Studies on α-Galactosidases (V)

The Multiplicity of the α-Galactosidase from Brewer's Bottom Yeast as Shown by Starch Zone-Electrophoresis

By

Kazumasa WAKABAYASHI*

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In the previous paper[1], the author has observed that α-galactosidases from various sources were resolved into several components on paper chromatography and that, even when these components had the same origin, they showed considerably different aglycon specificities in most cases. These facts were therefore taken as evidence for multiple forms of each α-galactosidase. The result was quite similar to that which HASH and KING[2] obtained for the multiplicity of an aryl β-glucosidase of *Myrothecium verrucaria* by paper electrophoresis.

Recently, NISIZAWA *et al.*[3] have also found that starch zone-electrophoresis reveals the complex nature of the β-glucosidase of *Irpex lacteus*, although the hydrolytic properties of each component were not investigated in detail.

These findings led the present author to attempt the precise examination of the behaviour of α-galactosidases on starch zone-electrophoresis. The present paper reports the results of the work with the α-galactosidase of bottom yeast.

* Assistant Professor of Chemistry, Faculty of Engineering, Shinshu University, Nagano, Japan.

Materials and Methods

1. *Preparation of enzymes*

i) *Initial solution*

The initial solution was prepared in almost the same way as described in a previous paper[4].

ii) *Purification by acetone and ammonium sulfate*

Purification of the enzyme was carried out in the same way as described in a previous paper[4]. The initial solution was first fractionated at pH 4.6, in the cold, with acetone of 30 and 55 per cent final concentration. The precipitate formed at 55 per cent acetone was dissolved in a small volume of water. This solution was then fractionated with ammonium sulfate at 0.5 and 0.95 saturation. The precipitate formed at 0.95 saturation was dissolved in a little water and dialyzed against approximately 50 volumes of acetate buffer (0.01 M, pH 4.8) for two days. The dialyzed solution was used as a purified enzyme solution.

2. *Substrates*

Phenyl α-D-galactoside (m. p. 138~140°C), *m*-cresyl α-D-galactoside (m. p. 150~151°C), *p*-cresyl α-D-galactoside (m. p. 144~145°C), *p*-nitrophenyl α-D-galactoside (m. p. 167~168°C), melibiose (commercial, superior quality) and galactobiose* ($[\alpha]_D^8 = +154.7°$, in water).

3. *Zone electrophoresis*

Zone electrophoresis was carried out with potato starch as the supporting medium in an apparatus similar to that of KUNKEL and SLATER[6]. The commercial potato starch was washed thrice with three volumes of 0.05 N NaOH, then with distilled water until the alkali was completely removed, and finally several times with the buffer used for the electrophoresis. This starch preparation was used directly as supporting medium. A small portion of the starch was washed again with water and dried for use in solidifying the enzyme sample. Changes in pH at the electrodes were avoided by renewing the buffer solution in the electrode vessels through of siphons from a

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* Galactobiose was prepared by utilizing the transgalactosylation reaction which occurred during the hydrolysis of melibiose by the α-galactosidase of brewer's bottom yeast[5].

tank containing 5 *l.* of the same buffer. The runs were made at about 15°C. The starting sample containing an appropriate amount of α-galactosidase was kneaded with dried starch and introduced into a 1 cm. slit cut crosswise in a 40 × 5 × 1 cm. block of the starch, and located nearly at the centre of the block. To estimate the approximate relative electroosmotic flow, a small amount of 1 per cent of moist dextrin (Merck) was introduced with starch into a 1 cm. slit located near the anodic end of the block. On completion of each run, strips 0.5 cm. wide cut from each end of the block were discarded, and the remaining block was then cut crosswise into sections 1 cm. wide, each of which was extracted twice with 5 or 10 ml. distilled water. The extracts were concentrated to 2 ml., if necessary, under reduced pressure at 25~30°C, and suitable aliquots were analyzed for protein, carbohydrate and α-galactosidase activity.

4. *Assay procedures*

i) *Estimation of protein*

Protein concentrations were determined by the method of LOWRY *et al*[7]. 5 ml. of the alkaline copper solution were well mixed in test-tubes with 0.5 ml. aliquots of each enzyme sample. The mixture was allowed to stand for 10 minutes at 30°C. 0.5 ml. of approximately 2-fold diluted FOLIN reagent was then added rapidly. After standing at 30°C for 20 minutes, the absorption of the mixture at 750 mμ was determined in a photo-electric colorimeter. Egg albumin (Wakô Co.) was employed as standard.

ii) *Estimation of carbohydrate*

Total carbohydrate analysis was carried out by the anthrone method[8], using glucose as standard. Each 4.5 ml. of the anthrone reagent (sulfuric acid: 66%) was pipetted into test-tubes of 3 cm. diameter which were then cooled in ice water and a 0.5 ml. aliquot of enzyme sample layered over the acid with ice-cooling. The contents were shaken while still being cooled, brought to room temperature, and then heated for exactly 10 minutes in a boiling water bath. After cooling again, the colour intensity of the mixture was measured at 628 mμ.

iii) *Estimation of enzyme activities*

Enzyme activities were in general determined in almost the same way as described in a previous paper[1]. A 0.012 M solution of *p*-nitrophenyl α-galactoside (1 part) was mixed with enzyme solution (1 part) and 0.05 M

acetate buffer of pH 4.8 (2 parts), and incubated at 30°C. After an appropriate period, the reaction was stopped by adding dilute sodium carbonate solution, and the amount of *p*-nitrophenol liberated was measured by the absorption at 400 mμ. The activity was usual expressed by this quantity, but K* or f** were calculated if needed. In certain cases K was obtained from the amount of reducing sugars liberated into the reaction mixtures as determined by SHAFFER-HARTMANN-SOMOGYI'S[9] method.

For comparing of the activities of each enzyme fraction against substrates with different aglycons, the value of K for each substrate was used.

Results

1. *Enzyme activities*

The specific activities of the initial solution and the preparation partially purified with acetone and ammonium sulfate are shown in Table 1.

Table 1. The specific activities of α-galactosidase preparations from bottom yeast.

Enzyme preparation	Substrate	K* × 10^3	Specific activity (100 × K / log 2 × g)
Initial solution	*p*-Nitrophenyl α-galactoside	6.90	0.446**
Partially purified preparation	*p*-Nitrophenyl α-galactoside	27.75	15.759***

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

** 0.69 / 0.301 × 5.14 = 0.446, where 5.14 indicates mg. protein estimated from the KJELDAHL nitrogen.

*** 2.775 / 0.301 × 0.585 = 15.759, where 0.585 indicates mg. protein estimated by the method of LOWRY *et al*[7].

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* K is the average velocity constant of the first order reaction obtained from the amount of *p*-nitrophenol liberated under the standard conditions.

** f is given by the following equation[10] :

$$f = 100 \times K / \log 2 \times g,$$

where g is mg. protein per ml. enzyme solution estimated by the method of LOWRY *et al.*[7] or from the KJELDAHL nitrogen.

2. *Fractionation by zone electrophoresis*

Zone electrophoresis was carried out using buffers (acetate, phosphate, borate-phosphate) of pH from 4.8 to 8.0 and ionic strength from 0.036 to 0.096.

i) *Electrophoresis at pH 4.8 and 5.8*

Results obtained from the electrophoresis at pH 4.8 with the initial solution are presented in Fig. 1, where the enzyme activity and the protein concentration of eluates are plotted as a function of the distance from the starting point.

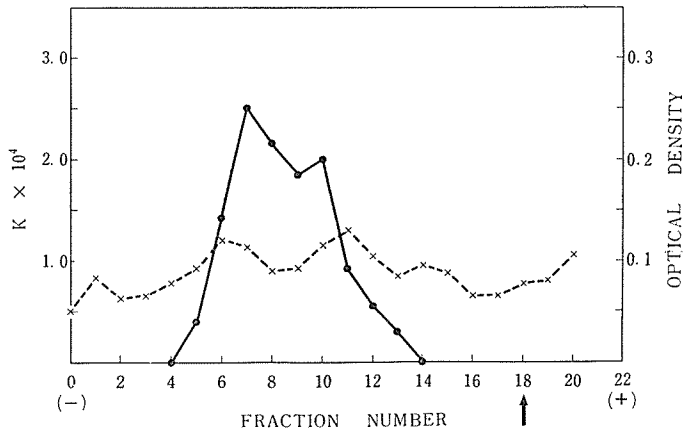

Fig. 1. Distribution of protein and activity in the zone electrophoresis of a crude α-galactosidase preparation.

—●— Enzyme activity as expressed by the velocity constant, K.
---×--- Protein concentration as expressed by the optical density at 750 mμ.
Arrow represents the point where initial solution was applied.
Conditions:
Buffer; acetate, pH 4.8, ionic strength 0.05. Electric power; 300 volts, 10~12 mA. Duration; 24 hours. Enzyme; initial solution, 2.5 ml. Eluate; 5 ml. for each section. Reaction mixture for activity measurement; 1.0 ml.

Results obtained from the electrophoresis at pH 5.8 are presented in Fig. 2.

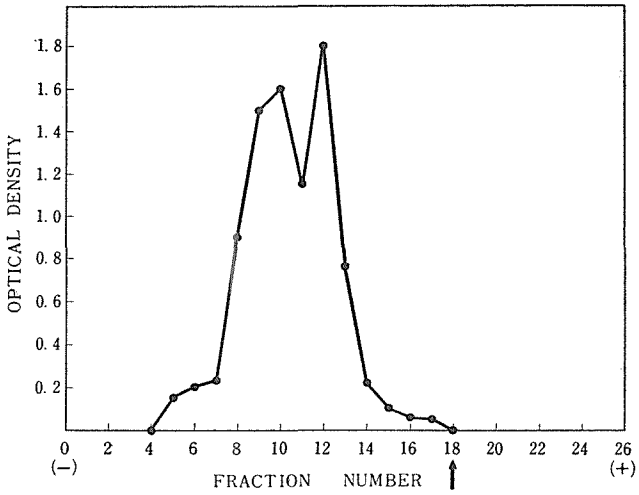

Fig. 2. Distribution of activity in the zone electrophoresis of a crude α-galactosidase preparation.

—●— Enzyme activity as expressed by the optical density at 400 mμ.
Arrow represents the point where initial solution was applied.
Conditions:
Buffer; phosphate, pH 5.8, ionic strength 0.096. Electric power; 300 volts, 12 mA. Duration; 21 hours. Enzyme; initial solution, 2.5 ml. Eluate; 5 ml. for each section. Reaction mixture for activity measurement; 0.4 ml. Incubation time; 40 hours.

As seen from Figs. 1 and 2, the α-galactosidase activity showed at least two peaks, although they separated incompletely. The protein was resolved into several components, which were not parallel to those of enzyme activity.

ii) *Electrophoresis at pH 8.0*

Two experiments were carried out with the initial solution using the buffers different both in nature and ionic strength, that is, phosphate buffer of ionic strength, 0.096 and borate-phosphate buffer of ionic strength, 0.036. The results are presented in Figs. 3 and 4. Single peaks were obtained in both cases. These ionic strength at this pH seem, therefore, to be inadequate for the resolution of the α-galactosidase of bottom yeast.

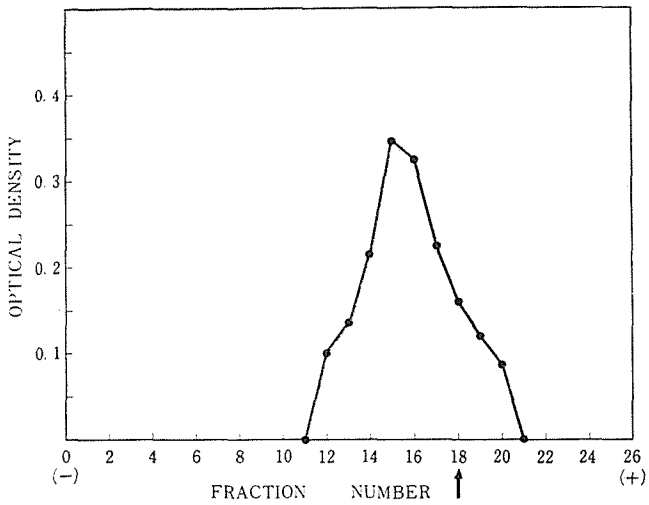

Fig. 3. Distribution of activity in the zone electrophoresis of a crude α-galactosidase preparation.

—●— Enzyme activity as expressed by the optical density at 400 mμ.
Arrow represents the point where initial solution was applied.
Conditions:
Buffer; phosphate, pH 8.0, ionic strength 0.096. Electric power; 300 volts, 12 mA. Duration; 20 hours. Enzyme; initial solution, 1.0 ml. Eluate; 5 ml. for each section. Reaction mixture for activity measurement; 0.4 ml. Incubation time; 18 hours.

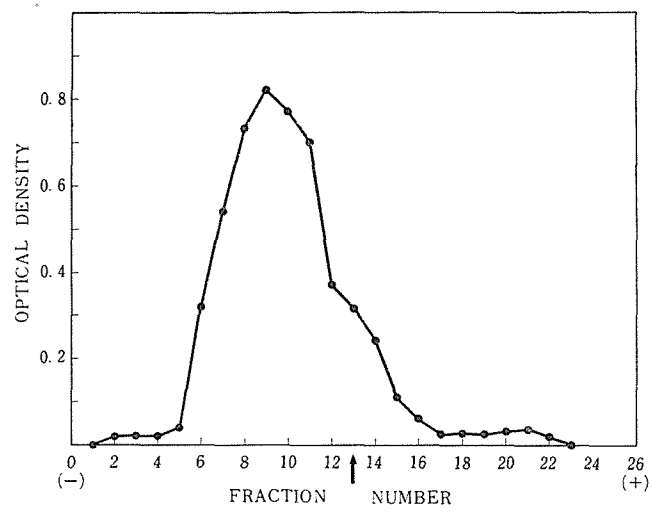

Fig. 4. Distribution of activity in the zone electrophoresis of a crude α-galactosidase preparation.

—●— Enzyme activity as expressed by the optical density at 400 mμ.
Arrow represents the point where initial solution was applied.
Conditions:
Buffer; borate-phosphate, pH 8.0, ionic strength 0.036. Electric power; 300 volts, 8 mA. Duration; 18 hours. Enzyme; initial solution, 2.5 ml. Eluate; 5 ml. for each section. Reaction mixture for activity measurement; 0.4 ml. Incubation time; 17 hours.

iii) *Electrophoresis at pH 7.0*

Fig. 5 shows the pattern obtained from the electrophoresis of the initial solution where phosphate buffer of ionic strength 0.06 was used.

As seen from the figure, the α-galactosidase activity showed the presence of at least four enzyme components. The component of slowest mobility, however, represented the major part.

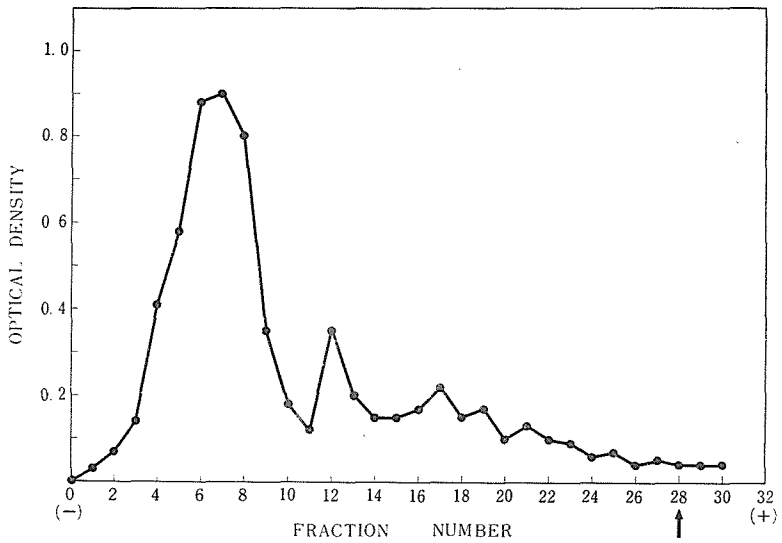

Fig. 5. Distribution of activity in the zone electrophoresis of a crude α-galactosidase preparation.

—●— Enzyme activity as expressed by the optical density at 400 mμ.
Arrow represents the point where initial solution was applied.
Conditions:
Buffer; phosphate, pH 7.0, ionic strength 0.06. Electric power; 300 volts, 8 mA. Duration; 40 hours. Enzyme; initial solution, 2.0 ml. Eluate; 5 ml. for each section. Reaction mixture for activity measurement; 0.4 ml. Incubation time; 48 hours.

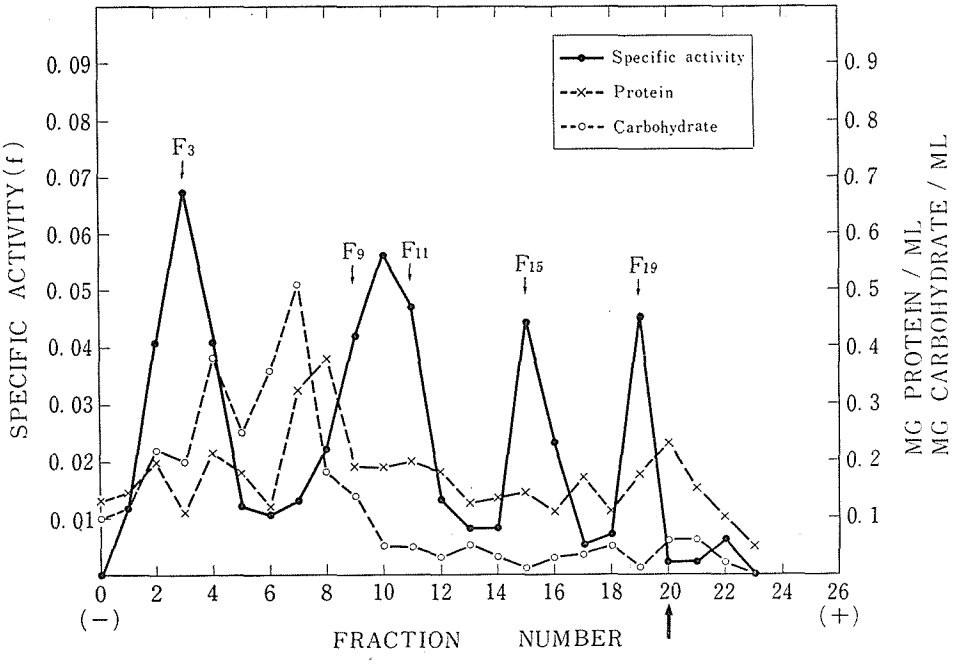

Fig. 6. Distribution of protein, carbohydrate and activity in the zone electrophoresis of a crude α-galactosidase preparation.

Arrow represents the point where initial solution was applied.
Conditions:
Buffer; phosphate, pH 7.0, ionic strength 0.096. Electric power; 300 volts, 12 mA. Duration; 24 hours. Enzyme; initial solution, 2.5 ml.
Eluate; 2 ml. concentrated for each section. Reaction mixture for activity measurement; 1.0 ml.

Further electrophoretic studies showed that the resolution of components could be improved by using phosphate buffer of ionic strength 0.096 at pH 7.0 for 24 hours at 300 volts. The results are shown in Fig. 6.

At least four components of α-galactosidase were evident and their separation was almost complete. Protein and carbohydrate both also showed a characteristic pattern of distribution and no definite relation could be found between the amount of either and the α-galactosidase activity.

Since the distribution pattern of α-galactosidase activity was reproducible when the initial solution was used, three similar experiments were carried out with the crude extract under the same conditions as used for Fig. 6. The eluates obtained from each corresponding starch section were combined

and concentrated to 5 ml. under reduced pressure at 25~30°C. Fractions 3, 9, 11, 15 and 19 were tested for their α-galactosidase activity against various substrates. The results are shown in Table 2.

The activity ratios of fractions 3 and 9 were so similar that the similarity of their aglycon specificities could be assumed, although the fractions migrated with considerably different mobilities, while the ratios for the other three fractions were somewhat different not only from each other but also from those for the first two.

Table 2. Enzyme activities of five fractions from the initial solution against α-galactosides with different aglycons.

Enzyme fraction	α-D-Galactoside							
	Phenyl		*p*-Cresyl		*m*-Cresyl		*p*-Nitrophenyl	
	K	r	K	r	K	r	K	r
3	0.237	1	0.373	1.57	0.225	0.95	0.936	3.95
9	0.335	1	0.423	1.26	0.339	1.01	1.332	3.97
11	0.375	1	0.475	1.27	0.374	1.00	1.320	3.52
15	0.305	1	0.313	1.03	0.263	0.86	1.170	3.84
19	0.193	1	0.311	1.61	0.470	2.44	1.227	6.36
(Initial)*	0.770	1	0.934	1.21	0.74	0.96	2.28	2.95

Enzyme fraction	α-D-Galactoside			
	Glucosyl		Galactosyl	
	K	r	K	r
3	0.417	1.76	0.623	2.63
9	0.625	1.87	0.901	2.68
11	0.794	2.12	0.675	1.80
15	0.563	1.85	0.395	1.29
19	0.766	3.97	0.591	3.06
(Initial)*	0.992	1.29	—	—

Note:
K, the value is multiplied by 10^4.
r, activity for phenyl α-galactoside is taken as 1.
* Figures are cited from a previous paper[11].

iv) *Reelectrophoresis of the eluates from the fraction 19 in Fig. 6*

The eluate from fraction 19 in Fig. 6 was kept in the ice-box (0~2°C) for three months, and then subjected to zone electrophoresis to examine whether the α-galactosidase activity in this extract can be resolved into additional components. The electrophoresis was carried out under the same conditions as the first electrophoresis. As shown in Fig. 7, the component migrated as a single peak over 24 hours.

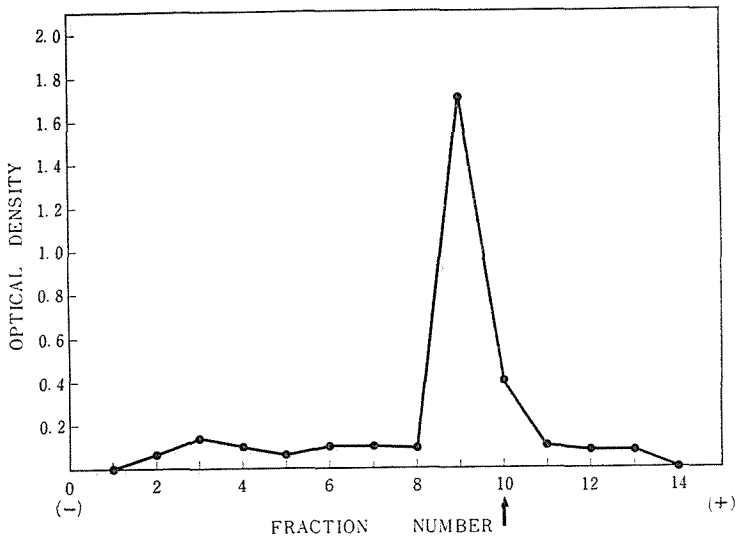

Fig. 7. Distribution of α-galactosidase activity on reelectrophoresis of the eluate from fraction 19 of Fig. 6.

—●— Enzyme activity as expressed by optical density at 400 mμ.
Arrow represents the point where eluate from fraction 19 was applied.
Conditions:
Buffer; phosphate, pH 7.0, ionic strength 0.096. Electric power; 300 volts, 12 mA. Duration; 24 hours. Enzyme; eluate from the fraction 19 in Fig. 6, 2.5 ml. Eluate; 2 ml. for each 2 cm. section. Reaction mixture for activity measurement; 0.8 ml. Incubation time; 8 days.

v) *Electrophoresis of a preparation partially purified with acetone and ammonium sulfate*

Fig. 8 shows the results obtained from the electrophoresis of a purified enzyme preparation, the activity of which was nearly 35 times as high as that of the initial solution. The experimental conditions were the same as

for Fig. 6. However, the α-galactosidase preparation was resolved into only two components.

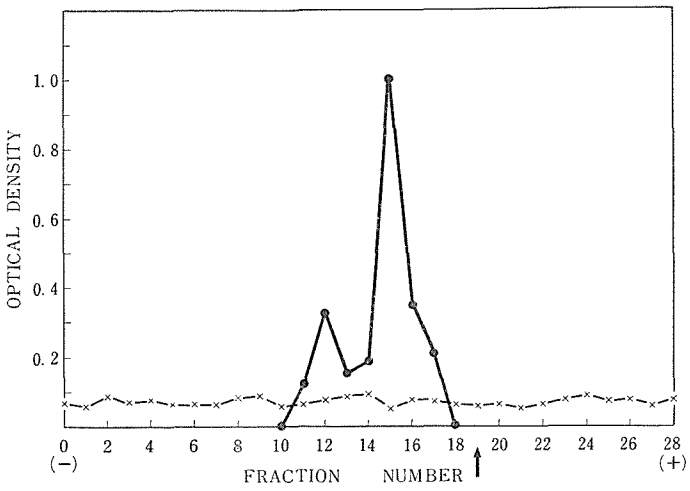

Fig. 8. Distribution of protein and α-galactosidase activity in the zone electrophoresis of a preparation partially purified with acetone and ammonium sulfate.

—●— Enzyme activity as expressed by optical density at 400 mμ.
—×— Protein concentration as expressed by the optical density at 750 mμ.
Arrow represents the point where partially purified enzyme solution was applied.
Conditions:
Buffer; phosphate, pH 7.0, ionic strength 0.096. Electric power; 300 volts, 12 mA. Duration; 24 hours. Enzyme; partially purified solution, 2.5 ml. Eluate; 2 ml. concentrated for each section. Reaction mixture for activity measurement; 0.4 ml. Incubation time; 28 hours.

Discussion

In order to confirm the result obtained in the previous paper[1], namely, the complex nature of the α-galactosidase of bottom yeast as shown by paper chromatography, crude extracts were subjected to starch zone-electrophoresis at different values of pH and ionic strength. Although the enzyme was incompletely resolved at pH 4.8 (acetate buffer, $\mu = 0.05$), pH 5.8 (phosphate buffer, $\mu = 0.096$), pH 8.0 (phosphate buffer, $\mu = 0.096$, and borate-phosphate buffer, $\mu = 0.036$) (Figs. 1~4), it was fairly completely separated into at least four components at pH 7.0 (Figs. 5 and 6). This

resolution was at its best when phosphate buffer of pH 7.0 and ionic strength 0.096 was used (Fig. 6).

Some possible causes for the resolution of α-galactosidase activity other than multiplicity might be considered: (a) the selective adsorption of enzyme on other proteins present in the enzyme solution; (b) the partial denaturation of enzyme molecules to different degrees during the electrophoresis, concentration and storage of the enzyme solution; (c) uneven perturbation of enzyme migration through electroconvection during electrophoresis.

None of these possibilities, however, would explain the presence of multiple components in the α-galactosidase for the following reasons. The activity ratios of the fractions against hexosyl α-galactosides were rather different, although most of them were similar for aryl α-galactosides. In particular, the ratios for the fraction 19 were noticeably different from the others (Table 2). This fact may well indicate the independence of each component. Such an assumption may be further supported by the fact that the amount of protein in the components into which it was resolved by the same electrophoresis did not parallel the α-galactosidase activity; in addition, one of the components migrated as a single peak on reelectrophoresis under the same conditions. It may be taken as further evidence for the multiplicity of this enzyme that a few components at least could be removed from a crude extract by fractionation with acetone and ammonium sulfate, as illustrated by the electrophoresis of a partially purified enzyme solution (Fig. 8).

Thus, the α-galactosidase of bottom yeast has been resolved by starch zone-electrophoresis into at least four components, the aglycon specificities of most of them being rather different from one other.

These, however, when they were subjected to electrophoresis under other conditions, would be resolved into additional components which might have a different aglycon specificity. There is, consequently, no reason to believe that the components obtained in the present work are ultimate single individuals.

The distribution of total carbohydrates, like that of total proteins, did not parallel the α-galactosidase activity. This suggests that there is no definite relationship between enzyme activity and the carbohydrates.

Summary

The α-galactosidase of brewer's bottom yeast has been resolved, using phosphate buffer of pH 7.0, and ionic strength 0.096, into at least four components by starch zone-electrophoresis. Since each seemed to be rather characteristic in aglycon specificity and since their resolution, for other reasons as well, could not be explained as due to artifacts occurring during the course of electrophoresis, it is probable that each component was initially present in the bottom yeast.

Acknowledgements

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