Conversion of Pepsinogen into Pepsin is Not a One-Step Process

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By incubation of pepsinogen with pepstatin at pH 2.5, the first 'active' protein generated on activation is trapped in an inactive complex. The first activation peptide liberated has been identified as residues 1–16 from the pepsinogen sequence. This suggests a sequential mechanism rather than a one-step formation of pepsin.

Pepsinogen converts itself into pepsin (Kassell & Kay, 1973) by undergoing limited proteolysis in which an *N*-terminal segment is cleaved off. At pH values below 3 the activation follows an intramolecular mechanism, whereas between pH 3 and 5 the reaction is intermolecular (Al-Janabi *et al.*, 1972).

Two reaction pathways are possible for this transformation. (1) Pepsinogen is converted into pepsin directly, liberating the activation segment as an intact polypeptide. In practice, this peptide has never been detected, and it has been assumed that this is due to digestion of the peptide to smaller pieces by the active pepsin formed. (2) Pepsinogen is converted into pepsin by a sequential process in which peptides are successively removed from the *N*-terminus until all 44 residues in the activation segment have been removed, e.g.:

Pepsinogen → peptide a + intermediate protein A

Intermediate protein A → peptide b + intermediate protein B

Intermediate protein B → peptide n + pepsin

The former possibility has been widely accepted as correct, with little experimental substantiation, but both possibilities, in addition to generating active pepsin, account for the number of activation peptides usually observed.

It should be possible to distinguish between them, if the active product could be trapped into an inactive complex as soon as it is formed. This would prevent further digestion of the entire activation segment in case (1) and further activation in case (2), so that it should be possible to isolate in case (1) the intact activation segment, whereas in case (2) a smaller peptide, such as peptide a, should be recovered.

The complete sequence of pig pepsinogen is known (Stepanov *et al.*, 1973; Sepulveda *et al.*, 1975) and the activation segment sequence (residues 1–44) is:

Pepstatin does not bind to pepsinogen at pH values between 2.5 and 5 (J. Kay & C. W. Dykes, unpublished work), but it is an extremely potent inhibitor of pepsin in the same pH range (Aoyagi *et al.*, 1971). This should serve as a useful trapping agent as long as intermediate protein A is active and therefore capable of being inhibited. If case (2) is correct, then intermediate protein A must be active (or it would not be possible to form pepsin).

Materials and Methods

Pig pepsinogen was purified to homogeneity from pig stomachs (Ryle, 1970) with a final step of chromatography on polylysine-Sepharose (Kay, 1972). Pepstatin was given very generously by Professor H. Umezawa.

Proteolytic activity measurements were made against haemoglobin at pH 2 (Anson, 1948) and Azocoll at pH 5.3 (Kay, 1975). Activation experiments with pepstatin were performed by adding pepstatin (6.2 mg/ml in methanol; 0.14 ml) to 32 ml of 3.2 M-HCl and mixing with a Vibramixer. This solution was cooled to 4°C and pepsinogen (36 mg/ml in 4 ml of water) was added (pepstatin/pepsinogen molar ratio 1.4:1). After being stirred for 2.5 h at 4°C the mixture was spun in a bench centrifuge to remove a small amount (<1%) of insoluble material. The supernatant was freeze-dried and the resultant powder was extracted (3 × 2 ml) with 0.05 M-sodium formate buffer, pH 3.5. Most of the 'protein' was insoluble, whereas the supernatant contained mostly 'peptide'. The latter was applied to a polylysine-Sepharose column (1.8 cm × 16 cm) in 0.05 M-formate buffer, pH 3.5.

Variations in the above conditions were performed (1) by adding 0.09 ml of pepstatin solution so that the pepstatin/pepsinogen molar ratio was 0.9:1, and (2) by incubation in 0.05 M-formate buffer, pH 4.1.

Leu-Val-Lys-Val-Pro-Leu-Val-Arg-Lys-Lys-Ser-Leu-Arg-Gln-Asn-Leu-Ile-Lys-Asp-Gly-
 Lys-Leu-Lys-Asp-Phe-Leu-Lys-Thr-His-Lys-His-Asn-Pro-Ala-Ser-Lys-Tyr-Phe-Pro-Glu-
 Ala-Ala-Ala-Leu-Ile-Gly-Asp~~~~~Pepsin~~~~~Pro-Val-Ala

Results

After activation of pepsinogen in the presence of pepstatin (pepstatin/pepsinogen molar ratio 1.4:1), the freeze-dried material was extracted with formate buffer, giving an insoluble and a supernatant fraction.

(a) Insoluble material

This was completely inactive against haemoglobin at pH 2 and Azocoll at pH 5.3. *N*-Terminal analysis with dansyl chloride showed both isoleucine and leucine, and polyacrylamide-gel electrophoresis at pH 8.1 revealed two bands, one with a mobility similar to that of pepsinogen and the other major band with a mobility intermediate between those of pepsinogen and pepsin.

The amino acid composition is similar to the composition calculated from the sequence of residues 17–370 in pepsinogen (Table 1).

When the pepstatin/pepsinogen molar ratio was lowered to 0.9:1, this insoluble 'protein' fraction had 10% of the specific activity of pepsin against haemoglobin and Azocoll. The composition of this material, with pepsin for comparison, is also included in Table 1.

(b) Supernatant

This was chromatographed on polylysine-Sepharose at pH 3.5. Peptides are not retarded on this column, which acts as a mixed affinity/ion-exchange resin for pepsinogen, pepsin and their derivatives

(Kay, 1972). A peptide fraction (A) passed unretarded through the column, and a second fraction (B) was eluted only on application of a linear NaCl gradient. Fraction B was found to be very similar to the insoluble material already described.

Samples of fraction A were taken for amino acid analysis and *N*-terminal analysis with dansyl chloride (Table 2). The 'crude' compositions are similar to that calculated from the sequence of residues 1–16 in pepsinogen, and it seems to make little difference to the results whether the activation is performed above or below pH 3. The compositions show alanine and valine from the excess of pepstatin added, since this compound is not retarded either on polylysine-Sepharose. It has an acylated *N*-terminus and so is unreactive to dansyl chloride. The yield of 'crude' peptide obtained varied from batch to batch, being within 55–100% of the theoretical value.

After removal of the excess of pepstatin by chromatography on a Bio-Gel P2 column (1 cm × 100 cm) in 0.5% NH₄HCO₃, the purified peptide material had the composition: Lys, 2.86; Arg, 1.90; Asp, 1.00; Ser, 1.00; Glu, 1.07; Pro, 0.89; Gly, 0.16; Val, 2.84; Leu, 3.94; this corresponds to that of residues 1–16 in pepsinogen. *N*-Terminal analysis showed leucine as the major residue, but very faint amounts of lysine and valine were also detected. The *C*-terminal residue was found to be leucine also, from a time-course of digestion with carboxypeptidase A. High-voltage paper electrophoresis at pH 6.5 (pyridine/acetic acid/water, 25:1:475, by vol.) and t.l.c. (in butan-1-ol/acetic acid/water/pyridine, 15:3:12:10, by vol.) both

Table 1. Amino acid compositions of the insoluble 'protein' fractions

Samples were hydrolysed for 24 h *in vacuo* in 6M-HCl at 105°C. Values are not corrected for losses on hydrolysis.

Amino acid	Amino acid composition (mol of residue/mol)			
	'Protein' from pepstatin+pepsinogen (molar ratio 1.4:1)	Expected from sequence of residues 17–370	'Protein' from pepstatin+pepsinogen (molar ratio 0.9:1)	Pepsin (residues 45–370)
Lys	8.05	7	1.37	1
His	2.96	3	1.07	1
Arg	3.29	2	1.88	2
Asp	42.3	45	44.6	42
Thr	23.0	27	25.2	26
Ser	37.2	44	40.0	43
Glu	27.9	27	28.3	26
Pro	16.7	17	17.1	15
Gly	35.7	36	39.1	35
Ala	22.0	20+1*	20.7	17+1*
Val	23.0	22+2*	21.5	22+2*
Met	3.4	4	3.7	4
Ile	24.3	27	22.6	26
Leu	32.4	29	27.7	26
Tyr	15.5	17	15.0	16
Phe	16.0	16	14.0	14

* Including 1 and 2 mol of alanine and valine from 1 mol of pepstatin bound/mol of pepsin.

Table 2. *Amino acid compositions of the 'peptide' fractions*

Samples were hydrolysed for 24h *in vacuo* in 6M-HCl at 105°C. Values are not corrected for losses on hydrolysis.

Amino acid	Amino acid composition (mol of residue/mol)				
	Pepstatin/pepsinogen molar ratio 4:1.1		Expected from sequence of residues 1-16	Pepstatin/pepsinogen molar ratio 0.9:1 Activation at pH 2.5	Expected from sequence of residues 1-44
	Activation at pH 2.5†	Activation at pH 4.1			
Lys	2.68	2.18	3	6.60	9
His	0	0	0	1.76	2
Arg	1.62	1.45	2	1.45	2
Asp	1.00	1.00	1	3.97	4
Thr	0	0	0	1.45	1
Ser	1.03	1.17	1	2.37	2
Glu	1.12	1.24	1	2.43	2
Pro	0.95	0.99	1	3.14	3
Gly	0.73	1.13	0	1.91	1
Ala	1.03*	1.45*	0	2.82	4
Val	3.83*	4.64*	3	2.33	3
Ile	0	0	0	1.06	1
Leu	3.43	3.62	4	5.10	7
Tyr	0	0	0	1.15	1
Phe	0	0	0	2.60	2
No. of activations performed	8	1		1	
<i>N</i> -Terminus	Leu	Leu	Leu	Many	

* Including 1 and 2 mol of residues of alanine and valine/mol from excess of pepstatin.

† Mean values from the eight activations performed.

showed the presence of one major basic peptide, but in both cases very faint additional spots could be detected with ninhydrin.

When the pepstatin/pepsinogen molar ratio was 0.9:1, the composition of the 'crude' peptides obtained was much more similar to the expected composition from residues 1-44 (Table 2). Several *N*-termini were observed, a result similar to that obtained on activation of pepsinogen in the absence of pepstatin.

Discussion

No evidence in support of case (1) has been found. It has not been possible to isolate a peptide corresponding to intact residues 1-44 even when the pepstatin/pepsinogen molar ratio was less than 1:1. In this case several peptides were obtained and the 'protein' fraction was very similar to pepsin. Presumably the pepstatin can inhibit 90% of the pepsinogen activating to intermediate protein A but the remaining 10% can convert itself all the way into pepsin. This active pepsin could then split off the remaining activation segments exposed on the inhibited intermediate protein A, so that a mixture

of 90% inhibited pepsin + 10% active pepsin is obtained.

Thus it would seem that pepsinogen does not convert itself into pepsin in a one-step transformation, releasing the activation segment 1-44. By difference, it would appear that the alternative, progressive activation, scheme must be operative. Certainly the amino acid composition and *N*- and *C*-terminal analyses of the peptide fraction obtained on activation at a molar ratio of 1.4:1 all suggest it to have been derived from residues 1-16 in the pepsinogen sequence.

The use of pepstatin to stop the activation, of course, precludes identification of the subsequent steps in the transformation. If the activation does proceed in a stepwise fashion, it is uncertain whether a limited number of discrete steps are involved or whether a 'ragged' activation at many susceptible peptide bonds could occur. The extra spots observed on electrophoresis and chromatography and the other faint *N*-termini suggest this could be so. Though most (90%) of the pepsinogen molecules have undergone proteolysis at the leucine-16-isoleucine-17 bond as the first step of activation, releasing peptide 1-16 as peptide a, it would seem

that a small amount (<10%) may have undergone additional proteolysis within the 1-16 sequence.

It is noteworthy that a peptide from bovine pepsinogen, homologous to residues 1-16 from the pig protein, has been suggested (Harboe *et al.*, 1974) to be the pepsin inhibitor peptide first mentioned by Herriott (1941). It seems to make sense, from a control point of view, to have the first bond being hydrolysed on activation releasing the pepsin inhibitor so that it can exert its effect if required.

It could be argued that activation in the presence of a non-physiological molecule like pepstatin could alter the activation process. However, since only a very low molar excess was used and since such profound differences were found on increasing the ratio from 0.9:1 to 1.4:1, it would seem that such criticism is not valid.

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