ISOLATION AND CHARACTERIZATION OF A PEPTIDE DERIVED FROM THE ACTIVE SITE OF CHICKEN PEPSIN

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A peptide was isolated from chicken pepsin which contains the aspartic acid residue reacting with diazoacetyl-D,L-norleucine methyl ester in the presence of Cu^{2+}-ions. The peptide is N-terminated with isoleucine and contains (besides isoleucine) valine, aspartic acid, two threonines, serine, and leucine. In concurrent experiments a peptide of the same composition was isolated from the thermolysin digest of chicken pepsin and its sequence determined as Ile-Val-Asp-Thr--Gly-Thr-Ser-Leu. Since both peptides have entirely identical amino acid composition and other characteristics, the sequenced peptide corresponds to the peptide isolated from the active site of the enzyme.

It is known that diazoacetyl-D,L-norleucine methyl ester (DAN) in the presence of Cu^{2+}-ions esterifies the aspartic acid residue of the active site of acid proteases and thus irreversibly inhibits their enzymatic activity. Pepsin of hog[1], cattle[2], chicken[3], and man[4], chymosin[5], cathepsin D and E (ref.[6,7]), penicillopepsin[8], the acid protease from *Rhizopus chinensis*[9], *Cladosporium*[10] and other microorganisms are inhibited by this reagent. This type of reaction permits the mechanism of action of these proteases to be studied in more detail and represents today one of the criterions by which the character of newly isolated proteolytic enzymes is judged.

The aim of this study was to determine the composition and amino acid sequence around the aspartic acid residue reacting in chicken pepsin with the inhibitor (DAN).

EXPERIMENTAL

Material

Chicken pepsin was obtained by activation of chicken pepsinogen prepared in our Laboratory as described earlier[11]. Diazoacetyl-D,L-norleucine methyl ester was synthetized in this Institute. Hog pepsin was a product of Worthington Biochemical Corp., Freehold, N.J., U.S.A. Sephadex G-25 fine and G-10 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Methods

Amino acid analyses were carried out by the method of Spackman and coworkers[12] as modified by Benson and Patterson[13]. The N-terminal amino acid was determined by dansylation[14].

Inhibition by DAN: 140 mg of chicken pepsin was disolved in 60 ml of 0·1M acetate buffer, pH 5·1, and 2 ml of 0·1M copper acetate was added. After 10 min the solution was treated with 2 ml of methanol containing 34 mg of DAN. The inhibition was allowed to proceed 30 min at room temperature. The proteolytic activity of the mixture checked by the degree of hydrolysis of a 2% haemoglobin solution at pH 2 dropped to zero.

Peptic digestion: Inhibited chicken pepsin was freed of excess inhibitor by exhaustive dialysis against water and denatured by treating the solution with three volumes of acetone for 5 h at room temperature. The sample was concentrated to 16 ml in a rotary evaporator, the pH was adjusted to 3·0 by hydrochloric acid, and 8 mg of hog pepsin was added. The digestion was allowed to proceed overnight at 25°C. The digest was concentrated to 5 ml in a rotary evaporator, a small quantity of sediment was centrifuged off, and the digest was subjected to gel filtration on a Sephadex G-25 column equilibrated with water (280 ml). The peptides were eluted by water. The fraction containing small peptides retarded in the column was rechromatographed on a column of Sephadex G-10 (100 ml), equilibrated with acetic acid at pH 4, and lyophilized.

Diagonal electrophoresis was carried out according to Bayliss and coworkers[15] in a slightly modified arrangement. The mixture of peptides (0·6 mg) was applied as a spot on paper Whatman No 3 MM. The electrophoresis was carried out in the buffer acetic acid–pyridine–water, pH 5·3 (670 μS) in the vertical arrangement[16] at a potential gradient of 30 V/cm. The paper strip containing the separated peptides was cut out and exposed to vapors of a saturated aqueous triethylamine solution overnight at 37°C. The strip was subsequently dried at room temperature and sewn to another sheet of paper Whatman No 3 MM. Electrophoresis was then carried out under identical conditions. After staining of the electropherogram with a 0·2% ninhydrin solution all peptide spots were localized on the diagonal except for the peptide containing the esterified aspartic acid residue: This peptide, originally neutral, acquired a free carboxyl by the alkaline treatment with triethylamine and lay therefore off the diagonal, in the section close to the anodic side.

Preparative electrophoresis: 20 mg of the peptide mixture was applied as a 20 cm line on paper Whatman No 3 MM and electrophoresis was carried out as described above. The zone containing neutral peptides was cut out and exposed to vapors of aqueous triethylamine solution. After drying the paper strip was sewn to another sheet of paper Whatman No 3 MM and electrophoresis was carried out under the conditions described above. A new ninhydrin-positive zone moved out from the mixture of neutral peptides toward the anode; this zone corresponded to the position of the aspartic acid containing peptide on the diagonal peptide map. The peptide was eluted by water and purified by high voltage electrophoresis on paper at pH 1·9 and a potential gradient of 85 V/cm (ref.[17]).

RESULTS AND DISCUSSION

Chicken pepsin like other acid proteases is inhibited by DAN in the presence of Cu^{2+}-ions. It has been shown that this reagent specifically esterifies one aspartic acid residue only. The inhibitor binds to chicken pepsin at a molar 1 : 1 ratio. Only prolonged,

several hours lasting treatment with DAN leads to nonspecific reaction which makes the enzyme to inhibitor ratio increase to 1 : 2 (ref.[3]). These data were confirmed also in our experiments. We examined the inhibition of chicken pepsin at various pH-values and showed that the optimal pH of labeling is 5·1.

The analyses of the peptide obtained both by diagonal electrophoresis and in the preparative experiment showed the presence of impurities. The peptide was therefore purified by high voltage electrophoresis on paper at pH 1·9. The amino acid analysis of the purified peptide gave the following values (20-h hydrolysate): $Ile_{0.6}$, $Val_{0.6}$, $Asp_{1.1}$, $Thr_{1.7}$, $Gly_{1.1}$, $Ser_{1.0}$, $Leu_{1.0}$. Isoleucine was the N-terminal amino acid of the peptide.

During a systematic study of the thermolysin digest[18] of chicken pepsin, a peptide was obtained whose composition was the same as the composition of the peptide described above. The amino acid sequence of this peptide was determined as Ile-Val-Asp-Thr-Gly-Thr-Ser-Leu. From the study of the thermolytic digest the behaviour of the peptide on the Sephadex G−25 column was known; this considerably facilitated the isolation of the labeled peptide. The N-terminal isoleucine of the peptide is preceded in chicken pepsin most likely by alanine; the bond Ala-Ile can therefore be cleaved both by thermolysin and pepsin. Since the amino acid composition of the specifically labeled peptide exactly corresponds to the composition of the peptide isolated from the thermolytic digest, and since both peptides show the same electrophoretic behavior, it is obvious that both peptides are identical and that in both cases the same peptide, namely the peptide from the active site, was isolated.

As can be seen in Table I the amino acid sequences of peptides isolated from the active sites of various acid proteases are very similar. The position occupied by isoleucine is constant, likewise the position of the tetrapeptide sequence Asp-Thr-Gly-Thr. The amino acid sequence of the peptide isolated from chicken pepsin is the same as the sequence of the corresponding peptide from bovine and porcine pepsin.

TABLE I

Amino Acid Sequence of Peptides Containing the Reactive Aspartic Acid Residue and Derived from the Catalytic Site of Certain Acid Proteases

Chicken pepsin	Ile-Val-Asp-Thr-Gly-Thr-Ser-Leu
Hog pepsin[15]	Ile-Val-Asp-Thr-Gly-Thr-Ser
Bovine pepsin[2]	Ile-Val-Asp-Thr-Gly-Thr-Ser
Chymosin[5]	Asp-Thr-Gly-Thr-Ser-Lys-Leu
Penicillopepsin[8]	Ile-Ala-Asp-Thr-Gly-Thr-Thr-Leu
Rhizopus chinensis protease[9]	Asp-Thr-Gly-Thr-Thr-Leu

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