HOG PANCREATIC α-AMYLASE. PREPARATION AND CHARACTERIZATION

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Crystalline α-amylase (EC 3.2.1.1) was prepared from hog pancreas. The preparation obtained was resolved into two isozymes by chromatography on DEAE-cellulose. The molecular weight (51 500), amino acid composition, and terminal groups of both isozymes were determined. Both isozymes have a single polypeptide chain containing 460—465 amino acid residues. The amino acid composition of both isozymes is similar. None of them has a free N-terminal end group. Both isozymes are C-terminated with leucine. The molecule of each isozyme is cross-linked by 5 disulfide bonds and contains two sulfhydryl groups.

Amylases are the basal enzyme in starch metabolism which is a universal carbon source for most organisms. The presence of these enzymes has been revealed in every tissue of molds, bacteria, plants, and animals. The cleavage of starch by amylases from various sources has been used by mankind for hundreds of years and todate these enzymes are gaining more and more application in industry. The amylases are among the first enzymes prepared in crystalline state. Our knowledge of their structure and of the function of their active centers, however, is very meagre. This paper extends an earlier communication[1] on the separation of the isozymes of pancreatic α-amylase, published simultaneously by several laboratories[2,3].

EXPERIMENTAL

Methods

Preparation of Crystalline Amylase and Separation of Isozymes

Crystalline α-amylase was prepared from fresh hog pancreas[4]. The crystallization was effected in dialyzing tubing and was carried out 4—6 times. The separation of the isozymes was effected on a DEAE-cellulose (DE 11 Whatman) column, prepared according to the manifacturer's instructions and equilibrated in 0·05M Tris-HCl buffer, pH 7·75. The column was washed with 1M-NaCl in the same buffer and finally with the equilibration buffer until the conductivity of the effluent was the same as the conductivity of the buffer. Amylase crystals were dissolved in distilled water containing 5 mM-$CaCl_2$, pH 9·5 (adjusted by ammonia) and the solution was dialyzed 16 h against the equilibration buffer. The absorbance of the effluent at 280 nm, amylase activity[5], and the behavior of the eluted proteins on gel electrophoresis were examined.

Analytical Disc Electrophoresis

The electrophoresis was carried out at pH 9·0 in 7·5% polyacrylamide gel[6]. A constant current of 3 mA per gel (0·5 × 5·5 cm) was applied until the marker (bromophenol blue) migrated to the gel bottom. The proteins in the gel were stained with 1% Amido Black 10B in 7% acetic acid. The gels were destained electrophoretically.

Ultracentrifugation Analysis

The molecular weights of isozymes A_I and A_{II} were determined by the method of sedimentation equilibrium at high speed[7] in Model E Beckman-Spinco Ultracentrifuge. The initial protein concentration was 0·33 mg/ml of 0·1M tetraborate-HCl buffer, pH 8·0. The sedimentation coefficient of crystalline α-amylase was determined in the same buffer at 0·2—1·0% protein concentration. The determination was carried out in a single-compartment cell of optical path 12 mm at 21·7°C. The sedimentation coefficient was calculated by the method of Schachman[8].

Amino Acid Analysis

Protein samples were hydrolyzed 20, 48, and 72 h at 105°C in constant-boiling hydrochloric acid. The analyses were made in Model 120B Beckman-Spinco Amino Acid Analyzer according to Benson and Patterson[9]. The cysteine and cystine content was determined either as cysteic acid[10] after complete oxidation or, alternatively, the protein was completely reduced, treated with iodoacetic acid, and cysteine and cystine determined as S-carboxymethyl-cysteine[11]. Tryptophan was determined spectrophotometrically[12] or colorimetrically[13]. Four samples were used for each hydrolysis time. The molecular weight was calculated from the amino acid analysis data graphically[14].

Other Methods

The sugar content was determined according to Kim[15] with the following modifications. The protein (0·5—1·0 mg) was hydrolyzed in 0·75 ml of 0·25N-H_2SO_4 and 100 mg of Dowex 50—X2 in H^+-form, L-arabinose and norleucine were added as internal standards. The further steps, including filtration, neutralization, deionization, and reduction of the resulting sugars with sodium borohydride were carried out as described. The alditols were acetylated by acetic anhydride (100 μl) in anhydrous pyridine (50 μl). The solvent was rotary evaporated and the dried aldital acetates formed were extracted three times with 50 μl of chloroform; the extracts were evaporated at 40°C. Immediately before chromatography the samples were dissolved in 5—10 μl of chloroform and 0·2—0·5 μl of the solvent was injected in Model 900 Perkin Elmer Gas Chromatograph. The latter was equipped with flame ionization detectors and with stainless steel columns (10 feet long, 0·125 inch o.d.), packed with Gas chrom Q 60—80 mesh and 3% ECNSS-M (liquid phase). The conditions of separation were as follows: temperature, columns 190°C, isothermal runs, detectors 230°C, injectors 290°C, gas flow hydrogen generated by Elhygen Model E 150, 35 ml/min, nitrogen as carrier 54 ml/min. The SH-groups were determined colorimetrically with 5,5'-dithiobis (2-nitrobenzoic acids)[16]. The N-terminal amino group was estimated by the phenylisothiocyanate method[17]. For C-terminal end group analysis hydrazinolysis[18] and digestion with carboxypeptidase A (Worthington)[19] were employed. The amino acids liberated were quantitated in the analyzer. N-Acetyl groups were determined as dansylacetylhydrazides[20] after hydrazinolysis and dansylation.

RESULTS

Pancreatic α-amylase can be resolved into two components, A_I and A_{II} by chromatography on DEAE-cellulose. These components differ in electrophoretic mobility (component A_I moves faster). The designation of the isozymes complies with the "Recommendations 1976" of IUPAC-IUB Commission on Biochemical Nomenclature. Both components show the same specific amylase activity. The result of chromatography depends on the ratio of column size to sample quantity. The yield under the conditions described in Fig. 1 was 63%. The ratio of component A_I to A_{II} varies in the range 1 : 1 to 1·3 : 1 with individual preparations.

The symmetric boundary observed during sedimentation analysis indicates the homogeneity of the starting amylase preparation and of both components as regards their molecular weights. The apparent sedimentation coefficient of component A_I is $s_{20,w} = 4{\cdot}5$ and of component A_{II} 4·8. The sedimentation coefficients are independent of protein concentration in the range 0·2 – 1·0%. The molecular weights are: component A_I 48700 ± 3400, component A_{II} 48000 ± 3400.

The method of end group analysis used in this study failed to show the presence of a free N-terminal group. Hydrazinolysis liberated 0·8 mol of leucine/mol. The presence of C-terminal leucine is also evidenced by the results of carboxypeptidase digestion. The identification of a longer C-terminal sequence was impossible because of the liberation of nonstoichiometric amounts of isoleucine, tyrosine, and tryptophan. The treatment of the enzyme with diisopropylphosphofluoridate did not affect this nospecific cleavage. The presence of a sugar moiety was not found. The presence of 1·5 mol of SH-group per one mol of protein was found in the presence of denaturing

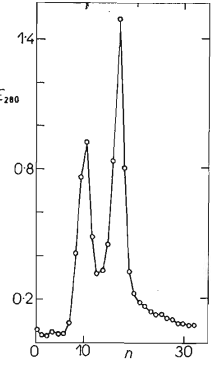

FIG. 1

Separation of 70 mg of α-Amylase on DE 11 Column (1·3 × 30 cm). Fractions 7—11 Contained Isoenzyme A_{II} and Fraction 14—18 Isoenzyme A_I

The sample was applied and the column was eluted by 0·05M Tris-HCl buffer, pH 7·75. Flow rate 18 ml/h. The fractions were collected at 20-min intervals.

agents (urea, sodium dodecylsulfate, and ethylenediaminotetraacetic acid). The presence of DNS-N-acetylhydrazide in both components was found after hydrazinolysis and dansylation; the quantity of the hydrazide indicates that one mol of protein contains about 0·1 mol of acetyl groups. The results of amino acid analysis are given in Table I. The method of graphical treatment of the data, by which the molecular weight of both components (51 500) was calculated, is shown in Fig. 2. The amino acid composition of both isozymes calculated on the basis of this molecular weight value, is shown in Table I.

DISCUSSION

Available information on hog pancreatic amylase is controversal. The data on N- and C-terminal groups and the possible existence of several peptide chains have been discussed[21]. It follows from our work that both isozymes are regular components of preparations obtained from mixed batches of pancreas. These isozymes are detectable in pancreas homogenates. The resolution of crystalline amylase into two components by chromatography on DEAE-cellulose is well reproducible. The separation of component A_{II} into two to four subcomponents has been observed in several runs. This separation is not reproducible and we have not been able to rechromatograph the subcomponents obtained. These components are not electrophoretically different. In our opinion the separation pattern depends on the exact equilibration of the sample and of the column and does not reflect the actual heterogeneity of the material. The stability of crystalline amylase is satisfactory. Even though the preparative runs were

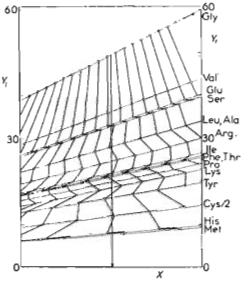

FIG. 2

Example of Part of Diagram Used for Determination of Probable Amino Acid Composition of Isozyme A_1 from Data of Amino Acid Analysis

The smaller multiples of values of individual amino acids obtained by analysis are plotted on axis Y_1, the larger multiples on axis Y_t. The most probable values lie on a perpendicular line assymptotic to a system of hyperbolas. The latter are obtained by connecting the intersections of lines parallel to integral numerical values of individual amino acids and a „fan" of lines connecting the input analytical data. The diagram shows the only interpretation of the analytical data in the range given by the determination of molecular weight in the ultracentrifuge. The numerical interpretation is shown in Table I.

carried out with the omission of special precautions to inhibit protease activities, the appearance of new N-terminal amino acids was not observed. Proteolytic cleavage obviously took place during storage of dialyzed and lyophilized samples and manifested itself by the appearance of new N-terminal amino acids, mainly glycine, tryptophan, leucine, and others. The presence of a sugar moiety was observed neither in crystalline amylase nor in the two isozymes. The presence of a small quantity of acetyl groups and the failure to isolate the N-terminal peptide by the conventional procedure[22] lead us to postulate that the presence of an N-acetylated amino acid at the N-terminus of the amylase molecule is very little probable. This postulate is in accordance with the results of Fabian[23]. The majority of other workers have assumed or shown the presence of an N-acetylated N-terminal amino acid. Our value

TABLE I

Amino Acid Composition of Isozymes of Pancreatic α-Amylase

The number of amino acid residues is based on a molecular weight of 51 500. The values of valine and isoleucine were obtained with 72-h hydrolysates. The values of serine and threonine were obtained by extrapolation to zero time of hydrolysis. The values of proline are means obtained with oxidized and S-carboxymethylated samples. Tryptophan was determined spectrophotometrically and colorimetrically (see Methods).

Amino acid	g/100 g		Numer of residues		Nearest integer	
	A_I	A_{II}	A_I	A_{II}	A_I	A_{II}
Lysine	4·52	4·62	18·2	18·6	18	19
Histidine	2·14	2·14	8·0	8·0	8	8
Arginine	7·84	7·49	25·9	24·7	26	25
Aspartic acid	14·10	13·78	63·1	61·7	63	62
Threonine	4·03	4·16	20·4	21·1	20	21
Serine	5·31	5·54	31·4	32·7	31	33
Glutamic acid	8·69	8·28	34·8	33·1	35	33
Proline	3·84	3·73	20·4	19·8	20	20
Glycine	5·66	5·45	51·0	49·2	51	49
Alanine	4·01	3·87	29·1	28·1	29	28
Cystine/2	3·53	3·48	11·9	11·9	12	12
Valine	7·67	6·97	38·2	36·2	38	36
Methionine	2·10	1·97	8·2	7·7	8	8
Isoleucine	5·25	4·79	23·9	21·8	24	22
Leucine	5·28	6·03	24·0	27·5	24	28
Tyrosine	5·37	5·16	16·9	16·3	17	16
Fenylalanine	6·32	6·06	22·1	21·2	22	21
Tryptophan	6·85	6·76	18·9	18·7	19	19
Total					*465*	*460*

of molecular weight of amylase based on the results of sedimentation analysis and on the graphical treatment of the data of amino acid analysis, is 51 500, *i.e.* in agreement with the majority of recorded data[24]. Based on this value the molecule contains 12 half-cystine residues. Of their number 10 are cross-linked by disulfide bonds and the remaining two half-cystines are either blocked or form an unstable disulfide bond. The differences in the amino acid composition of both isozymes are minor. We have not been able to show a difference in the number of aspartic acid residues in the two isozymes[25]. The results of the present study lead us to predict only minor differences n the primary structures of the two isozymes of hog pancreatic amylase.

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