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Purification and Properties of Wall-bound α-Glucosidase from Suspension-cultured Sugar-beet Cells

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Wall bound α-glucosidase (EC 3. 2. 1. 20) has been solubilized from suspension-cultured sugar-beet cells with Sumyzyme C and Pectolyase Y-23 and purified by a procedure including fractionation with ammonium sulfate, Sephacryl S-200 HR column chromatography, and CM-cellulose colum chromatography. The enzyme readily hydrolyzed maltose, nigerose, malto-oligosaccharides, and soluble starch, but hydrolyzed isomaltose more slowly. The enzyme hydrolyzed malto-oligosaccharides and soluble starch at a faster rate than maltose. The wall-bound α-glucosidase from sugar-beet cells is different from the enzymes extracted from the cells and seeds in substrate specificity.

Key words: *Beta vulgaris* L. cv. Tsukisappu, Sugar-beet, Wall-bound enzyme, α-Glucosidase, Protoplast.

INTRODUCTION

In a previous paper[15], we reported that four α-glucosidases are produced by suspension-cultured sugar-beet cells, grown in Murashige and Skoog medium[6] with shaking. The four enzymes can be extracted from the cells with buffer and NaCl[15]. After the solubilization of the four enzymes, about 40 % of the α-glucosidases produced by sugar-beet cells remained in the pellet. Therefore, the properties of the enzyme in the pellet can be examined. On the other hand, the insoluble α-glucosidases in water have also been found in sorghum and barley grain[12], and rice cells[14]. Water releases less than 2 % of the enzyme activity found by the direct incubation of milled grain with maltose and 22 % with 1 M NaCl. In rice cells, 89. 1 % of the enzyme activity remain in the pellet after the incubation with 2 M

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NaCl. About 70 % of α-glucosidase from sorghum grain is liberated using a combination of 8 M urea and 0.1 M sodium sulfite used to split disulfide bonds. However, barley grain and rice cells do not respond similarly. Membrane-bound α-glucosidases have also been reported in animals[2, 4, 8].

The fifth α-glucosidase in the pellet obtained after extraction of the soluble fraction with NaCl can not be solubilized with detergents or a combination of 8 M urea and 0.1 M sodium sulfite. Therefore, the enzyme is not a membrane component and is not insolubilized via a S-S-linkage. The enzyme must be firmly bound to the cell wall. In this study, we solubilized α-glucosidase from sugar-beet cell wall, purified the enzyme partially, and investigated some of its properties.

MATERIALS AND METHODS

1. *Materials*

Maltose (HHH), maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, and isomaltose (Hayashibara Biochemical Laboratories, Inc.), sodium carboxymethyl cellulose (CMC) and soluble starch (Ishizu Pharmaceutical Co., Ltd.), xylan (Sigma Chemical Co.), Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd.), and Sumyzyme C (Shinnihon Kagaku Kogyo Co Ltd.) were obtained from commercial sources.

2. *Cell culture*

Sugar-beet cells (*ca* 0.7 g wet wt) were inoculated into 125 ml of the medium of Murashige and Skoog in 500 ml conical flasks, and grown with gentle shaking (88 strokes per min) at 25℃ for 20 days in the dark.

3. *Enzyme assay*

α-Glucosidase activity was determined as follows. The reaction mixture containing 0.1 ml of 1 % maltose, 0.25 ml of 0.1 M acetate buffer, pH 4.5, and enzyme solution in a final volume of 0.5 ml was incubated at 37 ℃ for 1 hr. After incubation, the reaction was stopped by boiling for 5 min. The amount of glucose formed was measured by the method of Papadopoulos *et al.*[7], as modified by Dahlqvist[3]. One unit of α-glucosidase activity was defined as the amount of enzyme which liberates 1 μmol/hr of glucose from maltose under the conditions described above.

CMC- and xylan-hydrolyzing activities were determined by measuring the amount of reducing sugar liberated from the respective substrates according to the method of Somogyi[10]. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol/hr of glucose from the

respective substrate under the conditions described above.

4. *Determination of protein*

Protein was determined by the method of Warburg *et al.*[11]. The protein profiles in column chromatography were followed by measuring the absorbance of eluates at 280 nm.

RESULTS AND DISCUSSION

1. *Isolation of wall-bound α-glucosidase*

When sugar-beet cells were treated with the mixture of Sumyzyme C and Pectolyase Y-23, the wall-bound α-glucosidase was solubilized from the cells with the production of protoplasts. However, the mixture of Sumyzyme C and Pectolyase Y-23 has several forms of α-glucosidase and one of them emerged in the same position as the wall-bound α-glucosidase on Sephacryl S-200 HR column chromatography. Therefore, the α-glucosidases must be removed from the mixture of Sumyzyme C and Pectolyase Y-23.

Sumyzyme C (5 g) and Pectolyase Y-23 (150 mg) were dissolved in 25 ml of 0.1 M acetate buffer, pH 5.0, and the insoluble material was removed by centrifugation at 8,000 x*g* for 10 min. The solution was put on a Sephadex G-25 column (3.2 × 45 cm) to remove ammonium sulfate. The protein fractions were passed through a DEAE-Sephadex A-50 column (12.5 × 8 cm) equilibrated with 0.1 M acetate buffer, pH 5.0. The unadsorbed protein fraction was concentrated to 12 ml using a Bio-Engineering ultrafiltration device (G-05 T membrane; Bio-engineering Co.). The concentrate was filtered on a Sephadex G-100 gel column (1.8 × 146 cm) equilibrated with 0.1 M acetate buffer, pH 5.0. Protoplast-forming enzymes (Nos. 40～50) were separated from α-glucosidases (Fig. 1).

Sugar-beet cells (310 g) were suspended in 500 ml of 30 mM acetate buffer, pH 5.3, containing 0.5 M mannitol, 2 % sucrose, and the purified protoplast-forming enzymes (CMC-hydrolyzing activity; 2,400 μmol/min as glucose). The enzymic digestion was done at 30 ℃ with gentle stirring. After 2 hr, the formation of protoplasts was completed and 17.3 % of the enzyme activity in sugar-beet cells was solubilized (Fig. 2). The activity was measured after partial purification of the enzyme by fractionation with ammonium sulfate and Sephacryl S-200 HR column chromatography. Thus, considerable α-glucosidase activity must be lost during the digestion of the cells and the purification of the enzyme. Therefore, α-glucosidase in the cell wall pellet may be located in the wall and most of the enzyme may be liberated by the digestion of the wall that produces protoplasts.

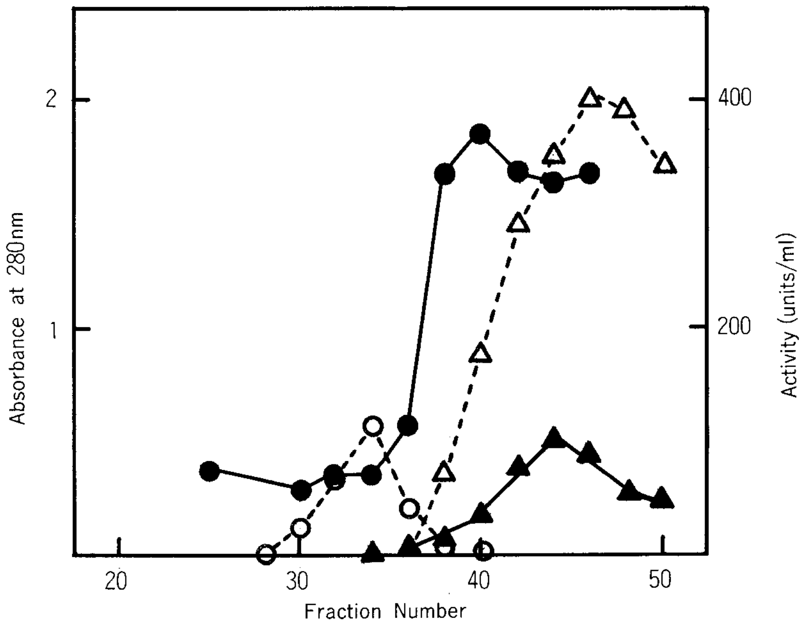

Fig. 1. Sephadex G-100 column chromatography of mixture of Sumyzyme C and Pectolyase Y-23.
The column was eluted with 20 mM acetate buffer, pH 5.0. The eluate was collected in 4.0 ml fractions. ●——●, Absorbance at 280 nm ; ○----○, α-glucosidase activity ; ▲——▲, xylan-hydrolyzing activity ; △----△, CMC-hydrolyzing activity.

The solubilized α-glucosidase solution was brought to 0.9 saturation with ammonium sulfate. The precipitate was collected by centrifugation at

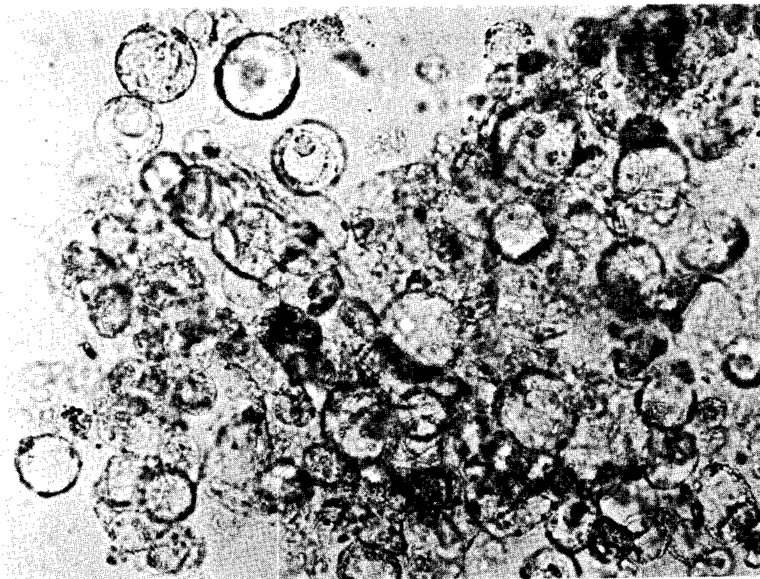

Fig. 2. Protoplasts from suspension-cultured sugar-beet cells.

8,000 x*g* for 10 min and dissolved in 50 mM acetate buffer, pH 4.5. The solution was put on a Sephacryl S-200 HR column (2.8 × 98 cm) equilibrated with 20 mM acetate buffer, pH 4.5, containing 150 mM NaCl (Fig. 3). The eluate with α-glucosidase activity was dialyzed overnight against 20 mM acetate buffer, pH 4.5. The dialyzed solution was put on a CM-cellulose column (1.6 × 10 cm) equilibrated with 20 mM acetate buffer, pH 4.5. The column was first eluted with the same buffer to wash off the unadsorbed protein, and then with a linear gradient of 0 ~ 1.0 M NaCl, also in the same buffer. The eluate with α-glucosidase activity was concentrated to 3 ml using an Amicon ultrafiltration device (PM-10 membrane; Amicon Co.). The concentrate was dialyzed overnight against 20 mM acetate buffer, pH 4.5, and used as an α-glucosidase preparation to determine the enzymatic properties. Table 1 shows a summary of the purification procedures.

2. *General properties of wall-bound α-glucosidase*

The pH optimum of the enzyme was 5.0. After 20 hr preincubation at 30 ℃ with 50 mM McIlvaine's buffer, the enzyme was stable in the pH range 4.5-7.0. The temperature optimum for the activity of the enzyme was 55 ℃

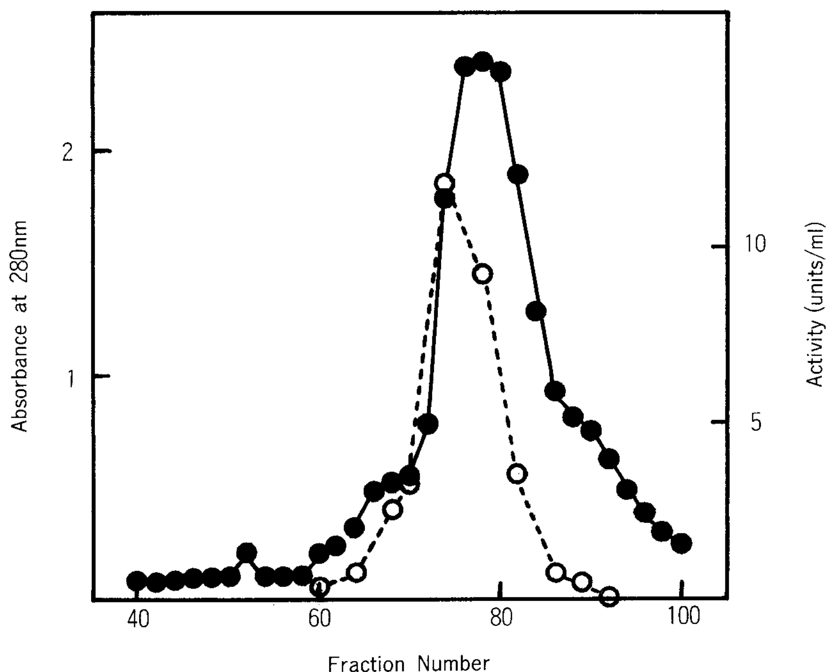

Fig. 3. Sephacryl S-200 HR column chromatography of wall-bound α-glucosidase from sugar-beet cells. The column was eluted with 20 mM acetate buffer, pH 4.5, containing 150 mM sodium chloride. The eluate was collected in 5.7 ml fractions. ●——●, Absorbance at 280 nm ; ○---○, α-glucosidase activity.

after a 60-min incubation. After a 15-min preincubation with 50 mM acetate buffer, pH 4.5, the enzyme was stable up to 50 ℃. The thermostability of the

Table 1. Summary of purification of wall-bound α-glucosidase from suspension-cultured sugar-beet cells

Procedure	Total protein (mg)	α-Glucosidase activity: Total activity (U)	Specific activity (U/protein, mg)	Yield (%)
Sephacryl S-200 HR chromatography	240	738.0	3.1	100
CM-cellulose chromatography	17.7	407.0	23.0	55.1

wall-bound enzyme was similar to those of the others of sugar-beet cells[15], as in rice cells[14], although a greater thermostability of wall-bound enzymes was reported for invertases[1, 5] and β-galactosidase[9].

3. *Substrate specificity*

The activity of the enzyme on various substrates was examined. Table 2 shows the relative rates of hydrolysis. The enzyme readily hydrolyzed maltose, nigerose, malto-oligosaccharides, and soluble starch, but hydrolyzed isomaltose weakly. For enzyme assays, we measured the amount of glucose liberated from the substrate as described in MATERIALS AND METHODS; that is, the hydrolysis values of maltose, nigerose and isomaltose were twice as high as those of the other substrate for the same degree of hydrolysis. Therefore, the result shows that the enzyme has higher substrate specificity for malto-oligosaccharide and soluble starch than

Table 2. Substrate specificity of wall-bound α-glucosidase from suspension-cultured sugar-beet cells

	Relative rate of hydrolysis (%)
Maltose	100
Isomaltose	14.1
Nigerose	70.6
Maltotriose	84.8
Maltotetraose	87.1
Maltopentaose	87.1
Maltoheptaose	110.9
Soluble starch	74.4

maltose. The four α-glucosidases from sugar-beet cells are divisible into two groups in terms of soluble starch and isomaltose hydrolyzing activities[15]. One group readily hydrolyzes soluble starch and isomaltose.

It hydrolyzes soluble starch at a faster rate than maltose as does the wall-bound enzyme from sugar-beet cells. Two α-glucosidases from sugar-beet seeds also exhibit very different properties in their soluble starch and isomaltose hydrolyzing activities as described above[13]. Therefore, the wall-bound enzyme from sugar-beet cells is similar to the enzymes extracted from the cells and seeds with buffer and NaCl in soluble starch-hydrolyzing activity, but is different from them in isomaltose-hydrolyzing activity.

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テンサイ培養細胞の細胞壁結合 α-グルコシダーゼの精製と性質

山崎良樹・今野晴義

テンサイ培養細胞には数種の α-グルコシダーゼが存在している．相等量の α-グルコシダーゼ活性は細胞破壊後，緩衝液と食塩で抽出されるが，まだかなりの α-グルコシダーゼ活性が細胞残渣に含まれたままである．その酵素は，界面活性剤や S-S 結合切断試薬では遊離されなかったが，細胞壁分解酵素により可溶化された．テンサイ培養細胞にスミチーム C とペクトリアーゼ Y-23を作用させると，細胞はプロトプラストになり，細胞壁由来の成分のみを分離できた．可溶化された部分から α-グルコシダーゼを硫安分画，セファクリル S-200 HR カラムクロマトグラフィー，CM-セルロースカラムクロマトグラフィーにより精製した．本酵素は，マルトース，ニゲロース，マルトオリゴ糖，可溶性澱粉によく作用したが，それらに比べイソマルトースに対する作用は弱かった．本酵素は，マルトオリゴ糖と可溶性澱粉にマルトースよりも強く作用した．本酵素以外にテンサイ培養細胞とテンサイ種子から数種類の α-グルコシダーゼが単離されているが，本酵素の基質特異性はそれらのものと異なっていた．

キーワード：*Beta valgaris* L. cv. Tsukisappu，テンサイ，細胞壁結合酵素，α-グルコシダーゼ，プロトプラスト