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The Specificity of Purified Porcine Pancreatic Elastase

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An electrophoretically homogeneous elastase preparation free from tryptic and chymotryptic activities was obtained by chromatography on DEAE-Sephadex and CM-cellulose. This preparation exhibits a narrower specificity towards peptide bonds than that observed by Naughton & Sanger (1961). With oxidized insulin B chain as substrate, the fastest breaks occur between alanine-14 and leucine-15 and between valine-18 and cysteic acid-19. The bond between glycine-23 and phenylalanine-24 is also efficiently hydrolysed. Other bonds hydrolysed are that between valine-12 and glutamic acid-13 and that between serine-9 and histidine-10. Oxidized insulin A chain is hydrolysed only at one of two points, between alanine-8 and serine-9 or between serine-12 and leucine-13, and the rate of hydrolysis is very low.

Naughton & Sanger (1961) reported that elastase purified by CM-cellulose column chromatography splits peptide bonds adjacent to neutral amino acids: they used oxidized A and B chains of insulin as substrates. Later studies revealed that their method of purification of elastase does not yield a homogeneous preparation (Gertler & Hofmann, 1967). Methods of obtaining pure elastase by batch separation were described by Baumstark, Bardawil, Sbarra & Hayes (1963), who used DEAE-cellulose, and by Ling & Anwar (1966), who used DEAE-Sephadex in combination with CM-cellulose chromatography. Similar methods were also used by Smillie & Hartley (1966). In view of the heterogeneity of elastase used by Naughton & Sanger (1961) in their specificity studies, it was decided to reinvestigate the specificity of elastase with a pure preparation. Our observations showed considerable differences from those reported by Naughton & Sanger (1961), and are presented in this paper.

MATERIALS AND METHODS

Materials. Twice-crystallized porcine elastase, prepared by the method of Lewis, Williams & Brink (1956), was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Oxidized A and B chains of insulin were obtained from Mann Research Laboratories Inc., New York, N.Y., U.S.A. The A chain (lot no. S3680) contained about 20% of B chain and was purified by high-voltage paper electrophoresis in 2M-pyridine–acetate buffer, pH 3·5, at 40v/cm. for 2hr. DEAE-Sephadex A-50 and Sephadex G-25 were the products of Pharmacia, Uppsala, Sweden, and CM-cellulose was obtained from H. Reeve Angel Inc., Clifton, N.J., U.S.A.

Elastase was assayed by the colorimetric method of Naughton & Sanger (1961) by measuring the dye released from Congo Red-dyed elastin.

Isolation of elastolytic products of insulin A and B chains. Oxidized A or B chain (5μmoles in 5·0ml. of 0·05M-$(NH_4)_2CO_3$ buffer, pH 8·8) was digested with 25μg. of elastase for 15min. or 24hr. The reaction was stopped by lowering the pH to 3·0 with 6M-HCl and the reaction mixture dried in a rotary evaporator under reduced pressure. Primary separation of the peptides was achieved by high-voltage paper electrophoresis on Whatman 3MM or 3HR filter paper in 2M-pyridine–acetate buffer, pH 3·5, at 40v/cm. for 2hr. The separated bands were located by staining guide-strips with ninhydrin, eluted with water and further purified by repeating the electrophoresis separately under the above conditions. If necessary the bands were refractionated by electrophoresis at pH 9·1 with 2% (w/v) $(NH_4)_2CO_3$ buffer for 90min. at 25v/cm.

Amino acid analysis. The peptides were hydrolysed with 6M-HCl in evacuated sealed tubes for 22hr. at 110° and analysed for amino acids by the method of Spackman, Stein & Moore (1958) in a Beckman–Spinco amino acid analyser or by the method of Piez & Morris (1960) in a Technicon amino acid analyser.

Calculation of yields. All yields reported were calculated from the analysis of the purified peptides.

Identification of N-terminal residues. *N*-terminal residues were identified by the cyanate method of Stark & Smyth (1963).

Purification of elastase. A 100mg. portion of twice-crystallized porcine elastase was dissolved in water by raising the pH to about 10·0 (10·0–10·3) with 0·1M-NaOH and titrated back to pH 8·7 with 4% (w/v) boric acid. After being adjusted to 0·01M-Na^+ concentration, the solution (40ml.) was applied to a column (40cm. × 2·5cm.) of DEAE-Sephadex A-50 equilibrated with 0·01M-sodium borate buffer, pH 8·7. The column was eluted first with 500ml. of 0·01M-sodium borate buffer, pH 8·7. Elastase appeared with the front in a total volume of about 70ml. (fraction *A*) followed by a non-proteolytic component (fraction *B*).

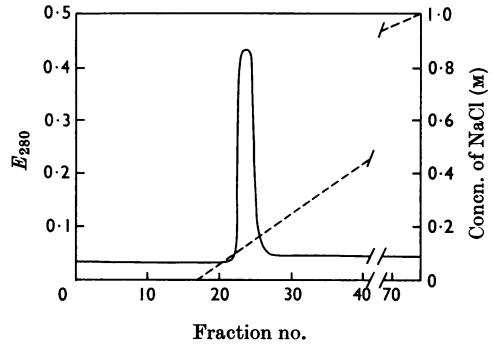

Fig. 1. CM-cellulose chromatography of elastase. Fraction *A*, containing elastase, from DEAE-Sephadex A-50 column (72·5ml.) was adjusted to 0·02M-Na^+ with sodium acetate, the pH was brought to 5·0 with acetic acid, and the fraction was applied to a CM-cellulose column (22cm. × 0·9cm.) that had been equilibrated with 0·02M-sodium acetate buffer, pH5·0. The column was washed with 250ml. of 0·02M-sodium acetate buffer, pH5·0. The column was then developed with a linear gradient of 0–1·0M-NaCl in a total volume of 800ml. of 0·02M-sodium acetate buffer, pH5·0. Fractions (14·5ml.) were collected and the E_{280} was recorded with an Isco u.v. scanner. ——, E_{280}; ----, concn. of NaCl.

Subsequently the column was eluted with 0·1M-NaCl–0·01M-sodium borate buffer, pH8·7, and then with 0·2M-NaCl–sodium borate buffer, pH8·7. Two protein peaks were eluted, of which one was strongly proteolytic and the other had carboxypeptidase A activity.

CM-cellulose chromatography. Fraction *A* from the DEAE-Sephadex A-50 column was adjusted to 0·02M-Na^+ concentration, brought to pH5·0 with acetic acid and chromatographed on a CM-cellulose column with a linear NaCl gradient as described in Fig. 1. A single protein peak having elastase activity was eluted in fractions 23–25. These fractions were pooled and used in the experiments described above after desalting on a Sephadex G-25 column and freeze-drying. About 30% of the original crystalline enzyme could be recovered as elastase by this procedure. The preparation thus obtained was homogeneous on starch-gel electrophoresis in urea–formate buffer (Smithies, 1959) and on polyacrylamide-gel electrophoresis at pH 9·3 (Reisfeld, Lewis & Williams, 1962). It yielded a single *N*-terminal amino acid, namely valine (Brown, Kauffman & Hartley, 1967), and had an amino acid composition similar to that reported by Gertler & Hofmann (1967) (Table 1). The preparation had no detectable activity towards *N*-acetyl-L-tyrosine ethyl ester and benzoyl-L-arginine ethyl ester, showing that it was free from chymotryptic as well as tryptic activities. The proteolytic activity, as measured by the casein-digestion method (Kunitz, 1947), was 2·98units/mg. of freeze-dried powder [the reported value for the Gertler & Hofmann (1967) preparation is 2·75units].

Table 1. *Amino acid composition of elastase*

The protein samples were hydrolysed in 5·7M-HCl (glass-distilled, constant-boiling) in sealed evacuated tubes for 24, 48 and 72hr. at 110°. The amino acid analysis was performed with a Beckman–Spinco amino acid analyser. To compare our values with those reported by Brown *et al.* (1967) and Gertler & Hofmann (1967) for their purified elastase preparations, values are given in molar ratios, assuming 16 alanine residues/molecule.

Amino acid	Amino acid composition (no. of residues/molecule)					
	Found after hydrolysis for			Integral values		
	24hr.	48hr.	72hr.	Our results	Gertler & Hofmann (1967)	Brown *et al.* (1967)
Aspartic acid	22·9	21·8	22·9	23	23	23
Threonine	15·6	13·3	10·4	18*	18	18
Serine	21·2	18·5	16·8	22*	21	22
Glutamic acid	18·6	18·6	18·8	19	19	19
Proline	6·9	6·9	7·3	7	7	7
Glycine	25·9	25·2	25·3	25	24	26
Alanine	16·0	16·0	16·0	16	16	17
Half-cystine	7·5	6·5	5·3	8	8	8
Valine	24·2	24·8	25·6	26	26	26
Methionine	1·6	1·9	1·9	2	2	2
Isoleucine	8·7	8·7	9·2	9	9	10
Leucine	16·2	15·6	16·4	16	17	17
Tyrosine	9·8	9·7	9·6	10	10	10
Phenylalanine	2·6	2·9	3·0	3	3	3
Lysine	3·2	3·0	3·2	3	3	3
Histidine	5·1	4·9	5·4	5	6	5
Arginine	11·3	10·3	10·6	11	11–12	11
Tryptophan †	—	—	7·3	7	7	7

* Extrapolated to zero time of hydrolysis. Extrapolated values for serine and threonine from another set of similar experiments were 22·3 and 18·4 respectively.

† Determined by the method of Barman & Koshland (1967).

RESULTS

Action of elastase on insulin B chain

Digestion for 24 hr. When oxidized insulin B chain was digested with elastase for 24 hr., the products could be resolved into seven bands by high-voltage paper electrophoresis at pH 3·5 (Fig. 2*a*). The amino acid composition showed most of these bands to be mixtures of peptides. Therefore the separated bands were further purified by repeating the electrophoresis separately at pH 3·5. The bands 2 and 4 were further fractionated by electrophoresis at pH 9·1 as described in the Materials and Methods section. This resulted in the isolation of seven pure peptides in addition to free leucine and valine. The yields and analytical results of the purified peptides are summarized in Table 2. Increasing the enzyme concentration tenfold or the incubation time to 72 hr. had no effect on the pattern of hydrolysis.

Digestion of insulin B chain for 15 min. To obtain

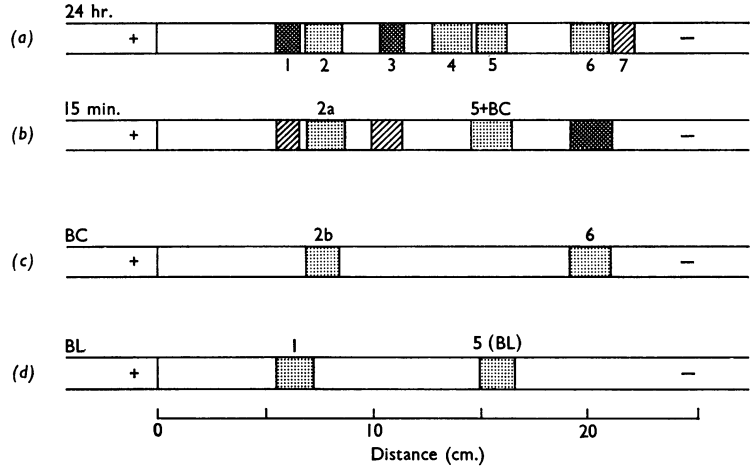

Fig. 2. Electrophoretic diagrams of elastase digests of insulin B chain and peptides BC and BL at pH 3·5, with 2 M-pyridine–acetate buffer. (*a*) 24 hr. elastase digest of insulin B chain; (*b*) 15 min. elastase digest of insulin B chain; (*c*) 24 hr. elastase digest of purified peptide BC; (*d*) 24 hr. elastase digest of purified peptide BL.

Table 2. *Peptides obtained by 24 hr. digestion of insulin B chain with elastase*

Band no.	Amino acid composition (experimentally determined molar ratios)	*N*-Terminus	Peptide yield after final purification (%)	Structure	Remarks
1	Leu, Val	Leu, Val	28·0 30·0	Free Leu and Val	From peptide BL*
2a	$CySO_3H$(1·00),Asp(1·00),Ser(0·92), Glu(1·93),Gly(1·03),Ala(1·01), Val(2·16),Leu(2·16),Phe(0·99), His(1·81)	Phe	4·5	Phe-Val-Asn-Gln-His-Leu-$CySO_3H$-Gly-Ser-His-Leu-Val-Glu-Ala	Separated at pH 9·1
2b	$CySO_3H$(0·86),Gly(1·94),Glu(1·00), Arg(1·00)	$CySO_3H$	38·0	$CySO_3H$-Gly-Glu-Arg-Gly	From peptide BC*
3	Glu(1·00),Ala(1·07)	Glu	4·1	Glu-Ala	—
4	$CySO_3H$(1·00),Asp(1·00),Ser(0·82), Glu(0·91),Gly(1·00),Val(1·09), Leu(1·09),Phe(0·91),His(0·91)	Phe	11·0	Phe-Val-Asn-Gln-His-Leu-$CySO_3H$-Gly-Ser	Purified at pH 9·1
5	Val(1·00),Leu(2·16),Tyr(0·85)	Leu	3·6	Leu-Tyr-Leu-Val	Peptide BL*
6	Thr(0·86),Pro(1·13),Ala(1·00), Tyr(0·86),Phe(2·00),Lys(1·00)	Phe	21·0	Phe-Phe-Tyr-Thr-Pro-Lys-Ala	From peptide BC*
7	Glu(1·00),Ala(0·90),Val(1·07), Leu(1·20),His(0·80)	His	1·6	His-Leu-Val-Glu-Ala	—

* See Table 3.

a clearer picture of the mode of action of elastase a 15min. digestion was attempted. The digest was subjected to high-voltage paper electrophoresis at pH 3·5. This gave two dark ninhydrin-positive bands having mobilities similar to those of bands 2 and 5 of the 24hr. digest (Fig. 2*b*); three relatively faint bands were also detected. The two dark bands were separately refractionated by electrophoresis at pH 9·1. The band '5+BC' (Fig. 2*b*) on refractionation gave two components, designated BL and BC.

Component BL had the composition corresponding to the sequence:

$$\overset{15}{\text{Leu-Tyr-Leu-}}\overset{18}{\text{Val}}$$

whereas component BC had the composition corresponding to the sequence:

$$\overset{19}{\text{CySO}_3\text{H*-Gly}}\text{.....}\overset{30}{\text{Ala}}$$

The band '2a' (Fig. 2*b*) after purification at pH 9·1 had the composition of the peptide:

$$\overset{1}{\text{Phe-Val}}\text{.....}\overset{14}{\text{Ala}}$$

These observations were strengthened by the identification of the expected *N*-terminal residues. The analytical results and the yields of these three peptides are summarized in Table 3.

Peptides 2a and BL (Table 3) are clearly the same as peptides 2a and 5 (Table 2) isolated from 24hr. digests. The appearance of these peptides 2a, BC and BL in short incubation periods (15min.) and the isolation of the peptide 2a from the 24hr. digest suggest that rapid breaks occur between alanine-14 and leucine-15 and between valine-18 and cysteic acid-19.

In a shorter incubation period (5min.) the peptide BC was associated with 32% of the peptide:

$$\overset{15}{\text{Leu-Tyr-Leu}}\text{.....}\overset{30}{\text{Ala}}$$

* Abbreviation: $CySO_3H$ (in amino acid sequences), cysteic acid.

as judged from the amino acid composition (moles/mole) of the peptide BC isolated from the 5min. digest: $CySO_3H$ (1·05), Thr (0·80), Glu (1·00), Pro (1·09), Gly (2·00), Ala (1·01), Val (0·32), Leu (0·64), Tyr (0·80), Phe (1·92), Lys (1·04), Arg (0·90). This indicated that the rate of hydrolysis of the bond between alanine-14 and leucine-15 is perhaps the fastest.

Redigestion of B-chain peptides. The purified peptide BC from a 15min. digest [free from the peptide:

$$\overset{15}{\text{Leu-Tyr-Leu}}\text{.....}\overset{30}{\text{Ala}}$$

(Table 3)] on redigestion with elastase gave two peptides (Fig. 2*c*):

$$\overset{19}{\text{CySO}_3\text{H-Gly-Glu-Arg-}}\overset{23}{\text{Gly}}$$

(yield 42·9%)

$$\overset{24}{\text{Phe-Phe-Tyr-Thr-Pro-Lys-}}\overset{30}{\text{Ala}}$$ (yield 20·0%)

The isolation of these two peptides in high yields from the elastase digest of the peptide BC suggests that elastase attacks the bond between glycine-23 and phenylalanine-24 fairly efficiently. This conclusion is further supported by the fact that the peptide BC could not be detected in either the 24hr. digests of insulin B chain or the 24hr. digests of the peptide BC.

Redigestion for 24hr. of the peptide BL isolated from a 15min. digest of insulin B chain with elastase gave rise to free leucine (yield 7·1%) and valine (yield 10·2%), and unhydrolysed peptide BL (yield 18·4%) was recovered (Fig. 2*d*).

Our attempts to locate the peptides:

$$\overset{10}{\text{His-Leu-Val-Glu-}}\overset{14}{\text{Ala}}$$

$$\overset{13}{\text{Glu-}}\overset{14}{\text{Ala}}$$

by redigestion of the peptide 2a (Table 3) with elastase were unsuccessful, although these two peptides were present in low yields in 24hr. digests of insulin B chain (Table 2, peptides 3 and 7). No

Table 3. *Peptides obtained by 15min. digestion of insulin B chain with elastase*

Band no.	Amino acid composition (experimentally determined ratios)	*N*-Terminus	Peptide yield after final purification (%)	Structure
2a	$CySO_3H$(1·01),Asp(1·00),Ser(0·92), Glu(1·96),Gly(1·04),Ala(1·01),Val(2·19), Leu(2·12),Phe(1·00), His(1·80)	Phe	13·8	Phe-Val-Asn-Gln-His-Leu-$CySO_3H$-Gly-Ser-His-Leu-Val-Glu-Ala
BL	Val(1·00),Leu(2·03),Tyr(0·94)	Leu	11·8	Leu-Tyr-Leu-Val
BC	$CySO_3H$(1·04),Thr(0·80),Glu(0·99), Pro(1·11),Gly(2·00),Ala(1·00),Tyr(0·80), Phe(1·91),Lys(1·04),Arg(0·89)	$CySO_3H$	10·5	$CySO_3H$-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala

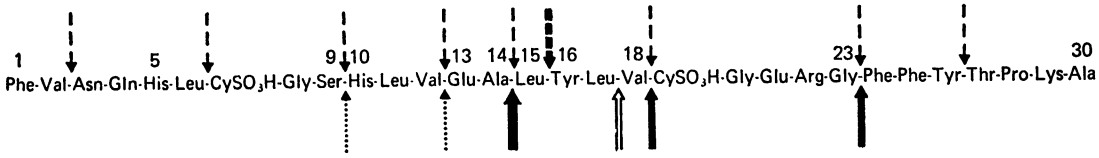

Fig. 3. Specificity of action of elastase on insulin B chain. ——→, Breaks reported by Naughton & Sanger (1961); ⟶ and ····→ breaks reported in the present paper.

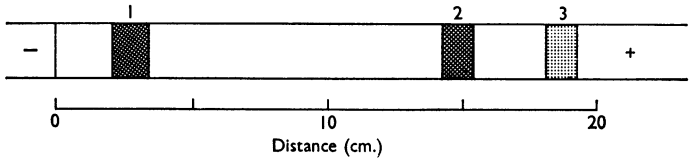

Fig. 4. Electrophoretic diagram of elastase digest of insulin A chain at pH 3·5.

Table 4. *Peptides obtained by 24 hr. digestion of insulin A chain with elastase*

Band no.	Amino acid composition (experimentally determined ratios)	*N*-Terminus	Peptide yield after final purification (%)	Structure
1	$CySO_3H$(0·78),Asp(2·12),Glu(2·30), Leu(2·00),Tyr(1·45)	Leu	2·6	Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-$CySO_3H$-Asn
2a	$CySO_3H$(1·84),Glu(2·13),Gly(1·00), Ala(1·12),Val(1·00),Ile(1·00)	Gly	4·2	Gly-Ile-Val-Glu-Gln-$CySO_3H$-$CySO_3H$-Ala
2b	$CySO_3H$(1·76),Asp(1·76),Ser(2·20), Glu(1·98),Val(1·00),Leu(2·00),Tyr(1·43)	Ser	0·9	Ser-Val-$CySO_3H$-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-$CySO_3H$-Asn
3a	$CySO_3H$(3·65),Asp(1·98),Ser(1·82), Glu(4·00),Gly(1·00),Ala(1·04),Val(1·88), Ile(0·98),Leu(1·94),Tyr(1·70)	Gly	7·5	Insulin A chain
3b	$CySO_3H$(2·71),Ser(1·64),Glu(1·76), Gly(1·00),Ala(1·00),Val(1·82),Ile(0·91)	Gly	0·9	Gly-Ile-Val-Glu-Gln-$CySO_3H$-$CySO_3H$-Ala-Ser-Val-$CySO_3H$-Ser

hydrolysis of the purified peptide 2a with elastase was detected.

All the observations described above show that the most preferred points of attack by elastase are between alanine-14 and leucine-15 and between valine-18 and cysteic acid-19. The bond between glycine-23 and phenylalanine-24 is also hydrolysed efficiently. The hydrolysis of other bonds is slow. The points of cleavage in insulin B chain are summarized in Fig. 3.

Mode of hydrolysis of insulin A chain

The action of elastase on insulin A chain was very slow. Separation of the products at pH 3·5 after 24 hr. hydrolysis showed three bands, of which unhydrolysed insulin A chain formed the darkest band (Fig. 4). The amino acid composition together with *N*-terminal analysis showed band 1 to be the peptide:

$$\overset{13}{\text{Leu-Tyr}}\ldots\ldots\overset{21}{\text{Asn}}$$

whereas band 2 could be resolved into two components by electrophoresis at pH 9·1:

$$\overset{1}{\text{Gly-Ile}}\ldots\ldots\overset{8}{\text{Ala}}\ \text{(peptide 2a, Table 4)}$$

$$\overset{9}{\text{Ser-Val}}\ldots\ldots\overset{21}{\text{Asn}}\ \text{(peptide 2b, Table 4)}$$

Band 3 also gave two components on electrophoresis at pH 9·1: unhydrolysed insulin A chain (peptide 3a, Table 4) and

$$\overset{1}{\text{Gly-Ile}}\ldots\ldots\overset{12}{\text{Ser}}\ \text{(peptide 3b, Table 4)}$$

These results, summarized in Fig. 5 and Table 4, show that only two breaks occur in the molecule as a result of elastase action: between alanine-8 and serine-9 and between serine-12 and leucine-13. Only one break seems to occur per molecule, because the peptide:

$$\overset{9}{\text{Ser-Val-CySO}_3\text{H-Ser}}\overset{12}{}$$

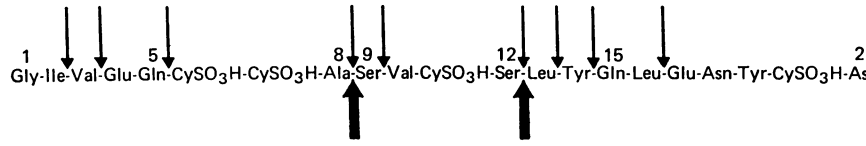

Fig. 5. Specificity of elastase on insulin A chain. ⟶, Breaks reported by Naughton & Sanger (1961); ⟶, breaks reported in the present paper.

was not detected. The yields of different peptides and other analytical results are given in Table 4.

DISCUSSION

DEAE-cellulose and DEAE-Sephadex ion-exchangers have commonly been used for purifying elastase (Baumstark *et al.* 1963; Smillie & Hartley, 1966; Ling & Anwar, 1966). Gertler & Hofmann (1967) combined DEAE-cellulose column chromatography with CM-cellulose column chromatography to obtain a preparation homogeneous on polyacrylamide-gel electrophoresis; it exhibited about 0·5% of trypsin and traces of chrymotrypsin activities. No chymotryptic and tryptic activities were detected in the elastase preparation described in the present paper.

The peptide results show that the major points of hydrolysis by elastase in the insulin B chain are the bonds between alanine-14 and leucine-15 and between valine-18 and cysteic acid-19. The bond between glycine-23 and phenylalanine-24 is also efficiently hydrolysed. Naughton & Sanger (1961) reported that the fastest break brought about by their elastase preparation was between leucine-15 and tyrosine-16. They also reported the liberation of free leucine and valine from the *N*-terminal end of insulin B chain, whereas in the experiments reported in the present paper these amino acids were liberated from the peptide:

$$\overset{15}{\text{Leu-Tyr-Leu-Val}}\overset{18}{}$$

The total number of breaks observed was less than that reported by Naughton & Sanger (1961). No hydrolysis of the bonds between valine-2 and asparagine-3, between leucine-6 and cysteic acid-7, between leucine-15 and tyrosine-16 and between tyrosine-26 and threonine-27 was observed. The difference in the pattern of hydrolysis with the insulin A chain was marked. Naughton & Sanger (1961) isolated 15 peptides in addition to free leucine, valine, alanine and tyrosine. The preparation described in the present paper yielded only four peptides and no free amino acids.

The action of elastase resembles, in general, that of the α-lytic enzyme, and more so the combined action of the α- and the β-lytic enzymes of soil bacteria of the *Sorangium* sp. (Whitaker, Roy, Tsai & Jurášek, 1965). It is noteworthy that the α-lytic enzyme, like elastase, is a serine proteinase (Smillie & Whitaker, 1967).

These observations suggest that elastase has narrower specificity requirements than those reported by Naughton & Sanger (1961), although in general it seems to attack peptide bonds adjacent to neutral amino acids. The variety of peptides isolated by Naughton & Sanger (1961) might have resulted from the heterogeneity of their enzyme preparation (Gertler & Hofmann, 1967). During the purification of elastase, we observed two more proteolytic peaks by elution of the DEAE-Sephadex column with increasing sodium chloride concentration. One of these peaks was strongly proteolytic and the other resembled carboxypeptidase A in its properties. It appears that the carboxypeptidase A component is responsible for the appearance of large amounts of free amino acids when crystalline elastase is used for the digestion of elastin (Ling & Anwar, 1966).

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REFERENCES

Barman, T. E. & Koshland, D. E., jun. (1967). *J. biol. Chem.* **242**, 5771.

Baumstark, J. S., Bardawil, W. A., Sbarra, A. J. & Hayes, N. (1963). *Biochim. biophys. Acta*, **77**, 676.

Brown, J. R., Kauffman, D. L. & Hartley, B. S. (1967). *Biochem. J.* **103**, 497.

Gertler, A. & Hofmann, T. (1967). *J. biol. Chem.* **242**, 2522.

Kunitz, M. (1947). *J. gen. Physiol.* **30**, 291.

Lewis, U. J., Williams, D. E. & Brink, N. G. (1956). *J. biol. Chem.* **222**, 705.

Ling, V. & Anwar, R. A. (1966). *Biochem. biophys. Res. Commun.* **24**, 593.

Naughton, M. A. & Sanger, F. (1961). *Biochem. J.* **78**, 156.
Piez, K. A. & Morris, L. (1960). *Analyt. Biochem.* **1**, 187.
Reisfeld, R. A., Lewis, U. J. & Williams, D. E. (1962). *Nature, Lond.*, **195**, 281.
Smillie, L. B. & Hartley, B. S. (1966). *Biochem. J.* **101**, 232.
Smillie, L. B. & Whitaker, D. R. (1967). *J. Amer. chem. Soc.* **89**, 3350.
Smithies, O. (1959). *Advanc. Protein Chem.* **14**, 65.
Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.
Stark, G. R. & Smyth, D. G. (1963). *J. biol. Chem.* **238**, 214.
Whitaker, D. R., Roy, C., Tsai, C. S. & Jurášek, L. (1965). *Canad. J. Biochem.* **43**, 1961.