DE NOVO BIOSYNTHESIS OF β-FRUCTOFURANOSIDASES IN AEROBICALLY AGED ONION DISKS

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Introduction

In almost all the studies on the enzyme development by the ageing (incubation) of plant tissue disks, intact disks or cell-debris fractions have been employed for the enzyme activity measurements and characterizations of the enzymes, because of the difficulty in solubilizing the cell-wall bound enzymes developed. As reported in a previous paper of this series[34], the present author could effectively solubilize and release the cell-wall bound β-fructofuranosidases from the cell-debris of onion disks with aqueous sodium chloride solutions, and found that this procedure is very useful for obtaining more detailed informations on the enzymes newly developed in aged tissue disks. This procedure was also used in the work described below.

The present paper deals with the development of β-fructofuranosidase activities in onion disks during aerobic incubation in the presence or absence of individual chemicals affecting the enzyme development. In some experiments, disc-electrophoretical examinations were employed to obtain a detailed knowledge of the enzyme development. Thus, the development of β-fructofuranosidases in onion disks was confirmed to be ascribed to *de novo* synthesis of the enzyme molecules in a similar manner as in other plant tissues.

Experimental

Materials and Methods

(1) Methods for Sample Preparation and Ageing

Onion bulbs (*Allium cepa* L. cult. var. Sapporo-Yellow) were obtained from commercial sources. The bulbs were separated into scales and cores with a knife. The cores were sliced on a microtome into slices 1 mm thick.

All the experiments described below were carried out aseptically.

After the disks of ca. 0.5 mm^2 punched from the slices were washed thoroughly with sterilized and deionized water, a poriton (25 g) of them was taken into a 200 ml-flask containing sterilized and deionized water (150 ml) or one of the sterilized solutions of test chemicals (150 ml) to incubate at 25°C for several periods under the aerobic conditions on a reciprocal type shaker. The medium in the flask was renewed at every 10 hours.

The incubated (or aged) disks were washed with water and homogenized thoroughly with a mixer and then in a mortar. The homogenate was repeatedly washed with water by centrifugation (1000 × g, 10 min.) to free from sugars. A portion (50 mg, dry basis) of the sedimented debris thus obtained was suspended in 0.5 M sodium chloride solution (10 ml), and still stood overnight in an ice box to solubilize enzymes. The filtrate (10 ml) from the debris suspension was used for the determination of β-fructofuranosidase activity.

(2) Assay for β-Fructofuranosidase Activity

After a mixture (final vol., 1 ml), composed of 0.02 M sucrose solution in the McIlvaine buffer (pH 4.5, 0.5 ml) and the enzyme solution (0.5 ml), was incubated at 37°C for 30 min., the enzymic reaction was terminated by addition of SOMOGYI-NELSON's reagent (1 ml), and the reducing sugars released from sucrose were measured by SOMOGYI-NELSON's method[35].

Enzyme activity 1 unit (1 U) was defined as the amount of enzyme hydrolyzing sucrose 1 μmole per minute at 37°C in 0.01 M sucrose solutions (pH 4.5).

(3) Disc Electrophoresis

Disc electrophoresis on polyacrylamide gel was carried out under the conditions of 2 mA/tube and migration for 2 to 3 hours according to the methods as reported by DAVIS[10] and REISFELD *et al.*[30]. To locate the positions of protein bands, the gel was once immersed in 1% Amido Schwarz solution in 7% (v/v) acetic acid aq., then washed with 7% (v/v) acetic acid aq. to visualize only the colored protein bands.

Results

I. Effects of Cut-widths of Disks on Developmept of β-Fructofuranosidase Activity by Ageing

Two kinds of experiments were conducted to examine the location of β-fructofuranosidase activities developed in the aerobically aged onion disks.

In the first experiment, the core part of onion bulbs was cut into the disks of 0.3–0.5, 1.0–1.5, 2.0–2.5, or 3.0–3.5 mm thick, respectively, and the disks thus prepared were incubated in water at 25°C for 5, 10, 24, 48, 72, or 98 hours, then the enzyme activities developed in the incubated disks were determined. The results showed that the thinner the disks were, the larger the enzyme activities per unit weight of the disks were (Fig. 1).

In the second experiment, the core part of onion bulbs was cut into 4×4×4-mm cubes, which were incubated in water at 25°C for 10 or 24 hours. The incubated cubes were separated into 1-mm thick slices and 2×2×2-mm cubes by slicing off their six exterior surface parts, and then, the slices and cubes thus obtained were assayed for β-fructofuranosidase activities, respectively. The enzyme activities of fresh, unincubated, disks

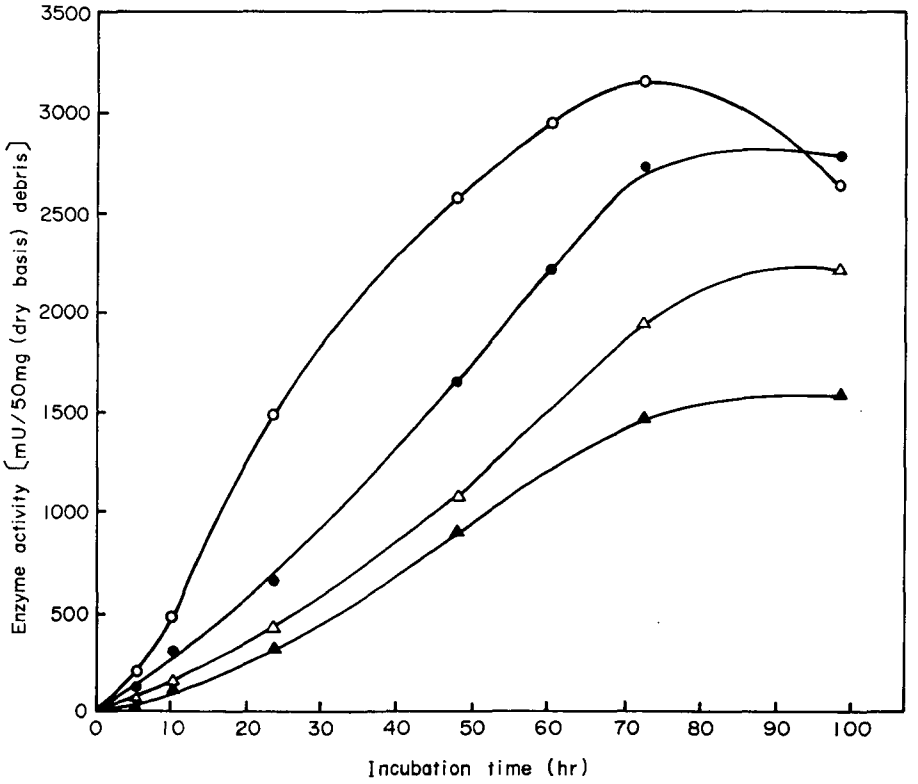


Fig. 1. Development of β-Fructofuranosidase Activity in Aged Onion Disks of Different Thickness

Thickness of disks: ○——○ 0.3–0.5 mm, ●——● 1–1.5 mm, △——△ 2–2.5 mm, ▲——▲ 3–3.5 mm.

Disks were incubated in water at 25°C.

were also measured. The enzyme activities per unit weight in the exterior part of the incubated disks were found to be larger than those in the fresh, unincubated, disks. However, the enzyme activities in the central part of the incubated disks were barely detected. These facts suggest that the β-fructofuranosidase development occurs predominantly in the exterior portion of the onion disks well aerated.

II. Effects of Chemical Substances on Development of β-Fructofuranosidase Activity

(1) Amino Acids and Their Analogs

Effects of amino acids and their analogs on the β-fructofuranosidase development were examined in use of L-proline, L-hydroxyproline, L-methionine, L-ethionine, DL-phenylalanine, DL-*p*-fluorophenylalanine, L-methionine sulfoximine, and others.

Prior to the main experiments, these amino acids and analogs were

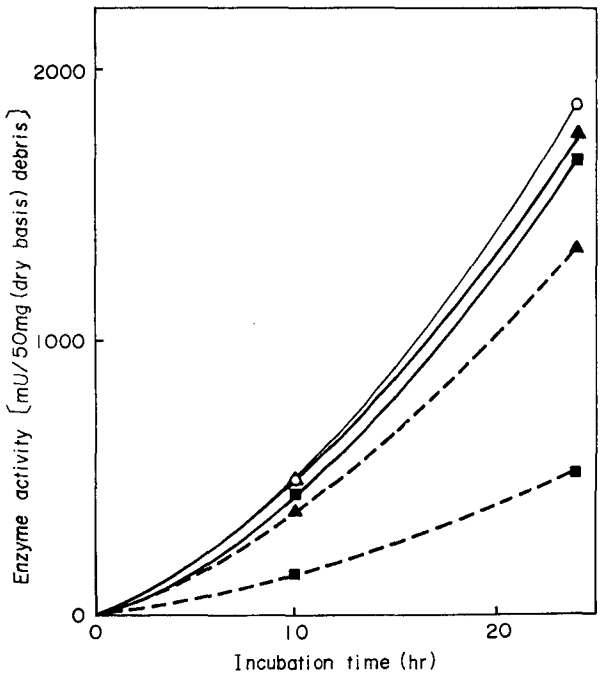


Fig. 2. Effects of Proline and Hydroxyproline on Development of β-Fructofuranosidase Activity

Disks of 1 mm thickness were incubated in water (O——O), or in L-proline (——) or L-hydroxyproline (- - -) solutions at 1 mM (■) or 0.1 mM (▲) concentrations.

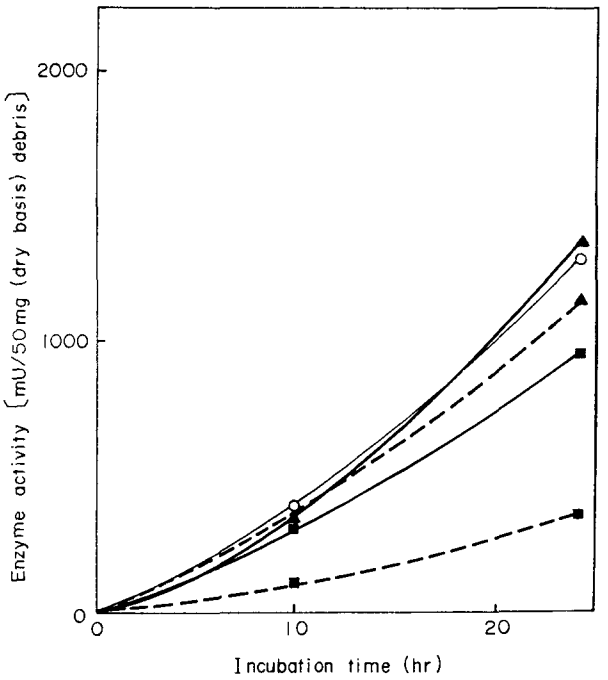


Fig. 3. Effects of Methionine and Ethionine on Development of β-Fructofuranosidase Activity

Disks of 1 mm thickness were incubated in water (○——○), or in L-methionine (——) or L-ethionine (- - -) solutions at 1 mM (■) or 0.1 mM (▲) concentrations.

ascertained to be ineffective to sucrose hydrolysis.

The enzyme development was unaffected by L-proline and DL-phenylalanine (in 1 mM and 0.1 mM solutions) even at a longer period of incubation for 24 hours (Figs. 2 and 4). Also, L-alanine, α-aminoisobutyric acid, and glutamic acid, not illustrated in the figures, gave the similar results.

Any inhibitory effects of L-methionine to the enzyme development were not observed in the concentration of 0.1 mM, but an appreciable inhibition of ca. 27.7% was observed after the 24-hours incubation in the case of 1 mM (Fig. 3).

In the concentration of 0.1 mM, L-hydroxyproline, DL-*p*-fluorophenylalanine, and L-ethionine gave the inhibitory effects of 27.5, 5.0, and 13.0%, respectively, and those in the 1 mM-concentration were 71.8, 72.9, and 72.8% (Figs. 2, 3, and 4). An inhibitor for glutamine metabolism, methionine sulfoximine, showed the largest inhibition of 90.0% in its 1 mM solution (Fig. 5).

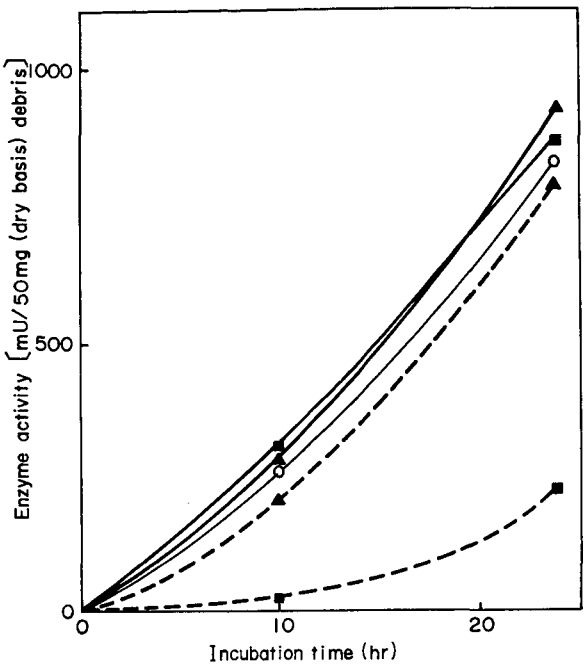


Fig. 4. Effects of Phenylalanine and *p*-Fluorophenylalanine on Development of β-Fructofuranosidse Activity

Disks of 1 mm thickness were incubated in water (○——○), or in DL-phenylalanine (——) or DL-*p*-fluorophenylalanine (- - -) solutions at 1 mM (■) or 0.1 mM (▲) concentrations.

The inhibitory effects to the enzyme development were also examined with use of the incubation mixtures containing both L-hydroxyproline and L-proline or both DL-*p*-fluorophenylalanine and DL-phenylalanine. The coupled two amino acids were added at the same time to the incubation mixtures until the predetermined concentrations were attained, and then the incubation was allowed to start.

The inhibition by L-hydroxyproline (1 mM concentration) was eliminated in the presence of L-proline in the final concentrations of 0.1 mM or 1 mM (Table 1). The inhibitory effect by DL-*p*-fluorophenylalanine (1 mM concentration) was completely abolished with the addition of DL-phenylalanine in the final concentration of 1 mM, but it was restored by 64% in the 0.1 mM concentration (Table 2).

(2) Sugars

At first, the measurements of β-fructofuranosidase activity were con-

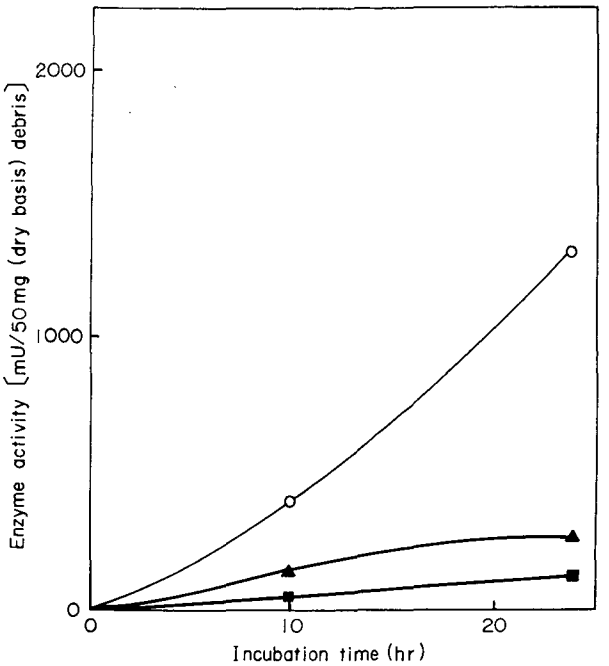


Fig. 5. Effects of Methionine Sulfoximine on Development of β-Fructofuranosidase Activity

Disks of 1 mm thickness were incubated in water (○——○), or in L-methionine sulfoximine (——) solutions at 1 mM (■) or 0.1 mM (▲) concentrations.

firmed to be undisturbed by any of the sugars used in the following experiments. Then, the effects of sugars on the enzyme development were examined. The onion disks were incubated in the sugar solutions of 0.05 M, which is equivalent to the actual concentration of reducing sugars

TABLE 1. Effects of Proline on Inhibition Produced by Hydroxyproline

Testing media	β-Fructofuranosidase activity [mU/50 mg (dry basis) debris]
Control (Water)	830
1 mM L-Hydroxyproline	516
1 mM L-Hydroxyproline+0.1 mM L-Proline	935
1 mM L-Hydroxyproline+1 mM L-Proline	926
0.1 mM L-Proline+0.1 mM L-Proline	840

Disks were incubated at 25°C for 24 hr. in the presence of one or two amino acids described in the Table.

TABLE 2. Effects of Phenylalanine on Inhibition Produced by *p*-Fluorophenylalanine

Testing media	β-Fructofuranosidase activity [mU/50 mg (dry basis) debris]
Control (Water)	650
1 mM DL-*p*-Fluorophenylalanine	190
1 mM DL-*p*-Fluorophenylalanine+0.1 mM DL-Phenylalanine	312
1 mM DL-*p*-Fluorophenylalanine+1 mM DL-Phenylalanine	632
1 mM DL-Phenylalanine+1 mM DL-Phenylalanine	710

Disks were incubated at 25°C for 24 hr. in the presence of one or two amino acids described in the Table.

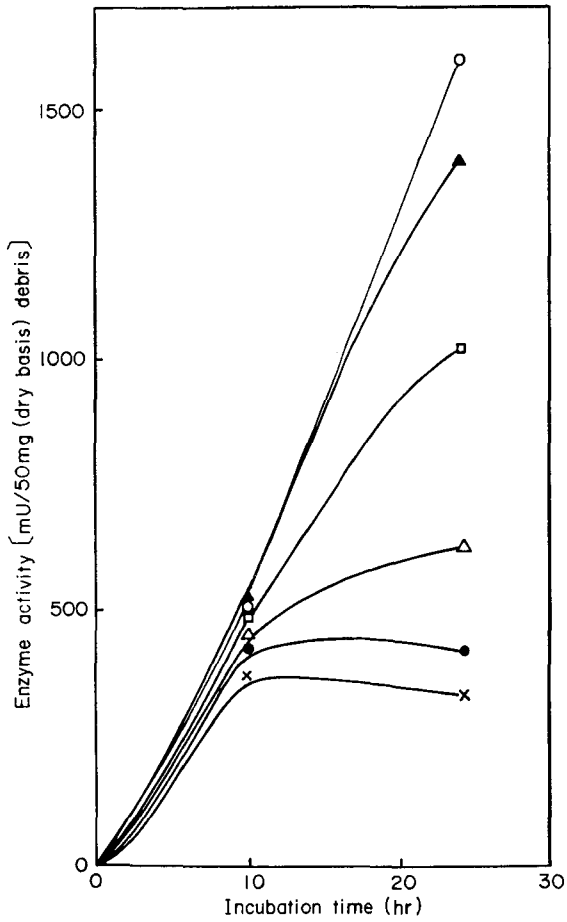


Fig. 6. Effects of Sugars on Development of β-Fructofuranosidase Activity

Disks were incubated at 25°C in the solutions containing one of the following sugars, respectively: ▲——▲, lactose; □——□, xylose; △——△, galactose; ●——●, maltose; ×——×, glucose; ○——○, control.

in onion bulbs.

The enzyme development was to varying extent inhibited by some of the sugars tested. Glucose, maltose, and galactose were highly active and lactose and xylose were moderately effective (Fig. 6). However, mannit, sorbit, and xylit (not illustrated in the figure) were not inhibitory.

In turn, quantities of glucose in the incubation media were tested for the β-fructofuranosidase development. After 24-hours incubations, the enzyme development was suppressed by 60.8, 79.1, 86.3, and 88.3% in the

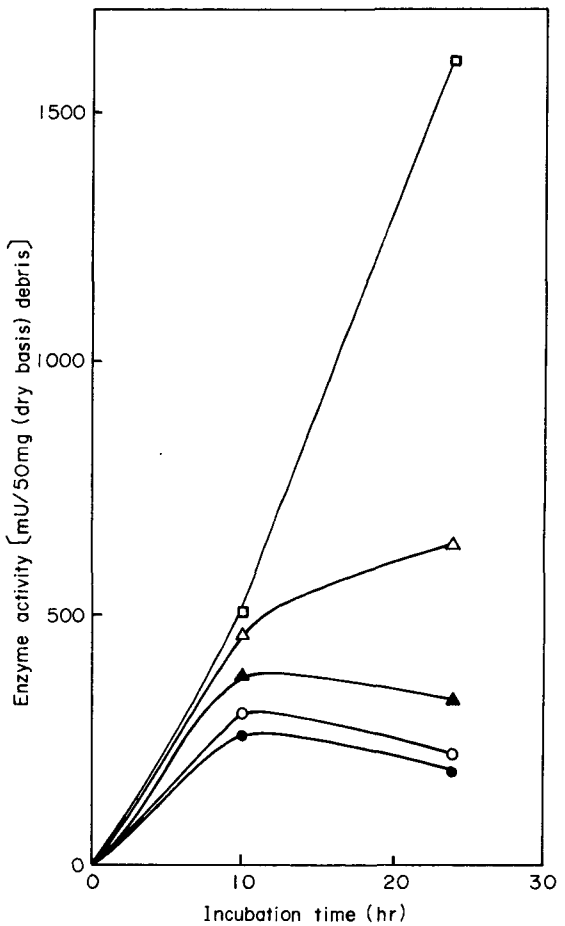


Fig. 7. Effects of Concentrations of Glucose on Development of β-Fructofuranosidase Activity

Disks were incubated at 25°C in the solutions containing glucose in the following concentrations, respectively: △——△, 10 mM; ▲——▲, 50 mM; ○——○, 100 mM; ●——●, 500 mM; □——□, control.

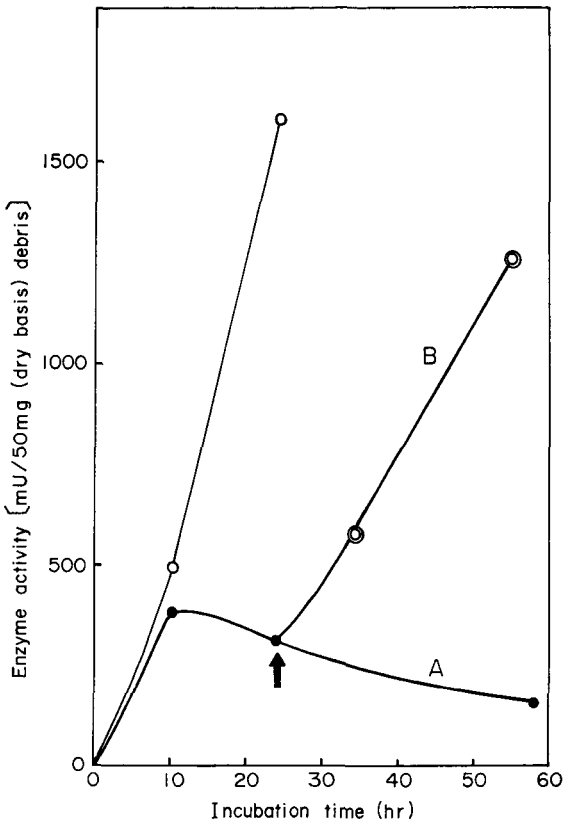


Fig. 8. Effects of Glucose on Development of β-Fructofuranosidase Activity

After disks were pre-incubated at 25°C for 24 hr. in a 50 mM glucose medium (●——●), one-half of the disks was allowed to incubate successively in the same medium (A) (●——●), while the other half was transferred to a water medium (↑) and incubated without glucose (B) (○——○). ○——○: control (water medium).

glucose solutions of 10, 50, 100, and 500 mM, respectively (Fig. 7). When the disks pre-incubated in the 50 mM glucose solution were transferred to water medium and continued to incubate in it, the enzyme development was restored (Fig. 8).

(3) Antibiotics

Effects of some inhibitors specific for protein synthesis, such as cycloheximide, blasticidin-S, and chloramphenicol, were studied on the β-fructofuranosidase development in the aged onion disks. Prior to the main

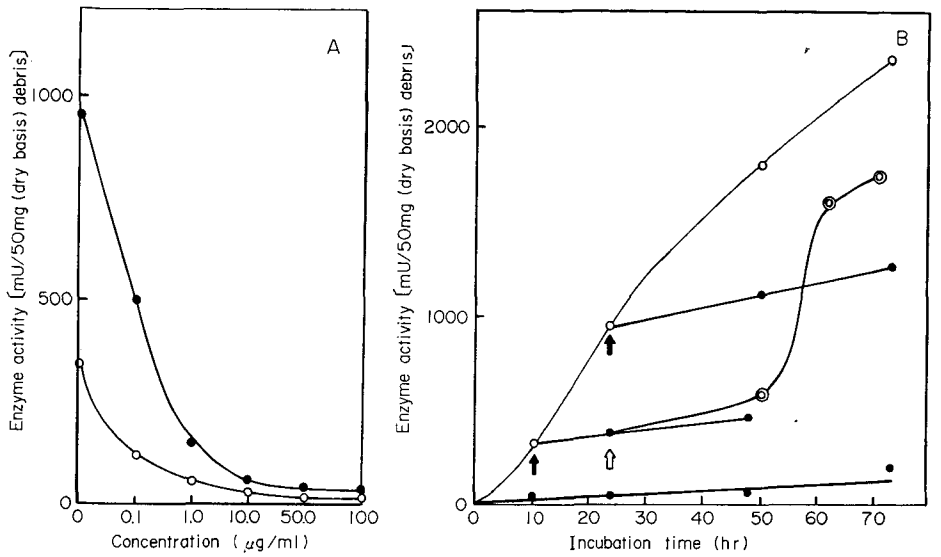


Fig. 9. Effects of Cycloheximide on Development of β-Fructofuranosidase Activity

A: Disks were incubated at 25°C for 10 and 24 hr. in cycloheximide solutions of various concentrations.
●——●, 24-hr. incubation; ○——○, 10-hr. incubation.

B: Arrows (↑) indicate transfer of disks from a water medium to a cycloheximide solution (10 μg/ml). Another arrow (⇧) indicates re-transfer of disks from a cycloheximide solution (10 μg/ml) to a water medium.
○——○ and ◎——◎, incubation in water; ●——●, incubation in cycloheximide solution.

experiments, it was confirmed that these antibiotics did not interfere the assays for enzyme activity.

In the concentration of 0.1 μg/ml, cycloheximde inhibited the enzyme development by ca. 50% at the incubation for 24 hours and by ca. 60% at 10 hours, respectively. In the concentration range from 10 to 100 μg/ml, this substance blocked almost completely the enzyme development (Fig. 9–A). When the disks pre-incubated in water for 10 or 24 hours were transferred to cycloheximide solutions (10 μg/ml) to incubate successively, the enzyme development was definitely retarded. However, when the disks thus incubated were again transferred to the other water media without cycloheximide to re-incubate, the enzyme development was re-started (Fig. 9–B).

Also, blasticidin-S almost perfectly blocked the β-fructofuranosidase

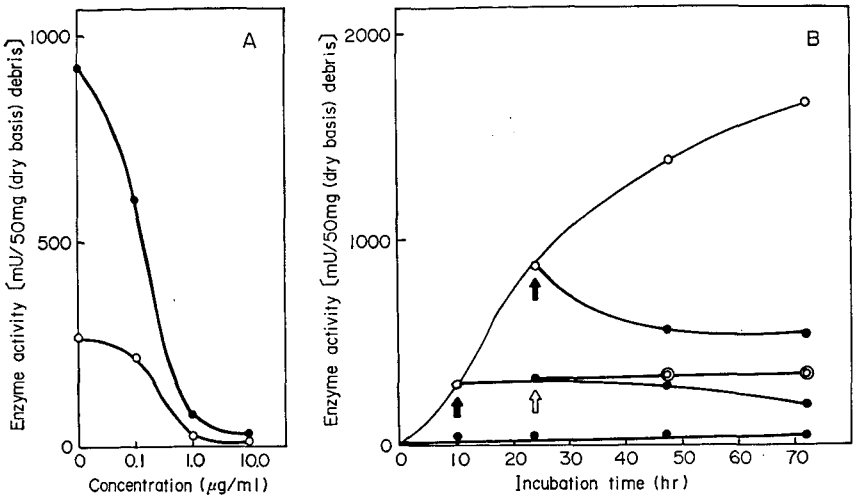


Fig. 10. Effects of Blasticidin-S on Development of *β*-Fructofuranosidase Activity

A: Disks were incubated at 25°C for 10 and 24 hr. in blasticidin-S solutions of various concentrations.
●——●, 24-hr. incubation; ○——○, 10-hr. incubation.

B: Arrows (⇑) indicate transfer of disks from a water medium to a blasticidin-S solution (10 μg/ml). Another arrow (⇧) indicates re-transfer of disks from a blasticidin-S solution (10 μg/ml) to a water medium.
○——○ and ◎——◎, incubation in water; ●——●, incubation in blasticidin-S solution (10 μg/ml).

development in its concentrations over 1 μg/ml (Fig. 10–A), and this inhibitory effect was irreversible (Fig. 10–B).

In the other experiments not shown in the figures, chloramphenicol solutions (6 mM) were found to be inhibitory for the enzyme development by 61.0% in the incubation for 10 hours and by 42.6% for 24 hours, respectively.

(4) Adenosine Triphosphate and 2, 4-Dinitrophenol

Adenosine triphosphate (ATP), one of the high-energy phosphates essential for protein synthesis, and 2, 4-dinitrophenol (2, 4-DNP), an uncoupling agent in the oxidative phosphorylation from adenosine diphosphate to ATP, were used to experiment their effects on the enzyme development. Prior to the main experiments, it was confirmed that the assays for *β*-fructofuranosidase activity were not interferred by these two substances.

In the incubations for 24 hours in use of 1 mM solutions of the testing chemicals, ATP stimulated 1.6-fold the enzyme development (Fig. 11–A),

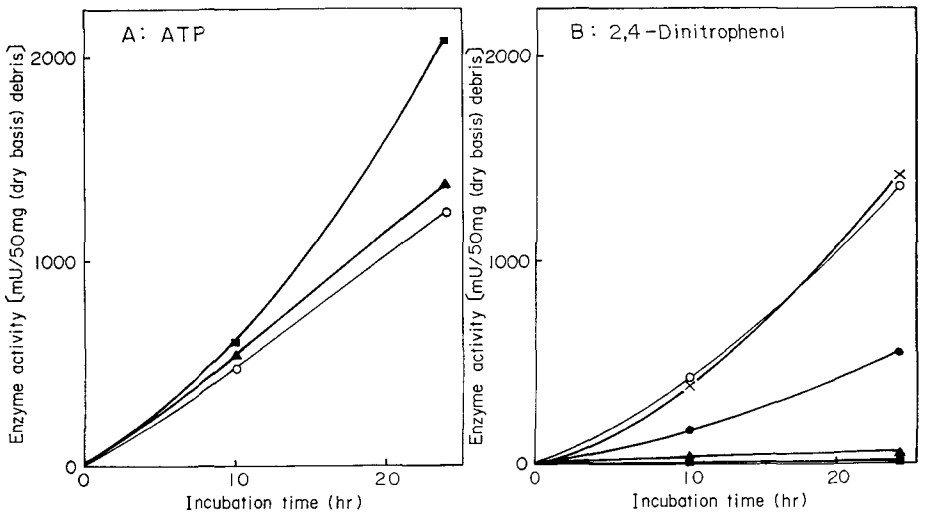


Fig. 11. Effects of ATP and 2, 4-Dinitrophenol on Development of β-Fructofuranosidase Activity

A: Disks were incubated in a water medium without ATP (○——○), or in 1 mM (■——■) or 0.1 mM (▲——▲) ATP solutions.

B: Disks were incubated in the media containing 2,4-dinitrophenol. ×——×, 0.01 mM; ●——●, 0.05 mM; ▲——▲, 0.1 mM; ■——■, 1 mM 2,4-dinitrophenol solutions; ○——○, control (water medium).

while 2, 4-DNP inhibited it by ca. 100% (Fig. 11-B). In the other studies, the competition between ATP and 2, 4-DNP for the enzyme development was experimented. It was found that the inhibition appeared in 0.05 mM 2, 4-DNP solutions could not be restored by an additional re-incubation in 1 mM ATP solutions. However, more detailed experiments on this subject must be made in future studies.

III. Electrophoretic Examinations for Effects of Chemical Substances on Development of β-Fructofuranosidase Activity

The responses of the onion disks to some chemicals in the β-fructofuranosidase development were examined electrophoretically. The enzyme preparations obtained from the disks incubated in the presence of respective chemicals were run electrophoretically on polyacrylamide gel, and then protein bands on the gel were inspected for their locations and assayed for β-fructofuranosidase activities.

In a similar way as described in "Methods for Sample Preparation and Ageing", core part of the onion bulbs was cut into disks (thickness 1 mm, area 0.5 cm^2) and incubated in the presence or absence of chemicals.

The disks incubated (100 g) for 72 hours were thoroughly homogenized with cold water in a mixer and in a mortar. The resulted homogenate was washed repeatedly with distilled water, and then with 0.2 M sodium chloride solution by centrifugation (1000 × g, 10 min.). The sedimented debris was extracted twice with 0.5 M sodium chloride solution (250 ml). The combined extract was concentrated to 2 ml with a collodion bag and centrifuged (10,000 × g, 15 min.). The supernatant (enzyme preparation) was electrophorized on polyacrylamide gel to examine for protein patterns by a similar method as described in "Disc Electrophoresis". Each of the protein bands on the gel was cut into slices and homogenized in the McIlvaine buffer (pH 4.5). After still-standing in an ice box, the homogenate was centrifuged (10,000 × g, 15 min.) and the supernatant was assayed for β-fructofuranosidase activity.

(1) **Blasticidin-S**

Fig. 12 shows disc-electrophoretic protein patterns of the enzyme preparations from the disks aged in the presence or absence of blasticidin-S. In the enzyme preparations from the fresh, not aged, disks or from the disks aged for 72 hours in an aqueous medium with blasticidin-S (10 μg/ml), three major protein bands were equally detected on the gel. None of them gave any significant β-fructofuranosidase activity. However, in the enzyme preparation from the disks aged without blasticidin-S, an additional, slowly migrating, band was clearly detected and this protein was proved to be active for β-fructofuranosidase.

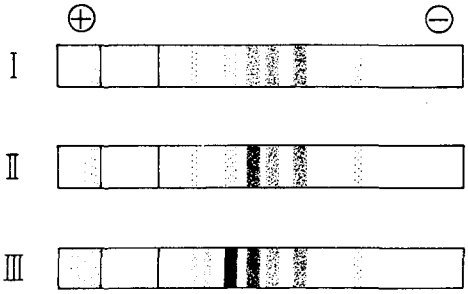


Fig. 12. Electrophoretic Protein Patterns of β-Fructofuranosidase Preparations on Polyacrylamide Gel

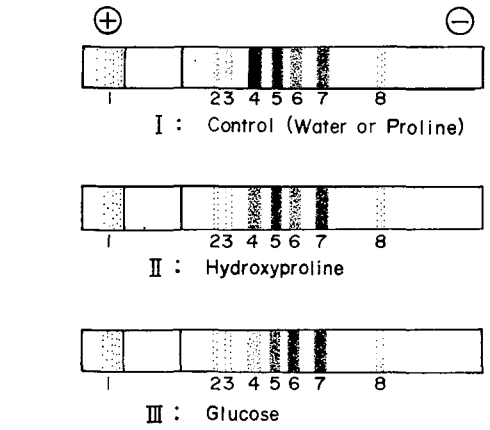
Disc electrophoresis was carried out on pH 4.3 polyacrylamide gel at 2 mA/tube for 2.5 hr..

Preparations of β-fructofuranosidase are as follows:

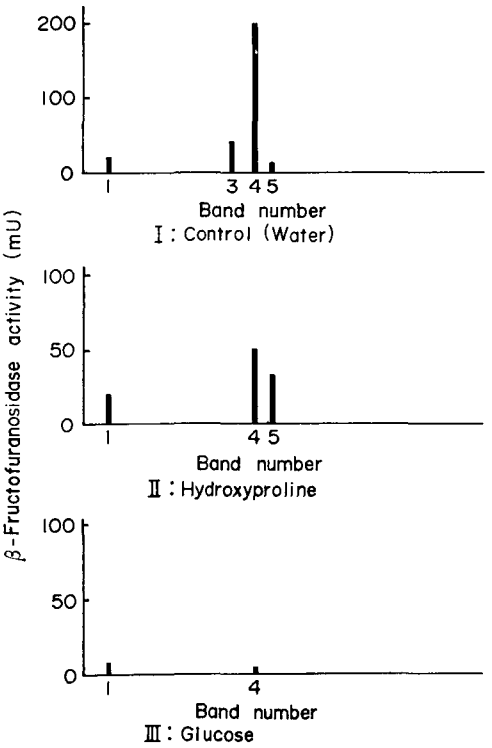
I: from fresh, not aged, disks.

II: from aged disks (incubated in the presence of blasticidin-S).

III: from aged disks (incubated in the absence of blasticidin-S).



(1) Electrophoretic Protein Patterns



(2) Distribution of β-Fructofuranosidase Activity

Fig. 13. Electrophoretic Protein Patterns and Activity Distribution of β-Fructofuranosidase Preparations on Polyacrylamide Gel

Preparations of β-fructofuranosidase were obtained from the disks aged (incubated) for 72 hr.:

I: in water medium or in 1 mM proline solution. (Both enzyme preparations gave indistinguishable protein patterns.)

II: in 1 mM hydroxyproline solution.

III: in 50 mM glucose solution.

It was suggested from these results that the aerobic ageing of onion disks induces *de novo* synthesis of the active protein(s) of β-fructofuranosidase, and that the biogenesis of enzyme is inhibited by blasticidin-S.

(2) Hydroxyproline and Glucose

In Figs. 2. and 6, it has been already shown that the development of β-fructofuranosidase activity was affected by hydroxyproline or glucose. Again, such a phenomenon was disc-electrophoretically investigated (Fig. 13).

The protein patterns of β-fructofuranosidase preparations obtained from the disks aerobically aged in the presence of hydroxyproline or glucose were almost identical with that of the enzyme from the disks aged in an aqueous medium, and four major protein bands (No. 4, 5, 6 and 7) were equally detected on the electrophoretograms.

A considerably higher β-fructofuranosidase activity was found in No. 4 band than in the others. Among No. 4 bands, the highest activity was detected in the control enzyme preparation and about a quarter of this activity was measured in the preparation from hydroxyproline treatment. The preparation from glucose treatment, however, showed only a extremely lower activity. These facts allowed to reconfirm the results illustrated in Figs. 2 and 6.

Discussion

Until now, it has been reported that aerobic incubation (ageing) of the disks from storage tissues of higher plants induces increases in respiration[1,8,9,18,22,24,29,32,33,36~38], mitochondria numbers[4,21], RNA synthesis[7,20,41], amino acid incorporation into protein molecules[8,9,23,31,42], and activities of various enzymes[13,16,17,25~28], or results in changes of metabolic pathways[2,3,7,19,31].

With respect to increases of the activities of carbohydrate metabolizing enzymes, or β-fructofuranosidases (invertases), in the aged tissue disks, MACDONALD and DEKOCK (1958)[24] found for the first time a positive relation between respiratory drifts and increased concentrations of reducing sugars, and they suggested the presence of enhanced invertase activities in the disks. Thereafter, the increased invertase activities were repeatedly observed by MACDONALD, BACON, and VAUGHAN in the roots of sugar beet[5,6,40], red beet[6,23,39,41], turnip[6], carrot[6,39], and chicory[6], and in the tubers of potato[39], dahlia[6], and Jerusalem artichoke[6]. The similar findings were also obtained in Jerusalem artichoke tubers by EDELMAN and HALL[11,12,13], in beet roots by PALMER[29], and in the stems of immatured sugar cane by GLASZIOU *et al.*[6].

From the incubation experiments of tissue disks in the presence of inhibitors for protein synthesis[11,13,23,41], it was suggested that the increases of invertase activities are not derived from activation of inactive form of the enzymes but from novel synthesis of the enzyme molecules; and also the newly developed invertases in red beet, potato, and carrot were found to exist in both cell wall and soluble fractions[39,40], but that in Jerusalem artichoke only in the former fraction[11].

In almost all the studies described above, intact aged disks or cell-debris fractions, instead of free enzymes, were employed for assaying the enzyme activities. The present author, however, used the enzymes solubilized and freed from the cell-debris fractions, because adoption of the latter method was proved to be advantageous not only for measuring the enzyme activities but also for electrophoretically examining the properties of the enzymes.

At first it was ascertained that aeration is indispensable for the β-fructofuranosidase development by the incubation of onion disks, because larger levels of enzyme activities were detected on the well-aerated surface layers of the disks (Fig. 1).

Then, L-hydroxyproline, DL-*p*-fluorophenylalanine, and L-ethionine were found to be inhibitory for the development of β-fructofuranosidase activities (Figs. 2, 3, and 4). The inhibition with L-hydroxyproline (1 mM) was completely reversed in the presence of L-proline (1 mM and 0.1 mM) (Table 1). The inhibitory effect of DL-*p*-fluorophenylalanine (1 mM), however, was antagonized perfectly in the presence of DL-phenylalanine (1 mM) but imperfectly in that of 0.1 mM concentration (Table 2).

On electrophoretograms, three kinds of enzyme preparations, obtained from the disks incubated in the media containing water alone, proline, or hydroxyproline, respectively, gave similarly four identical major protein bands (Fig. 13). Among these four bands, the highest β-fructofuranosidase activity was found in the most slowly migrating band, whose enzyme activities were then compared with each other among the three enzyme preparations. The activity of the enzyme from the disks incubated in hydroxyproline solution was found to be about a quarter of those from the incubations in water alone or proline solution. Thus, it was speculated from these facts that hydroxyproline is mis-incorporated, competitively with proline, into protein to form the enzyme molecules having comparatively lower activities. Any informations, however, were not obtained whether or not the complete hydroxyproline replacement results in perfect disappearance of the enzyme activities, because all of the three enzyme

preparations described above gave almost the same electrophoretic patterns and any specific protein bands derived from the hydroxyproline incubation were not observed.

Thirdly, such inhibitors for protein synthesis as chloramphenicol, blasticidin-S, and cycloheximide were proved to be inhibitory for the β-fructofuranosidase development in the aerobic incubation of onion disks. The inhibitory effects were larger in the latter two than in the former (Figs. 9 and 10). On disc-electrophoretograms, the enzyme preparation obtained from the disks incubated in the absence of blasticidin-S gave four major protein bands, among them only the most slowly migrating one was significantly active for β-fructofuranosidase. Contrary to this, the slow and β-fructofuranosidase active band was scarcely detected in the enzyme preparations derived from the fresh and un-incubated disks or from the incubated ones in the presence of blasticidin-S (Fig. 12). These facts show that blasticidin-S inhibits the development of β-fructofuranosidase molecules.

The findings as mentioned above made it possible to conclude that the β-fructofuranosidase development in onion disks by aerobic incubation is derived from *de novo* synthesis of the enzyme molecules in an analogous manner as in other plants.

Summary

When the disks prepared from the core part of onion bulbs were aerobically aged (incubated) with water, an increased β-fructofuranosidase activity was observed in cell debris fraction, especially in those from the exterior portions of the disks well aerated. Effects of some chemicals on the development of enzyme activity were investigated and the following results were obtained: (1) The enzyme activity development was decreased to varying extent in the presence of glucose, maltose galactose, xylose, or lactose, and the largest effect was exerted by glucose; (2) The activity development was depressed to a considerably larger extent with hydroxyproline, *p*-fluorophenylalanine, ethionine, or methionine sulfoximine. These depressions were suggested to be due to the competitive inhibitions of these amino acids to the incorporation of proline, phenylalanine, or methionine into enzyme molecules, respectively; (3) Since the activity development was inhibited by antibiotics such as cycloheximide, blasticidin-S, or chloramphenicol, it was suggested that increase of the enzyme activity is originated from formation of enzyme proteins; (4) The activity development was suppressed by 2, 4-dinitrophenol, whereas stimulated by adenosine tri-

phosphate; (5) The novel formation of β-fructofuranosidase-active protein(s) was disc-electrophoretically confirmed in the examinations of the enzyme specimens obtained from the disks aged in the presence or absence of blasticidin-S, hydroxyproline, or glucose. From these experimental results, it was established that the development of β-fructofuranosidase activity in the aged onion disks is originated from *de novo* biosynthesis of enzyme proteins.

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