Chapter 3.

The number and types of proteases found in the culture filtrate of *A. nidulans*.

3.1. Background

Cohen (1973a) identified four proteases, present in the culture filtrate of an *A. nidulans biA1* strain, which were distinguished on the basis of electrophoretic mobility, inhibitor sensitivity, substrate preference, and pH optimum. These proteases were designated α, β, γ and ε. β and γ were active at both neutral and alkaline pH (pH 5-6 and 8-9 respectively), while α and ε were active only at alkaline pH (Cohen 1973a). Proteases β, γ and ε were susceptible to inhibitors of serine proteases. Protease α was not susceptible to any of the inhibitors tested. It was observed that regions of proteolytic activity designated β1 and β2 were converted to enzyme β during storage at 4°C, indicating that protease β may occur as two precursor forms. The intracellular protease β was found in mycelia grown in repressing and derepressing conditions, indicating it was predominantly intracellularly located (Cohen 1973a). Protease γ was also synthesised as a precursor, designated δ, and conversion occurred *in vivo*. Ansari and Stevens (1983) detected three proteases, in mycelia grown under nitrogen-limiting conditions, two of which they purified and found to have inhibition profiles specific to serine proteases.

The derepressing conditions under which the *A. nidulans* extracellular proteases are produced include carbon-, nitrogen-, or sulphur-nutrient-limiting conditions. The studies of Cohen (1973a) and Ansari and Stevens (1983) examined the spectrum of extracellular proteases produced under nitrogen-limiting conditions. However, no information regarding the extracellular proteases produced under carbon-, or sulphur-nutrient-limiting conditions has been published.

3.2. Rationale and aims.

To understand any aspect of a system, it is necessary to know what components make up that system. As little was known of the structural genes that comprise the extracellular protease system in *A. nidulans*, confirming and extending our knowledge of the number and types of extracellular proteases produced was the objective of the work presented in this chapter.

To obtain this data, a biochemical approach was chosen. This involved employing one dimensional polyacrylamide gel electrophoresis (1D PAGE) to analyse filtrate obtained from cultures grown under conditions under which extracellular proteases were known to be produced, and finding a suitable method for the detection and identification of the proteases. This approach would enable us to answer a few basic questions:

1. How many, and what types of extracellular proteolytic enzymes are produced by *A. nidulans*?

2. Do the proteases produced under the different nutrient limiting conditions differ in any way? *i.e.* are different proteases produced under the different conditions?

3.3. Results.

3.3.1. Empirical determination of suitable methodology.

3.3.1.1. SDS-PAGE to detect extracellular proteases.

Culture filtrate from repressing and derepressing conditions was concentrated and the proteins separated by electrophoresis through a denaturing gel of 12.5%. Fewer easily visible bands were observed in the sample obtained from a culture subjected to nitrogen limiting conditions, compared to the bands visible in the sample obtained from a wildtype *A. nidulans* culture in repressing conditions (fig. 3.1.). Many more bands are visible in the derepressed sample than can be attributed to extracellular proteases, therefore SDS-PAGE alone is not a suitable technique for determining the numbers and types of extracellular proteases produced by *A. nidulans*.

In conjunction with SDS-PAGE, milk overlays (zymograms) were used to detect proteolytic activity in the filtrate of *A. nidulans* cultures subjected to nitrogen limiting conditions. In order to detect proteolytic activity it was necessary to renature the proteins after electrophoresis in denaturing conditions. After renaturation using the method of Saul *et al.* (1990), two regions of proteolytic activity were detected using the milk overlay method of Foltmann *et al.* (1985) (fig. 3.2.). I found that I could not consistently renature the proteases after SDS-PAGE. In addition, any protease that consisted of more than one protein (*e.g.* the PepB protease of *A. niger* (Inoue *et al.* 1991)), or proteases that required the binding of a ligand, such as metalloproteases, would not be detected using this method. Therefore, I decided to use native PAGE in conjunction with milk overlays for the analysis of the proteases produced by *A. nidulans*.

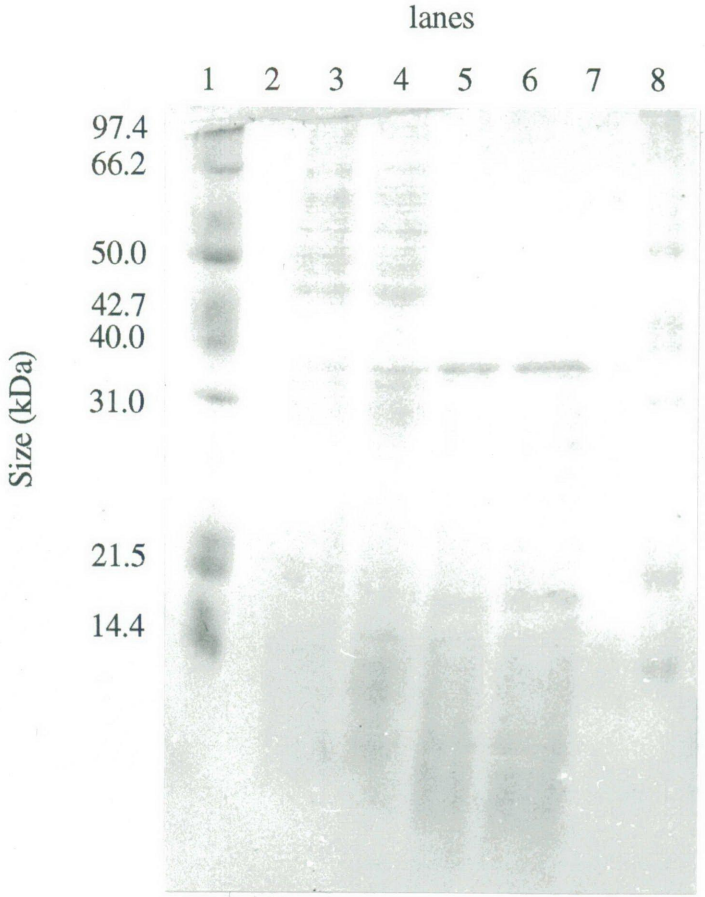

Figure 3.1. SDS-PAGE of culture filtrate from the wildtype strain MH2 after transfer to protease-repressing and protease-derepressing conditions for 4 hours. Protease repressing conditions (lanes 3 and 4) are not nutrient-limiting. Protease derepressing conditions (lanes 5 and 6) used in this experiment were nitrogen-limiting conditions. Sizes of the protein molecular weight standards (lanes 1 and 8). are marked on the left of the figure. No protein was run in lanes 2 and 7. It can be observed in this SDS-PAGE result that a different spectrum of proteins is present in culture filtrate produced under the two different conditions examined. Protease-repressed cultures were grown in minimal media containing 1% glucose as a carbon source, 10 mM ammonium as a nitrogen source. Protease-derepressed cultures were grown in minimal media containing 1% glucose, and lacking a nitrogen source. Minimal media used in this experiment was made with a salt solution containing inorganic sulphur.

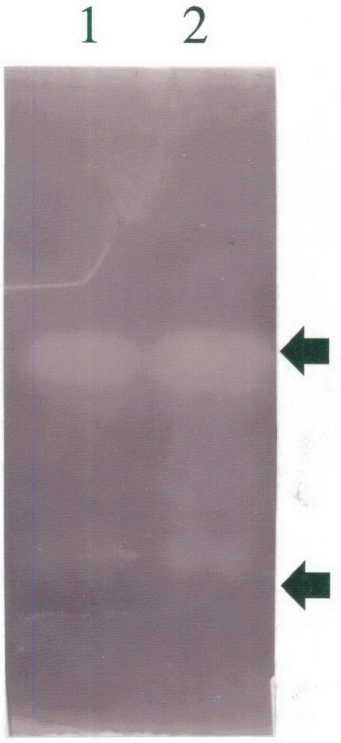

Figure 3.2. Milk overlay used to detect protease activity in a protein gel which had been renatured after SDS-PAGE. Both lanes contain culture filtrate obtained from a protease-overproducing strain MK86 after 16 hours in carbon-limiting conditions. Arrows mark the two regions of proteolytic activity detected.

3.3.1.2. Native PAGE to detect extracellular proteases.

The milk overlay protease detection method of Foltmann *et al.* (1985) was chosen from a number of recently published protease detection methods. This method has the advantage of being sensitive and can detect the activity of proteases active at both acid and alkaline pH.

Initial experiments used culture filtrate from cultures which were grown in unbuffered media at pH 6.5. In the case of filtrate from carbon- and nitrogen-limiting conditions, mycelia was transferred after 16 hours growth in mimimal media (made from sulphur containing salt solution) containing 1% glucose and 10 mM ammonium tartrate, to nutrient-limiting conditions for 16 hours in the case of carbon limitation and 4 hours in the case of nitrogen limitation, and the filtrate from the second incubation used. To obtain filtrate from sulphur-limiting conditions, conidia were inoculated directly into media which was limiting for sulphur, and allowed to grow for 20 hours. All incubations took place on an orbital shaker (1.5 x 100 rpm) at 37°C. Prior to analysis, culture filtrate was dialysed against milli-Q-H_20 to remove low molecular weight compounds. The filtrate was then lyophilised, and resuspended in a smaller volume to concentrate the sample. Non-denaturing 1D PAGE using a Tris-glycine buffer system (with a stacking gel at pH 8.3, and resolving gel at pH 9.5) was used to separate the proteins, and milk overlays (pH 5.3) were used to detect protease activity. Gels containing 7.5-12.5% polyacrylamide were tested, and the best resolution was found with 12.5% polyacrylamide gels. A variety of amounts of concentrated culture medium were also tested. A sample containing 1 unit protease activity (determined by assays for proteolytic activity at pH 7.2, see section 2.17.1.), gave the most satisfactory results with regards to band separation and detection. Although culture medium with greater amounts of proteolytic activity resulted in increased strength of the weaker bands, it also resulted in increased overlaps between the regions of higher activity causing these bands to become less distinct.

Initial experiments examined the proteases present in the culture filtrate of wildtype strains under protease derepressing conditions. The number of proteases present in filtrate obtained from cultures of *A. nidulans* grown at neutral pH (pH 6.5, unbuffered media) under carbon-, nitrogen-, or sulphur-nutrient limiting conditions differed. Six regions of proteolytic activity were observed in culture filtrate from carbon starvation conditions, while 2 proteolytic bands were predominant in cultures from nitrogen or sulphur starvation conditions (fig. 3.3.).

To examine the effect of pH on the activity of the proteases produced under standard conditions (unbuffered media, pH 6.5) the activity of the proteases was also examined by detection of proteolytic activity at pH values of 3, 5, 7 and 9. For this experiment culture filtrate from carbon starved cultures was used, as it was the condition under which the highest number of proteases were observed when detection was carried out at pH 5.3. No regions of proteolytic activity which had not been detected at pH 5.3 were detected at other pHs. No proteolytic activity was observed at pH 3. All regions of proteolytic activity were most active at pH 7. As all regions of proteolytic activity were easily observed at pH 5.3, this detection pH was maintained for further experiments.

3.3.2. The effect of different nutrient-limiting conditions.

Initial observations suggested that there may be some difference in the protease spectrum produced under the different nutrient-limiting conditions (see 3.3.1.) though all samples contained equal amounts of protease activity as determined by protease enzyme assays (fig. 3.3.). Experiments performed in the analysis of the *prtA* gene replacement strains (see section 5.3.3.) showed that the incubation time in derepressing conditions affected the number of proteases observed, *e.g.* after 4 hours of nitrogen limitation only two bands are observed, but after 24 hours six bands are clearly visible (fig. 5.10.). It was shown that all 6 bands of proteolytic activity were

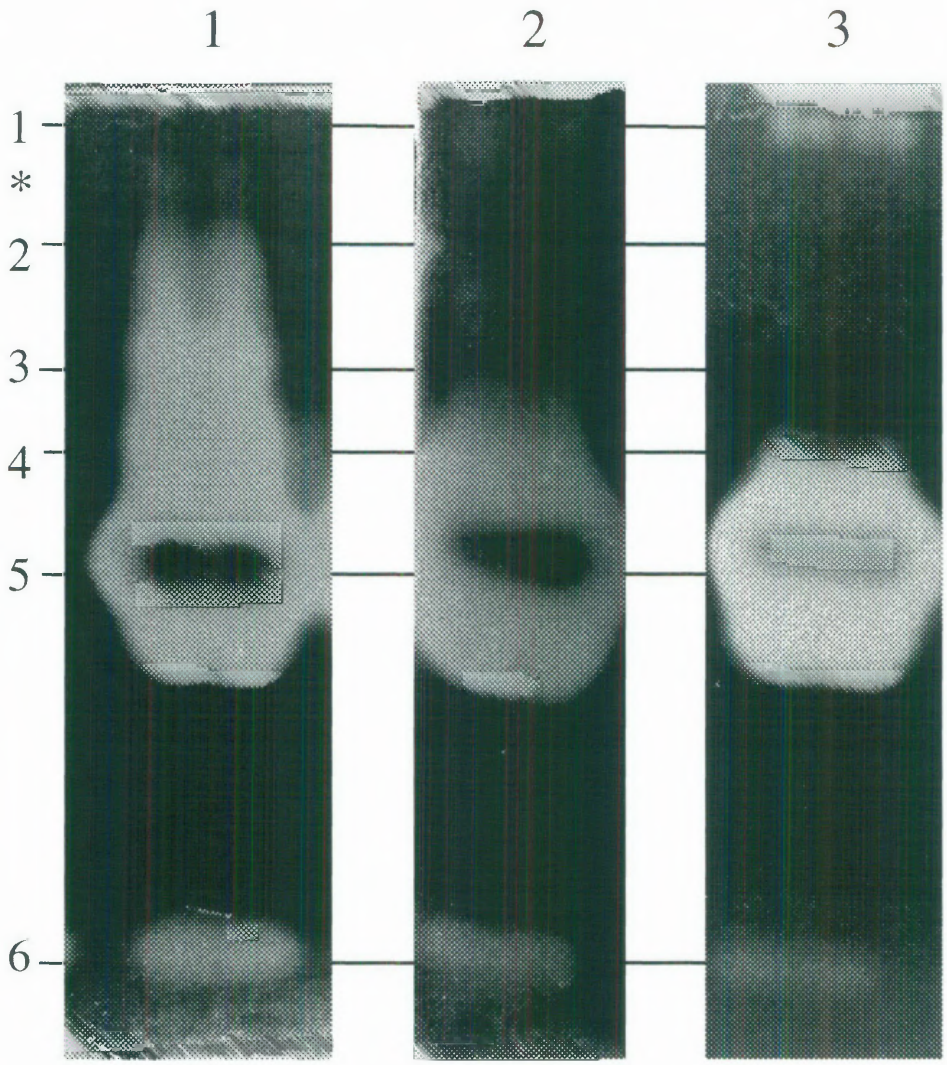

Figure 3.3. The spectrum of proteases observed in the culture filtrate of wildtype strains of *A. nidulans* under nutrient-limiting conditions. Lane 1. 16 hours carbon-limiting conditions. Lane 2. 4 hour nitrogen-limiting conditions. Lane 3. 20 hours sulphur-limiting conditions. Bands of proteolytic activity are designated 1-6 as labelled. The faint band marked with an asterisk was not observed in the majority of zymograms and therefore may be an artifact observed on this particular zymogram, or a band of protease activity correponding to a protease whos activity under the conditions used in this experiment is at the limits of detection and consequentially was not often detected.

present in filtrate from cultures subjected to carbon-limiting conditions for 16 hours and nitrogen-limiting conditions for 24 hours.

Increasing the incubation time with the milk overlay from 16 hours to 40 hours also enhanced the visibility of the fainter bands. With increased incubation time all six bands of proteolytic activity were detected in the filtrate of cultures subjected to 4 hours nitrogen-limitation, or grown for 20 hours in sulphur-limiting conditions. Extending the incubation time with the milk overlay appears to extend the limits of detection. Longer periods of time may be necessary for the detection of proteases whose activity is not detectable after 16 hours. Extending the incubation time also allowed for a greater period of activity by the predominant proteases, and like greater amounts of filtrate, resulted in increased overlap and a reduction in the distinction between bands.

To determine whether the pH of the media was affecting the results obtained under the different nutrient-limiting conditions, the pH of the media was determined before and after incubation with *A. nidulans* mycelium (Table 3.2). Under derepressing conditions the pH of the media was found to be much lower than the original pH of 6.5. The pH of the media, after growth of wildtype strains under carbon-, nitrogen-, and sulphur-nutrient limiting conditions, for 16, 4, and 20 hours respectively, did not differ greatly from the original pH of 6.5. Therefore it is unlikely that the different protease spectrums produced by wildtype strains under the different nutrient-limiting conditions can be attributed to differences in the pH of the media.

Table 3.2. pH of unbuffered media (pH 6.5) after inncubation with *A. nidulans* mycelia.

Medium Type	Nutrient Source			Length (hrs) of inncubation	pH after growth	pH before growth
	carbon 1% glucose	nitrogen 10 mM ammonium	sulphur 0.1% thiosulphate			
Liquid Media #	+	+	+	20	3.60 ±0.42	6.5
	+	+	+	4	4.29 ±0.33	6.5
	+	+	+	20	2.64 ±0.09	6.5
	+	+	-	20	6.75 ±0.46	6.5
	+	-	+	164	6.08 ±0.36	6.5
	-	+	+	16	6.53 ±0.15	6.5
Solid Media * + 1% skim milk	+	+	-	48	5.3 to 5.6	6.5
	+	-	+	48	~6.2	6.5
	-	+	+	48	~8	6.5
	-	-	+	48	~8	6.5

The pH for liquid medium (#) was determined using a pH meter and is the average of 6 cultures. The pH of solid medium was determined using narrow range pH paper

3.3.3. Protease inhibition studies.

It had been established (section 3.3.2.) that six regions of proteolytic activity are found in culture filtrate obtained from mycelia under carbon-, nitrogen- or sulphur-nutrient limiting conditions. A preliminary examination of the *xprE*, *F* and *G* mutants (see 1.2.6. for description) using PAGE in conjunction with milk overlays, had shown that the *xprG1* mutation resulted in the overexpression of all six regions of proteolytic activity under conditions of carbon limitation (Katz *et al.* 1996). Because of this, culture filtrate from a strain carrying the *xprG1* mutation grown under nitrogen limiting conditions was used in the protease inhibition study described below.

The identity of the proteases was determined with protease inhibitors. For each inhibitor experiment an untreated control was included for comparison. The effects of the various inhibitors is shown in figure 3.4., and is summarised in Table 3.1. Bands were designated 1-6 in order of increasing mobility (see fig. 3.3.).

The inhibition data shows that the enzymes responsible for bands 2, 3, 4, 5 and 6 are all sensitive to inhibitors known to affect serine proteases. Bands 2, 3, and 4 show identical susceptibility patterns to the inhibitors used in this study and therefore appear to be forms of the same enzyme. These bands were also observed in mycelial extracts from repressed cultures, and their intensity increased in mycelial extracts from cultures limiting for nitrogen (section 6.3.5.). This indicated that bands 2, 3 and 4 could be due to a predominantly intracellular protease.

The predominant band observed in culture filtrate obtained from mycelia grown under nitrogen limiting conditions was designated band 5 and it is another serine protease. Compared to the intracellular protease, it is more susceptible to inhibition by PMSF and aprotinin, and less susceptible to inhibition by trypsin inhibitor and PCMPS, all of which are inhibitors of serine proteases. The distinguishing feature of the third serine protease, band 6, is that it is more sensitive to inhibition by leupeptin and TLCK than either of the other serine proteases. The remaining band of activity, band 1, appears to be a metallo-proteinase as it is inhibited by the metal-chelating agents EDTA and 1, 10-o-phenanthroline. The aspartic protease inhibitor, pepstatin A, had no effect on the activity of any of the proteases detected using this technique.

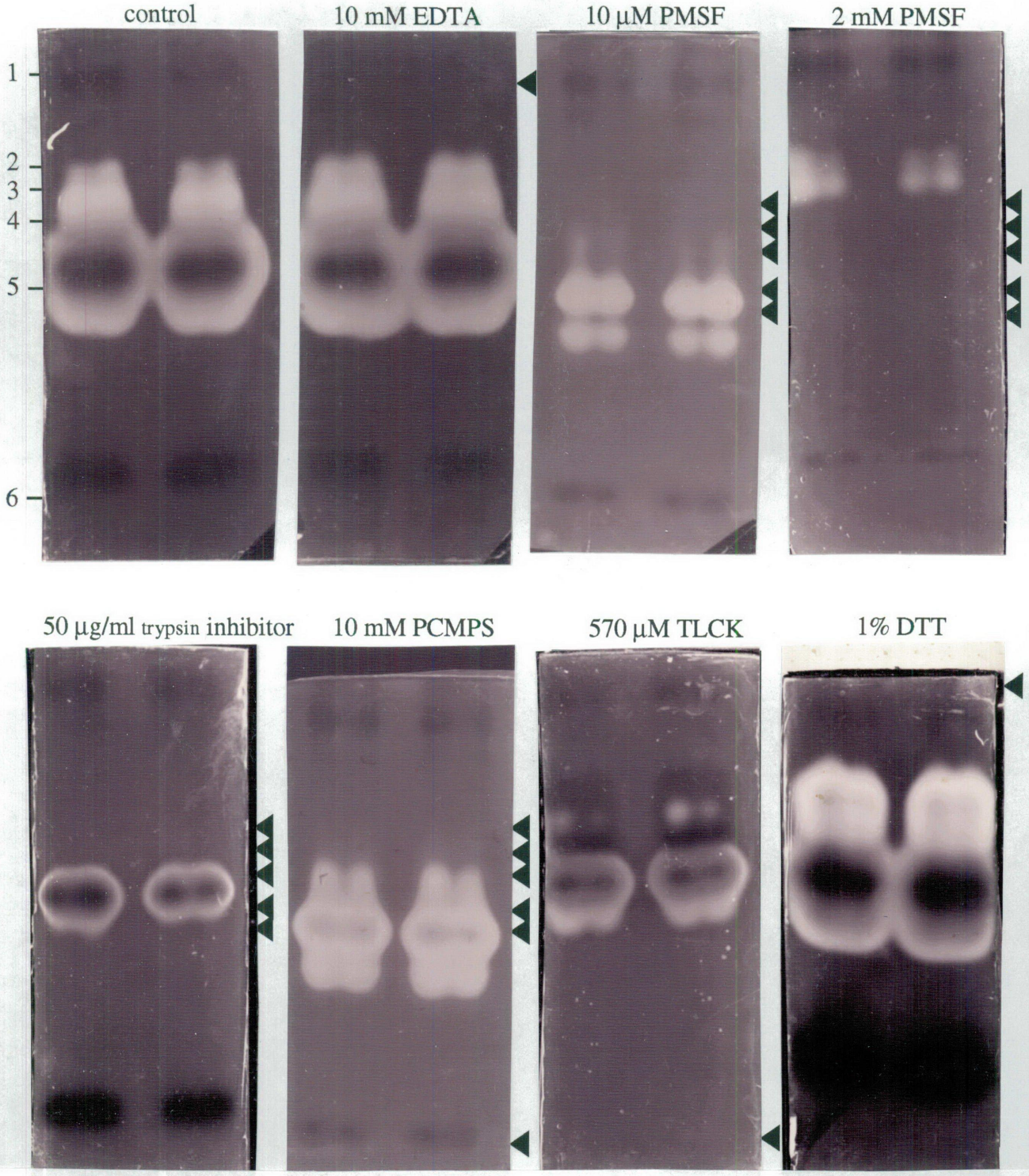

Figure 3.4. Effects of a variety of protease inhibitors on the proteases observed in the culture filtrate of *A. nidulans*. After electrophoresis, gels were exposed to inhibitors for 1 hour prior to protease detection. For each inhibitor experiment an untreated control was included for comparison. Numbers on the left of the control gel represent the numbers to which the regions of proteolytic activity are referred. Arrows indicate the region(s) of proteolytic activity affected. Abbreviations: EDTA = ethylenediaminetetra acetic acid, PMSF = phenylmethylsulfonylfluoride, PCMPS = p-chloromercuriphenylsulfonic acid, TLCK = Nα-p-tosyl-L-lysine chloromethyl ketone, DTT = dithiothreitol.

Table 3.1. The effect of different protease inhibitors on the activity of the proteases of *A. nidulans*.

INHIBITOR	Bands of protease activity					
	1	2	3	4	5	6
10 mM EDTA	-	+	+	+	+	+
10 μM 1,10-o-phenanthroline	-	+	+	+	+	+
1% DTT	-	+	+	+	+	+
2 mM PMSF	+	±	±	±	-	±
2 μg/ml aprotinin	+	±	±	±	-	-
50 μg/ml trypsin inhibitor	+	-	-	-	±	±
10 mM PCMPS	+	-	-	-	±	±
8 μM leupeptin	+	±	±	±	±	-
570 μM TLCK	+	+	+	+	+	-
1 μM pepstatin A	+	+	+	+	+	+

Bands are numbered as in figure 3.3. Protein gels were incubated in inhibitor solution for 1 hour prior to protease detection, activity is denoted +, total inhibition -, partial inhibition ±. For each inhibitor experiment an untreated control was included for comparison. DTT = dithiolthreitol, PMSF = phenylmethylsulfonylfluoride, PCMPS = p-chloromercuriphenylsulfonic acid, EDTA = ethylenediaminetetraacetic acid, TLCK = Nα-p-tosyl-L-lysine chloromethyl ketone

3.3.4. Detection of acid protease activity in the culture filtrate of derepressed *A. nidulans* cultures.

Native PAGE in conjunction with milk overlays and the use of protease inhibitors enabled us to identify three serine proteases and one metallo-protease present in derepressed *A nidulans* culture filtrate (section 3.3.3.). As aspartic proteases known as aspergillopepsins are known in many *Aspergillus* species it was surprising that I was not able to identify proteolytic activity corresponding to this class of acid proteases (Matsubara and Feder 1971).

A number of factors may have contributed to my inability to detect acid proteases in the filtrate of *A. nidulans* cultures subjected to derepressing conditions. If *A. nidulans* possesses any acid protease genes they may be regulated with regards to environmental pH. Consequentially media at pH 6.5 may not be suitable for the production of acid proteases. In general acid proteases are stable at acid pH values ranging from 2 to 6, and purification of these enzymes are usually carried out in this pH range (Matsubara and Feder 1971). Therefore, the preparation and electrophoresis conditions that I used may not have been suitable for acid proteases. It is also possible that, although *A. nidulans* is able to grow in environmental conditions ranging from pH 2.5 to pH 10.5 (Rossi and Arst 1990), in this *Aspergillus* species acid proteases may be weakly expressed or not expressed at all.

To detect acid protease activity in *A. nidulans* culture filtrate, modifications were made to the previous methods. As it is well known that *A. niger* produces acid proteases, so *A. niger* was examined along with *A. nidulans* as a means of determining whether the methodology was suitable for the detection of acid proteases. The *Aspergillus* cultures were grown for 16 hours in liquid culture media (minimal medium containing 1% glucose as a carbon source, 10 mM ammonium as a nitrogen source) at pH 6.5, prior to transfer to nitrogen-limiting medium buffered to pH 3 with 0.15 M citric acid, tri-sodium phosphate buffer. The culture filtrate was subsequently dialysed against 0.15 M citric acid tri-sodium phosphate buffer pH 3. Proteins contained in the filtrate were then electrophoresed through a low pH discontinuous PAGE system which stacks at pH 5.0 and resolves at pH 3.8 (Hames 1981). The gel was loaded with six samples from each species, alternating duplicates of each. After electrophoresis the gel was cut to give three gels containing two samples from *A. niger* and two from *A. nidulans*. The gels were equilibrated to the pH used in the overlays. Three different overlays were used in an effort to detect acid protease activity: the milk overlay of

Foltmann *et al.* (1985) at pH 5.3, which had been reported as suitable for the detection of the *A. niger* protease, a milk overlay buffered with 0.15 M citric acid tri-sodium phosphate pH 3, and an overlay containing haemoglobin buffered with 0.15 M citric acid tri-sodium phosphate pH 3. An extremely faint region of proteolytic activity was detected in the lanes containing the *A. niger* filtrate of the haemoglobin containing overlay. No proteolytic activity was detected with either of the other overlays. This experiment was repeated again once and again no proteolytic activity was detected.

3.3.5. A comparison of proteases present in culture filtrate and mycelial extracts.

Based on the protease inhibition data, I believed that the regions of proteolytic activity numbered 2-4 corresponded to the predominantly mycelium-bound, constitutively expressed protease designated β by Cohen (1973). To support the assertion that this enzyme corresponded to Cohen's protease β. I compared the electrophoretic mobility of proteases present in filtrate from protease-derepressed cultures, with those present in mycelia grown under protease-repressing conditions (fig. 3.5.). It was reported that after freezing, protease β was converted to the more active form, and this form has the greatest electrophoretic mobility of the three forms of protease β (Cohen 1973). As observed in Figure 3.5. the intracellular samples, which were not frozen during processing, contain bands of proteolytic activity which have similar electrophoretic mobilities to bands 2 and 3 found in culture filtrate, suggesting that bands 2-4 correspond to Cohen's protease β.

It was observed in the protease detection experiments that protease activity could be observed as either a clearing or precipitation of the milk protein present in the milk overlay. Protease activity in the regions of bands 1 and 6 were usually observed as a clearing, while bands 2-5 were observed as both clearings or precipitations, and sometimes as clearings surrounded by regions of precipitation (for example see band 5 of the control panel in fig 3.4). The reason for the different effects of the proteases on milk protein is unknown, although subtle differences in the pH of the gel and/or overlay may play a role.

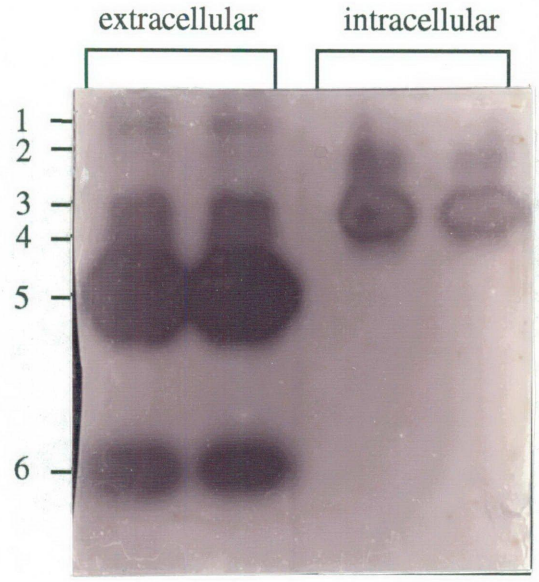

Figure 3.5. Zymogram comparing the proteases present in intracellular and extracellular samples obtained from wildtype strain MH2. The intracellular sample was obtained from mycelium subjected to protease-repressing conditions (incubated in minimal medium containing 1% glucose, 10 mM ammonium). The extracellular sample was obtained from a culture exposed to 16 hours carbon limiting conditions. It is of note that the intracellular protease is present in the intracellular sample in the less active forms that have slower electrophoretic mobilities (bands 2 and 3) than the more active forms which are present in the extracellular sample (bands 3 and 4). Proteolytic regions are numbered as for Table 3.1. Samples were loaded in duplicate with 10% of the total volume of culture filtrate or intracellular filtrate respectively. Minimal media used in this experiment was made using an inorganic sulphur containing salt solution.

3.4. Discussion.

Six regions of proteolytic activity were detected in culture filtrate obtained from mycelia grown under carbon-, nitrogen-, and sulphur-nutrient limiting conditions. Bands designated 5 and 6 were prominent under all conditions, whereas bands 1, 2, 3, and 4 were only clearly visible in filtrate from carbon-starved cultures. Increased detection time, or increased sample size was necessary for the detection of these bands in sulphur- or nitrogen-limited culture filtrates. It is possible that the difference in detectability is a function of the level of derepression, or the ratios of the individual proteases with regards to each other may differ depending on the stimulus. Carbon starvation results in high levels of all proteases, whereas nitrogen- and sulphur-nutrient limitation results in high levels of proteases corresponding to bands 5 and 6, and low levels of all other proteases. Increased starvation time or detection time is required to detect the less abundant proteases under these conditions.

The amount of filtrate used was determined in assays for proteolytic activity at pH 7.2. The amount used as a sample for electrophoresis was equivalent to that which would give an absorbance reading of 1.0 in the protease assay. Thus samples from all cultures contained, in crude terms, equivalent amounts of protease activity, as measured at pH 7.2. When equivalent samples of filtrate from the carbon-, nitrogen-, and sulphur-nutrient limiting conditions were compared the spectrum of protease detected was not the same for the different conditions. This showed that, although the same amount of proteolytic activity was contained in each sample, the ratio of the different enzymes differed depending on which nutrient-limiting condition was used to derepress protease production. This observation suggests that either there are differences in the ratios of the proteases produced under the different nutrient-limiting conditions, or that at different stages during derepression different ratios of the proteases are produced and that the points at which the samples are taken for carbon-, nitrogen-, and sulphur-nutrient limiting conditions are not equivalent.

The inhibition data shows that at least four different proteases are found in the culture filtrate of *A. nidulans* when it is grown at pH 6.5, in conditions limiting for either

carbon, nitrogen, or sulphur. Inhibitor studies have shown that three are serine proteases, and one is a metallo-protease.

Despite some apparently contradictory results Cohen's band α probably corresponds to my band 1. Contrary to my results, Cohen (1973) did not observe activity corresponding to enzyme α at neutral pH and observed that when EDTA was added prior to electrophoresis mobility of this enzyme was affected but its activity was not. I observed increased proteolytic activity of band 1 at close to neutral pH (pH 7). The results of this study suggest that the absence of α protease activity observed by Cohen (1973) at pH 5-6 may have been due to a reduction in activity to a level below detectability. This was overcome in our experiments by increased sensitivity of the method of detection and/or higher enzyme concentrations. An altered protein mobility due to chelation of the active metal ion may be due to changes in the overall charge of the protein. As protease inhibition by EDTA is reversible, exposure to metal ions after electrophoresis would result in reactivation. I believe the metallo-protease, band 1, probably corresponds to α in Cohen's notation. Two different types of metallo-protease genes have been isolated from *Aspergillus* species. The *A. oryzae* and *A. fumigatus* metallo-protease genes do not cross-hybridise, and sequence analysis has shown that there is little similarity between the two genes. The mature *A. fumigatus* protein is almost double the size of the *A. oryzae* metallo-protease (Tatsumi *et al.* 1991, Sirakova *et al.* 1994, Markaryan *et al.* 1994).

Three bands (2, 3 and 4) with identical inhibitor susceptibility patterns were observed, and evidence indicating that this region of proteolytic activity is due to multiple forms of a predominantly mycelium-bound serine protease was obtained. It is likely that bands 2, 3 and 4 are the allozymes of the enzyme β observed in a previous study of *A. nidulans* proteases (Cohen 1973). It is possible that the mycelium-bound serine protease identified in this study is a homologue of the *A. niger pepC* gene, which encodes an intracellular serine protease (Frederick *et al.* 1993).

The band I have designated 5 is the region which shows the highest level of proteolytic activity at pH 5.3. Like γ and δ, reported to be allozymes by Cohen (1973), band 5 is the most sensitive to inhibition by PMSF. With the use of inhibitors which reduced the activity in this region I was able to observe two distinct bands, which I believe

correspond to the allozymes γ and δ. Band 6 is a third serine protease, with an inhibition profile compatible with the results published for protease ε (Cohen 1973). A subtilisin-like extracellular serine protease homologous to *pepD* of *A. niger* has been cloned from *A. nidulans*. This gene, *prtA*, may encode the either of the serine protease activities corresponding to bands 5 or 6. A homologue of the serine carboxypeptidase, which has been shown to be an extracellular protease in *A. niger* (van den Hombergh *et al.* 1994), is another possible candidate which could be responsible for either extracellular serine protease.

Using this technique we were unable to detect any proteolytic activity which could be attributed to an acid protease in culture filtrate from *A. nidulans*. Foltmann *et al.* (1985) designed their protease detection method as alternative to haemoglobin zymograms for the detection of acid proteases, and showed that this method could detect the activity of fungal acid proteases, including the aspergillopepsin of *A. niger*. Therefore it would be reasonable to expect that this method would also be suitable for the detection of acid proteases from *A. nidulans*. Purification of acid proteases are often carried out in the pH 2 to 6 range where acid proteases are most stable (Matsubara and Feder 1971). Therefore the original preparation and electrophoresis methods used may not have been suitable for the detection of acid proteases. My attempts at detecting acid proteases in the filtrate of *A. nidulans* cultures using low pH purification and detection methods proved unsuccessful. Though I was able to detect acid protease activity in the filtrate from an *A. niger* culture, this result was not repeated, indicating that the methods used require further refinements. As I also cloned an aspergillopepsin gene from *A. nidulans*, and was unable to detect transcripts from this gene (see chapter 4.), the attempt to detect acid protease activity was not continued. While the methodology used to detect acid protease activity may not have been suitable, it is also possible that *A. nidulans* may not produce any acid proteases, or the conditions under which it produces such enzymes are different from those tested.