Studies on α-Galactosidases (VI)

Multiplicity of the α-Galactosidase from Brewer's Bottom Yeast as Shown by Chromatography on Calcium Phosphate Columns

By

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In the previous papers of this series[1][2] the complexity of the α-galactosidase in an extract from bottom yeast was demonstrated by the author using paper chromatography and starch zone-electrophoresis. The same enzyme has now been chromatographed on calcium phosphate columns, and the results will be presented in this paper. They also support the presence of multiple components in the α-galactosidase.

Materials and Methods

1. *Preparation of enzyme solution*

A crude extract from bottom yeast was prepared in the same way as described in a previous paper[3].

2. *Preparation of hydroxylapatite*

The hydroxylapatite was prepared by the method of TISELIUS *et al*[4]. One litre each of 0.5 M aqueous solutions of calcium chloride and of secondary sodium phosphate were allowed to drop simultaneously from two burets at a rate of 120 drops per minute into a beaker with stirring. The resultant precipitate was treated with dilute sodium hydroxide solution and with phosphate buffer of pH 6.0 to obtain hydroxylapatite. It can be stored for a fairly long period in 0.001 M phosphate buffer of pH 6.0.

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3. *Column chromatography*

A chromatographic tube (35 × 1.5 cm.) fitted with a cooling jacket was plugged with a glass filter at its lower end. A suspension of the hydroxylapatite which had been washed several times with phosphate buffer (0.05 M, pH 6.0) was introduced into the column and allowed to settle. The column was then equilibrated with the same buffer (20~21°C) for 15 hours.

A 2.5 ml. aliquot of the crude extract from bottom yeast which had been dialyzed against nearly 50 volumes of phosphate buffer (0.05 M, pH 6.0) in the cold for 24 hours was applied to the column (12×1.5 cm.) and then eluted stepwise with appropriate volumes of phosphate buffer of increasing concentration at pH 6.0. Effluents were collected into test tubes by a fraction collector (Toyo Roshi Co., Ltd.). The column was cooled with tap water at 20~21°C during elution.

4. *Enzyme assays*

i) *Estimation of transferring activity*

Enzyme assays were made under the conditions given in the table.

Galactose released was determined per 1 ml. of reaction mixture by the SHAFFER-HARTMANN-SOMOGYI method[5], and the phenol liberated simultaneously was estimated as described in a previous paper[3]. The extent of transfer was calculated from the equation:

$$\frac{\text{(phenol liberated)} - \text{(galactose liberated)}}{\text{(phenol liberated)}} \times 100$$

ii) *Estimation of enzyme activity and of protein*

Enzyme solution containing 0.05 M acetate buffer of pH 4.8 (3 parts) was incubated with 0.012 M substrate solution (1 part) at 30°C.

1.0 ml. aliquots of reaction mixture were removed at intervals and reducing power and liberated aglycon were measured by the SHAFFER-HARTMANN-SOMOGYI[5] and colorimetric methods, respectively. From the extent of hydrolysis the average reaction constant (K) was obtained, assuming the reaction to be first order. The ratio of the values of K was compared to give the aglycon specificity.

Protein concentration was determined by the method of LOWRY *et al*[6]. Egg albumin (Wakô Co.) was used as standard.

5. *Substrates and acceptors*

Phenyl α-D-galactoside (m. p. 138~140°C), *m*-cresyl α-D-galactoside (m. p. 150~151°C), *p*-nitrophenyl α-D-galactoside (m. p. 167~168°C), melibiose (commercial superior quality) and galactobiose* ($[\alpha]_D^8 = +154.7°$, in water) were used as substrates, and methanol, *n*-butanol, glycerol and galactose as acceptors.

Results

1. *Enzyme activity*

The specific activity of the α-galactosidase of the crude extract against *p*-nitrophenyl α-galactoside is presented in Table 1.

Table 1. The specific activity of the crude extract from bottom yeast.

Reaction mixture:

0.048 M *p*-Nitrophenyl α-galactoside	1.0 ml.
0.1 M Acetate buffer, pH 4.8	2.0 ml.
Enzyme	1.0 ml.

Temperature, 30°C.

Substrate	K* × 10^3	Protein (mg/ml)	Specific activity** (f)
p-Nitrophenyl α-galactoside	7.14	7.08	0.335

* K is obtained from the amount of *p*-nitrophenol liberated.

** $f = 100 \times K / \log 2 \times g = 0.335$, where g indicates mg. protein per ml. enzyme solution, estimated from the KJELDAHL nitrogen.

2. *Activities of enzyme fractions from column chromatography*

i) *Hydrolytic activities of fractions*

The crude extract was fractionated into three components by stepwise elution with phosphate buffer of pH 6.0. They each eluted with the buffers of 0.125 M, 0.15 M and 0.175 M respectively, as shown in Fig. 1. Most of

* Galactobiose was isolated from the reaction mixture after melibiose had been hydrolyzed by an extract from bottom yeast[7].

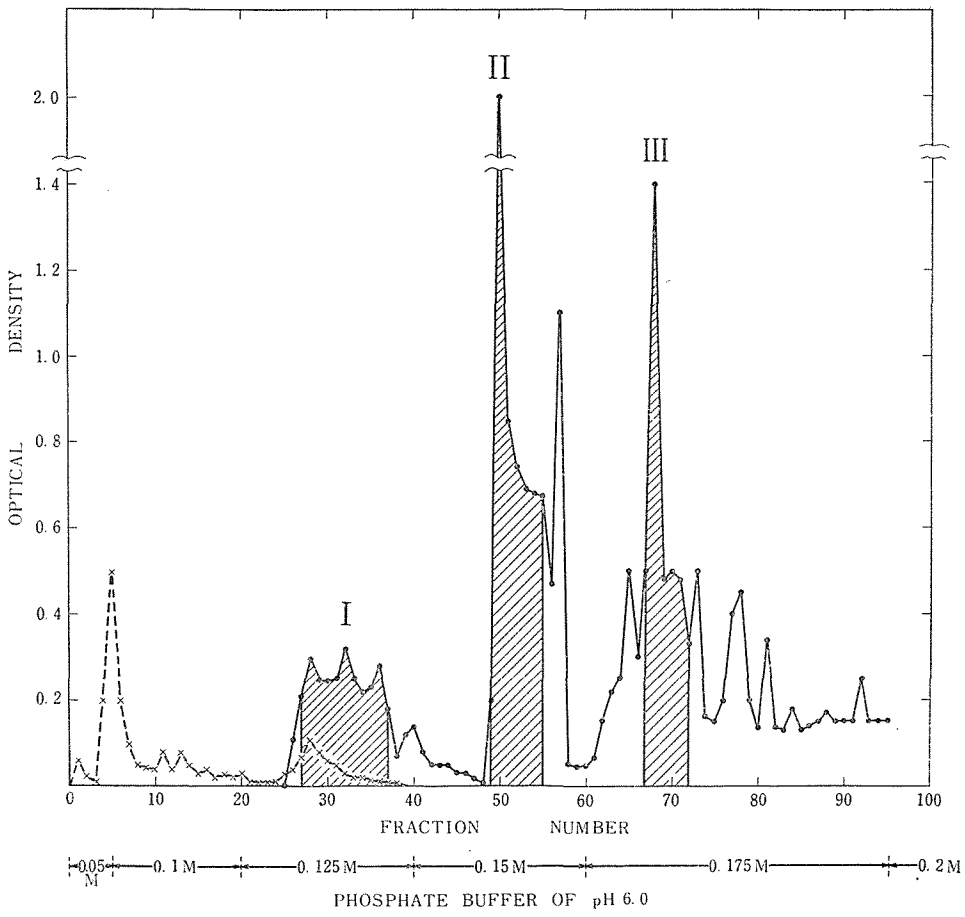

Fig. 1. Diagram of the chromatographic behaviour of the crude α-galactosidase from bottom yeast on stepwise elution.

—●— α-Galactosidase activity as expressed by the optical density at 400 mμ.

--×-- Protein concentration as expressed by the optical density at 750 mμ.

Conditions:

Initial solution, 2.5 ml.

Each fraction, 4 ml.

Reaction mixture:

Effluent	0.3 ml.
0.048 M *p*-Nitrophenyl α-galactoside	0.1 ml.

Incubation time, 16 hours.

Temperature, 30°C.

the protein was eluted with buffers of lower concentration, especially at 0.05 M to 0.1 M and at around 0.125 M. The enzyme activity, therefore, did not parallel the amount of protein.

Since resolution of the α-galactosidase activity such as is shown above was reproducible under the same conditions, the chromatographic procedure was repeated twice. The fractions corresponding to each of the shaded portions of Fig. 1 were combined, dialyzed against distilled water in the cold for 2 days and concentrated to 15 ml. under reduced pressure at 30°C. These fractions (I, II and III) were each redialyzed against nearly 50 volumes of acetate buffer (0.05 M, pH 4.8) in the cold overnight, and tested for their α-galactosidase activities. The results are shown in Table 2.

Table 2. Enzyme activities of fractions I, II and III against α-galactosides with different aglycons.

Enzyme fraction	α-D-Galactoside									
	Phenyl		*m*-Cresyl		*p*-Nitrophenyl		Glucosyl		Galactosyl	
	K	r	K	r	K	r	K	r	K	r
I	0.288	1	0.253	0.88	1.56	5.42	1.02	3.54	1.89	6.56
II	0.637	1	0.779	1.22	3.39	5.32	3.13	4.92	3.49	5.48
III	0.526	1	0.436	0.83	0.976	1.85	1.31	2.49	1.37	2.61
(Initial)*	0.77	1	0.74	0.96	2.28	2.95	0.992	1.29	—	—

Note:
K, value multiplied by 10^4.
r, activity for phenyl α-galactoside taken as 1.
* Figures cited from a previous paper[8].

Activity ratios of fractions I and II were not very different from each other, but were considerably different from those of fraction III.

ii) *Transferring activities of enzyme fractions*

The transferring activities of fractions I, II and III were compared using the same substrate and acceptors for each fraction. Phenyl α-galactoside was used as substrate, and methanol, *n*-butanol, glycerol and galactose as acceptors. The results are shown in Table 3.

The ratio of the extent of α-galactosyl transfer in the presence of different acceptor to the extent of transfer in the control was uniformly in the

range between 1.2 to 2 in all three fractions, the differences being too small to warrant the assumption of different transferring activities for these fractions.

Table 3. Extent of transgalactosylation shown by fractions I, II and III in the presence of acceptors.

Reaction mixture:
0.048 M Phenyl α-galactoside 1.0 ml.
Acceptor (methanol, 5 M; *n*-butanol, 2.5 M; glycerol, 2 M; galactose, 0.03 M) 1.0 ml.
Enzyme solution containing 0.05 M acetate buffer of pH 4.8 3.0 ml.

Temperature, 30°C.

Enzyme fraction	Acceptor	Reaction time	Phenol liberated	Galactose liberated	Galactose transferred	Mean	Ratio*
		(min.)	(%)	(%)	(%)	(%)	
I	None	2790	31.0	22.7	26.7	29.7	1.0
		4320	44.5	30.0	32.6		
	Methanol	2790	22.0	13.6	38.2	41.8	1.40
		4320	34.3	18.8	45.3		
	n-Butanol	2790	19.6	11.4	41.8	39.9	1.34
		4320	30.4	18.8	38.1		
	Glycerol	2790	25.2	15.1	40.1	44.1	1.48
		4320	40.0	20.8	48.0		
	Galactose	2790	27.4	17.2	37.2	34.8	1.17
		4320	37.0	25.0	32.4		
II	None	1440	48.0	32.3	32.7	30.4	1.0
		1920	55.8	40.2	28.0		
	Methanol	1440	43.2	22.6	47.8	48.4	1.59
		1920	58.0	29.6	49.0		
	n-Butanol	1440	37.0	21.2	42.7	44.9	1.47
		1920	46.8	24.8	47.0		
	Glycerol	1440	43.1	16.5	61.8	60.8	2.00
		1920	52.7	21.2	59.8		
	Galactose	1440	43.2	28.2	34.7	36.1	1.19
		1920	50.8	31.7	37.5		

III	None	1980 3100	48.6 59.6	32.1 37.3	33.9 37.4	35.7	1.0
	Methanol	1980 3100	49.5 61.6	26.7 28.9	46.1 53.1	49.6	1.39
	n-Butanol	1980 3100	52.0 57.1	19.7 23.1	62.1 59.5	60.8	1.70
	Glycerol	1980 3100	50.0 60.0	20.7 23.7	58.6 60.5	59.6	1.67
	Galactose	1980 3100	47.6 54.4	22.8 28.8	52.1 47.1	49.6	1.39

* The extent of transfer without acceptor is taken as 1.

3. *Rechromatography of the three fractions*

The three fractions were subjected to rechromatography under unaltered conditions after dialysis against phosphate buffer (0.05 M, pH 6.0) in the cold for 24 hours. The fractions I, II and III were again found only in the effluents of phosphate buffers of 0.125 M, 0.15 M and 0.175 M, respectively, as in the first chromatography. They were therefore not resolved into additional components, but eluted as single components, although the amounts of enzyme in the effluents decreased considerably. The results are shown in Figs. 2, 3 and 4.

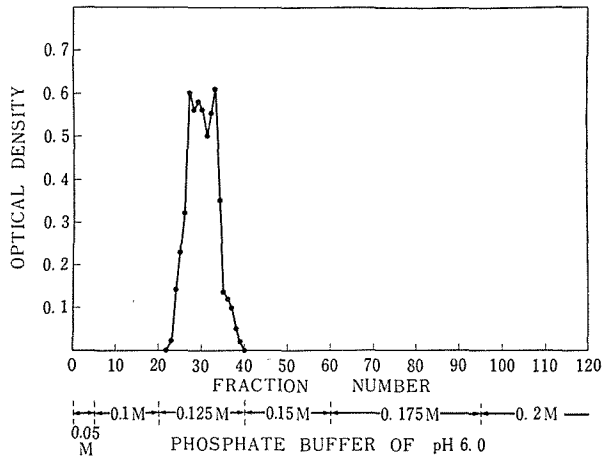

Fig. 2. Diagram of the rechromatography of fraction I in Fig. 1.
—●— α-Galactosidase activity as expressed by the optical density at 400 mμ.
Conditions: Fraction I, 2.5 ml.; each fraction, 4 ml. Reaction mixture: Effluent, 0.3 ml.; 0.048 M *p*-nitrophenyl α-galactoside, 0.1 ml. Incubation time, 12 days. Temperature, 30°C.

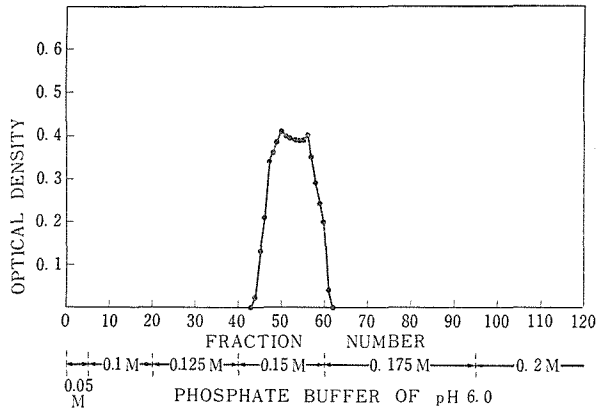

Fig. 3. Diagram of the rechromatography of fraction II in Fig. 1.
—●— α-Galactosidase activity as expressed by the optical density at 400 mμ.
Conditions: Fraction II, 2.5 ml.; each fraction, 4 ml. Reaction mixture: Effluent, 0.3 ml.; 0.048 M *p*-nitrophenyl α-galactoside, 0.1 ml. Incubation time, 5 days. Temperature, 30°C.

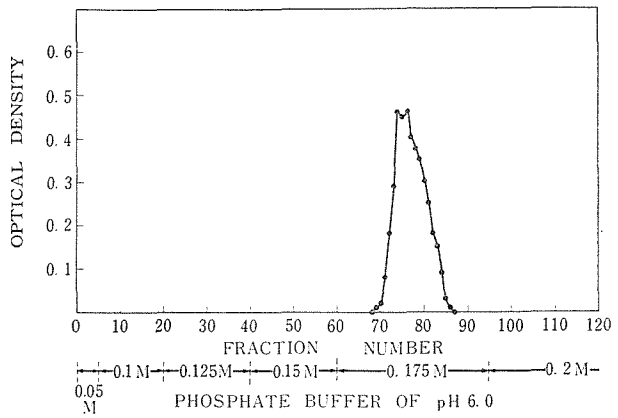

Fig. 4. Diagram of the rechromatography of fraction III in Fig. 1.
—●— α-Galactosidase activity as expressed by the optical density at 400 mμ.
Conditions: Fraction III, 2.5 ml.; each fraction, 4 ml. Reaction mixture: Effluent, 0.3 ml.; 0.048 M *p*-nitrophenyl α-galactoside, 0.1 ml. Incubation time, 10 days. Temperature, 30°C.

Discussion

A crude α-galactosidase preparation from bottom yeast was resolved into at least three distinct fractions by column chromatography with phosphate buffer of increasing concentration at pH 6.0 (Fig. 1). The relative activities of two of the fractions against α-galactosides of different aglycon were similar, but considerably different from that of the third. Since, however, the concentration of phosphate buffer at which the first two were eluted from the column were rather different, the α-galactosidases in the two fractions can not be of the same physical nature, even if their aglycon specificities are similar. The three components have, therefore, not been produced by artifact during chromatography, but must each be different originally. This assumption is supported by the facts that the three components could be rechromatographed as single components by phosphate buffer of the same concentration as that with which they were eluted from the first column and that the amounts of resolved protein did not parallel the α-galactosidase activity. These components, however, showed almost the same activities for the transfer of the α-galactosyl residue from substrates to acceptors (Table 3).

Fraction 19 obtained from a crude extract by starch zone-electrophoresis hydrolyzed *p*-nitrophenyl and hexosyl α-galactosides more easily than other α-galactosides. In this respect, therefore, its aglycon specificity is similar to those of the components I and II obtained in the present work. On the other hand, the aglycon specificities of component 3 and 9 obtained by zone electrophoresis are similar to that of component III obtained by column chromatography in hydrolyzing the α-galactosides mentioned above relatively slowly (cf. Table 2 both in the preceding paper[2] and in the present work).

However, since α-galactosidase preparations, although it was obtained from bottom yeast by similar procedures, were resolved into four distinct components by starch zone-electrophoresis, but into three components by column chromatography, it is not at present clear whether any of the components obtained by electrophoresis are to be identified with any of those obtained by column chromatography, even if the relative activities of such components against several substrates appear to be similar. It seems likely that the α-galactosidase of bottom yeast contains a variety of species which are very similar in their chemical and physical natures, but different enough to be resolved into a number of groups by one fractionation method and into other different groups of species by other methods. The components ob-

tained on fractionation may therefore not represent actual single forms, but groups of individuals which resemble each other closely in certain properties. At any rate, it seems very difficult to isolate a single form and to identify it as actually single.

Summary

The α-galactosidase from bottom yeast could be fractionated into at least three components by chromatography on calcium phosphate columns. Observations such as the different relative activity of each component against substrates, the identical behaviour of each component on rechromatography as on initial chromatography and the lack of parallelism between activity and amount of protein on resolution were made. These suggested that the resolution could not be due to artifacts occurring during chromatography, but was instead due to differences between the nature of the components. However no evidence for their ultimate singleness has been obtained in this work.

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