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ISOLATION BY AFFINITY CHROMATOGRAPHY OF NEUTRAL PROTEINASE FROM *CLOSTRIDIUM HISTOLYTICUM*

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SUMMARY

1. Neutral proteinase was isolated from concentrate of the culture filtrate of *Clostridium histolyticum* by affinity chromatography.

2. Four chromatographic agents were prepared by substituting the ligands caproyl-Gly–Leu, caproyl-Leu-NH_2, Gly–Leu and Leu-NH_2 into Sepharose 4B but of these only Sepharoyl-Cap-Gly–Leu proved effective. This agent separated the neutral proteinase from the bulk of the protein in the crude starting material with high efficiency.

3. The neutral proteinase is inhibited by metal chelating agents but not by DFP.

INTRODUCTION

Characterization of the neutral proteinase of *Clostridium histolyticum* has been hampered by difficulty in isolating this enzyme[1]. Since the neutral proteinases have a highly characteristic substrate specificity[2] and some are inhibited by partial substrates[3] it seemed to us that it might be possible to develop an improved method of purification based on affinity chromatography using partial substrates as ligands[4].

Cl. histolyticum neutral proteinase readily hydrolyses carbobenzoxyglycyl leucine amide (Z-Gly–Leu-NH_2) at the Gly–Leu bond[2] and we found that, under comparable conditions, it did not perceptibly hydrolyse Z-Gly–Leu or Leu-NH_2. In fact, these partial substrates inhibited digestion of casein by the enzyme. We therefore prepared chromatographic adsorbents in which Gly–Leu or Leu-NH_2 were attached either directly or through the chain extender[6], ε-aminocaproic acid, to a solid support, Sepharose 4B[5], which did not impede the movement of the enzyme through a chromatographic column.

Sepharoyl-Gly–Leu did not retard the enzyme and Sepharoyl-Leu-NH_2,

Abbreviation: Cap, caproyl.

although binding the enzyme, did not, on elution, effect separation from other protein also bound. The insertion of the chain extender did not significantly improve the chromatographic behaviour of the Leu-NH_2 analogue but greatly improved that of the Gly–Leu derivative. As shown in Fig. 1, this agent very efficiently separated the *Cl. histolyticum* neutral proteinase from the bulk of the accompanying protein; the collagenase[7] and the thiol-activated proteinase[8], present in the starting material were eluted separately from the neutral proteinase.

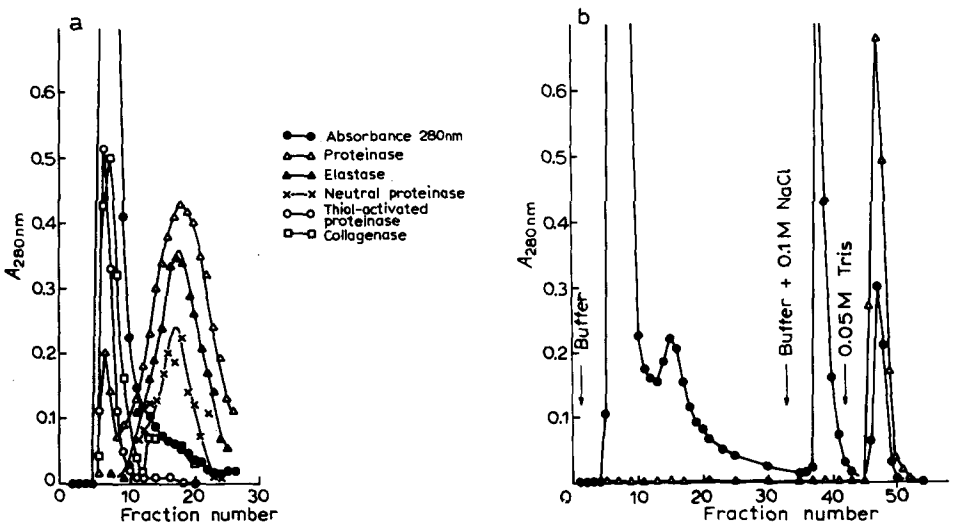

Fig. 1. Chromatography of neutral proteinase on Sepharoyl-Cap-Gly–Leu. Columns (200 mm × 9 mm) of the substituted Sepharose were prepared in 1 mM HEPES buffer adjusted to pH 7.2 with Tris and containing 1 mM $CaCl_2$. The columns were loaded with 20 mg of the partly purified enzyme. Elution was carried out with (a) the above buffer only or (b) the above buffer followed by, where indicated, (i) the above buffer made 0.1 M in NaCl and (ii) 0.05 M Tris, 1 mM $CaCl_2$, pH 10. Fractions eluted with pH 10 buffer were promptly adjusted to pH 7.4 with concentrated HEPES solution. Flow rate, 0.5 ml/min. Fraction volume 4 ml.

Some variation was found in the chromatographic behaviour of separately prepared batches of Sepharoyl-Cap-Gly–Leu. Thus in some cases, illustrated in Fig. 1a, the enzyme eluted from the column separately from the unwanted protein on continued elution with buffer (see legend to Fig. 1). In other cases, shown in Fig. 1b, proteinase together with some unwanted protein, remained bound to the column. In these latter cases, elution with 0.1 M NaCl in buffer removed much of the remaining unwanted protein and the enzyme could then be eluted with 0.05 M Tris, pH 10. Attempts to elute the proteinase with buffer containing Z-Gly–Leu-NH_2, Z-Gly–Leu, Gly–Leu or Gly–Leu-NH_2 were unsuccessful. Recoveries and activities of proteinase are presented in Table I; it can be seen that purifications ranged from 12 to 20-fold which can be compared with an overall purification of 12-fold in previous work[1] using a combination of gel filtration and ion exchange chromatography. A notable feature of the purification by affinity chromatography, not evident in Fig. 1, was the removal of a yellow protein that could not be separated from the proteinase in the above-mentioned study[1]. The homogeneity of the enzyme purified by affinity

chromatography was not examined by gel electrophoresis so that its purity is not known. However, in view of the efficiency of the purification by affinity chromatography it seems likely that this method of fractionation, in combination with other methods, would give enzyme pure enough for full characterization.

TABLE I

PURIFICATION OF PROTEINASE

	Protein concentration (mg/ml)	*Absorbance (276 nm)*	*Proteolytic activity**	*Proteolytic activity per unit absorbance at 276 nm*	*Recovery of proteolytic activity (%)*
Crude enzyme	1	—	0.400	—	
ECTEOLA-cellulose-treated enzyme (starting material)	1	1.23	0.470	0.38	Approx. 100
Peak enzyme (Fig. 1a)	—	0.047	0.480	10.2	Approx. 100
Purified by affinity chromatography (Fig. 1b)	—	0.120	0.580	4.92	Approx. 50

* Proteolytic activity expressed as absorbance at 276 nm found for casein digestion as set out in Experimental.

We were unable to ascertain the cause of the variability in the chromatographic behaviour of the Sepharoyl-Cap-Gly–Leu. It did not seem to be related to variation in the degree of substitution of the ligand in the Sepharose as batches behaving as described showed degrees of substitution with leucine falling within the range 2–4 μmoles of leucine/ml of bed volume. It was found also that the absorbent became progressively less effective with use, and could be used only three or four times. This was associated with a loss of leucine from the substituted Sepharose, suggesting that slow digestion of the chromatographic substrate occurred during chromatography. Perhaps the use of the D-analogues would have overcome this[6].

It was found in the present and previous work[1] that the activity against casein could be directly related to the activity against Bz-Gly–Leu-NH_2 and elastin, regardless of the method of fractionation. This observation, and the finding that other neutral proteinases show elastolytic activity[9], suggested that the bulk, if not all, of the caseinolytic and elastolytic activity shown by our preparation was due to a neutral proteinase. In agreement with this observation it was found that the activity of the crude enzyme against casein, Bz-Gly–Leu-NH_2 and elastin was substantially inhibited ($> 80\%$) by EDTA and 1,10-phenanthroline, whereas diisopropyl fluorophosphate (DFP) inhibited (about 10%) activity against elastin and did not inhibit activity against casein. Since this enzyme is inhibited by 1,10-phenanthroline, like other neutral proteinases, it is probably a zinc metallo-enzyme[3] stabilized by calcium ions[10]. Although there may have been present some other elastolytic enzyme inhibited by DFP[11] we were not able to detect a second elastolytic activity either in this study or a previous work[1] using other methods of fractionation.

EXPERIMENTAL

Crude enzyme was prepared from culture filtrate of *Cl. histolyticum* NCTC 503, essentially according to MacLennan *et al.*[12]. Pigment and some impurity of high molecular weight were removed from the crude enzyme by batchwise treatment with ECTEOLA-cellulose (10 g/g of freeze–dried crude enzyme) suspended in buffer containing 0.1 M NaCl. The enzyme solution was dialysed against 10^{-4} M $CaCl_2$ and freeze–dried, thus providing the starting material for fractionation.

In preparation of chromatographic agents, the ligands, ε-aminocaproylglycylleucine and ε-aminocaproylleucine amide were prepared *via* their carbobenzoxy derivatives from *p*-nitrophenyl-ε-carbobenzoxyamino caproate (m.p. 70 °C). While the protected derivatives were crystalline solids (ε-carbobenzoxyaminocaproylleucine amide, white needles from acetone, m.p. 128 °C, and ε-carbobenzoxyaminocaproylglycylleucine, white needles from ethanol–ether, m.p. 106.5 °C) which gave satisfactory elementary analyses, the free peptides could not be induced to crystallize. However, they showed a single ninhydrin-positive spot on thin-layer chromatography in the system silica gel–butanol–acetic acid–water (80:20:20, by vol.) and gave the appropriate amino acid analyses after acid hydrolysis.

Fractions were tested for activity against casein using a modification of the method according to Hagihara *et al.*[13]. Enzyme solution (0.1 ml) was incubated at 35 °C for 5 min with 0.5 ml of 2% casein (nach Hammarsten, Merck, Darmstadt) in 15 mM Tris containing 1 mM $CaCl_2$. The digestion was stopped by addition of 4 ml of 0.1 M trichloroacetic acid buffered with 0.2 M sodium acetate and 0.3 M acetic acid. Precipitation was completed by incubation at 35 °C for 30 min, the precipitate removed by filtration (Whatman filter paper No. 3) and absorbance of the filtrate measured at 276 nm. Neutral proteinase was assayed by incubating 0.2 ml of enzyme solution with 0.3 ml of 10 mM Bz-Gly–Leu-NH_2 in 1 mM Tris–HCl buffer containing 1 mM $CaCl_2$ for 1 h at 35 °C and estimating the degree of hydrolysis by the ninhydrin method of Rosen[14]. Thiol-activated proteinase was assayed using Bz-arginyl-naphthyl-NH_2 (with cysteine)[15], collagenase using collagen[16] and elastolytic activity using elastin[13] as substrates. All digestion mixtures were adjusted to pH 7.4 (ref. 17) with Tris and contained 1 mM $CaCl_2$.

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REFERENCES

1 McQuade, A. B. and Crewther, W. G. (1968) *Biochim. Biophys. Acta* 167, 619–620
2 Morihara, K. and Tsuzuki, H. (1971) *Arch. Biochem. Biophys.* 146, 291–296
3 Feder, J. and Schuck, J. M. (1970) *Biochemistry* 9, 2784–2791
4 Cuatrecasas, P. and Anfinsen, C. B. (1971) *Annu. Rev. Biochem.* 40, 259–278
5 Axen, R., Porath, J. and Ernback, S. (1967) *Nature* 214, 1302–1304
6 Cuatrecasas, P., Wilchek, M. and Anfinsen, C. B. (1968) *Proc. Natl. Acad. Sci. U.S.* 61, 636–643
7 Kono, T. (1968) *Biochemistry* 7, 1106–1114
8 Mitchell, W. M. and Harrington, W. F. (1968) *J. Biol. Chem.* 243, 4683–4692
9 Morihara, K., Tsuzuki, H. and Oka, T. (1968) *Arch. Biochem. Biophys.* 123, 572–588

10 Monier, R., Litwack, G., Somlo, M. and LaBouesse, B. (1955) *Biochim. Biophys. Acta* 18, 71–82
11 Takahashi, S., Seifter, S. and Binder, M. (1970) *Biochem. Biophys. Res. Commun.* 39, 1058–1064
12 MacLennan, J. D., Mandl, I. and Howes, E. L. (1953) *J. Clin. Invest.* 32, 1317–1322
13 Hagihara, B., Matsubara, H., Nakai, M. and Okunuki, K. (1958) *J. Biochem. Tokyo* 45, 185–194
14 Rosen, H. (1957) *Arch. Biochem. Biophys.* 67, 10–15
15 Riedel, A. and Wunsch, E. (1959) *Z. Physiol. Chem.* 316, 61–70
16 Seifter, S., Gallop, P. M., Klein, L. and Meilman, E. (1959) *J. Biol. Chem.* 234, 285–293
17 Mandl, I., MacLennan, J. D. and Howes, E. L. (1953) *J. Clin. Invest.* 32, 1323–1329