Pakistan J. Sci. Ind. Res., Vol. 18, Nos. 1–2, February–April 1975

ISOLATION AND PURIFICATION OF AMYLASE FROM RAT PANCREAS

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(Received April 4, 1974; revised November 21, 1974)

Abstract. The enzyme amylase was purified from rat pancreas. Three enzymatically active fractions were obtained upon chromatography on Amberlite IRC 50. One of these fractions was further separated into four isoenzymes. The mol. wt. of these were found to be 45,000, 33,110 and 16,220. The isoenzyme with 45,000 mol. wt. was crystallized and recrystallized. The specific activity of the crystalline isoenzyme was found to be 175, which is 3,000 times higher than the specific activity of the original homogenate.

The enzyme amylase belongs to the hydrolases group of enzyme, its systematic name being α-1,4-glucane-4-glucanohydrolyase (3.2.1.1).[1] The hydrolytic amylase can be divided into three groups according to its action[2] and distribuion in nature.[3]

1. α-Amylase (3.2.1.1). shows the dextrinizing effects, is found in plants, pancreas and saliva of animals .
2. β-Amylase (3.2.1.2). shows the saccharifying effects, is found in wheat, barley, soyabean and other plants.
3. γ–Amylase shows the formation of dextrin from glycogen in animals.

Amylases have been purified from differents sources. De-Pinto and Campbell[4] purified the enzyme from *Bacillus macerans*. Mahmoud and Attia[5] purified the amylase by adsorption on different types of soil clay.

Amylase were chromatographed on various ion exchange resins. Toda and Akabari[6] had chromatographed Taka amylase A on DEAE–cellulose column after precipitation and removing the precipitates by dialysis. Relatively stable proteins of low molecular weight and high isoelectric point have been chromatographed on finely divided Amberlite IRC 50.[7] The electrophoretic study of the wheat α-amylases (3.2.1.1). showed better resolution in the form of three bands indentical with the salivary amylase separated in the same way while β-amylase (3.2.1.2.) gave only one band.[8]

Enzyme of the rat pancreas was purified on two coupled column of Sephadex G-25 and DEAE-cellulose in tris-HCl buffer pH 8.2. Filtration on Sephadex G-100 and later purification on Biogel P-60 and CM cellulose after which they showed homogeneity by disc electrophoresis.[9] In a gel fitleration human serum amylase[10] and pancreatic amylase have been reported to behave similarly.[11]

The molecular weight of the enzyme amylase can be determined either by gel filtration, centrifugation, sedimentation or diffusion. Wilding[12] used dextrin Sephadex G-100 for the human amylase and found the molecular weight to be 45,000. Vandermers and Christopher[9] in thier studies on the chromatographic purification, molecular weight determination and amino acid composition reported a moclecular weight of 43,000 of rat pancreatic amylase by filtration on Sephadex G-100 and Biogel P-150.

Fischer and Bernfeld[13] have also reported the stability and the inactivation of α-amylase from hog pancreas. They stated that pure pancreatic α-amylase was stable whereas impure preparation of this enzyme were inactive due to proteolysis. McGeachin and Reynolds[14] suggested that hog liver amylase is different from that found in serum and other tissues. Later McGeachin *et al.*[15] noted that both salivary as well as pancreatic amylase are structuraly different from that of liver amylase. Procine pancreatic amylase has been fractionated by column chromatography and has been demonstrated to be comparising two isoenzymes.[16,17]

Several crystallization are necessary for complete removal of impurities. Heatley[18] conducted the spontaneous crystallization of amylase from pancreatic juice of rat and reported that recrystallization increases the purity as compared to crude crystals (not recrystallized) but once the maximum specific activity is attended further recrystallization seems unlikely to change purity.

Material and Method

Source of Enzyme. Male albino rats 3–4 months of age, and about same weight, obtained from the animal colony of Jinnah Postgraduate Medical Centre, were used. Pancreas of the 12 rats killed by hitting thier heads against the table were used for purification. The tissue was chilled and then homogenized in a glass homogenizer at 0°C for 10 min in normal saline. The homogenate was dilued to a concentration of 1 g tissue in 10 ml of total suspension (10% homogenate).

Hydrolysed starch (substrate amylose) and ammonium sulphate were obtained from Merck. Amberlite IRC 50 (analytical grade) was manufactured by Rohm and Haas Co., Resinous Product Division B.D.H. Sephadex G–200 was purchased from Pharmacia Uppsala, Sweden. Crystalline reference proteins, i.e. serum albumin, egg albumin, pepsin and chymotrypsin were obtained from Nutrional Biochemical Co. All other chemicals and

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reagents were obtained from commercial sources and were of highest purity available.

Assay for Enzyme Activity. Amylase activity was determined by Caraway's method.[19] One unit of enzymatic activity represented the amount of amylase which digest 5 mg starch in 15 min at 37°C.

Determination of Protein and Specific Activity. Protein concentration was estimated by reading the sample in the Unicamp spectrophotometer SP 800 at 280 nm against distilled water and specific activity was calculated as follows:

$$\text{Specific activity} = \frac{\text{Activity/ml}}{\text{mg protein/ml} \times (\text{O.D. at 280 nm})}$$

Enzyme Purification and Crystallization

The method used for enzyme purification and crystallization is as follows:

Step 1. To 150 ml 10% pancreatic homogenate, 113 g $(NH_4)_2SO_4$ was added, and this 75% saturated solution was left overnight in a refrigerator. The precipitate was removed by centrifugation at 30,000 rev/min at 4°C in Sorvale super speed R.C. 2 refrigerated centrifuge. The activity and specific activity of both the supernatent and residue (dissolved in normal saline) was determined by the method described.

Step 2. The enzyme solution (i.e. supernatent) was subjected to ion exchange chromatography requiring Amberlite IRC-50. The method of Hirs *et al.*[20] was modified for the purification of resin. To 50 g Amberlite IRC-50, 200 ml 0 05N NaOH was added. The suspension was heated for 15 min and washed to neutral with distilled water. The solution was then treated with 250 ml 0.05N HCl for 15 min and finally washed several times with distilled water till neutralized.

Step. 3. The resin was suspended in 0 01M phosphate buffer pH 7.0 and was packed in a column (40×2.5 cm). The clear enzyme solution about 5 ml was allowed to be adsorbed on the surface of resin. The enzyme was eluted with 0.001M phosphate buffer and 50–60 fractions of 5 ml each were collected and their protein content were determined as described. A graph between the optical density and the number of fraction eluted was plotted. The fraction belonging to same protein peaks were pooled and their specific activities were determined as described in the method.

Step 4. Molecular weight of amylase was determined by molecular sieving on Sephadex G-200 according to the method described by Andrews.[21] Following reference proteins were selected to cover the range of molecular weight expected: serum albumin (mol wt 70,000), egg albumin (mol wt 55,000), pepsin (mol wt 35,000), and chymotrypsin (mol wt 25,000). Dextrin blue (mol wt 200,000) was used as a marker of the front. A few mg of these proteins and marker was dissolved in 2 ml distilled water and applied on Sephadex G-200 column.

Step 5. Gel chromatography was carried out by suspension of 5 g Sephadex G-200 in 0.001M phosphate buffer pH 7, and left overnight in refrigerator and packed in a column of (60×1.5 cm). The elution was carried out with 0 001M phosphate buffer pH 7. Fraction of 5 ml each were collected. A graph between the mg/ml protein and the number of fraction eluted was plotted. The fraction belonging to the same protein peak were pooled and the specific activity in all of these fractions were determined.

Step 6. Log molecular weight of standard protein were plotted against ml eluted. The molecular weight of the enzyme was determined by interpolation of the same standard curve.

Step 7. The active fraction so obtained from the Sephadex G-200 column were pooled and saturated with 75% $(NH_4)_2SO_4$ was left in the refrigerator for the enzyme to crystallize. The turbidity which appeared after fortnight did not show amylase activity and was discarded by filtration on Whatman filter paper No. 1. Few more crystals of $(NH_4)_2SO_4$ were added to the saturate clear supernatent enzyme solution. Crystals were made visible after maintaining solution at yield of 4°C for 15 days. The maximum yield of the crystalline enzyme was obtained after 25 days. The solution containing the crystals was brought to room temperature. The crystals were removed and dried on a filter paper. The protein content, enzyme activity and specific activity of these crystals were determined by dissolving 1 mg crystalin 5 ml distilled water and were preserved for further investigation.

Results

In the present study number of attempts were made to purify the pancreatic amylase of albino rats. The results of the various step of purification are as follows:

Specific Activity of the Crude Enzyme. A 10% homogenate of pancreatic amylase was found to have an activity and specific activity of 1.65 and 0.6 respectively. The protein concentration was 28 mg/ml. After precipitation with 75% $(NH_4)_2SO_4$ the activity and specific activity were again determined in both the supernatent and the residue (Table 1).

Separation on Anion Exchange Column. Clear supernatent solution (5 ml), with specific activity of 0.52 was subjected to ion exchange Amberlite IRC 50. Fig. 1 shows the graph plotted between the optical density and the fractions so eluted. Three different proteins were separated having the specific activity of 4.43, 4.81 and 2.25, respectively. The protein content of each fraction was in the order of 0.46, 0.8 and 1.28 mg/ml. The second fraction having specific activity 4.81 and the protein content of 0.8 mg/ml was further subjected to 75% $(NH_4)_2SO_4$ precipitation. The activity, specific activity and protein content in the supernatent and residue was found to be 3.8, 2.8 units/ml 6.3, 5.6, 0.6 and 0.5 mg/ml respectively.

Gel Filtration. The four protein fractions so obtained when 5 ml enzyme solution was chromatographed on Sephadex G-200 as described in the

method has been shown in Fig. 2. A graph between the mg protein/ml and the no. of fraction eluted was plotted. Figure 2 shows four peaks belonging to different protein so obtained after chromatography. Activity, protein content and the specific activity were determined in each peak (Table 2).

Molecular Weight Determination. Molecular weight was determined on Sephadex G-200 column calibrated with standard proteins as described in the method and shown in Fig. 3.

Crsytallization. Each of the protein fraction obtained from Sephadex G-200 column was saturated with 75% $(NH_4)_2SO_4$ repeatedly for three weeks as described in the method. The crystals thus obtained were photographed (Figs. 4, 5, and 6). The activity, specific activity and protein concentration of the enzyme at various steps involved in the purification are summarized in Table 3. Overall yield per cent and fold purification at each step of the enzyme purification is shown in Table 4.

Table 1. The Activity, Protein Concentration, the Specific Activity of Supernatent and the Residue Obtained After Precipitation of the Crude Enzyme With 75% $(NH_4)_2SO_4$.

Enzyme solution	Activity (units/ml)	Protein concn (mg/ml)	Specific activity
Supernatent	5.7	11.0	0.52
Residue	4.8	24.3	0.22

Table 2. The Activity, Specific Activity, Protein Content and the Molecular Weight of the Four Different Proteins Obtained After Gel Filtration on Sephadex G–200.

Fraction No.	Activity (units/ml)	Protein concn	Specific activity	Mol wt
1	3.20	0.26	12.3	—
2	3.25	0.08	35.5	45,000
3	6.40	0.22	29.9	33,110
4	5.25	0.12	40.3	16,220

Table 3. The Activity, Protein Concentration and Specific Activity Obtained After Each Step in Purification.

Enzyme solutions	Activity (units/ml)	Density at 280 nm	Specific activity
Crude enzyme	1.65	28.00	0.06
Precipitation with ammonium sulphate	5.20	11.00	0.52
Anion exchange chromatography	3.90	0.80	4.81
Precipitation with ammonium sulphate	3.80	0.60	6.30
Gel filtration Sephadex G-200	5.25	0.08	35.50
1st crystallization	7.50	0.06	142.00
2nd crystallization	8.50	0.05	175.00

Table 4. Enzyme Purification Procedures Showing Yield % (overall) and Fold Purification at Each Step.

Preparation	Volume (ml)	Protein (mg/ml)	Activity (units/ml)	Total activity	Specific activity	Overall yield %	Fold purification
Crude extract	150	28.00	1.65	247.00	0.06	100	1
Precipitation with ammonium sulphate	35	11.00	5.70	100.50	0.52	80	9
Anion exchange chromatography	20	0.80	3.90	78.00	4.81	30	80
Precipitation with ammonium sulphate	15	0.60	3.80	57.00	6.30	23	105
Gel filtration on Sephadex G-200	5	0.08	5.25	26.25	35.50	17	592
1st crystallization	1	0.05	7.50	7.50	142.00	8.5	2367
2nd crystallization	1	0.05	8.50	8.50	175.00	5	3000

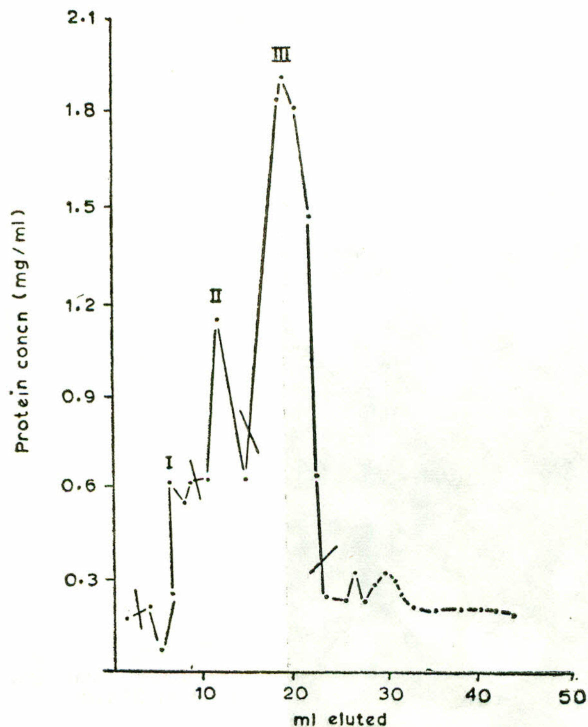

Fig. 1. Chromatography on Amberlite IRC-50 column (40 ×2·5 cm). Elution was carried out with 0·01M phosphate buffer pH 7·0 concentretion of protein in mg/ml are plotted agaisnt the volume of elute.

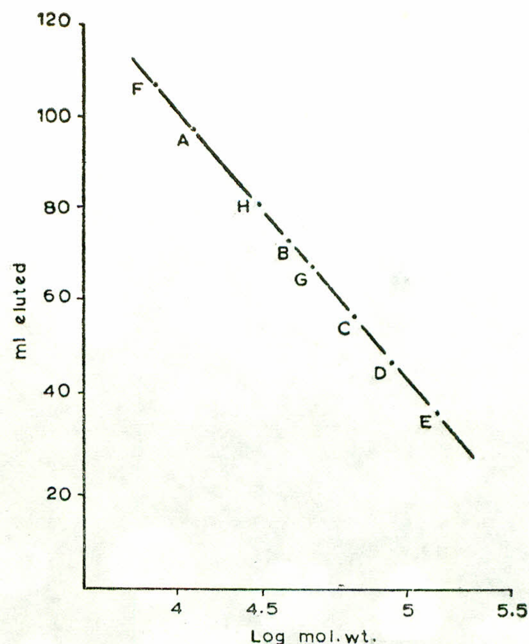

Fig. 3. Gel filtration on Sephadex G–200. A=Chymotrypsin (mol wt 25,000). B=Pepsin (mol wt 35,000)· C=Egg albumin (mol wt 25,000). D=Serum albumin (mol wt 70,000). E=Dextrine blue (mol wt 200,000). F=Amylase (mol wt 16,220). G=Amylase (mol wt 45,000). H=Amylase (mol wt 33,110).

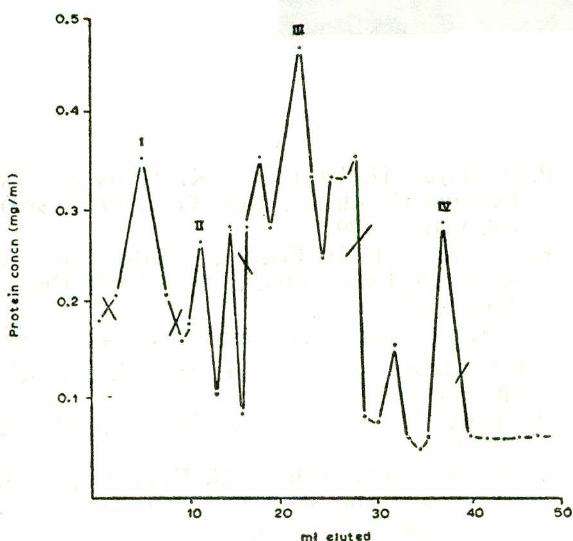

Fig. 2. Gel filtration on a Sephadex G-200 column (62×1·5 cm). Volume of the elute is plotted against concentration of protein. Four enzymatically active proteins could be separated.

Discussion

Amylase from animal sources have been investigated. The amylase from pancreas of rat was purified by Vandermeers and Christopher[9] and also from human saliva and pancreas by Wilding.[10] In the present study amylase was purified from pancreas of adult rats. The enzyme was purified by the usual techniques of protein purification and suitable condition for the crystallization and recrystallization was worked out.

Ion exchange chromatography revealed the presence of the three enzymatically active proteins. One of these protein when further subjected to purification by gel filtration on Sephadex G-200 was further separated into four isoenzymes of amylase. The molecular weight of the three of the isoenzymes were 45,000, 33,110 and 16,220. These results are not in agreement with those of Vandermeers and Christopher[9] who also purified rat pancreatic amylase by these techniques, but obtained different number of isoenzyme. The molecular weight of the isoenzymes isolated by them, however, are the same as found in the present studies. The number of enzymatically active proteins obtained appear to vary from the source of origin. Thus the result in the present studies cannot be compared with those of Peterson and Bendich[7] and Toda.[6]

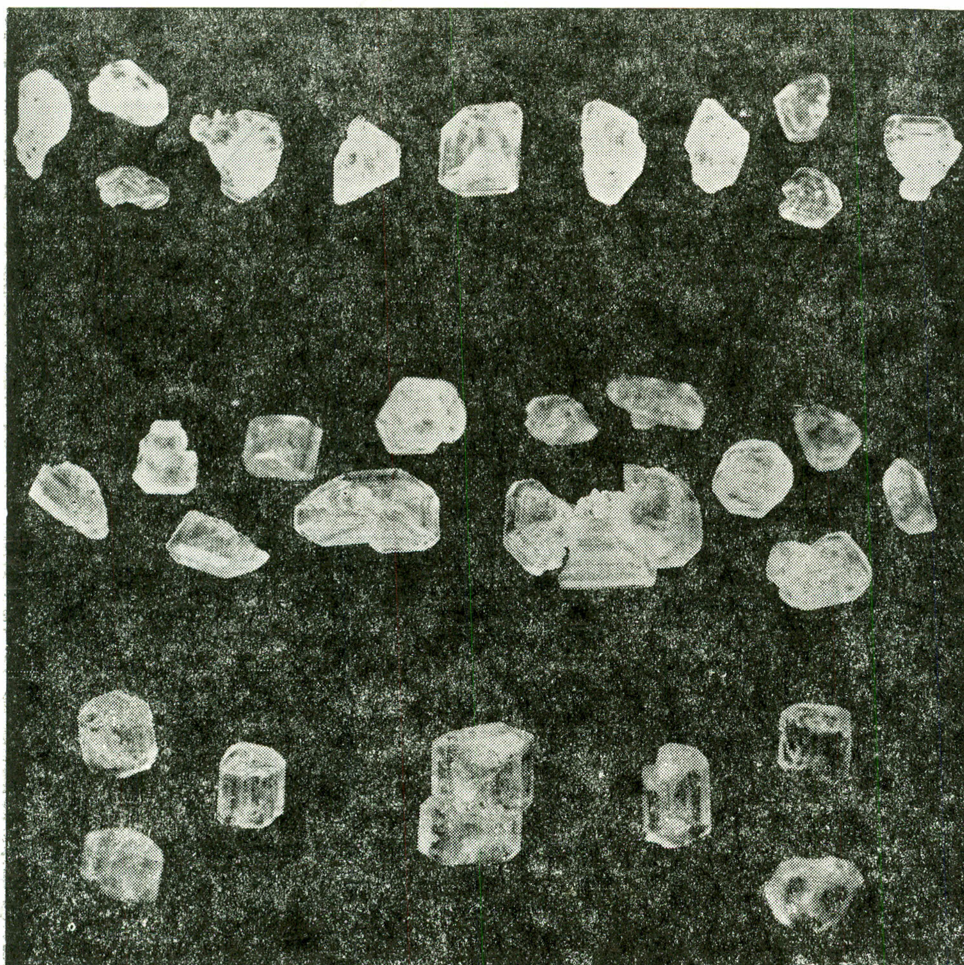

Fig. 4. Rat pancreatic isoamylase crystals (X5) (mol wt 16,220).

Fig. 5. Rat pancreatic isoamylase crystal (X5). (mol wt 33,110).

Fig. 6. Rat pancreatic isoamylase crystals (X5) (mol wt 45,000).

The crystallization and recrystallization of the isoenzyme solutions increased the specific activity. The maximum specific activity once obtained at recrystallization was not further increased by repeated recrystallization. A similar finding has been reported by Heatley[18] who isolated and recrystallized the rat pancreatic amylase.

From the present studies it becomes clear that the number of isoenzymes isolated depends upon the source of the enzyme. The yield per cent and fold purification is very isgnificant.

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