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Crystalline Pancreatic Amylase. II. Improved Method for its Preparation from Hog Pancreas Glands and Additional Studies of its Properties[1]

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A new procedure for the preparation of crystalline pancreatic amylase from swine is described briefly. The new method offers several distinct advantages over earlier methods reported for the purification and crystallization of pancreatic amylase. The yields of highly active crystalline amylase are materially increased while the new process is much less laborious and much less time consuming than earlier procedures. Additional properties of crystalline pancreatic amylase not previously reported are presented and discussed.

Introduction

Crystalline pancreatic amylase was prepared from hog pancreatin in 1931.[2] The yields of the crystals were small and they lost their amylase activity so rapidly that it was difficult to study the properties of the active crystalline amylase. In 1947, Meyer, *et al.*,[3] reported the preparation of larger yields of the crystalline amylase and a study of certain of its properties. A modification of the original procedure[2] has resulted in a method for the much more rapid and much less laborious preparation of crystalline pancreatic amylase with marked increases in the yields. This method is described briefly. Additional properties of pancreatic amylase, not previously reported, also are presented and discussed.

Experimental

Purification Procedures

Initial Material.—Commercial hog pancreatin, preferably undiluted, is a good starting material. The work reported here was carried out with a number of different samples of undiluted pancreatin[4] and also with pancreatin prepared in the laboratory from frozen pancreas glands of hogs and from ordinary commercial pancreatin. All manipulations are carried out in ice-baths with cold solutions and in diffused daylight.

Step 1.—Forty grams of pancreatin is mixed in an ice-bath with 400 ml. of an ice-cold phosphate solution; 0.008 *M* phosphate, 0.003 *M* calcium chloride, *p*H 7.0. A smooth paste is obtained first with a few ml. of the solution and then the entire mixture is stirred slowly, so as not to cause foam, with an electric stirrer in the ice-bath for one hour. The mixture is then centrifuged at approximately 0° in a refrigerated centrifuge.[5] Approximately 350 ml. of a clear amber extract is obtained.

Step 2.—The extract from step 1 is poured with constant stirring into eight times its volume of an ice-cold mixture of 95% ethanol[6] (840 ml.) and filtered anhydrous ethyl ether (1960 ml.) held in an ice-bath. The mixture is stirred during the addition of the extract and then is allowed to stand in the ice-bath for 20 minutes. A pale yellow oily precipitate settles out. The clear supernatant liquid is decanted off and discarded.

Step 3.—The oily precipitate from step 2 is taken up with constant stirring in 160 ml. of the ice-cold phosphate solution described in step 1 and stirred in an ice-bath until a smooth emulsion is obtained. The formation of a smooth emulsion is important for good yields and takes considerable time.

Step 4.—The emulsion from step 3 is poured with constant stirring into 900 ml. of cold 95% ethanol[6] held in an ice-bath. The mixture is allowed to settle for 10 minutes in the ice-bath. It is then centrifuged at approximately 0°.[5] The clear supernatant solution is discarded.

Step 5.—An ethanol–phosphate solution is prepared by mixing 1 volume of 95% ethanol[6] with 0.90 volume of the phosphate solution described under step 1, above. The precipitate obtained in step 4 is broken up with a smooth glass rod and held in an ice-bath while 560 ml. of the ice-cold ethanol–phosphate solution is added with constant stirring with an electric stirrer. The stirring in the ice-bath is continued until a uniform suspension is obtained. The suspension is centrifuged at approximately 0°.[5] The clear supernatant solution is saved; the precipitate is discarded.

Step 6.—The clear supernatant solution from step 5, above, is poured with constant stirring into 700 ml. of a mixture of equal volumes of ethanol[6] and filtered anhydrous ethyl ether, held in an ice-bath. If a precipitate does not settle in 5 minutes, more filtered ice-cold ethyl ether, 30 to 40 ml., may be added. The mixture is kept in the ice-bath for 15 minutes and then is centrifuged at approximately 0°.[5] The supernatant solution is discarded.

Step 7.—The centrifuge cup is inverted on filter paper in the refrigerator for a few minutes to remove traces of the solvent. The precipitate is then transferred to a cold tared watch glass and dried in a sulfuric acid desiccator held in the refrigerator.

The dried purified amylase is a light amber colored amorphous solid. The yield from 40 g. of pancreatin is approximately 2,500 mg. or approximately 6% of the original solids. The saccharogenic activity[7-10] of the purified amylase at this stage will depend upon that of the initial material but is usually between 6,000 and 11,000 mg. maltose equivalents[7-10] per mg. purified amylase. The yield of purified amylase represents 70 to 85% of the active amylase in the original pancreatin.

Crystallization Procedure. First Crystallization.—The dried pancreatic amylase is weighed and transferred to a small graduated centrifuge tube held in an ice-bath. Ice-cold phosphate solution at *p*H 7.0, described in step 1, above, is added to give approximately 80 mg. of amylase preparation per ml. of solution. The mixture is stirred with an electric stirrer in an ice-bath until no more solid appears to dissolve, usually for 3 or 4 hours. The mixture is then centrifuged at approximately 0°.[5] The saturated supernatant solution is decanted into a centrifuge tube. The tube is covered with parafilm[11] and placed in the refrigerator to crystallize. Usually crystallization takes place in 1 or 2 days. The completeness of the crystallization can be tested by placing the mother liquor aside in the refrigerator. As a rule, no further crystals appear even after a week of standing. If available, a drop of a suspension of crystals of pan-

(1) This investigation was supported, in part, by a research grant from the National Institutes of Health, Public Health Service, and in part by a grant from the Corn Industries Research Foundation.

(2) M. L. Caldwell, L. E. Booher and H. C. Sherman, *Science*, **74**, 37 (1931).

(3) K. H. Meyer, Ed. H. Fischer and P. Bernfeld, *Helv. Chim. Acta*, **30**, 64 (1947); *Arch. Biochem.*, **14**, 149 (1947); *Experientia*, **3**, 106 (1947).

(4) The authors wish to thank the Takamine Laboratory, Inc. for generous gifts of undiluted pancreatin of high amylase activity.

(5) Manufactured by the International Equipment Company, Boston, Massachusetts.

(6) All ethanol used in this work was treated as follows. Eighty grams of sodium hydroxide is added to 5 liters of 95% ethanol. After the mixture has stood for 12 hours or longer, 3 ml. of a saturated solution of silver nitrate is added dropwise with stirring. The mixture is filtered and distilled. The first and last few ml. are discarded.

(7) H. C. Sherman, M. L. Caldwell and M. Adams, THIS JOURNAL, **50**, 2529, 2535, 2538 (1928).

(8) R. B. Alfin and M. L. Caldwell, *ibid.*, **70**, 2534 (1948).

(9) L. L. Phillips and M. L. Caldwell, *ibid.*, **73**, 3559 (1951).

(10) M. L. Caldwell, S. E. Doebbeling and S. H. Manian, *Ind. Eng. Chem., Anal. Ed.*, **8**, 181 (1936).

(11) Purchased from Eimer and Amend, New York, N. Y.

creatic amylase is added to the solution to favor crystallization. Stirring the solution, gentle shaking or scratching the sides of the tube also help induce crystallization.

Washing of Crystals to Remove Electrolytes.—The crystals are centrifuged from the phosphate solution and washed rapidly three times with approximately twice their volume of ice-cold redistilled water. Determinations have shown that the total solids of the third and fourth washings are almost identical. Therefore, three washings are considered sufficient to remove most of the contaminating electrolytes.

Activity Measurements.—An amylase solution obtained by stirring washed crystals with cold redistilled water in an ice-bath for longer periods of time, for 1 hour or more, may now be used for activity measurements based upon total solids. Activity measurements also are often based upon total nitrogen.

Recrystallizations.—Washed crystals, occupying a volume of approximately 1 ml., are suspended in 5 ml. of ice-cold water in a small beaker placed in an ice-bath. Ice-cold 0.1 *M* ammonium hydroxide solution is then added dropwise with stirring until the solution reaches *p*H 8.9. The suspension is then kept in the ice-bath with constant stirring for three hours. At the end of the three hours, crystals should still be present showing that the supernatant solution is saturated. The suspension is then centrifuged. The clear supernatant saturated solution is decanted into a small beaker held in an ice-bath and neutralized to *p*H 6.9 by the dropwise addition with stirring of ice-cold 0.1 *M* acetic acid. The solution is then seeded and placed in the refrigerator to crystallize. It takes from two to three days for all the crystals to come down. The new crystals are centrifuged off and washed three times with ice-cold redistilled water in the manner already described, and the amylase activity and total solids or total nitrogen determined as before. The activity of these crystals is slightly higher than that after the first crystallization, showing that impurities have been removed in the recrystallization. The crystals left undissolved at *p*H 8.9, above, may be mixed with another crop of crystals for recrystallization.

The crystals obtained from the first recrystallization are recrystallized twice more in the same manner. Each time it takes 2 to 4 days for the crystals to come down. Each time the crystals are centrifuged off, washed with cold water and amylase activity and total solids or nitrogen determined. Increases in activity are observed after the second but not after the third or fourth recrystallizations. Therefore, three crystallizations are deemed sufficient.

Yield.—Approximately 200 mg. of three times crystallized pancreatic amylase with a saccharogenic activity[7-9] of approximately 16,000[12] is usually obtained initially from 40 g. of undiluted pancreatin. This yield represents approximately 10% of the active amylase in the original pancreatin. This yield can be increased materially by the preparation of several lots of the purified amorphous amylase and then by carrying out several consecutive crystallizations so that the mother liquors remaining from one crop of crystals can be processed with the next. In addition, the supernatant solutions obtained in the crystallizations and the wash solutions can be combined and precipitated with the 1:1 alcohol–ether mixture as described in step 6 and then crystallized.

Properties of Crystalline Pancreatic Amylase. Crystalline Form.—The crystals of pancreatic amylase obtained here are similar in form to those previously described.[2,3]

Ultimate Analysis.—The crystalline pancreatic amylase, after three crystallizations, gave the following average values on ultimate analyses[13]: C, 49.46; H, 7.18; N, 15.52; S, 1.33; P, 0.00; ash, 0.71. These data agree closely with data reported by Meyer, *et al.*,[3,14] for their crystalline hog pancreatic amylase with the exception of sulfur. Meyer, *et al.*,[3,14] report no sulfur or only insignificant traces of sulfur. The crystalline amylase obtained here invariably has contained sulfur. The above data are average values for 6 different crops of crystals obtained from several lots of commercial pancreatin[4] and from pancreatin prepared in the laboratory from frozen pancreas glands of hogs. These crystals, obtained from frozen glands and processed entirely in the laboratory, gave the following ultimate analysis: C, 51.61; H, 7.04; N, 15.18; S, 1.33; P, 0.00 and ash, 0.79. It is interesting to note that pancreatic amylase contains no phosphorus.

The ash of 3 times crystallized hog pancreatic amylase, obtained from frozen pancreas glands that had been processed entirely in the laboratory, was examined by spectrographic analysis.[15] It showed traces of calcium, 0.10 to 0.15%, and traces of magnesium, 0.05%, but no discernible traces of heavy metals.

Electrophoresis.—Crystalline pancreatic amylase, crystallized three times, was found to be homogeneous electrophoretically. The protein gave a sharp boundary and migrated as a homogeneous substance; no evidence of asymmetry was observed either in the ascending or in the descending columns after 2, 5, or 8 hours of electrophoresis[16] in phosphate buffer solutions at *p*H 6.5 or at *p*H 7.9 or in barbiturate buffer at *p*H 8.6. Countercurrent electrophoresis for 14 hours also failed to show any evidence of more than one component in the amylase solutions. In all cases, the crystalline amylase was dissolved in water and dialyzed at 0° to equilibrium against the buffer to be used before being subjected to electrophoresis. No change was found in the activity of the amylase solutions after the dialyses. The data for the electrophoresis and the photographs of the ascending and descending columns are similar to those published by Meyer[3] and are omitted for the sake of brevity.

Sedimentation in the Ultracentrifuge.—Sedimentation measurements[16] in the ultracentrifuge with solutions of three times crystallized pancreatic amylase which had been dialyzed at 0° for 5 hours against a phosphate buffer of ionic strength of 0.2 μ and at a *p*H of 6.5 also indicated that crystalline pancreatic amylase is homogeneous. Similar results have been reported by Meyer[3] for his crystalline pancreatic amylase.

Identity of Protein and Amylase.—Three times crystallized pancreatic amylase was extracted 5 times successively at 0° with small portions of cold freshly redistilled water. The saccharogenic activity of the 5 extracts, based upon their total solids, remained constant at 16,000 mg. maltose equivalents per mg. solid.[7-9,12] These data show that the solubility in water of the amylase and of the crystalline protein were the same under the conditions of these experiments.

Absorption Spectra.—Solutions of crystalline pancreatic amylase, prepared by successive extractions of three times crystallized amylase with cold redistilled water and giving a constant amylase activity of 16,000 mg. maltose equivalents per mg. solid[7-9,12] were diluted with water, or with phosphate buffer or with acetate and analyzed in the ultraviolet with a Beckman spectrophotometer. They showed a maximum absorption at 2,800 Å. and a minimum absorption at 2,500 Å. These results are typical of proteins in general and are in agreement with data reported by Meyer, *et al.*,[3] for their crystalline pancreatic amylase.

Relation between Optical Density and Amylase Concentration.—The data given in Table I show the influence of different concentrations of pancreatic amylase obtained by successive extractions of the crystals as described above, upon the optical densities of its solutions. When the maximum optical densities at 2,800 Å. were plotted against amylase concentration, a linear relationship was obtained. This linearity, obtained with successive extracts of the crystals, increases the evidence that the crystalline pancreatic amylase is a homogeneous protein. In addition, such data plotted on a large scale as a calibration curve make it possible to determine the concentration of crystalline pancreatic amylase in a solution of unknown concentration simply by measuring its optical density and comparing this with the calibration curve.

(12) The crystals produce 16,000 times their weight of maltose equivalents from 1% Lintner soluble potato starch in 30 minutes at 40° under certain specified conditions[7] and at a concentration of 1.3×10^{-5} mg. amylase per mg. starch.

(13) These analyses were carried out by Dr. Adalbert Elek, Microanalytical Laboratories, Los Angeles, California. To avoid possible contamination with sulfur, the purified preparations from which these crystals were obtained and the crystals were dried under vacuum and not over sulfuric acid.

(14) Ed. H. Fischer and P. Bernfeld, *Helv. Chim. Acta*, **31**, 1831 (1948).

(15) The spectrographic analyses were kindly carried out by Mr. John Dunbar in the laboratory of Professor T. I. Taylor, Columbia University.

(16) The electrophoresis and sedimentation measurements were kindly carried out by Dr. Maxine McKenzie in the laboratory of Professor Dan Moore, Columbia University.

TABLE I

ULTRAVIOLET ABSORPTION SPECTRA OF CRYSTALLINE PANCREATIC AMYLASE IN SOLUTIONS OF DIFFERENT CONCENTRATIONS[a]

Amylase solution					Optical densities 1×10^3
Solvent	Amylase concn., mg./ml.	pH	Absorption	λÅ.	
A, Redistilled water	0.06	6.3	Max.	2800	150.5
			Min.	2500	58
B, Phosphate, 0.01 *M*;	.56	7.0	Max.	2800	1242
and chloride, 0.02 *M*	.28	7.0	Max.	2800	630
	.14	7.0	Max.	2800	320
	.07	7.0	Max.	2800	179
	.035	7.0	Max.	2800	94

[a] Solutions were obtained by successive extractions with cold water of three times crystallized amylase and dilution with water (A) or with phosphate and chloride (B). The solutions all showed the same high amylase activity[7-9,12] of 16,000 mg. maltose equivalents per mg. solid at a concentration of 1.3×10^{-5} mg. per mg. starch.

Stability of Aqueous Solutions.—Saturated solutions of crystalline pancreatic amylase that had been crystallized 3 times did not show any loss of amylase activity when made up in redistilled water, and allowed to stand with no added electrolytes at 5° for 8 weeks. Similarly, dilute solutions, containing only 2 mg. of 3 times crystallized pancreatic amylase per ml., also showed no loss of amylase activity after standing at 5° for 10 weeks at *p*H 7.0 to *p*H 7.2 in the presence of 0.01 *M* phosphate and 0.02 *M* sodium chloride. Highly purified amorphous preparations of pancreatic amylase are much less stable on standing in aqueous solution and upon dialysis. The findings of Fischer and Bernfeld[17] indicate that the higher stability of the recrystallized amylase in solution and upon dialysis is due to the removal of a protease impurity.

Attempts to obtain dry pancreatic amylase by freezing solutions of the crystalline amylase at low pressures, by lyophilizing them, produced fluffy soluble products but caused losses of 30% or more of the amylase activity. These high losses of activity were observed even when the amylase solutions were frozen very rapidly by being added dropwise to flasks immersed and swirled constantly in freezing mixtures. Apparently, freezing disrupts some necessary arrangement or arrangements of active groupings[18,19] in the protein molecule.

Enzymic Homogeneity.—Pancreatic amylase exerts different kinds of enzymic activity: saccharogenic or sugar forming activity[7-9]; amyloclastic or liquefying activity[7-9]; glucosidase or dextrinase activity.[8,9,18-20] Studies with highly purified amorphous preparations of pancreatic amylase have given evidence[8,18-20] that these different activities all are properties of the amylase itself and are not due to contaminating carbohydrases. This conclusion has now been confirmed and strengthened by data obtained with crystalline pancreatic amylase. The 3 times crystallized amylase gave no evidence of selective activation or of selective inactivation of these different types of activity. Treatments that had a favorable or an unfavorable influence upon the stability or upon the activity of one kind of enzyme action had a similar favorable or unfavorable influence upon each of the others. The data with the crystalline amylase are very similar to those already reported[8,18-20] for amorphous preparations and are omitted here for the sake of brevity.

Maltase Activity.—When used in relatively very high concentrations, 500 or more times that required for amylase activity measurements, and when allowed to react with 1% maltose at 40° for 24 hours or more, highly purified amorphous preparations of pancreatic amylase showed traces of maltase activity.[20] The data given in Table II indicate that 3 times crystallized pancreatic amylase also will have slight action on maltose if sufficiently high concentrations of amylase and substrate react for sufficiently long periods of time. However, the data show that for practical purposes the glucose obtained in the hydrolysis of starches and of their components by pancreatic amylase is not due to any significant extent to the action of pancreatic amylase on maltose formed during the reaction.

Protease Activity.[21]—Highly purified but amorphous preparations of pancreatic amylase exert protease activity.[17,22,23] Three times crystallized pancreatic amylase was examined for protease activity by the method of Kunitz[24] using casein as substrate; 0.5% casein (Hammersten), 0.01 *M* phosphate, *p*H 7.6. The amylase was dissolved in 0.01 *M* phosphate, 0.02 *M* chloride, *p*H 7.2. No evidence of protease activity was found even when 1900 times the unit concentration[12] of the three times crystallized amylase reacted with the casein for 20 minutes at 40°. In comparable measurements, highly purified preparations of the amylase before crystallization exerted marked protease activity.

These results confirm and extend indirect evidence by Fischer and Bernfeld[17] that protease impurities are removed from pancreatic amylase by repeated recrystallization. Also, as Fischer and Bernfeld[17] suggest, the removal of protease impurities probably explains the fact that recrystallized pancreatic amylase is much more stable than less pure preparations upon standing in aqueous solution.

TABLE II

MALTASE ACTIVITY OF CRYSTALLINE PANCREATIC AMYLASE

Amylase[a] concn., relative[b]	Time of hydrolysis of maltose[c] in hours: 5	24	50
	Glucose formed per mg. maltose mg.		
1	0	0	0
12.5	0	0	0
125.0	0	0.01	0.02
500.0	0	.03	
630.0	0	.06	.07
930.0	0	.10	.14

[a] Pancreatic amylase: 3 times crystallized, at unit concentration produced 16,000 mg. maltose equivalents per mg. amylase in 30 minutes at 40° from Lintner soluble potato starch, 1%; 0.02 *M* chloride; 0.01 *M* phosphate; *p*H 7.2. [b] Unit concentration = 1.3×10^{-5} mg. amylase per mg. starch or per mg. maltose. [c] Maltose: 1%; 0.02 *M* chloride; 0.01 *M* phosphate; *p*H 7.2; 40°. Maltose, highly purified, prepared by action of β-amylase and recrystallized; $[\alpha]^{25}D$ 131.25; reducing value 98.5% determined by iodometric method.[10]

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