IDENTIFICATION OF AN ENZYMATICALLY ACTIVE INTERMEDIATE IN THE ACTIVATION OF PORCINE PEPSINOGEN

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1. Introduction

The proteinases of the gastric juice are secreted as inactive precursors which are irreversibly converted into the usually known enzymes by limited proteolysis releasing from 42–45 amino acid residues [1–4]. To initiate this process no other activating agents than hydrogen ions are required. It is generally accepted today that the initial stage in the activation process is a conformational change of the zymogen molecule from inactive to active conformation [5–8], but for the subsequent pathway of the activation, different views have been reported.

Bustin and Conway-Jacobs [9] coupled porcine pepsinogen A to Sepharose in such concentrations that interactions between individual pepsinogen molecules would be very limited. After exposure to acid, active enzyme and new N-terminal isoleucine were observed. Al-Janabi et al. [10] showed by activation of porcine pepsinogen A in solution at pH 2 that the conversion of alkali-stable pepsinogen to an alkali-labile product follows a first order reaction. Sanny et al. [11] found that the initial cleavage product formed during activation at pH 2 had the N-terminal sequence Ile–Gly–. They concluded that during activation of porcine pepsinogen at pH 2 the peptide bond Leu_{44}–Ile_{45} is the first to be cleaved. This is in contrast to experiments of Dykes and Kay [12]. At pH 2.5 they activated porcine pepsinogen in presence of the inhibitor pepstatin [13] and from the amino acid composition of the peptides produced during the reaction they inferred that peptide bond Leu_{16}–Ile_{17} was cleaved first. For the intermediate (which was inhibited in their experiments) they suggested the name 'pseudopepsin'.

The experiments reported in this paper confirm that at pH 2 the predominant initial cleavage occurs at peptide bond 16–17. This cleavage is the results of an intramolecular reaction and we show that the pseudopepsin has proteolytic activity and is alkali-labile.

2. Experimental

Porcine pepsinogen A was from Worthington (lot No. PG 1GB). Amino acid analyses were performed with a Durrum D-500 analyzer.

2.1. *Milk clotting assay*

Skim-milk powder (12 g) was reconstituted with 50 ml water. After addition of 50 ml 0.4 M sodium acetate, pH 4.9, the final pH was 5.3. The assay was carried out at 30°C in bifurcated tubes as described by Foltmann [5]. Clotting times from 1–15 min were found to be inversely proportional to enzyme concentration. The activity which gave a clotting time of 1 min was used as milk-clotting unit (U). The specific activity was calculated as U/ml A_{280}.

To determine the maximum activity of the preparation, activation in solution was carried out for 20 min at 25°C with 2.3 mg pepsinogen/ml in 0.015 M HCl containing 0.015 M KCl. The specific activity of this mixture was 22 U/ml A_{280}. The activity of the activation mixtures are expressed as percent of this maximum activity.

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2.2. *Activation of pepsinogen in solution*

To investigate the appearance of milk-clotting activity, activation experiments were carried out with low concentrations of pepsinogen (0.08–0.32 mg/ml) in salt-free solutions at 0°C (ice-bath). For each determination 100 μl pepsinogen solution was rapidly mixed with 100 μl 0.02 M HCl (magnetic stirring), the resulting mixture was found to have pH 2.1, independent of the pepsinogen concentration used. After the required period of activation, the reaction was stopped by rapid addition 900 μl 0.1 M sodium citrate, pH 5.3. Aliquots of such solutions were used for the milk clotting assay. To check that no further activation took place at pH 5.3 the following test was made: a sample of pepsinogen was activated for 40 s as described above; the activity of this solution was measured several times during a working day period, and no change in activity was observed.

For alkaline inactivation of enzymes formed during the activation of pepsinogen, 200 μl of an activation mixture was mixed with 200 μl 0.5 M Tris, pH 8.5. The solution was left for 20 min at 25°C and the milk clotting activity was determined after adjustment to pH 5.3 with 700 μl sodium citrate 0.1 M, pH 4.25.

To determine formation of protein with new N-terminal amino acid sequences during the activation of pepsinogen, reaction mixtures containing 0.8 mg pepsinogen (20 nmol) in 2.5–10 ml 0.01 M HCl were precipitated by rapid addition of 50% trichloroacetic acid to a final concentration of 20%. The mixtures were left for at least 30 min at 0°C, the precipitates were collected by centrifugation, washed with 3 × 1 ml ice cold acetone and dried. The N-terminal amino acid sequences were then determined as described by Klemm et al. [14]. In this procedure the liberated residues are indentified by quantitative amino acid analysis after conversion with hydriodic acid [15].

2.3. *Activation of Sepharose-bound pepsinogen*

Sepharose 6B® from Pharmacia was CNBr-activated according to Marsh et al. [16] (CNBr, purum, from Fluka was used). Porcine pepsinogen was coupled to the matrix as described by Bustin and Conway-Jacobs [9]. After acid hydrolysis the concentration of pepsinogen bound to Sepharose was determined by amino acid analysis.

Sepharose-pepsinogen (0.3–0.5 g wet wt) was packed in columns 6 mm diameter. Activation was initiated by addition of 0.5 ml 0.1 M HCl followed by 1.1 ml 0.01 M HCl and the flow was stopped. The exposure to acid varied from 30 min–20 h, then the column was washed with 1 ml 0.5 M sodium hydrogencarbonate, pH 9.8, containing 1.3% SDS (the coupling buffer used for sequencing). With 300 μl of this buffer the content of the column was transferred to tubes used for Edman degradation. Unactivated material was prepared for sequencing in the same way, but without addition of acid.

3. Results and discussion

The conversion of pepsinogen into active enzymes was observed through the increase of milk-clotting activity; this assay was carried out at pH 5.3. We cheked that no further activation occurred at pH 5.3, and so we were able to assay the active enzymes in presence of zymogen. Furthermore the conversion of pepsinogen was studied by determinations of N-terminal amino acid sequences of protein in solutions and of Sepharose-bound material.

The results of the sequencing experiments are shown in fig.1. In unactivated preparations of Sepharose-bound pepsinogen only the N-terminal sequence of the zymogen was observed. After exposure to acid, the

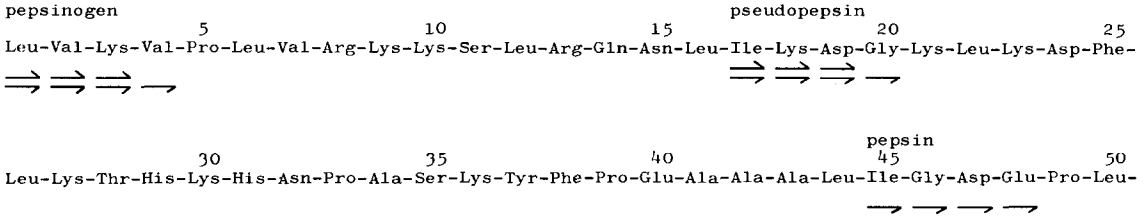

Fig.1. N-Terminal sequence of porcine pepsinogen A [18–20]. (⟶) Indicates the sequence found in activated Sepharose-bound pepsinogen. (⟶) Indicates the sequences observed after activation of pepsinogen in solution.

N-terminal propart of the zymogen remains coupled to the matrix, but in addition to this, the only N-terminal sequence was Ile–Lys–Asp–, corresponding to residues 17–19, the N-terminal sequence of pseudopepsin. There was no difference whether the pepsinogen had been exposed to acid for 30 min or for 20 h, and the same sequence was found in preparations containing from 0.9–1.8 mg pepsinogen/wet wt Sepharose.

Figure 1 further shows the N-terminal sequences determined after activation in solution. Only residues that could be assigned to the N-terminal sequences of porcine pepsinogen, pseudopepsin and pepsin were observed. Therefore it is most likely that the sequential activation of pepsinogen, at pH 2, somprises only two cleavage steps. If pseudopepsin is cleaved at another bond that the one preceding pepsin, the product disappears very fast compared to pseudopepsin.

Both pseudopepsin and pepsin have isoleucine as N-terminal residue. We can therefore quantitate the cleavage of pepsinogen by determination of trichloroacetic acid precipitated material with N-terminal isoleucine. The cleavage rate of pepsinogen is presented in fig.2. The pepsinogen gradually disappears, but after 30 min as well as after 60 min 10% of uncleaved pepsinogen was left. The relative amount of cleavage was corrected for this apparently unreactive material. Within the range of pepsinogen concentrations used in the experiments presented in fig.2 the rate of cleavage is independent of the concentrations. This indicates that with the present conditions of activation, the rate limiting step is a first-order reaction, representing either a conformational change or intramolecular cleavage of the zymogen.

The formation of N-terminal isoleucine represents the sum of preudopepsin and pepsin. The ratio between the two proteins was calculated from the ratio between lysine and glycine liberated at Step 2 of the Edman degradation and between glycine and glutamic acid liberated at Step 4. The quantitative analyses showed a background of 10–15% of glycine and alanine, so the accuracy of the method only allows presentation of results as integers of 10 such as shown in table 1. These results confirm that the predominant

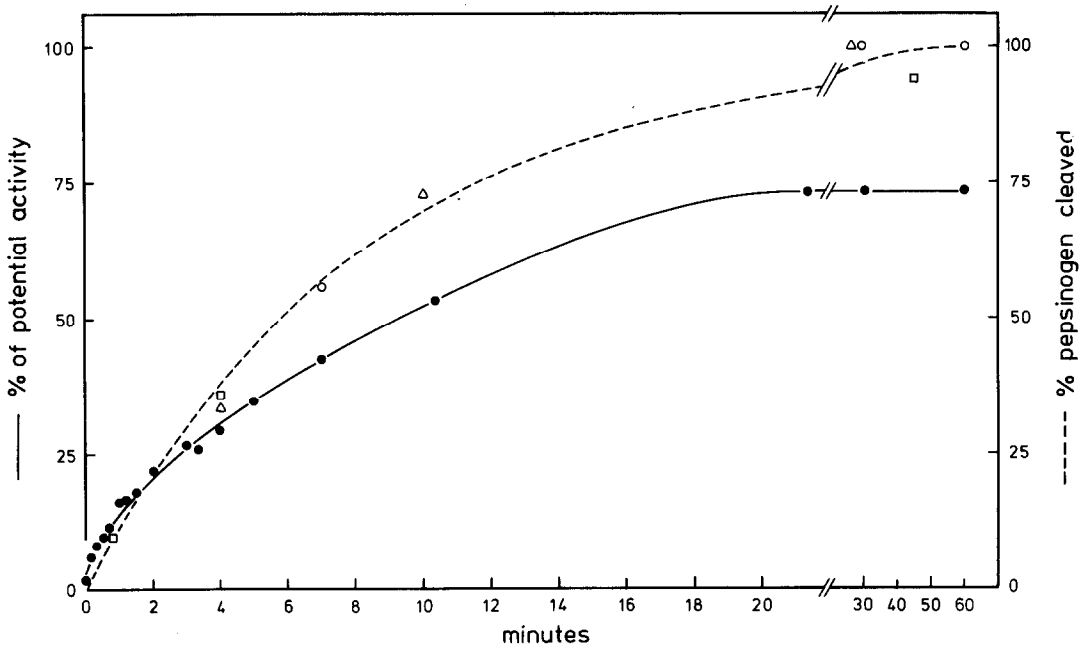

Fig.2. Appearance of milk-clotting activity during activation of pepsinogen 0.08 mg/ml, (•) (solid line). Appearance of N-terminal isoleucine by activation of pepsinogen (○) 0.08 mg/ml, (□) 0.13 mg/ml, (△) 0.16 mg/ml. The dashed-line curve shows the course of a first-order reaction with the velocity-constant 0.12 min^{-1}.

Table 1
Activation of porcine pepsinogen A

Pepsinogen concentration (mg/ml)	Percent pseudopepsin of cleaved pepsinogen					
	Activation time (min)					
	0.8	4	7	10	30	60
0.08	–	–	70	–	70	60
0.13	100	80	–	–	–	–
0.16	–	80	–	60	40	–
0.32	–	–	–	–	30	–

–Not determined

Pseudopepsin and pepsin were determined by quantitative sequencing of the first four residues. The relative amounts of pseudopepsin are expressed as percent of the sum of pseudopepsin and pepsin at any given time.

initial cleavage product, at pH 2, is pseudopepsin. They further suggest that the subsequent formation of pepsin is concentration-dependent. In more concentrated solutions, pseudopepsin is converted more rapidly into pepsin, and this may explain why Sanny et al. [11] observed only pepsin in their experiments with activation of pepsinogen in higher concentrations than we used here.

The rate of appearance of milk-clotting activity during activation of porcine pepsinogen, 0.08 mg/ml, is presented in fig.2. The specific activity reaches a plateau which is 73% of the maximum specific activity obtained by full activation in a more concentrated solution. From table 1 it is seen that there is 70% pseudopepsin in the mixture when the plateau activity is reached. Therefore we can conclude that pseudopepsin has milk-clotting activity. We also found that it is alkalin-labile; after exposure to pH 8.5 at 25°C for 20 min less than 5% of the milk clotting activity was left.

Comparison of the appearance of new N-termini and determination of enzymatic activity through the milk-clotting assay is complicated for several reasons: we do not know the exact activity of pseudopepsin relative to pepsin, and we do not yet know if the propart peptide (inhibitor peptide [1,17]) dissociates slowly from pseudopepsin or pepsin in our assay. However, it is our experience that the inhibition appears weaker in citrate than in acetate, therefore the effect has been minimized by the use of citrate to adjust the pH to 5.3 after activation. Furthermore the activity is compared with the maximum activity of an activation mixture also containing inhibitor peptides. The low value of the activity at the plateau in fig.2 could be explained by a lower activity of pseudopepsin compared with the activity of pepsin or by stronger inhibition of pseudopepsin.

Through we cannot account for all quantitative aspects of the activation, the results unambiguously demonstrate the presence of enzymatically active pseudopepsin as an intermediate in the activation of porcine pepsimogen at pH 2. Other pathways of activation may dominate at higher values of pH, but experiments in progress indicate that activation at pH 2 of bovine pepsinogen and prochymosin is analogous to that of porcine pepsinogen.

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