LEUCYL-PEPSIN A PRODUCT OF HOG PEPSINOGEN ACTIVATION BY PROTEINASES OF ASPERGILLUS ORYZAE

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Summary.
Under the action of Asp.oryzae proteinases at pH 5.0 hog pepsinogen is converted to leucyl-pepsin ("neopepsin") - the enzyme which is nearly identical with the product of autocatalytic pepsinogen activation being only one amino acid residue longer. This shows that Leu-Ile peptide bond is cleaved in the course of pepsinogen activation by pepsin in acid solutions. This result suggests redundancy of N-terminal amino group for maintaining of the active center of pepsin.

Introduction.

Activation of pepsinogen as well as of many other zymogens is a result of limited proteolysis directed both by the zymogen structure and the specificity of the activating enzyme (I). Asp.oryzae proteinase complex has been shown to transform hog pepsinogen into active proteinase,"neopepsin", which is very similar but not identical to "conventional" pepsin (2). It is the purpose of this paper to elucidate the structural relation between "neopepsin" and pepsin.

Materials and Methods.

Hog pepsinogen was purified by DEAE-cellulose chromatography (3). The mixture of Asp.oryzae proteinases -"oryzin" (Moscow Factory of Enzyme Preparations) was used without any fractionation. Proteolytic activity was assayed by

milk-clotting at pH 5.6 (4), hydrolysis of hemoglobin at pH 2.0 (5) and hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine at pH 4.0 (6). For determination of N- and C-terminal amino acids pepsin and neopepsin inactivated by phenol treatment were used (7). DNP-technique used was as described earlier (8), hydrazinolysis was applied to recover amino acids from DNP-derivatives of leucine and isoleucine, which afterwards were analysed on Amino Acid Analyzer.

To determine the N-terminal sequences of amino acids the modification of Edman's procedure was used (9). Methylthiohydantoins cleaved off were identified by thin-layer chromatography (I0) and mass-spectrometry (II). Mass-spectra of methylthiohydantoins eluted from thin layer plates were measured on MI-I3o5 mass-spectrometer supplied with device ensuring the evaporation of the substance in the ionization zone.

The activation was performed by addition of 25 ml of 0.I per cent "oryzin" solution in 0.I M acetate buffer, pH 5.0 to 500 mg of pepsinogen dissolved in I00 ml of the same buffer. After 20 hr. of incubation at 37° nearly quantitative activation was achieved as judged by the milk-clotting assay. The mixture was chromatographed on DEAE-cellulose (Fig.I). Fraction 3 was desalted on Sephadex G-25 and lyophylized.

Results.

From various proteinases tried only "oryzin" was effective giving practically quantitative yield of the active enzyme. Under the same conditions less than one per cent of spontaneous activation was observed. Even deliberate addition of pepsin to the zymogen at pH 5.0 does not accelerate the activation.

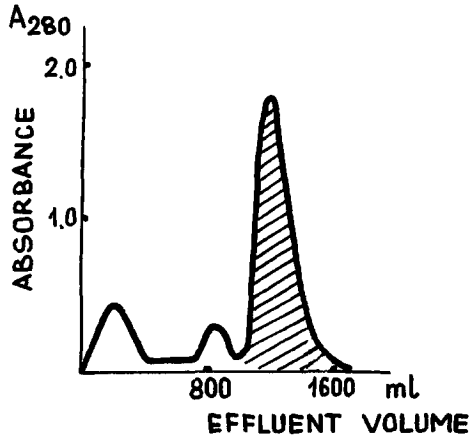


Fig.I. Chromatography of the products of pepsinogen activation on DEAE cellulose.
The mixture prepared by activation of 500 mg of pepsinogen was applied to 3.2 x 30 cm column of DEAE cellulose pre-equilibrated with 0.I N acetate buffer, pH 5.6. The column was eluted with NaCl gradient with the use of mixing chamber containing 650 ml of 0.I N acetate buffer, pH 5.6 and reservoir chamber containing 0.5 M NaCl. The peak revealing milk-clotting activity is cross-hatched.

The peak I om the chromatogramm (Fig.I) contains the peptides cleaved off in the course of the activation and the components of "oryzin". The peak 2 corresponds to residual pepsinogen. The position of the peak 3 which contains the active material coincides with that of pepsin. The properties of this artificially produced enzyme ("neopepsin") and those of pepsin are compared below.

		Neopepsin	Pepsin
Proteolytic activity			
against	hemoglobin	I00	=I00
	Acetyl-L-Phe-L-Tyr	I00	=I00
	milk-clotting	I00	=I00

Proteolytic activity after 20 min preincubation at pH 8	0	0
Content of basic amino acids	$Lys_1Arg_2His_1$	$Lys_1Arg_2His_1$
C-terminal sequence	Val-Ala	Val-Ala
N-terminal amino acid	Leu	Ile

Hence, the only difference found between neopepsin and pepsin is that in N-terminal amino acids. Evidently, their polypeptide chains should have somewhat different length. The equal content of basic amino acids shows that neopepsin cannot be much longer than pepsin. Otherwise, the very pronounced accumulation of basic amino acids in the N-terminal part of pepsinogen would increased their content in neopepsin.

To determine the N-terminal sequence of neopepsin step-wise cleavage of amino acids as methylthiohydantoins was applied. The following results were obtained in representative experiment starting with 4 μmol of inactivated neopepsin.

Degradation cycle	I	2	3	4	5
Methylthiohydantoin identified	Leu	Ile	Gly	Asp	Glu
Yield (μmol)	I.37	I.03	0.5	0.I5	0.I2

Methylthiohydantoins cleaved off in the first two cycles were identified by mass-spectrometry. To illustrate this procedure the relative intensities of characteristic peaks in mass-spectra of methylthiohydantoin cleaved off in the second cycle and of reference substances are given below:

m/e		Methylthiohydantoin Leu	Ile	Isolated after the 2nd cycle
I86	M^+	I00[x]	I00[x]	I00[x]
I57	$(M-C_2H_5)^+$	0	7	8
I43	$/M-CH(CH_3)_2/^+$	I60	4	6

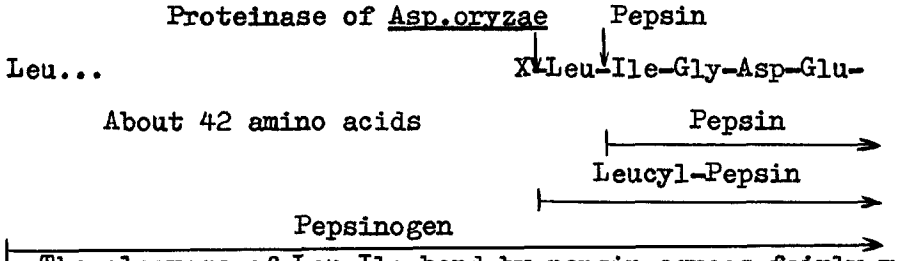
[x]) The intensities of parent ions are assumed to be =I00.

To check this identification, the residual protein after the first degradation cycle was dinitrophenylated and hydrolysed. From the hydrolysate DNP-isoleucine was isolated and free isoleucine identified after hydrazinolysis of DNP-derivative.

All these data show that neopepsin has the following N-terminal sequence: Leu-Ile-Gly-Asp-Glu- which is only one leucine residue longer than that of pepsin (I2): Ile-Gly-Asp-Glu- This allows us to change designation "neopepsin" for more descriptive "leucyl-pepsin".

Discussion.

From the data reported here some conclusions concerning pepsinogen activation might be drawn. Hydrolysis of peptide bonds adjoining to eventual N-terminals of pepsin and leucyl-pepsin might be visualised as follows:



The cleavage of Leu-Ile bond by pepsin agrees fairly well with the known specificity of the enzyme (I3). We suppose that the hydrolysis of X-Leu bond is produced by the neutral proteinase of Asp.oryzae (I4) which attacks the bonds formed by amino groups of hydrophobic amino acids.

Recently G.E.Perlmann and co-workers established N-terminal sequence of 41 amino acid residues in hog pepsinogen (I5,I6) . These authors claimed that the bond Glu-Ile is hydrolysed during the activation of pepsinogen. Our data

contradict this suggestion and support the alternative assumption according to which there are one or two amino acid residues between C-terminal glutamic acid of 4I amino acid fragment and the N-terminal amino acid of pepsin, i.e. isoleucine. We assume that leucine is one of these additional amino acids.

Leucyl-pepsin and pepsin possess essentially the same proteolytic activity. Hence, the N-terminal amino group cannot be of crucial importance for the function of pepsin. Redundancy of α-amino group of pepsin is in contrast with the important role of N-terminal amino group in the active center formation of serine proteinases (I). On the other hand one can compare the activation of pepsinogen with the activation of procarboxypeptidase A, which also proceeds by the rupture of different bonds in the rather extended segment of the polypeptide chain.

References.

I.M.Ottesen, Ann.Rew.Biochem.,36, part 2.,55 (I967).
2. V.M.Stepanov, E.A.Timokhina, FEBS 5th Meeting, Abstracts, p.267, Praha,I968.
3. N.P.Pyatnitzky, E.A.Timokhina, N.A.Rakova, V.M.Stepanov, Prikladnaya biokhimiya i mikrobiologiya,3,I89 (I967).
4. L.M.Ginodman in Aktualnye voprosy sovremennoi biokhimii, vol.2, p.54, I962, Medgis, Moscow.
5. D.Northrop, M.Kunutz, P.Herriott, Crystalline Enzymes (Russ. transl.), p.299, I947, Izdatinlit, Moscow.
6. N.G.Lutzenko, L.M.Ginodman, V.N.Orekhovich, Biokhimiya, 32,223 (I967).
7. V.M.Stepanov, T.I.Greil, Biokhimiya,28,540 (I963).
8. V.M.Stepanov, E.A.Timokhina, Biokhimiya,32,590 (I967).
9. V.M.Stepanov, V.F.Krivtzov, Zhurn. Obsch. Khim.,35,53 (I965).
IO. V.M.Stepanov, J.I.Lapuk, Zhurn.Obsch.Khim.,36,40 (I966).