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Author(s)	SHIOMI, Norio
Citation	Journal of the Faculty of Agriculture, Hokkaido University, 58(3), 321-342
Issue Date	1977-03
Doc URL	https://hdl.handle.net/2115/12905
Type	departmental bulletin paper
File Information	58(3)_p321-342.pdf



PURIFICATION AND CHARACTERIZATION OF β -FRUCTOFURANOSIDASES SOLUBILIZED AND RELEASED FROM CELL DEBRIS OF AGED ONION SLICES

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Received Apr. 30, 1976

Introduction

It is well known that β -fructofuranosidase (or invertase, saccharase) is widely distributed in higher plants, and many workers have studied it plantphysiologically. For instance, HATCH and GLASZIOU⁹⁾ investigated on the relationship between invertase activities and growth rates or sugar contents in storage tissues of immatured sugar cane in order to know the role of invertase *in vivo*, while HELLEBUST *et al.*¹²⁾ reported a positive correlation between invertase activity and cell elongation in corn radicle tips.

In general, the higher plant invertases are known to be present in both soluble and insoluble types, e.g., in corn coleoptiles¹³⁾, sugar cane¹⁰⁾, endocarp of pods of Kentucky Wonder pole beans¹⁸⁾, grape berries^{2,3)}, and tissue cultures of ten species of higher plants²¹⁾. The soluble enzymes are also called free, intracellular, or protoplasm invertases, while the insoluble ones are termed cell wall bound, cell debris associated, or outer space invertases.

On the other hand, it has been reported that washing (or ageing, aerating) of the slices or disks of higher plant tissues with water results in development of invertase activities in the following plant tissues: beet roots^{4,5,22~24)}, carrot roots^{5,7,8,22)}, potatoes²²⁾, Jerusalem artichoke tubers^{5~8)}, chicory roots⁵⁾, dahlia tubers⁵⁾, turnip roots⁵⁾, and *Compositae* plants⁴⁾. In grape²⁾, carrot, potato, and red beet^{22,23)} the newly developed enzymes are again of soluble and insoluble types, but in Jerusalem artichoke⁶⁾ they are only of insoluble type.

Trials of solubilization of the insoluble enzymes have been conducted to investigate more fully their properties: EDELMAN *et al.*^{6,8)} failed in solubilization of the insoluble enzymes from cell debris fractions in Jerusalem artichoke tubers by several physical, chemical, and enzymatic treatments;

and VAUGHAN *et al.*²³⁾ did not succeed in the similar experiments in use of buffer solutions or ethyl acetate in red beet enzymes. ARNOLD³⁾, however, reported that borate buffer (pH 8.5) was effective for solubilising the bound β -fructofuranosidases of grape berry pulps but a variety of other chemicals, including sodium chloride solution, were almost all ineffective; and STRAUS²¹⁾ found that toluene treatment was effective in release of a portion of enzymes from the cell walls of tissue-cultured tissues of higher plants.

In a previous paper¹⁹⁾, the present author briefly reported on *de novo* development of the cell debris associated β -fructofuranosidases in the onion slices aged by washing treatment with water, effective solubilization of these insoluble enzymes with 0.2~0.5M sodium chloride solutions, and some properties of the partially purified preparations of solubilized enzymes. The present paper deals with details of the experiments described above and further studies concerning them.

Experimental

Materials and Methods

Materials

Onion (*Allium Cepa* L. cult. var. Sapporo-Yellow) bulbs used for enzyme purification were obtained from commercial sources. Sucrose (Wako Pure Chemical Industries, Ltd.), maltose (Wako Pure Chemical Industries, Ltd.), raffinose (Merck Co.), melibiose (Merck Co.), and inulin (Wako Pure Chemical Industries, Ltd.) were also obtained from commercial products.

Methods

(1) Tests on Distribution of β -Fructofuranosidase Activities in Onion Slices

After onion slices (1~2 mm thickness) were washed with tap water for five days, a portion (150 g) of them was homogenized three times in cold water, giving ca. 600 ml of juice (crude aqueous extract) and ca. 25 g of cell debris.

The juice was centrifuged at $10,000\times g$ for 15 minutes. All of the centrifugation, unless otherwise indicated, was carried out under this condition. The supernatant, freed from pellets by centrifugation, was dialyzed against tap water and the resulted dialyzate was centrifuged. The supernatant was assayed for β -fructofuranosidase activities. The precipitate as well as the pellets described above were solubilized with 0.5M NaCl solution and then assayed.

The cell debris described above was suspended in 0.5M NaCl solution, filtered, and washed successively with the same salt solution, then with a small quantity of distilled water. Assays for β -fructofuranosidase activities were conducted on the combined solution of filtrates and washings (0.5M NaCl-extract) and on the insoluble debris (residual cell debris).

(2) Preparation of β -Fructofuranosidases I and II

Slices (300 g, 1~2 mm thickness) from the onion bulbs pre-washed with tap water for five days were homogenized in cold water. Cell debris was thoroughly re-homogenized in a mortar and washed with distilled water. Then, the debris was suspended in one lit. of 0.2M NaCl solution and stillstood over night in an ice box. The debris obtained after squeezing through muslin, was washed with the same solution, and was re-suspended to extract with 750 ml of 0.5M NaCl solution. The extract was concentrated to five ml with a collodion bag. After centrifugation (10,000 \times g, 15 minutes; all of the centrifugation was conducted under this condition), the resulted supernatant was chromatographed with 0.5M NaCl solution on a polyacrylamide column (3 \times 30 cm) pre-equilibrated with the same solution. The eluate, not containing any traces of nucleic acid-like substances, was concentrated to ten ml with a collodion bag, and was dialyzed against the McIlvaine buffer (pH 4.5) diluted 20 times with distilled water.

After centrifugation, the supernatant was chromatographed, with the McIlvaine buffer (pH 4.5) diluted 20 times with 0.3M NaCl solution, on a column (3 \times 37 cm) of Sephadex G-200 pre-washed with the same solution. Five-ml fractions were collected and the enzyme fractions from No. 23 to 30 were combined. The enzyme fraction was concentrated and re-chromatographed on a similar column. Dialyzed fraction No. 28, proved to be of the highest specific activity, was called β -fructofuranosidase I and used for enzyme tests. On the other hand, the precipitate obtained after centrifugation was dissolved in four ml of 0.5M NaCl solution and one ml-portion of the resulted solution was chromatographed on a Sephadex G-200 column (3 \times 37 cm) by the same way as described above. Five-ml fractions were collected. The fractions from No. 30 to 33 were pooled and concentrated to two ml with a collodion bag. The concentrated enzyme preparation was designated as β -fructofuranosidase II and used for enzyme reaction tests.

(3) Assay for β -Fructofuranosidase Activities

The following assay method was employed as a general method: A reaction mixture, consisting of 0.5 ml of 0.02M sucrose solution in the

McIlvaine buffer (pH 4.5) and 0.5 ml of the enzyme solution, was incubated at 37°C for 30 minutes. After the enzyme reaction was terminated by addition of one ml of SOMOGYI-NELSON's reagent, reducing sugars released were determined by SOMOGYI-NELSON's method²⁰.

In case of the residual cell debris, however, the debris (100 mg) was incubated with 0.02M sucrose solution (2.5 ml) and the McIlvaine buffer (pH 4.5, 2.5 ml) at 37°C for 30 minutes by stirring with a mechanical shaker. After the digest was heated in boiling water for one minute, the cell debris was removed and reducing sugars released were determined as described above.

One unit of the enzyme activity was defined as an amount of the enzyme which hydrolyzes one μ mole of sucrose per minute at 37°C in 0.01M sucrose solution in the McIlvaine buffer (pH 4.5).

(4) Determination of Protein

Protein was determined by the FOLIN-CIICALTEU reagent according to the procedure of LOWRY *et al.*¹¹.

(5) Disc Electrophoresis

The partially purified β -fructofuranosidases were subjected to disc electrophoresis on a polyacrylamide gel (pH 4.3) at room temperature for three hours at two mA per tube by the method of DAVIS¹⁶. Staining of protein bands was made with a solution of Amido Schwarz in 7% acetic acid. Stain at the gel-portion other than protein bands was decolorized by washing with 7% acetic acid.

(6) Paper Chromatography

By the use of a solvent system, n-butanol - acetic acid - water (4:1:2), paper chromatography of sugars was carried out on Toyo-Roshi No. 51 filter paper by triple ascending technique. Anisidine phosphate or aniline phthalate was used as the spray reagents for detecting sugars, and the paper-chromatograms were heated at 110°C for five minutes to complete the coloration.

Results

I. Development and Distribution of β -Fructofuranosidase Activities in Onion Slices by Washing Treatments with Water

(1) Development of β -Fructofuranosidase Activities in Onion Slices by Washing with Tap Water

Experiments on the development of β -fructofuranosidase activities in

TABLE 1. Development of β -Fructofuranosidase Activities in
 Onion Slices by Washing Treatment

(Comparison between the Washed and Unwashed Slices)

Fraction	Washed slices				Unwashed slices				
	Total activity (U)	Total protein (mg)	Volume (ml)	Specific activity (mU/mg protein)	Total activity (U)	Total protein (mg)	Volume (ml)	Specific activity (mU/mg protein)	
Juice (Crude aqueous extract)									
{ Supernatant*)	Supernatant	1.36	.183	201	7.43	***) { 0.15	193	100	0.78
	Precipitate**)	0	—	—	—				
{ Pellets**)		1.07	20	30	53.50	0.03	59	20	0.51
Cell debris									
{ 0.5M NaCl-extract		34.68	29	75	1,195.86	0.29	90	200	3.22
{ Residual cell debris		5.16	—	2.18****)	—	0	—	2.45****)	0
Total		42.27	—	—	—	0.47	—	—	—

*) The supernatant was dialyzed and re-centrifuged.

***) The precipitate and pellets were solubilized with 0.5M NaCl solution.

****) The supernatant showed so very low activities that every measurements were made on a precipitate obtained after 90% saturation with ammonium sulfate.

*****) Grams on dry basis.

The entire experiments are carried out according to the similar method as described in the "Methods, (1)".

45 g of onion slices washed with flowing tap water for five days were carried out. On each of the fractions obtained from homogenates of the washed slices and unwashed ones, the enzyme activities were assayed and compared with each other.

As shown in Table 1, it was found that the total enzyme activities measured in the washed onion slices were about 90 times larger than those in the unwashed ones, showing development of β -fructofuranosidases takes place by the washing treatment. About 94% of the activity was localized in the cell debris fraction and 82% in the 0.5M NaCl-extract from this fraction. In the washed slices the specific activities of the enzyme, extractable with 0.5M NaCl solution, were about 370 times larger than those in the unwashed ones.

(2) Influence of Washing Procedures on Development of β -Fructofuranosidase Activities in Onion Slices

Influence of washing procedures on the development of β -fructofuranosidase activities were experimented, in which one half of the onion

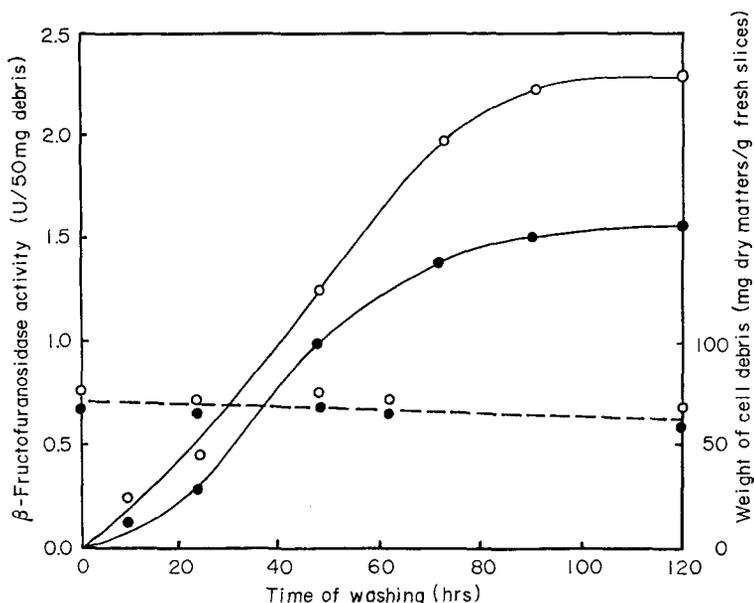


Fig. 1. Influence of Washing Procedures on Development of β -Fructofuranosidase Activity in Onion Slices

- Washed with flowing tap water
- Washed with still water
- β -Fructofuranosidase activity
- Weight of cell debris

slices was washed with flowing tap water at 25°C for every scheduled times and another half was washed, or incubated, in a vessel under the same conditions, except by mechanical stirring with still, not flowing, water. Then, the respective slices thus differently treated were harvested at every scheduled times, and cell debris was prepared by fractionations according to the similar method as described in the "Methods". After weighing, the cell debris was extracted with 0.5M NaCl solution, and the enzyme activities in the resulted extract were assayed.

The results obtained are shown in Fig. 1. Both of the two different washing treatments described above yielded the cell debris-dry matters of the same weight. The development of β -fructofuranosidase in case of the still-water procedure was much faster than that in the flowing-water one; the highest levels of enzyme activities were attained within three to five days, and the levels were 2.3 U/50 mg debris in the former method and 1.6 in the latter one.

(3) Distribution of β -Fructofuranosidase Activities in Onion Slices

Examinations on the distribution of β -fructofuranosidase activities developed in onion slices by the washing treatments with flowing tap water were made according to the method as described in the "Methods, (1)".

As shown in Table 2, only very small β -fructofuranosidase activities were found in the supernatant and pellets from the juice (crude aqueous

TABLE 2. Distribution of β -Fructofuranosidase Activities in Onion Slices

Fraction	Total activity (U)	Total protein (mg)	Volume (ml)	Specific activity (mU/mg protein)	
Juice (Crude aqueous extract)					
{ Supernatant* Pellets**)	Supernatant	4.53	610	670	7.43
	Precipitate**)	0	—	—	—
		3.56	66	100	53.94
Cell debris					
{ 0.5M NaCl-extract Residual cell debris	115.60	96	250	1,204.17	
	17.20	—	—	—	
Total	140.89	—	—	—	

*) The supernatant was dialyzed and re-centrifuged.

***) The precipitate and pellets were solubilized with 0.5M NaCl solution, and then their β -fructofuranosidase activities were assayed.

Details: see text.

extract) of washed slices (3.2% and 2.5% of the total, respectively). Almost all the enzyme activities distributed in the cell debris (94.3% of the total), from which 87.0% of the activities were extractable with 0.5M NaCl solution.

Hence, it may be concluded that, in the onion slices washed with flowing tap water, nearly all the β -fructofuranosidase is present in a binding form with any portions of the cell debris and that this enzyme can be effectively solubilized and released with 0.5M NaCl solution.

II. Purification of β -Fructofuranosidases

(1) Relation between Release of β -Fructofuranosidases and Sodium Chloride Concentrations

Effects of NaCl concentrations on the release of β -fructofuranosidases from the cell debris were tested.

A portion (100 mg) of the cell debris treated with acetone was suspended in ten ml of 0.1, 0.2, 0.3, 0.4, 0.5, or 1.0M NaCl solutions and stillstood over night in an ice box. The resulted extracts were, after removal of the debris, assayed for β -fructofuranosidase activities and protein contents.

Results are shown in Table 3.

TABLE 3. Effects of Sodium Chloride Concentrations on Release of β -Fructofuranosidases from Cell Debris

NaCl concentration (M)	Enzyme activity (U/100 mg debris)	Protein (mg/100 mg debris)	Specific activity (mU/mg protein)
0.1	0.0214	0.474	45.1
0.2	0.0328	1.020	32.2
0.3	0.0371	0.900	41.2
0.4	0.531	1.200	442.5
0.5	0.553	1.200	460.8
1.0	0.555	1.380	402.2

Yields of the proteins solubilized with 0.2~0.3M NaCl solutions were approximately doubled compared to that with 0.1M solution, but any significant increases in the yields were not observed in 0.4~1.0M concentrations. Recoveries of the enzyme activity and specific activity were increased about ten-fold when the NaCl concentration was changed from 0.3M to 0.4~1.0M.

Therefore, 0.5M NaCl solution was chosen as the most suitable extractant for releasing the β -fructofuranosidases of high specific activities. Also, for practical purposes, it is more desirable that the proteins of com-

paratively lower activities are previously removed from the cell debris by treatment with 0.2~0.3M NaCl solutions.

(2) Purification of β -Fructofuranosidases I and II

Purification of the β -fructofuranosidases, released with 0.5M NaCl solution from the pre-washed cell debris, was carried out as described in the "Methods, (2)".

Elution patterns of the released two β -fructofuranosidases in Sephadex G-200 column chromatography are shown in Fig. 2~4. Recoveries of proteins and enzyme activities in each step of the purification process are summarized in Table 4.

In this manner, two main enzyme fractions, β -fructofuranosidases I and II, could be partially purified. Specific activities of the two enzymes were 15.4 and 23.1 U/mg protein, respectively.

(3) Disc Electrophoresis of β -Fructofuranosidases I and II

To test purity of the partially purified enzymes, electrophoresis was conducted on polyacrylamide gels at pH 4.3 for three hours. Two pieces of gels were run for each of the enzymes, and one was tested for protein with Amido Schwarz and the other was assayed for β -fructofuranosidase activity.

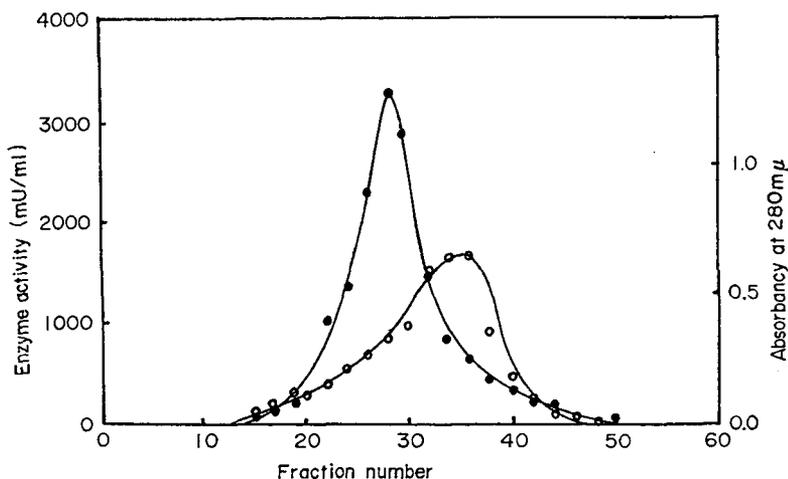


Fig. 2. Elution Pattern in Sephadex G-200 Column Chromatography of Onion β -Fructofuranosidase I

Five-ml fractions were collected.

●—●, β -Fructofuranosidase activity.

○—○, Protein concentration measured at 280 m μ .

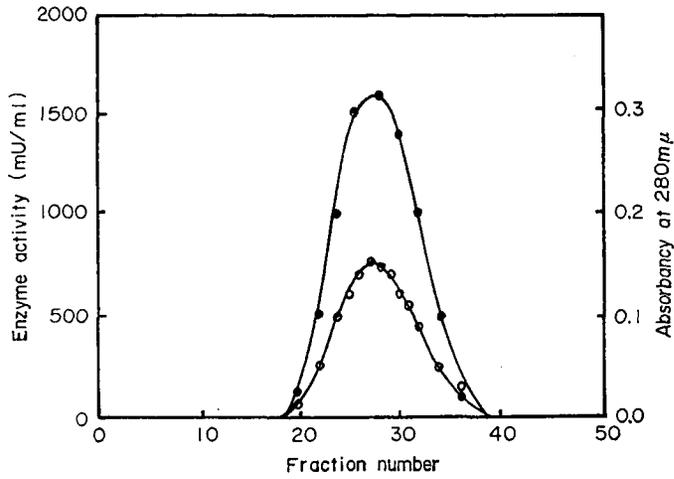


Fig. 3. Elution Pattern in Sephadex G-200 Re-column Chromatography of Onion β -Fructofuranosidase I
Five-ml fractions were collected.
●—●, β -Fructofuranosidase activity.
○—○, Protein concentration measured at 280 m μ .

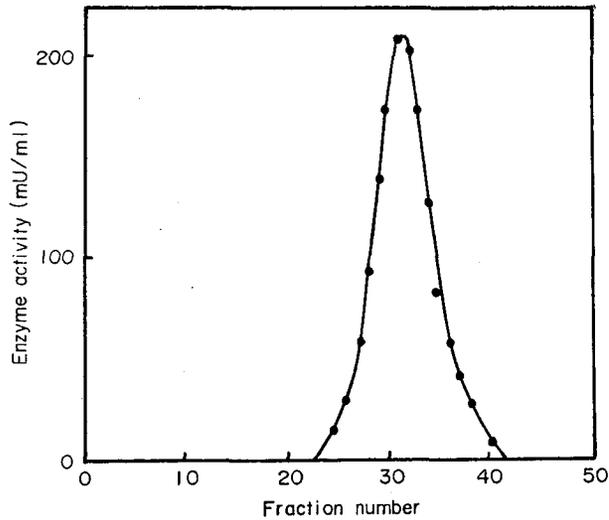


Fig. 4. Elution Pattern in Sephadex G-200 Column Chromatography of Onion β -Fructofuranosidase II
Five-ml fractions were collected.
●—●, β -Fructofuranosidase activity.
Protein measurements were not made owing to the extremely low concentrations of proteins in each of the fractions.

TABLE 4. Purification Process of β -Fructofuranosidases

Step	Total activity (U)	Total protein (mg)	Volume (ml)	Specific activity (U/mg protein)
0.5M NaCl-extract*)	236.5	93.3	770	2.53
Polyamide eluate	200.0	72.0	100	2.78
Supernatant**)	142.0	68.1	14.0	2.09
1st Sephadex G-200 Fr. No. 23~30	89.6	9.20	40.0	9.74
2nd Sephadex G-200 Fr. No. 28 (β -Fructofuranosidase I)	8.47	0.55	5.00	15.4
Precipitate**)	39.7	2.40	4.00	16.5
Sephadex G-200 Fr. No. 30~33 (β -Fructofuranosidase II)	3.58	0.155	2.00	23.1

*) From the cell debris pre-treated with 0.2M NaCl solution.

***) Obtained after dialysis and centrifugation of the concentrated eluate from polyamide chromatography.

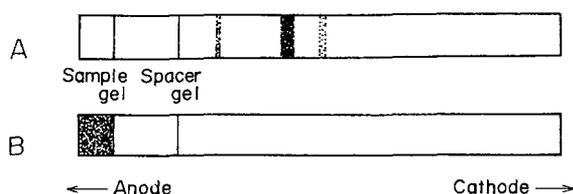


Fig. 5. Diagrammatic Representation of Protein Bands Separated by Electrophoresis on Polyacrylamide Gel

β -Fructofuranosidase (200 μ g protein) was applied to the gel electrophoresis (pH 4.3) and run for 3 hrs at 2 mA/tube.

Protein bands were stained with Amido Schwarz.

A: β -Fructofuranosidase I

B: β -Fructofuranosidase II

Electrophoretic patterns are shown in Fig. 5. The β -fructofuranosidase I preparation gave three protein bands on the electrophoretogram, and one of the bands showed β -fructofuranosidase activity but the other two not. The β -fructofuranosidase II preparation did not migrate on the acrylamide gel under the experimental conditions, and the enzyme activity was found only in sample gel portion.

Because the enzyme II preparation used in the electrophoresis contains NaCl but the enzyme I not, effects of NaCl on the migration of protein on the gel were tested. The migration of the enzyme I, however, was hardly affected by addition of NaCl (0.2M) to the sample gel portion.

III. Some Properties of β -Fructofuranosidases I and II

(1) Solubility in Sodium Chloride Solution

As the β -fructofuranosidase I is soluble in water but the β -fructofuranosidase II sparingly soluble, the latter enzyme was examined for its solubility in NaCl solutions.

The enzyme II preparation (1750 mU, 0.2 ml) was added to six solutions containing different amounts of NaCl, respectively. Then, the final concentrations of NaCl were adjusted to 0.02, 0.1, 0.25, 0.3, 0.4, and 0.5 M in 5 ml-solutions. The solutions thus prepared were centrifuged after standing over night in an ice box. The precipitate, after solubilized with 0.5 M NaCl solution, and the supernatant were measured for β -fructofuranosidase activities.

As shown in Fig. 6, the solubility of β -fructofuranosidase II is largely influenced by NaCl concentrations. In the 0.02 M NaCl-treatment, only a trace of the enzyme activity was detected in the supernatant and almost all the activity was present in the precipitate; in the 0.5 M NaCl-treatment, an opposite result was obtained.

Consequently, for solubilizing the enzyme and for furnishing the complete enzyme reactions, the concentrations of NaCl are necessary to be held, at least, at 0.25 M or above. Also, the concentration of NaCl in a reaction mixture of the enzyme I must be held at 0.25 M or above to experiment

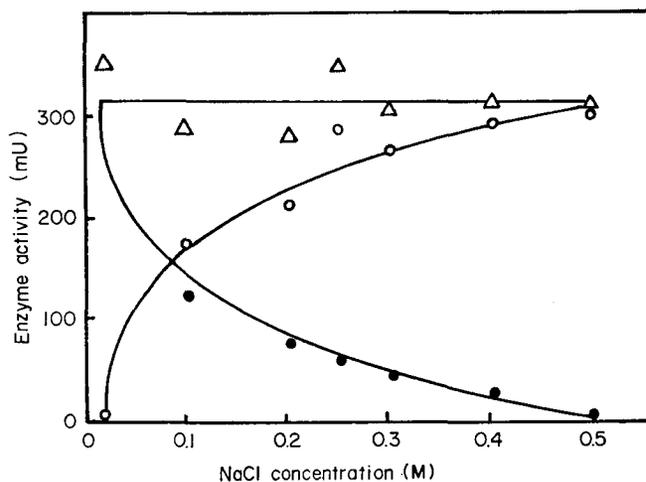


Fig. 6. Solubility of β -Fructofuranosidase II in NaCl Solution

- , Enzyme activity of supernatant fraction
 - , Enzyme activity of precipitate fraction
 - △—△, Total enzyme activity
- Experimental details are given in the text.

under the same conditions as in the enzyme II, although the former enzyme is freely soluble in water without NaCl.

(2) pH-Dependence

In Fig. 7, pH-dependence curves of the β -fructofuranosidases of both types acting on sucrose are shown. The enzyme I showed the optimum pH at 4.5 (in the presence or absence of 0.25M NaCl), similar to that of the enzyme II.

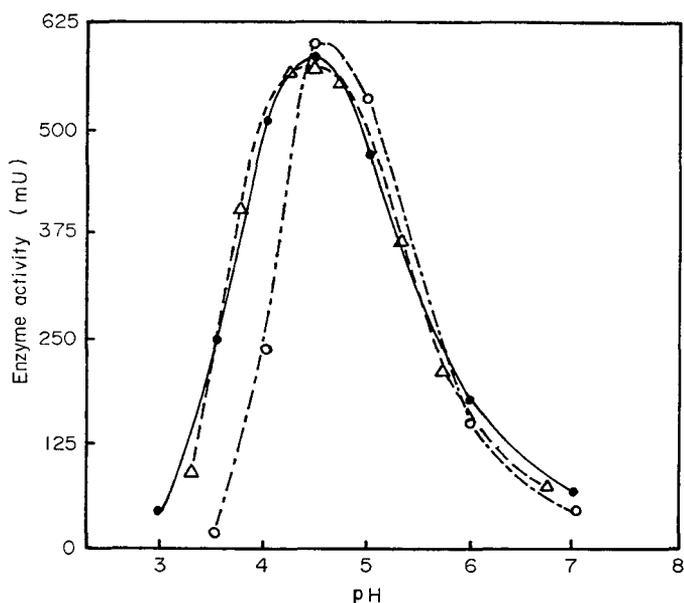


Fig. 7. Effects of pH's on Activity of β -Fructofuranosidases.

The β -Fructofuranosidases I and II were incubated with 0.01M sucrose in the McIlvaine buffer at 37°C for 30 min. over the pH-range 3~7, respectively.

●—●, β -Fructofuranosidase I

○---○, β -Fructofuranosidase I (in the presence of 0.25M NaCl)

△---△, β -Fructofuranosidase II (in the presence of 0.25M NaCl)

(3) pH-Stability

Effects of pH's on the stability of the β -fructofuranosidases I and II were investigated. The enzyme solutions were adjusted to various pH's, kept at 30°C for 30 min., cooled to 0°C, readjusted to pH 4.5, and then assayed for β -fructofuranosidase activities.

Results are shown in Fig. 8. The enzyme I, in the presence of NaCl, and the enzyme II were stable at the pH-range of 5~7. In the absence

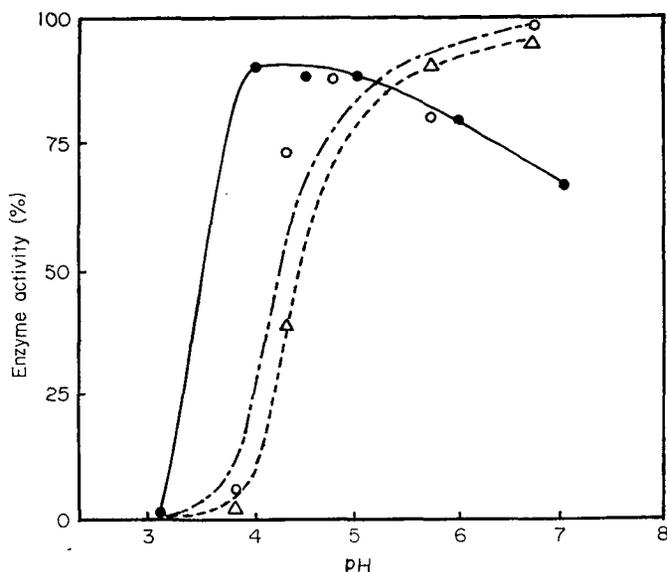


Fig. 8. Effects of pH's on Stability of β -Fructofuranosidases

Experimental details are given in the text.

●—●, β -Fructofuranosidase I (200 mU)

○---○, β -Fructofuranosidase I (160 mU, in the presence of 0.25 M NaCl)

△---△, β -Fructofuranosidase II (280 mU, in the presence of 0.25 M NaCl)

of NaCl, the enzyme I was stable at the pH-range of about 4~5 but unstable at pH 3.

(4) Thermal Stability

Stability of the β -fructofuranosidases at various temperatures was tested. The β -fructofuranosidase activity was determined after an aliquot of the enzymes was pre-incubated for 30 min. at 30, 37, 45, or 60°C, respectively.

Results are shown in Fig. 9. The two enzymes were labile on heating. The enzyme I was thermally inactivated at about 45°C both in the presence or absence of NaCl. The enzyme II was also completely destroyed on heating at 60°C for 30 min..

(5) Substrate Specificity

By the use of some substrates, the β -fructofuranosidases I and II were examined for their substrate specificity. Sucrose, raffinose, melibiose, maltose, and inulin were used as the substrates, whose final concentrations in reaction mixtures were all set at 0.01M, except 0.1% for inulin. A reaction mixture, consisting of 0.5 ml of the substrate solution in the McIlvaine

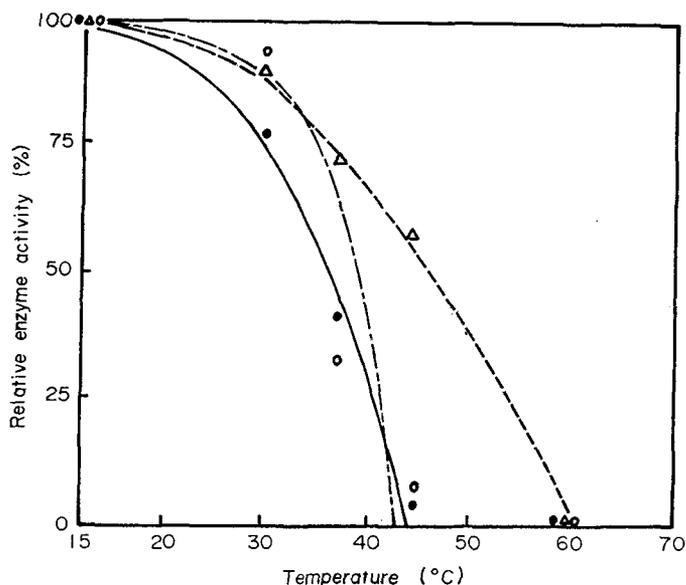


Fig. 9. Effects of Temperatures on β -Fructofuranosidases

Experimental details are described in the text.

●—●, β -Fructofuranosidase I (500 mU)

○---○, β -Fructofuranosidase I (300 mU, in the presence of 0.25 M NaCl)

△---△, β -Fructofuranosidase II (448 mU, in the presence of 0.25 M NaCl)

buffer (pH 4.5) and 0.5 ml of the enzyme solution, was incubated at 37°C for 30 minutes.

An increase of reducing sugars was observed in the digests from sucrose and raffinose, but not from maltose, melibiose, and inulin. Also, in the incubations at 37°C for 24 hours, fructose and glucose were paper-chromatographically confirmed in the digest from sucrose, and fructose and melibiose in that from raffinose. Glucose was scarcely detected in the digest from maltose. Any detectable reaction products were not obtained from melibiose and inulin.

Accordingly, it was verified that the tested two enzymes are specifically able to attack sucrose and raffinose.

(6) Substrate Concentration and Rate of Enzymatic Hydrolysis

Effects of concentrations of sucrose and raffinose on the rates of reactions by the β -fructofuranosidases I and II were investigated, and the MICHAELIS constants for both of the enzymes were calculated by means of the LINEWEAVER-BURK plots.

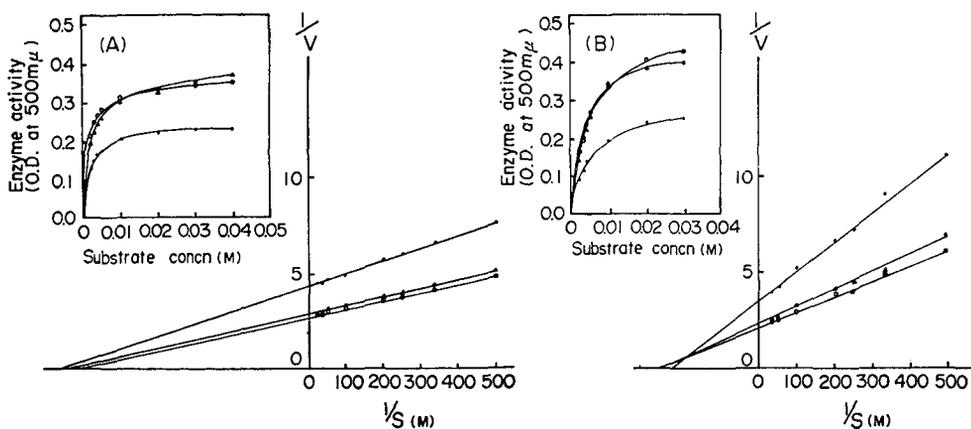


Fig. 10. Effects of Substrate Concentrations on Reaction Rate

A: Sucrose B: Raffinose

●—●, β -Fructofuranosidase I

○—○, β -Fructofuranosidase I (in the presence of 0.25 M NaCl)

▲—▲, β -Fructofuranosidase II (in the presence of 0.25 M NaCl)

Results are shown in Fig. 10. The MICHAELIS constants for each of the substrates were proved to be somewhat different: 1.49 mM (the enzyme I in the absence of NaCl), 1.64 (I in the presence of NaCl), and 1.56 (the enzyme II) in sucrose; 4.16 mM (I in the absence of NaCl), 3.85 (I in the presence of NaCl), and 3.85 (II) in raffinose at pH 4.5. In general, the presence of NaCl did not largely affect the MICHAELIS constants.

(7) Effects of Inhibitors

Inhibition of several substances on the β -fructofuranosidase activities was examined under the general assay conditions with the enzymes of 375 mU/ml.

Results are shown in Table 5. The β -fructofuranosidase I was inhibited either with pCMB (6×10^{-4} M) and HgCl_2 (5×10^{-5} M) by about 100% and 92% in the absence of NaCl, respectively. Also, the inhibitions were lessened in the presence of NaCl. The β -fructofuranosidase II was sensitive to both of the heavy metal inhibitors in the presence of NaCl: pCMB, 80% inhibition; HgCl_2 , 74% inhibition.

The other substances tested (at 5×10^{-4} M) hardly affected on the activities of both of the enzymes.

Thus, any significant differences in the effects of inhibitors were not found between the enzymes of two types.

TABLE 5. Effects of Inhibitors on β -Fructofuranosidases

Inhibitor		Relative activity		
		β -Fructofuranosidase I		β -Fructofuranosidase II
		in the absence of NaCl	in the pre- sence of NaCl	in the presence of NaCl
pCMB	(6×10^{-4} M)	0	16.5	19.8
HgCl ₂	(5×10^{-5} M)	8.2	35.7	25.8
NaN ₃	(5×10^{-4} M)	100	106	102
CuSO ₄	(")	103	103	104
ICH ₂ COOH	(")	97.0	105	104
Aniline-HCl	(")	98.0	98.4	92.0
Uranyl acetate	(")	89.5	93.1	91.5
Control		100	100	100

Discussion

Until today, purification of the β -fructofuranosidases of higher plant origin and elucidation of their properties have been not extensively made as compared with those from microbial and animal sources. Also, properties of the plant β -fructofuranosidases bound with cell wall have been not fully studied on account of difficulties in solubilization of the bound enzymes.

In a preliminary work of this series¹⁹⁾, the present author showed that the onion β -fructofuranosidases of bound type, newly developed after washing (or aerating, ageing) with flowing tap water, could be solubilized with 0.2 to 0.5M NaCl solutions and partially purified. Thus, several properties of the solubilized enzymes were able to be clarified.

The results obtained from more detailed experiments along this line are given here and discussed below.

(1) Development of β -Fructofuranosidase Activity in Aged Onion Slices

When onion slices were washed with tap water, an increased β -fructofuranosidase activity was found to be developed in both cell debris and soluble fraction of the slices. The β -fructofuranosidase activities measured in the cell debris from the aged slices were larger 137-times on the basis of total activity and 371-times on the basis of specific activity than those from the untreated slices, while in the soluble fraction the corresponding values were only 14-times and 17-times, respectively (Table 1). These facts indicate that the washing of onion slices induces development of β -fructo-

furanosidases and that the newly developed enzymes are more abundantly locating in the cell debris than in the soluble fraction. The latter indication was also ascertained from the other experiments showing that the total enzyme activities in the cell debris and in the pressed juice are 132.8 U and 8.1 U (ratio, 16.4:1.0), respectively (Table 2).

The β -fructofuranosidase distribution between the cell debris and soluble fraction seems to be different from plant to plant. For instance, according to the data given by VAUGHAN and MACDONALD²²⁾, the ratios of the total enzyme activity of cell debris to that of soluble fraction in several plant tissue slices aged by washing for three days are as follows: 7.24:1 in carrot, 1.31:1 in potato, and 0.95:1 in red beet.

(2) Solubilization of β -Fructofuranosidases Bound with Cell Debris

As shown in a previous report¹⁹⁾ and in Tables 1, 2, and 3 in this paper, the onion β -fructofuranosidases bound with the cell debris could be successfully solubilized and released with 0.5M NaCl solutions.

After the present author's preliminary investigation¹⁹⁾ had been reported, several works reporting success in the solubilization of bound enzymes appeared. MASUDA and SUGAWARA¹⁵⁾ reported that the bound type-saccharase of sugar beet roots could be solubilized with NaCl or KCl solutions more than 0.3M or with MgSO₄ solutions above 0.1M concentrations. HAWKER¹¹⁾ found that treatment of the insoluble invertase associated with cell-wall fraction of grape berries with borate buffer (pH 8.5), polyethylene glycol (Carbowax 4000), or non-ionic detergents solubilized most of the invertase, with the exception of those of invertases from aged carrot disks and from corn coleoptiles. LITTLE and EDELMAN¹⁴⁾ demonstrated that solubilization of the acid invertase associated with cell-wall preparations from the aged slices of Jerusalem artichoke tuber was achieved at high ionic strengths at pH ca. 8. RICARDO and APREES¹⁷⁾, however, found that the cell-wall bound acid invertase from the aged disks of mature carrot roots could not be solubilized with Na₂HPO₄-KH₂PO₄, sodium citrate, sodium borate, or Na₂HPO₄-citric acid buffers.

(3) Purification of Solubilized β -Fructofuranosidases

In a preliminary experiments of the enzyme purification, a series of the following phenomena was observed: when the β -fructofuranosidases freed from the debris by the treatment with 0.5M NaCl solution were dialyzed against the McIlvaine buffer (pH 4.5) diluted with water, a large quantity of the enzymes re-precipitated. About 80% of the total enzyme activity was recovered in the precipitate, and the residual 20% remained

in the soluble fraction ($10,000 \times g$ -supernatant). Concentration and re-dialysis of the latter supernatant did no longer result in precipitation. These facts suggest that the enzymes released from the cell debris are separable into at least two types of β -fructofuranosidases, sparingly soluble and soluble ones.

It was also found that the NaCl concentrations necessary for releasing β -fructofuranosidases from the cell debris were somewhat higher than those for re-solubilizing the enzymes precipitated by dialysis. This suggests that the ionic strength of extractants largely affects the bond-structures between enzyme molecules and cell wall, and that the ionic strength necessary for breaking such a bond is larger than that for re-solubilizing the precipitated enzymes.

As can be seen from Table 4, 85% of the total enzyme activities in the "0.5M NaCl-extract" was recovered in the "polyamide eluate". Overall recoveries of the enzyme activities obtained after dialysis and centrifugation of the "polyamide eluate" were found to be 60% in the "supernatant" and 17% in the "precipitate". At the last step of purification, total activity, total activity yield, and specific activity of the partially purified β -fructofuranosidase I were 8.47U, 3.6%, and 15.4U/mg protein, respectively, and those values of the enzyme II were 3.58U, 1.5%, and 23.1U/mg protein.

On Sephadex G-200 column chromatography, the β -fructofuranosidase II showed a longer retention time equivalent to about 15 ml than the β -fructofuranosidase I (Fig. 2 and 3), but the molecular weight difference between both of the enzymes was found to be smaller than 10,000 in an experiment in use of marker proteins.

(4) Properties of Solubilized β -Fructofuranosidases

The β -fructofuranosidases I and II thus obtained showed different behaviours in solubility (I, water soluble; II, sparingly soluble), polyacrylamide gel-electrophoresis (I, migrated to cathode at pH 4.3; II, not migrated), and thermal stability (I, completely inactivated by heating at ca. 45°C for 30 minutes; II, at ca. 60°C). However, the hydrolytic characters of the enzymes I and II were identical. Both of the enzymes specifically hydrolyzed β -fructofuranosidic linkages in sucrose (I and II, K_m ca. 1.6 mM) and raffinose (I and II, K_m ca. 3.9 mM) at the same optimum pH 4.5, but did not hydrolyze maltose, melibiose, and inulin. The enzymatic hydrolysis was inhibited by pCMB and $HgCl_2$.

On account of deficit of available data, the properties of solubilized onion β -fructofuranosidases have not been able to compare in detail with

those of the solubilized enzymes from different plant sources.

Acid β -fructofuranosidases are generally recognized to be more widely distributed than alkaline ones in higher plants; the two β -fructofuranosidases solubilized and released from the aged onion slices also belong to the category of acid enzymes (opt. pH 4.5). This is equally true to the solubilized β -fructofuranosidases from grape berries (opt. pH 4.0)³⁾ and from sugar beet root (opt. pH 4.6)¹⁵⁾.

Both of the onion enzymes showed the same K_m value, 1.6 mM, in sucrose hydrolysis. This value was undistinguishable from K_m 1.53 mM in the sugar beet enzyme¹⁵⁾, but somewhat smaller than K_m 3.48 ± 0.52 mM in the grape enzyme³⁾.

Substrate specificities of the onion enzymes were nearly the same as that of the grape enzyme³⁾: the formers hydrolyze sucrose and raffinose, but do not hydrolyze maltose, melibiose, and inulin; the latter decomposes sucrose, raffinose, and β -methyl fructoside, but does not decompose melezitose, turanose, and benzyl- β -D-fructofuranoside.

Activities of the onion enzymes were inhibited by pCMB and $HgCl_2$, but not inhibited by another SH-reagent iodoacetic acid. Therefore, the question whether they are SH-enzymes or not could not be resolved. The sugar beet enzymes¹⁵⁾ were reported to be inhibited by the SH-reagents pCMB and $HgCl_2$.

(5) *In vivo* Location and Solubilization Mechanism of Cell-wall Bound β -Fructofuranosidases

As described above briefly, it has been reported that the cell-wall bound β -fructofuranosidases, found to exist originally in intact plant tissues or secondarily in tissue slices aged with water, were able to be solubilized successfully. However, either the information obtained about extractants effective to the solubilization or the views proposed on the solubilization mechanisms are not necessarily the same among various works. Also, *in vivo* location of such a cell-wall bound enzyme and *in vivo* bond structures between enzyme molecules and cell-wall components seem to be different among plant species.

Inorganic salt solutions such as NaCl, KCl, or $MgSO_4$ aq. were reported to be effective for solubilizing the bound enzymes in sugar beet roots¹⁵⁾. LITTLE *et al.*¹⁴⁾ found that alkaline buffer solutions of high ionic strengths were very useful for solubilizing the bound enzymes of Jerusalem artichoke tubers, and they proposed that enzyme molecules may be bound predominantly by salt linkages to some charged component of cell-wall and the enzyme release may take place from rupture of the salt linkages by the

action of buffer solutions. At the same time, they remarked that this feature not necessarily represents the condition and location of the enzymes *in vivo*. The solubilization of the present author's onion enzymes with use of NaCl solutions is presumed to be obeyed the mechanism proposed by LITTLE *et al.*.

Concerning grape berry β -fructofuranosidases, ARNOLD³⁾ found that borate buffer (0.2M, pH 8.5) was effective to the solubilization, whereas the other extractants, including NaCl, were without effect. He suggested that the mechanism, by which the solubilization of bound enzymes results, might be the complex formation between borate ion $B(OH)_4^-$ and some polysaccharide associated with cell-wall materials, followed by the release of free enzymes due to the ionization of the polysaccharide-borate complexes by pH effects. HAWKER¹¹⁾ found that the grape berry enzyme termed insoluble invertase was solubilized mostly from the cell-wall fraction by the treatment with borate buffer (pH 8.5), Carbowax 4000, or non-ionic detergents, and that, when grape berry homogenates were prepared in the presence of Carbowax 4000, non-ionic detergents, or bovine serum albumin, almost all the total invertase activity was recovered in the soluble form. Then, he suggested that the insoluble invertase from grapes is an artifact of extraction caused by formation of a tannin-protein complex and/or a protein-tannin-cell wall complex, and that the grape invertase is probably located in the soluble form in the cytoplasm or vacuoles of cells.

In contrast to grapes, he¹¹⁾ showed that the insoluble invertase from aged carrot root disks could not be solubilized with the three kinds of extractants described above. Also, RICARDO and APREES¹⁷⁾ evidenced that neither of the four kinds of buffers, including sodium borate, could solubilized the cell-wall bound acid invertase from aged carrot disks, and postulated that some structural bond, different from that in the case of grapes, exists between the enzyme molecules and insoluble cell-wall fraction.

Consequently, these facts make it necessary to study in more detail the following subjects in near future works: characters of β -fructofuranosidase-cell wall bondings, mechanisms of enzyme solubilization and release, role of the enzymes of various types in carbohydrate metabolism in plants, and so on.

Summary

An increased activity of β -fructofuranosidases was observed in the onion slices aged by washing with tap water. Almost all the increased

enzyme activity was localized in cell wall debris fractions, from which two acid β -fructofuranosidases of bound type could be solubilized and released effectively with 0.5M NaCl solution. The released enzymes were partially purified by polyacrylamide- and Sephadex G-200- column chromatography and characterized. The enzymes I and II thus obtained, having specific activities of 15.4 U/mg protein and 23.1 U, respectively, showed different behaviours in solubility (I, water soluble; II, sparingly soluble) and in gel-electrophoresis (I, migrated to cathode at pH 4.3; II, not migrated). Both of the enzymes, however, specifically hydrolyzed β -fructofuranosidic linkages in sucrose (I and II, Km ca. 1.6 mM) and raffinose (I and II, Km ca. 3.9 mM) at the same optimum pH 4.5, but not hydrolyzed maltose, melibiose, and inulin. The enzymatic hydrolysis was inhibited by pCMB and HgCl₂.

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