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THE IDENTITY OF THE ELASTASE-ASSOCIATED ACIDIC ENDOPEPTIDASE AND CHYMOTRYPSIN C FROM PORCINE PANCREAS

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Summary

The preparations of chymotrypsin C (EC 3.4.21.2) and an acidic endopeptidase from porcine pancreas have been repeated using published procedures. The acidic endopeptidase showed lower activity than chymotrypsin C in all comparative experiments, but it was possible to precipitate a fraction from the acidic endopeptidase preparation which contained all the protein and was identical with chymotrypsin C. It is concluded that the acidic endopeptidase is identical with chymotrypsin C but it is contaminated by an inert non-protein material to which it is firmly bound. The formation of a precipitate, at low ionic strength, from mixtures of chymotrypsin C and elastase (EC 3.4.21.11) is independent of the availability of the active site of either enzyme.

Introduction

The published preparations [1—4] of elastase (EC 3.4.21.11) from pig pancreas all depend upon the initial isolation of a euglobulin precipitate which forms when extracts of pancreas are dialysed against distilled water. This precipitate contains elastase and an acidic endopeptidase [2] together with other enzymes [4] that disappear if the precipitate is purified by dissolution in buffer and dialysis. Baumstark [5] has isolated the acidic endopeptidase and investigated its properties in detail. In particular he compared the acidic endopeptidase with the data reported [6] for another acidic enzyme, chymotrypsin C (EC 3.4.21.2), and concluded that, despite similarities, the enzymes were different. The aim of the present work is to isolate these enzymes by the methods used previously and to compare them directly.

Materials and Methods

Enzymes. Elastase was prepared from Trypsin 1-300 (Nutritional Biochemicals) by the method of Shotton [3]. Chymotrypsin C was prepared by the

method of Folk and Schirmer [6] except that pancreatin (B.D.H. Chemicals Ltd.) rather than fresh pancreas acetone powder was used as a source. The acidic endopeptidase was isolated from Trypsin 1-300 by the method of Baumstark [5] but all samples obtained in this work were contaminated by a decomposition product. The precipitated endopeptidase was obtained by dissolving the endopeptidase (20 mg) in 10 ml of 5 mM Tris/acetate buffer, pH 8, and bringing the solution to 65% saturation with $(NH_4)_2SO_4$. The precipitate which formed was collected and washed by centrifuging, dialysed against distilled water and lyophilised giving 15 mg of precipitated endopeptidase.

Active site-inhibited enzymes. Inhibited elastase and chymotrypsin C were prepared by incubating the enzymes with 4 mM *N*-acetyl-di-L-alanylalanine chloromethyl ketone [7] and 0.3 mM *N*-tosyl-L-leucine chloromethyl ketone, respectively, for 90 min at 25°C, pH 7. After this treatment both enzymes showed less than 5% of their initial activity. The solutions of inhibited enzyme were dialysed overnight against distilled water.

Kinetic measurements on enzymes. These ester hydrolyses were followed by a Radiometer pH stat apparatus consisting of a pH meter PHM26, titrator control TTT11, an automatic burette and a recorder. All such measurements were at 25°C in presence of 1 mM Tris to stabilise the instrument, the ionic strength was brought to 0.05 by addition of KCl. An aliquot of enzyme solution was added to the substrate solution in the pH stat and recording begun. Because the curve produced on the pH stat is influenced by instrumental factors during the first few seconds of reaction, the trace slope at zero time was determined by backward extrapolation of a least-squares fit of the later points to a cubic equation. The kinetic constants k_{cat} and K_m were determined by the method of Wilkinson [8]. Assays of elastase were made at pH 8 using 4 mM *N*-acetyl-di-L-alanylalanine methyl ester and assays of chymotrypsin C and endopeptidase were made at pH 8 using 1 mM *N*-benzoyl-L-tyrosine methyl ester.

The assay of elastase by its action on orcein-elastin (Sigma) was made in pH 8 0.07 M carbonate buffer containing 0.04 M NaCl. Each assay mixture contained 20 mg orcein-elastin, elastase solution and buffer to 4 ml. This was incubated at 37°C for an appropriate time, centrifuged, and the absorbance determined at 590 nm against a blank. Standard curves were prepared using known concentrations of enzyme.

Amino acid analysis. Samples of the protein were dissolved in 3 ml 50% HCl and hydrolysed at 110°C for 24 h in sealed tubes. The samples were lyophilised and analysed on a Locarte amino acid analyser.

Gel electrophoresis. A Shandon analytical electrophoresis apparatus was used with 0.5 cm diameter gel tubes.

Interaction between elastase and acidic enzymes. Solutions of elastase and the acidic enzyme were prepared in distilled water. The reaction mixtures were prepared by taking a fixed volume of the elastase solution and adding it to varying volumes of the solution of the acidic enzyme. The total volume was brought to 3 ml and the mixtures kept at 0°C for 30 min. The tubes were centrifuged ($20000 \times g$, 10 min) and the supernatant decanted. The precipitate was dissolved in pH 8 5 mM Tris/acetate buffer containing 0.1 M KCl. The supernatants and the solutions of the precipitates were then analysed for the

two enzymes. In the cases where one of the enzymes was inhibited only the active enzyme was assayed.

Results

Isolation of acidic endopeptidase. The results obtained when the euglobulin precipitate was subjected to chromatography at pH 8.9 on DEAE-cellulose are illustrated in Fig. 1a and are identical with those found on the previous isolation of this enzyme [5]. The breakthrough peak contained 60% elastase from its activity. Rechromatography of the endopeptidase fraction, after dialysis and lyophilisation, still gave a breakthrough peak which showed neither elastase nor endopeptidase activity (Fig. 1b). This peak was not observed previously. Dialysis and lyophilisation of the active fraction from this second chromatography gave the sample of endopeptidase used in this work.

Stability of endopeptidase. When the endopeptidase was subjected to chromatography on DEAE-cellulose at pH 8.9 an inert breakthrough peak was observed, corresponding to 25% of the total protein present on the basis of its

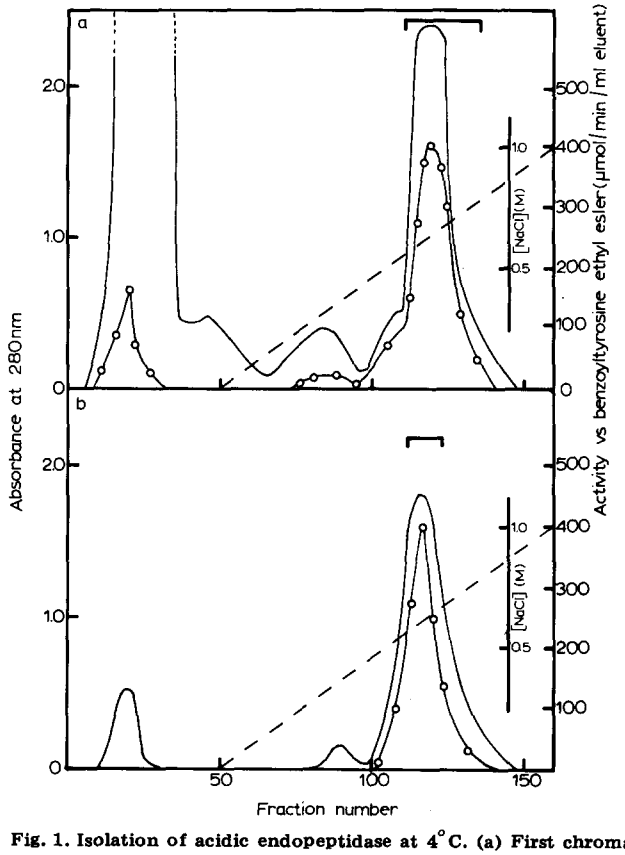

Fig. 1. Isolation of acidic endopeptidase at 4°C. (a) First chromatography of 400 mg elastase euglobulin in a 2.5 × 25 cm DEAE-cellulose column equilibrated with 0.07 M carbonate buffer, pH 8.9. Endopeptidase eluted by a NaCl gradient as shown. Fraction volume, 10 ml. Flow rate, 100 ml/h. (b) Second chromatography of the above fractions after dialysis under identical conditions. ———, absorbance; o———o, activity towards benzoyltyrosine ethyl ester; — — —, NaCl gradient.

ultraviolet absorbance. When the endopeptidase solution was stored for 40 h at 4°C, pH 8.9, before chromatography the breakthrough corresponded to 63% of the total protein. The protein in this peak was therefore formed from the endopeptidase under the chromatographic conditions.

Isolation of precipitated endopeptidase. This material was obtained in 75% yield by precipitation from the endopeptidase.

Isolation of chymotrypsin C. Although pancreatin was used instead of fresh pancreas powder the results obtained (Fig. 2) for the isolation of chymotrypsin C by chromatography on DEAE-cellulose at pH 8.0 were identical with those of Folk and Schirmer [6]. Rechromatography of the indicated fraction at pH 6.0 gave the protein as a single peak. It was precipitated with $(NH_4)_2SO_4$ (65% saturation), dissolved in distilled water, dialysed and lyophilised.

Ultraviolet spectroscopy. The extinction coefficients for the enzymes isolated in this work and previously are given in Table I. The extinction coefficient of the endopeptidase compares well with that found by Baumstark [5], and those of the precipitated endopeptidase and chymotrypsin C are very similar to those previously reported for chymotrypsin C [6].

Amino acid analyses. These are shown in Table II together with those previously reported for chymotrypsin C. Given that these are the results from single runs on an analyser there would appear to be strong similarities between all the enzymes. The major differences between the values reported here and those reported previously lie in the values for valine but our own values agree within the experimental error. Because of the hydrolysis conditions used results for cysteine and tryptophan were unreliable and are omitted.

Gel electrophoresis. The samples isolated in this work all showed precisely the same electrophoresis pattern, a major band together with a more mobile

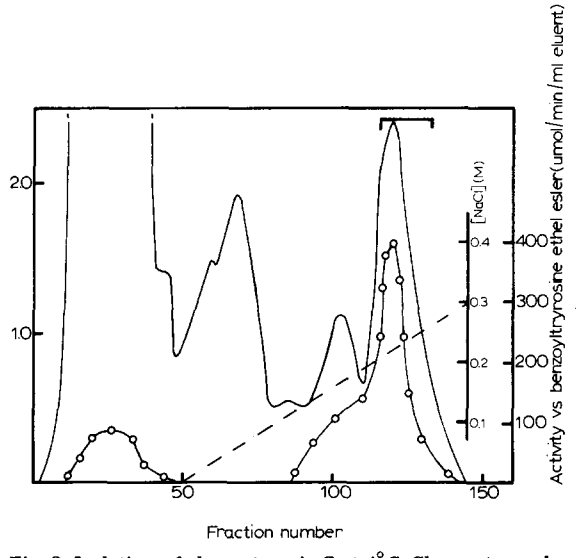

Fig. 2. Isolation of chymotrypsin C at 4°C. Chromatography of extract of pancreatin [6] on a 2.5 × 25 cm DEAE-cellulose column equilibrated with 5 mM Tris/acetate buffer, pH 8.0. Chymotrypsin C eluted by a NaCl gradient as shown. Fraction size, 7 ml; flow rate, 100 ml/h. ———, absorbance; o———o, activity towards benzoyltyrosine ethyl ester; — — —, NaCl gradient.

TABLE I

EXTINCTION COEFFICIENTS OF ENZYMES

Enzyme	($E^{1\%}_{1cm}$, 280nm)	Buffer
Endopeptidase *	16.6	pH 10, glycine, I = 0.1 M
Endopeptidase	16	pH 8.9, 0.07 M carbonate
Precipitated endopeptidase	22	pH 8.9, 0.07 M carbonate
Chymotrypsin C	22	pH 8.9, 0.07 M carbonate
Chymotrypsin C **	25	Not specified

* Ref. 5.
** Ref. 6.

minor contaminant. A typical separation is shown in Fig. 3. A mixture of the endopeptidase, precipitated endopeptidase and chymotrypsin C preparations gave an identical pattern to that of the individual components.

Electrophoresis in the presence of sodium dodecyl sulphate was used to determine the molecular weight of the enzymes. Again all three preparations gave identical results (Fig. 4). There was a major band at an apparent molecular weight of 30000 ± 3000 and two minor components at apparent molecular weights of 15000 and 16500. It was not clear whether these latter peaks were caused by the more mobile impurity seen on simple gels, or whether they represent components of the enzyme analogous to those formed from α-chymotrypsin under similar conditions.

The value found here for the molecular weights is in good agreement with a value of 28000—29000 for chymotrypsin C reported by Gratecos and Desnuelle

TABLE II

AMINO ACID COMPOSITION OF ENZYME (OMITTING TRYPTOPHAN AND CYSTEINE)

Residue	Number of residues per mol of *			
	Endopeptidase	Precipitated endopeptidase	Chymotrypsin C	Chymotrypsin C **
Ala	14	14	14	13—14
Arg	8	8	8	8
Asx	26	27	26	25
Glx	25	26	25	25
Gly	28	29	28	27
His	7	7	7	6
Ile	13	12	13	14
Leu	22	22	23	21
Lys	7	8	8	7
Met ***	1	1	1	1
Phe	4	4	4	4
Pro	14	16	16	15
Ser ***	24	24	24	22
Thr	18	17	17	16
Tyr ***	7	6	6	6
Val	20	19	19	23

* Normalised on number of residues found by Gratecos and Desnuelle [10].
** Ref. 10.
*** Corrected for breakdown during hydrolysis.

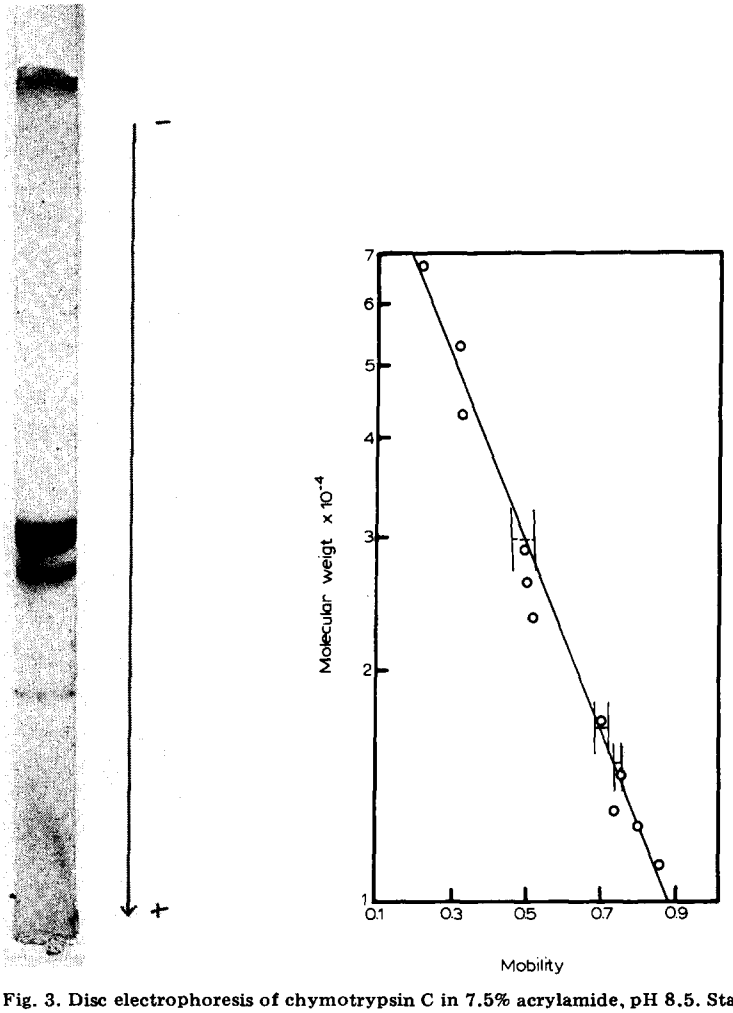

Fig. 3. Disc electrophoresis of chymotrypsin C in 7.5% acrylamide, pH 8.5. Stained with amido black.

Fig. 4. Determination of molecular weight by sodium dodecyl sulphate gel electrophoresis [9]. Gels at pH 7 contained 10% acrylamide, 0.27% bisacrylamide, 0.1% sodium dodecyl sulphate. Denatured sample (approx. 20 μg) applied to gel in 20% sucrose solution containing bromophenol blue. Current passed at 3—4 mA/tube until dye had traversed 80% of gel length. Stained with Coomassie brilliant blue. Standard proteins used were bovine serum albumin, glutamate dehydrogenase, ovalbumin, carbonic anhydrase, myoglobin, lysozyme, cytochrome c, elastase, trypsin and chymotrypsin.

[10] and in fair agreement with a value of 35300 for the acidic endopeptidase determined by Baumstark [5].

Kinetic specifity. The kinetic constants are given in Table III. The K_m values were identical, within the experimental error, for all the enzymes isolated here and for the reported values [11] for chymotrypsin C. The k_{cat} values were also very similar for the precipitated endopeptidase and chymotrypsin C although those for endopeptidase were lower. This last observation would be expected because of the contamination of endopeptidase by inert material which was observed on chromatography. Higher values of k_{cat} for chymotrypsin C were

TABLE III

KINETIC PARAMETERS FOR HYDROLYSIS OF SYNTHETIC ESTERS

pH 8.0, 1 mM Tris, 0.05 M KCl, 25°C. 3% methanol for acetyltyrosine and benzoylleucine esters, 6% methanol for benzoyltyrosine ester. Assumed molecular weight, 28500. Units: k_{cat}, s^{-1}; K_m, mM.

	Endopeptidase		Precipitated endopeptidase		Chymotrypsin C		Chymotrypsin C *	
	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m
Acetyltyrosine ethyl ester	60	17	100	18	92	13	170	20
Benzoyltyrosine ethyl ester	28	1.4	50	1.3	50	1.5	80	2.0
Benzoylleucine ethyl ester	15	0.4	20	0.4	15	0.32	28	0.4
Ac Ala_3OMe **	3	1	1.5	1	3	0.3	—	—

* Ref. 11, pH 7.9, 50 mM Tris, 0.1 M NaCl, 25° C, methanol as cited above.
** Elastase substrate.

reported previously but in a medium containing a higher concentration of buffer.

Assay of elastase. The assay of elastase based on the measurement of dye release on the hydrolysis of dyed elastin has been observed [12] to show an

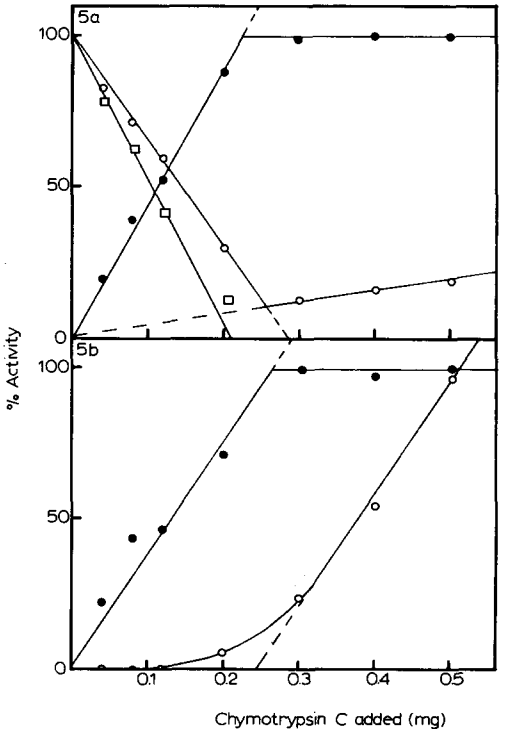

Fig. 5. Interaction of elastase with chymotrypsin C. 0.341 mg elastase, X mg chymotrypsin C in 3 ml water. Kept 30 min at 0° C, centrifuged. (a) Precipitation followed by measuring elastase activity. □——□, assay of elastase in supernatant with orcein-elastin; ○——○, assay of elastase in supernatant and ●——●, assay of elastase in precipitate with *N*-acetyl-di-L-alanylalanine methyl ester. All activities as percentage of elastase activity initially present. (b) Precipitation followed by assaying chymotrypsin C activity in supernatant ○——○ and in precipitate ●——●. Activity as percentage activity in final precipitate.

induction period which increases with decreasing elastase concentration. A rate assay based on the esterase activity of elastase against *N*-acetyl-di-L-alanylalanine methyl ester was therefore used in this work because this assay is linear over the full range of elastase concentrations.

Independence of assays. The addition of elastase to assay solutions of endopeptidase had no effect on the observed hydrolysis rate of benzoyltyrosine ethyl ester. The addition of acidic enzymes to assay solutions of elastase gave a slight increase in the rate of hydrolysis of *N*-acetyl-di-L-alanylalanine methyl ester which was exactly accounted for by the observed activity of the acidic enzymes towards this substrate.

Interaction of elastase and acidic enzymes at zero ionic strength. The results from the experiments with chymotrypsin C are shown in Fig. 5. The results for the precipitated endopeptidase, and for the interactions of active elastase with inhibited chymotrypsin C and of inhibited elastase with active chymotrypsin C were identical. The equivalence points in all these cases corresponded to 1.28 (± 0.2) mg elastase to 1 mg acidic enzyme and was the same, where both activities could be measured, whichever enzyme activity was used to determine it. However, if elastase was assayed using orcein elastin an equivalence point of 1.6 mg elastase to 1 mg acidic enzyme was found. The solubility of the euglobulin precipitate would appear to be very small since for the first 50% of the equivalence amount no activity of chymotrypsin C was observable in the supernatant (Fig. 5b).

The endopeptidase gave a much lower equivalence point of 0.65 (± 0.1) mg elastase to 1 mg endopeptidase, but the appearance of the assay curves was otherwise similar to those for chymotrypsin C.

A precipitate was observed when inhibited elastase and inhibited chymotrypsin C were mixed but it was not possible to quantify the interaction.

Discussion

The chymotrypsin C samples isolated in this work would appear to be identical with those isolated previously [6,10] in all properties tested. We attribute the lower value of k_{cat} found here to the lower buffer concentration used, we were unable to obtain reproducible results on our pH stat with the higher concentrations used earlier (Table III).

The preparation of acidic endopeptidase followed that of Baumstark [5] except for the presence of 10—20% inert protein in our material. In spite of the presence of this protein the properties of the enzyme were similar to those reported by Baumstark [5]. When samples of the enzyme were stored in solution at pH 8.9, 4°C for 40 h over 50% of the active enzyme was transformed into inactive protein. Since the chromatographic purification involved keeping the enzyme under these conditions for more than 10 h it was impossible to eliminate all the inert protein without altering the method.

The precipitated endopeptidase was prepared from the endopeptidase in order to duplicate the final stage of the chymotrypsin C preparation. A comparison of the 75% yield obtained with the differences in extinction coefficient indicates that all the ultraviolet-absorbing material in the endopeptidase sample was precipitated. Since the ratios of amino acid compositions in the two enzyme

samples are identical this implies that the material removed was not a peptide since such a peptide would have to contain ultraviolet-absorbing residues. In addition no protein material, other than residual endopeptidase could be detected in the supernatant remaining after the precipitation. The precipitated endopeptidase behaved identically with chymotrypsin C in all our experiments and we therefore conclude that it is identical with chymotrypsin C.

Chymotrypsin C and the precipitated endopeptidase each formed a euglobulin precipitate with elastase with an equivalence point of 1.6 molecules of elastase per molecule of acidic enzyme. Baumstark [5] found a 4 : 1 ratio using a dyed elastin assay. The use of this assay would overestimate the ratio, because of the induction period, but only by about 20%. He also showed, by dialysis experiments, that the interaction was strongly pH dependent. Since it is impossible to control pH at zero ionic strength we believe that most of the difference between our results and his lies in a difference in pH at which the association experiments were done caused by differences in the previous history of the elastase used. This work shows that it is possible to form the euglobulin precipitate when the active sites of both enzymes are blocked by inhibitors and that, if only one of the enzyme active sites is blocked, the stoichiometry of the precipitate is identical with that when both sites are free. This observation lends support to the concept of a euglobulin precipitate whose composition reflects the most favourable electrostatic interaction at a particular pH rather than any specific binding of one enzyme by another. The specificity of the endopeptidase towards precipitation with elastase rather than trypsin, shown by Baumstark [5], would then arise through better packing in the precipitate with elastase.

The precipitate from the endopeptidase reaction with elastase had a much lower stoichiometry than the chymotrypsin C precipitate. If the enzyme concentration was measured by its activity rather than its weight then a final ratio of 1.5 : 1 was obtained. It is concluded that the acidic endopeptidase of Baumstark [5] is chymotrypsin C contaminated by a material which does not absorb in the ultraviolet. A comparison of his molecular weight (35600) with that determined [10] for chymotrypsin C (28200) suggests that this contaminant, perhaps a polysaccharide, may form a tight complex with the enzyme.

All the acidic enzymes isolated in the work showed slight esterase activity towards *N*-acetyl-di-L-alanylalanine methyl ester which was not precipitated in the euglobulin in precipitation experiments. The enzyme responsible for this activity probably also causes the minor band observed on gel electrophoresis. It was not possible either to isolate it or to remove it from the chymotrypsin C samples.

In conclusion we have shown that the active material in the acidic endopeptidase of Baumstark is identical with chymotrypsin C. In this work the acidic endopeptidase was contaminated by two inert materials, one a decomposition or association product of the enzyme, the other non-proteinaceous. Work is continuing to determine the identity of the latter material.

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References

1 Lewis, U.J., Williams, D.E. and Brink, N.G. (1956) J. Biol. Chem. 222, 705—720
2 Lewis, U.J., Williams, D.E. and Brink, N.G. (1959) J. Biol. Chem. 234, 2304—2307
3 Shotton, D.M. (1970) Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 19, pp.113—140, Academic Press, New York
4 Loeven, W.A. (1963) Acta Physiol. Pharmacol. Neerl. 12, 57—76
5 Baumstark, J.S. (1970) Biochim. Biophys. Acta 220, 534—551
6 Folk, J.E. and Schirmer, E.W. (1965) J. Biol. Chem. 240, 181—192
7 Thomson, A. and Denniss, I.S. (1973) Eur. J. Biochem. 38, 1—5
8 Wilkinson, G.N. (1961) Biochem. J. 80, 324—332
9 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
10 Gratecos, D. and Desnuelle, P. (1971) Biochem. Biophys. Res. Commun. 42, 857—864
11 Keil-Dlouha, V., Puigserver, A., Marie, A. and Keil, B. (1972) Biochim. Biophys. Acta 276, 531—535
12 Naughton, M.A. and Sanger, F. (1961) Biochem. J. 78, 156—163
13 Schwert, G.W. and Takenaka, Y. (1955) Biochim. Biophys. Acta 16, 570—575