THE PRIMARY STRUCTURE OF PROCHYMOSIN (PRORENNIN) EC 3.4.4.3.
SOME TRYPTIC FRAGMENTS OF A MALEYLATED PREPARATION

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1. Introduction

Chymosin (EC 3.4.4.3) is the predominant protease in the fourth stomach of the young calf. For many years the term rennin has been used in English literature, but since mistakes often occur between rennin from the calf stomach and renin (EC 3.4.4.15) from the kidneys, we return to the older term chymosin, which previously has been used in European continental literature. Like other proteases from the digestive tract chymosin is secreted as an inactive precursor, which is transformed into active enzyme by a limited proteolysis. The chemistry of the enzyme and the kinetics of the activation process have been reviewed a few years ago [1]. This communication describes isolation and analyses of some peptides obtained after tryptic digestion of prochymosin in which the amino groups were blocked by reaction with maleic anhydride, and it is shown that these peptides constitute the *N*-terminal and the *C*-terminal amino acid sequences of the zymogen.

2. Experimental

Prochymosin was extracted from dried calf stomachs and purified as previously described [1]. From the chromatographic fractionation the dominating component, prochymosin-B, was used for the experiment.

Prochymosin was reduced and amino-ethylated according to Cole [2]. After dialysis and freeze-drying the sample was redissolved to give a 5% solution, and the free amino groups were blocked by reaction with maleic anhydride [3]. The surplus of maleic acid was removed by dialysis, and the preparation was freeze-dried once more.

460 mg of the freeze-dried preparation (corresponding to ca. 13 μmole) was digested with 9 mg of TCPK-trypsin (Merck, Germany) at pH 9, maintained by addition of 0.1 M NaOH. After 3 hr of digestion about 100 μmole of peptide bonds were hydrolyzed, and the digestion was stopped by adding 6 mg of Trasylol (pancreatic trypsin inhibitor, Merck). The digest was subjected to gel-filtration on a column of Sephadex G-75 (5 × 100 cm) equilibrated with a solution of 0.05 M ammonia in 40% ethanol. No peptides were obtained in pure state after the gel filtration. The fractions of the eluate which contained the low molecular weight peptides were concentrated in a rotatory vacuum evaporator and purified by high voltage paper electrophoresis. For description of buffers and apparatus see Ambler [4].

In order to locate peptides containing maleylated amino groups guidestrips from the first electrophoresis at pH 6.5 were demaleylated by exposure to the vapour of a pyridine/acetate buffer (pH 3.5) at 60° for 8 hr [3]. By this treatment the amino groups were unblocked and the guidestrips were examined by electrophoresis under the same conditions as before, but at a right angle to the first dimension. After staining with cadmium-ninhydrin [5] the demaleylated peptides were located as spots outside the diagonal of unchanged peptides.

For preparation of the peptides the appropriate bands of the main sheets were demaleylated and subjected to electrophoresis under the same conditions as the guidestrips. The peptides were eluted, and after quantitative analysis the amino acid sequence of the individual peptides was established by sequential

Edman degradation—dansylation [6] and the DNS-amino acids were identified by chromatography on polyamide layer sheets [7, 8].

3. Results

Fig. 1 shows a diagonal map of demaleylated low molecular weight peptides of the tryptic digest. The peptides marked Tm-1 to Tm-8 were isolated in yields of 2—3 μmole, while peptides corresponding to the dash lined spots were obtained in yields of only 0.1—0.3 μmole. The latter group of peptides represents unspecific cleavages.
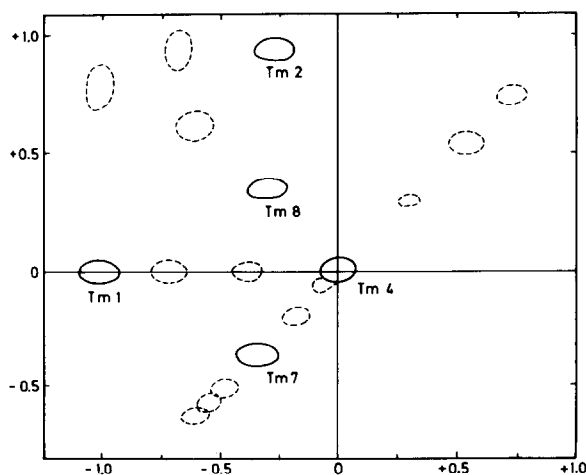

Fig. 1. pH 6.5 diagonal map of low molecular weight peptides from a tryptic digest of maleylated prochymosin. Conditions are described in the text. The mobilities are expressed relative to the mobility of an internal marker of 1-dimethylamino naphthalene-5-sulphonic acid (−1.0).

The analyses of peptide Tm-1 and Tm-2 are summarized in fig. 2.

Peptide Tm-1 is an off-diagonal peptide which does not contain any lysine, from which we may conclude that the N-terminal amino group has been maleylated. This is consistent with the observation that no ninhydrin positive zone is seen in the corresponding area of a guidestrip which has not been demaleylated. Consequently this peptide represents the N-terminal amino acid sequence of the original peptide chain. Since the peptide is neutral after demaleylation the glutamic acid cannot be amidated.

The first six residues of peptide Tm-2 were located directly by sequential Edman degradation—dansylation. The sequence of the remaining part of the molecule was established after a second tryptic digestion of the demaleylated peptide, from which the two basic peptides Tm-2, t-2 and Tm-2, t-3 were isolated. An overlapping sequence of the peptides Tm-1 and Tm-2 has eventually been provided by a peptide (AP—Cl) which has been isolated from a chymotryptic digest of activation peptides liberated during the conversion of prochymosin into chymosin [9].

The three peptides Tm-4, Tm-7 and Tm-8 turned out to represent amino acid sequences previously found in tryptic digests of active chymosin [10, 11]. The two diagonal peptides Tm-4 and Tm-7 have the sequences Phe—Asp—Pro—Arg and Glu—Tyr—Tyr—Ser—Val—Phe—Asp—Arg. The off-diagonal peptide Tm-8 has the sequence Ala—Asn—Asn—Leu—Val—Gly—Leu—Ala—Lys—Ala—Ile. The finding of high yields of a peptide with C-terminal isoleucine in a tryptic digest indicates with a high degree of evidence that this peptide forms the C-terminal sequence of the original peptide chain. An overlapping peptide between Tm-7 and Tm-8 has not been isolated from prochymosin, but since these two peptides are identi-
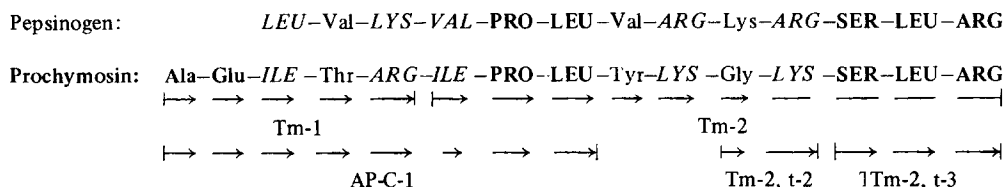

Fig. 2. The N-terminal amino acid sequence of prochymosin compared to that of porcine pepsinogen. Symbols used for comparison of the structures: Identical residues are in bold face and similar residues are in italics. Symbols used for the sequence study: —— quantitative amino acid composition; → sequential analysis by the "dansyl"-Edman techniques.

cal with the C-terminal sequence of chymosin we may take for granted that these peptides in fact represent the C-terminal amino acid sequence of prochymosin.

4. Discussion

From the observation that the N-terminal amino acids of prochymosin and chymosin were alanine and glycine respectively it has been assumed that the activation of prochymosin took place by liberation of a part of the N-terminus of the zymogen molecule. The detection of a C-terminal sequence in prochymosin which is identical with that of chymosin substantiates the general assumption that the zymogens of the acidic gastric proteases are converted into active enzymes by a limited proteolysis during which a peptide segment is cleaved from the N-terminal part of the peptide chain.

Previously it has been reported that there is a pronounced homology between the amino acid sequence of porcine pepsin and chymosin [11]. This homology has later been extended to the proteases from human gastric juice [12].

Comparison between the N-terminal amino acid sequence reported here and the N-terminal amino acid sequence of porcine pepsinogen found by Ong and Perlmann [13] shown in fig. 2 indicates a considerable degree of homology in the primary structure of the N-termini of the gastric zymogens. Three lysine and arginine groups are substituted for one another, but within the first 15 residues 4 positive charges are located the same way in the two zymogens.

From inhibition studies and studies of the enzymatic kinetics it is known that two or more dicarboxylic acids play a role in the enzymatic mechanism of the acidic proteases [14, 15]; further it is known that during the conversions of the zymogens of the gastric proteases into active enzymes, the isoelectric points are lowered due to a limited proteolysis liberating basic activation peptides [1, 13]. The homologous distribution of the positive charges in the zymogen molecules and in the active enzymes makes it tempting to suggest that these groups are important for the correct orientation of the amino acid residues in the active centre. The preponderant positive charges of the activation peptide may prevent the formation of the active centre in the zymogen, while the two arginine groups in the C-terminus which precede and terminate the sequence Tm-7 will orient the active dicarboxylic acids the right way when the activation peptide is removed.

Acknowledgement

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