for the Enzyme Commission
classification. Four different types of proteinases are recognised: aspartic, serine, cysteine and metalloproteinases, and each type has a characteristic set of functional amino acid residues arranged in a particular configuration to form the active site.

All types of proteinases catalyse the same reaction but have different mechanisms. They can also be distinguished from each other on the basis of their sensitivity to various inhibitors. The serine proteinases have a reactive serine residue; the aspartic proteinases have two catalytically essential aspartate residues; the cysteine proteinases have reactive cysteine residues and the metallo-proteinases require zinc for activity. At present there are some enzymes which do not fit into one of the four types of proteinases, e.g. the multicatalytic protease which has multiple subunits and at least four different proteolytic activities (Powers et al., 1993). As these new enzymes are characterised mechanistically, it is likely that most will be members of families within the four types of proteinases and will operate by the same general mechanism of peptide bond cleavage. However, there still remains the possibility that an entirely new mechanism of peptide bond hydrolysis remains to be discovered.

All four types of proteinases have been detected in fungi (Hartley, 1960; Barrett, 1977), although aspartic and serine proteinases predominate (North, 1982). Many fungi produce proteinases which are active at acidic pHs, and a large proportion of these have been shown to have properties consistent with aspartic proteinases. Most of these proteinases have molecular weights in the range of 30 000 to 45 000 and isoelectric points (pI values) below 5.1. They are usually able to hydrolyse a range of native proteins but the majority have little or no activity on small synthetic
substrates. Fungal species which produce extracellular acid proteinases often acidify the media in which they grow, and many of the enzymes are unstable above neutral pH.

The production of alkaline proteinases has been described for fungi of all major taxonomic groups. Most of those characterised were found to be serine proteinases. These proteinases generally have low molecular weights, in the range of 18 500 to 35 000. Most have low pI values, between 4.4 and 6.2, but pI values of 8.9 or higher have also been reported (North, 1982). The mechanism of catalysis has been extensively investigated and is shown in Figure 1.7. The hydroxyl group of the active site serine residue performs a nucleophilic attack on the carbonyl carbon of the scissile peptide bond thus forming a tetrahedral intermediate. A histidine residue in the active site serves as a general base accepting the proton from the serine residue. The acyl enzyme intermediate thus formed is broken down via a nucleophilic attack of a water molecule to complete the hydrolysis of the peptide bond (Neurath, 1984).

Although many distinct families of serine proteinases (EC 3.4.21.-) exist, the two best studied are the (chymo)trypsin and subtilisin (EC 3.4.21.14) families. These families are distinguished by a highly similar arrangement of catalytic His, Asp and Ser residues in radically different β/β (trypsin) α/β (subtilisin) protein scaffolds (Siezen et al., 1991). The (chymo)trypsin family is relatively well known, with well-characterised members such as chymotrypsin, trypsin, elastase, plasmin, and factor IX. Fewer members of the subtilisin family were known until fairly recently, when a surge of interest was driven by research and industrial applications.
Subtilases have been further divided into two main classes (I and II) based on multiple sequence alignment of the N-terminal catalytic domains. The distinction is based on characteristic sequence patterns and consensus residues. All subtilases contain the essential catalytic triad residues D32, H64 and S221. Most also contain N155 that helps to stabilise the oxyanion generated in the tetrahedral transition state (Carter and Wells, 1990). The conserved secondary structure consists of an internal core of seven parallel β-sheet strands and two buried helices, surrounded by five amphipathic helices and two anti-parallel β-sheet strands (Siezen et al., 1991). However, the
connections between conserved regions in the secondary structure are variable. They are generally on the surface of the molecule, allowing for variation in length and amino acid sequence. Alignment of class II subtilases is fairly unambiguous due to their high degree of sequence homology, even in the most variable regions, and the low incidence of insertions/deletions relative to proteinase K. Therefore, as suggested previously (Siezen et al., 1991), model structures of subtilisin class II proteinases can be derived directly from the proteinase K structure.

Proteinase K, isolated from the soil ascomycete *Tritirachium album* Limber, is the most extensively characterised member of this important group of serine proteinases. Its ability to digest native proteins, including keratin (Ebeling et al., 1974), has led to its widespread application in the preparation of nucleic acids. Other members of the subtilisin family have been extensively studied because of their practical application as additives to laundry detergents (Betzel et al., 1990), in the preparation of proteolytic creams for medicinal purposes, and in the food industry (Dolashka et al., 1992; Lyons, 1988).

1.7 Proteinase inhibition

Any compound which decreases the measured rate of hydrolysis of a given substrate is, in principle, an enzyme inhibitor (Salvesen and Nagase, 1989). There are several types of proteinase inhibitors, most of which are specific to the four types of proteases. Reversible inhibitors include simple competitive and transition-state inhibitors. These inhibitors usually contain substrate-like features and their potency depends on binding interaction with the enzyme. Irreversible inhibitors can be active-site directed inhibitors or mechanism-based inhibitors. Active site inhibitors
resemble the substrate and contain a reactive group which can react with amino acid residues at the active site of the proteases. Mechanism-based inhibitors, also known as suicide inhibitors, often contain a latent reactive group which is activated by enzyme catalysis. In this case, a normally innocuous reversible inhibitor is converted into a powerful irreversible inhibitor. Irreversible inhibitors usually inactivate proteases by first forming a reversible E•I complex followed by covalent bond formation and hence irreversible inhibition (Fersht, 1985; Powers et al., 1993). Proteinaceous protease inhibitors form a unique category of inhibitors found almost ubiquitously distributed in tissues of plants and animals (Murao et al., 1985). They have been widely used to elucidate proteinase mechanisms since the EI complex exists as a stable molecular species.

Inhibitors for serine proteases include simple substrate analogs, transition state analogs, alkylating agents which react with the active site histidine, acylating agents which react with the active site serine forming stable acyl enzymes, and mechanism-based inhibitors (Powers et al., 1993). Di-isopropyl-fluorophosphate (DFP) and phenylmethanesulphonyl fluoride (PMSF) are well known examples of low molecular weight inhibitors which act by irreversibly modifying the active site serine (Salvesen and Nagase, 1989). Bovine pancreatic trypsin inhibitor (BPTI) was discovered in 1930 as a proteinaceous inhibitor that inactivated trypsin in the pancreas (Murao et al., 1985). Such inhibitors do not hydrolyse under physiological conditions because the amino group that is released on cleavage of the peptide is constrained and cannot diffuse away from the active site of the enzyme (Fersht, 1985). Another example of a serine proteinase inhibitor is antipain, a peptide aldehyde which acts as a transition state intermediate by mimicking the tetrahedral intermediate formed during hydrolysis.
There is considerable interest in the design of highly specific irreversible enzyme inhibitors because of their potential use as therapeutic agents, and it is likely that more suicide inhibitors, which are unreactive in the absence of the target enzyme, will be developed for future use.

1.8 Proteinases of wood-inhabiting fungi

Fungi which grow on wood must obtain all their cellular nitrogen from wood, unless an alternative nutrient source is available. Proteinases may be essential for fungi to retrieve nitrogen from wood proteins, and may serve to recycle fungal nitrogen by autolysis (Levi et al., 1958). While cellulolytic and ligninolytic enzymes of wood-inhabiting fungi have been extensively studied, their proteolytic enzymes remain poorly characterised. The few available descriptions of proteinase production in artificial media have been reported for wood-decaying Basidiomycetes, *Schizophyllum commune*, *Postia placenta*, and *Trametes versicolor*, for the moulds *Aspergillus*, *Penicillium*, *Trichoderma*, and for the yeast *Aureobasidium pullulans*.

*Schizophyllum commune*, a weak white rot fungus, has been used as a model system to study the mechanisms of nitrogen-limited growth (Lilly *et al.*, 1990, 1991, 1994; Sessoms and Lilly, 1986). It possesses a complex system of proteolytic enzymes (Lilly *et al.*, 1994) which are thought to be involved in sustaining mycelial expansion during nitrogen-limited growth. Serine, metallo- and aspartic proteinases are produced by *S. commune*. Nitrogen deprivation results in a shift in the spectrum of proteinases produced: metalloprotease activity increases and serine proteinase activity decreases (Lilly *et al.*, 1994). When exponentially growing colonies are transferred to media low in nitrogen, radial expansion continues at nearly the same rate as in colonies transferred
to high nitrogen media. New growth is supported at the expense of existing mycelia. Proteins are degraded in older portions of the colony and proteolytically released amino acids subsequently appear in the colony margins (Lilly et al., 1991).

The proteinases produced by Postia placenta, a brown rot fungus, have acidic pH optima characteristic of aspartic proteinases (Matsushima et al., 1981; Micales, 1992). Proteinase formation by different strains of the brown-rot fungus P. placenta was examined to determine whether differences in proteolytic enzyme production could be correlated with the ability to decay wood (Micales, 1992). Although this work was not conclusive, the author suggested that the proteinases were associated with autolysis. Similarly, an association with nitrogen recycling has been reported for a range of decay fungi (Santamaria and Reyes, 1988; Venables and Watkinson, 1989), with the exception of T. versicolor. In this fungus, the spatial distribution of the proteinases at the margin of the agar-grown colony and the production of large quantities of proteinase before the onset of autolysis suggested that proteinases would be involved in primary attack of wood protein rather than autolysis (Venables and Watkinson, 1989).

Proteinases of moulds such as Aspergilli, have been extensively studied and reviewed by Cohen (1977). Much of the work examining proteinase production by Aspergilli has stemmed from the interest in these enzymes for applications in the food industry and enzymatic depilation in the tanning industry (Malathi and Chakraborty, 1991; Tatsumi et al., 1989). Therefore, many studies have used complex or undefined media. Nevertheless, all strains produced substantial levels of extracellular neutral and alkaline proteinase activity in response to protein starvation (Cohen, 1977). Mycelial extracts from Aspergillus nidulans have been shown to contain at least five
different proteinase activities (Cohen, 1973). There is an increase in proteinase activity after nitrogen starvation, and most intracellular activity is compartmentalised in vacuoles (Stevens and McLennan, 1983). The alkaline proteinase produced by *A. oryzae* has been cloned and expressed in *S. cerevisiae* (Tatsumi *et al.*, 1989).

Other research has investigated elastase production as a virulence determinant of *Aspergillus fumigatus* and *Aspergillus flavus*, opportunistic pathogens causing a variety of respiratory disorders (Frosco *et al.*, 1992; Denning *et al.*, 1993; Kolattukudy *et al.*, 1993; Moser *et al.*, 1994).

Production of an extracellular elastase was correlated with the ability to cause invasive aspergillosis in immunocompromised mice. The elastase was inhibited by PMSF and EDTA, and was classified as a subtilisin-like serine proteinase (Reichard *et al.*, 1990; Jaton-Ogay *et al.*, 1992). Closely related alkaline proteinases were secreted by other pathogenic species in contrast to non-pathogenic strains (Hanzi *et al.*, 1993).

*Trichoderma koningii*, *T. harzianum* and *Aureobasidium pullulans* secreted an alkaline proteinase completely inhibited by PMSF (Donaghy and McKay, 1993; Geremia *et al.*, 1993; Manonmani and Joseph, 1993). *Penicillium* species secreted aspartic proteinases (Matsushima *et al.*, 1981), although serine proteinases have also been detected (Chrzanowska *et al.*, 1993; Yamamoto *et al.*, 1993). These proteinases are thought to be involved in a variety of pathogenic, nutritional or developmental functions, and have been used in various industrial applications (e.g. the food industry, the detergent market).
To conclude, wood-inhabiting fungi have been shown to produce proteinases, and the production of extracellular serine proteinases has been reported for decay and mould fungi. However, most of the research interest has resulted from the industrial or medical importance of the fungi. None of the work cited was conducted on wood, and none has examined proteinase production by sapstaining fungi.

1.9 Research approach and objectives

The biological control of staining fungi can be approached in many ways. Some research groups have focused on biological control agents to outcompete the staining fungi (e.g. Benko, 1987; Stranks, 1976; Seifert et al., 1987), others have considered using colourless mutants which do not cause stain (Behrendt et al., 1995). Some work has been conducted to quantify and detect staining fungi on wood (Breuil et al., 1988, 1990, 1992), but little work has been conducted on the physiology of staining fungi especially when they are grown in "solid state" as on lumber. Many of these strategies for controlling sapstain would benefit from a better understanding of the physiology of the organism involved.

In common with all other living organisms, fungi require nitrogen in all aspects of their growth and metabolism. As discussed above, the major source of nitrogen in wood is protein, and fungi would require extracellular proteinases to break down these proteins into more assimilable forms of nitrogen. Little information is available on the proteases of staining fungi. The work carried out on wood-inhabiting fungi has usually involved growth on artificial media (Venables and Watkinson, 1989; Micales, 1992). Virtually no work has been done on the range of enzymes...
active when fungi grow on solid wood substrates such as lumber and trees. An understanding of proteinase production by staining fungi in wood would be essential for subsequent manipulation and disruption of the proteolytic enzyme systems. Furthermore, characterising microbial enzymes has important practical applications. It can provide new enzymes for use in commercial applications or supply information that permits the improvement of existing enzymes.

In Canada, *Ophiostoma piceae* (Münch) H. & P. Sydow is the most commonly isolated sapstaining fungus, and was therefore chosen as the model organism. Understanding more about the growth and metabolism of *O. piceae* is also considered important because of its possible involvement with oak decline in central and eastern Europe, and its close relationship to the Dutch elm disease pathogens *O. ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier (Brasier and Kirk, 1993). The genera *Ceratocystis* and *Ophiostoma* also include plant pathogens causing rot of sweet potatoes, wilt of coffee and rubber; and human pathogens causing sporotrichosis (Spatafora and Blackwell, 1994; Upadhyay, 1993). The ophiostomatoid fungi are thus of obvious economic importance in forest products, forestry, crop plants and medical mycology.

The model substrate chosen for this study was lodgepole pine, *Pinus contorta* var. *latifolia* Dougl., since it is a high-volume lumber in B.C. that is highly susceptible to sapstain development. Aspen, *Populus tremuloides* Michx., was chosen as a representative hardwood species that is subject to distinct discoloration due to its intrinsic light colour.

The overall goal of the work was to understand how *O. piceae* retrieves nitrogen from wood. This information may be useful for controlling the growth of staining fungi in wood. Physiological
studies on wood are complicated by the intrinsic variability of wood, and the difficulty of enzyme extraction and fungal manipulation. Therefore, the approach used throughout this work was to conduct parallel studies in artificial media and in wood.

The research was divided into three specific objectives:

A. **Characterisation of the proteinases secreted by *O. piceae***
   - Optimise culture conditions for proteinase production
   - Determine the number and type of proteinases produced under various conditions in wood and artificial media

B. **Purification and characterisation of the major serine proteinase***
   - Biochemical properties
   - Catalytic properties
   - Stability under various conditions
   - Sensitivity to inhibitors
   - Cleavage specificity on natural and synthetic substrates

C. **Targeted inhibition of serine proteinases on wood***
   - Identify types of proteinases produced by staining fungi on the basis of sensitivity to inhibitors
   - Application of specific inhibitors to stop fungal growth on wood
Chapter 2.

General methodology

2.1 Fungal strain

*Ophiostoma piceae* (Münch) H. & P. Sydow strain 387N, isolated from softwood chips at the MacLaren Mill (Mason, Québec, Canada), was obtained from the culture collection of Forintek Canada Corporation (Ste. Foy, Québec, Canada).

2.2 Growth conditions for staining fungi in culture

2.2.1 Solid media

Skim milk agar (1% skim milk, 1.5% agar) and malt extract agar (2% malt extract, 1.5% agar) (Difco, Detroit, USA) were used for growth of staining fungi. The former was used to determine proteinase activity by observing clearing zones during growth.

2.2.2 Liquid media

The synthetic liquid medium contained in each litre: 0.4 g CaCl$_2$·2H$_2$O; 1.0 g KH$_2$PO$_4$; 0.8 g Na$_2$HPO$_4$; 0.5 g MgSO$_4$·7H$_2$O; 3.0 g potassium hydrogen phthalate; the micronutrients of Vogel (1956); filter-sterilised vitamins of Montenecourt and Eveleigh (1977). The carbon source was 2% soluble starch unless otherwise specified. The nitrogen source was NH$_4$NO$_3$ 1.6 g/l for inorganic media and soybean proteins for organic media unless otherwise stated. Soybean proteins were supplied as 280 ml/l of unsweetened soya drink (Sunrise Markets, Vancouver,
B.C., Canada) that contained 3.1% protein, 1.4% fat, and 0.9% carbohydrate. The media were sterilised by autoclaving before the vitamins were added. The final pH of the media was 5.8 ± 0.2.

The inoculum (9.75 mg/l dry weight) was pre-grown for three to six days in the same medium as that used for the corresponding experiment. These inoculum cultures were prepared from 3 mm cores of the fungus grown on 2% malt extract agar plates. Cores were stored at -80°C in 10% glycerol. Most studies were performed in 300 ml glass Erlenmeyer flasks containing 60 ml liquid medium. Unless otherwise specified, cultures were grown in the dark at 23°C on a rotary shaker set at 250 rpm.

2.3 Fungal biomass determinations

The total fungal biomass in liquid culture was measured by filtering and washing the mycelia on pre-weighed glass fibre filters, drying the filters in a microwave (4 minutes on high setting), cooling in a desiccator and weighing. Total fungal protein was determined using the biuret assay (Herbert et al. 1971), and bovine serum albumin (BSA) as the calibration standard. Total ergosterol in extracts from freeze-dried mycelia (10-30 mg) or wood powder (1.5 g) was determined by HPLC analysis (Seitz et al. 1977; Nilsson and Bjurman, 1990) using a Nova-Pak C18 reverse phase HPLC column on a Waters 625 HPLC system (Millipore Corporation, Bedford, MA, USA). The method involved a one hour reflux with 60 ml of a methanol and ethanol mixture (5:1, v/v) containing 10% KOH, followed by extraction with 60 ml petroleum ether. The petroleum ether was evaporated and samples redissolved in methanol (1.5 ml) prior to HPLC analysis (Gao et al., 1993). Ergosterol (Aldrich Chemical Co., Milwaukee, WI, USA: product number E200-0) was used as the calibration standard.
2.4 Wood material

2.4.1 Hardwood and softwood blocks

A lodgepole pine (*Pinus contorta* var. *latifolia*) tree, approximately 45 year old, was harvested during winter in the UBC Alex Fraser Research Forest at Gavins Lake, B.C. Sapwood blocks (30 × 10 × 5 mm³) were cut with the 5 × 10 mm² face on the transverse plane, and the 30 × 10 mm² face on the radial longitudinal plane. Lodgepole pine samples were further separated and classified on the basis of distances above ground level (Sections I-VI, 3-0 m) and radial distances from the cambium (1) to the heartwood (6). An aspen tree, approximately 70 years old, was harvested during winter in Northern Alberta. Samples were also prepared from the sapwood as described for pine. Pine and aspen samples were packaged, and sterilised by gamma irradiation (Gamma cell 220, Atomic Energy of Canada, Ottawa, Canada) to receive a total dose of 2.5 mRad. Sterilisation was carried out at the Biomedical Research Centre, UBC. Branches, about 3 years old, were collected from a second lodgepole pine tree, and sterilised as described above. All the wood samples were kept at -10°C.

2.4.2 Inoculation of wood blocks

*O. piceae* was grown in liquid medium for 4 days. The biomass was harvested by centrifugation and resuspended in 0.8% NaCl. Samples were homogenised (Omni Homogenizer model 2000, Omni International, Waterbury, USA) and centrifuged to obtain a washed pellet of cells. The cells were made up to a final concentration of 2.5 mg/ml dry weight. Aliquots (20 µl) were used to inoculate sterile wood blocks. Blocks were incubated
in sealed Petri dishes at 23°C for 3 to 21 days. High humidity was maintained by using water
soaked filter paper in the bottom of the Petri dishes.

2.4.3 Sap pressed from wood chips

A lodgepole pine tree, approximately 40 years old, was collected from the same site at the
research forest in B.C. It was debarked and chipped. To obtain a liquid pressate the chips
were compressed in the screw feeder of a thermo-mechanical refiner which had a
compression ratio of 5:1. In this, the assistance of Dr. H. Cisneros and S. Johal of the Pulp
and Paper Research Institute of Canada, Vancouver, B.C., is gratefully acknowledged.

2.4.4 Milled wood powder

Wood blocks were milled into a powder using a micro mill (Bel-Art Products, Pequannock,
NJ, USA) cooled by liquid nitrogen.

2.4.5 Wood protein samples

Wood powder was homogenised (Omni Homogenizer model 2000, Omni International), or
ground with a mortar and pestle, in a 1% sodium dodecyl sulphate (SDS) solution, and
centrifuged to remove wood fibre. Sap pressed from lodgepole pine chips was clarified by
centrifugation and then freeze dried. Material was resuspended in phosphate buffered saline
(PBS), and was extracted three times with equal volumes of diethyl ether, and once with
hexane. Protein was precipitated from the aqueous phase using 100% ammonium sulphate.
The pellet was resuspended in PBS and desalted by ion retardation. Protein solutions were
concentrated using an ultrafiltration membrane with a molecular weight cut-off of 3000 Da (Amicon, Danvers, MA, USA).

2.5 Proteinase activity assays

Azocoll (<50 mesh; Calbiochem, La Jolla, CA, USA) was used as the substrate in a spectrophotometric assay to determine proteolytic activity in filtrates from liquid culture or in wood (Chavira et al. 1984). Assays were conducted in 1 ml reaction volumes in a water bath with agitation of 320 rpm, using 4 mg/ml azocoll prepared in 0.1 M Tris-Cl (pH 8) or 0.1 M MES-Tris-acetate (Ellis and Morrison, 1982) and 0.09 M CAPS (Sigma, St. Louis, MO, USA) for a range of pH's. Assays were generally conducted at 37°C, where proteolytic activity was greater than at room temperature. Reactions were terminated with 50 μl of 50% (w/v) trichloroacetic acid (TCA). After centrifugation, supernatants were transferred into a 96 well plate and the absorbance at 520 nm (A_{520}) was measured using a Thermomax microplate reader (Molecular Devices Corporation, Menlo Park, CA, USA). Each well received 200 μl of sample giving a path length of 0.7 cm. One unit (U) of proteinase activity was defined as the amount of enzyme that produced a rate of increase in absorbance of 0.1 O.D. units per min.

The cleavage specificity of the proteinase was determined using the following model substrates from Sigma: N-α-benzoyl-DL-Arg-p-nitroanilide (BAPNA) for trypsin-like activity; N-α-benzoyl-L-Tyr-p-nitroanilide (BTPNA) for chymotrypsin-like activity; N-acetyl-Ala-Ala-Ala-p-nitroanilide (ATAPNA) for elastase-like activity; succinyl-Ala-Ala-Pro-Leu-p-nitroanilide and succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (sAAPF) for subtilisin-like or chymotrypsin-like activity;
and L-Leu-\textit{p}-nitroanilide for amino-peptidase activity. Assays were conducted at 0.3 to 1 mM substrate concentrations (Sarath et al., 1989) in 20 mM Tris-Cl, pH 8 at 37°C. After completion of the reaction, 200 µl of the reaction mixture was transferred to a 96-well plate and the absorbance at 405 nm was measured using the microplate reader. In quantitative assays, the initial rate was measured using 1 mM sAAPF (DelMar et al., 1979) at different pH and temperature values. The rate was determined as the slope in the linear portion of a graph of absorbance against time, and was expressed as 10^{-3} times the rate of change in O.D. per minute (mOD/min). The assay plots were usually linear for the first ten minutes.

2.5.1 Kinetic constants

\( K_m \) and \( V_{max} \) values were determined graphically, from the initial rates of hydrolysis of sAAPF at five separate substrate concentrations, according to the method of Lineweaver and Burk (1934). The catalytic constant (\( k_{cat} \)) was calculated from the maximum velocity (expressed in moles per minute) using an enzyme loading of 0.6 µg and a molecular weight of 33 kDa, according to the formula: \( k_{cat} = (V_{max})/|E| \).

2.6 Inhibition studies

Various compounds were examined for their effects on the proteinase enzymes in culture filtrates and on the purified enzyme, or on the growth of the fungus in wood and in artificial media. Samples were pre-incubated for 30 min at room temperature in 0.1 mM Tris-Cl, pH 8 with the following compounds: ethylenediaminetetraacetic acid (EDTA), SDS, dithiothreitol (DTT), 2-mercapto-ethanol (Bio-Rad Laboratories, Richmond, CA, USA), aminoethylbenzene-
sulphonylfluoride (AEBSF) (Calbiochem), aprotinin, tartaric acid, turkey egg white inhibitor, soybean trypsin inhibitor, ethyleneglycoltetraacetic acid (EGTA), pepstatin, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), 1,10-phenanthroline, PMSF, phosphoramidon, 3,4-dichloro-isocoumarin (DCI), DFP, and tosyl phenylalanyl chloromethylketone (TPCK), antipain, chymostatin, N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), salicylic acid, H$_2$O$_2$, ZnSO$_4$·7H$_2$O, Pb(NO$_3$)$_2$ and CuCl$_2$·2H$_2$O (Sigma Chemical Co., St. Louis, MO, USA). PQ-8, a currently used commercial anti-sapstain formulation, containing the detergent dodecyl benzene sulphonic acid (DDBSA), copper and the chelating agent 8-hydroxyquinolinolone, was obtained from ISK Biosciences, Memphis, TN, USA. Residual proteinase activity was subsequently determined using azocoll or sAAPF as substrates.

2.7 Electrophoretic analyses

Isoelectric focusing (IEF pH 3-9; IEF pH 4-6.5), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE (gradient 8-25%) were performed on the PhastSystem (Pharmacia, Uppsala, Sweden). Protein concentrations were measured by the Bradford microassay (Bio-Rad), using BSA as the standard (Bradford, 1976). Separation by SDS-PAGE (Laemmli, 1970) and native PAGE was performed at a constant current of 10.0 mA for a total 70 V·h. Separation by IEF was performed at a constant current of 2.0 mA for a total of 431 V·h. The pI and MW markers were from the Pharmacia kits for IEF and SDS-PAGE, respectively. For activity assays, isoelectric focusing and native gels were run in duplicate: one gel was silver stained for proteins according to the manufacturer's
recommendations (Pharmacia) and the other was used for a contact print zymogram. Protein bands were quantified using the Discovery Series Model DNA 35 densitometer (PDI, New York, USA).

Proteolytic bands were detected using unprocessed gelatin-coated X-ray film (Zhu et al. 1990, Cheung et al., 1991). X-Omat RP XRP-1 X-ray film (Kodak, Rochester, USA) was incubated with its gelatin surface in contact with the gels for 4 to 15 minutes at 37°C. After incubation, the film was washed under a stream of water. Clear zones, where the gelatin had been hydrolysed, indicated the presence of proteinase activity.

2.8 N-terminal sequencing and amino acid analyses

Protein samples were boiled for 5 minutes in SDS-PAGE sample buffer and run under constant voltage of 110 V using a Mini-PROTEAN II cell (Bio-Rad). Separated proteins were electroblotted onto an Immobilon-P PVDF membrane (0.45 mm pore size, Millipore) using the Trans-Blot electrophoretic transfer cell (Bio-Rad) for 1 h at 100 V or 3 h at 45 V. Proteins on the membrane were stained with 0.02% (w/v) Ponceau S, and bands were cut out. The amino acids were analysed on an Applied Biosystems Model 420 A/H (Applied Biosystems, Forster City, CA), and the N-terminal sequence was determined by automated protein sequencing on a pulsed liquid sequenator (model 473, Applied Biosystems). The sequence was compared to known protein sequences using the BLAST (Basic Local Alignment Search Tool) system developed by the National Centre of Biotechnology Information at the National Library of Medicine, Bethesda, MD, USA (Altshul et al., 1990). The BLAST system was accessed using electronic mail to carry
out the search using the BLAST heuristic algorithm for sequence alignments.

For N-terminal sequencing of autolytic products, proteins were separated by discontinuous SDS-PAGE according to Schägger and von Jagow, (1987) using a 10% T, 3% C separating gel and a 4% T, 3% C stacking gel. Tricine (0.1M) was used as the trailing ion in the cathode buffer. After electrophoresis, proteins were blotted onto an Immobilon-P SQ PVDF sequencing membrane (Millipore) using the Trans-Blot cell (Bio-Rad) for 3 h at 45 V in 10 mM pH 11 CAPS (Sigma) transfer buffer containing 20% methanol. The method of Schägger and von Jagow (1987) was found to be useful for separating and blotting proteins smaller than 20 kDa prior to sequencing.

Amino acid composition analysis was also performed on lodgepole pine sapwood samples. In this case, wood powder (25 mg) was added to a rimless pyrex test tube with 3 ml of 6 M HCl. The mixture was frozen in an acetone/dry-ice mixture, the atmosphere changed to nitrogen, and the tube sealed using a flame. Samples were hydrolysed at 110°C for 24 hours. After cooling, samples were suction-filtered and a 10 µl aliquot of the filtrate was derivatised using phenyl-thioisocyanate. Norleucine was added as an internal standard and the mixture was analysed with an amino acid analyser (model 420 H, Applied Biosystems) using a reverse phase column.

N-terminal sequencing and amino acid analysis was performed by Sandy Kielland/Nadja Spitzer at the Protein Microchemistry Facility at the University of Victoria, B.C. Amino acid
composition analysis was also performed by Dr. Krystyna Piotrowska at the Nucleic Acid -
Protein Service at the University of British Columbia, B.C.
Chapter 3.

Growth, nutrition and proteinase activity of

*Ophiostoma piceae* in culture and on wood

3.1 Introduction

Work conducted on the physiology of Ophiostomatales has been sparse, with most comprehensive investigations being performed on the pathogenic species of *Ophiostoma* - *O. ulmi*. Detailed analyses of the physiology of saprophytic species was conducted by Käärik in the 1950s and 1960s on isolates predominantly from Swedish forest products. This work described the effects of growth factors on the growth and sporulation of several *Ophiostoma* species in artificial media (Käärik, 1960; Mathiesen-Käärik, 1960). Ecological studies on these fungi have been carried out on an *ad hoc* basis in various research organisations, often published only as internal reports (e.g. Chung and Smith, 1986; Sutcliffe and Chan, 1992). Thus, the work reported in this chapter was necessary to establish optimum growth conditions and proteolytic activity for *O. piceae* grown in artificial media and in wood.

Lodgepole pine and aspen are commercially important softwoods and hardwood species respectively, and are susceptible to discoloration by staining fungi (section 1.1). For both groups of trees, storage of nitrogen as protein has been shown (section 1.4). However, the storage proteins of deciduous hardwoods, especially poplar, have received more attention than those of evergreen conifers. Specific storage proteins with molecular weights of 32 and 36 kDa have been identified in many different genera that accumulate during the late summer or
early autumn and are highly abundant throughout the winter (Langheinrich and Tischner, 1991). However, the utilisation of these proteins by sapstaining fungi has not been demonstrated.

Regulation of proteolytic activity is a critical aspect in the physiology of an organism growing in an environment that offers little nitrogen. Little information is available on the proteinases of staining fungi when grown in artificial media and no information is available when grown on wood. Fungi which degrade insoluble polymers outside the cell and take up breakdown products would require a means of regulating extracellular enzyme activity. According to Cohen (1980), there are several different mechanisms by which regulation occurs. The two most likely mechanisms for regulation of proteinases secreted by _O. piceae_ involve induction or derepressed synthesis. Induction, where the substrate or an effector molecule initiates synthesis of the enzyme, has been postulated to be involved in proteinase production by _Neurospora crassa_ grown in the presence of BSA and other soluble proteins (Drucker, 1975). In this system, carbon, nitrogen or sulphur starvation would not induce proteinase production. For derepressed synthesis, synthesis of proteinase production is induced by nutrient limitation of carbon, nitrogen or sulphur. Derepressed synthesis is thought to be the simplest system for regulation of extracellular catabolic enzymes and has been demonstrated for the production of neutral and alkaline proteinases of _Aspergillus nidulans_ (Cohen, 1973).

In this chapter the nutrient requirements, optimum growth conditions and proteolytic activity of the staining fungus _O. piceae_ were evaluated.¹ Conditions under which proteinases were

secreted during growth in culture and in wood were determined, and regulation of the activity was considered during growth in culture and in wood. The proteolytic activity was quantified and the electrophoretic pattern was analysed under various conditions. The effects of altering the nitrogen source supplied in culture or in wood were assessed in terms of their impact on proteolytic activity.²

²Some of the data concerning the nitrogen in pine was presented as a conference proceedings at the International Research Group of Wood Preservation conference in Orlando, Florida: Abraham, L.D. and Breuil, C. 1993. Organic nitrogen in wood: growth substrates for a sapstain fungus. IRG Doc No. IRG/WP/10019
3.2 Materials and methods

3.2.1 Culture medium

The initial work used the synthetic media (section 2.2.2) with the following modifications to give the semi-synthetic medium: for each litre, 1 ml of filter-sterilised vitamin solution (Tan and Breuil 1986) was used instead of the vitamin solution prepared as described by Montenecourt and Eveleigh (1977), NH₄NO₃ was used at 0.8 g/l and yeast extract was added to 0.01%.

3.2.2 Temperature and pH experiments

These studies were carried out using semi-synthetic media with initial pH set at 5.1. Five replicate flasks for each temperature were used in two separate experiments to determine growth over a broad and narrow temperature range, respectively. The pH experiments were carried out at 23°C using semi-synthetic media adjusted to the required pH by the addition of NaOH or HCl. The biomass was determined after 3 and 5 days of growth.

3.2.3 Nutritional experiments

To determine carbon source requirements, filter-sterilised solutions of starch, sucrose, glucose, maltose, or raffinose were added to the semi-synthetic media to give final concentrations of 2% (w/v). The amount of starch remaining in the culture filtrate was
determined colorimetrically (Garcia-Alvarado et al., 1992). In nitrogen utilisation experiments, starch was used as the carbon source and inorganic nitrogen sources (NaNO₃, NH₄Cl, NH₄NO₃) or organic nitrogen sources (urea, BSA, collagen, acid-hydrolysed casein, arginine, asparagine, glutamine, proline) were added to give nitrogen equivalents of 28 mg/100 ml media. Nitrogen sources were also combined in the synthetic media to give utilisable nitrogen equivalents of 28 and 65 mg/100 ml. BSA and ammonia, in the form of ammonium nitrate, were added separately, or as mixtures at a ratio of 1:1 or 1:3. Ammonia depletion was monitored by the phenolhypochlorite method (Solorzano 1969), and nitrate by the method of Cawse (1967). BSA concentrations were determined by an indirect enzyme-linked immunosorbent assay (ELISA) (Kendall et al., 1983) using a monoclonal antibody to BSA obtained from Sigma (product number B-2901).

3.2.4 Wood analysis and inoculation

Lodgepole pine and aspen wood blocks (section 2.4.1) were soaked for 2 h in sterile solutions of BSA at 5% (w/v), ammonium nitrate at 4.6% (w/v), or water. After equilibration overnight, wood blocks were inoculated with *O. piceae* (section 2.4.2) and incubated for 3 to 14 days. Inoculated and control wood blocks were milled (section 2.4.4) and samples of 40 to 100 mg dry weight were individually weighed into tubes for proteolytic assay (section 2.5). Nitrogen analyses of milled wood samples were conducted by micro-Kjeldahl (Kjeldahl, 1883; Pomeranz and Moore, 1975). Statistical analysis was conducted using SYSTAT 5.2 for Macintosh (Evanston, IL, USA). Results were examined by analysis of variance (ANOVA) with post-hoc comparisons using Tukey HSD tests. Ammonia analyses were also conducted
on wood powders. A known quantity of powder was homogenised in deionised water for 1 min with cooling using a Polytron Brinkman homogeniser (Brinkman, Mississauga, Ontario, Canada). The extractable ammonia was then determined in the liquid fraction using an indophenol colorimetric assay (Sheiner, 1976).

3.2.4.1 Extraction of proteinase from wood

Wood powder, prepared from pine sapwood colonised by O. piceae for 18 days, was extracted in buffer overnight at 4°C. The extraction buffer was 0.1 M Tris-Cl, pH 8, containing 0.5% Triton X-100, 5 mM sodium ascorbate, 5 mM Na₂S₂O₅, 5 mM DTT, 5 mM MnCl₂, 20 mM MgCl₂, 1 mM CaCl₂, and 10% glycerol (Lewinsohn et al., 1991). The wood slurry was ground with silica sand in a chilled mortar, and the liquid was collected by vacuum filtration. The pellet precipitated between 50 and 90% ammonium sulphate was resuspended in PBS.
3.3 Results

3.3.1 Growth on solid media

At 23°C on malt extract agar with an initial pH of 5.0, the radial growth rates were $1.7 \pm 0.4$ mm/day (mean ± S.D., triplicate plates). The maximum radial growth after 4 days was observed at 23°C. No growth occurred on plates incubated at 37°C after 8 days incubation, even after they were transferred back to 23°C for a further 14 days. At 4°C, growth could only be detected after 1 week. After 14 days, however, radial growth was better at 4°C (0.4 mm/day) than at 32°C (0.1 mm/day). Proteolytic activity was shown by clear zones on skim milk agar after 5 days incubation at 23°C.

3.3.2 Growth in liquid medium

*O. piceae* has two growth forms in liquid medium: hyphal and yeast-like. The hyphal form was predominant on 2% malt extract, and the yeast-like form was predominant on semi-synthetic media. Growth on malt extract resulted in 10% lower fungal biomass but stronger pigmentation.

The growth curve, determined by dry weight, showed an initial 24 h lag phase in synthetic medium, followed by a rapid increase in biomass which corresponded to nitrogen depletion (*Figure 3.1*). After day 3, the growth slowed and the biomass reached a maximum dry weight:
Figure 3.1 Growth of *O. piceae* strain 387N at 23°C, in synthetic inorganic liquid medium at pH 6.1. Vertical bars represent 95% confidence intervals for the means: dry weight, n=4; protein, n=3; ergosterol, n=3 or 2 (the maximum coefficient of variation was 20.6%). (A) Fungal dry weight (solid circles), depletion of starch (open circles) and depletion of ammonia (open diamonds). (B) Biomass measurement by protein (solid circles) and ergosterol (open circles).

of 5 mg/ml at day 4. This coincided with the depletion of the nitrogen source, ammonia. The amount of nitrogen available for growth was approximately 0.3 g/l. The carbon source was completely depleted after 6 days. The growth profile was also measured by determining the
protein and ergosterol contents of the cells. As for fungal dry weight, the highest amount of protein (0.84 mg/ml) was recorded at day 3. However, the protein expressed as a percentage of the biomass was high at 23% on day 2 and then reached a stationary level of 15% on day 4. The ergosterol content reached a maximum of 23.8 mg/ml culture during the stationary phase of growth (Figure 3.1). All three parameters used to measure fungal growth showed a similar pattern comprising lag, exponential and stationary growth phases. Dry weight was chosen as the parameter for monitoring growth in subsequent experiments since it proved to be the most convenient and accurate to measure.

3.3.3 Optimal pH and temperature for liquid cultures

After 2 days growth in semi-synthetic liquid medium, highest biomass values were obtained with cultures grown at 23°C (Figure 3.2). At low temperatures, as noted on solid medium, the organism grew slowly initially. However, after 6 days the biomass obtained at 14°C was comparable to growth at 23°C. At 35°C growth was minimal after 2 days, but increased rapidly when the temperature was lowered to 23°C. From light microscopic observations, the fungus seemed to be more in the yeast form at higher temperatures and more in the mycelium form at lower temperatures.

An initial pH of 6.1 resulted in the greatest amount of biomass if cultures were harvested after 3 to 5 days (Figure 3.3). The pH dropped by about 1.5 units over this period. If cultures were harvested later, a higher initial pH resulted in higher amounts of biomass. Increasing acidity
appeared to enhance the ratio of yeast-like cells over hyphae. Acceptable growth was obtained over a pH range of 3 to 9.

Figure 3.2 Growth response of *O. piceae* strain 387N to temperature in semi-synthetic liquid medium with an initial pH of 5.1. Dotted lines show results from the initial experiment for day 2 (open circles) and day 6 (solid circles). Solid lines show results from a more refined experiment for day 2 (open circles) and day 3 (solid circles). Vertical bars represent 95% confidence intervals for each mean of 5 replicate cultures.
3.3.4 Carbon and nitrogen requirements

On agar plates, the carbon sources resulting in the most rapid growth rates were, in decreasing order, starch, maltose, raffinose, sucrose and then glucose. In semi-synthetic liquid media, the fungus was able to utilise monosaccharides, disaccharides, trisaccharides and polysaccharides like starch (Figure 3.4). Starch was chosen as the carbon source for subsequent experiments because it is an important source of carbon in wood.
Figure 3.4 Growth of *O. piceae* strain 387N on various carbon sources after 3 days (shaded bars) and 5 days (solid bars) of incubation at 23°C, in semi-synthetic liquid medium with an initial pH of 6.1. The control (CON) represents growth with no added carbon source. MX, 2% malt extract; MALT, 2% maltose; STAR, 2% starch; SUCR, 2% sucrose; GLUC, 2% glucose; RAFF, 2% raffinose. The maximum coefficient of variation was 4.6%.

*O. piceae* used ammonium, but not nitrate, as an inorganic nitrogen source (Figure 3.5). Dry weight obtained after growth on sodium nitrate was comparable to the level measured when no nitrogen source was available. The fungus was able to utilise all the organic sources of nitrogen tested, with casein resulting in the highest fungal biomass. When supplied with identical total levels of nitrogen, the same amount of fungal biomass was obtained with ammonium and urea. Growth was approximately the same with all the different amino acids supplied. Except for asparagine, the pH of the medium decreased with fungal growth for all nitrogen sources. The greatest decrease in pH was recorded for media supplemented with ammonium and urea.
Figure 3.5 Growth of *O. piceae* strain 387N on various nitrogen sources after 2 days (darkly shaded bars), 4 days (shaded bars) and 7 days (solid bars) incubation at 23°C, in semi-synthetic medium with an initial pH of 6.1. The control (CON) represents activity in cultures with no added nitrogen. N1, NaNO₃; N2, NH₄NO₃; N3, NH₄Cl; ASN, asparagine; GLN, glutamine; ARG, arginine; PRO, proline; CAS, casamino acids; BSA, bovine serum albumin. All nitrogen sources were added to give nitrogen equivalents of 28 mg/100 ml. The average of duplicate cultures are shown (maximum coefficient of variation was 12.7%).

3.3.5 Effect of different nitrogen sources on the proteolytic activity

In liquid media supplemented with various nitrogen sources, proteolytic activity in the filtrates was assayed at pH 5 and pH 8. Proteinase activity was minimal in media supplemented with easily assimilable nitrogen (e.g. ammonium, urea and single amino acids)
Small amounts of activity were detected at day 4, when ammonia was depleted. Similarly, activity was measured in filtrates of cultures grown in media devoid of nitrogen (CON) or supplemented with a non-assimilable source of nitrogen such as NaNO₃ (Figure 3.6).

**Figure 3.6** Azocoll assay showing proteolytic activity in culture filtrates of *O. piceae* at pH 8. Cultures were supplemented with various nitrogen sources and grown for 2 days (darkly shaded bars), 4 days (shaded bars), or 7 days (solid bars) at 23°C, in semi-synthetic liquid medium with an initial pH of 6.1. The control (CON) represents activity in cultures with no added nitrogen. BSA, bovine serum albumin; N1, NH₄NO₃; N2, NH₄Cl; ASN, asparagine; GLN, glutamine; ARG, arginine. All nitrogen sources were added to give nitrogen equivalents of 28 mg/100 ml. Proteinase activity units are defined as the amount of enzyme which produced an A₅₂₀ of 0.1 units/ (ml · min) at 37°C and 320 rpm.

As expected, the best activity was recorded when protein was used as the source of organic nitrogen (e.g. BSA, collagen). This activity was approximately 300 fold greater than in control cultures lacking a nitrogen source.
In all filtrates, proteinase activity measured at pH 8 was higher than at pH 5. The optimal pH for activity was confirmed in a more detailed study, where assays were conducted at a range of pH values, for cultures grown with BSA as the nitrogen source. The optimal pH for activity was 8 for each day of growth. For all pH's, the relationship between activity and days of growth followed a trend similar to that shown for pH 8 (Figure 3.7).

**Figure 3.7** Fungal biomass (open diamond) and depletion of starch (open circles) during growth of *O. piceae* strain 387N at 23°C, in synthetic liquid media with an initial pH of 6.1 containing BSA as sole nitrogen source. Vertical bars represent 95% confidence intervals for each mean of 4 replicate cultures. Proteinase activity units (solid circles) are defined as the amount of enzyme which produced an $A_{520}$ of 0.1 units/(ml · min) at 37°C and 320 rpm (maximum coefficient of variation was 19.9%).
The total proteolytic activity in the culture filtrate increased from day 1 to day 9. However, when the activity was expressed as a function of the biomass present, the maximum activity was observed at days 2 and 3. BSA was utilised by the cultures during the first 5 days of growth, whereas starch was not depleted (Figure 3.7). Using ELISA to measure BSA, we found that the initial concentration of 0.2% BSA was reduced to 0.015% by day 7. These results were in agreement with the depletion of BSA (pI 4.8) as seen in electrophoretic gels (Figure 3.8).

**Figure 3.8** Separation by PhastGel IEF 3-9 of the extracellular proteins obtained after growth of *O. piceae* 387N on BSA supplemented media. Lanes 1, 6: pI markers; lane 2: day 1; lane 3: day 3; lane 4: day 6; lane 5: day 9. (A) silver-stained gel showing location of proteins. (B) X-ray film overlay of the gel after 4 minutes incubation at 37°C, showing proteolytic activity.
Electrophoresis of culture filtrates by IEF showed a major protein band which focused at a pI value of 5.6 and corresponded to the site of clearing on the zymogram. The amount of protein and the size of the clearing zone increased with growth (Figure 3.8). Similarly, a single major band of clearing was obtained for native-PAGE gel overlays. Other proteinase bands at pI 5.2 and 7.6 were detected when the incubation time for the IEF gels in contact with the overlay was increased from 15 to 30 minutes.

In order to investigate the regulation of the proteinase, nitrogen was supplied in the form of both ammonium and protein. An increase in proteinase activity was detected which coincided with ammonia depletion after four days of growth (Figure 3.9).

![Figure 3.9](image_url)  
**Figure 3.9**  Fungal biomass (solid circle) and depletion of ammonia (open square) during growth of *O. piceae* strain 387N in media supplemented with equal proportions of nitrogen as ammonia and BSA. Proteinase activity units (open circles) are defined as the amount of enzyme which produced an $A_{320}$ of 0.1 units/(ml - min) at 37°C and 320 rpm.
When higher amounts of nitrogen were added to media containing both ammonia and BSA, such that the ammonium exceeded the nitrogen required for ten days of growth, proteinase activity was not detected at all during ten days of growth.

3.3.6 Nitrogen content of lodgepole pine and aspen sapwood

3.3.6.1 Untreated blocks

The moisture content of the wood expressed as a percentage of dry weight was 111 ± 11.5 and 125 ± 6.8 % for aspen and pine wood blocks, respectively. Nitrogen contents of lodgepole pine sapwood samples taken from different positions within the tree were very similar, although there seemed to be a slight decrease in nitrogen content of about 30 ppm with increasing height (from VI 1 to I 1). However only the value from the lowest sample (VI 1) was statistically greater than some of the samples at higher heights (III 1 to I 1). Samples taken from the cambium to the heartwood showed that the part closest to the cambium (VI 1) and that closest to the heartwood/sapwood boundary (VI 6) had slightly higher nitrogen contents than the samples between these regions.

Analyses of the ammonia content of pine and aspen showed that it accounted for less than 5% and 2% respectively, of the total nitrogen present (Table 3.2). Although pine contained less total nitrogen than aspen, it contained almost double the amount of extractable ammonia.
Table 3.1  Nitrogen content\(^1\) in lodgepole pine sapwood samples from various heights and radial distances in the tree as determined by Kjeldahl analyses and expressed as ppm N dry weight

<table>
<thead>
<tr>
<th></th>
<th>VI1</th>
<th>VI2</th>
<th>VI3</th>
<th>VI4</th>
<th>VI5</th>
<th>VI6</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI1</td>
<td>483 ± 14(^{tx})</td>
<td>447 ± 9(^{a})</td>
<td>458 ± 16(^{ab})</td>
<td>452 ± 4(^{a})</td>
<td>463 ± 5(^{abc})</td>
<td>475 ± 8(^{bc})</td>
</tr>
<tr>
<td>V</td>
<td>461 ± 4(^{xy})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>465 ± 18(^{yy})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>452 ± 2(^{y})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>455 ± 7(^{y})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>449 ± 4(^{y})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\)Mean ± S.D., n = 4; ANOVA of the radial and vertical samples were carried out separately, P<0.005;
Tukey test, P<0.05; values with the same letter are not statistically different

Organic nitrogen levels were difficult to quantify. SDS-PAGE of extracts from mature pine sapwood, sapwood from branches and sap pressed from pine wood chips (section 2.4.3) showed protein bands in the molecular range of 14 to 21 kDa, with minor bands between 31 and 45 kDa (gel not shown). Amino acid composition analysis of acid-hydrolysed pine sapwood samples indicated relatively high levels of Asx, Glx, Ser, Gly and Ala (Table 3.2). Asx and Glx are frequent in vegetative storage proteins (Wetzel and Greenwood, 1991). Such proteins would have been hydrolysed during acid digestion prior to amino acid analyses. The highest yield obtained was about 0.986 μg amino acids per mg wood. This corresponded to about 170 ppm nitrogen per mg wood.
Table 3.2 Amino acid composition of lodgepole pine sapwood samples. Results are expressed as mass percentage of amino acids in each sample.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>VI 3</th>
<th>II 1</th>
<th>II 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASX</td>
<td>10.5</td>
<td>8.3</td>
<td>10.1</td>
</tr>
<tr>
<td>GLX</td>
<td>12.4</td>
<td>11.0</td>
<td>13.2</td>
</tr>
<tr>
<td>SER</td>
<td>9.5</td>
<td>14.5</td>
<td>11.8</td>
</tr>
<tr>
<td>GLY</td>
<td>5.8</td>
<td>16.8</td>
<td>6.4</td>
</tr>
<tr>
<td>HIS</td>
<td>1.0</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>ARG</td>
<td>4.5</td>
<td>3.3</td>
<td>5.0</td>
</tr>
<tr>
<td>THR</td>
<td>6.8</td>
<td>5.9</td>
<td>6.4</td>
</tr>
<tr>
<td>ALA</td>
<td>9.4</td>
<td>8.5</td>
<td>8.7</td>
</tr>
<tr>
<td>PRO</td>
<td>5.0</td>
<td>4.1</td>
<td>4.8</td>
</tr>
<tr>
<td>TYR</td>
<td>1.8</td>
<td>2.8</td>
<td>1.1</td>
</tr>
<tr>
<td>VAL</td>
<td>6.3</td>
<td>6.0</td>
<td>7.2</td>
</tr>
<tr>
<td>MET</td>
<td>0.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ILE</td>
<td>11.8</td>
<td>2.4</td>
<td>5.1</td>
</tr>
<tr>
<td>LEU</td>
<td>5.1</td>
<td>11.6</td>
<td>5.3</td>
</tr>
<tr>
<td>PHE</td>
<td>3.1</td>
<td>7.4</td>
<td>5.7</td>
</tr>
<tr>
<td>LYS</td>
<td>6.1</td>
<td>5.8</td>
<td>7.9</td>
</tr>
<tr>
<td>CYS</td>
<td>ND¹</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TRP</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Total amino acids (ng) | 197.9 | 121.5 | 116.2 |
Sample size (mg)       | 24.1  | 25.4  | 27.8  |

¹not determined

3.3.6.2 Blocks treated with solutions of nitrogen

In experiments with addition of nitrogen to the wood, the nitrogen content of pine and aspen blocks increased significantly after 2 h soaking in solutions of NH₄NO₃ and BSA (Table 3.3). The sapwood of both wood species took up two times more nitrogen when soaked in ammonium nitrate than when soaked in BSA.
Table 3.3 Nitrogen content of lodgepole pine and aspen sapwood samples after soaking in nitrogen solutions.

<table>
<thead>
<tr>
<th>Sample description</th>
<th>TKN(^1) (ppm dry weight)</th>
<th>Extractable ammonia (ppm N dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pine unsoaked control</td>
<td>549</td>
<td>25</td>
</tr>
<tr>
<td>soaked in dH(_2)O</td>
<td>537</td>
<td>19</td>
</tr>
<tr>
<td>soaked in 4.6% NH(_4)NO(_3)</td>
<td>4495</td>
<td>ND(^2)</td>
</tr>
<tr>
<td>soaked in 5.0% BSA</td>
<td>2242</td>
<td>ND</td>
</tr>
<tr>
<td>Aspen unsoaked control</td>
<td>807</td>
<td>12</td>
</tr>
<tr>
<td>soaked in dH(_2)O</td>
<td>638</td>
<td>7</td>
</tr>
<tr>
<td>soaked in 4.6% NH(_4)NO(_3)</td>
<td>5900</td>
<td>ND</td>
</tr>
<tr>
<td>soaked in 5.0% BSA</td>
<td>2587</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^1\)Total Kjeldahl nitrogen
\(^2\)not determined

For the ammonium nitrate the nitrogen content was more than seven times that in the original wood. For BSA it was about four times greater. Control blocks soaked in water lost 12 and 169 ppm TKN from pine and aspen blocks, respectively. This probably represents the loss of soluble nitrogen.

3.3.7 Growth and proteolytic activity on pine and aspen sapwood

*O. piceae* grew actively on aspen and lodgepole pine. Visual differences in growth were observed between growth on wood blocks soaked in organic and inorganic nitrogen solutions. BSA appeared to accelerate growth and pigmentation on pine and aspen. Asexual reproductive structures, synnemata, were observed on the surface of wood after three days. Discoloration of pine and aspen was noticeable after 3 days, and intensified over two weeks.
Figure 3.10 Aspen and pine wood blocks 14 days after inoculation with *O. piceae*. From left to right, the blocks were uninoculated controls (A), soaked for 2 h in sterile solutions of water (B), 4.6% NH₄NO₃ (C) and 5% BSA (D)

Findings were similar for blocks soaked in water, although growth did not appear to be as rapid as on blocks soaked in BSA. In contrast, growth of *O. piceae* on wood soaked in inorganic nitrogen was white and filamentous on the surface of the blocks with little pigmentation, even after two weeks of fungal growth (*Figure 3.10*).

Initial assays indicated that proteolytic activity in wood was higher at pH 8 than at pH values less than 7. Therefore, subsequent assays were conducted at pH 8. Proteolytic activity in inoculated wood increased with time and reached its maximum after six or nine days. The level of proteolytic activity in pine (*Figure 3.11 A*) was approximately ten times higher in the
BSA-soaked wood than in the water-soaked wood, whereas the activity in NH₄NO₃-soaked wood was less than in water-soaked wood.

![Graph](image)

Figure 3.11 Proteolytic activity in lodgepole pine (A) and aspen (B) sapwood samples after colonisation by Ophiostoma piceae 387N at 23°C. Proteinase activity units are defined as the amount of enzyme which produced an A₅₂₀ of 0.1 units/g dry wood/min at 37°C, pH 8 and 320 rpm. Activity in wood soaked for 2 h in 5% BSA (solid squares), in water (solid circles) and in 4.6 % NH₄NO₃ (open triangles). The points plotted are the mean values of 4 determinations with standard deviations shown by error bars.

In aspen the pattern was similar (Figure 3.11 B), although the levels of activity were higher in all cases. In untreated or water-soaked wood, proteolytic activity was up to ten times higher
in aspen than in pine. This was not accounted for by a ten times increase in biomass levels which were measured by ergosterol. Ergosterol contents in pine and aspen were 38 and 65 μg/g wood respectively, after 14 days growth of *O. piceae*.

The presence of the proteinase in the wood was confirmed by IEF analyses of protein extracts from infected pine. A protein band that corresponded to the site of clearing on the zymogram, focused at a pI value of 5.6 which was comparable to the proteinase produced in liquid medium. Maximum proteolytic activity in wood extracts was detected at pH 8 (*Figure 3.12*), which again correlated with the optima pH for the proteinase produced in liquid medium.

![Graph showing the effect of pH on proteinase activity](image)

**Figure 3.12** The effect of pH on proteinase activity extracted from pine wood blocks colonised by *O. piceae* for 18 days. Proteinase activity units (U) are defined as the amount of enzyme which produced an A₅₂₀ of 0.1 units/min per ml extract at 37°C and 320 rpm.
3.4 Discussion

Our results showed that *O. piceae* can grow on a defined synthetic media supplemented with vitamins. Various carbon sources were assimilated equally well, including carbon polymers such as starch. Starch is generally recognised to be an important storage carbohydrate in trees, but soluble sugars can also constitute a major proportion of the total non-structural carbohydrates present (Cranswick *et al.* 1987). Further work in our laboratory (Gao *et al.*, 1994) has examined the ability of *O. piceae* to utilise wood lipids as carbon sources. Triglycerides and fatty acids were identified as nutrients for this fungus. Although *O. piceae* could degrade xylan and carboxymethyl cellulose when present in artificial media (data not shown), it was unable to utilise avicel, solka floc and wood cellulose and lignin. Generally, staining fungi cause minimal structural damage to wood (section 1.1) because they are unable to degrade the structural polysaccharides and lignin.

Like most *Ophiostoma* species (Käärik 1960), *O. piceae* utilised ammonia or organic nitrogen, but not nitrate, as a nitrogen source. Similarly, synthetic medium supplemented with nitrate did not support the growth of 41 wood-rotting basidiomycetes (Highley and Kirk, 1979). Growth and ammonium utilisation when ammonium nitrate was used as the sole nitrogen source provided a measure of the nitrogen requirement for *O. piceae*. Approximately 0.3 mg ammonia-N was assimilated to give 5 mg/ml biomass. Therefore the N-requirement of *O. piceae* was about 0.06 mg N/mg dry weight, which is consistent with known values for the nitrogen composition of fungi and in agreement with values of 0.05 and 0.08 mg N/mg dry weight determined for basidiomycete fungi (Kalisz *et al.*, 1986).
Apparently proteins (e.g. BSA) may be metabolised both as carbon and nitrogen sources, since starch was not utilised for the first 5 days when both BSA and starch were supplied in the medium (Figure 3.7). *O. piceae* is an ascomycete, but found in the same habitat as the decay fungi which are basidiomycetes. Therefore, it is interesting that basidiomycete fungi have also been shown to utilise protein as sole source of carbon, nitrogen and sulphur (Kalisz *et al.*, 1986). These nutritional requirements of *O. piceae* are similar to those found in previous investigations on sapstaining fungi (Käärik 1960, 1974).

Ambient temperature, moisture content and pH are other parameters that influence the rate of development of sapstain on wood. *Ophiostoma* species are mainly found in temperate climates. *O. piceae* is a mesophilic fungus that grows well at moderate temperatures between 14 and 32°C. The organism did not grow at 35°C and did not survive at this temperature for more than one week. After prolonged growth at 6°C, its biomass was similar to the amount detected at higher temperatures (Figure 3.2). This would explain why serious staining problems can occur when the wood is stored at 3 to 8 °C, or when the trees are cut in the early spring (personal communications from saw mill staff).

Apart from the effect of environmental factors, the form and concentration of nitrogen in wood was found to influence the wood discoloration which is caused mainly by the melanin in hyphal cell walls. When wood was soaked in an inorganic nitrogen solution, there was very little discoloration compared to wood in which protein was the major nitrogen source. The effect of the nitrogen source on pigmentation has also been demonstrated in liquid culture (A. Brisson, UBC, personal communication) where the nutrients available for growth can be
more readily defined and manipulated. Although the reasons for this observation are not fully understood, it is possible that the catabolism of proteins may result in the formation of products which are needed for melanin biosynthesis. These precursors may not be formed from the utilisation of inorganic nitrogen.

Fungal proteinases play a major role in nutrition, development and pathogenesis, and are produced by fungi of all major taxonomic groups (North 1982). When cultures of *O. piceae* were supplemented with protein, e.g. BSA, proteinase activity per unit of fungal biomass increased during the active growth of the organism, reaching a maximum between days two and three, and dropped sharply after day three. The low amount of proteinase activity detected on day one (*Figure 3.7*) could be due to the binding of the enzyme to the substrate present in the medium, or to the fungal cell wall, or to an extracellular sheath attached to the cell wall. These mechanisms have been reported in other fungi (Kalisz et al., 1987), and may confer a competitive advantage to an organism growing in a solid substrate such as wood. The presence of a hyphal sheath in liquid cultures of *O. piceae* has been observed (Hoffert, 1995). In addition, production of extracellular membranous structures, which may resemble a sheath, were observed during growth of *O. piceae* in wood (Luck et al., 1990).

The role or function of a proteinase must be directly related to its location *in situ*. Enzymes and their products are unlikely to diffuse away from their hyphae when they are growing in wood, and a fungal sheath is probably "instrumental" in retaining enzymes and products of digestion close to the hyphae (Venables and Watkinson, 1989). A polysaccharide hyphal sheath is a morphological feature of many staining and decay fungi, occurring around the
growing points and along the length of hyphae (Eriksson et al., 1990). It appears to be involved in the support and transportation of fungal enzymes (Palmer et al., 1983), and in the creation of a micro-environment for optimal enzyme activity (Eriksson et al., 1990). Naturally immobilised enzymes would offer advantages to organisms growing on solid substrates in ensuring efficient utilisation of substrate macromolecules such as proteins (Kalisz et al., 1987). It may also provide a form of protection against dehydration and environmental injury (Green et al., 1992), and assist in the attachment of the hyphae to the wood cell wall (Ruel and Joseleau, 1991).

Traces of proteinase activity were detected in liquid cultures of O. piceae supplemented with easily assimilable organic nitrogen sources like amino acids and urea. Similarly, very little activity was recorded when inorganic nitrogen was added to the media. In contrast, when no nitrogen source was present in the media, proteolytic activity was detected; however, the activity was about 300 fold lower than with organic nitrogen. When combined inorganic and organic nitrogen sources were supplied in culture, proteinase production was only observed after the ammonium had been depleted. Proteinase production under conditions of nitrogen starvation suggests that the major regulatory mechanism for control of proteolytic activity was derepression. Results in wood appeared to support this form of regulation.

Proteinase activity in wood soaked in ammonium nitrate was consistently lower than in wood soaked in water, suggesting a preferential use of inorganic nitrogen in the presence of protein. However, in wood the results were more difficult to interpret since the distribution of added nitrogen was unknown, and the form of nitrogen prior to the addition of nitrogen was not
completely defined. After pine sapwood had been soaked in inorganic nitrogen and infected by *O. piceae*, very little proteinase activity was detected. However, when the wood was soaked in protein solutions, three fold differences in proteinase levels were detected. Interestingly, proteolytic activity in untreated aspen was about 10 fold higher than in pine. Yet the total nitrogen content of aspen was not quite double that of pine, and the total biomass produced on aspen was only twice that on pine. Therefore, it appears that factors other than the nitrogen content may influence the growth and proteolytic activity of *O. piceae*. The presence of natural endogenous proteinase inhibitors associated with proteinases has often been reported (DeMartino, 1989) and may explain some of the differences observed in wood. Fungal inhibitors were previously shown to be present in pine sapwood (Bjurman, 1986), and extremely potent, specific proteinase inhibitors have been isolated in the past from such sources as the seeds of leguminous plants, grains and potatoes (Murao et al., 1985). Other factors such as differences in the pH, extractive content or type of protein substrate may also explain some of the differences in activity on pine and aspen.

Qualitative and quantitative data on the nitrogenous compounds in lodgepole pine have confirmed the presence of proteins and amino acids. The nitrogen content of pine and aspen determined by the Kjeldahl method agreed with previous data (Merrill and Cowling 1966), falling in the expected range of less than 0.1% of the dry weight of wood. Ammonium nitrogen represented less than 5% of the total nitrogen in pine and less than 2% of the total nitrogen in aspen. Wood samples were not analysed for nitrate because it was not a potential nitrogen source in these experiments with *O. piceae*, however, previous studies have shown that it is usually present at 1 to 2 percent of the total nitrogen (Käärik, 1960). Proteins were
shown to be present in mature pine wood, branch wood, and sap pressed from green chips of lodgepole pine. The proteins extracted from wood were mainly low molecular weight proteins which may be more amenable to extraction than higher molecular weight, hydrophobic proteins. Proteins are probably the most important nitrogen reserves in trees (van den Driessche, 1984), comprising 75 to 80% of the nitrogen in bark and 50 to 60% of the nitrogen in the wood of young apple trees. However, woody tissues are heavily lignified and contain relatively few living cells per unit mass. Furthermore, woody tissues generally contain high levels of phenolic compounds which often create difficulties in isolating and quantifying proteins from wood. Phenolics interfere with many of the protein assay procedures. In addition, the presence of wood extractives appears to further complicate extraction protocols. However, all evidence suggests that the protein in wood was likely responsible for proteinase production by *O. piceae*.

Proteinases secreted by *O. piceae* during growth on wood or in liquid culture were also evaluated after electrophoresis. The use of X-ray film following native PAGE or IEF was a very quick, and effective way to localise the proteolytic band after non-denaturing electrophoretic separation. Compared to the traditional zymogram procedure (Brown *et al.*, 1982) using a second substrate gel containing skim milk, the use of X-ray film was considerably cheaper and more convenient. Furthermore, the results were immediately visible after rinsing the film whereas the traditional overlay requires staining before zones of clearing are distinct.

In the case where protein was the sole source of nitrogen, this work showed that an alkaline proteinase with a pI of 5.6 was produced. This proteinase appeared to function in the primary
retrieval of nitrogen during growth on wood and in artificial media. The proteolytic activity measured spectrophotometrically in culture filtrates was in agreement with the results from native and isoelectrofocusing gels followed by overlays. A major proteinase band with a pI of 5.6 and optimum activity at pH 8 was detected after 24 hours of growth, and increased throughout the growth. In general the pH of wood varies from 4 to 6, but there can be considerable heterogeneity between different tissues. Wood pH is measured on wood powder (sawdust) in a suspension. It is difficult to know how closely such measured pHs correspond to actual microenvironmental conditions (Rayner and Boddy, 1988). For example, the pH of the parenchyma cells may be quite different from the other woody cells. Similarly, the pH in the microniche of the hyphal sheath may also be different than in the rest of the wood in order to create optimal conditions for enzyme reactions.

Late in the stationary growth phase, two other minor proteinase bands were detected in culture filtrates, at pI 7.6 and pI 5.2. Similar results were found in cultures grown on other sources of protein such as casein, gelatin, collagen and soybean. The proteinase activity profile of *O. piceae* was linked to growth, in contrast to reports on the proteinase activity of *Penicillium roqueforti*, which produces acid proteinases independently of biomass (Petrovic et al., 1991). In general, in wood, as in synthetic media, two types of proteinases may be produced -- one to break down wood protein into easily assimilable compounds, and the other to recycle fungal protein when the external source of nitrogen is depleted (Micales, 1992). Recycling and translocation of nitrogenous compounds within the fungal cell may provide a nitrogen pool. Lilly and co-workers (1991) suggested that under nutrient limitation, fungi may not necessarily sporulate, but may divert all energy resources to continued hyphal extension in
order to increase the chance of reaching a fresh nutrient supply. Then, autolytic breakdown of proteins in older mycelia is the major source of translocatable nitrogen (Fenn and Kirk, 1981).

In summary, in this chapter the growth characteristics of *O. piceae* on its natural substrate, wood and in artificial media were described. The production of extracellular proteolytic enzymes during growth under various conditions was examined. Proteinases appeared to be secreted under conditions of starvation, and in the presence of protein when no other simple nitrogen sources were available. Proteinase production was associated with active growth, suggesting an essential physiological role in primary retrieval of nitrogen. Production of proteinases during growth on wood confirmed that protein was the major nitrogen source in wood, and analyses confirmed the minimal content of ammonia in pine and aspen. Electrophoretic separation of secreted proteins suggested that one major proteinase was produced with an acidic pl and an alkaline pH optimum.
Chapter 4.

Isolation and preliminary characterisation of a subtilisin-like serine proteinase secreted by *Ophiostoma piceae*

4.1 Introduction

The introduction to this thesis outlined the classification of proteinases according to their catalytic mechanism (section 1.6) and the use of inhibitors to distinguish the four types of proteinases (section 1.7). One of the useful steps in the classification of a newly discovered proteinase is the exposure of the enzyme to a limited number of “standard” inhibitors. For each type of proteinases specific inhibitors acting on the crucial amino acid or metal ion at the active site are known. For example, sensitivity to PMSF in addition to DFP permits the identification of serine proteinases, E-64 can be used to assay for cysteine proteinases and pepstatin for aspartic proteinases. Similarly, chelating agents such as EDTA and 1,10 phenanthroline identify metalloproteinases (Dunn, 1989). Once the initial testing has established which types of proteinases are present, the response to a broader range of inhibitors can provide more data on the mechanism of action.

While inhibitors are often useful in determining the type of proteinase in crude extracts, for definitive testing purified enzymes should be used. In general, the purification of proteolytic enzymes presents the normal challenges associated with the purification of all proteins. In addition there are specific problems, such as autolysis, inherent in dealing with the purification of this class of enzymes (DeMartino, 1989). Many proteinases can be purified with a
combination of conventional steps. The proposed purification steps can be selected on the basis of their resolving power, sequence compatibility, capacity for amount of sample (in terms of volume and protein concentration), cost, protein yield and the necessity of preserving the activity of the proteinase (Harris, 1989). In the early stages of a purification strategy, capacity and low cost are important while at the later stages high resolution is important.

For the purification of an extracellular protein, the first stage is generally clarification (e.g. centrifugation), usually followed by a concentration step (e.g. ultrafiltration or precipitation). Chromatography techniques succeeding the primary separation techniques can include hydrophobic interaction, ion exchange or affinity chromatography. Chromatofocusing and gel filtration are usually only considered towards the end of the purification strategy when the samples are smaller (DeMartino, 1989). Assays for yield and degree of purification will indicate whether the yield is acceptable and whether one technique is more effective than another. Analysis by gel electrophoresis will also indicate the purity of the protein and how many contaminants are present (Harris, 1989).

Initial work, described in Chapter 3, showed the production of proteinases during growth of *O. piceae* 387N in protein-supplemented liquid culture and in blocks of lodgepole pine and aspen. The results showed that the major proteinase produced had an alkaline pH optimum, and this suggested that the enzyme was a serine proteinase. In this chapter inhibitors were used to confirm the secretion of a serine proteinase during active fungal growth in wood in liquid culture, and to determine whether any other types of proteinases were produced. Results on the purification and characterisation of the major proteinase in terms of electrophoretic properties,
catalytic properties, amino acid content and N-terminal sequence are presented. The stability of
the proteinase was characterised under various pH/temperature regimes and the results
compared with data for other proteinases. Some of the characteristics which may influence
both the function and practical application of the purified proteinase are detailed.¹

¹These data were submitted for publication: Abraham, L.D., and Breuil, C. 1995. Isolation and characterization
of a subtilisin-like serine proteinase secreted by the sap-staining fungus Ophiostoma piceae. Enzyme and
Microbial Technology. Accepted.
4.2 Materials and methods

4.2.1 Chromatography of proteinase

4.2.1.1 Analytical separations

Fungal cultures grown in the synthetic medium containing 3% starch and 0.6% soybean protein (section 2.2.2) were harvested after 3 to 6 days growth. Culture filtrates were partially concentrated by ultrafiltration using the Minitan system equipped with four PLCC Minitan plates with a molecular weight cut-off of 5 kDa (Millipore), or the Amicon system fitted with a Diaflo YM3 membrane with a 3 kDa cut-off (Amicon). Proteins were then precipitated using ammonium sulphate at 90% saturation, and the pellets resuspended in Tris-Cl (0.1 M, pH 8) and stored at -20°C. Various chromatographic separations were tested using the FPLC (fast protein liquid chromatography) system (Pharmacia).

Anion exchange chromatography was conducted using a MonoQ HR5/5 column (Pharmacia) equilibrated with 20 mM Tris-Cl, pH 8. Samples were first desalted using a Bio-gel P6 column (Bio-Rad) equilibrated with the equilibration buffer. Elution from the anion exchange column was carried out by applying a linear salt gradient from 0 to 0.5 M NaCl in the buffer. Gel filtration was conducted using a Superose 12 HR 10/30 column (Pharmacia) equilibrated with 20 mM Tris-Cl, pH 8. Chromatofocusing was conducted using a MonoP HR 5/20 column (Pharmacia) equilibrated with 25 mM piperazine-Cl, pH 6.3. Elution was carried out using 1/10 diluted Polybuffer 74-Cl (Pharmacia) at pH 4.5. Hydrophobic interaction chromatography was performed on a Phenyl-Superose HR 5/5 column (Pharmacia) equilibrated with 100 mM Tris-Cl,
1.7 M ammonium sulphate, at pH 8. Proteins were eluted by applying a decreasing linear salt gradient from 1.7 to 0 M ammonium sulphate in the buffer.

4.2.1.2 Preparative separations

Larger scale purifications were carried out by preparative hydrophobic interaction chromatography. The culture filtrate after 5 days of growth (approximately 5.8 l) was collected by filtration and ammonium sulphate was added immediately to give 2 M salt. The sample was loaded onto a 4.8 × 17 cm phenyl-Sepharose column (Pharmacia) equilibrated with 100 mM Tris-Cl, 1.7 M ammonium sulphate, at pH 8. Elution was carried out by applying a decreasing linear salt gradient from buffer containing 1.7 to 0.5 M salt. The fractions containing the peak of proteinase activity were pooled, and precipitated using ammonium sulphate at 90% saturation.

4.2.2 Determination of glycosylation

Glycosylation in the purified proteinase was determined using the digoxigenin (DIG) glycan detection kit (Boehringer-Mannheim, Mannheim, Germany). Transferrin and creatinase served as positive and negative controls for glycoproteins, respectively.

4.2.3 pH and thermal stability studies

The proteinase was mixed with 0.1 M MES-Tris-Acetate broad range buffer (Ellis and Morrison, 1982) at a ratio of 1:10 by volume. After incubation at set pHs or temperatures,
residual proteinase activity was assayed at 37°C, in 0.1 M Mes-Tris-Acetate (pH 8) using sAAPF as substrate (section 2.5). Half lives were determined by interpolating the least-squares linear fit of a plot of the ln of remaining activity versus time.
4.3 Results

4.3.1 Increasing proteinase production in liquid culture

When BSA (0.2%) was used as the nitrogen source in liquid media, proteinase activity in the culture filtrate reached 2.5 U/ml after 9 days of growth (section 3.3.5). To increase yields of proteinase, the medium composition was modified to support more fungal biomass. Higher concentrations of protein (0.6% soyprotein) and starch (3%) were used to increase fungal growth by four times. This increase in biomass to 20 mg/ml was accompanied by elevated levels of proteinase activity which reached 14 U/ml. A time course study showed that proteinases were produced during active fungal growth (Figure 4.1). When the proteinase activity was expressed relative to the amount of protein measured in the culture filtrate, the specific enzyme activity remained constant during the stationary phase of growth (Figure 4.1).

4.3.2 Inhibition of proteinases produced in culture and in wood

The proteinases produced by O. piceae were classified according to the effects that inhibitors had on proteolytic activity (Table 4.1). Total inhibition of activity in culture filtrates was obtained with PMSF, a known inhibitor of serine proteinases. An inhibitor specific for chymotrypsin-like serine proteinases, TPCK, and classical metalloproteinase inhibitors (1,10-phenanthroline and phosphoramidon) enhanced rather than inhibited proteinase activity. However, all other serine proteinase inhibitors caused some degree of inhibition (23 to 47%) at the concentrations used. Significant inhibition (88%) was also obtained with the chelating agent
EDTA. Aspartic and cysteine proteinase inhibitors had no inhibitory effect on activity, even with assays performed at acidic pH values.

Figure 4.1 Fungal growth (dry weight) and extracellular proteinase production by *O. piceae* strain 387N in synthetic medium (section 2.2) containing soybean protein as a nitrogen source. Proteinase activity units (U) are defined as the amount of enzyme that produced a rate of increase in A₅₂₀ of 0.1 units/min at 37°C and 320 rpm. A similar trend was observed in duplicate experiments.

Proteinases produced by *O. piceae* strain 387N on wood (section 3.3.7) showed a similar inhibition pattern. There was no inhibition by 1,10-phenanthroline, pepstatin or E-64. However significant inhibition (greater than 50%) was observed after incubation with PMSF and EDTA. These results suggested that extracellular proteinases secreted by *O. piceae* when the organism grew both in wood containing protein as the nitrogen source and in liquid culture were mechanistically similar.
Table 4.1  Effects of proteinase inhibitors on the hydrolysis of azocoll by the culture filtrate of *O. piceae* strain 387N after growth on soybean protein

<table>
<thead>
<tr>
<th>Type of proteinase</th>
<th>Inhibitor</th>
<th>$A_{520 \text{ nm}}$ No inhibitor</th>
<th>$A_{520 \text{ nm}}$ With inhibitor</th>
<th>% Inhibition$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspartic</td>
<td>pepstatin$^2$</td>
<td>0.80</td>
<td>0.80</td>
<td>0.0</td>
</tr>
<tr>
<td>cysteine</td>
<td>E-64$^3$</td>
<td>0.75</td>
<td>0.83</td>
<td>-10.7</td>
</tr>
<tr>
<td>metallo</td>
<td>EDTA$^4$</td>
<td>0.75</td>
<td>0.09</td>
<td>88.0</td>
</tr>
<tr>
<td></td>
<td>1,10-phenanthroline$^5$</td>
<td>0.75</td>
<td>0.86</td>
<td>-14.7</td>
</tr>
<tr>
<td></td>
<td>phosphoramidon$^6$</td>
<td>0.75</td>
<td>0.80</td>
<td>-6.7</td>
</tr>
<tr>
<td>serine</td>
<td>PMSF$^7$</td>
<td>0.57</td>
<td>0.00</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>AEBSF$^8$</td>
<td>0.57</td>
<td>0.30</td>
<td>47.4</td>
</tr>
<tr>
<td></td>
<td>DCI-100$^9$</td>
<td>0.57</td>
<td>0.43</td>
<td>24.6</td>
</tr>
<tr>
<td></td>
<td>DFP$^{10}$</td>
<td>0.53</td>
<td>0.41</td>
<td>22.6</td>
</tr>
<tr>
<td></td>
<td>TPCK$^{11}$</td>
<td>0.50</td>
<td>0.70</td>
<td>-40.4</td>
</tr>
</tbody>
</table>

$^1$% Inhibition was calculated as the percentage difference between the absorbances of the reactions with and without inhibitors. The values for no inhibitor controls represent the activity where only the inhibitor was absent.

$^2$pepstatin (1 μM in 1% DMSO), $^3$E-64 (10 μM), $^4$EDTA (10 mM), $^5$1,10-phenanthroline (10 mM with 1% methanol) and $^6$phosphoramidon (10 μM), $^7$PMSF (2 mM in 1% DMSO), $^8$AEBSF (2 mM), $^9$DCI (100 μM in 1% DMSO), $^{10}$DFP (100 μM in 1% 2-propanol), $^{11}$TPCK (100 μM in 1% ethanol)

*TPCK and PMSF are also known to affect cysteine proteinases*
4.3.3 Purification of a serine proteinase

In order to further characterise the major proteinase produced by *O. piceae*, various chromatographic methods were tested for purifying the proteinase from culture filtrates. The separation of a proteolytic peak from other proteins was obtained by hydrophobic interaction chromatography on the FPLC system (*Figure 4.4*). Recovery of the proteinase activity in the peak was between 86 and 100% in duplicate experiments. This was in contrast to separations by anion exchange (MonoQ HR5/5), chromatofocusing (MonoP HR5/20) and gel filtration (Superose 12 HR10/30) where recoveries of proteinase activity were low, about 20 to 50% of applied activity. This was possibly due to autolysis of the proteinase, a phenomenon that has been examined further in Chapter 5.

To purify large quantities of the proteinases for subsequent characterisation, preparative hydrophobic interaction chromatography was used as a first step. In preparation for hydrophobic interaction chromatography, ammonium sulphate was added to culture filtrates immediately after harvesting by filtration. This was also found to stabilise the proteolytic activity in the crude culture filtrate. After chromatographic separation at 4°C, the single peak containing the proteolytic activity was concentrated for storage by precipitation with ammonium sulphate (*Table 4.2*). This procedure resulted in a 2.2 fold purification, which was low because the protein of interest was the major protein from the outset. Additional hydrophobic interaction chromatography on FPLC resulted in an increase of specific activity from 243 to 280 U/mg protein.
Figure 4.2 FPLC fractionation of proteins in the culture filtrate of *O. piceae* strain 387N using a Phenyl Superose HR5/5 column. The proteins (1.7 mg) were loaded in 1.7 M ammonium sulphate and eluted with 75 ml of a decreasing salt gradient (dotted line) from 1.7 to 0 M ammonium sulphate which was applied at a flow rate of 0.5 ml/min. Elution of the proteins was monitored at 280 nm. The arrow indicates the only peak showing proteinase activity using azocoll as substrate.

Table 4.2 Purification of the extracellular proteinase produced by *O. piceae* strain 387N

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vol (ml)</th>
<th>Total Activity (U)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (activity) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>5100</td>
<td>56 100</td>
<td>510</td>
<td>110</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Preparative hydrophobic interaction chromatography</td>
<td>845</td>
<td>33 000</td>
<td>150</td>
<td>220</td>
<td>2.0</td>
<td>59</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
<td>10.3</td>
<td>25 750</td>
<td>106</td>
<td>243</td>
<td>2.2</td>
<td>46</td>
</tr>
</tbody>
</table>
4.3.4 Properties of the purified proteinase

4.3.4.1 Electrophoretic properties

Electrophoretic analyses of the culture filtrate showed one major extracellular protein in the culture filtrate of *O. piceae* when grown in the modified medium (section 4.3.1). A 33 kDa protein was observed by SDS-PAGE (*Figure 4.3*, lane 2), and a pI 5.6 protein was shown by IEF (*Figure 4.4A*, lane 2). The major band after IEF corresponded to a proteinase that is able to degrade gelatin coating the X-ray film used as an overlay (*Figure 4.4B*, lane 2). A second proteinase band at pI 5.2 was detected when the incubation time for the overlay was increased from 3 to 10 minutes.

*Figure 4.3* SDS-PAGE (8-25% PhastGel) showing the purification of the major proteinase secreted by *O. piceae* strain 387N after growth on media supplemented with soymilk protein. Lane 1, low molecular weight (MW) standards (Pharmacia) with MW expressed in kDa; lane 2, culture filtrate after 6 days of growth; lane 3, protein of 33 kDa obtained from culture filtrate after purification using hydrophobic interaction chromatography. Proteins were loaded at 300 ng/lane and stained using silver as recommended by Pharmacia.
Figure 4.4 IEF (PhastGel 3-9) monitoring the purification of the extracellular proteinase from *O. piceae* strain 387N. Lane 1, wide range pH standards (Pharmacia); lane 2, culture filtrate after 6 days of growth; lane 3, protein band of 33 kDa obtained from culture filtrate after purification using hydrophobic interaction chromatography. Proteins were loaded at 500-600 ng/lane. (A) Silver-stained gel showing location of proteins and proteinase (arrow), (B) X-ray film overlay of the gel after 3 min incubation at 37°C showing the proteolytic activity (arrow).

To determine the pH and molecular weight of the purified proteinase, protein preparations obtained after the second round of hydrophobic interaction chromatography were analysed by SDS-PAGE and IEF. On SDS-PAGE a major band of 33 kDa (*Figure 4.3*, lane 3) and minor bands of lower MWs were observed. These minor bands could be degradation products of the proteinase with similar hydrophobic properties, or protein fragments of the proteinase formed after the purification. A major protein band at a pH value of 5.6 was observed on IEF gel with a minor band at a pH of 5.2 (*Figure 4.4*, lane 3). These values were confirmed using IEF gels with
a narrow ampholyte range of pH 4.5 to 6. Most of the proteolytic activity shown by the X-ray film overlay was associated with the band at a pI of 5.6. The purified enzyme was not glycosylated as shown by the lack of reaction with the colorimetric substrate (NBT-BCIP) using the digoxigenin (DIG) glycan detection kit.

The MW and pI values for the purified proteinase were the same as those for the major protein in culture filtrates after growth on soyprotein *(Figure 4.3, lanes 2 and 3; Figure 4.4, lanes 2 and 3)*. Furthermore, the electrophoretic pattern on SDS-PAGE and IEF was the same as that found for the major proteinase produced on BSA-supplemented medium (section 3.3.5). This confirmed that the same major alkaline serine proteinase was produced when protein was present as a nitrogen source.

4.3.4.2 Catalytic properties

The optimal activity of the purified proteinase was found to be between pH 7 and 9 when assayed on azocoll at 37°C *(Figure 4.5A)*, and to be at 40°C when assayed at pH 8 *(Figure 4.6A)*. The range of pHs for the optimal activity indicated that the proteinase belonged to the alkaline protease family. Proteinase activities dropped off rapidly at pH values greater than 10. The pH was determined at the start of the reaction and remained constant over the course of the reaction.
Figure 4.5 The effect of pH on the activity of the extracellular proteinase purified from \textit{O. piceae} as determined at 37°C using azocoll (A) and succinyl-Ala-Ala-Pro-Phe-\textit{p}-nitroanilide (B) as substrates. Mes-Tris-Acetate (0.1 M) was used from pH 3 - 9 and CAPS (0.09 M) was used from pH 8.3 - pH 10.5 for activity using azocoll. The proteinase activity units (U) determined in CAPS buffer were adjusted by a factor of 1.38 to normalise the activity at pH 8.3 to that observed in Mes-Tris-Acetate buffer. Reaction rates on the substrate succinyl-Ala-Ala-Pro-Phe-\textit{p}-nitroanilide was expressed as $10^3$ times the rate of change in O.D. per min (mOD/min). Points plotted are the mean values of 2 to 4 determinations.
To investigate the cleavage specificity, model substrate peptides were used (Dunn, 1989). The proteinase had no activity on the substrates BAPNA, BTPNA, and ATAPNA used to detect trypsin-, chymotrypsin- and elastase-like activities, respectively. The aminopeptidase substrate L-Leu-\textit{p}-nitroanilide was not hydrolysed. However, activity was observed using the subtilisin substrates succinyl-(\textit{Ala})\textsubscript{2}-Pro-Phe-\textit{p}-nitroanilide (sAAPF) and succinyl-(\textit{Ala})\textsubscript{2}-Pro-Leu-\textit{p}-nitroanilide. Phenylalanine was more readily hydrolysed than leucine in the P1 position on the substrate. The kinetic constants for hydrolysis of sAAPF were calculated from Lineweaver-Burk plots (Table 4.3). The catalytic constant was calculated from the maximum velocity as described in section 2.5.1. For comparison the table also includes values for another fungal proteinase and a mammalian proteinase. The substrate sAAPF is a better substrate for chymotrypsin and the proteinase from \textit{Metarhizium anisopliae} both in terms of \(K_m\) and \(k_{cat}\).

**Table 4.3** Kinetic constants for the hydrolysis of succinyl-Ala-Ala-Pro-Phe-\textit{p}-nitroanilide by the proteinase purified from \textit{O. piceae}, chymotrypsin and a cuticle degrading proteinase from \textit{Metarhizium anisopliae}

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>Substrate concentration range (mM)</th>
<th>(K_m) (mM)</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(k_{cat}/K_m) (M(^{-1}).s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{O. piceae} protease</td>
<td>0.06 - 1</td>
<td>3</td>
<td>2.3</td>
<td>7.7 \times 10(^2)</td>
</tr>
<tr>
<td>chymotrypsin(^1)</td>
<td>0.02 - 2</td>
<td>0.11</td>
<td>39.4</td>
<td>3.6 \times 10(^4)</td>
</tr>
<tr>
<td>Pr1 proteinase of \textit{M. anisopliae}(^1)</td>
<td>0.02 - 2</td>
<td>0.27</td>
<td>28.1</td>
<td>1.0 \times 10(^4)</td>
</tr>
</tbody>
</table>

\(^1\)St. Leger \textit{et al.}, 1987

Temperature and pH activity profiles on sAAPF (Figures 4.5B, 6B) were similar to those obtained on azocoll.
Figure 4.6 The effect of temperature on the activity of the extracellular proteinase purified from *O. piceae* as determined at pH 8 using azocoll (A) and succinyl-Ala-Ala-Pro-Phe-<i>p</i>-nitroanilide (B) as substrates. Reaction rates on the substrate succinyl-Ala-Ala-Pro-Phe-<i>p</i>-nitroanilide was expressed as $10^3$ times the rate of change in O.D. per minute (mOD/min). Points plotted are the mean values of 2 to 4 determinations.

4.3.4.3 Stability after exposure to various pH and temperature regimes

The proteinase isolated from *O. piceae* was exposed to pH's from 3 to 11 before being assayed for residual activity at pH 8, 37°C. Greater than 80% of the initial activity was recovered after a 4 h exposure to pH's 6 to 11 at room temperature, whereas 73% of the initial activity was recovered after 4 h at pH 5 and no activity was detected after incubation at pH 3. In fact, after
just 5 min exposure to pH 3 at room temperature, only 40% of activity was recovered. At 37°C, the proteinase was even less stable across the pH range. Less than 30% of the original activity remained after 1 h incubation for all pH's tested (Figure 4.7). At 37°C, the proteinase was most stable at pH 6, at which it had a half life of 30.4 min (Table 4.4).

![Graph](image)

**Figure 4.7** Effect of pH on the stability of the proteinase isolated from culture filtrates of *O. piceae* strain 387N. Protein concentration during the incubation was at 0.04 mg/ml with 0.1 M ammonium sulphate. Incubations of 60 min duration were carried out at a range of pH's in MES-Tris-Acetate (0.1 M), at room temperature (square symbols) or at 37°C (circles). Samples were then assayed at 37°C, pH 8 using succinyl-Ala-Ala-Pro-Phe-p-nitroanilide as substrate. The points plotted are the mean values of 3 determinations, with standard deviations shown by error bars.
The thermostability of the proteinase isolated from *O. piceae* was evaluated at pH 6 and 8.5. The results showed that the proteinase was relatively thermolabile (Table 4.5). At room temperature, the half life of the proteinase at pH 8 was 6.7 days. However, as the temperature increased, the half life decreased rapidly (*Figure 4.8*). At temperatures higher than 40°C the half life was always less than 5 minutes.

**Table 4.4** Half lives of the serine proteinase purified from culture filtrates of *O. piceae* strain 387N at different pH's. Enzyme preparations were exposed to a range of pHs in MES-Tris-Acetate buffers (0.1 M) at 37°C for 0 to 60 min before being assayed at 37°C, pH 8 using succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. Protein concentration was at 0.04 mg/ml with 0.1 M ammonium sulphate during the pre-incubation.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>t_{1/2} (min)</th>
<th>Correlation coefficient$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3.4</td>
<td>less than 5</td>
<td></td>
</tr>
<tr>
<td>pH 5.1</td>
<td>15.1</td>
<td>0.99</td>
</tr>
<tr>
<td>pH 6.1</td>
<td>30.4</td>
<td>1.00</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>27.3</td>
<td>1.00</td>
</tr>
<tr>
<td>pH 8.4</td>
<td>20.3</td>
<td>1.00</td>
</tr>
<tr>
<td>pH 8.9</td>
<td>20.4</td>
<td>1.00</td>
</tr>
<tr>
<td>pH 11.5</td>
<td>17.7</td>
<td>0.99</td>
</tr>
</tbody>
</table>

$^1$Correlation coefficients for the best-fit lines to the In residual activity versus time.
Figure 4.8. Effect of temperature on the stability of the proteinase isolated from culture filtrates of *O. piceae* strain 387N. Protein concentration during the incubation was at 0.04 mg/ml with 0.1 M ammonium sulphate. Enzyme preparations were incubated at 23 to 50°C for 5 min at pH 6 (squares) or at pH 8.5 (circles) before being assayed at 37°C, pH 8 using succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrate. The points plotted are the mean values of 3 determinations, with standard deviations shown by error bars.
Table 4.5  Half life of the proteinase isolated from *O. piceae* at pH 6 and 8, at temperatures ranging from 23 to 40°C. Protein concentration during the incubation was at 0.04 mg/ml with 0.1 M ammonium sulphate. Residual activity was assayed at 37°C, pH 8 using succinyl-Ala-Ala-Pro-Phe-p-nitroanilide as substrate. A plot of ln residual activity (%) versus incubation time was used to interpolate half lives.

<table>
<thead>
<tr>
<th>pH</th>
<th>Condition</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>Correlation coefficient&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>23</td>
<td>160.8</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>8.1</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>0.6</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.1</td>
<td>1.00</td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>76.8</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>7.7</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>0.9</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.2</td>
<td>1.00</td>
</tr>
</tbody>
</table>

<sup>1</sup>Correlation coefficient for the best-fit lines to the ln residual activity versus time
4.3.4.4 *Effect of inhibitors*

The effects of selected serine proteinase inhibitors and chelating agents on the purified proteinase were examined. DFP, specific for the active site of serine proteinases, inhibited proteolytic activity in a dose-dependent manner (*Figure 4.9*), confirming that this proteinase was a serine-type proteinase.

![Graph showing residual proteolytic activity](image)

**Figure 4.9** Residual proteolytic activity after 30 min incubation with DFP. The activity was measured on azocoll (open circles) and succinyl-Ala-Ala-Pro-Phe-p-nitroanilide.
Other classic serine proteinase inhibitors similarly caused a reduction in activity on protein (azocoll) and peptide substrates (Table 4.6). However, two high molecular weight inhibitors which are known to inhibit some serine proteinases, aprotinin and soybean trypsin inhibitor, had no effect on the purified proteinase. Activity was reduced significantly by chelating agents, such as EDTA and salicylic acid (Table 4.7). This was in contrast to other classical metallo-proteinase inhibitors (1,10-phenanthroline) which enhanced, rather than inhibited, proteinase activity. Proteolytic activity was also inhibited by SDS, while reducing and oxidising agents had little effect (Table 4.7).

Table 4.6 Effects of serine proteinase inhibitors on the hydrolysis of azocoll and succinyl-Ala-Ala-Pro-Phe-p-nitroanilide by the proteinase purified from O. piceae strain 387N

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>% inhibition&lt;sup&gt;1&lt;/sup&gt; azocoll</th>
<th>sAAPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF</td>
<td>0.2</td>
<td>ND&lt;sup&gt;2&lt;/sup&gt;</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>AEBSF</td>
<td>2</td>
<td>33</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>40</td>
<td>39</td>
</tr>
<tr>
<td>antipain</td>
<td>0.01</td>
<td>ND</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>ND</td>
<td>98</td>
</tr>
<tr>
<td>chymostatin</td>
<td>0.01</td>
<td>ND</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>ND</td>
<td>100</td>
</tr>
<tr>
<td>aprotinin</td>
<td>equimolar&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>soybean trypsin inhibitor</td>
<td>equimolar</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>turkey egg white inhibitor</td>
<td>equimolar</td>
<td>78</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>1</sup>the difference in absorbance between inhibited and uninhibited enzyme, expressed as a percentage of the uninhibited enzyme
<sup>2</sup>not determined
<sup>3</sup>equimolar with proteinase
Table 4.7 Effects of different reagents on the hydrolysis of azocoll by the proteinase purified from O. piceae strain 387N

<table>
<thead>
<tr>
<th>Type</th>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>% inhibition(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chelators</td>
<td>EDTA</td>
<td>1</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>EGTA</td>
<td>1</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>TPEN</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>salicylic acid</td>
<td></td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>tartaric acid</td>
<td></td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>detergents</td>
<td>SDS</td>
<td>0.35</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.5</td>
<td>100</td>
</tr>
<tr>
<td>reducing agents</td>
<td>DTT</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2-mercaptoethanol</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>oxidising agents</td>
<td>H(_2)O(_2)</td>
<td>8.8</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^1\) the difference in absorbance between inhibited and uninhibited enzyme, expressed as a percentage of the uninhibited enzyme

4.3.4.5 Effect of metal ions and buffer salts

Mercury, cobalt, nickel, copper, zinc and lead resulted in a decrease in proteolytic activity on azocoll (Table 4.8). However, only mercury caused a significant reduction in activity when measured on a short peptide substrate. Similarly, sodium citrate, sodium carbonate and sodium phosphate appeared to affect activity on azocoll but not on sAAPF. In contrast, divalent ions such as calcium, manganese and magnesium increased activity on both substrates. A commercial antisapstain formulation, PQ-8, was included in these experiments since it contains oxine copper as its prime active ingredient (section 2.6). It resulted in 96% inhibition on azocoll when the concentrate (5.4% Cu-8, 55-65% DDBSA) was used at a 1000 times dilution.
The assay using the sAAPF was performed at a higher ammonium sulphate concentration than the azocoll assay. This is known to stabilise the proteinase. Therefore, inhibition of activity measured by the sAAPF assay was more likely to be at the active site rather than an effect on stability of the enzyme. Ammonium sulphate increased the rate of hydrolysis of sAAPF even at concentration of 1 M. However, at the same concentrations it caused a decrease in the hydrolysis of azocoll.

**Table 4.8** Effect of metal ions and buffer salts on the hydrolysis of azocoll and succinyl-Ala-Ala-Pro-Phe-p-nitroanilide by the proteinase purified from *O. piceae* strain 387N

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>% inhibition(^1) azocoll</th>
<th>% inhibition(^1) sAAPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl(_2)</td>
<td>10</td>
<td>-15.9</td>
<td>-16.5</td>
</tr>
<tr>
<td>CuCl(_2)</td>
<td>10</td>
<td>23.4</td>
<td>-1.9</td>
</tr>
<tr>
<td>CoCl(_2)</td>
<td>10</td>
<td>61.4</td>
<td>0.9</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>10</td>
<td>-20.7</td>
<td>-3.9</td>
</tr>
<tr>
<td>MnCl(_2)</td>
<td>10</td>
<td>-14.4</td>
<td>-40.8</td>
</tr>
<tr>
<td>HgCl(_2)</td>
<td>10</td>
<td>100</td>
<td>80.0</td>
</tr>
<tr>
<td>NiCl</td>
<td>10</td>
<td>13.6</td>
<td>-17.5</td>
</tr>
<tr>
<td>KCl</td>
<td>10</td>
<td>-10.6</td>
<td>ND(^2)</td>
</tr>
<tr>
<td>CaSO(_4)</td>
<td>10</td>
<td>-8.6</td>
<td>ND</td>
</tr>
<tr>
<td>ZnSO(_4)</td>
<td>10</td>
<td>100</td>
<td>-6.8</td>
</tr>
<tr>
<td>Ca(NO(_3))(_2)</td>
<td>10</td>
<td>-21.1</td>
<td>ND</td>
</tr>
<tr>
<td>Pb(NO(_3))(_2)</td>
<td>10</td>
<td>59.7</td>
<td>ND</td>
</tr>
<tr>
<td>AgNO(_3)</td>
<td>10</td>
<td>-10.2</td>
<td>ND</td>
</tr>
<tr>
<td>Ba(OH)(_2)</td>
<td>10</td>
<td>-1.5</td>
<td>ND</td>
</tr>
<tr>
<td>Na-acetate</td>
<td>100</td>
<td>3.5</td>
<td>-9.7</td>
</tr>
<tr>
<td>Na-carbonate</td>
<td>100</td>
<td>12.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Na-H carbonate</td>
<td>100</td>
<td>-3.6</td>
<td>-9.7</td>
</tr>
<tr>
<td>Na-citrate</td>
<td>100</td>
<td>64.3</td>
<td>-5.8</td>
</tr>
<tr>
<td>Na-phosphate</td>
<td>100</td>
<td>14.8</td>
<td>-1.9</td>
</tr>
</tbody>
</table>

\(^1\)Inhibition was calculated as the percentage difference between the absorbances of the reactions with and without inhibitors. A negative sign indicates the reaction was enhanced not inhibited.

\(^2\)ND not determined
Table 4.9 Amino acid composition of the extracellular proteinase purified from *O. piceae* strain 387N compared to those reported for serine proteinases in the subtilisin and (chymo)trypsin families

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>O. piceae proteinase&lt;sup&gt;1&lt;/sup&gt;</th>
<th>proteinase K&lt;sup&gt;2&lt;/sup&gt;</th>
<th>subtilisin BPN&lt;sup&gt;3&lt;/sup&gt;</th>
<th>bovine trypsin&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mole %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASX</td>
<td>10.4 ± 2.0</td>
<td>11.3</td>
<td>10.2</td>
<td>9.7</td>
</tr>
<tr>
<td>GLX</td>
<td>7.3 ± 1.0</td>
<td>4.4</td>
<td>5.5</td>
<td>6.2</td>
</tr>
<tr>
<td>SER</td>
<td>10.9 ± 1.9</td>
<td>13.5</td>
<td>13.5</td>
<td>14.6</td>
</tr>
<tr>
<td>GLY</td>
<td>16.3 ± 0.8</td>
<td>12.4</td>
<td>12.0</td>
<td>11.1</td>
</tr>
<tr>
<td>HIS</td>
<td>1.2 ± 0.2</td>
<td>1.5</td>
<td>2.2</td>
<td>1.3</td>
</tr>
<tr>
<td>ARG</td>
<td>1.2 ± 0.1</td>
<td>4.4</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>THR</td>
<td>6.8 ± 1.0</td>
<td>8.0</td>
<td>4.7</td>
<td>4.4</td>
</tr>
<tr>
<td>ALA</td>
<td>17.2 ± 2.9</td>
<td>12.0</td>
<td>13.5</td>
<td>6.2</td>
</tr>
<tr>
<td>PRO</td>
<td>2.5 ± 0.3</td>
<td>3.3</td>
<td>5.1</td>
<td>4.0</td>
</tr>
<tr>
<td>TYR</td>
<td>2.8 ± 0.5</td>
<td>5.5</td>
<td>3.6</td>
<td>4.0</td>
</tr>
<tr>
<td>VAL</td>
<td>6.8 ± 1.3</td>
<td>6.2</td>
<td>10.9</td>
<td>5.3</td>
</tr>
<tr>
<td>MET</td>
<td>0.5 ± 0.0</td>
<td>1.8</td>
<td>1.8</td>
<td>0.9</td>
</tr>
<tr>
<td>ILE</td>
<td>4.4 ± 1.1</td>
<td>3.3</td>
<td>4.7</td>
<td>7.5</td>
</tr>
<tr>
<td>LEU</td>
<td>6.3 ± 1.1</td>
<td>4.7</td>
<td>5.5</td>
<td>4.4</td>
</tr>
<tr>
<td>PHE</td>
<td>1.3 ± 0.5</td>
<td>2.2</td>
<td>1.1</td>
<td>6.2</td>
</tr>
<tr>
<td>LYS</td>
<td>4.0 ± 0.9</td>
<td>2.9</td>
<td>4.0</td>
<td>1.8</td>
</tr>
<tr>
<td>1/2 CYS</td>
<td>ND&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.8</td>
<td>0.0</td>
<td>5.3</td>
</tr>
<tr>
<td>TRP</td>
<td>ND</td>
<td>0.7</td>
<td>1.1</td>
<td>1.8</td>
</tr>
</tbody>
</table>

<sup>1</sup>Mean of 4 separate determinations ± S.D.
<sup>2</sup>Jany and Mayer, 1985
<sup>3</sup>Tsuchiya et al., 1992
<sup>4</sup>Miller et al., 1974
<sup>5</sup>not determined
4.3.4.6 Amino acid composition and N-terminal sequence

The proteinase was rich in glycine, alanine, serine and aspartic acid/asparagine but low in histidine, arginine, tyrosine, proline, methionine, isoleucine, phenylalanine and lysine (Table 4.9).

The N-terminal sequencing of the 33 kDa protein yielded a sequence of 18 amino acids, A Y T T Q T G A P W G I S R L L H K (Figure 4.10). Using the BLAST system, we found the following proteins with similar sequences: a serine proteinase from Schizosaccharomyces, an alkaline proteinase from Aspergillus fumigatus, an alkaline proteinase from Trichoderma harzianum, an elastase from Metarhizium anisopliae, a serine proteinase from Acremonium chrysogenum. These enzymes are from the subtilisin family of serine proteinases sometimes referred to as subtilases. Based on its NH2-terminal sequence, the proteinase purified from *O. piceae* can be classified as a subtilisin class II serine proteinase.

### Figure 4.10

N-terminal sequence of the proteinase purified from *O. piceae* strain 387N and comparison with sequences reported for fungal serine proteinases that are in the subtilisin class II family. Black boxes show residues that are conserved in these enzymes.
4.4 Discussion

Initial experiments reported in this chapter were conducted on crude culture filtrate and wood powders after growth of *O. piceae*. They showed that the proteinases produced in protein-supplemented liquid media and on wood were sensitive to PMSF and EDTA. Inhibitors for other types of proteinases did not cause a reduction in proteolytic activity. We concluded that the proteinase(s) produced by *O. piceae* in liquid media and wood were serine-type proteinases with a requirement for metal ions. Many proteinases classified as serine proteinases have been shown to be sensitive to EDTA. They include fungal proteinases from *Aspergillus fumigatus*, *A. flavus* (Frosco *et al.*, 1992; Kolattukudy *et al.*, 1993), *A. niger* (Barthomeuf *et al.*, 1989), *Aureobasidium pullulans* (Donaghy and McKay, 1993), and *Trichoderma koningii* (Manonmani and Joseph, 1993), and a bacterial proteinase from *Xanthomonas maltophilia* (Debette, 1991).

The basis for the classification of metalloproteinases is the presence of a metal ion (usually zinc) which participates in catalysis. Therefore 1,10-phenanthroline is usually preferred as a diagnostic indicator of metallo-proteinases because it has a much higher stability constant for zinc than for calcium. In contrast, chelating agents such as EDTA can inactivate both the zinc-dependent metalloproteinases and some calcium-stabilised proteinases from other classes (Salvesen and Nagase, 1989).

Studies investigating the conformational integrity of subtilisin have shown that calcium chelating agents lead to autolytic digestion (Wells and Estell, 1988). It is thought that the removal of enzyme-bound calcium by chelation increases the flexibility of the protein, and
thereby its rate of autolysis (Siezen *et al.*, 1991). This may explain the sensitivity of the proteinase to inhibition by EDTA and will be explored further in the following chapter.

Subsequent experiments to purify the proteinase from *O. piceae* used liquid media. Initial attempts to purify the proteinase were frustrated by repeated losses in enzyme activity. It was found that good activity recoveries were possible after separation by hydrophobic interaction chromatography, where the proteinase eluted at about 1 M ammonium sulphate. Other studies have reported the use of ammonium sulphate to prevent self digestion (Kolattukudy *et al.*, 1993). The conformation of the protein in high salt concentrations may not be favourable for autolytic digestion. Our experiments showed that the purified proteinase was stable in 1 M ammonium sulphate for several days at room temperature. However, autolysis of the proteinase may have occurred during separations when ammonium sulphate was not present, leading to poor activity recoveries after gel filtration, anion exchange and chromatofocusing. Interestingly, the rate of hydrolysis of a peptide substrate was enhanced in the presence of ammonium sulphate. However, at the same concentration of ammonium sulphate, activity could not be detected on the protein substrate azocoll.

Ammonium sulphate appeared to affect the substrate-enzyme interaction in ways which were dependent on the nature of the substrate. Previous research has shown that the lack of inhibition of subtilisin by bovine pancreatic trypsin inhibitor is most likely caused by a steric hindrance (Mitsui, 1985). Steric collision of the inhibitor and the proteinase occurs at sites far from the reactive site of the inhibitor (and thus the active site of the enzyme) preventing binding. Similarly, ammonium sulphate could affect the conformation of the proteinase and
the substrate such that steric hindrances of this kind affected digestion of larger protein substrates but not small peptide substrates. Interestingly, a high molecular weight proteinase inhibitor, turkey egg white inhibitor, caused significant reduction in activity on azocoll, but no inhibition on the peptide substrate. This suggests that the peptide was hydrolysed as a substrate in preference to the inhibitor or pseudo-substrate.

The purified proteinase preparation was analysed by SDS-PAGE and IEF. Although a single band at 33 kDa was observed on SDS-PAGE gels, a major band at pI 5.6 and a minor band at pI 5.2 were observed on IEF gels. A single polypeptide giving 2 bands on IEF may be an artifact or due to microheterogeneity of the protein (Gianazza and Righetti, 1980). Artefact formation during IEF has been largely attributed to protein-ampholyte interactions. Microheterogeneity among proteins is now recognised to be widespread, even though its underlying causes are not fully understood. Microheterogeneity can, for example, represent alternative stable conformations of the same polypeptide. These may arise from post-translational modifications such as phosphorylation and glycosylation. Glycosylation was not detected in the purified proteinase, and the presence of carbohydrate in purified fungal serine proteinases has been reported in only a few instances (North, 1982; Tunlid et al., 1994). Interestingly, serine proteinases from A. fumigatus, A. flavus and A. oryzae have been shown to contain a putative glycosylation site in their protein sequences, but glycosylation has not been shown (Ramesh et al., 1994).
It is probable that the structure and properties of this proteinase have evolved to suit protein digestion in wood, which proceeds at ambient temperatures and at pH values between 4 and 7. Proteolytic activity of the purified proteinase dropped off at pHs greater than 10 as described for alkaline proteinases from *Aspergillus flavus*, *Aureobasidium pullulans*, *Verticillium suchlasporium* (Malathi and Chakraborty, 1991; Donaghy and McKay, 1993; Lopez-Llorca, 1990). However, the proteinase appeared to be stable over a wide pH range *in vitro*, being inactivated at pH values less than 3. Inactivation at lower pH values was irreversible. The inactivation may be conformational, involving misfolding, aggregation and adsorption; or chemical, involving deamidation of Asn/Gln residues, β-elimination of Cys residues and hydrolysis of peptide bonds at Asp residues (Tatsumi *et al.*, 1994). It is unlikely that autolysis was involved, since the enzyme did not have any detectable activity at pH 3. Similar pH stability results have been found for proteinase K, where changes were observed in the secondary structure below pH 3 (Dolashka *et al.*, 1992).

The proteinase was extremely unstable at temperatures above 23°C. Since fungal growth is optimal at this temperature (section 3.3.3), the physiological function of the enzyme *in vivo* is unlikely to be affected by its thermolability. In contrast, other fungal subtilases, including proteinase K, are remarkably heat stable (Betzel *et al.*, 1988). The thermostability of proteinase K has been partially attributed to the presence of two disulphide bonds (Betzel *et al.*, 1990). While there seems to be agreement that disulphide bridges contribute to the overall stability of a protein, attempts to introduce new S-S bonds to enhance thermostability of subtilases have generally met with little success (Siezen *et al.*, 1991). Thermostability of
proteins is determined by factors such as structural stability or resistance to chemical
degradation processes. However, in the case of proteinases, resistance to autolysis plays a
major role (van den Burg et al., 1990).

Amino acid composition analysis indicated that subtilisin BPN' and proteinase K from the
subtilisin family of serine proteinases have amino acid compositions similar to that of the
proteinase from *O. piceae*. In contrast, serine proteinases such as bovine trypsin from the
(chymo)trypsin family appear to have a significantly lower content of alanine and a higher
content of phenylalanine.

High homology at the N-terminus is sufficient to distinguish two definite classes of subtilases
(Siezen et al., 1991). Class I subtilases include proteinases from Gram positive and Gram
negative bacteria, as well as mammalian, insect and yeast proteinases. Class II subtilases
include many fungal proteinases including proteinases from *Tritirachium album, Aspergillus
oryzae*, and *Acremonium chrysogenum*. In addition to having homology with the N-terminus of
the class II subtilases, the proteinase purified from culture filtrates of *O. piceae* was shown to
display similar properties to those reported for this class of serine proteinases. These included
the inhibition pattern and cleavage specificity. Furthermore, polyclonal antibodies against the
subtilisin class II enzyme, proteinase K, cross-reacted with the proteinase purified from *O.
piceae*. These results support the classification of the purified enzyme as a subtilisin class II
serine proteinase.
In summary, for the first time, an enzyme from the sap-staining fungus *Ophiostoma piceae* was described and purified. The subtilisin-like serine proteinase isolated was produced as the major protein in the extracellular filtrate during growth on protein-supplemented medium. It appears to be actively secreted during growth on wood where similar inhibition patterns and biochemical profiles were obtained. The properties of the proteinase, particularly the autolysis and substrate specificity will be explored further in Chapter 5.
Chapter 5.

Autolysis and substrate specificity of a proteinase purified from *Ophiostoma piceae*

5.1 Introduction

During the purification of the pI 5.6 proteinase from *O. piceae*, the proteinase was found to be unstable under certain conditions. Autoproteolytic cleavage was considered a likely explanation for losses of activity. This observation prompted a detailed examination of this phenomenon in attempts to identify conditions which favoured stability or degradation of the isolated proteinase. It was also necessary to understand autolysis in order to carry out substrate specificity experiments. Therefore the two objectives for this section were the evaluation of the autolysis and substrate specificity as part of the functional characterisation of the proteinase purified from *O. piceae*.

Autolysis or autoproteolysis is influenced by several factors, including the cleavage specificity of the proteinase. Autolysis has been observed in different types of proteinases. Recent studies have characterised autoproteolysis in aspartyl proteinases of retroviruses (Rose *et al.*, 1993), neutral proteinases (van den Burg *et al.*, 1990), and class I subtilases (Kim *et al.*, 1990; Braxton and Wells, 1992; Jang *et al.*, 1993). The identification of the cleavage sites of autoproteolysis is critical for designing mutations which can slow the rate of autolysis. A considerable research

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1These data were submitted for publication: Abraham, L.D., and Breuil, C. 1995. Factors affecting autolysis of a subtilisin-like serine proteinase secreted by *Ophiostoma piceae* and identification of the cleavage site. *Biochimica et Biophysica Acta.* In press.
effort has focused on improving the stability of the subtilases in order to enhance their application, particularly in the detergent industry. However, autoproteolytic cleavage sites have not been previously identified for class II subtilases. In this chapter, the role of autolysis in stability was examined and factors which affect autolysis evaluated. By characterising the enzyme fragments produced by autolysis, the position of autoproteolytic cleavage for subtilase class II enzymes was determined.

The second experimental objective was to determine the relative activity of the purified proteinase towards various specific substrates in order to define the specificity, and compare it with that of proteinase K. To achieve this purpose, simple synthetic substrates in which there is only one bond susceptible to enzymatic hydrolysis can be used as described by Walsh and Wilcox (1970) and Sarath et al. (1989). Amino acid esters and amides blocked at the N-terminal were used in spectrophotometric assays. The mechanism of action was also evaluated using the oxidised B-chain of insulin which has served for over 30 years as the substrate of choice for the initial screening of the specificity of a newly discovered proteinase (Dunn, 1989).

The insulin sequence contains a selection of peptide bonds in an unfolded structure so that all the peptide bonds should be freely accessible (Dunn, 1989). Much of the previous published work to determine points of cleavage in oxidised insulin B-chain has used HPLC, paper chromatography or TLC separation of peptides after digestion, followed by amino acid analysis and/or N-terminal sequencing of the separated peptides. These procedures are both time-consuming and laborious. In this work, peptides generated by digestion of insulin with the proteinase from O. piceae were separated by HPLC coupled to an on-line to electrospray ionisation mass spectrometer (ESI MS).
The cleavage sites were determined by matching the peptide masses with the theoretical cleavage of insulin, and by sequencing the peptides using tandem MS.²

Finally the functional interaction of the purified proteinase was studied with native proteins, including extracts from wood. Protein extracts were incubated with enzyme preparations and analysed electrophoretically after various incubation times. The final part of this study was designed to demonstrate that the function of a proteinase purified from a staining fungus is to breakdown wood proteins and use these proteins as a source of nitrogen.

5.2 Materials and methods

5.2.1 Proteinase preparation and purification

The proteinase used for this study was purified to homogeneity, according to SDS-PAGE, by hydrophobic interaction chromatography (section 4.3.3). The purified proteinase was stored at -20°C in 0.1 M Tris-Cl (pH 8) buffer containing 1 M ammonium sulphate.

5.2.2 Circular dichroic spectroscopy

Circular dichroic (CD) measurements were made on a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co. Ltd., Tokyo, Japan). Spectra were recorded between 200 and 350 nm using cuvettes of 10 mm or 1 mm pathlength. Protein concentrations for near (250 - 350 nm) and far (200 - 250 nm) UV spectra were 0.4 - 0.8 mg/ml and 0.09 mg/ml, respectively. CD data were expressed in terms of ellipticity measured in degrees (1 mdeg = 0.001 deg). Proteinase K (Boehringer Mannheim) and the proteinase isolated from O. piceae were prepared for CD spectroscopy as described previously (Genov and Shopova, 1978). Briefly, they were inactivated with PMSF and the intact proteinases were separated from autolytic products by gel filtration using a Superose 12 column equilibrated with 0.1 M phosphate buffer (pH 7) containing 0.5 M KCl on the FPLC (Pharmacia).

The melting temperatures (Tm), defined as the midpoint in the thermally induced transition from the folded to the unfolded state, were determined in 0.1 M phosphate buffer (pH 7) containing
0.5 M KCl for the PMSF-inactivated proteinases. Spectral changes were recorded at 221 nm as the temperature was increased using the JTC-340 temperature control programme (Japan Spectroscopic Co. Ltd., Japan) and the Neslab RTE-110 temperature controller (Neslab Instruments Inc., Newington, NH, USA). The effects of calcium (27 mM) and ammonium sulphate (1 M) on the Tm of the proteinase from O. piceae were also determined in 20 mM Tris-Cl buffer (pH 8). Re-folding of the proteinase in 20 mM Tris-Cl (pH 8) with 1 M ammonium sulphate was assessed by recording spectra at 221 nm while decreasing the temperature.

5.2.3 Generation of autolytic products

Autolytic degradation products were generated by desalting samples (83 μg - 2.6 mg) at room temperature on a Bio-gel P6 polyacrylamide gel column (1 ml)(Bio-Rad) equilibrated with the desired buffer. Alternatively, they were generated by adding active proteinase to proteinase inactivated by incubation with 2 mM PMSF for 30 min at room temperature, or by boiling in buffer containing 2.5% SDS and 5% β-mercaptoethanol for 5 min. Before use, these inactivated substrates were transferred to 0.1 M Tris-Cl (pH 8) or 25 mM piperazine-Cl (pH 6.3) containing 10 mM EDTA by ultrafiltration using the Microcentricon 10 (Amicon). Inactivated, washed proteinase substrates (86 μg) were digested with 0.5 μg active proteinase at room temperature. The digestion was stopped by the addition of equal volumes of SDS-PAGE sample buffer and boiling for 5 minutes.

Glycerol, BSA, DTT, MgCl₂, CaCl₂, and ammonium sulphate were tested as protectants against autolysis during heating. The proteinase was mixed with 0.1 M MES-Tris-Acetate buffer, pH 8
containing the protectants at a ratio of 1:10 by volume. After incubation at set temperatures, proteins remaining were examined by SDS-PAGE (section 2.7), and residual proteinase activity was assayed at 37°C, in 0.1 M Mes-Tris-Acetate (pH 8) using sAAPF as substrate (section 2.5). Half lives were determined by interpolating the least-squares linear fit of a plot of the ln of remaining activity versus time.

5.2.4 Assay of activity against p-nitrophenyl esters

Various phenyl ester substrates were purchased from Sigma and prepared at 2.4 mM in dioxane. They were always prepared fresh on the day of use. A methanolic buffer (30 mM Tris-Cl, 20% methanol, 0.1 M CaCl₂, pH 7.5) was added to each well in a 96 well plate. Enzyme dilutions were prepared for the purified O. piceae proteinase and proteinase K, in 20 mM Tris-Cl, 0.1 M CaCl₂, pH 7.5 immediately prior to use. Dilutions were adjusted to be in the linear range of the assay. The reaction was initiated by the addition of the substrate, to give a final concentration of 79 μM. Four replicate wells were used for each of three enzyme concentrations. The progress of each reaction was followed, at 405 nm at room temperature for 10 min with shaking, using a Thermomax microplate reader (Molecular Devices Corporation). Each well received 200 μl of sample giving a path length of 0.7 cm. The initial reaction rate was determined as the slope in the linear portion of a graph plotting absorbance against time, and was expressed as 10⁻³ times the rate of change in O.D. per minute (mOD/min). The assay plots were usually linear for the first five minutes.
5.2.5 Insulin-B chain digestion

An aliquot (100 μl) of bovine insulin B-chain (Sigma) prepared at 1 mg/ml in 0.1 M Tris-Cl, pH 8 containing 2 mM CaCl₂, and 2 μg O. piceae proteinase were incubated at room temperature. Substrate cleavage sites were determined after stopping the reaction at 2 min, 5 min, 1 h and 24 h.

5.2.5.1 HPLC conditions

At the specified times, a 5 μl aliquot was removed from the reaction mixture and diluted 10 times with HPLC solvent A (pH 2.2) to stop the reaction. Peptides in an aliquot (20μl) were separated on an Ultrafast Microprotein Analyser (Michrom BioResources Inc., Auburn, CA, USA) by reverse phase HPLC on a 1 x 150-mm Reliasil C-18 column (5 μm, 300-Å). Chromatography solvents were 2% acetonitrile (MeCN), 0.05% trifluoroacetic acid (TFA) (solvent A) and 80% MeCN, 0.045% TFA (solvent B). The column was developed with a linear gradient from 5% to 50% solvent B in 30 min, followed by a gradient from 50 to 80% solvent B in 2 minutes. The flow rate was 50μl/min, and the UV absorbance was measured at 214 nm in a 300-nl flow cell with a pathlength of 2 mm. A post column flow split diverted 85% of the column eluate for fraction collection, and 15% to an ion spray mass spectrometer (Hess et al., 1993).

For analysis by tandem MS (MS/MS), column fractions containing the peak of interest were each injected onto a concentrator HPLC column (0.5 x 150-mm Reliasil C18 column) at a flow rate of 20 μl/min. A 10 to 50% gradient of solvent B was applied over 12 min, followed by a gradient from 50% to 90% solvent B in 1 minute, with all the eluate fed into the MS.
5.2.5.2 MS conditions

Mass spectra were recorded on a PE-Sciex API III triple quadrupole MS (PE-Sciex, Thornhill, Ontario, Canada) equipped with an Ion Spray ion source. The ion spray voltage was approximately 5000 V and the nebulizer gas pressure was 40 psi. All LC/MS experiments were done in a single quadrupole operating mode using quadrupole 3 of the mass analyser. The mass range from 175 to 2200 Da was scanned with a step size of 0.5 Da and a dwell time of 1 ms per step.

For LC/MS/MS the spectrum was obtained by selectively introducing an ion of a single mass-to-charge ratio from the first quadrupole (Q1) into the collision cell (Q2) and observing the daughter ions in the third quadrupole (Q3). Thus, Q1 was locked on a particular m/z ratio, and the Q3 scan range was adjusted to cover the range from 50 to higher than the m/z ratio selected. Conditions: collision gas thickness = 4.3 x 10^{14} molecules/cm^2 (CGT = 430), collision gas was N\textsubscript{2}/Ar mix in 10/90 proportion, step size = 0.5 or 1, orifice energy = 80, dwell time = 1ms.

5.2.6 Preparation of proteins from poplar

A three year old branch was removed in April from an eight year old *Populus deltoides/trichocarpa* (hybrid DT 49-177) grown in Agassiz, B.C. The bark and cambium layers were peeled back and the differentiating xylem was removed by scraping. The wood was ground with silica sand in a mortar and pestle with liquid nitrogen. Chilled buffer (50 mM Tris-Cl, pH 8 containing 5 mM MgCl\textsubscript{2}) was added, mixed gently, and the solution clarified by repeated centrifugation at 10000 g. The supernatant was dried under vacuum (Speedvac, Savant,
Farmingdale, NY, USA) and resuspended in 20 mM Tris-Cl containing 27 mM CaCl$_2$ immediately prior to proteolytic digestion.

5.2.7 Proteinase activity against proteins

Protein substrates including BSA, casein, globulins from pumpkin seeds (Cucurbita pepo) and edestin from hemp seeds (Sigma) were prepared at 4 mg/ml in 20 mM Tris-Cl, pH 8. The purified O. piceae proteinase and proteinase K were added at 1:200 ratio (w/w) and incubated with the substrate at room temperature for 5 to 150 min. Proteins extracted from poplar xylem were digested for 2 to 5 h at an enzyme to substrate ratio of 1:50. Reactions were terminated by boiling in SDS-PAGE sample buffer. Proteins in the digested samples were separated by SDS-PAGE.
5.3 Results

5.3.1 Autolysis of the proteinase

5.3.1.1 Autolysis

Preliminary work suggested that the proteinase may be unstable. Proteinase activity losses were found after desalting into various buffers for FPLC techniques. To determine if low activity recovery was due to protein degradation, proteinase preparations were desalted into various buffers, and the protein bands observed on SDS-PAGE gels (Figure 5.1). By SDS-PAGE, a major protein band at 33 kDa was observed when the purified proteinase was inactivated by 2 mM PMSF and desalted into buffer (25 mM piperazine-Cl, pH 6.3 + 10 mM EDTA) (Figure 5.1, lane 4). However, when PMSF was not added, two lower molecular weight protein bands of about 14 and 19 kDa were observed in addition to the 33 kDa band (Figure 5.1, lane 3). This distinct change in the protein banding pattern was accompanied by a proteolytic activity loss of about 80% (Table 5.1). PMSF inhibited the formation of smaller molecular weight proteins, which suggested that these were proteolytic degradation products from the major proteinase. The fragments were considered to be the major degradation products with the sum of the individual molecular weights being the molecular weight of the native protein.
Figure 5.1 SDS-PAGE (8-25% PhastGel) showing autolysis of the proteinase isolated from *O. piceae* strain 387N during gel filtration, generating low molecular weight cleavage products. Lane 1, low molecular weight (MW) standards (Pharmacia) with MW expressed in kDa; lane 2, proteinase purified by hydrophobic interaction chromatography prior to gel filtration; lanes 3 to 8, proteins bands obtained after desalting into piperazine-Cl, pH 6.3 + 10 mM EDTA (lane 3); into piperazine-Cl, pH 6.3 + 10 mM EDTA with prior inactivation of the proteinase with 2 mM PMSF (lane 4); into piperazine-Cl, pH 6.3 + 10 mM EDTA + 0.15 M ammonium sulphate (lane 5); into piperazine-Cl, pH 6.3 + 10 mM EDTA + 10 mM CaCl₂ (lane 6); into piperazine-Cl, pH 6.3 (lane 7); into Mes-Tris-Acetate, pH 2.4 (lane 8). Proteins were desalted at 5 mg/ml and loaded onto gels at 100 ng/lane. Staining was carried out using silver as recommended by Pharmacia.
Table 5.1  Generation of autoproteolytic products from the proteinase isolated from *O. piceae* strain 387N during buffer exchange on a gel filtration column (1 ml) at room temperature. Proteinase preparations were applied at the specified concentrations, and eluted with the equilibration buffer. Protein bands observed after silver staining of the gel were quantified by densitometry.

| Salt             | Concentration (mM) | pH  | Additive          | Protein concentration (mg/ml) | Activity recovered (% ± S.D. 
(%) | Quantity of protein (%) in major bands observed after SDS-PAGE (kDa) |
<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperazine-Cl</td>
<td>25</td>
<td>6.3</td>
<td>None</td>
<td>5.0</td>
<td>19.8 ± 0.5</td>
<td>57 29 14</td>
</tr>
<tr>
<td>Piperazine-Cl</td>
<td>25</td>
<td>6.3</td>
<td>10 mM EDTA</td>
<td>5.0</td>
<td>20.1 ± 1.5</td>
<td>51 28 21</td>
</tr>
<tr>
<td>Piperazine-Cl</td>
<td>25</td>
<td>6.3</td>
<td>10 mM EDTA 10 mM CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5.0</td>
<td>106.4 ± 1.3</td>
<td>99</td>
</tr>
<tr>
<td>Piperazine-Cl</td>
<td>25</td>
<td>6.3</td>
<td>10 mM EDTA 0.15 M (NH&lt;sub&gt;4&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>5.0</td>
<td>99.4 ± 2.3</td>
<td>99</td>
</tr>
<tr>
<td>Piperazine-Cl</td>
<td>25</td>
<td>6.3</td>
<td>10 mM EDTA 2 mM PMSF&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5.0</td>
<td>3.4 ± 0.5</td>
<td>100</td>
</tr>
<tr>
<td>Piperazine-Cl</td>
<td>20</td>
<td>9.5</td>
<td>None</td>
<td>8.0</td>
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<td>99</td>
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<tr>
<td>MES-Tris-Acetate</td>
<td>100</td>
<td>2.4</td>
<td>None</td>
<td>5.0</td>
<td>3.2 ± 0.8</td>
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</tr>
<tr>
<td>Na-acetate</td>
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<td>5.0</td>
<td>25.5 ± 2.5</td>
<td>24 36 32</td>
</tr>
<tr>
<td>Tris-Cl</td>
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<td>8.0</td>
<td>None</td>
<td>8.0</td>
<td>88.8 ± 4.1</td>
<td>98</td>
</tr>
</tbody>
</table>

<sup>1</sup>S.D. indicates the standard deviation

<sup>2</sup>samples were pre-treated with PMSF prior to loading on the column
In order to ascertain whether the loss of activity after heating was due to irreversible inactivation or autolysis of the purified proteinase, protein preparations were analysed by SDS-PAGE after incubation for various times at elevated temperatures. The native protein band at 33 kDa decreased with increased exposure time, but no protein breakdown products were detected (Figure 5.2).

Figure 5.2 SDS-PAGE (8-25% PhastGel) showing thermal instability of the proteinase isolated from O. piceae strain 387N. Lane 1, low molecular weight (MW) standards (Pharmacia) with MW expressed in kDa; lanes 2 to 4, proteinase preparations incubated at 55°C for 5 min with no additive, with 0.9 M (NH₄)₂SO₄ and with 29 mM CaCl₂ (lanes 2 to 4, respectively); proteinase preparations incubated at 40°C with 29 mM CaCl₂ for 0, 10 and 30 minutes (lanes 5 to 7, respectively); lane 8, proteinase preparation incubated at 40°C for 30 minutes without any additive. Proteins were incubated at 0.4 mg/ml and 0.5 ml were loaded on each lane. Staining was carried out using silver as recommended by Pharmacia.
After incubation of the purified proteinase at 55°C for 5 min or at 40°C for 30 min, the protein band at 33 kDa was not observed (Figure 5.2, lanes 2 and 8). This observation was consistent with studies on autolysis of other proteinases (Braxton and Wells, 1992; van den Burg et al., 1990) that reported difficulties in isolating breakdown products under similar conditions.

These results suggested that loss of proteinase activity after incubation at higher temperatures was not due to irreversible inactivation but that elevated temperatures resulted in loss of native protein as a result of autolysis. Autolysis of the proteinase occurred at temperatures up to 80°C as indicated by the disappearance of the 33 kDa band on SDS-PAGE gels (gel not presented).

5.3.1.2 Factors which affect autolysis

Glycerol (1.95 M), BSA (18.4 mM), DTT (0.9 mM), MgCl₂ (9 mM), CaCl₂ (9 to 27 mM), and ammonium sulphate (0.76 M), were all tested as protectants against thermal inactivation. MgCl₂, BSA and DTT had little effect as thermoprotectants at 40°C (Table 5.2). However, glycerol, CaCl₂, and ammonium sulphate significantly extended the half life of the proteinase. The half life of the proteinase without additives was about 7.7 min at 40°C, whereas an half life of 2.3 h was found in the presence CaCl₂. At 45°C the half life increased from 1.9 minutes for the unprotected enzyme to 9.4 min, 40.4 min and greater than 2 h when incubated with glycerol, CaCl₂ (27 mM) and ammonium sulphate, respectively.

The observed effect of "thermoprotectants" on the half life of the proteinase was further analysed by examining the protein bands by SDS-PAGE. In the presence of CaCl₂ at 40°C
there was no significant observable decrease in the native protein band at 33 kDa over 30 minutes (*Figure 5.2*, lanes 5 to 7) whereas the native protein band had disappeared after 30 minutes at 40°C for the unprotected enzyme. At 55°C, ammonium sulphate provided the most protection against autolysis (*Figure 5.2*, lane 3), followed by CaCl₂ (*Figure 5.2*, lane 4), and then glycerol.

Ammonium sulphate and CaCl₂ were similarly shown to prevent autolysis of the purified proteinase during buffer exchange at room temperature. The appearance of lower molecular weight bands (*Figure 5.1*, lanes 3 and 7) was inhibited when ammonium sulphate and CaCl₂ were present in the equilibration buffer (*Figure 5.1*, lanes 5 and 6 respectively), and most of the applied activity was recovered after gel filtration (Table 5.1).
Table 5.2 Effect of thermoprotectants on the half life of the proteinase isolated from *O. piceae* strain 387N, at pH 8, for temperatures ranging from 40 to 50°C. Protein concentration during incubation was at 0.04 mg/ml with 0.1 M ammonium sulphate. Residual activity was assayed at 37°C, pH 8, using succinyl-Ala-Ala-Pro-Phe-\textit{p}-nitroanilide as substrate. A plot of ln residual activity (%) versus incubation time was used to interpolate half lives.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Additive</th>
<th>Concentration (mM)</th>
<th>$t_{1/2}$ (min)</th>
<th>Correlation coefficient*</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>None</td>
<td>NA</td>
<td>7.7</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>18.4</td>
<td>8.8</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>MgCl$_2$</td>
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<td>9.3</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>DTT</td>
<td>0.9</td>
<td>6.5</td>
<td>1.00</td>
</tr>
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<td>46.5</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
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<td>140.0</td>
<td>0.97</td>
</tr>
<tr>
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<td>170.0</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>760.0</td>
<td>65.3</td>
<td>0.98</td>
</tr>
<tr>
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<td>None</td>
<td>NA</td>
<td>1.9</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>1950.0</td>
<td>9.4</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>CaCl$_2$</td>
<td>27.0</td>
<td>40.4</td>
<td>0.99</td>
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<tr>
<td></td>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>760.0</td>
<td>123.0</td>
<td>0.97</td>
</tr>
<tr>
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<td>$&lt;1.0$</td>
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</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>1950.0</td>
<td>1.7</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>CaCl$_2$</td>
<td>27.0</td>
<td>5.2</td>
<td>1.00</td>
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<tr>
<td></td>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>760.0</td>
<td>13.7</td>
<td>0.98</td>
</tr>
</tbody>
</table>

NA not applicable

*correlation coefficients for the best-fit lines to the ln residual activity versus time.
5.3.1.3 Mechanism of autolysis

Exposure to elevated temperatures causes a shift from the native, catalytically active enzyme to the unfolded, catalytically inactive enzyme. The Tm values thus provide an estimate of relative thermodynamic stability. The transition temperature for the proteinase isolated from O. piceae was determined by following the changes in absorbance induced by temperature. The Tm for the proteinase from O. piceae was 37.1°C in 20 mM Tris-Cl (pH 8) (Figure 5.3) and 40.3°C in 0.1 M phosphate buffer (pH 7) with 0.5 M KCl (Figure 5.4). These transition temperatures were much lower than the 66.3°C determined for proteinase K in phosphate buffer. The addition of CaCl₂ (27 mM) increased the Tm for the proteinase isolated from O. piceae from 37.1°C to 49.4°C (Figure 5.5), and that of ammonium sulphate (1 M) shifted it further to 50.7°C (Figure 5.6). The thermal unfolding was not reversible. Although the proteinase was inactivated by PMSF, the possibility of autolysis during the melting process cannot be ruled out at this point.

It has been speculated that autolysis could proceed by an intramolecular mechanism, in which each molecule of enzyme digests itself, or by an intermolecular mechanism, in which molecules of proteinase digest other molecules of proteinase. If each molecule of proteinase digested itself, the rate of autolysis should be independent of the applied protein concentration. During gel filtration experiments, the amount of autocatalytic degradation was found to depend on the protein concentration applied. At lower protein concentrations (0.25 mg/ml), degradation products were not observed as major protein bands, and greater than 85% of the applied proteinase activity was recovered (Table 5.1). Higher protein concentrations (5 mg/ml) desalted under identical conditions, resulted in the recovery of less
than 20% of the applied activity as well as the identification of the aforementioned 19 and 14 kDa breakdown products on SDS-PAGE gels (Figure 5.1, lane 7). These bands accounted for 29% and 14%, respectively, of the total protein in the preparation. These data suggested that, under the experimental conditions used, autolysis was an intermolecular rather than an intramolecular event.

To further investigate the ability of the proteinase to act intermolecularly, proteinase samples were inactivated with PMSF prior to digestion with small amounts of active proteinase. When the PMSF-inactivated substrate was transferred into 0.1 M Tris-Cl (pH 8), and digested with active proteinase, no degradation was observed, even up to 48 h. However, when the PMSF-inactivated substrate was transferred into 25 mM piperazine-Cl (pH 6.3) containing 10 mM EDTA, and digested with active proteinase, the 19 and 14 kDa degradation products were observed on SDS-PAGE gels after 10 min digestion at room temperature. These studies indicated that, at pH 6.3 in the presence of a calcium chelator, the enzyme’s conformation was susceptible to proteolytic attack by active proteinase. When active proteinase was used to digest proteinase which had been inactivated by boiling with SDS, four breakdown products were observed with approximate molecular weights of 18, 17, 13 and 12 kDa. This indicated that the sites of proteolytic cleavage in a linearised substrate were different from those found with the same substrate in a folded state, and suggested that folding rendered these sites inaccessible to enzymatic attack.
Figure 5.3 Melting curve and first derivative for the proteinase purified from culture filtrates of *O. piceae* strain 387N in 20 mM Tris-Cl (pH 8). Spectra were determined at a protein concentration of 0.1 mg/ml in a 1 mm cell. Spectra are the average of three scans.
Figure 5.4 Melting curve and first derivative for the proteinase purified from culture filtrates of *O. piceae* strain 387N in 0.1 M phosphate buffer (pH 7), 0.5 M KCl. Spectra were determined at a protein concentration of 0.1 mg/ml in a 1 mm cell. Spectra are the average of three scans.
**Figure 5.5** Melting curve and first derivative for the proteinase purified from culture filtrates of *O. piceae* strain 387N in 20 mM Tris-Cl (pH 8) containing 27 mM CaCl₂. Spectra were determined at a protein concentration of 0.1 mg/ml in a 1 mm cell. Spectra are the average of three scans.
Figure 5.6 Melting curve and first derivative for the proteinase purified from culture filtrates of *O. piceae* strain 387N in 20 mM Tris-Cl (pH 8) containing ammonium sulphate (1M). Spectra were determined at a protein concentration of 0.1 mg/ml in a 1 mm cell. Spectra are the average of three scans.
Figure 5.7  N-terminal amino acid sequence of the 14 kDa fragment from the autolysis of the 33 kDa proteinase isolated from *O. piceae* strain 387N. The sequence is compared to corresponding sequences (Siezen et al., 1991) of two class II subtilases: proteinases T and proteinase K from *Tritirachium album*, and two class I subtilases: subtilisin BPN' and Carlsberg from *Bacillus subtilis*. X represents unidentified residues and the arrow shows the newly identified cleavage sites for subtilase class II enzymes by comparison with that reported for class I enzymes (Kim *et al.*, 1990; Braxton and Wells, 1992).
5.3.1.4 Identification of autolytic cleavage site

To determine the site of autocatalytic degradation, the N-termini of the 14 kDa and 19 kDa autolysis products were sequenced. The N-terminus of the 19 kDa fragment was A Y T T Q T G A P W G I S R L L H K, which was identical to the N-terminus of the native protein. The first 24 residues of the second fragment were defined with the exception of positions 6 and 17 (Figure 5.7). There was a high degree of similarity with corresponding regions in proteinase K and proteinase T, with 13 and 16 amino acids identical to those in proteinase K and proteinase T, respectively. Proteinase K and proteinase T are both produced by T. album, and are classified as class II subtilases which display a high degree of similarity at the N-terminus to the proteinase isolated from O. piceae. The position for autolytic cleavage was found to exist at the N-side of Ser$^{170}$, when abiding by the numbering system pre-established for proteinase K. Although the entire amino acid sequence of the proteinase from O. piceae is unknown at this point, this site is likely to be near residue 170 based on the sizes of the cleavage products.

5.3.2 CD spectroscopy for structural comparison with proteinase K

The structures of the proteinase from O. piceae and proteinase K were assessed by near and far UV CD (Figure 5.8). Proteinase K and the proteinase from O. piceae display a similar pattern in the far UV region, dominated by a large negative band at 221 nm, arising mainly from $\alpha$-helices (Dolashka et al., 1992). The similar spectra in this region suggest these proteinases have comparable secondary structural contents in solution. According to the X-ray model (Betzel et al., 1988), proteinase K contains 32.4% $\alpha$-helix and 32% $\beta$-sheet. Since
the secondary structure of the proteinase from *O. piceae* is similar to that of proteinase K, many of the properties of *O. piceae*’s proteinase can be interpreted without detailed knowledge of its crystal structure.

The near UV spectra for the two proteinases are quite different, indicating differences in their aromatic amino acid contents. Proteinase K has several peaks in the aromatic region of the near UV with positive peaks at 292 and 304 nm, and a negative peak centred at 274 nm. The CD spectrum of the proteinase from *O. piceae* in the near UV spectral range has positive bands at 281 and 291 nm, and a shoulder at 260 nm. According to analyses by Kolvenbach *et al.*, 1990, all three types of aromatic residues, tryptophan, tyrosine and phenylalanine, contribute to the CD spectra in this region. They also speculated that the two disulphide bonds present in proteinase K contribute to the observed signals in the near UV spectral region.

![Figure 5.8](image)

**Figure 5.8** Far and near UV circular dichroic spectra of proteinase K (solid) and the proteinase isolated from *O. piceae* (dotted) measured in 0.1 M phosphate buffer (pH 7) containing 0.5 M KCl, in a cell with a pathlength of 1 mm (far) and 1 cm (near). Spectra are the average of three scans.
5.3.3 Proteinase active site specificity

As described in Chapter 4 (section 4.3.4.2), no activity was detected on the model substrates BAPNA, BTPNA, and ATAPNA used to detect trypsin-, chymotrypsin- and elastase-like activities respectively. However, activity was observed using the subtilisin substrates succinyl-(Ala)$_2$-Pro-Phe-$p$-nitroanilide and succinyl-(Ala)$_2$-Pro-Leu-$p$-nitroanilide. Phenylalanine was more readily hydrolysed than leucine in the P1 position using the nomenclature of Schechter and Berger (1967) as described in section 1.6.

The active site specificity on model substrates was also assessed from the relative rates of hydrolysis of $p$-nitrophenyl ester substrates (Table 5.3). The *O. piceae* proteinase appeared to preferentially hydrolyse aromatic (Tyr, Leu, Phe) or apolar (Gly, Ala) residues at the P1 site. The best in a series of substrates was CBZ-Gly-$p$-nitrophenyl ester. Ala, Tyr, Leu and Phe were hydrolysed in decreasing order. Val and Pro were poor substrates. The results also illustrated the importance of the N-acyl substituent of the amino acid esters. Replacing a benzyl group (CBZ) with an aliphatic group (BOC) decreased the rate of hydrolysis of otherwise similar substrates by up to 10 times. Presumably the CBZ group provides a useful aromatic binding site for the enzymes. The only case where there was little difference in the CBZ and BOC substrates was for phenylalanine. Proteinase K displayed a similar specificity to the proteinase from *O. piceae* on these substrates (Table 5.3).
Table 5.3 P1 specificity of the *O. piceae* proteinase and proteinase K on p-nitrophenyl ester substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Proteinase activity (U')</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>O. piceae</em> proteinase</td>
</tr>
<tr>
<td>CBZ-Gly NP</td>
<td>57.4</td>
</tr>
<tr>
<td>CBZ-Ala NP</td>
<td>19.9</td>
</tr>
<tr>
<td>CBZ-Tyr NP</td>
<td>17.1</td>
</tr>
<tr>
<td>CBZ-Leu NP</td>
<td>11.9</td>
</tr>
<tr>
<td>CBZ-Phe NP</td>
<td>3.2</td>
</tr>
<tr>
<td>CBZ-Val NP</td>
<td>0.4</td>
</tr>
<tr>
<td>CBZ-Pro NP</td>
<td>0.3</td>
</tr>
<tr>
<td>BOC-Gly NP</td>
<td>5.2</td>
</tr>
<tr>
<td>BOC-Phe NP</td>
<td>3.0</td>
</tr>
<tr>
<td>BOC-Ala NP</td>
<td>2.3</td>
</tr>
<tr>
<td>BOC-Leu NP</td>
<td>2.3</td>
</tr>
</tbody>
</table>

1Units represent the initial reaction rate expressed as $10^3$ times the rate of change in O.D. per minute (mOD/min)

5.3.4 Cleavage specificity on insulin

The digestion of insulin by the *O. piceae* proteinase was analysed by LC/MS after incubation times ranging from 2 min to 24 h. After 2 min of digestion (data not shown) major peaks were C, D and E, with the last corresponding to the molecular weight of undigested insulin. MS chromatograms (TIC) of 5 min, 1h, and 24 h digestion times are shown in *Figure 5.9* as A, B, and C, respectively. Only peaks containing peptide masses that matched various fragments of insulin B-chain, as determined by LC/MS or tandem MS, are labelled. After 5 min of digestion, the major fragments identified as peaks C and D corresponded to the primary cleavage of insulin (peak E) between Leu$^{15}$ and Tyr$^{16}$. The remaining peaks labelled in *Figure 5.9* A correspond to peptides resulting from cleavage at secondary sites of fragments C and D. Upon increasing digestion times, the observation made in *Figure 5.9* A was
confirmed in Figure 5.9 B and C which show the disappearance of peaks C, D and E with time and the relative increase of smaller fragments. The proposed fragments of insulin are shown in Figure 5.10.

Figure 5.9 Mass chromatograms (TIC) of proteolytic digests of insulin B-chain after 5 minutes (A), one hour (B) and overnight incubations (C). All labelled peaks were identified by LC/MS or tandem MS.
The location of the cleavage sites giving rise to the observed peaks was determined by matching the masses of the peptides with masses of various fragments of insulin. Where ambiguities existed, or where more than one insulin fragment had the same mass as the observed peptide mass, confirmation of the peptide sequence was resolved by sequencing by tandem MS as shown in Figure 5.11 for the analysis of peak 19 in Figure 5.9. The daughter ions obtained were mainly type b ions, where the charge is retained on the amino terminus of the ion to form an acylium ion; or type-y ions where the charge is retained on the carboxy terminus (Siuzdak, 1994). Amino acid composition analyses of selected proteolytic fragments were in agreement with the sequences determined by tandem MS (data not shown).

The results indicated that fragments C and D resulted from the initial cleavage between Leu\textsuperscript{15} and Tyr\textsuperscript{16}, which happens to be the observed primary cleavage site for proteinase K and subtilisins Carlsberg and Novo (Kraus et al., 1976). Some of the other cleavage sites (Figure 5.10) have previously been identified for other subtilisin-like serine proteinases, including subtilisin (Morihara and Tsuzuki, 1969) and proteinase K (Kraus et al., 1976), but there appears to be unique differences between the digestion patterns (Figure 5.12). For example, no evidence was obtained in this study for cleavage after Tyr\textsuperscript{16} or Tyr\textsuperscript{26}, which were common cleavage sites for several other serine proteinases. Cleavage after His\textsuperscript{10}, Val\textsuperscript{18} and Arg\textsuperscript{22} was unusual when compared to the other digestion patterns, and may be unique target sites for the \textit{O. piceae} serine proteinase.
Figure 5.10 Digestion of insulin B chain (peak E) by the proteinase isolated from *O. piceae* strain 387N. All peptides shown were separated by HPLC and their position in the insulin B-chain determined by MS or tandem MS or tandem MS/amino acid analysis. Amino acid residues at the P1 position are underlined and in bold. Dotted and solid arrows represent the partial or complete breakdown of fragments, respectively. The minor peaks observed and identified have not been shown on this diagram. B in the amino acid sequences in the diagram represents cysteic acid (R=CH₂SO₃H).
Figure 5.11 Tandem MS spectra for the parent ion m/z 1091. The daughter ions generated by the fragmentation process resulted in different classes of fragment ions. Fragment ions of the type-b (top row) and type-y (bottom row) are generated if the charge is retained on the N terminus and C terminus of the peptide, respectively.
**Figure 5.12** Sites of cleavage of oxidised insulin B chain by various serine proteinases. Initial cleavage by the *O. piceae* proteinase represents the sites determined after 5 minute digests. Complete digests indicate the sites cleaved after extended digests overnight. B in the diagram represents cysteic acid (R=CH₂SO₃H). Splitting sites for the remaining proteinases were taken from Morihara and Tsuzuki, 1969; Kraus *et al.*, 1976.
5.3.5 Substrate specificity on proteins

Results detailed in Chapters 3 (section 3.3.5) and 4 (sections 4.3.4.1 and 4.3.4.2) indicated that the purified proteinase was able to degrade azocoll and gelatin coating X-ray films. Casein present in skim milk was also degraded when it was used as a overlay following non-denaturing electrophoresis. Analysis by SDS-PAGE also showed the disappearance of proteins when casein, BSA and plant proteins, such as globulins and edestin, were digested by the *O. piceae* proteinase. Most of the bands in the preparation of casein had been digested to peptides or amino acids that were not detected on the gels after only 5 min incubations (*Figure 5.13*). The hydrolysis of casein by proteinase K, at equal enzyme loadings, was more complete than that of the proteinase from *O. piceae*. Similar differences in the extent of hydrolysis were observed for proteins extracted from wood (*Figure 5.14*). Both proteinases appeared to be most active on the higher molecular weight components, with the gels showing an accumulation of lower molecular weight products as digested proceeded. Interestingly, there was little change in the 2 h and 5 h digestion patterns for both the *O. piceae* proteinase and proteinase K, suggesting the digest had reached completion or conditions were no longer suitable for prolonged enzyme activity.
Figure 5.13 SDS-PAGE (8-25% PhastGel) showing degradation of casein by the proteinase purified from *O. piceae* strain 387N and proteinase K. Lane 1, low molecular weight (MW) standards (Pharmacia) with MW expressed in kDa; lane 2, casein undigested control; digestion with *O. piceae* proteinase for 5 min (lane 4) and 10 min (lane 3); digestion with proteinase K for 5 min (lane 6) and 10 min (lane 5).

Figure 5.14 SDS-PAGE (8-25% PhastGel) showing degradation of proteins extracted from poplar by the proteinase purified from *O. piceae* strain 387N and proteinase K. Lanes 1 and 8, low molecular weight (MW) standards (Pharmacia) with MW expressed in kDa; crude protein extracts prior to digestion (lanes 2, 7); protein extracts after digestion by *O. piceae* proteinase for 2 h (lane 3) and 5 h (lane 4); protein extracts after digestion by proteinase K for 2 h (lane 5) and 5 h (lane 6).
5.4 Discussion

As discussed in the preceding chapters, the subtilisin-like serine proteinase secreted by *O. piceae* was the major protein secreted under conditions where protein is supplied as the nitrogen source, and is likely a key enzyme in the physiology of this fungus. Therefore the factors which influence autolysis and substrate specificity of the proteinase may be critical to the growth and survival of this fungus.

The results for the *O. piceae* proteinase suggested that under conditions of heating, altered pH or partial depletion of protein-bound ions by EDTA, the structure of the proteinase was perturbed or relaxed, becoming a more suitable substrate for proteolysis. Many proteins can be partially unfolded by relatively minor environmental changes, such as a modest elevation in temperature or a shift in pH (Geisow, 1991). In the case of proteinases, this unfolding may be sufficient for it to become a substrate for autoproteolysis. Our results indicated that autolysis proceeded by an intermolecular mechanism. This mechanism might be accentuated at high temperatures where either populations of both active folded and inactive unfolded proteins exist in concert, or all enzymes are active but partially unfolded.

Autolysis has been reported for many of the subtilisin-like serine proteinases, often leading to multiple protein bands in purified samples (Frosco *et al.*, 1992; Reichard *et al.*, 1990) or discrepancies in size of proteins determined by SDS-PAGE and gel filtration (Bajorath *et al.*, 1988; Kolattukudy *et al.*, 1993). Calcium ions and ammonium sulphate contributed significantly to the stability of the functional enzyme folded conformation as indicated by the
higher melting temperatures when they were present. The use of ammonium sulphate solutions for storage of proteinases to prevent autolysis has been reported previously (Kolattukudy et al., 1993), but it has not been the subject of extensive investigations, in contrast to calcium. The mechanism of stabilisation by calcium is thought to be through a contribution to the overall stability of the surface regions of the enzyme, and a reduction in the flexibility of the protein, both of which reduce its susceptibility to partial unfolding and subsequent autolysis (Betzel et al., 1990). Recent work with subtilisin BPN' (Braxton and Wells, 1992) demonstrates that molecules do not need to unfold fully for autolysis, but that the rate-limiting process involves local unfolding.

Calcium ions are required by some proteinases for folding of the polypeptide chain and hence for activity (e.g. proteinase K and thermitase). It has been suggested that this may have originated as a protective action by the cells against intracellular proteolysis (Betzel et al., 1990). Intracellular calcium levels are too low to produce folded, active enzyme, whereas extracellular levels are high enough to activate secreted proteinases. Significantly, calcium is present in wood at levels greater than that typical in fungal cells, with 550 ppm calcium detected in lodgepole pine.

The sites of autoproteolysis in subtilisin BPN' were shown to be a region of high mobility as estimated from crystallographic B-factors (Braxton and Wells, 1992). Fontana and co-workers (1986) showed that cleavage sites did not occur within the segments of regular secondary structure (such as helices), but instead at loops or turns characterised by the highest degree of flexibility. The loop containing the identified proteolysis site has also been
identified as being actively involved in the binding of Ca\(^{2+}\) ions in many subtilases (Siezen et al., 1991). Therefore, it seems likely that during the gel filtration experiments reported in this study, protein-calcium interactions were disrupted, causing some unfolding and subsequent proteolysis.

The position for autolytic cleavage of the proteinase isolated from *O. piceae* was identified as the N-side of Ser\(^{170}\) according to the numbering system of proteinase K. This cleavage site occurs in a variable region located in a loop on the surface of the molecule connecting conserved elements of the \(\beta\)-pleated sheet structure (Siezen et al., 1991). Previous studies on subtilisin BPN’ (Braxton and Wells, 1992), a subtilisin class I enzyme, identified an autoproteolysis site at the N-side of Thr\(^{164}\), in a similarly accessible exposed loop structure near the active site. Since *O. piceae*’s proteinase has been characterised as a class II subtilase, these results suggested that the autoproteolysis sites in class I and II subtilases are similar.

In most other subtilase class II enzymes, Ser\(^{170}\) follows after a conserved alanine residue at position 169, suggesting a P1 cleavage specificity for autoproteolysis. Interestingly, subtilisin BPN’ rapidly cleaves Ala\(^{48}\)-Ser\(^{49}\) and Ser\(^{163}\)-Thr\(^{164}\) (Braxton and Wells, 1992), indicating that one autolysis site in subtilisin BPN’ also contains alanine in the P1 position. As discussed by Braxton and Wells (1992), these sites are not necessarily cleaved on the basis of primary specificity. Factors influencing the globular nature of proteins, such as folding, flexibility, and stereochemistry may be the critical determinants of stability, and cleavage specificity alone may not determine the suitability as a proteinase substrate.
Proteolysis is generally directed and limited to the cleavage of specific peptide bonds in the target protein. One of the keys to this selectivity is the accessibility of the scissile peptide bond to the processing proteinase. Compact protein domains are usually resistant to proteolysis, in contrast to more flexible surface loops and interdomain regions that can be exposed to the active site of proteinases (North, 1982). In addition, many proteinases are specific for the amino acid side chain at the P1 position, using the nomenclature of Schechter and Berger, 1967 (section 1.6). For example, trypsin cleaves after positively charged amino acids, namely lysine and arginine. Chymotrypsin cleaves after large hydrophobic residues such as tryptophan, tyrosine and phenylalanine, and elastase cleaves after alanine and serine amino acids with small side chains. In some cases, the specificity may be determined by amino acids further removed from the scissile bond.

The advantage of using simple model substrates to investigate specificity is that the observed kinetics are usually relevant to a single cleavage event. The use of small peptide substrates also eliminates problems associated with the structural conformation of proteins because oligopeptides usually do not have stable well-defined structure, and exist as random, extended chains. However, several studies have indicated the role of secondary enzyme-substrate interactions. These include for example, the effects of elongation of the peptide substrate resulting in a dramatic changes in binding and catalytic efficiency (Dunn, 1989). Therefore, in assaying the cleavage specificity, factors which influence either the binding or catalysis will affect the results. For this reason the synthetic substrates have a major drawback in that they are generally soluble only in organic solvents which may have a significant influence on catalysis. Homopolymers of single amino acids were soluble in different solvents, making
true comparisons impossible. The phenyl ester substrates had an additional drawback that spontaneous hydrolysis occurred in the substrate. This was mentioned by Walsh and Wilcox, 1970 who recommended simply subtracting the rate of the background reaction from the assay rate. The spontaneous hydrolysis was affected significantly by temperature. Therefore it was better to perform the assay at 23°C rather than 25°C or higher. It was also necessary to perform the experiment with appropriate controls. An additional problem was that reproducibility depended greatly on precise technique. There was always quite a bit of variation between replicate samples, and linear reaction rates were often not observed. Despite these problems, the results from this assay suggested that the *O. piceae* proteinase had a specificity similar to that of proteinase K. Aromatic or apolar residues at the P1 site were preferred. Leucine at the P1 site was a better ester substrate than phenylalanine at the P1 site. However, this was in contrast to the results using the model substrates Suc-(Ala)₂-Pro-Phe-*p*-nitroanilide and Suc-(Ala)₂-Pro-Leu-*p*-nitroanilide (section 4.3.4.2). When using insulin as the substrate, leucine at the P1 site was also the first bond to be cleaved.

The cleavage specificity of the proteinase on insulin indicated a broad specificity leading to doubts concerning the homogeneity of the proteinase preparation. Therefore, LC/MS was used to conclusively demonstrate the purity of the enzyme used for digestion. Only one protein species was present upon injection of the proteinase preparation. The only other species detected in the TIC were peptides that are probably due to autolytic degradation of the proteinase. Therefore the observed broad specificity could be attributed to a single proteinase. Aromatic residues were hydrolysed preferentially at the P1 site. Although alanine was the most likely residue at the P1 site for autoproteolysis, cleavage after alanine was not observed.
Interestingly, the proteinase was also able to cleave after polar, positively charged or negatively charged residues. Although the first bond to be cleaved had Leu at the P1 site, cleavage was not observed after Leu\textsuperscript{6}, Leu\textsuperscript{11}, or Leu\textsuperscript{17}. The lack of cleavage may be influenced by the P4 residue, which was hydrophobic for several of the cleavage sites observed. Furthermore, cleavage after Phe\textsuperscript{24} and Phe\textsuperscript{25} was observed, but there was no evidence of cleavage after Phe\textsuperscript{1}. This suggests the need for more than one amino acid for binding and subsequent cleavage. In fact, work with proteinase K has shown that the smallest peptide hydrolysable by this subtilisin class II enzyme is a tetrapeptide (Kraus et al., 1976).

In general, the binding site of subtilases can be described as a surface channel or crevice accommodating at least six amino acid residues (P4 to P2\textsuperscript{'}) of a polypeptide substrate. Substrate binding is predominantly determined by the binding of the P1 and P4 residues in two pockets on either side of the backbone strand 125 to 128. In many subtilisins, including proteinase K, both pockets are large and hydrophobic, giving rise to a broad specificity with a preference for aromatic or large nonpolar P1 and P4 substrate residues (Siezen et al., 1991).

In this study, ESI MS offered a rapid, simple and accurate means of obtaining molecular weight information, which was sensitive in the low picomole range. Analysis by tandem MS provided sequence information allowing a direct correlation to sites of cleavage. Using this technique it was possible to follow the digestion of insulin over time and identify the primary and secondary cleavage points in a relatively short time frame. With the development of more sensitive instruments and instruments capable of auto MS/MS, cleavage specificities of new proteinases could be determined with even greater rapidity. This method could be further
developed as a general strategy for characterising sites of cleavage using other enzymes and substrates.

That *O. piceae*’s proteinase had broad specificity was consistent with the enzyme’s function when produced by the fungus during growth in wood where a variety of protein substrates are available. Substrate specificity may indicate the range of proteolytic events in which a proteinase might be able to participate, as discussed by North (1982). However, it cannot always be related directly to activity on the physiological substrate. Therefore, it was considered important to investigate activity on proteins extracted from wood. Degradation of proteins extracted from the xylem tissue of poplar was observed after incubation with the enzyme for two hours. Disappearance of protein bands on SDS-PAGE gels indicated most proteins in the extract were susceptible to the action of the proteinase. Similar results have been recently reported for xylem proteins from American Elm (*Ulmus americana* L.) after colonisation by the dutch elm disease fungus *Ophiostoma ulmi* (Eshita et al., 1994). Other proteins hydrolysed by the proteinase include gelatin, collagen, albumin, edestin, globulins and casein. These data support the conclusion that the proteinase has a broad specificity and is able to degrade a variety of substrates, including physiological substrates.

In summary, this chapter addressed two key factors in the action of the isolated proteinase: its autolysis and substrate specificity. Under conditions of heating, altered pH or partial depletion of protein-bound ions, the structure of the proteinase was susceptible to autoproteolysis. Glycerol, calcium ions and ammonium sulphate increased the thermal stability. The major autoproteolytic cleavage site was identified and by comparison with
other subtilases, located in an outer exposed loop. The secondary structure was similar to that of proteinase K, and aspects of the substrate specificity were also comparable to proteinase K. The proteinase had a broad specificity with a slight preference for hydrophobic or aromatic amino acids at the P1 site. It was able to hydrolyse esters and a number of different proteins, including proteins isolated from the xylem of poplar.
Chapter 6.

Targeted inhibition of proteolytic enzymes
produced by sapstaining fungi

6.1 Introduction

As discussed in section 1.2, sapstain of lumber is caused mainly by *Ophiostoma* and *Ceratocystis* species. For this reason, *O. piceae*, a frequently isolated sapstaining fungus, has been used for most of the work described in this thesis so far. However, black yeasts such as *Aureobasidium*, and dark moulds such as *Alternaria* can also cause discoloration of sapwood (section 1.2). In addition, the green moulds, *Trichoderma* and *Penicillium* species, which sporulate on the surface of lumber, also contribute to the decrease in wood value. These other groups of sapstain and mould fungi would become increasingly important if members of the Ophiostomatales were prevented from colonising lumber. The ecology of these fungi has been covered to some extent (Seifert and Grylls, 1991; Seifert, 1993), but there is little information on the physiology and the biochemical characteristics of these organisms, especially proteolytic enzyme production in solid wood. Therefore, before attempting to inhibit the proteinase enzymes as an anti-sapstain strategy, it was necessary to evaluate the number and type of proteinases produced by other staining and mould fungi.¹

¹Some of these data have been published or presented at conferences:
Previous studies have shown that a range of staining fungi produce proteinases and aminopeptidases when growing in protein-supplemented artificial media (Breuil and Huang, 1994; Banerjee et al., 1995a). Carboxypeptidase activity has also been detected in *O. piceae* growing in wood and liquid media (Banerjee et al., 1995b). Aminopeptidases and carboxypeptidases catalyse the hydrolysis of amino acid residues from amino and carboxy termini of protein and peptide substrates respectively. They are found in organelles, in the cytoplasm and in secreted forms in the media (Taylor, 1993). As with proteinases, peptidases can be classified according to susceptibility to inhibitors, location, and optimal pH for activity.

In these studies, proteinase production was correlated with primary growth, suggesting that the role of the secreted proteinases for sapstain fungi is to breakdown proteins to retrieve nitrogen for growth and reproduction. The same may not be necessarily true for decay fungi (Micales, 1992; Venables and Watkinson, 1989). Whether decay and staining fungi produce proteinases as a means of nitrogen retrieval from the substrate or from older portions of the mycelium, inhibition of proteinases in wood may slow fungal colonisation. Many of the extracellular proteinases produced by staining fungi are inhibited by the serine proteinase inhibitor PMSF and are also affected by the chelating agent EDTA (Breuil and Huang, 1995). EDTA, amastatin, bestatin and 1,10-phenanthroline have been found to inhibit aminopeptidase activity (Banerjee et al., 1995a).

The *in vitro* properties and localisation of a proteinase (Hoffert et al., 1995) may provide some indication of its function. However, with these data alone, it is difficult to draw more than
speculative conclusions about its in vivo role. In addition, alterations to proteinase levels during physiological responses or developmental changes allow us to predict functions. A more direct approach is dependent on the ability to manipulate proteinase activity in vivo. Many proteinase inhibitors are now available but they are not always specific for individual proteolytic enzymes, and there is often the danger that processes other than those involving proteinase activity might be affected (North, 1982). Despite these limitations, these compounds were evaluated for their effect on growth and proteinase production using the model organism O. piceae. This evaluation is presented in the present chapter, as is the screening of proteinase production by different staining fungi.

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\(^2\)Some of the data concerning the testing of inhibitors were presented as a conference proceedings at the International Research Group of Wood Preservation conference in Helsingør, Denmark: Abraham, L.D., Bradshaw, D.E., Byrne, T., Morris, P.I., and Breuil, C. 1995. Targeting fungal proteinases to prevent sapstain on wood. IRG Doc No. IRG/WP/10097
6.2 Materials and methods

6.2.1 Fungal strains and culture conditions

The fungal strains *Ophiostoma piceae* 387E, 387H, 387I, 387J and 387N, *Ophiostoma ainoae* 701A, *Ophiostoma piliferum* 55H, *Ophiostoma populatum* 671A, *Cephaloascus fragrans* 307I, *Phialophora botuliospora* 707A, *Alternaria tenuis* 2G, *Cladosporium cladosporioides* 273D, *Aureobasidium pullulans* var. melanogenus 132Q and *Trichoderma harzianum* E58 were obtained from the Forintek Canada Corp. culture collection (Ste. Foy, Québec, Canada). *Ophiostoma piceae* 212735 was from the Agriculture Canada culture collection. All fungal strains were maintained on malt extract agar slants at 4°C. The fungi were pre-grown in a synthetic medium containing ammonium nitrate as the nitrogen source and starch as carbon source (section 2.2.2), before being inoculated on wood blocks (section 2.4.2) or in liquid synthetic medium containing protein. Fungal biomass in wood was determined by ergosterol (section 2.3).

6.2.2 Proteolytic activities

Proteinase activity was determined in infected wood powders or in culture supernatant using azocoll (section 2.5). Inhibitors were added during a 30 min pre-incubation as described previously (section 2.6). Aminopeptidase activity was determined using 50 mM L-Leu-p-nitroanilide as substrate (Masuda et al., 1975). The assays were carried out in Tris-Cl buffer pH 7.0 at room temperature for 24 hours on a platform shaker. After centrifugation the
supernatant was transferred to a 96-well plate and the absorbance was measured at 405 nm. One arbitrary unit of aminopeptidase activity was defined as the amount of enzyme which produced an increase absorbance reading of 0.01 O.D. units per min.

6.2.3 Testing inhibitors of the proteinase in artificial media

The synthetic medium (section 2.2) was supplemented with either NH₄NO₃, 2 g/l; or skim milk, 10 g/l as the nitrogen source, and solidified with 20 g/l agar. Inhibitor solutions were filter sterilised and added to the medium just prior to pouring the agar plates. Three replicate plates were used for each inhibitor at each concentration.

The inoculum was prepared by growing *O. piceae* in the synthetic medium containing inorganic nitrogen for 8 days. The biomass was harvested by centrifugation and resuspended in phosphate buffered saline (PBS). Samples were homogenised (Omni Homogeniser model 2000, Omni International) and centrifuged to obtain a washed pellet of cells. The cells were made up to a final dry weight concentration of 1 mg/ml. Aliquots (10 µl) were used to inoculate the centre of each agar plate. The plates were incubated in the dark at room temperature for 5 days, and the diameter of each colony was measured. Percent inhibition of growth was calculated relative to growth on media containing no inhibitor.
6.2.4 Testing inhibitors of the proteinase in wood

The ASTM D4445-84 test was used in these experiments. This is a standard laboratory test for screening chemicals to control sapstain and mould on unseasoned lumber. The details of the testing procedure are specified, including a visual evaluation of stain of the wood samples to provide a quick final assessment. Lodgepole pine sapwood blocks (5 cm × 2 cm × 0.5 cm) were labelled and sterilised as before (section 2.4.1). Six to eight replicate blocks were dipped in inhibitor solutions and placed on glass rods in Petri dishes to equilibrate for 24 h. A washed *O. piceae* inoculum prepared at 0.4 mg/ml was used to inoculate the wood blocks. An aliquot (250 μl) was applied down the centre of the wood block. Plates were incubated as before (section 2.4.2). After 14 days incubation, the degree of pigmentation and surface growth/sporulation was visually evaluated relative to untreated wood, and enumerated as percent inhibition.
6.3 Results

6.3.1 Proteolytic activity on solid media

A number of staining fungi were screened for proteinase production on artificial media containing skim milk as the nitrogen source. Zones of clearing, indicative of extracellular proteinase activity, were observed for all staining and mould fungi listed in section 6.2.1.

6.3.2 Inhibition of proteolytic activity in culture filtrates

Several of the strains tested on solid media were selected for liquid culture studies. As described by Breuil and Huang (1994), all the selected strains secreted proteinases into culture filtrates when grown in liquid medium supplemented with different protein sources such as soy milk, skim milk, or BSA. After incubation with a range of proteinase inhibitors, activity in the culture filtrates was reduced in all cases by PMSF (Table 6.1), although the inactivation was less for samples from O. populinum and C. cladosporioides. Enzyme activities in most samples were also affected by EDTA, with the exception of the samples of A. tenuis and T. harzianum. The proteolytic activity of C. cladosporioides was strongly inactivated by 1,10 phenanthroline and EDTA. E-64, a cysteine proteinase inhibitor, did not affect any of the samples, while pepstatin, an aspartic proteinase inhibitor, only affected the sample from O. populinum. The strong action of PMSF suggested that most of the samples contained serine proteinases. However, since several inhibitors inactivated the samples, at least to some extent, it was necessary to determine whether more than one proteinase was present in each sample.
6.3.3 Protein profiles

Electrophoretic analysis of culture filtrates from eight of the sapstaining strains was conducted after 3 to 5 days growth. X-ray film overlays following electrophoresis (section 2.7) showed that all samples contained more than one proteolytic band after IEF between pH 3 to 9, with the exceptions of *O. populnum* and *A. tenuis* which apparently produced only one proteinase with an alkaline pl (pl>9). All four strains of *O. piceae* produced a proteinase with pl 5.6, and minor proteinases with pl 5.2 and 8. Among the other proteolytic bands detected after IEF, the samples from *O. ainoae* showed two bands with acidic pl values, and that from *T. harzianum* showed at least five active bands. After separation by native PAGE, multiple proteolytic bands were again visible for most samples, but the number of bands was generally less, probably due to the fact that proteins with higher pl values would migrate to the cathode instead of the anode during separation.

6.3.4 Fungal growth and proteolytic activity in wood

All staining fungi used in this work were mesophilic and grew actively on lodgepole pine sapwood at 23°C. After two weeks of fungal growth, all the wood blocks showed some visual discoloration. Fungal biomass was determined by subtracting the ergosterol content in the non-inoculated wood from that in infected wood blocks. For most fungal strains, the ergosterol content increased during the first week and reached a constant value after seven or nine days, indicating that the fungal growth had reached stationary phase (*Figure 6.1* and Table 6.2).
Table 6.1 Effects of proteinase-inhibitors on the hydrolysis of azocoll by wood powders and culture filtrates\(^1\) that have been inoculated with different staining fungi

<table>
<thead>
<tr>
<th>Fungal samples</th>
<th>% inhibition(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1,10-Phenanthroline(^3)</td>
</tr>
<tr>
<td></td>
<td>wood filtrate</td>
</tr>
<tr>
<td>O. piceae 387E</td>
<td>+</td>
</tr>
<tr>
<td>387 J</td>
<td>-</td>
</tr>
<tr>
<td>387N</td>
<td>-</td>
</tr>
<tr>
<td>212735</td>
<td>-</td>
</tr>
<tr>
<td>O. ainoae 701A</td>
<td>+</td>
</tr>
<tr>
<td>O. populinum 671A</td>
<td>ND(^8)</td>
</tr>
<tr>
<td>A. tenuis 2G</td>
<td>-</td>
</tr>
<tr>
<td>C. cladosporioides 273D</td>
<td>ND</td>
</tr>
<tr>
<td>T. harzianum E58</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^1\) based on Breuil and Huang, 1994

\(^2\) Inhibition efficiency was calculated as follows:
- +++ inhibition efficiency between 75-100%;
- ++ inhibition efficiency between 75-50%;
- + inhibition efficiency between 50-25%;
- - inhibition efficiency less than 25%.

\(^3\) 1,10-Phenanthroline (10 mM with 1% methanol), \(^4\) PMSF (2 mM with 1% DMSO), \(^5\) EDTA (10 mM), \(^6\) Pepstatin (1µM with 1% DMSO), \(^7\) E-64 (10µM)

\(^8\) not determined
All strains produced proteolytic enzymes during growth on wood. Figure 6.1 shows the results for *O. piceae* 212375. Proteolytic activity, expressed per g dry wood, increased with time and reached its maximum after three to seven days, shortly before or at the beginning of the stationary phase as shown by the ergosterol content. During the stationary phase a decline in the activity was generally observed. A similar trend was observed for all the fungi grown in wood, although proteolytic activity peaked later at seven to nine days (Table 6.2). All measurements were repeated in a second set of experiment. Due to the heterogeneity of wood the values were slightly different, but the trend was the same in both sets of data. The aminopeptidase activity increased with time and reached a maximum at days nine or twelve.
(Figure 6.1 and Table 6.2). Contrary to the proteinase, the aminopeptidase activity often remained relatively constant or increased during the stationary growth phase.

6.3.5 Inhibition of proteinases produced in wood

The pattern of inhibition for proteinase activities in wood was similar to that found in culture filtrates. Cysteine and the aspartic proteinase inhibitors had no effect on the activity (Table 6.1), whereas PMSF strongly inhibited the activity of all the different samples, suggesting that the majority of the proteinases secreted by the fungi were of the serine type. Since most were also affected by the chelating agent, EDTA, but not by the metalloproteinase inhibitor, 1,10-phenanthroline, they were probably stabilised by calcium. The similarity of the inhibition patterns suggested that proteinases secreted during fungal growth on protein-supplemented liquid media were similar to those secreted during growth on wood in which protein was the major nitrogen source. The detection of serine proteinases produced by all sapstaining fungi in both cultivation systems suggested that this group of proteinases should be targeted for the application of selective inhibitors on wood. All potentially inhibitory compounds were screened against O. piceae since this model system was better understood. Furthermore, the concentrations of many of the inhibitors could be predicted by examining the data for inhibition of the purified subtilisin-like serine proteinase in vitro (section 4.3.4.4).
Table 6.2  Proteolytic activity and ergosterol contents in wood infected with staining fungi. Proteinase and aminopeptidase activities were assayed at pH 8.0 and pH 7.0, respectively

<table>
<thead>
<tr>
<th>Fungal strains</th>
<th>Activity and Biomass</th>
<th>3</th>
<th>7</th>
<th>Days after inoculation</th>
<th>9</th>
<th>12</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>proteinase¹</td>
<td>0.065 ± 0.028</td>
<td>1.355 ± 0.087</td>
<td>1.662 ± 0.178</td>
<td>1.388 ± 0.107</td>
<td>1.210 ± 0.100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aminopeptidase¹</td>
<td>0.130 ± 0.017</td>
<td>0.250 ± 0.028</td>
<td>0.370 ± 0.032</td>
<td>0.260 ± 0.025</td>
<td>0.320 ± 0.027</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ergosterol²</td>
<td>2.2</td>
<td>31.0</td>
<td>48.5</td>
<td>49.0</td>
<td>51.5</td>
<td></td>
</tr>
<tr>
<td>A. tenuis 2G</td>
<td>proteinase</td>
<td>0.057 ± 0.015</td>
<td>0.210 ± 0.102</td>
<td>0.632 ± 0.128</td>
<td>0.528 ± 0.085</td>
<td>0.685 ± 0.210</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aminopeptidase</td>
<td>0.103 ± 0.005</td>
<td>0.280 ± 0.070</td>
<td>0.427 ± 0.015</td>
<td>0.707 ± 0.022</td>
<td>0.953 ± 0.075</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ergosterol</td>
<td>ND³</td>
<td>17.2</td>
<td>ND</td>
<td>43.4</td>
<td>51.9</td>
<td></td>
</tr>
<tr>
<td>O. piceae 387E</td>
<td>proteinase</td>
<td>0.960 ± 0.127</td>
<td>1.100 ± 0.170</td>
<td>1.040 ± 0.088</td>
<td>0.875 ± 0.127</td>
<td>0.720 ± 0.090</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aminopeptidase</td>
<td>0.087 ± 0.018</td>
<td>0.198 ± 0.018</td>
<td>0.330 ± 0.032</td>
<td>0.243 ± 0.025</td>
<td>0.288 ± 0.027</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ergosterol</td>
<td>32.9</td>
<td>83.5</td>
<td>87</td>
<td>90</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>O. piceae 212375</td>
<td>proteinase</td>
<td>0.285 ± 0.030</td>
<td>0.883 ± 0.110</td>
<td>0.533 ± 0.043</td>
<td>0.540 ± 0.115</td>
<td>0.690 ± 0.070</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aminopeptidase</td>
<td>0.135 ± 0.020</td>
<td>0.228 ± 0.025</td>
<td>0.172 ± 0.028</td>
<td>0.143 ± 0.028</td>
<td>0.220 ± 0.015</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ergosterol</td>
<td>8.5</td>
<td>34.9</td>
<td>ND</td>
<td>37.8</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>O. piceae 387N</td>
<td>proteinase</td>
<td>0.022 ± 0.015</td>
<td>0.278 ± 0.085</td>
<td>0.783 ± 0.070</td>
<td>0.773 ± 0.133</td>
<td>0.483 ± 0.097</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aminopeptidase</td>
<td>0.082 ± 0.003</td>
<td>0.123 ± 0.027</td>
<td>0.233 ± 0.032</td>
<td>0.270 ± 0.018</td>
<td>0.222 ± 0.027</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ergosterol</td>
<td>trace</td>
<td>15.2</td>
<td>ND</td>
<td>ND</td>
<td>94.0</td>
<td></td>
</tr>
<tr>
<td>O. ainoae 701A</td>
<td>proteinase</td>
<td>0.962 ± 0.002</td>
<td>3.175 ± 0</td>
<td>2.387 ± 0.002</td>
<td>1.847 ± 0.003</td>
<td>1.742 ± 0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aminopeptidase</td>
<td>0.098 ± 0.018</td>
<td>0.212 ± 0.018</td>
<td>0.342 ± 0.032</td>
<td>0.255 ± 0.025</td>
<td>0.300 ± 0.027</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ergosterol</td>
<td>30.0</td>
<td>42.7</td>
<td>46.9</td>
<td>30.4</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

¹U/g dry wood (mean ± S.D.), ²ergosterol represents biomass as µg ergosterol per g dry wood, ³ND: Not determined
6.3.6 Effect of inhibitors on growth of *O. piceae* in artificial media

An artificial medium was used for growth of *O. piceae* in which nitrogen was available in organic form as protein or inorganic form as NH₄NO₃. Clearing zones were produced around fungal colonies in media supplemented with skim milk, indicating the production of extracellular proteinases. Slightly slower growth rates were observed on media supplemented with inorganic nitrogen (*Figure 6.2*). Therefore, when inhibitors were added to media containing either inorganic or organic nitrogen, it was possible to measure the effect of inhibitors on colony diameters and clearing zones due to protein hydrolysis.

*Figure 6.2* *O. piceae* strain 387N colonies (C) on media supplemented with skim milk (A) and NH₄NO₃ (B). Clearing zones (arrow) were produced on organic medium due to the activity of secreted proteinase(s).
The four general groups of inhibitors tested were heavy metals, chelators, detergents and serine proteinase inhibitors. PQ-8, a currently used antisapstain formulation, was also included as a commercial control. Results for PQ-8 are presented separately because it contains more than one possible active ingredient, and the results are therefore given for the diluted formulation. Concentrations for all inhibitors were either based on recommendations in the literature or according to the results obtained using the purified enzyme (section 4.3.4.4 and 4.3.4.5).

The heavy metal salts ZnSO$_4$•7H$_2$O, Pb(NO$_3$)$_2$ and CuCl$_2$•2H$_2$O, inhibited growth on media containing protein or inorganic nitrogen, suggesting that they were toxic to fungal growth rather than inhibitory to the proteinase (Table 6.3). EDTA, tartaric acid and SDS similarly prevented growth on both media, while other chelators, TPEN and EGTA, were not as effective. At the highest concentration EGTA caused a maximum inhibition of 15% on organic medium. TPEN gave inconsistent results but with an inhibition of less than 1% at the highest concentration on organic medium. Specific serine proteinase inhibitors, AEBSF and antipain, did not disrupt growth on either medium. In contrast, another serine proteinase inhibitor, chymostatin, prevented fungal growth on the organic nitrogen medium but permitted growth on inorganic nitrogen medium, suggesting specific inhibition of the proteolytic system. PQ-8 resulted in 82% and 11% inhibition on the organic and inorganic media, respectively, when the concentrate (5.4% Cu-8, 55-65% DDBSA) was used at 1000 times dilution. This was the same dilution at which the purified proteinase was inhibited (section 4.3.4.5). Since growth was dramatically affected on organic medium, but only
inhibited to a lesser extent on inorganic medium, it suggested that at these low concentrations, PQ-8 was acting by specific inhibition of the proteolytic system.

Table 6.3 Effect of heavy metals, chelators, serine proteinase inhibitors, and detergents on growth of *O. piceae* strain 387N on media supplemented with organic and inorganic nitrogen.

<table>
<thead>
<tr>
<th>Type</th>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>% inhibition organic</th>
<th>% inhibition inorganic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy metals</td>
<td>CuCl₂·2H₂O</td>
<td>10</td>
<td>100.0</td>
<td>94.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>27.2</td>
<td>76.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>0.0</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>Pb(NO₃)₂</td>
<td>100</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>61.5</td>
<td>56.4</td>
</tr>
<tr>
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6.3.7 Effect of inhibitors on growth of *O. piceae* on lodgepole pine

Compounds applied to lodgepole pine samples inoculated with *O. piceae* were visually evaluated for their effect on fungal growth, sporulation, and pigmentation. In general, chelators were not effective inhibitors of *O. piceae* growth in wood, although EDTA did have some effect on pigmentation at higher concentrations (Table 6.4). The detergent, SDS, was more promising because it affected growth and pigmentation at the higher concentrations. Similar to findings in artificial media, PQ-8 was an effective inhibitor of growth. However, specific serine proteinase inhibitors, AEBSF and antipain, did not prevent growth of *O. piceae* on wood. Chymostatin was not tested on wood due to its prohibitive cost and its instability.

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6.4 Discussion

The species obtained from the Forintek culture collection have been frequently isolated on lumber in both eastern and western Canada (Seifert and Grylls, 1991). All the selected strains secreted some extracellular proteinases in solid agar media, liquid media, and wood. In liquid media supplemented with protein as a nitrogen source, proteinase production by staining fungi has been associated with fungal growth (Breuil and Huang, 1994). In this study, the level of proteolytic activity varied among the different organisms, but the maximum proteinase activity was recorded when the fungi were actively growing, again suggesting that the enzymes played a major role in nutrition. The decline in proteinase activity during stationary phase (Figure 6.1) suggested that conditions in wood were altered during the growth of the fungus such that the proteinase was inactivated, or unstable and degraded. The release of fatty acids during growth on lodgepole pine (Gao et al., 1994) would result in a decrease in pH which may then cause instability or inactivation of the proteinase (section 4.3.4.3). Degradation of the proteinase may be part of the the recycling strategy of an organism growing in a nitrogen limited environment. Reuse of the nitrogen from extracellular enzymes would also be mediated by proteinases. Therefore, it is possible that more than one proteolytic enzyme is present at different stages of growth and that some of them may have different roles.

The aminopeptidase activity was measured throughout the different phases of fungal growth, with a maximum activity during stationary phase. It is likely that these enzymes are also involved in the terminal stages of peptide digestion. The first step in protein hydrolysis is
carried out by proteinases, converting proteins into peptide residues that may be further degraded by peptidases into amino acids directly available for uptake and metabolism.

Ergosterol could be used as a convenient measure of fungal biomass in wood. However, in liquid medium, the level of ergosterol per fungal biomass determined by dry weight, is not constant during fungal growth. For *O. piceae* the ergosterol content in the mycelium at the stationary phase was twice the amount in the mid-exponential growth phase (Gao *et al.*, 1993). Consequently, it seems more accurate to express the proteinase activity per unit dry wood, and to correlate the activity to the growth phase of the fungus as indicated by ergosterol. Our results thus showed that the proteinases were synthesised while the fungi were actively growing. The highest proteinase activity in wood was recorded for the green mould, *T. harzianum*, as had been previously found for liquid media when the activity was expressed as U/mg biomass (Breuil and Huang, 1994). *Trichoderma* spp. have been studied extensively as producers of cellulases rather than proteinases. The existence of multiple enzymes of the same type in the cellulase complex of *Trichoderma* has been the subject of numerous debates (Nakayama *et al.*, 1976; Labudova and Farkas, 1983; Luderer *et al.*, 1991). Limited proteolysis of a common enzyme precursor has been suggested to explain the multiplicity of enzymes, and to explain the number of cellobiohydrolases with identical N-terminal sequences (Chen *et al.*, 1993).

The fungal proteinases produced were classified by determining their susceptibility to a limited number of inhibitors. The inhibition pattern for proteinases secreted during growth on wood was almost identical to the pattern obtained for proteinases secreted in liquid media
(Breuil and Huang, 1994). All samples were strongly inhibited by the irreversible serine proteinase inhibitor PMSF. Electrophoretic analysis showed that many of the strains produced several proteinases. At least one other extracellular proteinase with an alkaline pI was observed during growth of *O. piceae* in wood and in media (section 3.3.5). Intracellular proteinases are almost certain to exist to fulfil other functions (section 1.7). Aminopeptidases were also present, associated with the cell pellet rather than as secreted enzymes (Banerjee et al., 1995a). Therefore, the selective inhibition of a single proteinase might not inhibit growth completely. However, if inhibition of proteinases causes a significant reduction in the availability of nitrogen, the fungus may enter an exploratory-type of growth phase where it commits energy to further exploration rather than consolidation and exploitation of the existing mycelial domain (Rayner and Boddy, 1988). The effect of an exploratory-type growth on wood may be a reduction in mycelial biomass per unit surface area of wood which may result in little or no discoloration. Therefore, since all strains produced at least one serine proteinase, it was considered that targeted inhibition of this group may be a possible route for developing antisapstain compounds.

The detailed inhibition characteristics of the serine proteinase secreted by *O. piceae* showed that it was sensitive to a range of serine proteinase inhibitors *in vitro* (section 4.3.4.4). However, many of the specific serine proteinase inhibitors were too expensive, toxic or unstable to be used in tests in artificial media or on wood. Inhibitors from *Streptomyces* spp. such as antipain and chymostatin have been cited as being of particular value in these types of experiments, since side effects have not been previously reported (North, 1982). Chymostatin, a specific reversible inhibitor of serine proteinases, was effective in preventing growth on
protein-supplemented medium. Unfortunately, this compound was both too unstable and expensive to test on wood samples.

Chelating agents were also effective inhibitors when used in crude filtrates, wood powders, or applied to the purified proteinase. The effect of EDTA could most likely be attributed to its chelation of calcium ions, which are important for maintaining the active folded form of the proteinase and preventing subsequent autolysis. However, the chelators did not consistently perform well in artificial media or when they were applied to wood. This apparent discrepancy in results may be attributed to the presence of many cations in media and wood (Fengel and Wegener, 1989) which could saturate the chelating capacity of the chelator at the applied concentrations. It may also reflect the enhanced stability of proteinases when they were secreted in a complex microenvironment, as compared to being tested in purified form.

In contrast to the relative ease with which enzymes can be tested in purified form, practical difficulties arise when testing enzyme inhibitors in wood or culture. On wood it can be difficult to assess the effectiveness of an inhibitor since many factors are unknown at the outset of the experiment. For example, the distribution may not be uniform if the compound is lipophilic or binds preferentially to certain wood components. The stability of the inhibitor solution in wood and its bioavailability have generally not been determined.

The ideal antisapstain product (section 1.1) would be affordable, effective and target fungi causing discoloration with no adverse effects on man or the environment. In Canada the wood treating industry has made an effort to use formulations containing environmentally
friendly chemicals. However, all existing formulations suffer from several of the following problems: high fish toxicity, skin reactions among workers, difficulties in handling in cold weather, corrosion of equipment, unwanted discoloration of the wood, and high costs. Thus none of the currently used antisapstain chemicals fulfil all the demands of the ideal product as defined earlier. PQ-8, a formulation containing 5.4% Cu-8 and 55-65% DDBSA, is one of the few antisapstain chemicals that is approved for use on wood in contact with food. Therefore it was included in the experiments as a currently used commercial control.

Interestingly, PQ-8 appeared to function in these tests as a specific proteinase inhibitor. The mechanism of action of PQ-8 has not been definitively established (Mike Freeman, ISK Biosciences, personal communication), but the formulation does contain both a detergent and a chelating agent. Other detergents and chelating agents were identified as inhibitory to the proteinase (section 4.3.4.4) and to fungal growth (section 6.3.6). While these results are still preliminary, they point to the opportunities that exist for enhancing the performance of antisapstain chemicals through knowledge of their interaction with wood, and with the staining fungi which grow in wood.

In summary, all staining fungi tested produced serine proteinases and many strains secreted multiple proteinases. Although the secreted fungal proteolytic enzymes could be effectively inhibited in vitro, definitive testing on wood and commercial application were constrained by the current lack of specific, cheap, stable, non-toxic proteinase inhibitors. Nevertheless, using the approaches described in this chapter, and related studies, it is possible to achieve a better
understanding of the physiology of sapstaining fungi in order that control strategies can be formulated and developed at a later stage.
This project was directed at a significant economic problem for the lumber industry: sapstain. The discoloration of sapwood is caused by pigment-producing fungi which do not destroy the structural integrity of lumber, but instead cause aesthetic damage to wood. The lack of information available on the physiology of sapstaining fungi was the motivation for a research programme. The overall approach was as follows: by examining the physiology and biochemistry of staining fungi, specific enzyme systems important in the nutrition or pigmentation pathway could be identified. Once these systems were understood, they might be manipulated or inactivated to disrupt growth or pigmentation. Targeting biocides that have activity only against identified systems could lead to highly specific protectants which have little or no general toxicity. Mechanism-based inhibitors targeted at cellulases produced by decay fungi have been designed in accordance with this strategy (Namchuk et al., 1992).

The project described in this thesis, a component of the research programme described above, was designed to address the issue of nitrogen utilisation by staining fungi. More specifically, it was focused on examining the proteolytic enzymes required by staining fungi to retrieve nitrogen from proteins in wood.

Proteinases were produced by a range of staining fungi during growth in protein-supplemented liquid cultures. Detailed analyses of growth and proteolytic enzyme production by *O. piceae* indicated that proteinases were not produced during active growth when inorganic nitrogen was
supplied as the sole nitrogen source. These results suggested that the proteolytic enzymes played a role in primary retrieval of nitrogen rather than in recycling nitrogen during autolysis of fungal mycelium. When the fungi were grown in wood, proteinases were detected during active growth, suggesting that protein was the major nitrogen source available in wood. This work also confirmed the low amounts of total nitrogen available in wood and the presence of proteins in pine sapwood.

The proteolytic enzyme systems of staining fungi were further characterised in artificial media, where fungal and enzyme manipulation was considerably easier. Inhibition of proteinases by PMSF indicated that serine proteinases were produced by all staining fungi, although many produced several proteinases. In the model system examined, \textit{O. piceae} produced one major proteinase in wood and in protein-supplemented cultures. This enzyme was classified as a subtilisin-like class II serine proteinase. It was similar in secondary and tertiary structure to other subtilisin class II enzymes such as proteinase K. However, in terms of its physiological role, it appeared to be well suited to function in wood. In particular, the proteinase had broad specificity, in that it was able to cleave a wide range of peptide bonds. This would be advantageous if different protein substrates were available in wood at various times of the year.

The inhibition pattern of the purified enzyme was studied in detail to determine the sensitivity to heavy metals, detergents, chelators, oxidising agents, reducing agents and specific proteinase inhibitors. Chelation of calcium ions appeared to promote autoproteolysis or autolysis. Autolysis was the major factor affecting the stability of the enzyme. Factors such as pH and
temperature also appeared to affect the degree of folding and hence the sensitivity to autoproteolytic degradation.

This study of this new proteinase has analysed some factors implicated in its stability and autolysis. Importantly, the site of autoproteolytic cleavage for subtilisin class II enzymes was identified. By changing the amino acid composition at this site, it may be possible to slow down or prevent autolysis in this group of proteinases.

Since serine proteinase production was a general feature of staining fungi growing in wood, inhibitory compounds were tested in preliminary studies in media and wood. Unfortunately, many of the commercially available proteinase inhibitors affected other physiological processes, rather than specifically inhibiting the proteinase. Therefore, when these compounds were tested on wood, conclusions were limited. Future definitive testing would require specific, stable and non-toxic proteinase inhibitors. Possible areas where such compounds may be identified include existing pesticides, in nature, in the medical field, or in novel biotechnology approaches.

An alternative approach may be to manipulate conditions to cause instability and autolysis of the proteinase. Compounds which affect the pH of wood may contribute to factors causing autolysis. Chelating agents were also tested for their effect on the enzyme activity in wood, but the initial results were not encouraging. It may be necessary to use much higher concentrations since wood contains significant quantities of ions. However, the results did suggest that chelation affected physiological processes other than proteolytic activity. Therefore, this would not be a selective or targeted approach.
The other possible protective action is the modification of proteins in wood such that they become unavailable for digestion by the proteinase. Research on chemical modification of wood to improve its durability has intensified in the past decade due to increased environmental concern over the use of tropical hardwoods and broad spectrum pesticides (Beckers et al., 1994). Acetylation has received more attention than any other modification process because of its relatively low cost, and the relatively low toxicity of the chemical materials and the final modified wood product (Wakeling et al., 1992). The improved resistance of acetylated wood to decay fungi is well documented, compared to the poor performance against stain and mould fungi (Wakeling et al., 1992). Treatment of wood with the aim of specifically targeting proteins has not yet been addressed. In the future this approach may be extended to the controlled release of a toxic chemical from its bonded site. Instead of permanent bonding, chemical could be reacted through labile bonds that would hydrolyse under conditions that occur during biological attack such as heat, moisture or changes in pH (Rowell, 1991).

Existing organophosphorus pesticides developed against acetylcholine esterase (Fest and Schmidt, 1982) could be screened against isolated proteinases in vitro, and against fungal growth in media and in wood tests. The proteinase purified from *O. piceae* was shown to have esterase activity and was effectively inhibited by one of the earliest organophosphorus compounds DFP synthesised as a nerve poison during the Second World War (Eto, 1974). About 140 phosphorus compounds are or were used as practical pesticides in the world, and less toxic organophosphates have been developed (Fest and Schmidt, 1982).

Naturally occurring proteinase inhibitors may offer compounds with the desired specificity and little general toxicity. Proteinase inhibitors have been found in animals, plants and
microbes. Most of the well characterised inhibitors act as pseudo-substrates by combining irreversibly with the enzymes without cleaving the peptide bond at the reactive site (Neurath, 1984). In many cases it is not clear why proteinase inhibitors having substrate-like structures are not degraded by the proteinase. In *Streptomyces* subtilisin inhibitor, structural features such as a salt bridge are necessary for inhibitory action (Kojima et al., 1994). The design of inhibitors has benefited from the study of natural products, yet our knowledge of subtilisin inhibitors is relatively poor compared with that of trypsin inhibitors (Terada et al., 1994).

Many proteases are involved in various human diseases, and these enzymes are targets for the development of inhibitors as therapeutic agents. Inhibition of HIV aspartic proteinase is an attractive target for therapy of AIDS which has met with some difficulties (Condra et al., 1995) but is still considered a valid approach (Richman, 1995). Within the serine proteases, potential target enzymes include neutrophil elastase, thrombin, dipeptidyl peptidase IV and granzymes. In the future, specific proteinase inhibitors will be tested clinically for the treatment of human disease (Powers et al., 1993), and the requirements for these inhibitors in terms of stability, low toxicity and specificity may render them suitable candidates for application on wood.

Another interesting and novel approach to controlling insects and fungal pathogens of woody plants has been recently reported (Klopfenstein et al., 1994). It involves the cloning of a proteinase inhibitor into poplar seedlings. Enzyme-linked immunosorbent assays demonstrated leaf expression of the proteinase inhibitor. Although using this approach to protect lumber may seem impractical at the present time, molecular approaches of this nature would require understanding the fungal enzymes involved in wood colonisation.
Although this thesis has addressed some aspects of the physiology of *O. piceae*, many questions remain unanswered:

- The secretion of other proteinases by *O. piceae* during other growth phases or morphological changes has not been addressed and merits further investigation. Similarly, the occurrence, localisation and identification of intracellular proteinases would be essential to a complete understanding of the physiology of this fungus.

- Interrelationships between the carbon and nitrogen cycle were not investigated. However, proteins were apparently used as a carbon and nitrogen source when starch and protein were supplied in culture media. This may be an interesting issue in the regulation of proteinases.

- The assumption was made that proteins from wood constituted the major nitrogen source available to the sapstaining fungi. However, in wood colonisation, succession patterns have been shown, whereby the fungi colonise lumber after bacteria and actinomycetes. Therefore, the biomass of these prior wood inhabitants may also serve as substrates.

- A combined genetic and biochemical approach may be used to obtain more precise information about the specific inactivation of a single proteinase. Several regions of the isolated subtilisin-like proteinase have been sequenced and shown to possess a high degree of homology (Siezen *et al*., 1991). Therefore DNA probes could be created to screen cDNA or genomic libraries of sapstaining fungi for the presence of subtilisin-like serine proteinase genes. Deletion mutants of *O. piceae* defective in the production or activity of the subtilisin-like serine proteinase could be generated. These mutants could be tested for growth in media containing protein as the sole nitrogen source, and for their ability to colonise and discolor wood. This would illustrate unequivocally the key role of this enzyme. A similar approach has
been reported for examining the virulence of *Aspergillus fumigatus* mutants deficient in a serine proteinase (Kolattukudy et al., 1993).

- Regulation experiments could also be conducted using a genetic approach. For example, overexpression studies could be conducted on the proteinase in *O. piceae* by the introduction of a more efficient promoter (Kim et al., 1995). Wild type and recombinant strains could then be compared for their growth characteristics on wood.

In conclusion, this work has contributed to an understanding of the nutritional factors involved in colonisation of wood by sapstaining fungi, particularly in terms of the nitrogen cycle. All the staining fungi tested secreted proteinases and were able to utilise protein as a source of nitrogen. The study of the extracellular subtilisin-like serine proteinase from *O. piceae* has provided information that may result in practical applications in the effort to improve existing methods to control staining fungi. In addition, understanding differences in nitrogen utilisation may help to explain succession patterns on wood. This may have important implications for the control of other fungi which cause extensive damage to wood and wood products. Finally, this work may provide some additional data for the research effort to control other diseases caused by ophiostomatoid fungi. Future consideration of physiological, genetic and ecological aspects of sapstain fungi will further increase the knowledge required for more effective control.
References


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1Also referenced as Mathiesen-Käärik, A.


¹Also referenced as Käärik, A.


