HOG PANCREATIC α-AMYLASE; N-TERMINAL SEQUENCE ANALYSIS

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Both isozymes of hog pancreatic amylase were digested with chymotrypsin, subtilisin, and pronase. These digests were passed over a column of sulfoethyl-Sephadex C-25 and peptides not containing free NH_2-groups thus isolated. It was shown that both isozymes have the same N-terminal amino acid sequence, pyrrolidonecarboxylic acid — tyrosine. No peptides with acetylated amino groups were found.

We have shown in a preceding paper[1] that hog pancreatic amylase (EC 3.2.1.1) represents two isozymes differing in electrophoretic mobility. These isozymes were prepared by chromatography on DEAE-cellulose. The isozymes isolated do not differ from each other or from the starting material in specific activity. Their molecular weights determined in the ultracentrifuge as well as amino acid compositions are identical within experimental error. The N-terminal amino acid of both isozymes and crystalline α-amylase cannot be identified either by the phenyl isothiocyanate method or by dansylation; this shows that the α-NH_2-group of the N-terminal amino acid is blocked.

The presence of an acetyl group in crystalline α-amylase or in its cyanogen bromide fragment has been demonstrated by various authors[2,3]. These findings led several workers to believe that the N-terminal amino acid of pancreatic α-amylase was acetylated. Contrarily, Fabian[4] isolated from a combined tryptic, chymotryptic, and nagarse digest of α-amylase pyrrolidonecarboxylic acid (pyrrolide-2-one-5-carboxylic acid) and believes that it is the N-terminal amino acid of the enzymes.

We have made an effort in this study to isolate peptides lacking a free amino group from various digests of both isozymes and thus to decide which hypothesis is correct.

EXPERIMENTAL

Material

Crystalline pancreatic α-amylase was prepared according to Fischer[5] and resolved into two isozymes by chromatography on a column of DEAE-cellulose[1]. The enzymic digestions were effected by the following enzymes: subtilisin (Novo, Denmark), chymotrypsin (twice crystallized,

prepared by activation of 7-times crystallized chymotrypsinogen), pronase (ex *Streptomyces griseus*, Koch-Light, England), trypsin (B-grade, Calbiochem. U.S.A.), carboxypeptidase A (DFP-treated, Worthington, U.S.A.).

Methods

Enzymic digestions were effected by the enzymes described above under the following conditions. The protein was dissolved in water (10 mg/ml) and the pH of the solution was adjusted to 8·3 by ammonium carbonate. The enzyme to substrate ratio (w/w) was 1 : 50. The digestion was allowed to proceed 4 h at 37°C. After this period the enzyme to substrate ratio was adjusted to 1 : 25 and the cleavage was allowed to proceed for 2 h more. At the end of this period the digest was diluted and lyophilized.

The peptides with blocked amino groups were isolated on a column of SE-Sephadex C-25 (1·5 × 10 cm, Pharmacia, Uppsala, Sweden), washed with 0·5M-HCl and distilled water until the washings gave a negative test for Cl^--ions. The lyophilized digest was dissolved in freshly boiled water (3 mg/ml); the pH of the solution was 6·5 ± 0·2. This solution was filtered through the ion-exchange column at a rate of 5 ml/h. Peptides which had not been held up were treated further. The electrophoresis of peptides on paper was carried out at pH 5·6 according to Mikes[6]. The peptides were chromatographed on paper in the system butanol–pyridine–acetic acid–water (30 : 20 : 60 : 4) (ref.[7]). The peptides and pyrrolidonecarboxylic acid were detected by chlorination according to Reindel and Hoppe[8]. The presence of acetyl groups in peptides or peptide fractions was tested after hydrazinolysis by the reaction with dansyl chloride[9]. 1-Acetyl-2-dansyl hydrazine was identified by two-dimensional chromatography on polyamide thin layer sheets in the system formic acid–water (1st direction) and benzene–acetic acid (2nd direction) (ref.[10]). Amino acid analyses were carried out on samples hydrolyzed at 110°C in constant-boiling hydrochloric acid 20 h in the presence of phenol. The analyses were made in Model 120B Beckman-Spinco Amino Acid Analyzer according to Benson and Patterson[11].

Digestion with carboxypeptidase A was carried out at pH 7·4 and 37°C, 4 h. The digest was evaluated chromatographically and electrophoretically and tyrosine liberated was quantitated in the amino acid analyzer.

RESULTS AND DISCUSSION

Both isozymes of hog pancreatic α-amylase were digested in separate experiments with trypsin, chymotrypsin, subtilisin, and pronase. Peptides with blocked amino group were isolated from the chymotryptic, subtilisin, and pronase digests. No such peptides were contained in the tryptic digest.

Chymotryptic Digest

The chymotryptic digests of both isozymes contained one nihydrin-negative peptide only; the latter showed after hydrolysis the presence of glutamic acid and tyrosine at a ratio of 1 : 1. The yield of the peptide was approximately 0·7 mol/mol protein (51 500). The peptide was negative on electrophoresis at pH 5·6. Carboxypeptidase A liberated from the peptide tyrosine and a ninhydrin-negative product; the latter gave glutamic acid after hydrolysis. The mobility of the product on electrophoresis was the same as that of a standard of pyrrolidonecarboxylic acid. The peptide did not

bear an acetyl group. The amino acid sequence of the peptide is PCA . Tyr (PCA = = pyrrolid-2-one-5-carboxylic acid). The peptide is a result of specific chymotryptic cleavage.

Subtilisin and Pronase Digest

Peptide PCA . Tyr was again isolated from these digests of both isozymes. Smaller quantities of two other peptides PCA . Tyr(Ala, Pro) and PCA . Tyr(Ala, Pro, Glu) were also isolated from the digests. Chymotryptic digestion of these peptides afforded PCA . Tyr.

None of the fractions which passed through the SE-Sephadex column contained an acetyl group in spite of the fact that a peptide with N-terminal N-acetylated serine has been isolated from another material by the same procedure[12]. We assume therefore that the N-terminal end group of both isozymes of α-amylase is pyrrolidonecarboxylic acid and that the second amino acid in the chain is tyrosine. The possibility that the PCA-containing peptides were formed by additional cyclization of N-terminal glutamine of a peptide from the middle part of the chain is very little probable since peptide PCA . Tyr was isolated from three different digests. The treatment of the peptides, which was carried out quickly under mild conditions also decreases the possibility of additional cyclization of glutamine to pyrrolidonecarboxylic acid.

A problem to be solved is whether pyrrolidonecarboxylic acid is present in the native molecule of the enzyme or whether it is formed in the process of its isolation. Experiments designed to prove the presence of N-terminal glutamine or glutamic acid during the various stages of α-amylase preparation were unsuccessful. Both isozymes of hog pancreatic α-amylase, which are very similar in many respects, have also an identical N-terminal sequence, *i.e.* PCA . Tyr.

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