Purification Method of Proteins by Preparative Gel Electrophoresis. Crystallisation of α-amylases.

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INTRODUCTION

Polyacrylamide gel electrophoresis is widely used for the preparation of proteins. There are two methods: one is the gel slice extraction method[1-4], in which the electrophoresis gel slab is cut into slices and the protein fractions are extracted by crushing the slices with a buffer solution. The other is the continuous elution method[5-8], in which protein is moved through the electrophoresis gel out into an elution chamber to be removed by a stream of elution buffer. The former is simpler and gives much sharper resolution than the latter. However, some undefined soluble gel components are extracted from the gel and they might interfere with further purification. Hence, the continuous elution method has been preferred[9-12].

However, in this method some difficulties are observed. One is the fact that only a small quantity of the sample can be applied. Secondly, the separation of each indiviadual protein fraction in the elutate is not always sufficient. To secure a sufficient separation and to prevent the dilution of the protein fractions by the flow of the elution buffer, the elution chamber is made as thin as possible. In some cases an elaborate flow rate monitor is attached to make the elution of the protein fractions uniform and to avoid excessive dilution of slowly migrating fractions. This makes the apparatus more complicated.

On the other hand, the gel slice extraction method is simple and can achieve sharp separation of pure protein fractions. The problems with this method are to enlarge the apparatus for a larger sample quantity and to remove the contaminated soluble gel components. There is certainly a limit to the size of the apparatus but the most difficult problem is the eliminiation of the soluble gel components.

There have been several attempts to devise a method for removing

the contaminants. Hjertén[13] reported an electrophoretic zone-sharpening method, by which the neutral polymers of ethyleneglycol and the undefined soluble components of polyacrylamide gel were separated from the protein fractions. Suzuno et al,[14] separated the soluble gel components from protein fractions by electrophoresis using a membrane of agarose or washed polyacrylamide gel.

Using a discontinuous buffer system with a sucrose gradient, we developed a simple and general electrophoretic method for removing the soluble gel components from protein fractions. The apparatus was the same as that used in the preparative gel electrophoresis method. In this method, all protein fractions with different mobilities on disc electrophoresis migrated in a sharp band in the same position as the tracking BPB, while the soluble gel components remained at the position, at which the sample was applied originally. Using BPB as marker, therefore, the protein fraction could easily be isolated. This procedure may be called "purification electrophoresis." From the extracts of the preparative electrophoresis gels, we purified by this procedure Taka-amylase A and human salivary α-amylase in the crystalline state with high yields.

MATERIALS AND METHODS

Materials. Tris (hydroxymethyl)-aminomethane (Tris) and thioglycolic acid were purchased from Sigma Chemical Co., acrylamide, N, N'-methylenebisacrylamide (BIS) and N, N, N', N',-tetramethylethylenediamine (TEMED) from Nakarai Chemicals, Ltd Japan, and Amido Schwarz 10 B from Merck. Other chemicals were all of analytical grade and prepared in Japan.

Crystalline bovine serum albumin was purchased from INC Nutritional Biochemicals Corp. Bovine serum was prepared from fresh bovine blood in our laboratory. Human serum was obtained from healthy adults. Red cells were isolated from healthy human blood by repeated centrifugation and washing with an isotonic saline solution. The hemolysate was prepared by adding a quantity of water 10 times the volume of the red cells and centrifuging. Saliva was collected from a healthy adult by chewing solid paraffin. Taka-diastase from *Aspergillus orizae* was purchased from Sankyo Co. Japan.

Assay method. Protein concentrations were estimated according to the method of Lowry et al[15]. Amylase activity was assayed according to the method of Noelting and Bernfeld[16], and expressed in terms of milligrams of maltose liberated in 3 min at 30°C and pH 6.7, using potato soluble starch as a substrate. The protein concentrations of the gel extracts were

not determined, because the soluble gel components caused high blank values in Lowry's assay method, and absorbed a wide range of ultraviolet light.

Analytical disc gel electrophoresis. Polyacrylamide gel disc electrophoresis was carried out by a slightly modified procedure[17] of Ornstein and Davis[18]. Glass tubes 7.5 cm long and 0.5 cm in diameter were used. A separation gel of 7.5% acrylamide 5.0 cm in height and a spacer gel 1 cm in height were prepared. The top of the gel was washed once with a running buffer solution of pH 8.3. The sample solution was prepared by adding 60 mM of thioglycolate solution of pH 6.7, and 30% sucrose solution to the protein solution at the ratio of 3:3:4. The sample solution of 0.05 or 0.1 ml containing 100 to 150 μg of protein was applied between the running buffer solution and the spacer gel. The protein fractions were stained by 1% Amido Schwarz 10 B in 7% acetic acid, and amylase activity was stained by the iodine-starch reaction as described below.

Activity stain. After electrophoresis, the gel was removed from the glass tube and incubated in a substrate solution (2g of potato soluble starch dissolved in a mixture of 90 ml of 2.0 M acetate buffer, pH 5.9, and 10 ml of 0.1 M $CaCl_2$) at 37°C for 30 min. Then the gel was removed from the substrate solution and incubated in a reaction solution (a mixture of 90 ml of 2.0 M acetate buffer, pH 5.9, and 10 ml of 0.1 M $CaCl_2$) at 37°C for 10 to 120 min, depending on the amylase activity. Finally, the gel was immersed in a 7% acetic acid diluted iodine-potassium iodide solution, containing 0.5 ml of 6% I_2 and 4% KI in 1 liter of 7% acetic acid. The amylase band appeared on the strongly blue colored background as a faded band. By this stain method, several picograms of human salivary α-amylase could be demonstrated as sharp bands.

Preparative gel electrophoresis.

Preparative gel electrophoresis was carried out as reported previously[4], using the gel slab electrophoresis apparatus. Its principle was the same as that of the disc electrophoresis of Ornstein and Davis.[18] A photograph and a diagram of the apparatus*) are shown in Fig. 1. The apparatus was composed of a gel chamber and upper and lower buffer vessels. For the preparative gel electrophoresis, a gel chamber 15 cm in length, 18 cm in height, and 3 cm in width was usually used, although gel chambers with different widths could be attached to the apparatus. The upper and the lower buffer vessels were each separated into two

*) Manufactured by the Joko-Sangyo Co., Tokyo.

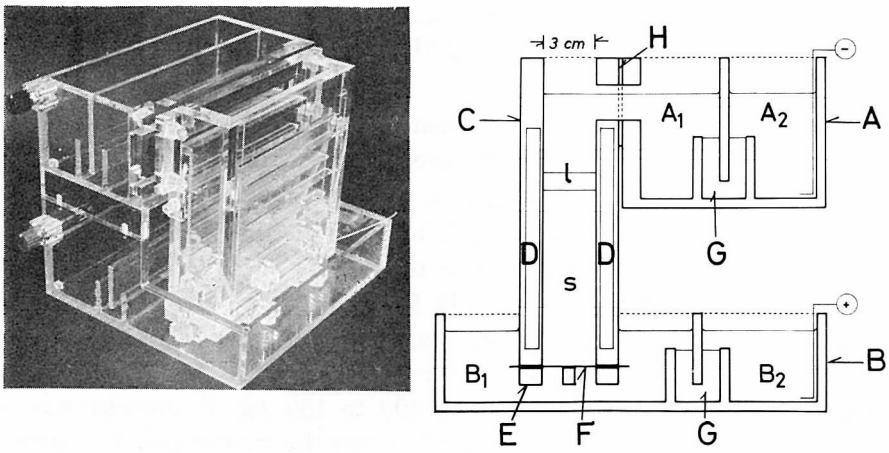


Fig. 1 Photograph and diagram of the apparatus of preparative gel electrophoresis.
A: Upper buffer vessel, B: lower buffer vessel, (A_1 and B_1: upper and lower atrial chambers, A_2 and B_2: upper and lower electrode chambers), C: gel chamber, D: cooling plates, E: supporting rack, F: cellophane membrane, G: gel bridge, and H: rubber gasket. s: Separation gel and l: spacer gel.

parts: an electrode chamber and an atrial chamber. The two chambers were connected by a polyacrylamide gel bridge.

The bottom of the gel chamber was supported by a sheet of cellophane membrane on a rack and the separation gel was prepared normally with 7.5% polyacrylamide to 10 cm in height. The spacer gel of 2.5% polyacrylamide of pH 6.7 was layered 1.5 cm in height. The running buffer was a Tris-glycine buffer of pH 8.3 containing a few drops of 0.001% BPB per liter. The buffer for the electrode chamber and the connecting gel bridge was 3 times more concentrated than the running buffer, to ensure a constant pH of the separation gel during the run. Protein samples were prepared to 10% in sucrose at pH 6.7, and layered on the spacer gel.

Electrophoresis was carried out at 400 V for 15 to 17 hours with cooling by water at 7°C circulated through the cooling plates of the gel chamber. When the tracking BPB band moved about 8 cm into the separation gel, the current was turned off and the separation gel slab was removed from the gel chamber and cut into 3 mm slices. A slice or several slices containing amylase activity identified by the activity stain method, were crushed in a porcelain mortar, and mixed with a buffer solution of twice their volume. The pH was adjusted to neutral by adding 2M acetic acid, and the suspension was stirred for 1 hour at 4°C and centrifuged at 10,000 rpm for 45 min. The precipitate was reextra-

cted using the same procedure as in the first extraction. Over 90% of the extractable protein was recovered by the first extraction. The combined extract was concentrated to about 10 ml by ultrafiltration through a Diaflo PM 10 membrane by an Amicon ultrafiltration cell. All preparation procedures were performed in a cold room at 4°C.

Purification electrophoresis: Removal of the soluble gel components by electrophoresis using a discontinuous buffer system with a sucrose gradient.

The soluble gel components contaminating the protein extract from the gel slices were separated electrophoretically using the same apparatus as in the preparative gel electrophoresis. The procedure was based on the same principle as disc electrophoresis concentrating charged molecules. To support the concentrated protein band during the electrophoresis, a sucrose gradient of 15 to 45% was used in the buffer solution. By this procedure, all the protein fractions and BPB were concentrated gradually to a sharp band which migrated into the supporting buffer solution, while the soluble gel components remained at the position where the sample was applied originally. Hence the protein fraction could

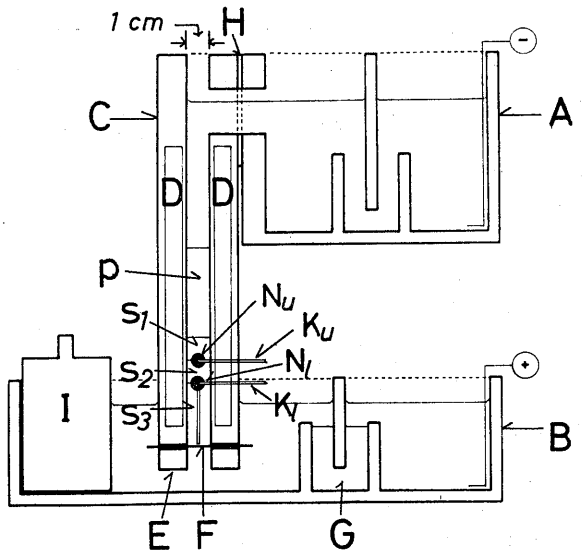


Fig. 2 Diagram of the apparatus for purification electrophoresis.

A and B: Upper and lower buffer vessels, C: gel chamber, D: cooling plates, E: supporting rack, F: cellophane membrane, G: gel bridge, H: rubber gasket, I: plastic block, Nu and Nl: upper and lower tube nipples, and Ku and Kl: plastic capillary tubes, s_1, s_2, and s_3: supporting buffer solution containing sucrose of 15%, 30%, and 45%, P: sample solution.

easily be recognized and eluted, using the tracking BPB band as a marker. In Fig. 2, the apparatus for the purification electrophoresis is presented diagrammatically.

In order to facilitate the preparation of the sucrose gradient and the application and removal of the sample solution, the gel chamber was provided with two nipples, as shown in Fig. 2, at 3 cm and 4 cm from the bottom of the cell. Plastic capillary tubes were inserted into the nipples and the one through the lower nipple reached to the bottom of the gel chamber.

The bottom of the gel chamber was closed by a cellophane membrane. The running buffer solution was poured into the gel chamber and cooled. The supporting buffer solutions containing sucrose of 15%, 30%, and 45%, were introduced through the lower capillary in layers 1 cm, 1 cm, and 3 cm high, respectively. The buffer solutions were prepared by mixing the stock buffer solution (Tris 5.98 g, 1 N HCl 48 ml, and water to 100 ml), water, and 60% sucrose solution in the proportions of 1:5:2, 1:3:4, and 1:1:6, respectively. The sample solution was concentrated to an appropriate volume by ultrafiltration and adjusted to pH 6.7 with 2 M acetic acid. Sucrose was added to 7.5% and the solution was layered with a syringe between the running and the supporting buffer solution. The atrial chamber of the lower vessel contained the supporting buffer solution of 45% sucrose to prevent disturbance of the sucrose gradient in the gel chamber. The running buffer solution was poured into the upper vessel and the electrode chamber of the lower vessel. Before the electrophoresis, the capillaries were stoppered.

Electrophoresis was carried out at 400 V under cooling at 7°C. When the tracking BPB band migrated to a position below the opening of the upper nipple, the current was turned off, and the upper part of the solution was removed through the upper capillary, and the BPB containing part was drawn out through the lower capillary. The latter solution was dialyzed against a buffer solution.

Other methods.

The turbidity obtained from the gel extract by the addition of ammonium sulfate was measured by a Shimadzu QR-50 spectrophotometer using a turbidimetric attachment. Turbidimetry was carried out at 600 nm with a controll of water 0 O.D. Nephelometry was carried out using a 65% saturated ammonium sulfate solution as a controll to infinity, and 0.200 mg/ml bovine albumin solution with 65% ammonium sulfate saturation to 0.200 O.D.

RESULTS

Soluble polymers of polyacrylamide gel.

We examined at first the extract of the usual 7.5% gel. As presented in Fig. 3, the gel extract showed considerable ultraviolet absorption, especially in the region below 250 nm. The UV absorption curve changed during the preservation period. On the other hand, when the gel was preserved for 1 to 24 hr., UV absorption of the extract changed only slightly. When the extracts from variously aged gels were dialyzed against water, their UV absorption decreased considerably. But the decrease was most marked in the extract of the one-hour-aged gel, and the least in that of the 24-hours-aged gel. From these facts, it is clear that the soluble gel components which absorb UV light are not yet in a difinite state but reacting and changing gradually for over 2 weeks.

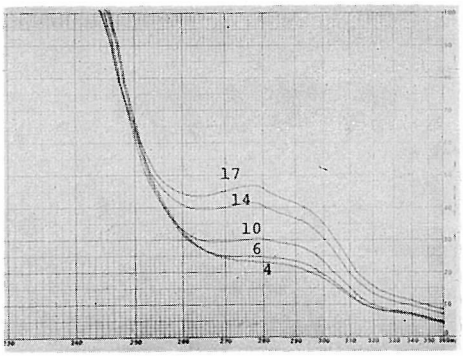


Fig. 3. UV absorption change of polyacrylamide gel extract by aging.

7.5% polyacrylamide gel was extracted with water of twice its volume. The extract was maintained at room temperature for the days indicated in the figure and UV absorption curves were recorded by spectrophotometer.

Fig. 4 shows the effects of ultrafiltration and dialysis of the gel extract. When the gel extract was ultrafiltrated and concentrated, the ultrafiltrate showed almost the same UV absorption as that of the original extract. But the concentrate showed increased absorption. When the gel extract, ultrafiltrate, and concentrate were dialyzed against water, their UV absorption deccreased considerably, except that of concentrate. When ammonium sulfate was added to the gel extract, turbidity appeared at about 55% saturation and increased with the addition of the salt. The turbidity did not decrease by the dialysis of the gel extract, as shown in Fig. 5. From these fact we may conclude that the UV absorbing

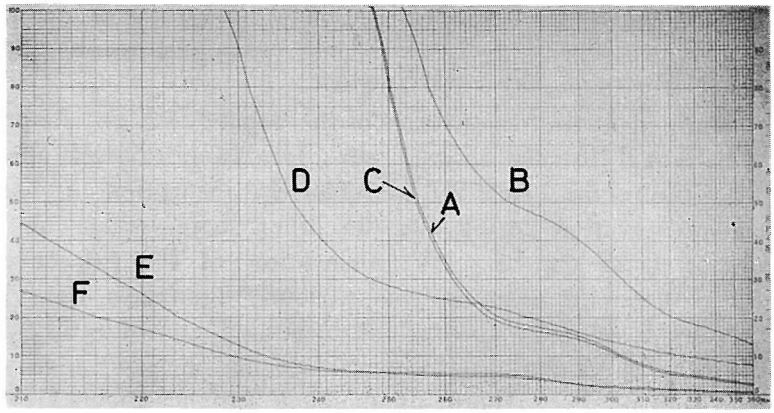


Fig. 4 Ultrafiltration and dialysis of the polyacrylamide gel extract.

7.5% polyacrylamide gel extract with water was concentrated by ultrafiltration through Diaflo PM 10 membrane by Amicon ultrafiltration cell. A: Original gel extract, B: concentrated gel extract, C: ultrafiltrate, D: the concentrate after dialysis against water, E: gel extract after dialysis, F: ultrafiltrate after dialysis.

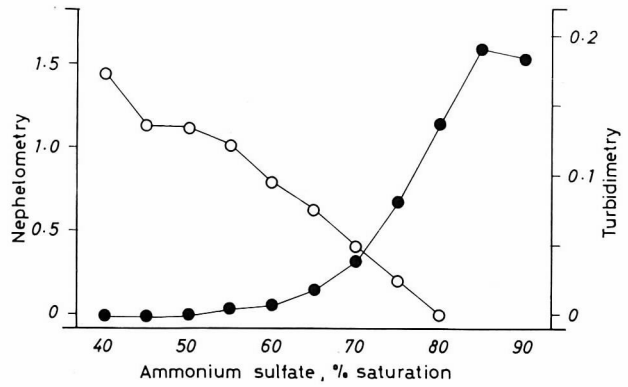


Fig. 5 Turbidimetry and nephrometry of precipitation of gel extract with ammonium sulfate.

Turbidimetry was carried out at 600 nm with a control of water 0 O.D. Nephrometry was carried out using a 65% saturation of ammonium sulfate as a control to infinity, and 0.20 mg/ml bovine serum albumin with 65% saturation ammonium sulfate to 0.200 O.D. -●-●-: Turbidity at 600 nm, -○-○-: optical density by nephelometry.

gel extract contains some soluble acrylamide polymers, of which considerable part can be dialyzed. But some part is not dialyzable and precipitated by ammonium sulfate saturation.

When the gel was formed from recrystallized acrylamide and BIS, the gel extract showed almost the same UV absorption as that of the

usual gel, notwithstanding the times of the recrystallisation. When the amount of BIS added was changed to 0.2, 0.4, 0.6, and 0.8% using 7.5% acrylamide, gel extracts showed very similar UV absorption. Only the dialysate of the extract of the gel with 0.4% BIS showed least absorption.

By an electrophoresis of the gel, the content of the UV absorbing soluble gel components decreased to some extent with the duration of the run. But the solution containing the substances migrated out from the gel showed almost the same UV absorption as that obtained by simple diffusion. This fact shows that we cannot expect to eliminate the contaminants by the preliminary electrophoresis before the addition of the sample.

Column chromatographies with Sephadex and other substances showed only slight separation of the UV absorbing gel components and bovine albumin. Hence they seem to be poorly applicable to the separation

Recovery of the protein fractions after the purification electrophoresis.

Using purification electrophoresis apparatus with a gel chamber of 15×18×1 cm, recovery of human serum protein was tested. 0.5 ml of normal human serum was diluted to about 40 ml with water and adjusted to pH 6.7 with 2 M acetic acid. Sucrose was added at a concentration of 7.5% and the final volume was increased to 50 ml with water. The running buffer solution and the supporting buffer solution with the sucrose gradient were introduced into the apparatus. The sample solution was layered between the running and the supporting buffer solutions. The height of the sample solution was about 3.5 cm.

Immediately after the current was applied, a sharp BPB band appeared at the top of the sample solution. The BPB band gradually became concentrated. After a few minutes, the BPB band rapidly diffused into the whole of the sample solution. The diffused BPB band gradually narrowed, became more concentrated, and moved downward. After 3 to 3.5 hr., the BPB band was contracted to about 2 mm, and reached the zone of the supporting buffer solution with 30% sucrose. At this moment, the current was turned off and the BPB-containing part of the solution was drawn out through the lower capillary. In Fig. 6-A, examples of the elution patterns of human serum are shown. From the figure, it can be observed that the tracking BPB band migrated a little faster than the protein. Owing to the diffusion during the electrophoresis, a discontinuity of pH could not be sharply maintained, and the protein fractions of the serum were not separated individually but distributed in a relatively

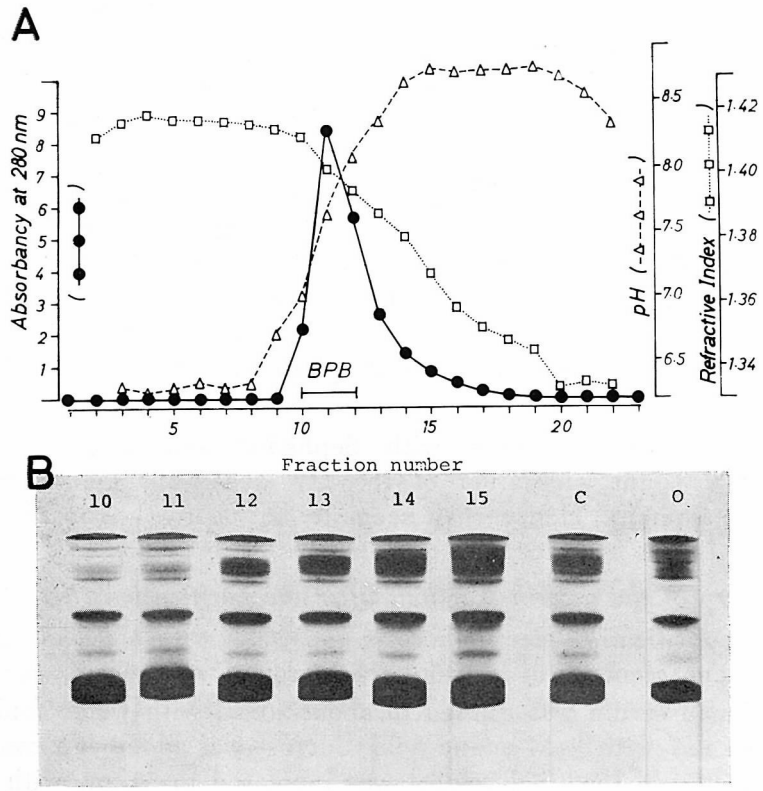


Fig. 6 Elution patterns of human serum after purification electrophoresis.

0.5 ml of human serum was diluted to 50 ml and applied. Electrophoresis was carried out at 400 V under cooling at 7°C. When the tracking BPB band reached a position between the upper and the lower tube nipples, the current was turned off and each 2 ml of fraction was drawn out through the lower capillary.

A: Elution pattern. -●-●- : Absorption at 280 nm, -△-△-: pH. PH values were obtained with a Hitachi-Horiba F-55-S pH meter by diluting the fractions 10-fold. -□-□-: Refractive index at room temperature as measured by an Abbe refractometer (Simadzu).

B: Disc electrophoresis patterns of the drawn out fractions (Fraction No. 10 to 15.), combined fraction (C), and the original human serum (O).

narrow zone. In Fig. 6-B, disc electrophoresis patterns of some eluted fractions are shown. From the figure, it can be seen that the lately eluted fractions were composed of relatively high concentrations of globulin frations. The protein patterns of the combined fraction was almost the same as that of the original untreated serum.

In Table I, results obtained by the procedure are shown. From 50 ml of the sample solution containing human serum, over 90% of the protein was recovered, concentrated in 12 ml. The recovery rates for the

Table. I Recovery of proteins and soluble gel component by purification electrophoresis.

Sample	Applied			Recovered			Yield
	ml	E_{280}		ml	E_{280}		%
		per ml	Total		per ml	Total	
Human serum	50	0.889	44.4	12	3.44	41.3	93
Human red cell hemolyzate	14	11.15	156	7.7	19.6	151	97
Bovine serum albumin	14	1.45	20.3	3.0	6.58	19.7	97
Bovine serum	14	3.15	44.1	8.2	6.09	49.9	113
Undefined soluble gel components*	10	0.831*	8.31*	42	0.143	7.43	89

*) Values of absorption at 240 nm.

other protein samples, human red cell hemolysate, bovine serum, and bovine serum albumin amounted over 95%.

Removal of the soluble gel components by purification electrophoresis.

The gel extract was prepared from 400 ml of 7.5% polyacrylamide gel block. The gel was crushed as finely as possible in a mortar. The

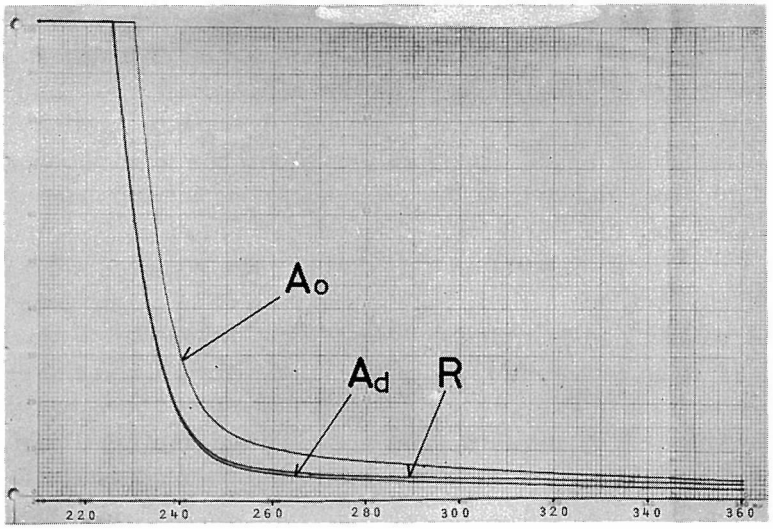


Fig. 7 UV absorption curves of the concentrated extract of polyacrylamide gel before and after purification electrophoresis.

Concentrated and dialyzed extract of polyacrylamide gel was subjected to purification electrophoresis at 400 V at room temperature. When the tracking BPB band reached a position between the upper and the lower tube nipples, the current was turned off and the solution was collected where the sample was applied originally. The recovered fraction was concentrated by ultrafiltration and dialyzed against water. The UV absorptions of the applied and the recovered samples were recorded by a spectrophotometer. Ao and Ad: The original and 1.7 times diluted applied sample, R: the recovered sample.

gel homogenate was suspended in an equal volume of water, stirred for 1 hr., and centrifuged at 12,000 rpm for 45 min. The extration was repeated 3 times. About 1.1 liters of the collected extract was concentrated to about 50 ml by ultrafiltration and dialyzed thoroughly against a large volume of water for 48 hr. with frequent exchanges. Fig. 7 shows the UV absorption of the concentrated extract. The absorption increased until 260 nm and very steeply at about 250 nm.

Ten ml of the concentrated gel extract was subjected to the purification electrophoresis in the same manner as the protein samples. When the tracking BPB band reached the supporting buffer solution of 30% sucrose, the current was turned off, and the gel extract was collected where the sample solution was applied originally. The fraction obtained was dialyzed thoroughly against water. The volume of the recovered solution was 42 ml. Its UV absorption curve is given in Fig. 7. From the figure, it may be observed that the absorption curves of the original and the recovered solutions are almost the same. Thus it is clear that the soluble acrylamide polymers extracted from the gel remained almost totally unmoved from the position of application.

Purification of amylases by preparative gel electrophoresis.

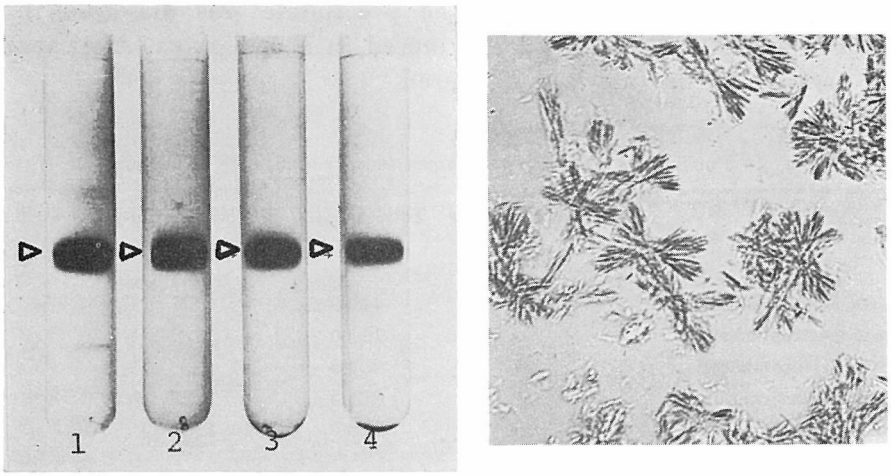
1. Taka-amylase A.

Taka-amylase A was purified from commercial Taka-diastase (Sankyo). Five grams of Taka-diastase powder was stirred into 30 ml of water for 30 min at room temperature. The slightly turbid solution obtained was centrifuged at 10,000 rpm for 10 min. Fifteen ml of the clear brown supernatant was subjected to the preparative gel electrophoresis with a gel chamber of 15×18×3 cm. When the tracking BPB band moved about 8 cm into the separation gel, the gel was removed and cut into 3 mm slices. Amylase activity was stained using a small part of each slice and usually demonstrated in 2 to 3 slices. They were extracted with twice their volume of 2 mM calcium acetate solution and the procedure was repeated twice. The combined extract was neutralized by 2 M acetic acid, concentrated by ultrafiltration, and dialyzed against 2 mM calcium acetate solution. The final solution obtained amounted to 14.2 ml.

Purification electrophoresis was carried out as described above. When the tracking BPB band reached the 30% sucrose fraction, the current was turned off and the fraction containing the tracking BPB band was collected. It was dialyzed against a 2 mM calcium acetate solution. The purified amylase solution amounted to 70 ml. It was concentrated to 25.5 ml by ultrafiltration, and 32 ml of cold acetone was added dropwise with gentle stirring. The precipitate was isolated by

Table. Ⅱ Purification procedure of Taka-amylase A from Takadiastase (Sankyo).

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	601	12230	43	100
Prep. electrophoresis	—	9060	—	74
Purifn. electrophoresis	163	8660	54	71
lst Crystallization	95	6240	65	51
2nd Crystallization	67	4460	67	36



A B

Fig. 8 Disc electrophoresis patterns in the purification steps of Taka-amylase A (A) and a picture of the recrystallized Taka-amylase A (B).

A-1: Crude extract of Taka-diastase (Sankyo), 2: extract obtained after preparative gel electrophoresis, 3: eluate obtained after purification electrophoresis, and 4: the first crystalline sample.

centrifugation and dissolved in a small volume of 2 mM calcium acetate solution. After centrifugation, cold acetone was added carefully to the supernatant, until a slight turbidity appeared. After 2 to 3 days, crystals of Taka-amylase A appeared. When the suspension was stored for another few days, the amylase crystals increased.

In Table II, the purification procedures of Taka-amylase A are summarized. From 601 mg of protein of crude extract from 2.5 g of Taka-diastase, 95 mg of crystalline enzyme were obtained. The recovery rate was 51%. In Fig. 8, disc electrophoresis patterns of the fraction obtained in the purification steps and a picture of the recrystallized

Taka-amylase A are shown.

2. Human salivary α-amylase.

Human salivary α-amylase was purified by the same procedure used for Taka-amylase A. Saliva was collected from a healthy adult by chewing solid paraffin. The crushed paraffin was filtered out with a gauze, and the filtrate was centrifuged at 10,000 rpm for 20 min in a cold room. The supernatant was used for the purification.

After the preparative gel electrophoresis, the amylase fraction was extrated from the gel and the extract was purified by purification electrophoresis. The purified amylase solution was dialyzed and concentrated, and cold acetone was added. The precipitate was dissolved in a minimal volume of 0.1% $CaCl_2$ and stored in a cold room. After several days, crystals of the amylase appeared.

Table. Ⅲ Purification procedure of human salivary α-amylase from saliva.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Saliva supernatant	253	44600	177	100
Prep. electrophoresis	—	19000	—	43
Purifn. electrophoresis	64	27400	433	61
Acetone fractionation	62	27200	443	61
Crystallization	40	23500	587	53

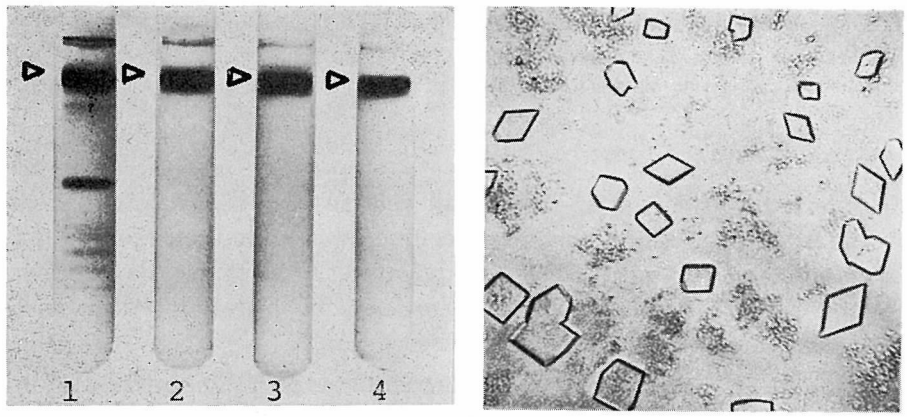


Fig. 9 Disc electrophoresis patterns in the purification steps of human salivary α-amylase (A) and a picture of the crystalline enzyme (B).

A-1: Supernatant of saliva, 2: extract obtained after preparative gel electrophoresis, 3: eluate obtained after purification electrophoresis, and 4: the crystalline enzyme.

Table III summarizes the purification steps. From 200 ml of human saliva containing 253 mg of protein, 40 mg of crystalline amylase was obtained. The activity recovery amounted to 53%. Fig. 9 shows the disc electrophoresis patterns of the fraction obtained in the purification steps and a picture of the crystallized enzyme. The crystalline sample showed one sharp and homogeneous band on disc gel electrophoresis.

DISCUSSION

Protein recovery of the preparative gel electrophoresis.

In our earlier experiments[19], we obtained highly purified spinach leaf aldolase using preparative gel electrophoresis with the gel slice extraction method. The spinach leaf extract was subjected to preparative gel electrophoresis and the extract obtained from the gel was fractionated by the addition of ammonium sulfate at between 25 and 50% saturation. The aldolase fraction obtained was fairly pure and migrated a nearly single band on analytical disc electrophoresis. It was thus purified 75-fold by a single electrophoresis, and its recovery was 41%.

In another experiment in our laboratory[20], bovine serum albumin was recovered at a rate of about 90% by several extractions of the gel, where 90% of the extractable protein was recovered in the first extraction.

Recoveries with the gel slice extraction method were not always high. We recovered only small percentage of potato phosphorylase activity. When the separation gel was prepared at a lower pH (6.7), however, usually more than 50% of phosphorylase activity was recovered. The enzyme might be denatured at a high pH by the procedure. Animal phosphorylases were also sensitive to higher pH values.[21]

As shown in Table II, the recovery of Taka-amylase A with preparative gel electrophoresis was 74%. On the other hand, that of human salivary α-amylase was 43% (see Table III). In the latter case, however, the total activity increased 1.4 times after purification electrophoresis. The same phenomenon was observed in other experiments with salivary amylase. Since the recoveries of protein by purification electrophoresis were very high, as shown in Table I and II, the real recovery of the salivary amylase with preparative gel electrophoresis should be near 65%. The gel extract might contain an inhibitory substance against salivary amylase, which would be removed by the purification electrophoresis.

With the continuous elution method, all protein fractions contained originally in the sample could be recovered. However, the separation of protein fractions was often not sharp, so that considerable portions of

the protein fractions were lost. Thus, the recoveries were usually 50 to 80% when the sample was purified several times.

With the gel slice extraction method, it is not difficult to achieve a recovery of 50%. Therefore, with regard to recovery, the gel slice extraction method is not inferior to the continuous elution method. Also the procedure is simpler and the resolution is better.

Removal of the soluble gel component.

Feldberg and Datta[22] used preparative gel electrophoresis as the last step of a procedure for the purification of threonine deaminase from *Rhodosprillum rubrum*. To remove the gel components, the sample solution obtained was applied to a DEAE-cellulose column. The column was then washed with a buffer until the absorbance at 225 nm was negligible. In our earlier experiment[20], however, the soluble gel componets could not be removed from bovine serum albumin by preliminary electrophoresis or by column chromatography on Sephadex G 25 or DEAE-cellulose. Lerch and Wolf[23] reported that after hydroxylapatite chromatography the eluate still contained impurities from the polyacrylamide. Suzuno et al[14] separated the soluble gel components by electrophoresis using a thin membrane of agarose or washed polyacrylamide gels. The procedure was complicated and the recovery of bovine serum protein was not over 70%.

Hjertén[13] reported an electrophoretic zone-sharpening method by which neutral polymers such as polyethyleneglycol, dextran, or the undefined soluble components of polyacrylamide could be removed. It was based on the fact, that the neutral substrances remained at the original position, whereas the charged substances migrated into the buffer solution with a sucrose gradient. The electrophoretic migration of a substance was strongly reduced when the substance migrated from a sucrose-free buffer into a buffer containing sucrose. The concentration of the charged substances could be followed visually if the sample was colored or absorbed UV light. The fractions were then analyzed by UV absorption or measurements of enzymatic activity.

Here we used the discontinuous buffer system with a sucrose gradient. All the protein fractions and BPB migrated as one sharp band due to the principle of the concentration by a discontinuous buffer system. The protein fraction could easily be collected without any additional equipment, as in the method of Hjertén[24]. For the separation of the soluble gel component, we used the same apparatus as in preparative gel electrophoresis. We named the procedure "purification electrophoresis." As shown in Table I, the recoveries of the protein samples were

very good, and the soluble gel component was almost completely removed.

Crystallization of α-amylases.

Taka-amylase A. Taka-amylase was originally crystallized by Fischer and de Montmollin[25] from "Clarase 900" by fractional precipitation with ammonium sulfate and acetone. In 1954, Akabori et al[26] reported a simple method for crystallization of the enzyme from Taka-diastase (Sankyo). They used fractional precipitation with ammonium sulfate and acetone, with rivanol (2-ethoxy-6, 9-diamino-acridinium lactate) as a specific precipitant. From 30 g of Taka-diastase, they obtained about 0.5 g of crystalline enzyme. The recovery rate was 33%.

In our experiment, 95 mg of crystalline Taka-amylase A was obtained from 2.5 g of Taka-diastase (Sankyo) in a 3-step purification procedure: preparative gel electrophoresis, purification electrophoresis, and acetone fractionation. The overall recovery was 51%.

Human salivary α-amylase. Human salivary amylase was originally crystallized by Meyer et al[27] in 7 steps of purification, including 4 acetone fractionation steps, 2 ammonium sulfate fractionation steps, and treatment with an anion exchange resin. The overall recovery was 24%. Mutzbauer and Schulz[28] simplified the procedure by the use of Sephadex column chromatography. From 1 liter of normal saliva, they obtained 115 mg of α-amylase. In our experiment, 40 mg of crystalline enzyme was obtained from 200 ml of normal human saliva by a procedure with only 3 steps, as in the case of Taka-amylase A. The overall recovery was 53%.

As mentioned above, preparative gel electrophoresis and the gel slice extraction method, including purification electrophoresis are very useful for the preparation of proteins. The method is very simple and the resolution is very sharp. If an appropriate buffer system is used, it is not difficult to obtain highly purified proteins at a high yield.

SUMMARY

Taka-amylase A from *Aspergillus orizae* and human α-amylase were crystallized in a three-step procedure:

(1) Crude extract of Taka-diastase (Sankyo) or saliva supernatant was applied to a gel slab type apparatus of preparative electrophoresis. The active fractions were extracted by crushing the gel slices with a buffer solution. (2) The soluble gel components, which were extracted from the gel slices together with protein fractions, were removed by electrophoresis with a discontinuous buffer system containing sucrose gradient

(purification electrophoresis). (3) After acetone fractionation, crystalline amylases were obtained. The overall recovery rates of Taka-amylase A and human salivary amylase were 51% and 53%, respectively.

Further usage of preparative gel electrophoresis and purification electrophoresis to obtaining pure porteins was examined.

REGERENCES

1) Kaltschmidt, E. and Wittmann, H.G.: Ribosomal proteins. VI. Preparative polyacrylamide gel electrophoresis as applied to the isolation of ribosomal proteins. *Anal. Biochem.*, **30**: 132-141, 1969.
2) Gordon, R.B., Brown, D.H. and Brown, B.I.: Preparation and properties of the glycogen-debranching enzyme from rabbit liver. *Biochim. Biophys. Acta*, **289**: 97-107, 1972.
3) Wada, M., Naito, H.K., Ehrhart, L.A. and Levis, L.A.: Polyacrylamide-gel block-electrophoresis of plasma lipoproteins. *Clin. Chem.*, **19**: 235-239, 1973.
4) Nakamura, S., Ogata, H., Takeo, K., Kuwahara, A., and Suzuno, R.: The effect of α_2-macroglobulin from bovine serum on bovine α-chymotrypsin. *Hoppe-Seyler's Z. physiol. Chem.*, **356**: 677-692, 1975.
5) Lewis, U.J. and Clark, M.O.: Preparative methods for disk electrophoresis with special reference of the isolation of pituitary hormones. *Anal. Biochem.*, **6**: 303-315, 1963.
6) Jovin, T., Chrambach, A., and Naughton, M.A.: An apparatus for preparative temperature-regulated polyacrylamide gel electrophoresis. *Anal. Biochem.*, **9**: 351-369, 1964.
7) Gordon, A.H. and Louis, L.N.: Preparative acrylamide electrophoresis: A single gel system. *Anal. Biochem.*, **21**: 190-200, 1967.
8) Hjertén, S., Jerstedt, S. and Tiselius, A.: Apparatus for large-scale preparative polyacrylamide gel electrophoresis. *Anal. Biochem.*, **27**: 108-129, 1969.
9) (a) Kohn, L.D., Rackman, P.M., Allen, R.H., and Jakoby, W.B.: Tartaric acid metabolism. V. Crystalline tartrate dehydrogenase. *J. Biol. Chem.*, **243**: 2479-2485, 1968. (b) Kohn, L.D. and Jakoby, W.B.: ibid VI. Crystalline oxaloglycolate reductive decarboxylase. *J. Biol. Chem.*, **243**: 2486-2493, 1968. (c) Kohn, L.D.: ibid. VIII. Crystalline tartronic semialdehyde reductase. *J. Biol. Chem.*, **243**: 4426-4433, 1968. (d) Scher, W. and Jakoby, W.B.: Maleate Isomerase. *J. Biol. Chem.*, **244**: 1878-1882, 1969.
10) Reed, R.E. and Hess, J.L.: Partial purification and characterization of aspartate aminotransferases from seedling oat leaves. *J. Biol. Chem.*, **250**: 4456-4461, 1975.
11) Hardman, J.K., Berger, H., and Goodman, M.: Tryptophan operon read-through-isolation and characterization of an abnormally long tryptophan synthetase α-subunit from a frame-shift mutant of Escherichia coli. *J. Biol. Chem.*, **250**: 4634-4642, 1975.
12) Curry, R.A. and Ting, I.P.: Purification and crystallization of three isoenzymes of malate dehydrogenase from zea mays seeds. *Arch. Biochem. Biophys.*, **158**: 213-224, 1973.
13) Hjertén, S. : A new method for the concentration of high and low molecular weight substances and for their recovery following gel electrophoresis, partition and precipitation experiments. *Biochim. Biophys. Acta.*, **237**: 395-403, 1971.
14) Suzuno, R., Takeo, K., Ogata, H., Kuwahra, A. and Nakamura, S.: Eliminating method of soluble polymer from acrylamide gel. *The Physico-Chemical Biol.*, **18**: 167, 1974 (Japanese).
15) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J.: Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.*, **193**: 265-275, 1951.
16) Bernfeld, P.: In *Methods in Enzymology* (Colowick, S.P. and Kaplan, N.O., edited) Vol. 1, pp. 149-154, Academic Press, New York, 1955.
17) Takeo, K. and Nakamura, S.: Dissociation constants of glucan phosphorylases of rabbit

tissues studied by polyacrylamide gel disc electrophoresis. *Arch. Biochem. Biophys.*, **153**: 1–7, 1972.
18) Ornstein, L. and Davis, B. J.: *Disc electrophoresis.* Preprint by Distillation Product Industries, 1962.
19) Takeo, K., Suzuno, R., Shimizu, J., and Nakamura, S.: Purification of spinach aldolases by preparative disc electrophoresis. *The Physico-Chem. Biol.*, **14**: 308–310, 1970.
20) Nakamura, S.: Fractionation of proteins by disc electrophoresis. —Gel slice technique—. *The Physico-Chem. Biol.*, **17**: 177–185, 1973.
21) Davis, C. H., Schliselfeld, L. H., Wolf, D.P., Leavitt, C.A., and Krebs, E.G.: Interrelationships among glycogen phosphorylase isozymes. *J. Biol. Chem.*, **242**: 4824–4833, 1967.
22) Feldberg, R.S. and Datta, P.: L-Threonine deaminase of Rhodosprillum rubrum. Purification and characterization. *Eur. J. Biochem.*, **21**: 438–446, 1971.
23) Lerch, B. and Wolf, G.: Isolation of phosphodiesterase from sugar beet leaves. *Biochim. Biophys. Acta*, **258**: 206–218, 1972.
24) Hjertén, S.: Zone electrophoresis in columns of agarose suspensions. *J. Chromatogr.*, **12**: 510–526, 1963.
25) Fischer, E.H. and de Montmollin, R.: Crystallization of the α-amylase of Aspergillus oryzae. *Nature*, **168**: 606–7, 1951.
26) Akabori, S., Ikenaka, T., and Hagihara, B.: Isolation of crystalline Taka-amylase A from "Takadiastase Sankyo." *J. Biochem.*, **41**: 577–582, 1954.
27) Meyer, K.H., Fischer, E.H., Staub, A., and Bernfeld, P.: Sur les enzymes amylopytiques X. Isolement et cristallisation de l'α-amylase de salive humaine. *Helv. chim. Acta*, **31**: 2158–2164, 1948.
28) Mutzbauer, H. and Schulz, G.V.: Die Bestimmung der molekularen Konstanten von α-Amylase aus Humanspeichel. *Biochim. Biophys. Acta*, **102**: 526–532, 1965.