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PURIFICATION AND PROPERTIES OF THE EXTRACELLULAR METALLO-PROTEINASES OF *CHROMOBACTERIUM LIVIDUM* (NCIB 10926)

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Summary

Four extracellular proteolytic enzymes (I—IV) (EC 3.4.22.—) were identified in static cultures of *Chromobacterium lividum* (NCIB 10926) by agar gel electrophoresis and isoelectric focusing. Proteinases I—III were freed of non-enzymic protein by chromatography on TEAE-cellulose and CM-cellulose. The enzyme mixture was then fractionated in a pH gradient by isoelectric focusing. All three enzymes were shown to be heat-labile metallo-enzymes. Optimal activity occurred at pH 5.6 for enzyme I and at pH 6.2 for enzymes II and III. Remazolbrilliant Blue-hide powder was a sensitive substrate for these enzymes. Proteinase I was also shown to degrade haemoglobin and casein effectively, but not myoglobin, ovalbumin or bovine serum albumin. Proteinases I—III exhibited molecular weight values of 75 000, 72 000 and 67 000 by exclusion chromatography and 71 000 and 66 000 by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis for enzyme I and II, respectively. The amino acid compositions of enzymes I and II were somewhat similar. Proteinase I was inhibited by EDTA, 1,2-di(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid or 1,10-phenanthroline and both Ca^{2+} and Co^{2+} were required for maximal activity. Mg^{2+} could substitute for Ca^{2+} or Mn^{2+} for Co^{2+}. The interrelationship of proteinases I—III is discussed.

Introduction

Many gram-negative organisms which grow on the surface of meat at ambient or chill temperatures exhibit extracellular proteolytic activity [1]. One such organism is *Pseudomonas fragi* and a metallo-proteinase (EC 3.4.22.—) was recently purified from broth culture and its properties characterized [2].

Abbreviation: EGTA, 1,2-di(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

The enzyme was active at neutral pH and exhibited a requirement for both Zn^{2+} and Ca^{2+}. During a recent survey carried out in these laboratories a highly proteolytic, achromogenic strain of *Chromobacterium lividum* was identified on meat during standard microbiological testing. The organism was subjected to culture on a preparative scale for an investigation of its extracellular proteinases. Details of the isolation, identification and culture conditions for this organism will be published elsewhere (Dainty, R.H. et al., unpublished).

Little information exists on the proteinases of *Chromobacterium* species although their protein-digesting abilities are normally checked during identification tests for this genus. The mesophilic *Chromobacterium violaceum* rapidly degrades gelatin and casein whereas strains of the psychrophilic *Chr. lividum*, which grow well at 20°C and not at all at 37°C, exhibit only a weak lytic action on casein-agar gels and are generally inactive against gelatin [3]. The *Chromobacterium* proteinases reported in this paper are quite distinct from any other microbial metallo-enzymes that have been studied up to the present time.

Materials and Methods

Materials were obtained from the following commercial sources: CM-cellulose (CM-52) (Whatman); Sephadex G-100 (Pharmacia); TEAE-cellulose (0.80 mequiv./g), whale myoglobin, ovalbumin (grade V) (Sigma); haemoglobin substrate powder (Worthington); bovine serum albumin (Kock-Light); casein (B.D.H.); ampholines, pH 3—10 (LKB); hydrolysed casein (Oxoid). Other chemical reagents were of Analar grade and were dissolved in distilled deionized water. Freeze-dried bacterial cultures were from N.C.I.B., Torry, or from this Institute's collection (MR80, MR82).

Maintenance and growth of bacterial cultures for enzyme production. Chr. lividum (NCIB 10926) was maintained by monthly subculture in cooked meat medium. Cultures were stored at 1°C after 48 h of growth at 20°C. Starter cultures and cultures for enzyme production were grown at 20°C without agitation in 5-l conical flasks containing 2 l of medium. The medium (pH 6.0) contained (g/l) casein hydrolysate (5.0), glucose (1.0), KH_2PO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), $CaCO_3$ (0.05). Starter cultures were inoculated with 0.5% (v/v) of the cooked meat culture and grown for 48 h. These were then used as the inoculum (2.5%, v/v) for the main batches of culture medium.

Batches of 25 flasks were sampled daily for the assay of extracellular proteinase activity. A maximum value was reached at about day 7 and the cells were then harvested by passage at 150—200 ml/min through an MSE continuous centrifuge operated at 16 000 rev./min. The crude enzyme was precipitated from the culture supernatants by the addition of solid $(NH_4)_2SO_4$ to 75% saturation. After stirring at 20°C for 16 h the precipitate was collected on a Buchner funnel by filtration through Whatman glass fibre discs. A supension of the crude enzyme in $(NH_4)_2SO_4$ could be safely stored at 4°C.

Small scale cultures of other strains were similarly grown and the supernatants tested for activity using the casein-agar plate assay (Dainty, R.H. et al., unpublished).

Enzyme assay. Proteolytic activity was determined routinely at 37°C with insoluble Remazolbrilliant Blue-hide powder as substrate using the automated

method of Newman and Etherington [4]. The buffer was 0.2 M sodium acetate, pH 5.5, containing 2 mM $CaCl_2$ and 0.2% (v/v) Triton X-100. One unit of activity is defined as the amount of dye released by 1 μg/ml of pure trypsin when this is assayed at pH 7.8 [4]. Enzyme samples were diluted to below 4 units/ml with 1 mM $CaCl_2$ in water prior to assay. Other potential protein substrates were examined at a final concentration of 1% (w/v) in 0.1 M sodium acetate buffer, pH 5.5, containing 1 mM $CaCl_2$. The reaction was terminated by the addition of an equal volume of 10% (w/v) trichloroacetic acid and after 10 min filtered through Whatman No. 42 filter paper. The tyrosine content of the filtrate was estimated with the Folin-Ciocalteu reagent [5].

Agar gel electrophoresis. Culture supernatants and preparations of the enzymes at various stages during purification were analysed by electrophoresis in agar gels. These were prepared from 1% (w/v) agar in 25 mM sodium barbitone buffer, pH 8.2. Zones of activity were subsequently detected after immersing the plates for 1 h in 0.2 M sodium acetate buffer, pH 5.5, containing 1 mM $CaCl_2$ and 0.2% (w/v) globin. Full details of this method have been published elsewhere [6].

Protein determination. The method of Lowry et al. [7] was used with bovine serum albumin as standard.

Enzyme purification. All operations were carried out at 4°C. The precipitate of crude enzyme obtained from 50 l of static culture broth was resuspended and dialysed against several changes of 20 mM Tris · HCl, pH 7.5, containing 1 mM $CaCl_2$. The solution was clarified by centrifuging and applied to a 4.5 × 30 cm column of TEAE-cellulose equilibrated to the same buffer. Buffer was pumped through the column at 11.7 ml/min to elute the unadsorbed enzyme which was then precipitated by the addition of solid $(NH_4)_2SO_4$ to 85% saturation. The suspension was stirred overnight and then collected by centrifuging. The pellet was resuspended and dialysed against several changes of 10 mM sodium acetate buffer, pH 5.5, containing 1 mM $CaCl_2$. Portions of the enzyme were then chromatographed on a 2.2 × 46 cm column of CM-cellulose with a 1000 ml linear gradient of 10—40 mM sodium acetate buffer, pH 5.5, containing 1 mM $CaCl_2$. The fractions in the single peak of activity were bulked, concentrated by pressure dialysis and then dialysed against 1 mM $CaCl_2$. Final purification was achieved by preparative isoelectric focusing.

Isoelectric focusing. Ampholines, pH 3—10, were incorporated into a sucrose gradient to 1% (w/v). The 110 ml column was used for the analysis of crude or partially purified enzyme preparations. For some preparations the final purification was achieved with the 440 ml column. The focusing time was generally 40 h with the column cooled to 4°C. The initial potential was 250 V rising to 800 V for the final 24 h. Fractions of 1 ml were collected from the small column and 4 ml from the large column. Immediately after the pH had been determined one drop of 0.1 M $CaCl_2$ was added to each fraction. The purified enzymes were dialysed against 1 mM $CaCl_2$ to remove the Ampholines and then frozen to −25°C.

Disc gel electrophoresis. The method of Reisfeld et al. [8] was used for cationic migration with a 7.5% (w/v) separating gel.

Molecular weight determination. A Sephadex G-100 column was equilibrated with 0.1 M sodium acetate buffer, pH 5.5, containing 0.1 M NaCl and

1 mM $CaCl_2$. A calibration curve was prepared using proteins of known molecular weight and the molecular weight values of the purified enzymes were calculated using Andrews method [9]. Molecular weight values were also determined by the use of sodium dodecyl sulphate-polyacrylamide gel electrophoresis with the slab technique of Sykes and Bailey [10].

Metal ion analysis. Pure enzyme protein was dialysed exhaustively against distilled, deionized water and then freeze-dried. Portions of 100—250 mg were ashed in a muffle furnace at 500°C until a constant weight was reached. The ash was taken up in 2 M HCl and individual metals determined by atomic absorption spectroscopy using a Perkin-Elmer model 305B spectroscope.

Amino acid analysis. Portions of pure freeze-dried, salt-free enzyme were hydrolysed in sealed ampoules at 110°C for 24 h in 5.7 M HCl. The amino acid compositions were determined with a Beckman Model 120C analyser using the two column system.

Effect of metal ions on enzymic activity. A stock solution of enzyme was prepared in 0.2 M sodium acetate buffer, pH 5.5. For some experiments the enzyme was fully activated by the inclusion of 2 mM $CaCl_2$. Alternatively, diffusible ions were removed by dialysis against several changes of water. To one volume of diluted enzyme was added one volume of pH 5.5 buffer containing the metal ion or metal-chelating agent and the mixture held at 0°C for 20 min. In some experiments a further two volumes of buffer containing metal ion were added to the chelator-enzyme mixtures and the enzyme held for an additional 20 min at 0°C. Activity was then determined in the standard manner but with $CaCl_2$ omitted from the assay buffer. Appropriate controls were prepared and these were assayed in the standard system. The control activity was 1.5—2 units/ml in these experiments.

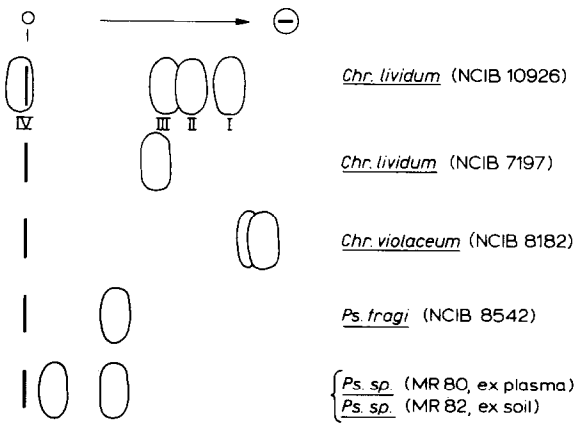

Fig. 1. Agar gel electrophoresis at pH 8.2 of bacterial culture supernatants. Zones of proteolytic activity were located by first immersing the gel plates for 1 h in 0.2% (w/v) globin in 0.2 M sodium acetate buffer, pH 5.5, containing 2 mM $CaCl_2$ and then incubating at 37°C. The undegraded protein was fixed and stained with Ponceau S. The locations of proteinases I—IV are as indicated.

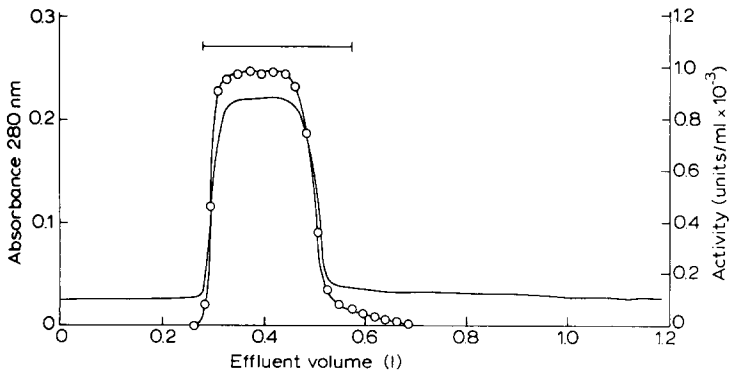

Fig. 2. Chromatography on TEAE-cellulose. The $(NH_4)_2SO_4$ precipitate from a 50 l batch of culture broth supernatant was run through a 4.5 X 30 cm column of TEAE-cellulose equilibrated to 20 mM Tris · HCl, pH 7.5/1 mM $CaCl_2$ to remove pigment and contaminating protein. The bulk of the proteinase activity emerged in the breakthrough peak. ———, protein; o———o, proteinase activity.

Results

Identification of enzymes in the culture supernatants. A preliminary examination of the extracellular proteinase activity indicated that this was maximal near pH 5.5 and agar gel electrophoretograms were therefore buffered to this pH. Four zones of activity could be demonstrated by this technique. Three zones were cathodic and one weaker zone remained near the origin. These proteolytic zones, were numbered I—IV in order of decreasing positive charge (Fig. 1) and zone 1 was generally found to be the strongest. Electrophoresis of proteolytically active cultures from other species of *Chr. lividum* and *Pseudomonas* revealed a single zone of activity which moved near to or behind enzyme

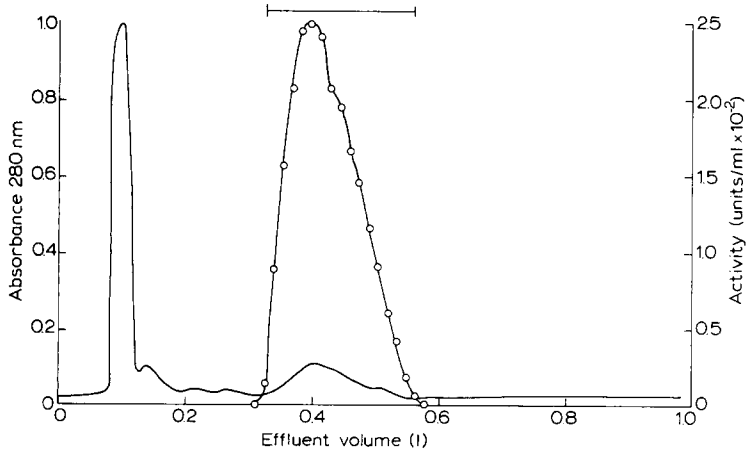

Fig. 3. Chromatography on CM-cellulose Chromatography of $32 \cdot 10^{-3}$ units of crude enzyme from the TEAE-cellulose column on a 2.2 X 46 cm column of CM-cellulose equilibrated to 10 mM sodium acetate buffer, pH 5.5, containing 1 mM $CaCl_2$. Activity was eluted by a linear 1000 ml gradient to 40 mM sodium acetate buffer, pH 5.5. ———, protein; o———o, proteinase activity.

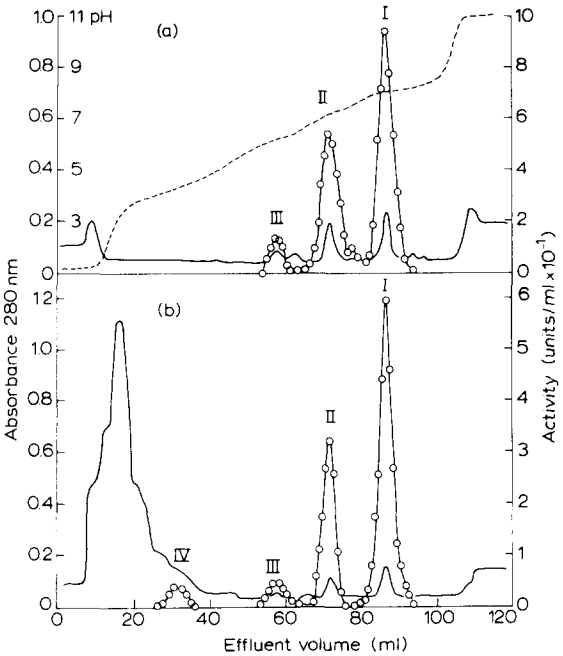

Fig. 4. Isoelectric focusing of *Chr. lividum* (NCIB 10926) proteinases in a pH 3—10 Ampholine gradient. (a) 1050 units of mixed enzyme after chromatography on TEAE-cellulose and CM-cellulose. (b) 550 units of crude enzyme from a $(NH_4)_2SO_4$ precipitate of culture supernatant. ———, protein; ○———○, proteinase activity; - - - - - -, pH.

III. A strain of *Chr. violaceum* exhibited a double zone of activity which was more cathodic than zone I.

Enzyme purification. The $(NH_4)_2SO_4$ precipitate of crude enzyme was always intensely pigmented. This dark-brown pigment together with much of the contaminating protein was removed from the enzyme mixture by adsorption onto TEAE-cellulose (Fig. 2). The enzyme activity in the unadsorbed protein was examined by agar gel electrophoresis and found to contain the proteinases I, II and III. The minor proteinase IV was bound to the column exchanger under these conditions. The three enzymes were weakly adsorbed to CM-cellulose and eluted together in a single asymmetric peak of activity (Fig. 3). Normally each preparation was divided into several smaller portions for chromatography at this stage of the purification.

Isoelectric focusing was used as the final purification step. The enzyme mixture from the CM-cellulose column was completely resolved in a pH 3—10 gradient (Fig. 4). Each enzyme was identified by agar gel electrophoresis and the following pI values were determined using the 110 ml column; enzyme I (8.05), enzyme II (7.15), enzyme III (6.15). Analysis of the original crude $(NH_4)_2SO_4$ precipitate resolved these three enzymes and also separated out proteinase IV (pI 4.35). The purified enzymes were examined by disc-gel electrophoresis and a single band of protein was found for each.

The complete data for the purification of proteinases I, II and III from a standard batch of 50 l of culture medium are given in Table I. Generally the

TABLE I

PURIFICATION OF *CHR. LIVIDUM* PROTEINASES

Step	Protein (mg)	Activity (units X 10^{-3})	Specific activity (units X 10^{-3}/mg)	Yield (%)	Purification (fold)
Culture supernatant (47.7 l)	—	205	—	100	—
75% Saturated $(NH_4)_2SO_4$ precipitate	2209	201	0.091	98	(1)
TEAE-cellulose chromatography	650	182	0.28	89	3.1
CM-cellulose chromatography	34	113	3.3	55	24.2
Isoelectric focusing (I	12	35	2.8	17)	
(II	15	27	1.8	13) 33	—
(III	—	6	—	3)	

activity in these static cultures reached a maximum of about 4 units/ml. The final specific activities for proteinases I and II were $2.8 \cdot 10^3$ and $1.8 \cdot 10^3$ units/mg, respectively, and the overall yields were 17% for enzyme I and 13% for enzyme II.

Activity and stability of the isolated enzymes. A pH vs. activity curve was constructed for each enzyme with a discontinuous buffer system consisting of sodium acetate and Tris/maleate buffers (Fig. 5). Proteinase I was most active near pH 5.6 and proteinases II and III were maximally active near pH 6.2. Temperature activity curves were constructed and the optimal activity for each enzyme was located at 45°C (Fig. 6).

Each enzyme was exposed to various pH values to determine its stability range (Fig. 7). At 20°C the three enzymes readily lost activity above pH 8.5 and proteinase I was much less stable at 30°C. The enzymes were more stable

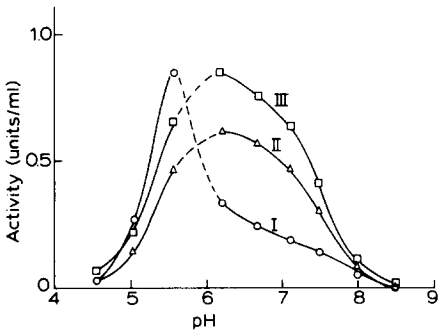

Fig. 5. pH vs. activity curves. pH vs. activity curves were constructed for proteinases I—III using the automated assay but with the working buffers prepared from 0.2 M sodium acetate, pH 4.5—5.6 and 0.2 M Tris/maleate, pH 6.2—8.5. 2 mM $CaCl_2$ was included as activator.

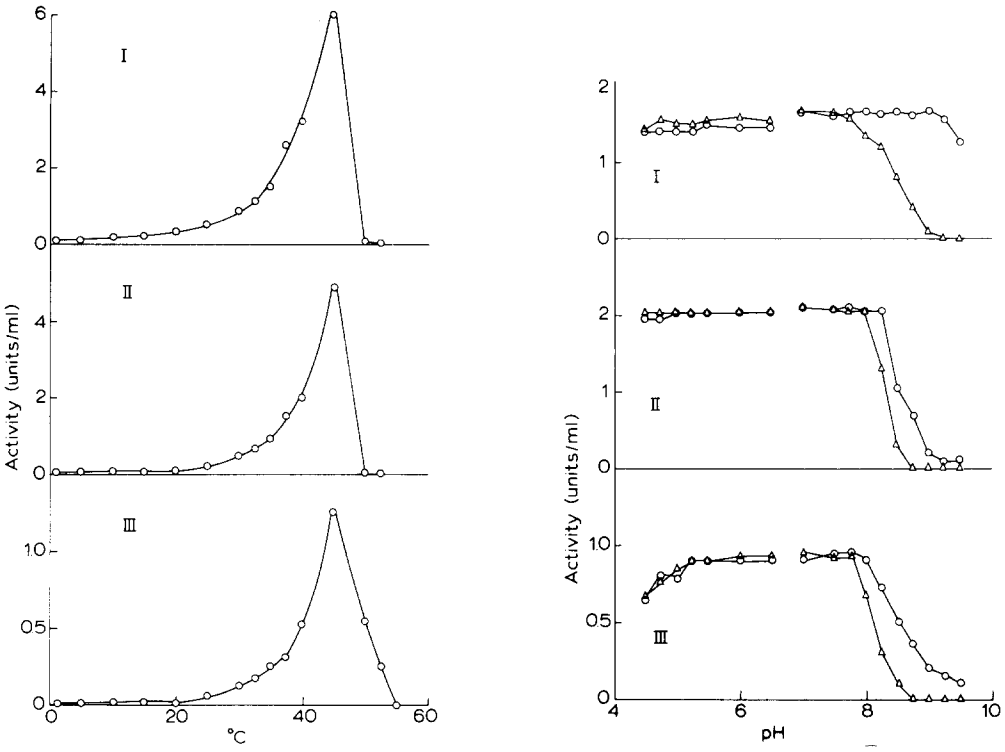

Fig. 6. Temperature vs. activity curves. Proteinases I—III were assayed in the automated system at different bath temperatures to determine the temperature optimum for each.

Fig. 7. pH-stability range of proteinases I—III. Enzymes were placed in cold (0°C) 0.2 M buffers prepared from either acetate or Tris and containing 2 mM $CaCl_2$. The enzyme solutions were heated for 5 min at either 20°C (○———○) or 30°C (△———△) and then immediately cooled to 0°C. The enzyme was diluted 10-fold with pH 5.5 buffer and then assayed for residual activity at pH 5.5

below neutrality although proteinase III lost some activity below pH 5. Temperature stability curves revealed that the enzymes were completely inactivated at 55°C in the presence of 2 mM Ca^{2+} (Fig. 8). The addition of 0.5% (w/v) gelatin extended the resistance of the enzymes to denaturation by 1—5°C.

Molecular weight determination. By gel permeation chromatography the molecular weights were calculated to be 75 000 for proteinase I, 72 000 for proteinase II and about 65 000 for proteinase III. The calculated values obtained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis for proteinases I and II were 71 000 and 66 000, respectively.

Action of proteinase I on different proteins. The time-course for the release of tyrosine from different proteins is shown in Fig. 9. Haemoglobin and casein were rapidly digested whereas myoglobin, bovine serum albumin and ovalbumin were only slowly degraded.

Amino acid composition. Proteinases I and II exhibited somewhat similar amino acid compositions (Table II). The sulphur-containing amino acids, methionine and cysteine, were absent from both proteins. Proteinase I contained more acidic residues than proteinase II but as proteinase I exhibited a

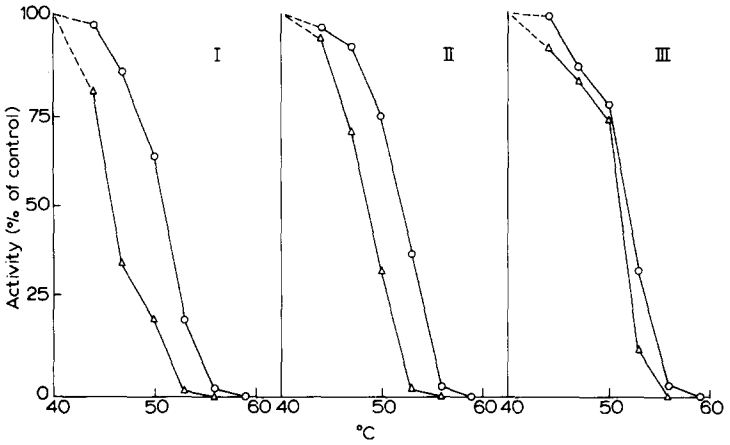

Fig. 8. Temperature inactivation of Proteinases I—III. Enzymes were dissolved in cold (0°C) 0.2 M sodium acetate buffer, pH 5.5, containing 2 mM $CaCl_2$ and with (○———○) or without (△———△) 0.5% (w/v) gelatin. The solutions were heated for 5 min at the specified temperatures, immediately cooled to 0°C and then assayed for residual activity at 37°C.

higher pI value then many of these residues must have been amidated.

Metal ion content. Only the major enzyme, proteinase I, was obtained in sufficient quantity for a detailed metal analysis. The data given in Table III show the presence of substantial amounts of Ca^{2+}, which may in part result from incomplete dialysis of the purified enzyme. After Ca^{2+} only Zn^{2+} was lo-

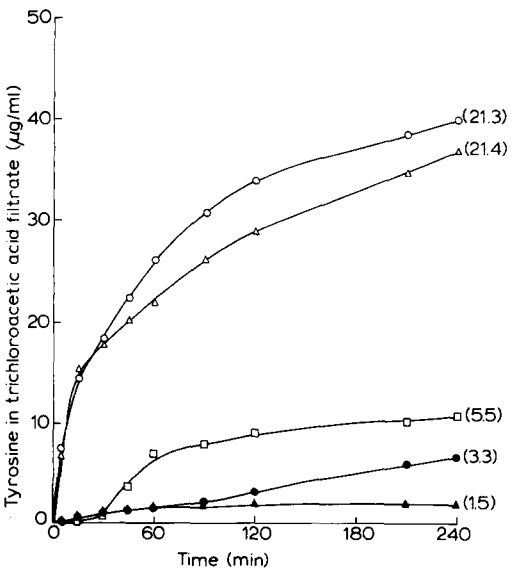

Fig. 9. The digestion of different proteins at 37°C by proteinase I. Each reaction mixture consisted of 10 mg/ml protein and 1 unit/ml enzyme in 0.2 M sodium acetate buffer, pH 5.5, containing 2 mM $CaCl_2$. The amount of Tyrosine soluble in 5% (w/v) trichloroacetic acid was determined and at 240 min the percentage of the total substrate Tyrosine in solution is indicated. ○———○, haemoglobin; △———△, casein; □———□, myoglobin; ●———●, bovine serum albumin; ▲———▲, ovalbumin.

TABLE II

AMINO ACID COMPOSITION OF PROTEINASES I AND II

Tryptophan was not determined.

Amino acid	Proteinase (residues/1000 residues)	
	I	II
Asp	122	96
Thr	139	106
Ser	126	97
Glu	50	53
Pro	34	66
Gly	137	202
Ala	136	141
Cys	0	0
Val	49	54
Met	0	0
Ile	39	30
Leu	15	32
Tyr	38	30
Phe	49	22
Lys	24	38
His	32	9
Arg	11	25

cated in substantial quantity but this was less than the stoichiometric ratio of 1 : 1. A small amount of Mg^{2+} was also identified.

Studies on metal ion activation. The greater part of these studies dealt with proteinase I. A large number of different metals were tested for their ability to reactivate enzyme which had been dialysed against water. Table IV shows that several group IA metals, particularly Ca^{2+}, could activate proteinase I. Ca^{2+} also activated proteinase II. The transition metals of group VIII, Co^{2+} and Ni^{2+} were partially effective. Zn^{2+}, which was found to be present in proteinase I was inhibitory to all three enzymes. Proteinase III appeared to remain fully active following dialysis.

Table V records the data obtained when proteinase I was treated with metal-chelating agents and then saturated with specific metal ions. EDTA inhibited

TABLE III

METAL ION COMPOSITION OF PROTEINASE I

Ashed samples were redissolved in 2M HCl and analysed by atomic absorption spectroscopy. An average molecular weight value of 73 000 was used in calculating the ratios.

Metal ion	gatom/mol
Ca^{2+}	1.60—1.80
Co^{2+}	<0.01
Fe^{2+}	0.05
Mg^{2+}	0.18
Mn^{2+}	<0.003
Ni^{2+}	0.03
Pb^{2+}	<0.003
Zn^{2+}	0.29—0.59

TABLE IV

EFFECT OF METAL IONS ON THE ACTIVITY OF *CHR. LIVIDUM* PROTEINASES

Dialysed enzyme was pretreated with an equal volume of 20 mM metal ion in the pH 5.5 buffer at 0°C for 20 min. The control was pretreated similarly with buffer only.

Metal ion	Activity (percent of control) of proteinase:		
	I	II	III
None	14	23	100
Ba^{2+}	67		
Bi^{3+}	55		
Ca^{2+}	102	100	100
Co^{2+}	81	77	100
Cu^{2+}	0		
Fe^{2+}	16		
Fe^{3+}	0		
Mg^{2+}	94		
Mn^{2+}	88		
Ni^{2+}	8		
Pb^{2+}	36		
Zn^{2+}	5	3	4
Zn^{2+} (40 mM)	0		

the enzyme completely and activity was not restored by Ca^{2+}. Ca^{2+}, however, did reverse inhibition by the Ca^{2+}-specific-chelating agent EGTA. Reactivation of EDTA-pretreated enzyme was most successful with a combination of Ca^{2+} and Co^{2+}.

The transition metals were specifically removed by 1,10-phenanthroline and this compound completely inhibited enzyme I. The addition of excess Co^{2+} was effective in restoring activity and this effect was enhanced to above 100%

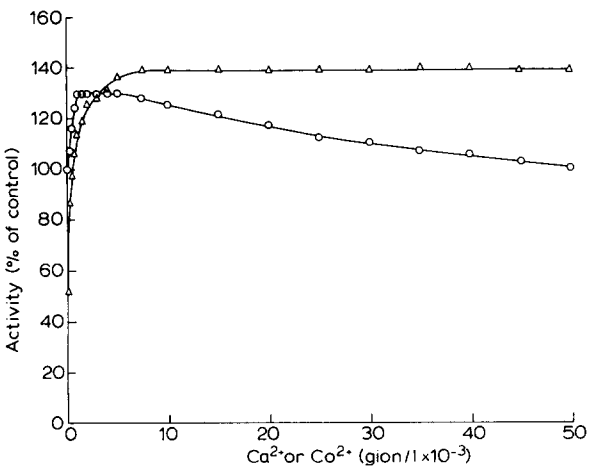

Fig. 10. The combined effect of Ca^{2+} and Co^{2+} on the activity of proteinase I. The enzyme was pretreated with both metal ions at the concentrations indicated. o———o, 1 mM Ca^{2+}, Co^{2+} varied; △———△, 2 mM Co^{2+}, Ca^{2+} varied.

TABLE V

EFFECT OF METAL IONS ON PROTEINASE I FROM *CHR. LIVIDUM* PRETREATED WITH METAL-CHELATING AGENTS

One volume of Ca^{2+}-activated enzyme was mixed with one volume of 2 mM metal chelator in the pH 5.5 buffer and allowed to stand at 0° for 20 min. Two volumes of 20 mM metal in buffer were then added and the mixture reactivated for 20 min. Metals were tested singly or in pairs.

Chelating Agent	Metal ions		Activity (percent of control)
	a	b	
EDTA	—	—	0
	Ca^{2+}	—	0
	Ca^{2+}	Co^{2+}	66
	Ca^{2+}	Mg^{2+}	0
	Ca^{2+}	Mn^{2+}	39
	Co^{2+}	—	20
	Co^{2+}	Mg^{2+}	30
	Co^{2+}	Mn^{2+}	24
	Mg^{2+}	—	0
	Mg^{2+}	Mn^{2+}	15
	Mn^{2+}	—	13
EGTA	—	—	9
	Ca^{2+}	—	91
1,10-phenanthroline	—	—	0
	Ca^{2+}	—	0
	Ca^{2+} (50 mM) *	—	0
	Ca^{2+}	Co^{2+}	110
	Ca^{2+}	Mg^{2+}	0
	Ca^{2+}	Mn^{2+}	74
	Co^{2+}	—	76
	Co^{2+}	Mg^{2+}	100
	Co^{2+}	Mn^{2+}	76
	Mg^{2+}	—	0
	Mg^{2+}	Mn^{2+}	40
	Mn^{2+}	—	35

* Ca^{2+} added to enzyme before addition of metal-chelating agent.

by Ca^{2+}. Good reactivation was also obtained by substituting Mg^{2+} for Ca^{2+} or Mn^{2+} for Co^{2+}.

Proteinases II and III were also totally inhibited by pretreatment with EDTA. The effect of 1,10-phenanthroline or EGTA on these two enzymes was not tested.

The combined effect of Co^{2+} and Ca^{2+} on the activity of proteinase I was tested further. Portions of dialysed enzyme were reactivated by the addition of either Ca^{2+} or Co^{2+} and the minimum optimum concentration of each metal determined. This was below 1 mM for Ca^{2+} and 2 mM for Co^{2+}. Further portions of enzyme were pretreated with either Ca^{2+} or Co^{2+} at these concentrations and then the second metal added at various concentrations (Fig. 10). In this experiment it was shown that activity could be enhanced to 130—140% of the control value. Ca^{2+} in excess, did not affect the enzyme adversely, but Co^{2+} was less effective above 6 mM.

Discussion

Static culture broths of *Chr. lividum* (NCIB 10926) contained at least four different enzymes, proteinases I—IV as determined by electrophoretic analysis in agar gel and by isoelectric focusing. The electrophoretograms revealed that these enzymes were different from the *Pseudomonas* and *Chr. violaceum* proteinases. Another strain of *Chr. lividum* (NCIB 7197) exhibited only a single proteolytic zone of activity nearly coincident with proteinase III. Proteinase IV was not investigated further but proteinases I—III were purified and each enzyme was shown to be inhibited by EDTA. These metallo-proteinases exhibited maximal activity below neutrality whereas most other microbial metallo-enzymes are mainly active at pH 7—8 or in the alkaline range [11]. The molecular size was also twice that of other enzymes in this group [12]. Proteinases I—III were also unusual in being very heat labile, despite each having a high temperature optimum for activity of 45°C.

Remazolbrilliant Blue-hide powder proved to be an exceptionally sensitive substrate for these proteinases as compared to the other enzymes tested previously [4]. Haemoglobin and casein were also shown to be susceptible substrates for proteinase I.

Proteinases I—III were fractionated and demonstrated to be very similar enzymes in terms of activity, stability and physical properties. By amino acid analysis proteinases I and II were found to be similar and both lacked any sulphur-containing amino acid. Many other microbial metallo-proteinases contain no cysteine and have little or no methionine [11].

Proteinase I was obtained in sufficient quantity for a detailed examination of its enzymic properties. The Ca^{2+}-specific-chelating agent EGTA inhibited proteinase I which clearly established a specific requirement for this metal ion. Ca^{2+} has been identified in many microbial enzymes where it appears to have an important role as a stabilizer to the enzyme's conformation, particularly for the thermo-stable proteinases [13]. Such a stabilizing role for Ca^{2+} in proteinase I was not established. However, solutions of the enzyme were found to be unstable in storage unless Ca^{2+} was present.

Proteinase I was completely inhibited by 1,10-phenanthroline in the presence of excess Ca^{2+}. This type of inhibitory response has been observed for many microbial proteinases, including collagenase and a second metal ion, Zn^{2+} has been identified as the activator in each enzyme [11,14]. Chemical analysis of these enzymes has confirmed the presence of both Ca^{2+} and Zn^{2+}. Pure proteinase I was also shown to contain these two metal ions but on testing the ions for their ability to reactivate the enzyme, Zn^{2+} was shown to be very inhibitory. This anomalous situation cannot be satisfactorily explained without further experimentation. Co^{2+} was identified as an important activator but was loosely bound, if present at all, in the enzyme when secreted since this ion existed at a trace level only in the ashed samples. *Pseudomonas aeruginosa* elastase-like proteinase is similarly activated by Co^{2+}, but not Zn^{2+}, in the presence of Ca^{2+} [15]. It has been found that Co^{2+} and Mn^{2+} can also effectively replace Zn^{2+} in a number of microbial proteinases [11].

Proteinases I—III were completely separated by isoelectric focusing and their specific activity values were subsequently determined using a buffer containing

added Ca^{2+} only. These measured values were lower than for the complete sample mixture before fractionation and the decrease can now be interpreted from the dual metal ion requirement for proteinase I. The technique of isoelectric focusing eliminates all but the most tightly bound of metal ions and therefore in order to restore full activity both Ca^{2+} and Co^{2+} are required. Further investigation is necessary to establish which metal ions are normally bound to the enzyme and to elucidate the activation mechanism.

Proteinases I—III remained unresolved by conventional ion-exchange techniques, but could be separated only by their electrophoretic differences. These differences were not artefacts brought about by the incorporation of different ions since proteinase II was shown to be a slightly smaller protein and the amino acid analyses were not identical. When some old stored preparations of partially purified enzyme were fractionated by isoelectric focusing it was observed that proteinase II was now frequently recovered as the major enzyme together with an enhanced quantity of proteinase III. Proteinases II and III are not normally observed in the active medium of fast growing aerated cultures, but proteinase I is always present (Dainty, R.H. et al., unpublished). However, proteinases II and III have been detected on re-examination of fermenter-produced proteinase I after storage for 1 year. A possible explanation of these changes in the enzyme patterns is that proteinases II and III are derived from proteinase I by limited autolysis. However, without further investigation of the enzyme structures the interrelationship remains obscure.

This investigation was undertaken using the medium from static culture broths. A detailed examination of the conditions for enzyme production in high yield from fermenter culture are to be described elsewhere (Dainty, R.H. et al., unpublished).

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