N-TERMINAL SEQUENCE OF SWINE PEPSINOGEN AND PEPSIN. THE SITE OF PEPSINOGEN ACTIVATION.

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SUMMARY. The sequence of 119 amino acids of swine pepsinogen comprising the fragment released during the zymogen activation as well as the N-terminal part of pepsin is established. The activation of swine pepsinogen is shown to be accompanied by specific cleavage of Leu-Ile bond in the sequence:

41 46
Ala Ala Ala Leu Ile Gly

where Ile-45 represents the N-terminal residue of pepsin. This sequence is attacked in the course of pepsinogen activation by external enzymes - neutral proteinases and elastase.

INTRODUCTION. Although the mechanism of pepsinogen activation became recently an object of vivid discussion (1-5) the sequence of amino acids of the zymogen preceding isoleucine residue which appears as the N-terminal of pepsin was not known. Pepsinogen activation is accompanied by the cleavage of N-terminal fragment containing as shown in this paper 44 amino acids. Ong and Perlmann established the N-terminal sequence of 41 amino acids of pepsinogen and presumed that it is Glu-Ile bond which is cleaved during pepsinogen activation although the presence of one-two amino acids between these residues was not excluded (6). This assumption was accepted in the literature (cf.7) but it has been shown in our laboratory that leucine rather than glutamic acid preceds pepsin N-terminal residue -

isoleucine in pepsinogen sequence (4). The study of an extended N-terminal sequence of swine pepsinogen allows us to describe in definite terms the change of primary structure during pepsinogen activation.

MATERIALS AND METHODS. The preparation of swine pepsinogen and of leucyl-pepsin has been described elsewhere (4). Amino acid sequences were determined using Beckman-Spinco Protein-Peptide Sequencer, Model 890. The runs were performed with 370 nmol of leucyl-pepsin and 300 nmol of pepsinogen dissolved in 40 per cent pyridine. 1-Chlorobutane used for the extraction of thiazolinones contained 5.10^{-5}M dithiotreitol. Thiazolinones were converted to the corresponding phenylthiohydantoins (PTH) by treatment with 1N HCl at 80° for 10 min. PTH were analysed by gas-liquid chromatography when necessary after conversion to trimethylsilyl derivatives (8).

Peptides corresponding to the sequence 45-119 (Fig.1) were isolated from chymotryptic (9-11) or thermolytic (12) hydrolysates of pepsin N-terminal fragment B-2. Their structures were studied by conventional procedures.

Pepsinogen activation with pancreatic elastase was performed as follows. To the solution of 300 mg of pepsinogen in 50 ml of 0.1 M acetate buffer, pH 5.0, 20 mg of elastase in 10 ml of the same buffer were added. The mixture was incubated 20 h. at 36°, then 10 mg of elastase were added and the solution was kept for 24 h. at 36°. At this time the activity of the mixture measured by milk-clotting showed almost quantitative conversion of the zymogen into active enzyme. In the control mixture without the addition of elastase only 2.5 per cent of pepsinogen were activated.

The mixture was applied on DEAE-cellulose column (2 x 40 cm)

Fig.1. The N-terminal sequence of swine pepsinogen (P.). The line C. shows the homologous sequences of calf prochymosin (13).

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                                                                  15
P. Leu Val Lys Val PRO LEU Val Arg Lys LYS SER LEU ARG Gln Asn
C. Ile Thr Arg Ile PRO LEU Tyr Lys Gly LYS SER LEU ARG Lys Ala
                                                                  30
P. LEU Ile Lys Asp GLY Lys LEU Lys ASP PHE LEU LYS Thr His Lys
C. LEU Lys Gly His GLY Leu LEU Gly ASP PHE LEU LYS
                                                                  45
P. His Asn Pro Ala Ser Lys Tyr Phe Pro Glu Ala Ala Ala Leu Ile
C.                                                  Gly Glu Val
                                                                  60
P. Gly Asp Glu PRO LEU Glu ASN TYR LEU ASP Thr Glu TYR PHE GLY
C. Ala Ser Val PRO LEU Thr ASN TYR LEU ASP Ser Gln TYR PHE GLY
                                                                  75
P. Thr ILE Gly Ile GLY THR PRO Ala Gln Asp PHE Thr Val Ile Phe
C. Lys ILE Tyr Leu GLY THR PRO Pro Gly Glu PHE
                                                                  90
P. ASP THR GLY SER SER Asn Leu TRP Val Pro Ser Val Tyr CYS Ser
C. ASP THR GLY SER SER Asp Phe TRP                   CYS Lys
                                                                 105
P. SER Leu ALA CYS Ser Asp HIS Asn Gln PHE Asn PRO Asp Ser Asp
C. SER Asn ALA CYS Lys Asn HIS Gln Arg PHE Asp PRO Arg
                           P                                  119
P. Ser Thr Phe Glu Ala Thr Ser Glu Glu Leu Ser Ilr Thr Tyr
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Fig.1a. The internal homology in swine pepsinogen.

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16                                                          28
LEU ILE Lys ASP Gly Lys LEU Lys Asp Phe LEU Lys THR
LEU ILE Gly ASP Glu Pro LEU Gly Asn Tyr LEU Asp THR
44                                                          56
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equilibrated with 0.1 M acetate buffer, pH 5.6. The elution was achieved by linear gradient from the starting buffer (300 ml) to 0.5 M NaCl in the same buffer (Fig.2). The peak containing the active enzyme was collected, desalted on Sephadex G-25 and lyophilized to give 146.7 mg of the protein. The enzyme was inactivated by phenol treatment (4) and used for N-terminal assay and the automatic sequencing. 14 mg of this protein gave after dinitrophenylation, acid hydrolysis and TLC 100 nmol of DNP-Ala, 100 nmol of DNP-Leu and 70 nmol of

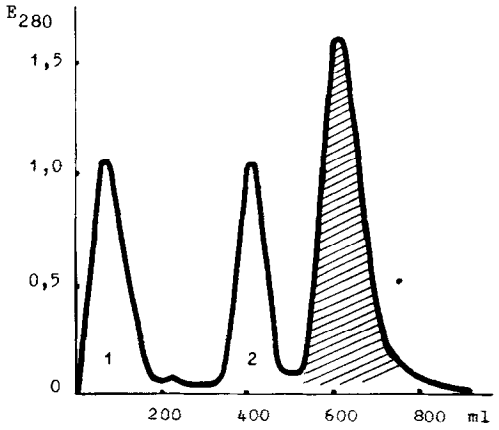

Fig.2. DEAE-cellulose chromatography of the mixture obtained by pepsinogen activation with elastase. Conditions as specified in the text. The peak which shows milk-clotting activity is cross-hatched. The peak 1 contains elastase and the peptides cleaved off in the course of activation. The peak 2 - pepsinogen .

DNP-Ile, the latter two derivatives being identified after hydrazinolysis and amino acid analysis. The content of basic amino acids of the enzyme obtained by elastase activation was equal to that of pepsin (Lys_1, His_1, Arg_2).

RESULTS AND DISCUSSION. Application of the sequenator to pepsinogen gave the sequence 1-50 (Fig.1) overlapping with that of pepsin. The sequence 1-39 confirmed the data obtained by Ong and Perlmann but the sequence Glu-Ala was found for the residues 40-41 rather than Ala-Glu suggested by these authors (6). The sequence 41-43 Ala-Ala-Leu for the first time revealed in this work was followed by Ile-45 - the N-terminal amino acid of pepsin. Identification of Leu as residue 44 once more confirmed the structure of leucyl-pepsin (4). The automatic sequencing of Leu-pepsin gave the sequence 44-89 which was in agreement with the structures of N-terminal nonapeptide (7), peptides of thermolytic hydrolysate 54-58, 59-61, 62-70, 71-

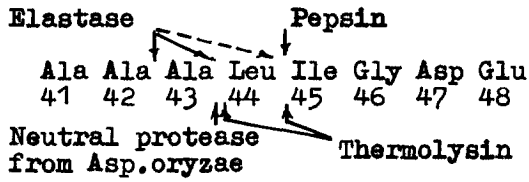

Fig.3. The activation site of swine pepsinogen.

82, 87-91 and of chymotryptic hydrolysate 60-71, 84-88 (9-12). The sequence determined with the sequenator overlaps with that of residues 87-119 established earlier in this laboratory (12).

The N-terminal sequence of swine pepsinogen and pepsin is obviously homologous with that of calf chymosin and prochymosin (13) (Fig.1). There is rather suggestive indication on the internal homology that appears when sequences 16-28 and 44-56 are compared (Fig.1a).

The sequence 74-82 coincides with peptides which contain the aspartic acid residue reacting with 1-p-nitrophenoxy-2,3-epoxy propane (14). Obviously, this inhibitor might interact with Asp-76 as well as with the aspartic acid residue in the sequence Ile-Val-Asp which reacts also with diazoacetyl inhibitors. Strong homology of the corresponding fragment of chymosin supports the suggestion on the essential role of Asp-76.

It is shown that pepsinogen activation is accompanied by the hydrolysis of Leu-Ile bond (Fig.3). This bond is flanked by two hydrophobic amino acids which is in agreement with the main features of pepsin specificity. It is tempting to consider the cluster of alanine residues 41-43 as functionally important trait of pepsinogen sequence. It might be suggested that the cluster of amino acid residues with short side chains promote the binding of the activating enzyme to the sequence containing the "hypersensitive" Leu-Ile bond. Peptide Ala-Ala-

Thr-Leu found by Kassell et al. in the activating mixture of bovine pepsinogen (15) is homologous with the sequence 41-44 of swine pepsinogen. It appears that Leu-Val bond hydrolysis accompanies the activation of bovine pepsinogen.

The N-terminal amino acids of pepsins are remarkably variable. Thus, valine is N-terminal in human (16) and bovine (17) pepsins, serine - in human gastricsin (16) and chicken pepsin (18), alanine - in swine pepsin B (19), glycine - in calf chymosin (20). Apparently, the N-terminal residue cannot determine the point of activating proteinase attack. The fact that the sequence preceding the N-terminal amino acid of pepsin bears rather characteristic traits evokes the suggestion that this part of the sequence governs the specificity of activation process.

Availability of this sequence for the proteolytic attack by external proteinases had been proved earlier by the activation of pepsinogen with neutral proteinases (Fig.3). So far as the cluster of alanine residues was found at the activation site, we applied pancreatic elastase for pepsinogen activation at pH 5.0. This enzyme performed the activation with high yield although rather slowly. The active product contained alanine, leucine and isoleucine as N-terminal amino acids. The application of the sequenator resulted in cleavage of following amino acids: 1st step Ala+Leu, 2nd step Leu+Ile, 3d step Ile+Gly, 4th step Gly+Asp, 5th step Asp+Glu.These data show that the activation product contains the mixture of leucyl-pepsin and alanyl-leucyl-pepsin which arise as a result of hydrolysis of Ala-Leu and Ala-Ala bonds of the zymogen. "Conventional" pepsin is also present although only trace amounts of corresponding amino acids were cleaved off during the automatic sequencing. The bond Leu-Ile might be hydrolysed by elastase or

eventually by pepsin derivatives appearing in the course of activation.

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