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Action of Human Pepsins 1, 2, 3 and 5 on the Oxidized B-Chain of Insulin

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Human pepsins 1 and 2 attack the B-chain of oxidized insulin at pH 1.7 at the same bonds as does human pepsin 3. At pH 3.5, pepsins 1 and 2 attack insulin B-chain at essentially the same bonds as at pH 1.7, but more slowly. For all three enzymes, the first bond to be hydrolysed is $Phe_{(25)}$–$Tyr_{(26)}$, followed simultaneously by $Glu_{(13)}$–$Ala_{(14)}$, $Leu_{(15)}$–$Tyr_{(16)}$ and $Tyr_{(16)}$–$Leu_{(17)}$. Human pepsin 5, however, attacks $Phe_{(24)}$–$Phe_{(25)}$ first of all, followed by $Leu_{(15)}$–$Tyr_{(16)}$ and $Tyr_{(16)}$–$Leu_{(17)}$. The results suggest that each pepsin has only one active site. Acid hydrolysis indicates that the sites of enzymic cleavage are not bonds with an inherent instability at low pH.

The isolation and purification in sufficient quantities (Roberts & Taylor, 1972, 1978) of the individual human pepsins 1, 2, 3 and 5, as classified by Etherington & Taylor (1967), has enabled comparative studies of the enzymes to be carried out.

The purpose of the present investigation was first to determine the sites of action of pepsins 1 and 2 on the B-chain of oxidized insulin; pepsin 1 is of particular interest as it is present in increased amount in patients with peptic ulcer (Taylor, 1970), in patients during stress (Walker & Taylor, 1976) and in cigarette-smokers with peptic ulcer, as compared with non-smokers (Walker & Taylor, 1977). Secondly, we have studied the order in which bond cleavage of insulin B-chain occurs with pepsins 1, 2, 3 and 5 in an attempt to ascertain whether the different enzymes attack different bonds initially, and whether pepsins 3 and 5, which each attack substrates such as human serum albumin with two pH maxima near pH 2.0 and 3.3 (Taylor, 1959), attack different bonds initially at these different pH values. Pepsin 3 is the major human pepsin and is homologous with pig pepsin A; pepsin 5 was first described as human pyloric pepsin (Taylor, 1956), and is homologous with the pig 'gastricsin' of Richmond *et al.* (1958) and pig pepsin C of Ryle & Porter (1959); subsequently two pepsins 5, fundic and pyloric, were demonstrated (Etherington & Taylor, 1967). Finally, the possibility that the bonds cleaved were more sensitive to proteolysis because they were more readily cleaved non-enzymically at acid pH was also investigated.

Experimental

Materials

Human pepsins were isolated from gastric juice by using the preparative agar-gel technique reported by Etherington & Taylor (1971) and Roberts & Taylor (1972). Pepsins 1, 2, 3 and 5 were classified by their relative mobilities as compared with a pig pepsin A marker (Roberts & Taylor, 1978). Pig pepsin A was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and bovine insulin (six times crystallized) was from Boots, Nottingham, U.K.

Methods

Preparation of the B-chain of oxidized insulin. Bovine insulin was oxidized by the method of Hirs (1956) and the B-chain separated out by selective precipitation as described by Sanger (1949). Amino acid analysis confirmed the composition of the oxidized B-chain (Etherington & Taylor, 1971).

Digestion of the B-chain. Known amounts of the individual pepsins were added to a solution of the B-chain of oxidized insulin (1.25mg/ml). Solutions were adjusted to a final pH of 1.7 or 3.5 with 0.1M-HCl. Enzyme:substrate ratios were of the order of 1:1000 (w/w) and are indicated in the text. Samples of the digest equivalent to 1.25mg of the B-chain were taken at various timed intervals after digestion at 37°C and the reaction was stopped by heating for 45min in a boiling-water bath (Sanger & Tuppy, 1951). The samples were then freeze-dried. The pH change during incubation was never more than 0.1 pH unit.

Electrophoretic and chromatographic separation of the digestion products of the B-chain of oxidized insulin. The freeze-dried digest was dissolved in 250μl of aq. 10.0% (v/v) acetic acid and applied to Whatman 3MM paper. It was then subjected to high-voltage electrophoresis (Miles Hi-Volt, Shoreham-by-Sea, Sussex, U.K.) at pH 5.2 in pyridine (20g/l)/acetic acid (10g/l) for 45–60min at 10mA/cm width and 55V/cm length. The application strips, along the line of electrophoresis, were then sectioned

off and stitched to a sheet of Whatman 3MM paper (46cm × 57cm) and subjected to descending chromatography in the solvent butan-1-ol/acetic acid/NH_3-free water (12:3:5, by vol.). The separated peptide fragments were located by staining with ninhydrin (Smith, 1969). The finally developed chromatogram constituted a 'fingerprint'.

A comparison of the staining intensity with ninhydrin, and of spot size, of a series of related di- and tri-peptides (e.g. glycyltyrosine, phenylalanylphenylalanine, leucyltyrosine, glycyltryptophan, histidylhistidine and leucylglycylglycine) indicates that the cross-hatched zones, e.g. peptides 1, 2, 3, 5, 6, 7 and 8 in Figs. 1–4, are approximately equivalent to between 0.10mg and 0.30mg of peptide and the open zones to less than 0.10mg. Exceptionally, where the undigested B-chain is evident (i.e. in Fig 2, pH3.5, ½h and 1h incubations), the cross-hatching may indicate up to 1.25mg of the B-chain, the amount added initially to the paper.

Peptide amino acid analysis. Amino acid analysis of the unstained peptides was carried out after location, separation and final elution from the paper (Blackburn, 1966) followed by peptide hydrolysis and amino acid analysis as described by Etherington & Taylor (1971). We were able to confirm the amino acid analyses of Etherington (1967) except for peptides 13 and 14, which are discussed in the Results section.

The system of nomenclature of the fragments was that reported by Etherington (1967). Table 1 shows the numbering and composition of each fragment as modified by Roberts (1975).

Identification of N-terminal residues. *N*-Terminal residues were identified by the dansyl chloride method, with separation of the dansyl-amino acids on polyamide sheets (Beale, 1969). Dansylation of the peptide fragments was used to confirm the numbering of each peptide fragment, and its position on the developed 'fingerprint'.

Calculation of the amounts of each pepsin used. The amount of human pepsin used in each experiment was calculated by comparison of its proteolytic activity (A_{700}) against the substrate haemoglobin at pH2.0 with the proteolytic activity (A_{700}) of known amounts of pig pepsin A.

Non-enzymic digestion of the B-chain of oxidized insulin. Acid digestion of the B-chain of oxidized insulin (1.25mg/ml) was carried out at pH1.0, pH1.7 and 3.5. Temperatures of hydrolysis were 37 and 110°C; at the latter temperature, hydrolysis was carried out in sealed de-aerated tubes. Hydrolysis was carried out over various timed intervals and the fragments produced were separated as for the enzymic hydrolysis.

Classification of the bonds cleaved during enzymic digestion. During the course of the timed enzyme breakdown of the oxidized B-chain of insulin, it became apparent that certain fragments released were, in fact, released by degradation of initial degradation products of the substrate. Thus a nomenclature of primary and secondary sites of action was adopted to differentiate such degradation. However, to avoid confusion, as the majority of workers refer to major and minor sites of action, the usage of major and minor will be restricted to the ease with which bonds are cleaved. Thus it is possible to have primary major and minor sites of cleavage on the intact B-chain, and secondary major and minor sites of cleavage of peptides released during degradation.

Table 1. *Peptides from peptic digests of insulin B-chain: numbering and composition (Etherington, 1967, as modified by Roberts, 1975)*

Amino acid sequence of the B-chain of oxidized insulin: 1, Phe; 2, Val; 3, Asn; 4, Gln; 5, His; 6, Leu; 7, $CySO_3H$; 8, Gly; 9, Ser; 10, His; 11, Leu; 12, Val; 13, Glu; 14, Ala; 15, Leu; 16, Tyr; 17, Leu; 18, Val; 19, $CySO_3H$; 20, Gly; 21, Glu; 22, Arg; 23, Gly; 24, Phe; 25, Phe; 26, Tyr; 27, Thr; 28, Pro; 29, Lys; 30, Ala.

Peptide number	Residues of the B-chain	*N*-Terminal (dansyl) group
1	26–30	Tyrosine
2	25–30	Phenylalanine
3	1–11	Phenylalanine
3a	2–11	Valine
4	5–11	Histidine
5	1–14 (15)	Phenylalanine
6	1–13	Phenylalanine
7	1–15 (16)	Phenylalanine
7a	2–4	Valine
7b	1–4	Phenylalanine
7c	16	Tyrosine
8	1–16	Phenylalanine
9	1, 25	Phenylalanine
11	14–15	Alanine
12	15–16	Leucine
13	16–25	Tyrosine
	17–25	Leucine
14	16–24	Tyrosine
	17–24	Leucine
15	17–23	Leucine
16	12–16	Valine
17	12–14	Valine
18	12–13	Valine

Results

Table 1 lists the various peptide fragments that have been identified during this study and indicates their amino acid composition and *N*-terminal group.

Human pepsin 1

The action of human pepsin 1 on the B-chain of oxidized insulin is shown in Figs. 1(*a*) and 1(*b*) at pH1.7 and 3.5 respectively. At pH1.7 after 30min

digestion peptides 1, 11 and 13 were liberated and remained visible up to 24h of incubation. *N*-Terminal analysis of peptide 13 showed that both tyrosine and leucine were present in approximately equal amounts. It was concluded therefore that this peptide was a mixture of residues 16–25 and 17–25. Thus primary sites of cleavage are at the bonds 25–26, liberating peptide 1, and 13–14, 15–16 and 16–17, liberating peptides 6, 7, 11 and 13. Between 2h and 24h peptides 5 and 3 were released, indicating secondary major cleavage of peptides 6 and 7 at $Leu_{(11)}$–$Val_{(12)}$ and $Ala_{(14)}$–$Leu_{(15)}$ bonds respectively.

Digestion at pH3.5, although initially less pronounced, clearly showed that a primary major site of cleavage is at the $Phe_{(25)}$–$Tyr_{(26)}$ bond, as peptide 1 was released after 30min and unchanged insulin was still evident. After 4h the well-defined fragments 6, 7 and 13 became visible, implying that the bonds $Glu_{(13)}$–$Ala_{(14)}$, $Leu_{(15)}$–$Tyr_{(16)}$ and $Tyr_{(16)}$–$Leu_{(17)}$ are either primary minor or secondary major sites of action at pH3.5. Peptide 13 was found, on dansylation, to consist of equal amounts of the residues 16–25 and 17–25, thus confirming the similar finding at pH1.7.

At pH3.5 after 24h, free phenylalanine and tyrosine were released, probably as a result of cleavage of the $Phe_{(24)}$–$Phe_{(25)}$ bond and of the $Tyr_{(16)}$–$Leu_{(17)}$ bond from peptide 13 (16–25). Since peptides 3a

Table 2. *Sites of action of human pepsins* 1, 2, 3 *and* 5 *on the B-chain of oxidized insulin*
Symbols: ⇐, primary major; ← primary minor or secondary major; ←-, secondary minor.

Individual human pepsins ...	1		2		3		5	
pH ...	1.7	3.5	1.7	3.5	1.7	3.5	1.7	3.5
1 Phe								
2 Val								
3 Asn								
4 Gln								
						←-		
5 His								
6 Leu								
7 $CySO_3H$								
8 Gly								
9 Ser								
10 His								
11 Leu								
	←	←	←		←	←		←
12 Val								
13 Glu								
	⇐	⇐	⇐	⇐	⇐	⇐	←	←
14 Ala								
	←	←	←		←	←		←
15 Leu								
	⇐	⇐	⇐	⇐	⇐	⇐	⇐	⇐
16 Tyr								
	⇐	⇐	⇐	⇐	⇐	⇐	⇐	⇐
17 Leu								
18 Val								
19 $CySO_3H$								
20 Gly								
21 Glu								
22 Arg								
23 Gly								
24 Phe								
		←-				←	⇐	⇐
25 Phe								
	⇐	⇐	⇐	⇐	⇐	⇐	←-	←-
26 Tyr								
27 Thr								
28 Lys								
29 Pro								
30 Ala								

(residues 2–11) or 7a (residues 2–4) were not identified it seems unlikely that the free phenylalanine was derived from $Phe_{(1)}$. The presence of peptide 5 indicated cleavage of $Ala_{(14)}$–$Leu_{(15)}$.

Human pepsin 2

The action of human pepsin 2 is shown in Figs 2(*a*) and 2(*b*) at pH1.7 and 3.5 respectively. At pH1.7

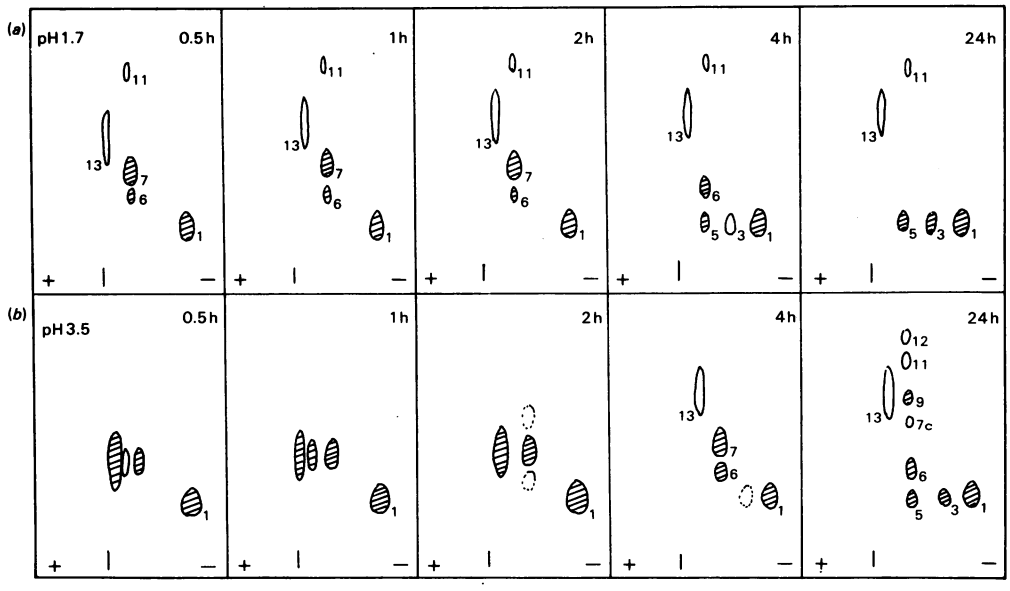

Fig. 1. *Digest of the B-chain of oxidized insulin by pepsin* 1
The peptide 'maps' ('fingerprints') are represented diagramatically. Cross-hatched zones indicate a high ninhydrin stain intensity. Numbering of the peptides is shown in Table 1. Unidentified spots are either unchanged insulin or fragments of the first 25 residues. The enzyme: substrate ratio was 1:2000 (w/w). (*a*) pH1.7; (*b*) pH3.5.

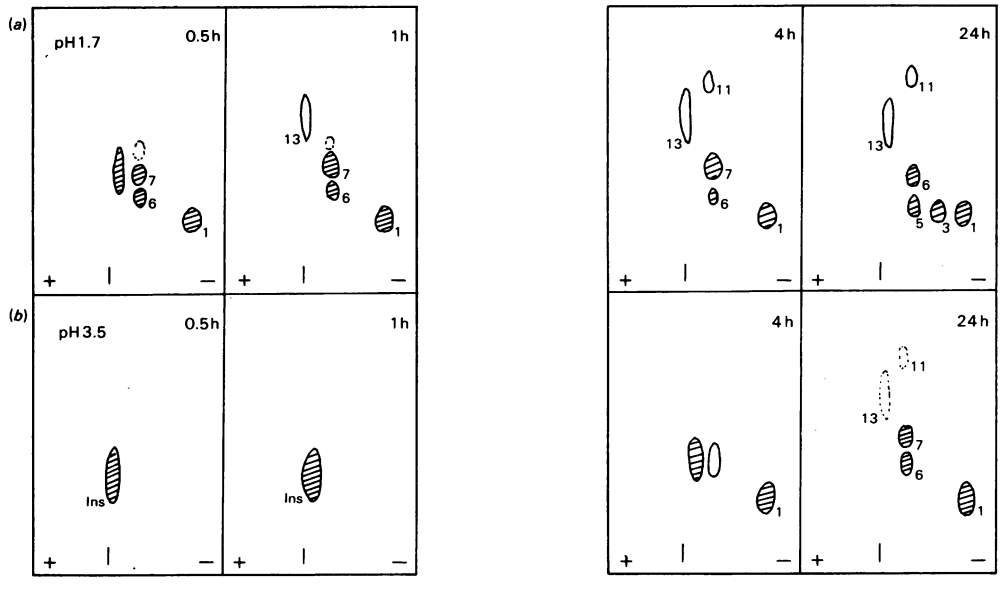

Fig. 2. *Digest of the B-chain of oxidized insulin by pepsin* 2
The details are as for Fig. 1, except the enzyme: substrate ratio was 1:5000 (w/w). (*a*) pH1.7; (*b*) pH3.5.

after 30min it was apparent that although cleavage had occurred, unchanged insulin was still present. Thus the peptides present indicate sites of primary major cleavage at the bonds $Phe_{(25)}$–$Tyr_{(26)}$ $Leu_{(15)}$–$Tyr_{(16)}$ and $Glu_{(13)}$–$Ala_{(14)}$. After 1h peptide 13 was evident, indicating cleavage of $Tyr_{(16)}$–$Leu_{(17)}$. Peptide 11 was then located after 4h, suggesting further cleavage of peptide 7 at $Glu_{(13)}$–$Ala_{(14)}$. At 24h the 'fingerprint' for pepsin 2 was similar to that for pepsin 1, but the presence of peptide 6 indicated

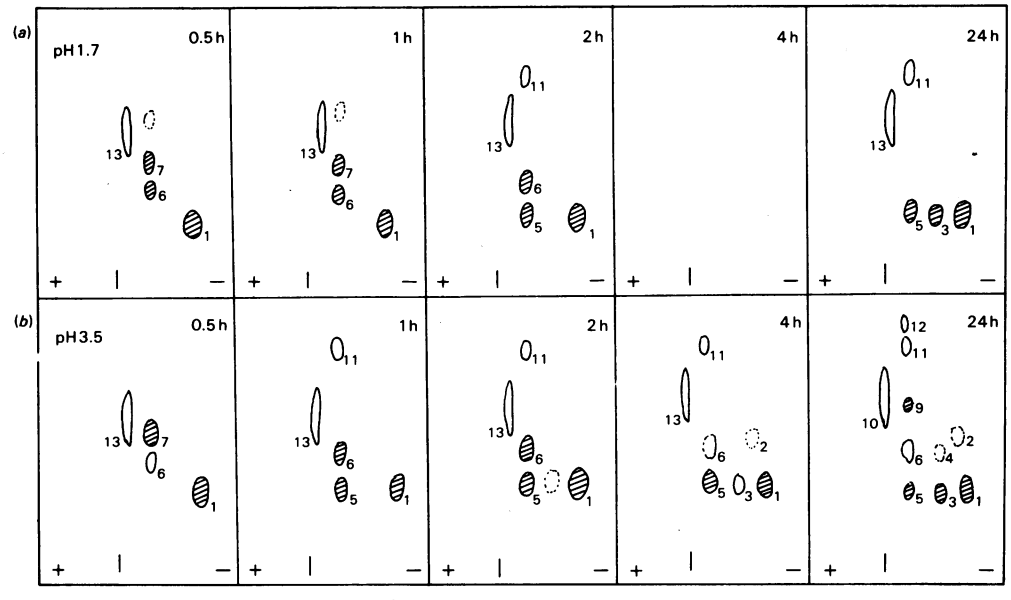

Fig. 3. *Digest of the B-chain of oxidized insulin by pepsin* 3
The details are as for Fig. 1, except the enzyme:substrate ratio was 1:1700 (w/w). (*a*) pH1.7; (*b*) pH3.5.

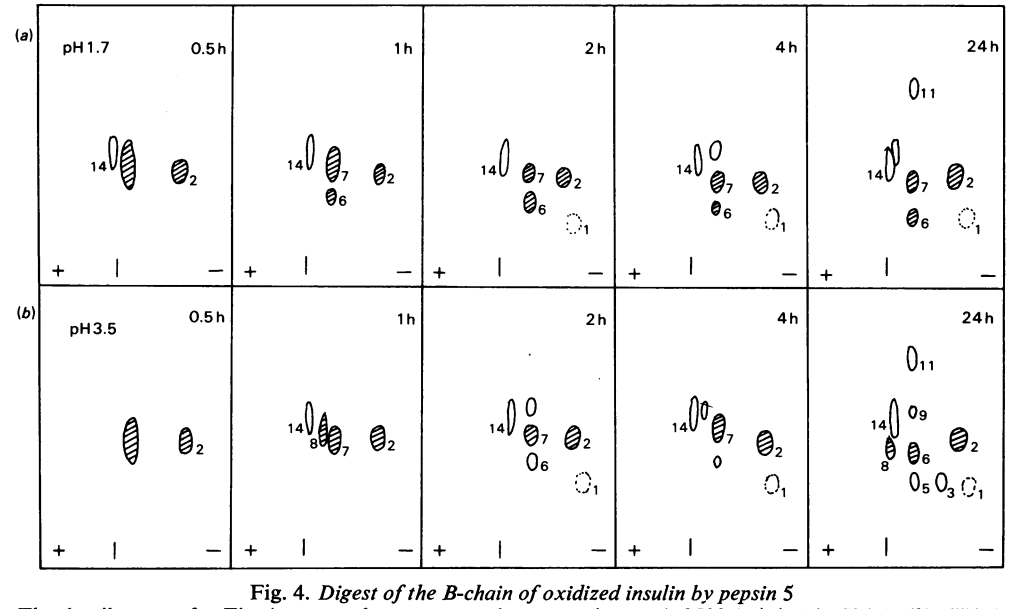

Fig. 4. *Digest of the B-chain of oxidized insulin by pepsin* 5
The details are as for Fig. 1, except the enzyme: substrate ratio was 1:2500 (w/w). (*a*) pH1.7; (*b*) pH3.5.

that hydrolysis at 11–12 was not complete. Peptide 13 consisted mainly of the tyrosyl derivative (16–25) with only small amounts (about 20%) of the leucyl derivative.

Enzymic breakdown of the substrate at pH3.5 was much slower than that for pepsin 1 although following the same pattern and confirming the bond $Phe_{(25)}$–$Tyr_{(26)}$ as a primary major site of action.

Human pepsin 3

The cleavage of the B-chain of oxidized insulin by human pepsin 3 at pH1.7 (Fig. 3*a*) was very similar to that shown for pepsin 1. After 30min peptides 1, 6, 7 and 13 were released. Hydrolysis of $Ala_{(14)}$–$Leu_{(15)}$ occurred earlier than with pepsin 1. Peptide 13 consisted of approximately equal amounts of the tyrosyl and leucyl derivatives at both pH1.7 and 3.5.

At pH3.5 (Fig. 3*b*) the developed 'fingerprints' show considerable similarity with pepsin 1 except that digestion of the substrate occurred much more rapidly, particularly at $Ala_{(14)}$–$Leu_{(15)}$. Unlike pepsin 1, pepsin 3 released peptides 2 and 4, at 4h and 24h respectively, indicating secondary minor cleavage at $Phe_{(24)}$–$Phe_{(25)}$ and $Glu_{(4)}$–$His_{(5)}$.

Human pepsin 5

The mode of action of human pepsin 5 on the oxidized B-chain (Figs. 4*a* and 4*b*) was essentially as reported by Etherington & Taylor (1971). However, the analysis from the timed digests showed more clearly the primary and secondary sites of cleavage.

At pH1.7 peptides 2 and 14 were liberated in 30min at pH1.7; thus $Phe_{(24)}$–$Phe_{(25)}$, $Leu_{(15)}$–$Tyr_{(16)}$ and $Tyr_{(16)}$–$Leu_{(17)}$ are the first bonds to be split. *N*-Terminal analysis of peptide 14 showed that both tyrosine and leucine were present in approximately equal amounts. It was concluded that this peptide was a mixture of residues 16–24 and 17–24. Then follow $Glu_{(13)}$–$Ala_{(14)}$ and later $Phe_{(25)}$–$Tyr_{(26)}$. At pH3.5 after 24h, bonds $Ala_{(14)}$–$Leu_{(15)}$ and $Leu_{(11)}$–$Val_{(12)}$ are additionally hydrolysed.

Acid digestion

There was no detectable hydrolysis of the B-chain at 37°C for up to 48h at pH1.7 and 1.0.

At 110°C hydrolysis did occur and was complete after 8h at pH1.7, whereas at pH3.5 after 8h no hydrolysis was detected.

Hydrolysis of the B-chain probably occurred from the *C*-terminal end, as alanine was the first amino acid evident (Hill, 1965). None of the peptides revealed by peptic hydrolysis was detected. Thus the enzymic sites of cleavage are not bonds preferentially cleaved at low pH owing to inherent bond instability at low pH.

Discussion

The general specificity of action of the human pepsins 3 and 5 on the B-chain of oxidized insulin was essentially as reported by Etherington & Taylor (1971). The series of timed digestions showed that human pepsin 3 split the B-chain initially at four primary bonds, namely $Glu_{(13)}$–$Ala_{(14)}$, $Leu_{(15)}$–$Tyr_{(16)}$, $Tyr_{(16)}$–$Leu_{(17)}$ and $Phe_{(25)}$–$Tyr_{(26)}$ at both pH1.7 and 3.5. Human pepsin 5 showed a different primary bond specificity in that the $Phe_{(24)}$–$Phe_{(25)}$ bond was cleaved preferentially, instead of $Phe_{(25)}$–$Tyr_{(26)}$, at the two pH values studied. Studies on acid digestion alone show clearly that the bonds cleaved are enzyme dependent and not sensitive to solutions of low pH.

The bond specificity of human pepsins 1 and 2 on the B-chain of oxidized insulin was very similar to that of pepsin 3. The differences that were apparent were in the rates of specific bond cleavage at pH3.5. Thus both pepsins 1 and 2 cleaved the B-chain much more slowly than did pepsin 3 at pH3.5, although the actual bonds cleaved were much the same. Thus pepsins 1, 2 and 3 would appear to have no significant bond specificity differences at the primary or secondary sites of cleavage on the B-chain of oxidized insulin, but pepsin 5 acts differently.

The hypothesis that pepsins 3 and 5 might have two separate active centres was originally advanced by Taylor (1959) as the simplest explanation of the then-available evidence, most of which arose from the existence of the two pH maxima near pH2.0 and 3.2, from the differential effect of inhibitors on the maxima and from the demonstration that different synthetic substrates were attacked at one or other of the maximum pH values. Subsequently, the hypothesis received support from the work of Erlanger *et al.* (1967), who showed the presence of two different β-aspartic acid residues modifiable by various group-directed inhibitors. However, it seemed that only one of the modified aspartic acid residues was involved in the catalytic activity of pepsin towards the substrate.

Hartsuck & Tang (1972) also concluded that inhibition of the pepsin catalytic activity was associated with one β-aspartic acid residue only and not two residues, as had been demonstrated earlier (Tang, 1971).

Kunimoto *et al.* (1972) inferred from the binding of pepstatin to pig pepsin, modified by *p*-bromophenacyl bromide and diazo-*p*-bromoacetophenone, that the two aspartic acid residues exist near one another in the three-dimensional molecule, as binding by pepstatin was only effectively blocked when both groups were substituted in the pepsin molecule. However, in their work proteolytic activity was measured by using the synthetic substrate *N*-acetyl-L-phenylalanyl-L-di-iodotyrosine, and the

blocking of the action of pepsin on such substrates does not mean that other peptide bonds may not be cleaved.

Meanwhile Etherington & Taylor (1971), from studies on the B-chain of oxidized insulin, now favoured the view that either the pepsins had two active sites, each with very nearly identical activity, or that there was a single active site at which substrate binding is greatly affected by pH. The present studies indicate that with all four human pepsins the sites of insulin B-chain attacked initially at pH 1.7 and 3.5 are the same for each enzyme, with the main differences arising from rates of cleavage. These results again suggest that each enzyme has only one active site.

If, therefore, the same active site is involved at pH 1.7 and 3.5, the slower proteolysis at pH 3.5 with pepsins 1 and 2 gives additional information about the order in which bonds are cleaved. Peptide 1 is clearly the first hydrolytic product, indicating that $Phe_{(25)}$–$Tyr_{(26)}$ is the bond which is first attacked by the active site. $Glu_{(13)}$–$Ala_{(14)}$ and $Leu_{(15)}$–$Tyr_{(16)}$ are next attacked, more or less simultaneously, closely followed by $Tyr_{(16)}$–$Leu_{(17)}$ (from the evidence of pepsin 2 at pH 1.7). The fact that peptides such as residues 16–30 are never found confirms that 25–26 is the first bond to be split for pepsins 1 and 2, and suggests the same order for pepsin 3 also. For pepsin 5, the first bond to be split is 24–25, followed by 16–17 and 15–16.

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