Column Chromatographic Fractionation of Apricot Emulsin*

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Crude emulsin of apricot (*Prunus armenica*) kernel was prepared by the method of tannin-fractionation. It was then purified by a fractional ammonium sulfate precipitation. The purified enzyme was further fractioned by adsorbing the enzyme on a CM-cellulose column and eluting it with the diluted McIlvaine's buffer solution. By this chromatography, six peaks of activities of β -glucosidase and β -xylosidase were developed. From one component of these peaks, petal-like crystals were obtained. The fractions thus obtained by chromatographic fractionation and crystallization were found differ with regard to the ratio of the β -glucosidase activity to the β -xylosidase one.

The specificity, purification and chemical nature of β -glucosidase, β -galactosidase and β -xylosidase have been investigated for many years by various Workers¹⁾. The complete purification of these enzymes, however, has not yet been reported.

Thus, Miwa *et al.*²⁾ have shown that the β -glucosidase, β -galactosidase and β -xylosidase of apricot emulsin can at least, be partially separated by fractional ammonium sulfate precipitation and other fractional method, although these enzymes are often considered to be the different activities of a single enzyme. For fungal enzyme, Morita³⁾ has reported the separation of β -xylosidase from β -glucosidase, but has not succeeded in the isolation of β -xylosidase.

The homogeneity of the enzyme preparation thus reported, however, has not been shown by the above investigators, and the purification has usually been based on a relatively fractional method with several precipitating reagents.

On the other hand, Jermyn⁴⁾ employed the column chromatographic technique for examining the homogeneity of his stachybotrys atra β -glucosidase.

This paper deals with the chromatographic fractionation and crystallization of the enzyme in apricot emulsin.

EXPERIMENTAL

(1) **Preparation of Crude Emulsin** Ten kg of fresh apricot kernels was defatted with an oil expeller, and the cakes dried and finely powdered. By applying the purification method of Helferich *et al.*⁵⁾, 75 g of crude emulsin was obtained from this powder. In this procedure, zinc sulfate treatment and tannin-fractionation method were operated at room temperature (15–20°C) but the acetone treatment was performed in an ice box to prevent the inactivation of enzyme. In the last step of this process the enzyme precipitate was washed with petroleum ether and dried under reduced pressure. The purity of the enzyme preparation in this stage was observed by the electrophoretic analysis under the conditions indicated in the foot column of Fig. 1, and the existence of four or more components were recognized. On the other hand, specific β -glucosidic activity designated

* The present author uses the general term emulsin for expressing the enzyme system of apricot emulsin.

1) J.B. Sumner and K. Myrbäck, "The enzyme" Vol. 1, Part I, Academic Press 1950, p. 584–630 (cited by Veibel).

2) T. Miwa and K. Tanaka, "Symposia on enzyme chemistry" (Japan), 1949, p. 19

3) Y. Morita, *J. Biochem.*, **43**, 7 (1956).

4) M.A. Jermyn, *Australian J. Biol. Sci.*, **8**, 541 (1955).

5) B. Helferich, S. Winkler, R. Gootz, O. Peters, and E. Günther, *Z. physiol. chem.*, **208**, 91 (1932). J.B. Sumner and K. Myrbäck, "The enzyme", Vol. 1, Part 1, Academic Press, 1950, p. 585 (cited by Veibel)

FIG. 1. Electrophoresis of Crude Apricot Emulsin (Hitachi electrophoretic apparatus)

Sample; after tannin-fractionation and dialysis: concn. of protein; 2%; buffer; $\Gamma/2=0.05$, pH 4.8 acetate buffer; time; 100 min.; temp.; 5°C

as per mg protein was determined throughout the purification process as shown in Table I.

(2) **Preparation of Purified Emulsin.** With the procedure as shown in Table I, further purification of crude emulsin was carried out. In this process, collodion casing was employed for the dialysis of enzyme solution, and it was found effective for the removal of the yellow color impurity. At the end of this purification process, the purity of this enzyme preparation was investigated by the electrophoretic analysis as shown in Fig. 2, and a slight separation

FIG. 2. Electrophoresis of Purified Apricot Emulsin (Spinco Model H electrophoretic apparatus)

Sample; after $(\text{NH}_4)_2\text{SO}_4$ -fractionation, sp. act. 31 (β -glucosidase): concn. of protein; 1.5%; buffer; $\Gamma/2=0.05$, pH 4.8 acetate buffer; time; 74 min.; temp.; 5°C

of three or more components was observed although the preparative separation of these components by the electrophoretic apparatus was not accomplished. The over-all purification designated as the specific β -glucosidic activity was found to be approximately 300-fold in this stage.

Further purification of the enzyme with ammonium sulfate was not successful. Therefore, the following column chromatographic fractionation of the enzyme was attempted.

(3) Chromatographic Fractionation of Purified Emulsin

1. **Preparation of cation-exchanging CM-cellulose** According to the briefly described procedure of Sober *et al.*⁶⁾, cation-exchanging CM-cellulose was prepared as follows: the mixture of 100 g cellulose

powder (Toyo Roshi Co., Ltd.) and one liter of 15% sodium hydroxide solution was heated on a boiling water bath for ten min.. After cooling, 20 g of monochloroacetic acid was added to the mixture and it was heated again for ten min.. The alkaline mixture was neutralized with glacial acetic acid and washed repeatedly with distilled water until the pH of the washings became 5.6.

2. **Test for adsorption of β -glucosidase on CM-cellulose by batch-operation** It could be shown that the β -glucosidase in the dialyzed solution was specifically adsorbed on CM-cellulose in the suitable pH (Fig. 3). In this test 5 ml of the dialyzed enzyme

FIG. 3. Adsorption of β -Glucosidase on CM-Cellulose (Batch operation)

Sample; ap. act. 4 (β -glucosidase): CM-cellulose; 1.5 ml (sediment-volume): buffer; 10 times diluted McIlvaine's buffer (0.02 M sodium phosphate-0.001 M citric acid): room temp. (30°C) —○— activity, —●— protein, ---●--- sp. act. β -glucosidase activity was determined by the method of Sumner.⁷⁾ Protein was determined by the method of Gornall *et al.*⁸⁾

solution (containing 185 units of β -glucosidase, sp. act. 4.0) was mixed with 2.5 ml of the diluted McIlvaine's buffer solution (0.02 M sodium phosphate-0.001 M citric acid) previously adjusted to various pH values

6) H.A. Sober and E.A. Peterson, *J. Am. Chem. Soc.*, **76**, 1711 (1954).

7) J. B. Sumner and G. F. Somers, "Laboratory Experiment in Biological Chemistry" 1949, p. 144.

8) A. G. Gornall, C. J. Bardawill and M. David, *J. Biol., Chem.*, **177**, 751 (1949)

(4.0, 4.5, 5.0, 5.5). The buffered enzyme solutions were poured onto the CM-cellulose which had been sedimented in centrifuge tubes (sedimentary volume, 1.5 ml) and shaken immediately. After thirty minutes the adsorbents were centrifuged off. The quantities of protein and β -glucosidase activity units in these supernatant solutions were determined. From these values the amounts of adsorbed protein and enzyme were calculated. pH values of the supernatant solutions were also determined and the values were dotted in Fig. 3.

3. Preparation of column The above mentioned CM-cellulose was packed into the glass tube (diam. 1.45 cm, height 50 cm CM-cellulose column) with distilled water, and it was washed with distilled water until the washing lost its turbidity. Since the volume of the CM-cellulose was slightly decreased by washing, the volume was made up to the height of 50 cm. The packed CM-cellulose was buffered with 200 ml of diluted McIlvaine's buffer solution (pH 4.8, 0.02 M sodium phosphate-0.001 M citric acid).

4. Adsorption of enzyme on CM-cellulose column The purified enzyme was dialyzed against distilled water and buffered with the same volume of diluted McIlvaine's buffer solution (pH 4.8, 0.02 M sodium phosphate-0.001 M citric acid). This buffered enzyme solution (12.5 ml, containing 100 mg of protein, 3100 units of β -glucosidase) was poured onto the column.

5. Fractional elution and assay of enzyme activity As the eluent, diluted McIlvaine's buffer solutions adjusted to pH 4.8 and pH 8.0 (0.02 M sodium phosphate-0.001 M citric acid) were prepared. The flask containing 200 ml of the pH 4.8 buffer solution was placed on a shelf, fitted in a height of 1.5 m from the upper end of the column, and connected to the column with a rubber tube. Then the buffer solution was continuously poured onto the column, and the pH of the buffer solution was slowly raised in steps from 4.8 to 7.0 by addition of the pH 8.0 buffer solution (when each 10 ml of the buffer solution flowed out from the column, the same volume of the pH 8.0 buffer solution was mixed into the flask). The effluent was collected in portions of 2 ml each and β -glucosidase and β -xylosidase activities were estimated by the following procedure. The quantity of protein in each fraction was determined by the method of Lowry *et al.*⁹⁾ For the measurement of β -glucosidase activity, the reaction mixture was prepared by mixing with 0.5 ml of 0.02 M salicin solution (salicin was dissolved with pH 4.4, 0.1 M

acetate buffer) and 0.04 ml of enzyme solution. After the incubation of the mixture at 30°C for one min., 0.5 ml of 0.1 N sodium hydroxide solution and 3 ml of dinitrosalicylic sugar reagent¹⁰⁾ were mixed successively, and the mixture was heated for five min. in boiling water. The optical density of this solution was taken in the Coleman universal spectrophotometer. By this procedure of enzyme assay, the unit of enzyme activity was not determined but the relative β -glucosidase activity was estimated. The β -glucosidase activity was measured by the same procedure except that O-cresyl β -D-xyloside (m.p. 161-2°) was employed as the substrate and the time of incubation was lengthened to thirty min..

By this column chromatography six (a, b, c, d, e and f) peaks of activities of β -glucosidase and β -xylosidase, respectively were observed as shown in Fig. 4, and the distinct shift of the ratios of β -glucosidase to β -xylosidase in each peak was recognized. The clear separation of β -glucosidase from β -xylosidase, however, did not occur.

(4) Crystallization As shown in Fig. 4 the effluent fractions were divided into six components (a, b, c, d, e and f), and each component was treated to crystallize with the procedure summarized in Table I.

In this way, petal-like crystals deposited from the component (a) and they were accompanied with a considerable quantity of amorphous matter. Most of the amorphous matter was separated from the crystals by centrifuging at low velocity of rotation. Fig. 5 shows the crystals from component (a). In other components no definite crystalline substance was found.

Recrystallization of the enzyme was not attempted, because of the insufficient amount of the crystals for this purpose.

(5) Enzyme Activities of Crystalline Preparation One drop of the crystalline suspension was dissolved with 10 ml of distilled water, and the β -glucosidase and β -xylosidase activities of one ml of this enzyme solution were measured by the procedure above mentioned. On the other hand, a small amount of the tannin-fractionated emulsin powder ("crude emulsin" in Table I) was dissolved with distilled water, and the insoluble matter was centrifuged out. This crude enzyme solution was diluted

9) O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, *J. Biol. Chem.* **193**, 265 (1951).

(10) J.B. Sumner, *J. Biol. Chem.* **65**, 393 (1925).

FIG. 4. Chromatography of Purified Apricot Emulsin.

Sample; 100mg protein, sp. act. 31 (β -glucosidase); column; 50 $\times$ 1.45 cm (CM-cellulose); buffer for elution; pH 4.8-7.0 (pH 8.0, 10 times diluted McIlvaine buffer was added slowly to pH 4.8, 10 times diluted McIlvaine buffer); effluent rate; 3-10ml/hr.; temp.; 20-22 $^{\circ}$ C; recovery of total protein; 80%.

with distilled water until one ml of this solution contains the corresponding amount of β -glucosidase activity to the above crystalline enzyme solution.

The β -xylosidase activity of this crude enzyme solution was measured, and compared with that of the crystalline enzyme solution. From this experiment a

TABLE I. PURIFICATION AND FRACTIONATION OF APRICOT EMULSIN

	Powder of apricot seeds; sp. act. 0.1 (extd. with distd. H ₂ O)
	↓
	Tannin-fractionation; Helferich's method ⁵⁾
	↓
Crude emulsin:	Enzyme powder; 50g, ext. (1000ml distd. H ₂ O)
	↓
	Extract; filter with the aid of animal charcoal
	↓
	Filtrate; sp. act. 1, yield 100%, dialyze against distd. H ₂ O (collodion casing, 2 days)
	↓
	Dialyzate; filter with the aid of animal charcoal
	↓
	Filtrate; sp. act. 4, yield 91%
	↓
	1st (NH ₄) ₂ SO ₄ -fractionation; 0.48-0.7 sat, pH 4.8, 30° C
	↓
	Precipitate; dissolve (minimum distd. H ₂ O), dialyze against distd. H ₂ O (collodion casing, 2 days)
	↓
	Dialyzate; filter with the aid of animal charcoal
	↓
	Filtrate; sp. act. 6, yield 64%
	↓
	2nd (NH ₄) ₂ SO ₄ -fractionation; 0.48-0.65 sat. pH 4.8, 30° C
	↓
	Precipitate; dissolve (minimum distd. H ₂ O), dialyze against distd. H ₂ O (collodion casing)
	↓
	Dialyzate; centrifuge, 4000 r. p. m., 20 min.
	↓
	Supernatant; sp. act. 23, yield 17%
	↓
	3rd (NH ₄) ₂ SO ₄ -fractionation; 0.48-0.6 sat. pH 4.8, 30° C
	↓
	Precipitate; dissolved (minimum distd. H ₂ O), dialyze against distd. H ₂ O (collodion casing, 1 day)
	↓
	Dialyzate; centrifuge 4000 r. p. m. 20 min.
	↓
	Supernatant
	↓
	4th (NH ₄) ₂ SO ₄ -fractionation; 0.5-0.6 sat. pH 4.8, 30° C
	↓
	Precipitate; dissolve (minimum distd. H ₂ O), dialyze against distd. H ₂ O, (collodion casing, 1 day)
	↓
	Dialyzate; sp. act. 31, yield 12%, add solid (NH ₄) ₂ SO ₄ (0.7 sat)
	↓
Purified emulsin:	Precipitate; (store in refrigerator), dialyze against distd. H ₂ O, nothing insol. matter
	↓
	Dialyzate
	↓
	Chromatographic fractionation
	↓
	Enzyme solution; (6 components), dialyze against 400ml of 0.7 sat (NH ₄) ₂ SO ₄ , outer soln change 3 times, ↓ centrifuge 7000 r. p. m. 15 min.
	Precipitate; dissolve (minimum H ₂ O), dialyze against distd. H ₂ O (collodion casing, 1 day)
	↓
	Dialyzate; centrifuge 4000 r. p. m. 10 min.
	↓

(Continuation of TABLE I)

↓
 Supernatant; add solid Na_2SO_4 (0.5 sat), add small amount of sat. $(\text{NH}_4)_2\text{SO}_4$
 ↓
 Slight turbid solution; add minimum amount of distd. H_2O until the white precipitate disappear in about 30 min.
 ↓
 Solution; slowly condensed with silica gel (in refrigerator, 5 days)
 ↓
 Crude crystal; (appear from (a) component only), add 10 times vol. of 0.7 sat. $(\text{NH}_4)_2\text{SO}_4$, centrifuge 1000 r.p.m. 2-3 min. (repeat 3 times), microscopic examination
 ↓
 Precipitate (purified crystal)

β -glucosidase activity was determined by the method of Sumner⁷⁾

Protein was determined by the method of Gornall et al⁸⁾ or Lowry et al⁹⁾

Specific activity was designated as β -glucosidase units per mg protein.

FIG. 5. Crystals from Apricot Emulsin.
 ×600

relatively strong β -xylosidase activity was proved in the crystalline preparation as shown in Fig. 6. However, the definite nature of the crystals still remains unsolved.

DISCUSSION

As mentioned above, crude apricot kernel emulsin was purified by the fractional ammonium sulfate precipitation with regard to the specific β -glucosidase activity as a criterion, but the complete purification of the enzyme by this procedure resulted in fail. The preparative separation of the components with the aid of Spinco Model H electrophoretic apparatus was not accomplished because of their extremely close mobilities. From the view point of these facts it is interpreted that apricot emulsin contains some components which have closely resembled physicochemical properties.

When the purified enzyme preparation was

FIG. 6. Comparison of the Activities of β -Glucosidase and β -Xylosidase with Crude and Crystallized Preparation.

crude enzyme; after tannin-fractionation: G; β -glucosidase activity; X; β -xylosidase activity. Enzyme activities were measured with the same procedure in the case of column chromatography

chromatographed with CM-cellulose column, it was apparently separated in six components, and the crystalline substance was obtained from component (a) although the other components were not crystallized. Therefore, the chromatographic fractionation seemed to be effective for the separation of the crystalline substance.

As to the question whether β -glucosidase and β -xylosidase of apricot emulsin is com-

pletely separated, the author could not isolate any perfectly single enzyme in this study, but the distinct shift of the ratio of β -glucosidase to β -xylosidase was effected by the column chromatography. The separation of the two enzymes was not complete even after crystallization. However, it is noteworthy that the crystals indicated a relatively strong β -xylosidase activity.

On the other hand six peaks of activities of β -glucosidase and β -xylosidase were de-

veloped by the column chromatography, but the difference in these fractions has not yet been determined.

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