# Invertase of Cell Walls from Cycad Pollen

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The data presented in the previous paper have reported that ribonuclease and phosphomonoesterase are eluted from cycad pollen cells with the various salt solutions (1). From the mode of elution of these enzymes the authors were occasioned to have conceived an idea that the enzymes exist in cell walls of pollen. Experiments were made to prove the concept and it was ascertained that ribonuclease, phosphomonoesterase, acid pyrophosphatase, and invertase activities were detected in cell wall fraction after the destruction of cells. It was then undertaken to characterize the invertase and to test the relationship between the enzyme and cell walls, because this enzyme in cell walls may be of not a small importance in germination and growth of pollen cells, especially in an artificial medium.

## METHODS

*Pollen cells and ribonuclease*. Methods for preparation of pollen cells and partially purified ribonuclease were the same as those described before (1).

*Assay of enzyme activity*. Ribonuclease and phosphomonoesterase activities were assayed by the same procedures as previously described (1).

For determining invertase activity, the reaction mixture containing 0.3 *ml* of 0.5 *M* sucrose, 0.5 *ml* of 0.1 *M* citric acid-0.2 *M* phosphate buffer, *pH* 4.0, 0.2 *ml* of enzyme and 0.5 *ml* of water was used. Incubation was made at 37°*C* for 30 *min* and the reaction was stopped by adding 0.5 *ml* of 1 *M* sodium carbonate. If necessary, the reaction mixture was centrifuged at 1000 $\times g$ for 5 *min.*

The reducing sugar contents liberated were measured by Somogyi's method (2). One unit of invertase was defined as the amount of enzyme that formed 1 $\mu$mole of glucose per *min* under the above conditions.

For determining acid pyrophosphatase activity, the reaction mixture containing 1.0 *ml* of 2 *mM* sodium pyrophosphate, 0.6 *ml* of 0.2 *M* acetate buffer, *pH* 5.0, 0.2 *ml* of enzyme and 0.2 *ml* of water was used. Incubation was made at 37°C for 15 *min* and the reaction was stopped by adding 1.0 *ml* of 0.3 *N* perchloric acid. If necessary, the reaction mixture was centrifuged at 1000 $\times g$ for 5 *min.* Orthophosphate contents liberated were measured by the modified Fiske-Subbarow's method (3).

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Determination of $Mg^{2+}$-dependent alkaline pyrophosphatase activity was the same as that for acid pyrophosphatase except in this case it contains 0.2 *ml* of 0.01 *M* $MgCl_2$ instead of water and 0.05 *M* Tris-HCl buffer, *pH* 9.0. One unit of pyrophosphatase activity was defined as the amount of enzyme that liberated 1 *μmole* of orthophosphate per *min* under the above conditions.

*The disintegration of pollen cells and preparation of cell wall and supernatant fraction.* Destruction of pollen cells to obtain cell wall fraction was carried out with a teflon-glass homogenizer. Pollen cells (4 *g*) were washed three times with 40 *ml* of water by centrifugation and finally filled up to 40 *ml* with water. Aliquots of the suspension were homogenized for appropriate length of time in an ice-bath and centrifuged at $1000\times g$ for 5 *min*. The precipitate was washed two times with water and designated as cell wall fraction. The supernatant and washings were combined and named supernatant fraction. These fractions were used only to examine the distribution of enzymes during disintegration of pollen cells, because cell wall fraction obtained was contaminated with ultraviolet absorbing materials, perhaps nuclei, which were removed by sonciation.

*Preparation of cell walls for purification of invertase and for adsorption experiments.* Pollen cells (2 *g*) were washed three times with 20 *ml* of water by centrifugation and finally filled up to 20 *ml*. Aliquots of cell suspension were disintegrated with a teflon-glass homogenizer for 10 *min* in an ice-bath and centrifuged at $1000\times g$ for 5 *min*. Precipitate was washed three times by centrifugation, filled up to 40 *ml* with water and mixed with an equal volume of 0.5 *M* sucrose. Cell suspension was sonicated at 20 *kc* for 2 *min* and washed three times by centrifugation. Precipitate was filled up to 20 *ml* with water and used as cell walls.

## RESULTS

### *Evidence for the presence of enzymes in cell walls*

Distribution of enzymes during disintegration of pollen cells was shown in Fig. 1. Ribonuclease, phosphomonoesterase, acid pyrophosphatase, and invertase activities were detected in both cell wall and supernatant fractions. Only negligible activity of $Mg^{2+}$-dependent alkaline pyrophosphatase was detected in cell wall fraction and the bulk of the activity was found in supernatant fraction. It may be possible that cell wall enzymes are released into supernatant during disintegration of cells and that interior enzymes are adsorbed secondarily to cell walls. However, if enzymes exist really in cell walls, activities must be detected when intact cells are directly incubated with substrates.

In fact, as shown in Table 1, ribonuclease, phosphomonoesterase, acid pyrophosphatase, and invertase activities were detected 13-42 % on cell surface to the whole cell activities except $Mg^{2+}$-dependent alkaline pyrophosphatase; the latter activity was observed only slightly 0.3 % on the surface. From these results it was suggested that $Mg^{2+}$-dependent alkaline pyrophosphatase was located only in cell interior and other enzymes were situated both in cell interior and cell walls.

### *Elution of invertase from cell walls*

Elution of invertase was carried out with various concentrations of NaCl solution. To 1 *ml* of cell wall suspension were added 1 *ml* of 0.1 *M* citric acid-0.2 *M* phosphate buffer,

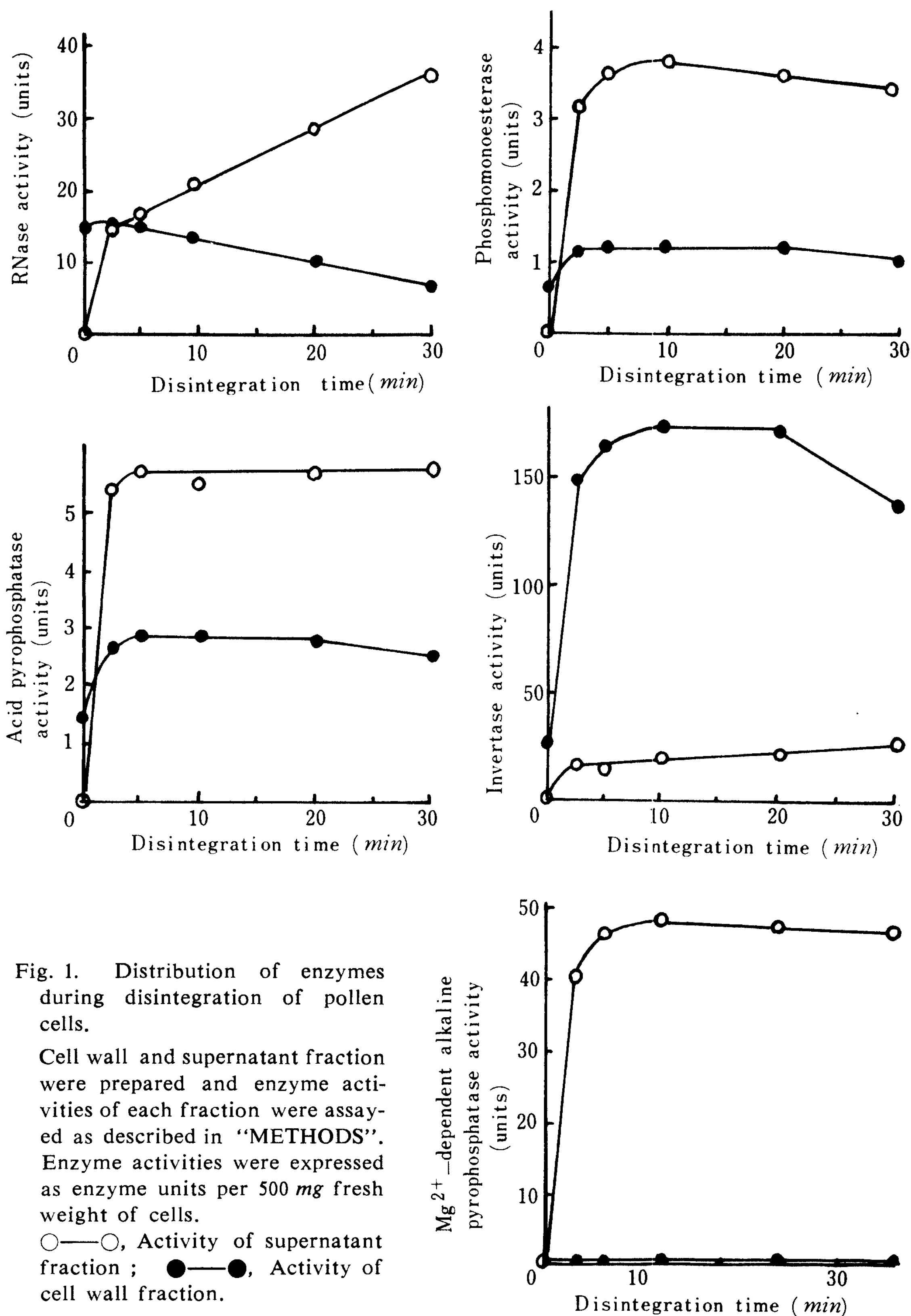


Fig. 1. Distribution of enzymes during disintegration of pollen cells.

Cell wall and supernatant fraction were prepared and enzyme activities of each fraction were assayed as described in "METHODS". Enzyme activities were expressed as enzyme units per 500 *mg* fresh weight of cells.
○——○, Activity of supernatant fraction ; ●——●, Activity of cell wall fraction.

Table 1. Enzyme activities detected at pollen cell surface

| Enzyme | (A) Activity at cell surface | (B) Activity of whole cells | (A)/(B) % |
|---|---|---|---|
| Ribonuclease | 14.65 | 35.15 | 41.7 |
| Phosphomonoesterase | 0.66 | 5.03 | 13.1 |
| Acid pyrophosphatase | 1.52 | 8.28 | 18.4 |
| Invertase | 28.3 | 193.3 | 14.6 |
| $Mg^{2+}$-dependent alkaline pyrophosphatase | 0.16 | 48.92 | 0.3 |

Enzyme activities detected at pollen cell-surface were determined by incubating intact cells with substrates and compared with those of whole cells. Activities of whole cells were shown by determining activity of cell suspension disintegrated with a teflon-glass homogenizer for 10 *min*. Enzyme activities were expressed as enzyme units per 500 *mg* fresh weight of cells.

*pH* 6.0, and a suitable volume of NaCl solution. Reaction mixture was incubated at 37℃ for 20 *min* and centrifuged at 1000 ×*g* for 1 *min*. As shown in Fig. 2, elution of invertase occurred above 0.2 *M* NaCl solution and reached the maximum level at 0.4 *M*. Finally about 60 % of cell wall activity was eluted. A suitable *pH* range of elution was between 5 and 7.

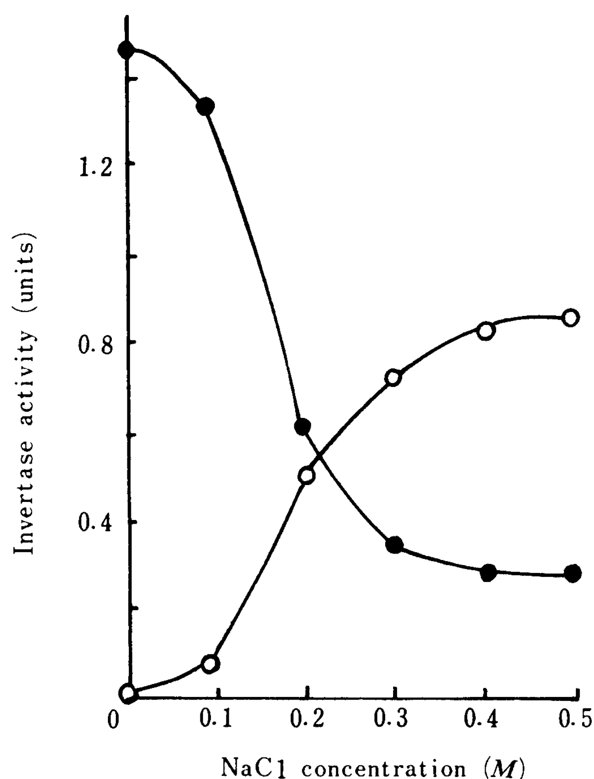


Fig. 2. Effect of NaCl concentration on elution of in vertase from cell walls.

Pollen cell walls were incubated in various concentrations of NaCl bufferized with citric acid-phosphate, *pH* 6.0, at 37°*C* for 20 *min* and centrifuged as described in text. Enzyme activity of eluate and precipitate were determined and expressed as enzyme units per cell walls obtained from 25 *mg* fresh weight of cells.

○——○, Activity of eluate; ●——●, Activity remaining in cell walls.

*Purification of invertase*

Cell walls obtained from 20 *g* fresh weight of pollen cells as described in "METHODS" were filled up to 200 *ml* with water, added with 160 *ml* of 1 *M* NaCl and 40 *ml* of 0.1 *M* citric acid-0.2 *M* phosphate buffer, *pH* 6.0 and incubated at 37°C for 20 *min.* Reaction Mixture was centrifuged at 6500×*g* for 20 *min.* To the precipitate was added 100 *ml* of 0.4 *M* NaCl containing the same buffer as above and the mixture was centrifuged. 418 *ml* of combined supernatant was dialyzed against 0.01 *M* citric acid-0.02 *M* phosphate buffer, *pH* 6.0. Precipitate formed during the dialysis was dissolved in 20 *ml* of 0.5 *M* NaCl and allowed to

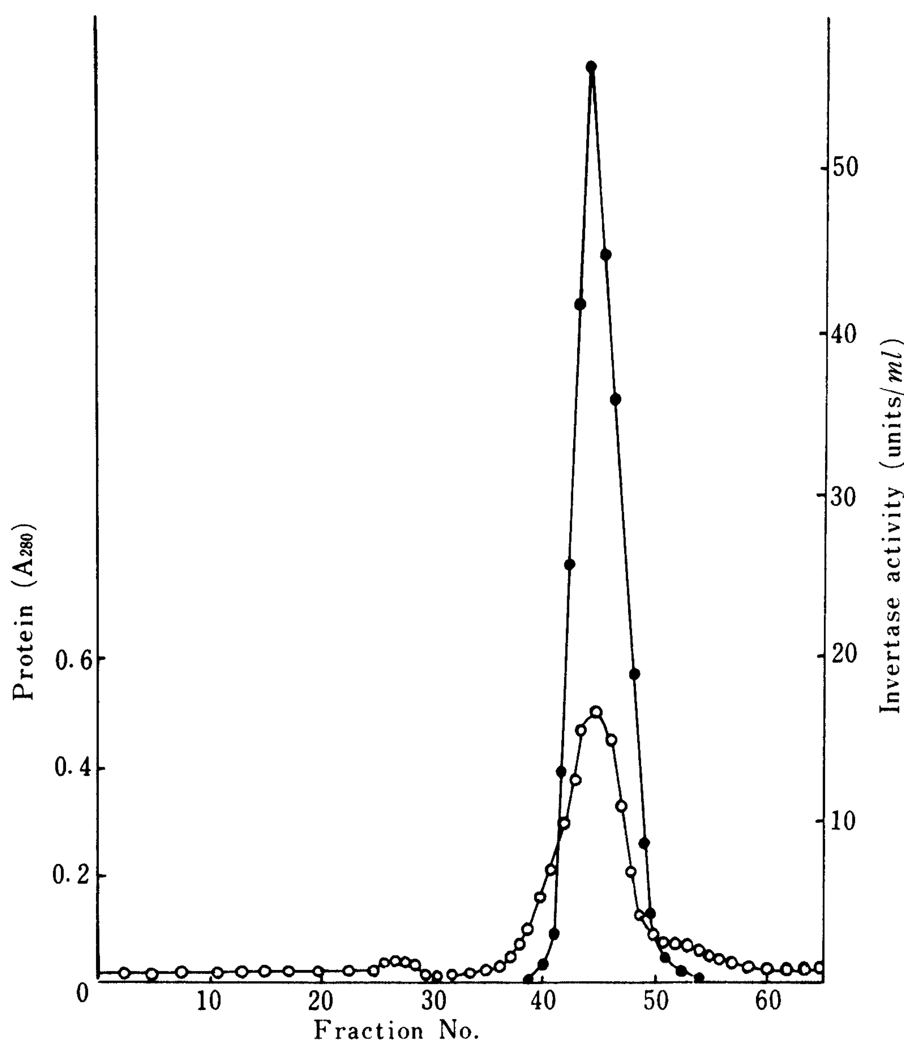


Fig. 3. The second gel filtration of invertase on a Sephadex G-100 column.

Column size, 1.9×95 *cm*; Fraction, 3.3 *ml*; Sample, Gel-filtrate on the first Sephadex G-100 column, $A_{280}$, 14.5; Elution buffer, 0.01 *M* citric acid-0.02*M* phosphate, *pH* 6.0, containing 0.4 *M* NaCl.

○——○, Protein; ●——●, Invertase activity.

Table 2. Purification procedure of invertase

| Fraction | | Volume (*ml*) | Protein ($A_{280}$) | Activity (units) | Recovery (%) |
|---|---|---|---|---|---|
| Cell wall fraction after homogenization | | 400 | — | 7960 | 100 |
| Cell wall homogenate after sonication | | 400 | — | 6006 | 75. 4 |
| Eluate with 0. 4 *M* NaCl | | 418 | 539. 2 | 3542 | 42. 7 |
| Precipitate after dialysis | | 20 | 104 | 1673 | 21. 0 |
| First gel-filtrate from Sephadex G-100 | | 50. 5 | 14. 5 | 845 | 10. 6 |
| Second gel-filtrate from Sephadex G-100 | | | | | |
| (Fraction No.) | (44) | 3. 3 | 1. 50 | 138 | 7. 4 |
| | (45) | 3. 3 | 1. 61 | 187 | |
| | (46) | 3. 3 | 1. 32 | 148 | |
| | (47) | 3. 3 | 1. 07 | 120 | |

pass through a column of Sephadex G-100, 3.5 × 55 *cm*, equlibrated to citric acid-phosphate buffer, *pH* 6.0, containging 0.4 *M* NaCl. The fractions with activity units of 6 per *ml* or greater than that were pooled (50.5 *ml*) and concentrated to about 1 *ml* with a collodion bag. The concentrated enzyme solution was passed through a column of Sephadex G-100, 1.9 × 90 *cm*, equilibrated with the same buffer described above.

The result of the second gel filtration was shown in Fig. 3. The typical purification procedure was summarized in Table 2. A considerable variation in the yield was recorded at the step of dialysis.

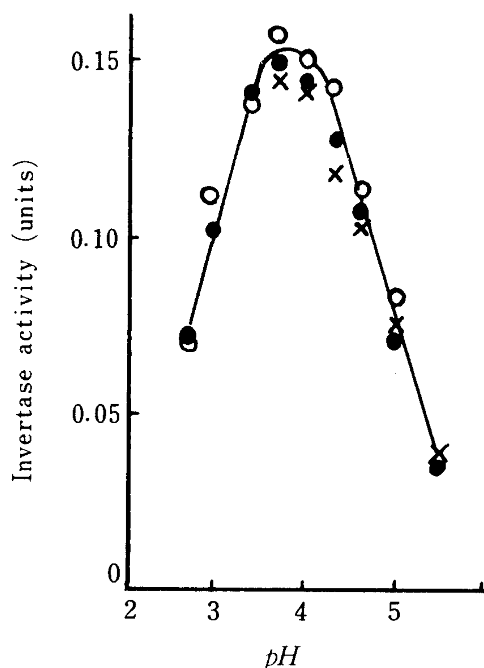


Fig. 4. Effect of *pH* on invertase activity.

Activity was assayed as described in "METHODS", but *pH* value was varied by buffers indicated; ○——○, citric acid-phosphate; ●——●, citric acid-citrate; ×——×, acetic acid-acetate.

*Properties of invertase*

Effect of *pH* on invertase activity was determined in varying *pH* values. As shown in Fig. 4, an optimum *pH* was about 4.0. Substrate specificity of invertase was determined with sucrose, raffinose, maltose, melibiose, trehalose, and melezitose and among these, sucrose and raffinose were sufficiently hydrolyzed, but other substrates were completely inert. By paper chromatographic analysis of hydrolyzates, from sucrose were given two spots of glucose and fructose, and from raffinose two spots of fructose and a disaccharide, melibiose. From this fact, it was recognized that this enzyme belong to $\beta$-fructofuranoside-fructohydrolase (EC

3.2.1.26). The Km value of invertase for sucrose under the standard assay conditions was about 3.5 *mM*.

*Adsorption of solubilized invertase to cell walls*

Adsorption was attempted by use of invertase purified by the procedure as shown in Table 2. Cell walls prepared as described in "METHODS" were treated with 0.1 *N* NaOH, 0.1 *N* HCl and 0.5 *M* NaCl prior to adsorption. For each reagent, treatment was made for 30 *min* at a room temperature with occasional shaking, and the cell suspension was centrifuged at 1000 ×*g* for 5 *min*. Precipitate was washed ten times with water by centrifugation and finally suspended in water.

Table 3. Adsorption of invertase to cell walls

| Source | Activity of invertase | | | | Activity ratio of adsorbed invertase to added invertase |
|---|---|---|---|---|---|
| | Bound originally to cell walls | Detected in cell walls after addition of enzyme | Not adsorbed to cell walls | Adsorbed to cell walls | |
| Cell walls treated with 0.1 *N* NaOH | 0 | 5.85 | 0.08 | 5.85 | 2.31 |
| Cell walls treated with 0.1 *N* HCl | 0 | 0.97 | 4.56 | 0.97 | 0.38 |
| Cell walls treated with 0.5 *M* NaCl | 1.23 | 6.14 | 0.64 | 4.91 | 1.94 |
| Intact cell walls | 5.28 | 11.04 | 0.61 | 5.76 | 2.28 |

To 1 *ml* of previously treated cell wall fraction obtained from 20 *mg* fresh weight of cells were added 1 *ml* of citric acid-phosphate buffer, *pH* 5.0, 2.7 *ml* of water and 0.3 *ml* of purified invertase (2.53 units). The reaction mixture was stood for 30 *min* at a room temperature with occasional shaking and centrifuged at 1000×*g* for 5 *min*. Supernatant and precipitate were filled up to 5 *ml* and each activity was expressed as enzyme units per 5 *ml* of reaction mixture. For determining activity of invertase bound originally to cell walls, reaction mixture not added with purified enzyme was used.

As shown in Table 3, cell walls treated with 0.1 *N* NaOH and 0.1 *N* HCl contained no activity. Invertase was bound fully to cell walls treated with NaOH and NaCl, but in case of cell walls treated with HCl, enzyme was adsorbed below 20 %. Because enzyme was also adsorbed to intact cell walls, it seems likely that binding site of intact cell wall is not saturated with protein and has an ability to attach additional proteins.

Unexpectedly, the activity was enhanced about 2-fold after the adsorption to cell walls (5.85 or 4.91). Some change might occur of structure and conformation of enzyme protein adsorbed to cell walls, but it is incomprehensible that invertase fraction not adsorbed to cell walls treated with HCl was also augmented to 4.56.

*Effect of pH on adsorption of solubilized invertase to cell walls*

In this experiment, cell walls treated with 0.1 *N* NaOH were used. They have two advantages; (1) cell walls do not or slightly contain invertase activity, and (2) cell walls have

Table 4. Effect of *pH* on adsorption of invertase to cell walls treated with 0. 1 *N* NaOH

| Reaction *pH* | Activity of invertase | | | | Activity ratio of adsorbed invertase to added invertase |
|---|---|---|---|---|---|
| | Bound criginally to cell walls | Detected to cell walls after addition of enzyme | Not adsorbed to cell walls | Adsorbed to cell walls | |
| 4 | 0. 11 | 5. 35 | 0. 11 | 5. 24 | 1. 98 |
| 5 | 0. 11 | 6. 29 | 0. 10 | 6. 18 | 2. 34 |
| 6 | 0. 11 | 6. 16 | 0. 19 | 6. 05 | 2. 29 |
| 7 | 0. 11 | 5. 78 | 0. 28 | 5. 67 | 2. 15 |
| 8 | 0. 11 | 5. 80 | 0. 22 | 5. 69 | 2. 16 |
| 9 | 0. 11 | 5. 65 | 0. 16 | 5. 54 | 2. 10 |

To 1 *ml* of cell wall fraction (obtained from 20 *mg* fresh weight of cells) treated with 0. 1 *N* NaOH were added 1 *ml* of citric acid-phosphate, *pH* 4-7 or Tris-HCl, *pH* 8-9, 2. 7 *ml* of water and 0. 3 *ml* of purified invertase (2. 64 units). Treatment of reaction mixture and presentation of enzyme activity were the same methods as described in Table 3.

an ability to bind invertase almost completely within the range applied. The buffer used was citric acid-phosphate, *pH* 4-6 and Tris-HCl, *pH* 7-9. As shown in Table 4, adsorption was not influenced by varying *pH* values and in all cases, invertase activity adsorbed was enhanced about 2-fold.

The properties of invertase being bound originally to intact cell walls, purified invertase and invertase readsorbed to cell walls treated with NaOH were compared with each other. The *pH* optimum, Km value, heat stability and optimal temperature on activity were not significantly different.

*Effect of pH on adsorption of solubilized ribonuclease to cell walls*

In this experiment, intact cell walls were used instead of cell walls treated with 0.1 *N* NaOH, and the result obtained was almost the same in both cases. Ribonuclease was eluted from intact pollen cells with 0.5 *M* sodium acetate and purified by the procedures described in the previous paper (1). As shown in Table 5, adsorption of ribonuclease was much different from that of invertase and depended strongly on *pH* of reaction mixture. The optimum *pH* on adsorption was below 4.0 and enzyme was little adsorbed above *pH* 6.0. The activity of enzyme adsorbed to cell walls was not enhanced; on the contrary, the activities not adsorbed above *pH* 6.0 were nearly 170 % of that of the starting materials.

## DISCUSSION

The enzymes in cell walls of higher plants have been studied by several groups of workers. The existence and the role of cell wall enzymes are increasing attention again. In a recent paper, Knox *et al.* have demonstrated that acid phosphatase, ribonuclease, esterase, and amylase activities are associated with pollen cell walls of ten species of flowering plants and the site of deposition is the cellulosic intine in the walls of several different structural types (4).

In case of cycad pollens, invertase, ribonuclease, phosphomonoesterase, and acid pyrophosphatase were detected at the surface of intact cells. Seemingly, the mode of binding to

Table 5. Effect of *pH* on adsorption of ribonuclease to cell walls

| Reaction *pH* | Activity of ribonuclease | | | | Activity ratio of adsorbed ribonuclease to added ribonuclease |
|---|---|---|---|---|---|
| | Bound originally to cell walls | Detected to cell walls after addition of enzyme | Not absorbed to cell walls | Adsorbed to cell walls | |
| 3 | 1.13 | 11.38 | 0.88 | 10.25 | 0.92 |
| 4 | 1.13 | 12.00 | 1.96 | 10.87 | 0.97 |
| 5 | 1.13 | 3.21 | 8.75 | 2.08 | 0.19 |
| 6 | 1.13 | 1.67 | 19.50 | 0.54 | 0.05 |
| 7 | 1.13 | 1.75 | 19.58 | 0.62 | 0.06 |
| 8 | 1.13 | 1.04 | 19.38 | — | — |
| 9 | 1.13 | 1.58 | 17.08 | 0.45 | 0.04 |

To 1 *ml* of cell wall fraction obtained from 20 *mg* fresh weight of cells were added 1 *ml* of citric acid-phosphate, *pH* 4-7, or Tris-HCl, *pH* 8-9, 2 *ml* of water and 1 *ml* of purified ribonuclease (11.17 units) and the reaction mixture was treated by the same methods as described in Table 3. Activity of each fraction was expressed as enzyme units per 5 *ml* of reaction mixture.

cell walls is different with respect to the sorts of enzymes and plants, because the conditions of extraction and adsorption are not fixed uniformly. For example, Edelman *et al.* could not induce any solubilization of invertase from the cell debris of Jerusalem artichoke by a variety of treatments; namely extraction with buffer, detergents, organic solvents, freezing and thawing, and sonication (5). Arnold found that a bound fraction of grape invertase was solubilized by treatment with 0.2 *M* borate buffer, *pH* 8.5, whereas several other aqueous salts were without effects (6). Nakagawa *et al.* could elute the bound enzyme of tomato cell wall with 250 *mM* phosphate buffer, *pH* 8.0 (7). On the other hand, cell wall invertase of cycad pollen cells was eluted 0.4 *M* NaCl containing citric-phosphate buffer between *pH* 5 and 7. It may be concluded that the enzymes and cell walls are combined with ionic bonds, because of the existence of the salt solutions effective enough to elute the most of the cell wall enzymes tested.

To elucidate the mechanism of binding between enzymes and cell walls adsorption of liberated enzymes must be attempted.

An example of success in the binding experiment was a work carried out by Nakagawa *et al* (7). They have dealt with the preparation of cell walls from tomato pericarp and with the nature of the *in vitro* binding of pure pectinmetylesterase and invertase to the purified cell walls, and found that the binding of both enzymes to cell walls were affected remarkably with the *pH* of cell wall suspension and the maximum adsorption was at *pH* 4.7. Jansen *et al.* have reported that avena cell walls are capable of binding added pectinmethylesterase in amounts up to approximately 200 times greater than that inherent to cell walls and also binding other proteins such as pepsin, peroxidase and $\alpha$-chymotrypsin (8). However, invertase from grape berry (6) and UDPG-pyrophosphatase from corn coleoptile (9) were not adsorbed by cell walls from the respective source.

In cycad pollen cells, adsorptions of partially purified invertase and ribonuclease were performed with cell walls untreated or treated with NaOH, NaCl and HCl. Invertase was adsorbed fully to cell walls untreated or treated with the alkali or the salt, but adsorption to cell walls treated with HCl was below 20 %. Since the ability to adsorb enzyme was recovered when HCl-treatment of cell wall was followed by NaOH, it may be suggested that invertase was adsorbed to site of $OH^-$-type in cell walls.

No significant change was observed of adsorption of invertase by varying *pH* values between 4 and 9, but adsorption of ribonuclease depended strongly on *pH*, optimum *pH* of the latter being below 4.0. This seems to indicate that adsorption of enzymes depends on an affinity between each enzyme protein and the binding site of cell walls.

When solubilized enzymes came in contract with cell walls, even if not adsorbed, invertase and ribonuclease activities were enhanced remarkably. But, no evidence to give the reason for this type of activation was obtained in so far as tested. Further experiments will be necessary to elucidate sufficiently this interesting observation.

*Acknowledgement* – The authors are indebted to Miss K. Yoshihara for her technical assistance.

## SUMMARY

This investigation was undertaken to clarify the presence of cell wall enzymes and binding of enzymes to cell walls. Cell walls prepared with homogenization and sonication were used in this experiment. They were free from $Mg^{2+}$-dependent alkaline pyrophosphatase activity which was deemed to exist only in cell interior.

The results obtained were summarized as follows.

(1) Ribonuclease, phosphomonoesterase, acid pyrophosphatase and invertase activities were detected in cell wall fraction and at intact cell surface. Especially, about 90 % of invertase activity in whole cells was in cell wall fraction.

(2) Invertase was eluted from cell walls with 0.4 *M* NaCl containing citric acid-phosphate, *pH* 6.0 and partially purified by means of dialysis and gel-filtration. The *pH* optimum, substrate specificity and Michaelis constant of invertase purified were not significantly different from those before solubilization.

(3) Readsorptions of solubilized invertase and ribonuclease were carried out with cell walls untreated or treated with NaOH, HCl and NaCl. Invertase was fully adsorbed to cell walls at *pH* 5.0 except cell walls treated with HCl. Adsorption of invertase was not affected by varying *pH* values between 4 and 9, but in case of ribonuclease, adsorption was maximal at *pH* 4.0 and enzyme was little adsorbed above *pH* 6.0.

(4) Invertase and ribonuclease were activated remarkably when they came in contract with cell walls, even if not adsorbed. The mechanism of activation has not been made clear.

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