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The First Step in the Activation of Chicken Pepsinogen is Similar to that of Prochymosin

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Chicken pepsinogen was incubated at pH2.5 with pepstatin. The zymogen activated itself by a sequential mechanism and an intact peptide derived from residues 1–26 in the protein was released in the first step. This peptide was found to inhibit the milk-clotting activities of pig and chicken pepsins and calf chymosin but to different extents.

Chicken pepsinogen has been purified and characterized (Bohak, 1973; Keilova & Kostka, 1975). Its main importance is that it is the one acid-proteinase zymogen that contains a free thiol group, and so it should be possible to introduce isomorphous replacements into the molecule to form useful derivatives for X-ray-crystallographic studies.

Pig pepsinogen and calf prochymosin convert themselves into the more active forms of their respective enzymes at acid pH by undergoing a limited proteolysis in which an *N*-terminal 'activation segment' is cleaved off. However, activation does not take place in a one-step transformation, but involves a sequential process in which peptides are successively removed from the *N*-terminus until the entire activation segment has been removed (Dykes & Kay, 1976). If the activations are allowed to proceed in the presence of the inhibitor pepstatin, which has no affinity for the zymogen forms (Kay & Dykes, 1976), but which combines immediately with the first active protein species as it is being formed, then the peptide released in the first step can be isolated. In the case of pig pepsinogen, the first peptide released was derived from residues 1–16 in the parent zymogen (Dykes & Kay, 1976), whereas in the case of prochymosin the first 27 residues were released as an intact peptide (Kay & Dykes, 1977). This has now been confirmed by other workers, using a different technique (Pedersen, 1976; Christensen *et al.*, 1977).

Thus we decided to find out whether chicken pepsinogen would activate itself in a manner analogous to that shown by pig pepsinogen, i.e. releasing about 16 residues as an intact peptide, or, like prochymosin, releasing about 27 residues. The sequences at the *N*-termini of the three proteins are:

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            1                   5                  10
Pig             Leu-Val-Lys-Val-Pro-Leu-Val-Arg-Lys-Lys-Ser-Leu-Arg-
Chick       Ser-Ile-His-Arg-Val-Pro-Leu-Leu-Lys-Gly-Lys-Ser-Leu-Arg-
Calf  Ala-Glu-Ile-Thr-Arg-Ile-Pro-Leu-Tyr-Lys-Gly-Lys-Ser-Leu-Arg-
            15                  20                  25
Pig         Gln-Asn-Leu-Ile-Lys-Asp-Gly-Lys-Leu-Lys-Asp-Phe-Leu-
Chick       Lys-Gln-Leu-Lys-Asp-His-Gly-Leu-Leu-Glu-Asp-Phe-
Calf        Lys-Ala-Leu-Lys-Glu-His-Gly-Leu-Leu-Glu-Asp-Phe-Leu-
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One or two of the peptides released on activation of pepsinogen can act as inhibitors of pepsin at pH values above 4 (Herriott, 1941). The peptide from residues 1–17 in cow pepsinogen (Harboe *et al.*, 1974) and peptides from 1–16 and 27–38 in pig pepsinogen (Anderson & Harthill, 1973; Wang & Edelman, 1971) are efficient inhibitors of the milk-clotting activity of pig (and cow) pepsin(s). In contrast, the pig peptide (residues 1–16) is unable to inhibit calf chymosin (Kay & Dykes, 1977). Thus we decided to examine the inter-species inhibitory activity of the chicken activation peptide.

Experimental

Chicken pepsinogen and pepsin were prepared as described previously (Keilova & Kostka, 1975; Kostka *et al.*, 1977). Chymosin was obtained by further purifying a crude commercial preparation (Koch–Light Laboratories, Colnbrook, Bucks., U.K.) by the method of Foltmann (1970). Pig pepsin was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Pepstatin was generously given by Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan. Automatic sequence analysis (Edman & Henschen, 1975) in a Beckman Spinco model 890C sequencer was done by using chemicals supplied by the manufacturers and Quadrol program no. 07172. The amino acid phenylthiohydantoin derivatives were identified by t.l.c. on silica-gel layer sheets and by g.l.c.

The proteolytic activity of chicken pepsin was measured at pH2 with 2% (w/v) haemoglobin as substrate (Keilova & Kostka, 1975). The milk-clotting activity of chicken pepsin, pig pepsin or chymosin was measured as described by McPhie (1976), with diluted skim milk (1ml of milk, 4ml of 0.1M-$CaCl_2$, 45ml of 0.2M-sodium acetate buffer, pH5.3) as substrate. Measurements were made at 25°C in an Opton PM-QII spectrophotometer at 500nm. The clotting time was taken from recorded curves at the point when $A_{500} = 0.4$ was reached.

The inhibitory activity of the activation peptide from chicken pepsinogen towards the milk-clotting activity of chicken pepsin, pig pepsin and chymosin was tested by preincubating each enzyme with different volumes of the peptide solution (0.5mg in 1ml of 0.2M-sodium acetate buffer, pH5.3) for 10min at room temperature (20°C) and then measuring the residual milk-clotting activity as described above.

The activation of chicken pepsinogen (10μmol) at pH2.5 in the presence of pepstatin (22μmol) was carried out as described for pig pepsinogen (Dykes & Kay, 1976), as was the subsequent freeze-drying and extraction of the freeze-dried powder with 0.05M-sodium formate buffer, pH3.5, to separate the insoluble 'protein' material from the soluble 'peptide' fraction. Dykes & Kay (1976) then used an affinity column, polylysine–Sepharose, to fractionate the activation peptide plus the excess of pepstatin from some 'protein' material that had become solubilized. However, since chicken pepsinogen has little affinity for polylysine–Sepharose under the conditions used (C. W. Dykes, unpublished work), an alternative fractionation procedure was adopted. Since the activation mixture of chicken pepsinogen plus pepstatin was totally without activity towards haemoglobin at pH2, it was possible to chromatograph the soluble extract at acid pH so as to prevent the possible formation of complexes between activation peptide and protein. Consequently the supernatant from the formate extraction was freeze-dried, redissolved in 0.3M-HCl and chromatographed on Sephadex G-100 (Fig. 1). Fractions A and B from this step contained high-molecular-weight material, and on subsequent chromatography (separately) on Sephadex G-50 both fractions emerged as one peak in the void volume of the column. Neither fraction was homogeneous as assessed by gel electrophoresis at pH8.3 or by *N*-terminal analysis, and it would seem likely that these fractions correspond to the 'protein' material described by Dykes & Kay (1976) for pig pepsinogen, i.e. mixtures of pepsinogen minus its activation peptide (pseudopepsin plus residual unactivated pepsinogen). Fraction D contained only salt. Fraction C was itself resolved into three subfractions (designated CI, CII and CIII) by chromatography on Sephadex G-25 (Fig. 2). Fraction CI was very similar to fraction B in its electrophoretic

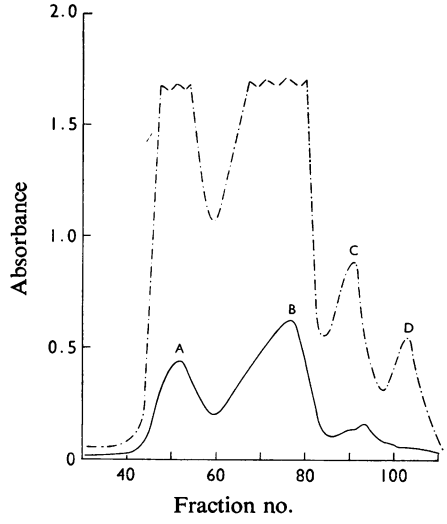

Fig. 1. *Chromatography on Sephadex G-100*
The extract (15ml) of the activation mixture was applied to a column (4cm×110cm) of Sephadex G-100, equilibrated in 1mM-HCl. Fractions (10ml) were collected. ——, A_{280}; –·–·, A_{230}.

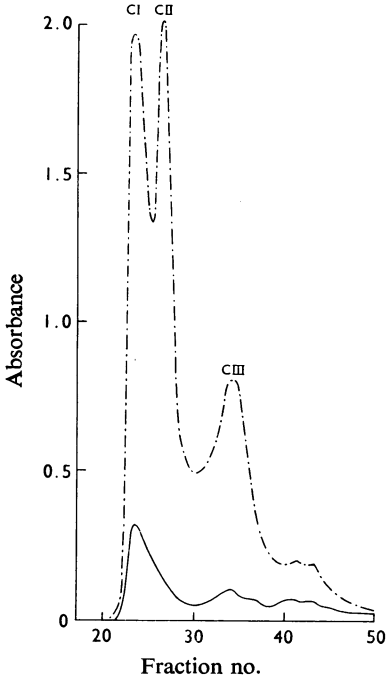

Fig. 2. *Chromatography of the activation peptide on Sephadex G-25*

Fraction C (from Fig. 1) was dissolved in 4ml of 0.2% NH_4HCO_3 and applied to a column of Sephadex G-25 (2.2cm×58cm) in the same buffer. Fractions (5ml) were collected. ——, A_{280}; –·–·, A_{230}. Fraction CII was rechromatographed on the same column, and a single peak was eluted in the identical position.

behaviour, and fraction CIII contained very little in the way of peptide material. Fraction CII was homogeneous as determined by end-group analysis, with serine being the only residue observed. The yield of peptide was 3.5μmol (35% of theoretical). The composition of this peptide (with the expected number of residues of each amino acid from residues 1–26 in chicken pepsinogen given in parentheses) was: lysine 4.85 (4); histidine 1.69 (2); arginine 1.69 (2); aspartic acid 2.30 (2); threonine 0.38 (0); serine 1.90 (2); glutamic acid 2.24 (2); proline 1.14 (1); glycine 1.97 (2); alanine 0.35 (0); valine 1.10 (1); isoleucine 0.93 (1); leucine 6.00 (6); tyrosine 0.28 (0); phenylalanine 0.87 (1). The complete sequence of peptide CII was elucidated by automatic Edman degradation and found to be:

Ser-Ile-His-Arg-Val-Pro-Leu-Leu-Lys-Gly-Lys-Ser-Leu-Arg-Lys-Gln-Leu-Lys-Asp-His-Gly-Leu-Leu-Glu-Asp-Phe-

This is in agreement with *N*-terminal 26 residues in chicken pepsinogen (Kostka *et al.*, 1977).

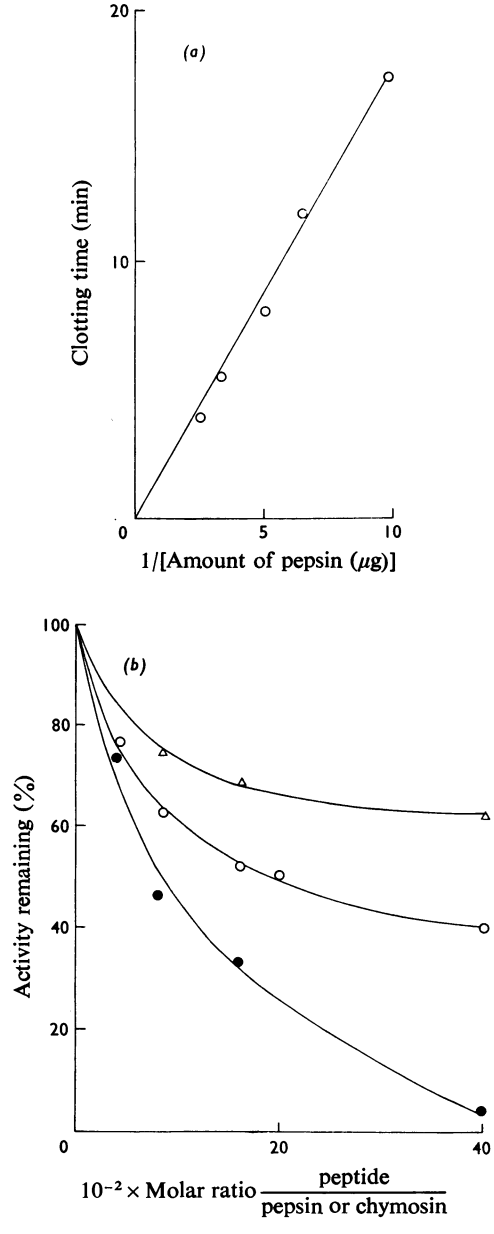

Fig. 3. *Inhibition of milk-clotting activity by the chicken activation peptide*

(*a*) The dependence of clotting time on the amount of pepsin used (400, 300, 200, 150, 100ng respectively). (*b*) Inhibition of the activity of chicken pepsin (200ng; ○), pig pepsin (100ng; ●) and calf chymosin (100ng; △) by various amounts of the chicken 1–26 activation peptide. Each enzyme was preincubated for 10min at room temperature with the solution of peptide (0.5mg in 1ml of 0.2M-sodium acetate buffer, pH5.3) before determination of the residual milk-clotting activity.

Before the ability of the activation peptide (CII) from chicken pepsinogen to inhibit the milk-clotting activity of pepsin or chymosin at pH5.3 could be tested, it was necessary to ensure that the peptide was not contaminated by the potent inhibitor, pepstatin. This was achieved by demonstrating that the proteolytic activity of chicken pepsin towards haemoglobin at pH2 was not affected by the presence of peptide CII.

In the milk-clotting experiments at pH5.3, the linear dependence of the clotting time on the amount of chicken pepsin used is shown in Fig. 3(*a*). Peptide CII inhibits the milk-clotting activities of chicken and pig pepsin and chymosin, but to different extents (Fig. 3*b*).

Discussion

The intact peptide corresponding to the *N*-terminal 26 residues in chicken pepsinogen was obtained in a yield (35%) comparable with that obtained for pig pepsinogen (Dykes & Kay, 1976). This, together with the lack of evidence for any other, shorter, peptide, would appear to suggest that chicken pepsinogen activates to release in the first instance a peptide containing the *N*-terminal 26 residues. This is analogous to the case for calf prochymosin and in contrast with the situation observed for pig (and cow) pepsinogens (Kay & Dykes, 1977). By comparison of the sequences, it can be seen that, whereas it is a Leu^{17}–Ile^{18} bond that is being hydrolysed in the pig (and cow) zymogen(s), it is a Leu–Lys bond that occupies this position in the chicken and calf proteins. While a Leu–Ile bond should be readily accommodated in the inherent, hydrophobic, active site (Fruton, 1976), a Leu–Lys bond might be expected to be more resistant to a peptic hydrolysis, so that the initial split is deferred to a subsequent, susceptible, bond.

The three-dimensional structures of the acid proteinases produced by three different micro-organisms have been elucidated and have been found to be so similar in their features (Subramanian *et al.*, 1977; Hsu *et al.*, 1977) that it may be predicted that the mammalian pepsins will be found to contain essentially the same structure. On this basis, the free amino group of the *N*-terminal isoleucine residue in pepsin would be so far removed in space from the active-site cleft of the protein that it would be impossible to form pepsin from pepsinogen (intramolecularly) in only one step. However, the distances involved are adequate to accommodate both the Leu^{17}–Ile^{18} bond and subsequently the Phe^{26}–Leu^{27} bond for hydrolysis in the active-site cleft of the same molecule (M. N. G. James, personal communication).

In contrast with the inhibitory capabilities of the pig 1–16 activation peptide, the chicken 26-residue peptide appeared to be able to inhibit chymosin, chicken pepsin and pig pepsin to varying extents. However, the molar ratios of chicken peptide/proteinase used were about 20–40 times higher than those used to test the inhibitory capacity of the pig peptide towards chymosin and pig pepsin (Kay & Dykes, 1977). It is possible, therefore, that chymosin might be sensitive to the pig 1–16 peptide at sufficiently high molar ratios.

Pig pepsin was inhibited almost completely by both peptides, but at different molar ratios. This may indicate an effect dependent on the difference in chain length of the two peptides (16 or 26 residues). There is a high degree of homology in the sequences, and it may be that the longer chicken peptide is not accommodated as readily in the active-site cleft. It would be interesting to see whether the inhibitory capacity of the chicken peptide for pig pepsin could be increased by shortening the chain length.

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