Multiple cysteine proteinase forms during the life cycle of *Dictyostelium discoideum* revealed by electrophoretic analysis

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Proteinases of the cellular slime mould *Dictyostelium discoideum* have been analysed using electrophoresis on polyacrylamide gels containing gelatin (gelatin/PAGE). Multiple proteinase forms were apparent in vegetative myxamoebae, but the presence of individual enzyme forms depended on the manner in which the cells were grown. Axenic cells had a characteristic A-pattern of proteinases consisting of six bands, the most active enzymes having apparent M_r values of 51000 and 45000 (these have been named ddCP51 and ddCP45, respectively). Some of the proteinases were also present in the medium, the major extracellular form was ddCP42, a 42000- M_r enzyme. Cells grown in association with bacteria had a distinct B-pattern with three main enzymes that had apparent M_r values of 48000, 43000 and 38000. All of the A- and B-pattern proteinases were most active at acid pH in the presence of dithiothreitol and were inhibited by various agents such as *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), leupeptin and chymostatin, which inactivate cysteine proteinases. One of the enzymes, ddCP30, was identified as cysteine proteinase B which had been purified and characterized previously [North, M. J. & Whyte, A. (1984) J. Gen. Microbiol. 130, 123–134]. During starvation of axenic cells in shaken suspensions some of the vegetative proteinases disappeared, ddCP42 was released from the cells and one new enzyme with an apparent M_r of 48000 appeared. Addition of cyclic AMP had little effect on these changes. When the axenically grown myxamoebae underwent development on filters, similar changes in band pattern were observed and the aggregation stage was characterized by the presence of three cysteine proteinase bands (apparent M_r values of 48000, 45000 and 43000). Proteinases, especially ddCP42, were released from the cells and could be collected from the buffer-saturated pads which supported the filters. The results demonstrate that cysteine proteinases are present throughout growth and development of *D. discoideum* and that the forms present are subject to nutritional and developmental regulation.

INTRODUCTION

The proteolytic system of the cellular slime mould *Dictyostelium discoideum* has been the subject of a number of recent studies (North, 1982a, 1985). In this organism, as in mammalian cells, there are lysosomal acid proteinases of both the aspartic and the cysteine type. It possesses other enzymes, including a neutral hydrolase active on peptide nitroanilides of the general structure X-Ala-Ala-Pro-Yaa-Nan (where X = acetyl or succinyl, Yaa = Leu, Phe or Ala) (North, 1982b), which may be a serine proteinase (M. J. North, T. M. Kyndt & S. J. McKay, unpublished work), and various peptidases, including leucine aminopeptidase (Firtel & Brackenbury, 1972; North & Cotter, 1984) and dipeptidyl aminopeptidases (Chan *et al.*, 1987). Cysteine proteinases (CPs) in *D. discoideum* are developmentally regulated and have attracted particular attention (North, 1985). Various investigators have shown that the vegetative myxamoebae of the growth phase contain high levels of cysteine proteinase activity and that this declines when development has been initiated by starvation. The developmental phase of the life cycle culminates in the

formation of fruiting bodies consisting of spores and vacuolated non-viable stalk cells, and the decrease in cysteine proteinase activity was reported to be greater in cells which were destined to become spores (pre-spore cells) than in those that became stalk cells (pre-stalk cells) (Fong & Rutherford, 1978). During spore germination the activity increases as myxamoebae emerge from the spore coat (Jackson & Cotter, 1984; North & Cotter, 1984). The overall pattern of regulation of cysteine proteinase activity suggested a role in nutrition and implied that the enzymes are of less importance during development.

Paradoxically, *D. discoideum* has at least three developmentally regulated genes which encode proteins that appear to be members of the cysteine proteinase family. The putative protein products of these genes have been termed CP1 (Williams *et al.*, 1985), CP2 [Pears *et al.*, 1985] (this protein has also been called pst-cath (Datta & Firtel, 1987)) and CP3 (Presse *et al.*, 1986a). The CP1 and CP2 genes are co-ordinately regulated and are expressed maximally at the time of cell aggregation. Their expression appears to be exclusive to the developmental phase, since no mRNA has been detected

Abbreviations used: E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; PAGE, polyacrylamide-gel electrophoresis; PDF, pad diluting fluid; PMSF, phenylmethanesulphonyl fluoride; TLCK, tosyl-l-lysylchloromethane; TPCK, tosylphenylalanylchloromethane; DTT, dithiothreitol; CP1, 2 and 3; cysteine proteinase 1, 2 and 3.

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in vegetative cells, and occurs during both spore- and stalk-cell differentiation (Pears & Williams, 1987). The CP3 gene is expressed maximally at a later stage of development (Presse *et al.*, 1986b), but its cell type specificity has not been reported. None of the products of these developmentally regulated CP genes has yet been identified.

In order to extend the analysis of cysteine proteinases in *D. discoideum*, a new analytical method has been used. In previous work, electrophoresis on polyacrylamide gels containing denatured haemoglobin was employed to differentiate the proteinases (North & Harwood, 1979) and provided supporting evidence for the decline in cysteine proteinase activity during development observed by other methods (Fong & Rutherford, 1978; Gustafson & Thon, 1979; North, 1982b). PAGE involving gels containing gelatin, in which proteins can be separated under reducing and denaturing conditions, has been used successfully in the analysis of the proteinases of other lower eukaryotes (Lockwood *et al.*, 1987a,b) and provides better resolution and sensitivity than the haemoglobin-gel method. Its value in the identification of the *D. discoideum* proteinases has been investigated, and in this paper we demonstrate that cysteine proteinases are detected specifically by this method. A number of important findings concerning the multiple forms of cysteine proteinase present in *D. discoideum* are described including their dependence on nutritional factors, a further aspect of which is considered in the accompanying paper (North, 1988).

MATERIALS AND METHODS

Materials

Gelatin (porcine type 1), tosyl-lysylchloromethane (TLCK), tosylphenylalanylchloromethane (TPCK), iodoacetic acid and bovine albumin were from Sigma (Poole, Dorset, England). Pepstatin, chymostatin, antipain, leupeptin and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64) were products of The Peptide Institute Inc. (Osaka, Japan) and obtained through Scientific Marketing Associates (London, England). PAGE 83 was purchased from BDH (Poole, Dorset, England). All other reagents were of the highest purity commercially available.

Organisms, growth and development

D. discoideum strain AX2 (A.T.C.C. 24397), which is capable of growth in axenic medium, was kindly provided by Dr. B. D. Hames (Leeds, England), and was used in all experiments unless otherwise indicated. The non-axenic strains, NC4 and V12M2, were both obtained from Dr. J. G. Williams (Imperial Cancer Research Fund Laboratories, South Mimms, England). All cultures were grown at 22 °C. Stocks were maintained by growth in association with *Klebsiella aerogenes* on SM agar (Sussman, 1966). Bacterially grown myxamoebae were obtained either after growth with live *K. aerogenes* on SM agar or from shaken cultures in which myxamoebae were inoculated into autoclaved suspensions of *K. aerogenes* (A_{650} 2.5) in 20 mM-sodium phosphate buffer, pH 6.0. Axenic cultures of myxamoebae were grown in HL5 medium (Watts & Ashworth, 1970); normally 86 mM-glucose was included. The cells were harvested by centrifugation at 500 *g* for 5 min. Bacterially grown cells were washed with water until the supernatant was free of

bacteria; axenic cells were washed once with water. Samples of medium were taken from the first supernatant and any remaining cells removed by centrifugation at 11 700 *g* in an MSE Microcentaur microcentrifuge.

Two systems were used for analysing developmental changes. Myxamoebae were resuspended at a density of 1×10^7 cells/ml in 50 ml of 20 mM-sodium phosphate buffer, pH 6.0, contained in a 250 ml Erlenmeyer flask and shaken at 120 rev./min at 22 °C. Samples were removed periodically and cell pellets were obtained by sedimenting at 500 *g* for 1 min in a bench centrifuge. To remove any remaining cells from the supernatant, samples were re-centrifuged for 2 min at 11 700 *g* in an MSE Microcentaur microcentrifuge. Alternatively, myxamoebae were deposited on Millipore nitrocellulose filters (5×10^7 /filter) supported by Whatman GF/D pads saturated with pad diluting fluid (PDF; 25 mM-KCl/5 mM-MgCl₂/50 mM-sodium/potassium phosphate buffer, pH 6.5) (North & Williams, 1978). Cells were washed from the filters into water and the cell pellet collected by centrifugation. Samples of PDF were obtained by squeezing the pads and then removing any solid material by microcentrifugation for 5 min. In all experiments samples were stored at -20 °C until required, with no apparent effect on activity.

Gelatin/PAGE analysis

The method used was an adaptation of that described by Heussen & Dowdle (1980) and was carried out essentially as described by Lockwood *et al.* (1987a). Proteinases were normally separated on 7.5% (w/v) acrylamide gels containing 0.2% (w/v) gelatin using a Bio-Rad Protean I system. The cell lysates were prepared in 0.1% (v/v) Triton X-100 and protein concentrations were determined by the method of Sedmak & Grossberg (1977) using bovine serum albumin (Sigma, Fraction V), as standard. Samples containing 30 µg of protein were diluted 2-fold in electrophoresis sample buffer [0.0625 M-Tris/HCl, pH 6.5, 2% (w/v) SDS, 5% (v/v) mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) Bromophenol Blue] and loaded into wells. Extracellular samples (medium, buffer, PDF) were similarly diluted 2-fold with electrophoresis sample buffer and 150 µl of the mix loaded per well. After electrophoresis overnight at 6 mA/gel, the gels were transferred to 2.5% (v/v) Triton X-100 for 1 h and then to an appropriate buffer at 25 °C to develop the proteinase bands. The standard buffer was 0.1 M-sodium acetate/acetic acid, pH 4.0, containing 1 mM-dithiothreitol (DTT). Other buffers used were 0.1 M-glycine/HCl, pH 2.0, 0.1 M-sodium acetate/acetic acid, pH 5.0, 0.1 M-sodium phosphate buffer, pH 6.0, and 0.1 M-Tris/HCl, pH 8.0. Some indication of the progress of band formation could be gained from the appearance of distinct zones which were more opaque than the rest of the gel. Band formation was stopped at an appropriate time by transferring the gel to stain [0.2% (w/v) PAGE 83 in 25% (v/v) propan 2-ol/10% (v/v) acetic acid]. After 2 h, the gel was destained in 10% (v/v) acetic acid. For a permanent record, the gels were either photographed or scanned at 550 nm using a Gelman DCD-16 scanner.

Inhibitor sensitivity and pH-dependence were determined by loading a single sample containing up to 1 mg of protein mixed with electrophoresis sample buffer on to a stacking gel with no wells and electrophoresis carried out as above. After Triton X-100 treatment, the

gel was cut into 2 cm-wide strips which were incubated separately in appropriate buffer (plus inhibitor) before staining and destaining as normal. Inhibitors were tested at pH 4.0 in the presence of 1 mM-DTT at the following concentrations unless otherwise indicated: pepstatin, leupeptin, antipain, chymostatin and E64 all at 20 μ g/ml; phenylmethanesulphonylfluoride (PMSF), EDTA, iodoacetic acid, TLCK and TPCK all at 1 mM.

RESULTS

Analysis of vegetative proteinases by gelatin/PAGE

Gelatin/PAGE analyses allowed the identification of a number of proteinases in *D. discoideum* (Fig. 1). In cells grown axenically with glucose (G cells), six major proteinases were detected. As the proteins were separated under reducing and denaturing conditions it was possible to obtain an apparent M_r for each form from their positions relative to those of standards of known M_r . At least eight independent determinations were made using gels containing 7.5%, 10% or 12% (w/v) acrylamide. There was no evidence that the presence of the gelatin affected the mobility of proteins, although there was a slight discrepancy between the apparent M_r of actin observed here (46000) and that reported elsewhere (42000) (Livi *et al.*, 1987). Initially the presence of strongly staining endogenous protein bands was a problem as they made it difficult to see proteinase bands distinctly. Actin caused particular problems as it is an abundant protein in *D. discoideum* and its electrophoretic mobility was similar to that of a number of the proteinases. To minimize this difficulty, a high concentration (0.2%, w/v) of Coomassie Blue (PAGE 83) was used to improve gelatin staining and the amount of sample protein was restricted to 30 μ g or less.

The proteinases in G cells have been named ddCP30, ddCP38A, ddCP42, ddCP45, ddCP51 and ddCP54 according to their apparent M_r [the prefix dd denotes the species, CP denotes a cysteine proteinase (see below) and ddCP38A has the suffix A to distinguish it from an enzyme with similar electrophoretic mobility, but differing properties in bacterially grown myxamoebae]. ddCP45 and ddCP51 were always the predominant

forms. The gel shown in Fig. 1(a) was incubated for a long period (8 h) to ensure that all possible bands developed, and consequently the ddCP38A and ddCP42 bands are more intense than normal. When cells were grown axenically in the absence of glucose (NS cells), the same proteinase forms were detected, but ddCP42 and ddCP38A contributed a greater proportion of the activity, and ddCP54 and ddCP30 were less active and cannot be seen in Fig. 1(a) (lane 2). Preparations of proteinase B, a 30000- M_r cysteine proteinase purified previously from G cells (North & Whyte, 1984), gave a band at an identical position to ddCP30.

Gelatin/PAGE proved to be a particularly convenient method for detecting extracellular proteinase activity. The growth medium from cultures of G cells contained high levels of ddCP42 and traces of a number of the other intracellular proteinases, including an additional proteinase named ddCP41 detectable in intracellular samples at lower pHs (Fig. 1b). The differences between the intracellular and extracellular patterns indicated that the appearance of proteinases in the medium was not due simply to cell lysis. The quantities of ddCP42 were also too large to be accounted for by leakage from damaged cells.

A previous electrophoretic analysis of the proteinases in bacterially grown myxamoebae and axenically grown myxamoebae did not reveal distinct nutrient-dependent differences (North & Harwood, 1979). The gelatin/PAGE technique, however, showed that the proteinase forms present in cells grown on *K. aerogenes* differed from those of axenic cells (Fig. 1c, lane 5). Three major bands were present in the former, ddCP38B, ddCP43 and ddCP48. ddCP48 was always a major activity; the quantities of ddCP38B and ddCP43 were more variable. The same bands were found whether the cells were grown in association with live bacteria on nutrient agar plates (Fig. 1) or in shaken suspensions of autoclaved bacteria (results not shown). ddCP30 was just detectable in most samples of bacterially grown myxamoebae, but none of the other forms characteristic of the axenically grown myxamoebae was apparent. The proteinases found in bacterially grown cells of strain AX2 were also present in the non-axenic strains NC4 and V12M2 (results not shown).

Fig. 1. Proteinase forms detected by gelatin/PAGE in *D. discoideum*

Samples were analysed by electrophoresis on gelatin-containing gels as described under Materials and methods. All three gels were incubated with 1 mM-DTT at pH 4.0 for (a) 8 h or (b,c) 4 h. (a) Lane 1, vegetative G cell extract; lane 2, vegetative NS cell extract. (b) Lane 1, cell-free medium from log-phase G cell culture; lane 2, cell-free medium from stationary-phase G cell culture. (c) Lane 1, vegetative G cell extract (log phase); lane 2, vegetative G cell extract (stationary phase); lane 3, developmental G cell extract (aggregation, 12 h); lane 4, developmental G cell extract (early culmination, 20 h); lane 5, extract from cells grown on *K. aerogenes*; lane 6, extract from developmental cells (bacterially grown) (aggregation 12 h). Unless otherwise indicated only the segment of the gel containing proteinase bands is shown.

For convenience the distinct proteinase patterns observed in axenically grown and bacterially grown myxamoebae will be referred to as the A- and B-pattern, respectively, and the enzyme forms which comprise the patterns as the A- and B-form proteinases.

Analysis of the proteinases present at the aggregation stage of development showed lower activity, but an unchanged proteinase pattern, for bacterially grown myxamoebae (Fig. 1c, lane 6). For developing axenically grown myxamoebae the proteinase pattern was simpler than in vegetative cells (Fig. 1c, lanes 3 and 4). The developmental changes are discussed in greater detail below.

Properties of the proteinase forms

All of the proteinases were most active over the acid pH range (Fig. 2). None of the A-form enzymes could be detected above pH 5, although the optimum pH varied from one enzyme to another. In axenic cell extracts all the enzymes were dependent on DTT for maximal activity. The B-form enzymes were also active in the acid pH range, but showed a broader pH-dependence; gelatin digestion occurred at pH 6, and low activity was

detectable at pH 8 (results not shown). All three B-form proteinases were maximally active in the presence of DTT, but they were less dependent on DTT than the A-form enzymes.

The A-form enzymes were all severely inhibited by the cysteine proteinase inhibitors antipain, chymostatin, TPCK, TLCK, leupeptin and E64 (Fig. 3). Scans for the latter three agents are not shown as no bands were apparent. Pepstatin reduced the activity of all forms at a high concentration (100 $\mu\text{g/ml}$), but had no effect at 20 $\mu\text{g/ml}$ at which concentration most aspartic proteinases would be inactivated. EDTA, a metallo-proteinase inhibitor, had no significant effect, and PMSF, a serine proteinase inhibitor, had a slight effect, but only with ddCP45. The B-form enzymes displayed some differences from the A-form enzymes and also from one another. All were severely inhibited by E64, leupeptin and TLCK (scans not shown), but chymostatin, antipain and TPCK were far less effective. The three enzymes were affected similarly by these agents. Both EDTA and pepstatin had a differential effect, reducing the activity of ddCP43 and ddCP38B, but not that of ddCP48, whereas PMSF was only slightly inhibitory, most noticeably

Fig. 2. pH-dependence of the myxamoebal proteinase forms

The Figure shows the inverse densitometric scans at 550 nm of gel strips incubated at different pHs. (a) G cell extract. (b) Extract of cells grown on *K. aerogenes*. (i) pH 2.0, (ii,iii) pH 4.0, (iv) pH 5.0, (v) pH 6.0. (i,ii,iv,v) 1 mM-DTT included. Apparent M_r values ($\times 10^{-3}$) are shown.

Fig. 3. Effect of proteinase inhibitors on the myxamoebal proteinase forms

The Figure shows the inverse densitometric scans at 550 nm of gel strips incubated at pH 4.0 with 1 mM-DTT in the presence of inhibitors at the concentrations specified under Materials and methods. (a) G cell extract. (b) Extract of cells grown on *K. aerogenes*. (i) No inhibitor, (ii) PMSF, (iii) EDTA, (iv) pepstatin (a, at 100 $\mu\text{g/ml}$), (v) TPCK, (vi) antipain, (vii) chymostatin. Apparent M_r values ($\times 10^{-3}$) are shown.

towards ddCP43. Thus, while the B-form enzymes had many of the characteristics expected of cysteine proteinases (DTT activation, inhibition by leupeptin and E64), their inhibition by EDTA and pepstatin and the relative resistance to antipain and chymostatin differentiates them from the more typical cysteine proteinases of the A-pattern.

A further distinction between individual A-pattern and B-pattern enzymes was apparent with ddCP38A and ddCP38B. The two enzymes differ in their stability at 50 °C, the latter being considerably more stable than ddCP38A or indeed any of the other cysteine proteinases (North, 1988).

Developmental changes in proteinase patterns

Starvation in phosphate buffer. Myxamoebae starved in buffered suspensions will undergo many of the processes that they normally carry out during the early stages of the developmental phase. Some developmentally regulated changes, including the expression of the CP1

and CP2 genes (Pears & Williams, 1987), can be induced prematurely by addition of cyclic AMP. Starvation of axenically grown cells resulted in a change in proteinase pattern (Fig. 4a). In the absence of cyclic AMP, there was a gradual decrease in intracellular proteinase activity. Most of the A-form proteinases were diminished after 2 h: ddCP54 and ddCP42 were lost completely and ddCP51 and ddCP30 were barely detectable after 8 h, although ddCP45 did remain. One new band became apparent within the first 2 h. It corresponded to an enzyme with an apparent M_r of 48000 which has been named ddCP48D (D for development), but it is not yet known whether it is distinct from the B-form proteinase of similar mobility. Its activity reached a maximum after 6–8 h and then declined. Addition of 1 mM-cyclic AMP after 90 min had little effect on the early changes, but there was a slower decrease in proteinase activity at later stages. Two proteinases were present after 12 h, ddCP48D and a 45000- M_r proteinase which may be ddCP45, although

Fig. 4. Changes in proteinase forms in shaken suspensions of myxamoebae

Cells were incubated in a shaken suspension in 20 mM-sodium phosphate, pH 6.0, and sampled periodically. To one culture (+) 1 mM-cyclic AMP was added after 90 min. The gels were incubated at pH 4.0 with 1 mM-DTT. (a) Cell extracts. (b) Samples of cell-free buffer; lane 1 shows an extract of cells freshly harvested from growth medium. The times of starvation (h) are shown.

the latter declined initially when the cells were starved, and so a second enzyme of similar mobility may have been formed.

During starvation the cells release proteinase activity into the buffer (North, 1982c). The major extracellular proteinase detected by gelatin/PAGE was ddCP42 (Fig. 4b), the enzyme found in growth medium (Fig. 1). Significant quantities of ddCP41 together with traces of ddCP51 and ddCP30 were also present. The addition of cyclic AMP caused a slight reduction in the amount of proteinase released, but had no effect on the forms detected.

Development on filters. Developmental expression in buffered cell suspensions is restricted, and for completion of the developmental phase the cells must be on a solid-air interface. The initial changes in proteinase pattern observed during development of axenically grown myxamoebae on filters were similar to those observed in buffer (Fig. 5a). There was an early loss of some of the major vegetative proteinases (ddCP54 and ddCP51 in particular) and the appearance of a new band, ddCP48D. A proteinase with a mobility corresponding to that of ddCP45 was retained throughout. Additional minor bands were present, including one with an apparent M_r of 43000 which can be seen more clearly in densitometric scans (Fig. 6), but the developmental proteinase bands were not always well resolved. The increased amounts of actin formed during development contributed to the difficulty in resolving the bands. The proteinases in developing cells had properties similar to

Fig. 5. Changes in proteinase forms during development on filters

Axenically grown myxamoebae (G cells) were incubated on filters as described under Materials and methods and sampled periodically. The gels were incubated at pH 4.0 with 1 mM-DTT. (a) Cell extracts. (b) Samples of cell-free PDF (whole gel shown). The times of development (h) are shown.

those of vegetative cells, that is they were dependent on DTT for activity and were inhibited by the same inhibitors, and were therefore considered to be cysteine proteinases.

During development, proteinase activity accumulated in the PDF with which the cells were in contact (Fig. 5b). The major proteinase released was ddCP42, although many of the other intracellular enzymes were also present. The level of extracellular activity reached a peak after 4 h of development and then declined slowly suggesting that autodigestion might have occurred. At the pH of the PDF (6.5) none of the enzymes showed significant activity towards gelatin, although it is known that proteinase B (ddCP30) is optimally active towards synthetic peptide derivatives at pH 6.0 (North & Whyte, 1984). The PDF also contained larger proteinases including, at later stages of development, enzymes which were detected near the top of the gel. The origin of the latter is unknown, but they may represent aggregates of smaller enzymes which were not dissociated by the comparatively mild denaturation conditions employed in the running of the gels.

Fig. 6. Densitometric scans of developmental proteinase bands

The Figure shows the inverse densitometric scans at 550 nm of individual lanes of a gel incubated at pH 4.0 with 1 mM-DTT. The samples were prepared from G cells developing on filters. The time of development in hours is shown. Apparent M_r values ($\times 10^{-3}$) are shown.

DISCUSSION

Although the technique was not initially intended for one type of proteinase, gelatin/PAGE has proved to be particularly suitable for analysing the cysteine proteinases of *D. discoideum*. The multiple forms which appear on gelatin gels are believed to exist within the cells and are not formed as a result of cell extract preparation or electrophoresis. No formation of new proteinase bands can be induced after cell extracts have been prepared (North, 1988). The different forms are of physiological significance, since the proteinase pattern was dependent on the manner in which the cells were grown, and there were differences between the patterns of intracellular and extracellular proteinases. The presence of one major extracellular form, ddCP42, suggests that its targeting differs from that of the other cysteine proteinases. The higher intracellular levels of ddCP42 in NS cells may reflect differences in the rates of secretion by NS and G cells. Perhaps the presence of glucose, like sucrose (Seshadri *et al.*, 1986), stimulates secretion.

The relationship between the proteinases reported here and those described previously has been partially solved. ddCP30 is proteinase B (North & Whyte, 1984), one of four cysteine proteinases identified previously (North &

Harwood, 1979). Proteinase C, which was active on haemoglobin gels at pH 5, may correspond to ddCP42 or ddCP38A, both of which were more active than the other proteinases at this pH (Fig. 2). Proteinase D could be any of ddCP45, ddCP51 or ddCP54, the haemoglobin-gel method having failed to resolve these forms from one another. The lack of resolution of the earlier method would also explain why the pattern observed in axenically grown myxamoebae was not previously distinguishable from that of cells grown on bacteria. Proteinase E, a 58000- M_r aspartic proteinase and the most active enzyme on haemoglobin (North & Harwood, 1979; North & Whyte, 1984), was not normally observed on gelatin gels. Further analyses have suggested that it is probably inactivated by the cysteine proteinases under the conditions used for gelatin/PAGE (M. J. North, D. A. Cotter & K. Franek, unpublished work).

The results have demonstrated that cysteine proteinase activity persists throughout the developmental phase, but that the enzyme forms change. The question of whether any of the developmental forms represents the product of a developmentally regulated CP gene has not been fully resolved. Clearly none of the vegetative enzymes corresponds to one of these products as the genes are not expressed in axenically grown cells (Pears *et al.*, 1985; Presse *et al.*, 1986b). The only new major band to appear during development was ddCP48D, but it was detected within 4 h and its appearance was not affected by the addition of cyclic AMP. In normal development, CP1 and CP2 mRNAs are not present until after 8 h (Pears *et al.*, 1985), but their time of expression in buffered cell suspensions can be affected by adding cyclic AMP (Pears & Williams, 1987). It is interesting that the appearance of ddCP48D was concomitant with the loss of ddCP51. This is very similar to one of the changes which occurs when bacteria are added to axenic cultures (North, 1988). As development progressed there was poorer resolution of the bands in the 35000–50000- M_r zone where the unprocessed CP1 and CP2 products might be expected to appear, but if these products were present then they must have relatively little activity towards gelatin after electrophoresis.

The origin of the different cysteine proteinases must at present remain a matter for speculation. Larger forms of mammalian lysosomal cysteine proteinase found extracellularly (Mort *et al.*, 1983; Troen *et al.*, 1987) and in secretory granules (Docherty *et al.*, 1984) are thought to represent precursor forms which have not been processed completely. For axenically grown myxamoebae of *D. discoideum*, however, the extracellular enzyme ddCP42 was apparently smaller than the predominantly intracellular forms (ddCP45 and ddCP51). Further studies will be necessary to establish whether the enzymes represent products of the same gene at different stages of maturation. On the other hand, there is as yet no evidence to rule out the involvement of multiple vegetative cysteine proteinase genes. It will now be of interest to establish the extent to which the different forms of proteinase revealed by gelatin/PAGE fulfil separate physiological roles and how the structural variations which underlie the differences in electrophoretic mobility relate to functional distinctions.

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