岡大資生研報 (Bull. Res. Inst. Bioresour. Okayama Univ.) 5 : 1-9 (1997)

Change in the Multiple Forms of α-Glucosidase from Spinach Seeds

Satoshi FURUI, Manabu SUGIMOTO and Yukio SUZUKI

Two molecular forms of α-glucosidase were isolated from spinach seeds after storage at 4℃ by CM-cellulose column chromatography and gel filtration. The molecular masses of α-glucosidase A and B were 78 kDa and 82 kDa by SDS-PAGE, and 62 kDa and 70 kDa by gel filtration, respectively. α-Glucosidase A had high activity not only toward malto-oligosaccharides but also toward α-glucans. The optimum pH was 4.5-5.5 and about 50% of the activity remained after incubation at 65℃ for 20 min. On the other hand, α-glucosidase B had high activity toward malto-oligosaccharides but faint activity toward α-glucans. The optimum pH was 5.0 and no activity was found after incubation at 65℃ for 20 min. The enzymatic and immunological properties of α-glucosidase A and B were similar to those of α-glucosidase I or II, and α-glucosidase III or IV, isolated from spinach seeds without 4℃ storage, respectively. These findings suggest that the α-glucosidase in spinach seeds is modified to be two molecular forms.

Key words : Spinach, α-Glucosidase, Multiple molecular forms.

INTRODUCTION

Multiplicity of carbohydrases has been reported in many plants and explained by genetic variation[2,8,11], proteolytic modification of polypeptide chain[5,14], or carbohydrate modification of polypeptide chain[10]. α-Glucosidase [EC 3. 2. 1. 20] is an exo-carbohydrase, which catalyzes the splitting of α-glucosyl residue from the nonreducing terminal of substrate to liberate α-glucose[4,16]. Multiple molecular forms of α-glucosidase with different substrate specificity have been found in plants[6,15,17,18]. Previously, we separated four molecular forms of α-glucosidase in spinach seeds by

Research Institute for Bioresources, Okayama University, Kurashiki 710, Japan
Received October 30, 1996

several kinds of chromatography[12]. Two of them had relatively high activity not only toward malto-oligosaccharides but also toward soluble starch, and the others had high activity toward malto-oligosaccharides but faint activity toward soluble starch. However, whether they are derived from genetic variation or post-translational modification remains unknown.

The four molecular forms of α-glucosidase might be modified into two molecular forms in spinach seeds. We isolated two molecular forms of α-glucosidase from spinach seeds after storage at 4℃ and compared their properties with those of the four α-glucosidases isolated from spinach seeds without storage to evaluate their differences.

MATERIALS AND METHODS

1. *Enzyme and protein assays*

Enzyme assay was performed as described previously[13]. The liberated glucose was assayed by the Tris-glucose oxidase-peroxidase method with a Glucose AR-II Test (Wako Pure Chemical Ind., Ltd.). Protein was measured by the method of Bradford[1] with bovine serum albumin as a standard.

2. *Estimation of molecular mass*

The molecular mass of the purified enzymes was estimated by SDS-PAGE[7] and gel filtration. SDS-PAGE was done on 10% acrylamide, and protein bands were stained with Coomassie brilliant blue R-250. The gel filtration was carried out by HPLC on a TSK_{gel} $G3000SW_{XL}$ column (0.78 × 30 cm, TOSOH Co., Ltd.) equilibrated with 0.05 M acetate buffer (pH 5.3) containing 0.2 M NaCl. The flow rate was 0.5 ml/min and the elution was monitored by absorption at 280 nm.

3. *pH-Activity, pH-stability, and temperature stability*

pH-Activity and pH-stability were measured from pH 2.0 to 9.0. The reaction mixture for pH-activity, which consisted of 0.2 ml of 0.5% maltose, 0.1 ml of enzyme solution (5 m units), and 0.2 ml of Britton-Robinson buffer, was incubated at 37℃ for 10 min. The reaction mixture for pH-stability, which consisted of 0.02 ml of enzyme solution (5 m units) and 0.08 ml of Britton-Robinson buffer, was kept at 4℃ for 24 h, then 0.2 ml of 0.5% maltose and 0.2 ml of 0.1 M acetate buffer (pH 5.0) were added and incubated at 37℃ for 10 min. Temperature stability was measured between 30 and 75℃. The mixture, which consisted of 0.1 ml of enzyme solution (5 m units) and 0.2 ml of 0.1 M acetate buffer (pH 5.0), was kept for 20 min, left to cool on ice, and after addition of 0.2 ml of 0.5 % maltose incubated at 37℃ for 10 min.

4. *Preparation of anti-serum against α-glucosidases*

The purified α-glucosidase A or B was mixed with Freund's complete adjuvant and the emulsified mixture was used to immunize a male rabbit by subcutaneous injection. Two weeks later, the emulsified mixture was injected into the rabbit again. Anti-serum was prepared as described previously[12].

5. *Preparation of enzyme extract*

Spinach seeds (*Spinacia oleracea* L. cv. Dash) obtained from Yamato Plantation Co., Ltd. (Nara, Japan) were stored at 4℃ for 4 months. The seeds (1 kg) were disrupted and extracted as described previously[12]. Solid ammonium sulfate was added to the filtrate to 85% saturation. The precipitate was collected by centrifugation, dissolved in 0.01 M acetate buffer (pH 5.3) containing 0.05 M NaCl, and dialyzed overnight against the same buffer at 4℃. The precipitate was removed by centrifugation and the supernatant was used as the enzyme extract.

6. *CM-cellulose column chromatography (1st)*

The enzyme extract was put on a CM-cellulose column (5.6 × 18 cm) equilibrated with 0.05 M acetate buffer (pH 5.3) containing 0.05 M NaCl. The column was washed with the same buffer and absorbed proteins were eluted with 0.3 M NaCl and 0.5 M NaCl in the same buffer. The enzyme activity was found in the fractions eluted with 0.3 M and 0.5 M NaCl. The two enzyme fractions were collected separately and concentrated by ultrafiltration through a UK-50 membrane (Advantec Toyo Co., Ltd.).

7. *CM-cellulose column chromatography (2nd)*

The enzyme solution eluted with 0.3 M NaCl on CM-cellulose column (1st) was dialyzed against 0.05 M acetate buffer (pH 5.3) and put on a CM-cellulose column (5.6 × 15 cm) equilibrated with 0.05 M acetate buffer (pH 5.3). The column was washed with the same buffer and absorbed proteins were eluted with a linear gradient from 0 M to 0.3 M NaCl in the same buffer. The enzyme fractions were collected, concentrated by Ultracent-30, and dialyzed against 0.05 M acetate buffer (pH 5.3) containing 0.05 M NaCl.

The enzyme solution eluted with 0.5 M NaCl on a CM-cellulose column (1st) was dialyzed against 0.05 M acetate buffer (pH 5.3) containing 0.3 M NaCl and put on a CM-cellulose column (5.6 × 15 cm) equilibrated with 0.05 M acetate buffer (pH 5.3) containing 0.3 M NaCl. The column was washed with the same buffer and absorbed proteins were eluted with a linear gradient from 0.3 M to 0.5 M NaCl in the same buffer. The enzyme fractions were collected, concentrated by Ultracent-30, and dialyzed against 0.05 M acetate buffer (pH 5.3) containing 0.05 M NaCl.

8. *Purification of α-glucosidase A and B*

The enzyme solutions eluted with a 0 M to 0.3 M NaCl gradient (α-glucosidase A) and eluted with a 0.3 M to 0.5 M NaCl gradient (α-glucosidase B) on CM-cellulose column (2nd), respectively, were put on a Toyopearl HW-55S column (2 × 70 cm) (TOSOH Co., Ltd.) equilibrated with 0.05 M acetate buffer (pH 5.3) containing 0.05 M NaCl. The enzyme fractions were collected, concentrated by Ultracent-30 (TOSOH Co., Ltd.), and rechromatographed to purify them.

RESULTS

1. *Purification of α-glucosidases*

Two α-glucosidases were purified from spinach seeds after storage at 4℃ for 4 months. By CM-cellulose column chromatography, two enzyme fractions, one eluted with 0.3 M NaCl (α-glucosidase A) and the other with 0.5 M NaCl (α-glucosidase B) were obtained. The purification procedure is summarized in Table 1. α-Glucosidases A and B were purified 6.06- and 26.9-fold, and the ratios of maltose-hydrolyzing activity *versus* soluble starch-hydrolyzing activity were 0.42 and 18.8, respectively. The purified enzymes were homogeneous by SDS-PAGE (data not shown).

Table 1. Purification of α-glucosidases from stored spinach seeds

Step	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)	Ratio[b]
Ammonium sulfate	2550	5040	1.98	1	100	2.14
CM-cellulose (1st)						
α-Glucosidase A	430	428	1.00	0.50	8.49	0.47
α-Glucosidase B	486	3020	6.21	3.14	59.9	5.33
CM-cellulose (2nd)						
α-Glucosidase A	82.2	270	3.28	1.66	5.36	0.41
α-Glucosidase B	160	2170	13.6	6.87	43.1	13.0
Gel filtration						
α-Glucosidase A	18.7	225	12.0	6.06	4.46	0.42
α-Glucosidase B	21.8	1160	53.2	26.9	23.0	18.8

[a] Maltose-hydrolyzing activity.
[b] Maltose-hydrolyzing activity/soluble starch-hydrolyzing activity.

2. *Properties of α-glucosidases*

The molecular masses estimated by SDS-PAGE were 78 kDa for α-glucosidase A and 82 kDa for α-glucosidase B, respectively. The molecular masses estimated by gel filtration were 62 kDa for α-glucosidase A and 70 kDa for α-glucosidase B, respectively.

The optimum pHs on hydrolysis of maltose were 4.5-5.5 and 5.0 for α-glucosidases A and B, respectively. Both enzymes were stable between pH 3.0 and 9.0. After treatment at 65℃ for 20 min in acetate buffer (pH 5.0), α-glucosidase A retained 52% of the activity, but α-glucosidase B lost activity. The effects of pH and temperature on hydrolysis of soluble starch for α-glucosidase A and B were similar to those on hydrolysis of maltose.

Table 2. Substrate specificities of α-glucosidases from stored spinach seeds. The reaction mixture (0.5 ml), which consisted of 0.2% substrate and enzyme (5 mU), was incubated at 37℃ for 10 min. Relative rates to maltose-hydrolyzing activity are shown.

Substrate	Relative activity (%)	
	α-Glucosidase	
	A	B
Maltose	100	100
Maltotriose	272	135
Maltotetraose	292	131
Maltopentaose	401	63
Soluble starch	239	1
Isomaltose	35	1
Pheny-α-D-glucoside	12	0
Methyl-α-D-glucoside	3	0
Sucrose	1	0

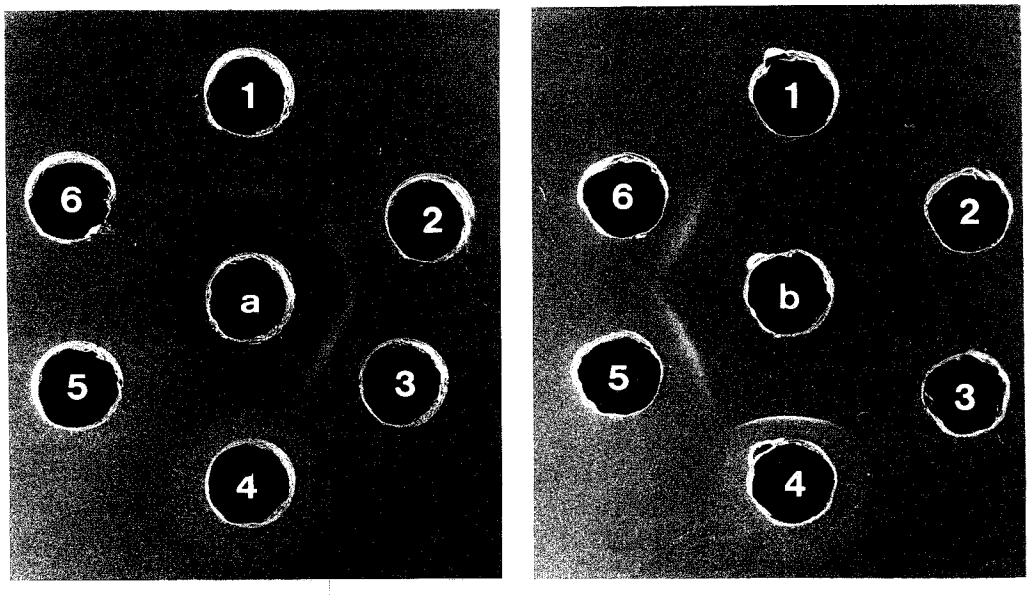

Fig. 1. Ouchterlony double diffusion of the purified α-glucosidases from spinach seeds. The center wells contained 30 μl of anti-α-glucosidase A-serum (a) and anti-α-glucosidase B-serum (b), respectively. The surrounding wells 1, 2, 3, 4, 5, and 6 contained 3.5 μg of α-glucosidase I, A, II, III, B, and IV, respectively.

The hydrolytic activities on various α-glucosides were examined and compared with those on maltose (Table 2). α-Glucosidase A had higher hydrolytic activity on maltotriose, -tetraose, and -pentaose than on maltose, and showed the highest activity on maltopentaose. α-Glucosidase A hydrolyzed isomaltose and phenyl-α-D-glucoside, but the activities on methyl-α-D-glucoside and sucrose were less than 10% of that on maltose. The hydrolytic activity of α-glucosidase B was the highest on maltotriose, and those on maltotriose to -pentaose decreased with the increase in the degree of glucose polymerization. The activities on soluble starch, isomaltose, phenyl-α-D-glucoside, methyl-α-D-glucoside, and sucrose were less than 1% of that on maltose.

The immunological comparison of the enzymes was done by Ouchterlony double diffusion[9]. Anti-α-glucosidase A-serum formed a precipitation line with α-glucosidase A, I, and II, and anti-α- glucosidase B-serum formed a precipitation line with α-glucosidase B, III, and IV (Fig. 1).

DISCUSSION

Previously, we isolated four molecular forms of α-glucosidase in spinach seeds[12]. α-Glucosidases I and II belong to α-glucosidase group III which hydrolyzes not only homogeneous malto-oligosaccharides but also α-glucans, and α-glucosidase III and IV belong to α-glucosidase group II which hydrolyzes homogeneous malto-oligosaccharides preferentially but not synthetic α-glucosides or sucrose[3]. However, only two α-glucosidases (α-glucosidase A and B) were separated from the seeds after storage at 4℃. Table 3 summarizes the properties of six forms of α-glucosidase in spinach seeds. α-Glucosidase A, I, and II showed the same molecular masses by SDS-PAGE and gel filtration, respectively. Soluble starch-hydrolyzing activity was about 2 times higher than maltose- hydrolyzing activity.

Table 3. Comparison of properties of α-glucosidases from spinach seeds

α-Glucosidase	Molecular mass by		Ratio[a]	Immuno-precipitation with
	SDS-PAGE	gel filtration		
I	78 kDa	62 kDa	0.54	anti-α-glucosidase A-serum
II	78 kDa	62 kDa	0.50	anti-α-glucosidase A-serum
III	82 kDa	190 kDa	16.1	anti-α-glucosidase B-serum
IV	82 kDa	70 kDa	17.8	anti-α-glucosidase B-serum
A	78 kDa	62 kDa	0.42	anti-α-glucosidase A-serum
B	82 kDa	70 kDa	18.8	anti-α-glucosidase B-serum

[a] Maltose-hydrolyzing activity/soluble starch-hydrolyzing activity.

Anti-α-glucosidase A-serum formed a precipitation line with α-glucosidases I and II. The difference between these three enzymes is that α-glucosidase A is eluted with 0.3 M NaCl on a CM-cellulose column and α-glucosidase I and II do not bind to the CM-cellulose column.

α-Glucosidase B, III, and IV showed the same molecular mass by SDS-PAGE. Maltose-hydrolyzing activity was about 18 times higher than soluble starch-hydrolyzing activity. The molecular mass of α-glucosidase III determined by gel filtration was different from those of α-glucosidase B and IV, and anti-α-glucosidase III-serum formed a precipitation specifically with α-glucosidase III[12]. However, anti-α-glucosidase B-serum formed a precipitation with α-glucosidase III and IV. These findings suggest that α-glucosidase III is a dimer or trimer of a polypeptide which has the same molecular conformation as α-glucosidase B or IV.

We analyzed the elution profile of α-glucosidase on a CM-cellulose column extracted from spinach seeds harvested in different seasons. Some of them showed the same elution profile as that of the stored seeds described. Therefore, α-glucosidase A might be derived from certain modification of α-glucosidase I and II, and α-glucosidase B might be derived from certain modification of α-glucosidase III and IV. The N-terminal amino acid sequence of α-glucosidase A and B could not be compared because they were blocked. Molecular cloning is in progress to investigate whether α-glucosidase A and B in spinach seeds is caused by genetic variation or post-translational modification.

Acknowledgments-This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan, and by a grant of the Ohara Foundation in Kurashiki with the purpose of promoting the advancement of agricultural sciences.

REFERENCES

1. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 : 248-254.
2. Brown, A. H. D. and Jacobsen, J. V. 1982. Genetic basis and natural variation of α-amylase isozymes in barley. Genet. Res. Camb. 40 : 315-324.
3. Chiba, S. 1980. Action and classification of α-glucosidase. J. Jpn. Soc. Starch Sci. 27 : 84-90.
4. Chiba, S., Hiromi, K., Minamiura, N., Ohnishi, M., Shimomura, T., Suga, K., Suganuma, T., Tanaka, A., Tomioka, S. and Yamamoto, T. 1979. Quantitative study on anomeric forms of glucose produced by α-glucosidase. J. Biochem. 85 : 1135-1141.
5. Hayashida, S., Nomura, T., Yoshino, E. and Hongo, M. 1976. Formation of active derivatives of

glucoamylase I during the digestion with fungal acid protease and α-mannosidase. Agric. Biol. Chem. 40 : 141-146.

6. Konishi, Y., Kitazato, S. and Nakatani, N. 1992. Partial purification and characterization of acid and neutral α-glucosidases from preclimacteric banana pulp tissues. Biosci. Biotech. Biochem. 56 : 2046-2051.
7. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacterio phage T4. Nature 227 : 680-685.
8. Mikami, B., Aibara, S. and Morita, Y. 1982. Distribution and properties of soybean β-amylase isozymes. Agric. Biol. Chem. 46 : 943-953.
9. Ouchterlony, O. 1967. In "Handbook of Experimental Immunology," Weir, D. W. ed. 463-492. Blackwell Scientific Publications, Oxford.
10. Pazur, J. H., Knull, H. R. and Cepure, A. 1971. Glycoenzymes : Structure and properties of the two forms of glucoamylase from *Aspergillus niger*. Carbohyd. Res. 20 : 83-96.
11. Shewry, P. R., Parmar, S., Buxton, B., Gale, M. D., Liu, C. J., Hejgaard, J. and Kreis, M. 1988. Multiple molecular forms of β-amylase in seeds and vegetative tissues of barley. Planta 176 : 127-134.
12. Sugimoto, M., Furui, S. and Suzuki, Y. 1995. Multiple molecular forms of α-glucosidase from spinach seeds, *Spinacia oleracea* L. Biosci. Biotech. Biochem. 59 : 673-677.
13. Sugimoto, M. and Suzuki, Y. 1993. Subsite affinities of α-glucosidase from *Mucor javanicus*. Biosci. Biotech. Biochem. 57 : 1378-1379.
14. Takahashi, T., Tsuchida, Y. and Irie, M. 1978. Purification and some properties of three forms of glucoamylase from a *Rhizopus* species. J. Biochem. 84 : 1183-1194.
15. Yamasaki, Y. and Suzuki, Y. 1979. Purification and properties of three forms of α-glucosidase from germinated green gram (*Phaseolus vidissimus* Ten.). Agric. Biol. Chem. 43 : 481-489.
16. Yamasaki, Y. and Suzuki, Y. 1980. α-Glucosidase and glucoamylase from molds. J. Jpn. Soc. Starch Sci. 27 : 74-83.
17. Yamasaki, Y. and Suzuki, Y. 1980. Two forms of α-glucosidase from sugar-beet seeds. Planta 148 : 354-361.
18. Yamasaki, Y. and Suzuki, Y. 1980. Two forms of α-glucosidase from rice seeds at the milky stage. Agric. Biol. Chem. 44 : 707-715.

ホウレンソウ種子に存在するα-グルコシダーゼの分子多型変化

古井　聡・杉本　学・鈴木幸雄

4℃保存したホウレンソウ種子から2種のα-グルコシダーゼをCM-セルロースカラムクロマトグラフィーとゲル濾過により精製した．α-グルコシダーゼAとBの分子量はそれぞれSDS-PAGEで78 kDa，82 kDa，ゲル濾過で62 kDa，70 kDaであった．α-グルコシダーゼAはマルトオリゴ糖だけでなく可溶性デンプンに対して強い加水分解活性を示した．至適pHは4.5-5.5であり，65℃，20分処理後に約50%の残存活性を示した．一方，α-グルコシダーゼBはマルトオリゴ糖に対して強い加水分解活性を示したが，可溶性デンプンに対してほとんど活性を示さなかった．至適pHは5.0であり，65℃，20分処理後に活性を消失した．α-グルコシダーゼAとα-グルコシダーゼBは，4℃保存を行わない種子に見出されたα-グルコシダーゼI，IIとα-グルコシダーゼIII，IVにそれぞれ酵素化学的性質，免疫化学的性質が類似していた．これらの結果，ホウレンソウ種子に存在するα-グルコシダーゼの分子多型は2種類に収束されることが示唆された．

キーワード：ホウレンソウ，α-グルコシダーゼ，分子多型