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# PURIFICATION AND PROPERTIES OF α-L-ARABINOFURANOSIDASE FROM PLANT *SCOPOLIA JAPONICA* CALLUSES

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## Summary

α-L-Arabinofuranosidase (α-L-arabinofuranoside arabinofuranohydrolase, EC 3.2.1.55) from the culture medium of *Scopolia japonica* calluses was partially purified.

Various properties of the enzyme were studied and the effects of lactones on the activity were determined.

## Introduction

We observed α-L-arabinofuranosidase (α-L-arabinofuranoside arabinofuranohydrolase, EC 3.2.1.55) activity in leaf, stem, and root preparations of a plant, *Scopolia japonica*, in addition to the suspension culture medium of the calluses of the plant. This is the first case to report the presence of this enzyme in any culture medium of plants.

L-arabinose-containing polysaccharides and glycoproteins are known to be present in plant cell walls [1—3] and the elucidation of the structure of these biopolymers should contribute to an understanding of the physiology of plant cells. Chemical methods have usually been used to determine the fine structure of the carbohydrate moiety, but for making more specific analysis of the carbohydrate component the use of glycosidases under milder conditions is necessary. α-L-arabinofuranosidase is a particularly useful enzyme for studies of the structure of these biopolymers. Although properties of α-L-arabinofuranosidases from bacteria [4] and fungi [5,6] have so far been studied, these enzymes were not used for structural studies of arabinose-containing molecules.

The present paper describes the purification of the enzyme from plant cells and some of its properties.

## Experimental

### *Materials*

*S. japonica* calluses were the generous gift of Dr. Hashimoto, Saitama Uni-

versity. L- and D-arabono-1,4-lactone were obtained from Koch-Light laboratories Ltd., England. L- and D-galactono-1,4-lactone were obtained from Sigma Chemicals Co., U.S.A. Sephadex G-150 and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals Co., Sweden. Catalase (beef liver, mol. wt. 240 000), aldolase (rabbit muscle, 158 000), bovine serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen A (bovine pancreas, 25 000) and cytochrome *c* (horse heart, 12 500) were purchased from Boehringer Mannheim Biochemicals Co., West Germany.

*Enzyme substrates.* *p*-Nitrophenyl-α-L-arabinofuranoside and *p*-nitrophenyl-α-L-arabinopyranoside were synthesized according to Fielding et al. [7]. Other *p*-nitrophenylglycosides were purchased from Seikagaku-Kogyo Co., Japan. Phenyl-β-L- and D-arabinopyranoside were purchased from Nakarai Chemicals Co., Japan.

*Methods*

*Enzyme assays.* *p*-Nitrophenyl-α-L-arabinofuranoside and other *p*-nitrophenylglycosides were used as substrates for enzyme assays. Enzyme samples were incubated at 37°C in 50 mM sodium citrate/phosphate buffer (pH 4.8) containing 1 mM substrate in final volume of 300 μl. After a known time 2 ml of 1 M $Na_2CO_3$ were added and the absorbance of the liberated *p*-nitrophenol was measured at 420 nm. A unit of enzyme was defined as the amount which would liberate 1 μmol of *p*-nitrophenol/min. The specific activity was expressed as enzyme units/mg of protein. The protein determinations were made by the method of Lowry et al. [8], using crystalline bovine serum albumin as a standard.

*Culture of plant calluses.* The calluses of *S. japonica* were cultured in suspension in Murashige's and Skoog's medium [9] containing 5% sucrose/0.5% casamino acid/1 ppm 2,4-dichlorophenoxyacetic acid at 27°C in the dark, using a rotary shaker at 120 rev./min.

*Procedure of purification of α-L-arabinofuranosidase.* The purification procedure was carried out at 4°C unless otherwise specified.

Step 1. Ultrafiltration. The 21st-day culture medium (12 l) was harvested by centrifugation at 16 000 × *g* for 20 min, concentrated and dialyzed against 50 mM sodium citrate/phosphate buffer (pH 7.0) with a Diaflo PM-10 membrane (Amicon Corp., U.S.A.).

Step 2. Heat treatment. The enzyme solution (protein, 3.7 mg/ml) was heated at 55°C for 20 min, and then was centrifuged at 46 000 × *g* for 1.5 h. The supernatant was lyophilized.

Step 3. $(NH_4)_2SO_4$ fractionation. The lyophilized sample (protein, 464 mg) was solubilized in 50 ml of 10 mM sodium citrate/phosphate buffer (pH 6.5). The solution was brought to 40% saturation at 0°C with solid $(NH_4)_2SO_4$ and centrifuged at 5700 × *g* for 20 min. The supernatant was further brought to 70% saturation at 0°C with $(NH_4)_2SO_4$ and again centrifuged at 5700 × *g* for 20 min. The precipitate thus formed was dissolved in 10 mM sodium citrate/phosphate buffer (pH 6.5)/0.2 M NaCl, and the resulting solution was dialyzed against the same buffer and concentrated with a Diaflo PM-10 membrane.

Step 4. Gel filtration on Sephadex G-150. A 5.0-ml portion (protein, 28 mg) of the enzyme solution was applied to a column (1.8 × 125 cm) of Sephadex

G-150, equilibrated with 10 mM sodium citrate/phosphate buffer (pH 6.5)/0.2 M NaCl. The column was eluted with the buffer and the eluate was collected. The fractions containing α-L-arabinofuranosidase were pooled and concentrated.

Step 5. Chromatography on DEAE-Sephadex. A column (1.2 × 20 cm) of DEAE-Sephadex A-50 was equilibrated with 10 mM sodium citrate/phosphate buffer (pH 7.5). A portion (protein, 15 mg) of the enzyme solution, previously dialyzed against the same buffer, was applied to the column and eluted with the same buffer. The non-retarded fraction was dialyzed against distilled water and concentrated.

Step 6. Isoelectric focusing fractionation. A column (LKB 8101) of 1.5% carrier ampholyte with pH range 6—9 (LKB Produkter AB, Sweden) was prepared by mixing ampholytes of pH range 6—8 and 7—9 with a linear gradient 0—47% of sucrose. Electrophoresis of the enzyme solution (protein, 3.0 mg) was carried out at 900 V for 92 h. After electrophoresis, the column was fractionated and then the pH, absorbance at 280 nm and enzyme activity were measured.

## Results

### *Occurrence of enzyme*

The distribution of α-L-arabinofuranosidase was examined in the 21st-day cultured cells of *S. japonica* calluses and the culture medium (Table I). The enzyme was mainly localized in the culture medium. The time course of the appearance of enzymic activity in the culture medium was studied with the growing callus cells (Fig. 1). The enzymic activity in the medium began to decrease after the growth of the calluses reached a plateau. Since the enzyme was relatively stable (see below), it was considered that this decrease may be due to proteases secreted from the cells. The 21st-day cultured medium in the logarithmic phase of the growth was harvested to minimize the effect of degrading enzymes and activities of several glycosidases were determined in the

TABLE I

DISTRIBUTION OF α-L-ARABINOFURANOSIDASE IN THE CULTURE MEDIUM AND INTRACELLULAR FRACTIONS OF *S. JAPONICA*

The suspension (200 ml) of the 21st-day culture of *S. japonica* calluses was filtered to separate the cells from the medium. The cells were homogenized in 50 mM sodium citrate/phosphate buffer (pH 7.0). The homogenate was centrifuged at 480 × *g* for 20 min, followed by collecting and suspending the precipitates in the buffer (fraction containing cell walls). Meanwhile, the supernatant was centrifuged at 27 000 × *g* for 30 min to obtain the supernatant (fraction containing cell sap) and the precipitates (fraction containing intracellular particles). And then, the enzymic activity was determined in each fraction, as described in Methods.

| Fraction | Total activity (units per 200 ml) | % |
|---|---|---|
| Cell walls | 0.150 | 16.9 |
| Intracelular particles | 0.014 | 1.6 |
| Cell sap | 0.149 | 16.8 |
| Extracellular medium | 0.575 | 64.7 |

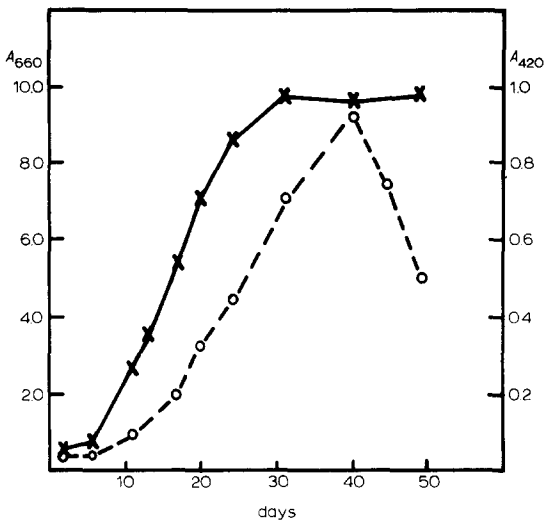


Fig. 1. The growth curve of *Scopolia japonica* calluses cultured in suspension and the activity of α-L-arabinofuranosidase secreted in the medium. *Scopolia japonica* calluses were cultured in suspension as described in Methods. An alqiuot of the suspension was taken out of the culture flask under a sterilized condition at adequate intervals. The cell growth was expressed by the turbidity measured at 660 nm (X) instead of counting actual cell number. The open circles (○) represent the enzymic activity.

medium harvested by centrifugation at 16 000 × *g* for 20 min (Table II). β-Galactosidase, α-L-arabinofuranosidase and α-mannosidase showed relatively high activities.

In addition, we detected α-L-arabinofuranosidase activities also in root, stem and leaf of *S. japonica* at the flowering time. Each tissue of the plant was sterilized with 70% ethanol and 10% hydrogen peroxide. Then it was homogenized in 10 mM sodium citrate/phosphate buffer (pH 6.0), followed by measuring the enzymic activity and the amount of protein. The specific activity of the enzyme observed in stem was found to be about 3 times higher than that in other tissues.

TABLE II

VARIOUS GLYCOSIDASES IN THE CULTURE MEDIUM OF *S. JAPONICA* CALLUSES

The suspension of *S. japonica* calluses cultured in suspension for 21 days was centrifuged at 16 000 × *g* for 20 min. An aliquot of the resulting supernatant was incubated with various *p*-nitrophenylglycosides at 37°C at pH 4.5 for adequate periods, followed by measuring the absorbance of liberated *p*-nitrophenol at 420 nm, as described in Methods.

| Glycosidases | Total activity (units/l of the medium) | Relative activity |
|---|---|---|
| α-L-arabinofuranosidase | 4.42 | 100 |
| α-L-arabinopyranosiase | 0.285 | 6.45 |
| α-Galactosidase | 0.274 | 6.20 |
| β-Galactosidase | 5.94 | 134 |
| α-Glucosidase | 0.063 | 1.4 |
| β-Glucosidase | 0.037 | 0.84 |
| α-Mannosidase | 0.775 | 17.5 |
| β-Mannosidase | 0.011 | 0.25 |
| α-L-fucosidase | 0.026 | 0.59 |

*Purification of enzyme*

After ultrafiltration, the enzyme solution was heated at 55°C for 20 min. In this procedure, no apparent loss of α-L-arabinofuranosidase activity was observed, but 97% of β-galactosidase activity was lost.

The enzyme preparation was carried in the following sequences; $(NH_4)_2SO_4$ fractionation, gel filtration on Sephadex G-150 (Fig. 2), chromatography on DEAE-Sephadex, and finally, isoelectric focusing fractionation (Fig. 3). The isoelectric point (p*I*) of the main fraction of α-L-arabinofuranosidase was 8.0 and those of the minor fractions were 6.0 and 5.7. Glycosidase activity contained in the isoelectric focusing fractions is shown in Table III. The most purified enzyme preparation (p*I* 8.0) contained small amounts of α-mannosidase, α-galactosidase, β-glucosidase and α-L-arabinopyranosidase, but no β-galactosidase. In the minor fraction (p*I* 6.0), α-mannosidase was predominant, although it still contained small amounts of other glycosidases including β-galactosidase.

The results of a purification are summarized in Table IV.

*Properties of α-L-arabinofuranosidase*

The main fraction (p*I* 8.0) obtained by the isoelectric focusing fractionation was used as the sample of α-L-arabinofuranosidase unless otherwise specified.

*Heat stability of α-L-arabinofuranosidase.* The heat stability of α-L-arabinofuranosidase was compared with that of β-D-galactosidase from the same source. α-L-Arabinofuranosidase was more stable than β-galactosidase.

*Effect of pH.* Effect of pH on the enzymic activity was studied, using 10 mM acetate buffer (pH 4.0—5.5), 10 mM sodium citrate/phosphate buffer (pH 2.5—8.0) and 10 mM Veronal-HCl buffer (pH 7.0—9.5). The enzyme was active covering the range pH 3.0—7.5 with the optimum pH 4.8. The remaining activ-

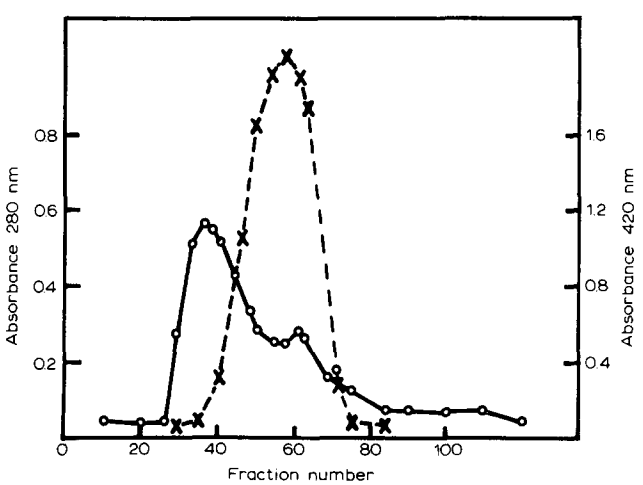


Fig. 2. Gel filtration of α-L-arabinofuranosidase on Sephadex G-150. The enzyme solution (protein, 28 mg/5.0 ml) in 10 mM sodium citrate/phosphate buffer (pH 6.5)/0.2 M NaCl after $(NH_4)_2SO_4$ fractionation was applied on a Sephadex G-150 column (1.8 × 125 cm) equilibrated with the same buffer. The column was eluted with the buffer at a flow rate of 14.3 ml/h. The eluate was collected in 5.0-ml fractions. The dotted line shows enzymic activity and the solid line the absorbance at 280 nm of protein.

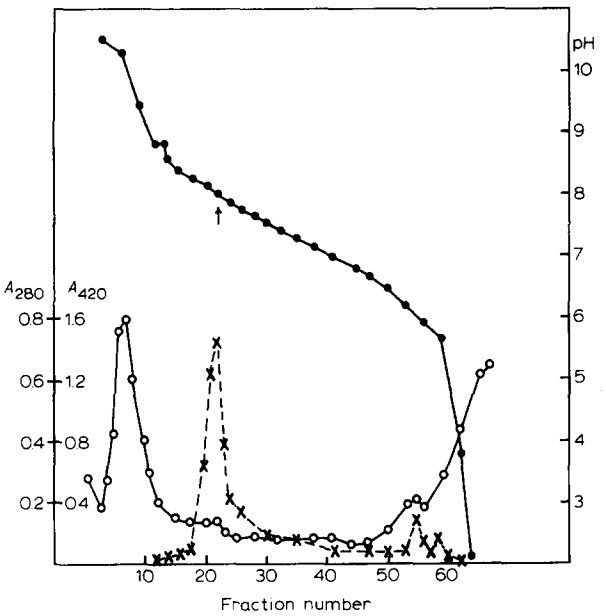


Fig. 3. Isoelectric focusing fractionation of α-L-arabinofuranosidase. The enzyme solution (protein, 3.0 mg) after the column chromatography on DEAE-Sephadex was electrophoresed in a column with pH gradient of 6—9 prepared with 1.5% carrier ampholyte. After the separation of the content of the column into 1.94 ml fractions, the activity of α-L-arabinofuranosidase (×), pH (•) and the absorbance at 280 nm of protein (○) was measured.

TABLE III

GLYCOSIDASE ACTIVITIES CONTAINED IN ISOELECTRIC FOCUSING FRACTIONS

The main fraction (p*I* 8.0) and the minor fraction (p*I* 6.0) of the isoelectric focusing fractionation were pooled and dialyzed against 10 mM sodium citrate/phosphate buffer (pH 5.5). Each glycosidase activity was measured as described in Methods.

| Glycosidases | Relative activity | |
|---|---|---|
| | Main fraction (p*I* 8.0) | Minor fraction (p*I* 6.0) |
| α-L-arabinofuranosidase | 100 | 100 |
| α-L-arabinopyranosidase | 6.9 | 7.0 |
| α-Galactosidase | 1.0 | 4.8 |
| β-Galctosidase | 0 | 8.2 |
| α-Glucosidase | 0 | 0 |
| β-Glucosidase | 1.4 | 7.2 |
| α-Mannosidase | 2.1 | 358 |
| β-Mannosidase | 0 | 0 |
| α-L-fucosidase | 0 | 0 |
| β-L-arabinopyranosidase * | 0 | n.d. ** |
| β-D-arabinopyranosidase * | 0 | n.d. ** |

* The enzyme sample was incubated with phenyl-β-L- or D-arabinopyranoside, followed by measuring the liberated arabinose according to the method of Park and Johnson [10].
** Not determined.

TABLE IV

SUMMARY OF PURIFICATION OF α-L-ARABINOFURANOSIDASE

| Step of purification | Total protein (mg) | Specific activity (U/mg protein) X $10^3$ | -Fold purification | Total activity (units) | Yield (%) |
|---|---|---|---|---|---|
| Culture medium | 14 144 | 3.75 | (1.0) | 53.0 | 100 |
| Ultrafiltration | 3 031 | 16.9 | (4.5) | 51.2 | 96.6 |
| Heat treatment | 1 282 | 40.9 | (10.9) | 52.4 | 98.9 |
| Ammonium sulfate (40—70% sat.) fraction | 469 | 88.3 | (23.5) | 41.4 | 78.1 |
| Sephadex G-150 fraction | 277 | 145 | (38.7) | 40.2 | 75.8 |
| DEAE-Sephadex A-50 fraction | 213 | 187 | (49.9) | 39.8 | 75.1 |
| Isoelectric focusing main fraction | 37.0 | 611 | (163) | 22.6 | 42.6 |

ity of the enzyme was measured after storage at various pH for 20 h at 4°C or 37°C. At both temperatures, activity was lost when stored at pH values less than 3.5. At 37°C, for example, the loss of activity at pH 3.0 was 57% of the activity at pH 5.0. When the enzyme was stored at pH 5.5 and 4°C for a month, the loss of activity was less than 5%.

*Molecular weight of α-L-arabinofuranosidase.* The molecular weight was estimated at 62 000 by gel filtration on Sephadex G-150 column, according to the method of Andrews [11].

*Kinetics.* From a Lineweaver-Burk plot of the enzymic action on the synthetic substrate, Michaelis constant ($K_m$) and maximum velocity ($V$) were determined to be 6.7 mM and 5.26 units/mg protein, respectively (Fig. 4).

*Effect of some inhibitors.* Addition of up to 0.2 M NaCl had no effect on the enzymic activity. Table V shows results of experiments using different ionic constituents and inhibitors. SH reagents were unable to reduce the activity of the enzyme and, therefore, it was concluded that the enzyme did not contain SH groups at the active site.

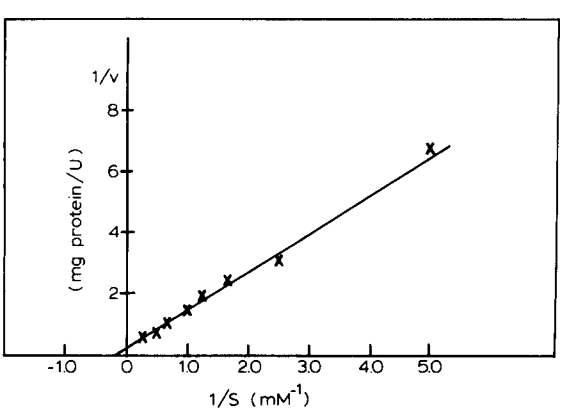


Fig. 4. Double reciprocal plots of initial velocity of the enzymic action and substrate concentration. The enzymic activity was assayed using various concentrations of *p*-nitrophenyl-α-L-arabinofuranoside.

TABLE V

EFFECT OF SOME INHIBITORS ON THE ACTIVITY OF α-L-ARABINOFURANOSIDASE

The enzymic activity was measured in the presence of the following compounds at 10 mM except the cases where concentrations were specified in parentheses.

| | Inhibiton (%) |
|---|---|
| Control | 0 |
| $MgCl_2$ | 6.5 |
| $CaCl_2$ | 7.4 |
| $CuCl_2$ | 90.4 |
| $ZnCl_2$ | 44.9 |
| $MnCl_2$ | 30.0 |
| EDTA * | 0 |
| Mercaptoethanol | 0 |
| Dithiothreitol | 0 |
| Glutathione (reduced) (1 mM) | 0 |
| Glutathione (reduced) (10 mM) | 13.4 |
| Cysteine (1 mM) | 1.9 |
| Cysteine (10 mM) | 19.6 |
| PCMB ** (0.5 mM) | 1.4 |
| PCMB (1.0 mM) | 3.1 |

* Ethylenediaminetetraacetic acid disodium salt.
** *p*-Chloromercuribenzoic acid.

*Effect of plant growth hormones and antibiotics.* Plant growth hormones (1 ppm 2,4-dichlorophenoxyacetic acid, indole-3-acetic acid and kinetin) and antibiotics (50 ppm Kanamycin and chloramphenicol) added in cultures showed no direct effect on the enzymic activity.

*Effect of some carbohydrates, their derivatives and lactones on α-L-arabinofuranosidase.* L-arabinose and D-arabinose showed some degree of inhibition. D-galactose, L-arabitol and D-galactitol also showed a little inhibition. On the other hand, phenyl-β-L-arabinopyranoside and phenyl-β-D-arabinopyranoside showed no effect under a condition (10 mM) as used in other cases.

The effect of some structurally-related lactones on the enzymic activity was also examined. L-arabono-1,4-lactone (the most similarly related compound tested) showed a strong inhibition (for example, 91% inhibition by 1 mM L-arabonolactone), while other lactones (D-arabono-1,4-lactone, L- and D-galactono-1,4-lactone) had no effect. These results showed that the enzyme was highly specific for the configuration of L-arabinofuranose (Fig. 5).


L-Arabinofuranose L-Arabono-1,4-lactone

Fig. 5. Structure of L-arabinofuranose and L-arabono-1,4-lactone.

## Discussion

The presence of araban-degrading enzyme in some plants has been previously reported [12,13]. For the first time, we observed the presence of α-L-arabinofuranosidase in both the cultured cells and the culture medium of *S. japonica*, in addition to those of *Nicotiana tabacum*. We also found the enzyme in the tissues of the intact plant of *S. japonica*. The enzyme was mainly localized in the 21st-day culture medium rather than in the cells (Table I).

α-L-Arabinofuranosidases from plant pathogenic fungi have been studied by many workers, who have discussed the possibility that the enzymes act as attacking agents when the fungi attack on the cell walls of target plants. On the contrary, the physiological role of α-L-arabinofuranosidase secreted extracellularly from plant calluses in culture is not revealed at all. In addition, it is not known why the activity of α-L-arabinofuranosidase and that of β-galactosidase are strong in the culture medium, while activities of other glycosidases are weak (Table II). Thus, we may suggest the following possibility. These glycosidases are first synthesized inside the cells and then secreted during the phase of the vigorous cell proliferation. They act on some specific structural materials of the cell walls, mainly socalled primary cell walls [1], to make the cell walls keep soft so that the cells are easy to continue the division. In fact, α-L-arabinofuranosidase was much secreted when *S. japonica* callus cells were growing vigorously in suspension culture, while the extracellular level of α-L-arabinofuranosidase began to decrease after the increase of number of cells reached a plateau under a given culture condition. A large amount of the activity of this enzyme was, however, found in root, stem and leaf of the original plant at the flowering time. Thus, in addition to the possibility mentioned above concerning the physiological role of α-L-arabinofuranosidase, it may also be suggested for the enzyme to play a fundamental role on the metabolism of polysaccharides and glycoproteins.

Concerning many arabinosidases including α-L-arabinofuranosidases from fungi, their isoelectric point, molecular weight, optimum pH and heat stability were reported [5,6,14,15]. So we concluded that the α-L-arabinofuranosidase from the plant is a new enzyme different from α-L-arabinosidase from fungi, when their properties were compared. As far as the studies hitherto reported are concerned, the enzymes from fungi were acidic proteins which showed a range of p*I* between 3.0—6.5. Their molecular weight were found to be between 16 000 and 63 000, except in *Scrolotinia fructigena* where the molecular weight was reported as 200 000 and 350 000. Most fungal enzymes were inactivated at 55 to 60°C. Making a clear contrast to the fungal enzymes, the α-L-arabinofuranosidase from *S. japonica* showed characteristically an isoelectric point of 8.0, a molecular weight of about 62 000 and high heat stability that 40% of total activity remained after a treatment at 65°C for 3 min at pH 7.0, for example.

In *S. japonica* calluses, an activity of α-L-arabinopyranosidase was also found. But such activity was considered to be mainly due to the broad specificity of β-galactosidase owing to the similarity of the configuration between both substrates, *p*-nitrophenyl-α-L-arabinopyranoside and *p*-nitrophenyl-β-D-galactoside, used, and also owing to the behavior during such processes of

purification including gel filtration on Sephadex G-150, SP-Sephadex column chromatography and others. However, the activity of α-L-arabinopyranosidase remained intact in the enzyme sample after a heat treatment which destroyed β-galactosidase. Incidentally, the activity of α-L-arabinopyranosidase was inhibited with L-arabono-1,4-lactone, a specific inhibitor for α-L-arabinofuranosidase, suggesting the possibility of the α-L-arabinopyranosidase activity to be included in α-L-arabinofuranosidase. No further research, however, was made on the activity of α-L-arabinopyranosidase.

This α-L-arabinofuranosidase was able to act on beet araban to release free arabinose exoenzymically. The details of these experiments together with the specificity of the enzyme to natural substrates will be reported elsewhere.

## Acknowledgement

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