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Effect of deglycosylation on the properties of β-fructofuranosidase *P*-1 from *Aureobasidium* sp. ATCC 20524

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Key words: β-Fructofuranosidase; Deglycosylation; *Aureobasidium*; Enzymatic stability

SUMMARY

Most of the carbohydrate moiety of β-fructofuranosidase *P*-1 from *Aureobasidium* sp. ATCC 20524 was removed by endo-β-*N*-acetylglucosaminidase F. A subunit of 94000 Da was observed in SDS-PAGE after deglycosylation. The K_m value for sucrose was not changed by deglycosylation but the stability at pH 4–5 and 50 °C was decreased. The deglycosylated enzyme was more sensitive to proteases such as pronase E and subtilisin than the native enzyme. It is considered that the carbohydrate moiety of β-fructofuranosidase *P*-1 contributes to the stability of the enzyme but is not essential in its catalytic function.

INTRODUCTION

Deglycosylation of certain enzymes has been carried out to investigate the significance of their carbohydrate moieties. Wang et al. [8] released the carbohydrate moiety of porcine pancreatic ribonuclease by a mixture of neuraminidase; β-galactosidase, α-mannosidase and β-*N*-acetylglucosaminidase and suggested the involvement of the carbohydrate moiety in the stabilization of surface conformation. Chu et al. [1] deglycosylated yeast invertase by endo-β-*N*-acetylglucosaminidase and showed that the deglycosylated enzyme was more sensitive to proteases. Takahashi et al. [6] suggested that the carbohydrate moiety of Taka-amylase A is not an essential participant in the catalytic activity. Yamamoto et al. [9] reported a decrease in thermal stability and an increased susceptibility to proteolysis with β-*N*-acetylhexosaminidase after deglycosylation. Takegawa et al. [7] and Maruyama et al. [5] reported the deglycosylation of glucoamylase and phospolipase B, respectively. There is, however, no report on the deglycosylation of β-fructofuranosidase which has high fructosyl-transferring activity.

In the present paper, we describe the effect of deglycosylation on the properties of β-fructofuranosidase from *Aureobasidium* sp. [2,3].

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MATERIALS AND METHODS

Microorganism and cultivation. Cultivation of *Aureobasidium* sp. ATCC 20524 for β-fructofuranosidase *P*-1 production was carried out in liquid culture (Sucrose 20%, yeast extract 2%, $NaNO_3$ 1%, K_2HPO_4 0.75%, $MgSO_4 \cdot 7H_2O$ 0.1%; pH 6.5–7) at 30 °C for 48 h as in previous reports [2,3]. Cells were harvested by centrifugation and lyophilised.

Purification of β-*fructofuranosidase P-1.* β-Fructofuranosidase *P*-1 was solubilised from dry cells by Kitalase (Wako) in McIlvain buffer (pH 5) at 40 °C for 2 h and then centrifuged [3]. β-Fructofuranosidase *P*-1 was purified to homogeneity by fractionations involving ethanol, $(CH_3COO)_2Ca$ and $(NH_4)_2SO_4$ and DEAE-Cellulofine A-800 and Sephadex G-200 column chromatography as described previously [3].

Endo-β-*N-acetylglucosaminidase treatment.* Purified β-fructofuranosidase *P*-1 (0.2 mg protein) was deglycosylated by 0.5 U endo-β-*N*-acetylglucosaminidase F (Sigma) in 40 μl of 25 mM acetate buffer (pH 5) at 37 °C for 12 h.

The molecular weight of deglycosylated β-fructofuranosidase *P*-1 was estimated by Sephadex G-200 gel filtration as described previously [3].

Electrophoresis. Sodium dodecyl sulfate–polyacryl-

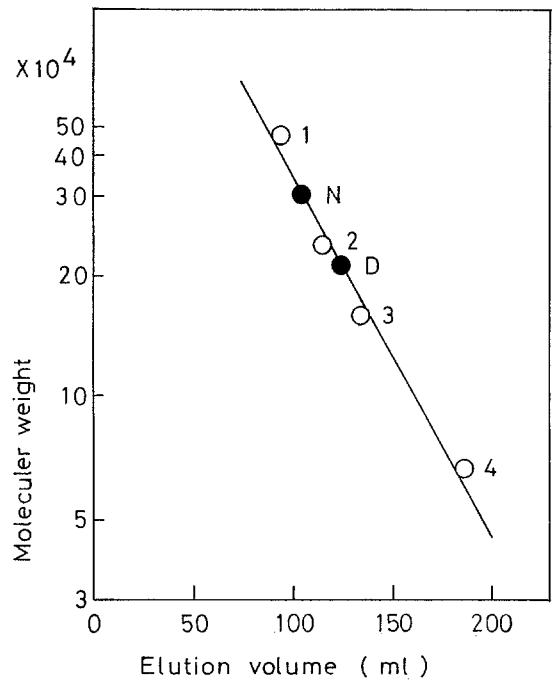

Fig. 1. Plot of logarithm of molecular weight of proteins against elution volume on Sephadex G-200. N, native β-fructofuranosidase *P*-1. D, deglycosylated β-fructofuranosidase *P*-1. Standard proteins: 1, ferritin (450000); 2, catalase (240000); 3, aldolase (158000); 4, albumin (bovine serum, 68000).

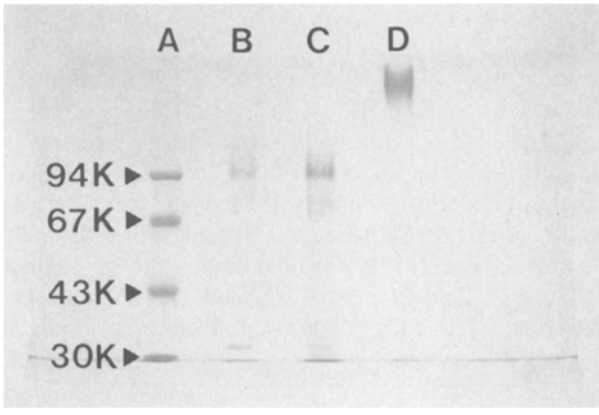

Fig. 2. SDS-PAGE of deglycosylated and native β-fructofuranosidase *P*-1. Lane A: molecular weight markers. Lanes B and C: deglycosylated β-fructofuranosidase *P*-1. Lane D: native β-fructofuranosidase *P*-1. Molecular weight markers: phosporylase b (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000). The endo-β-*N*-acetylglucosaminidase F protein band was detected at a position corresponding to a molecular weight of near 30000.

amide slab gel electrophoresis (SDS-PAGE) was carried out in 7.5% (w/v) acrylamide and 0.1% (w/v) SDS with a discontinuous Tris-glycine buffer system by the method of Laemmli [4]. After electrophoresis, the gel was stained with commassie blue. The following proteins were used as molecular weight markers: phosphorylase b (94000), bovine serum albumin (67000), ovalbumin (43000) and carbonic anhydrase (30000).

Protease digestion. Digestion of deglycosylated and native β-fructofuranosidase *P*-1 by proteases such as pronase E (Sigma) and subtilisin (Sigma) was carried out as follows. The reaction mixture employed 5 U of the enzyme (deglycosylated or native β-fructofuranosidase *P*-1) and 1 mg protease (pronase E 4.4 U, subtilisin 11.2 U) in 1 ml 40 mM Tris-HCl buffer (pH 7.5) and digestion was carried out at 37 °C for 90 min.

β-Fructofuranosidase activity assay. The enzymatic activity was assayed in a mixture consisting of 0.1 ml of enzyme solution (deglycosylated or native β-fructofuranosidase *P*-1), 0.4 ml of 75% sucrose, 0.5 ml of 0.15 M McIlvain buffer (pH 5) at 50 °C for 20 min using high performance liquid chromatography (HPLC) and glucose oxidase methods as described in a previous paper [3]. One unit of enzyme activity was defined as the quantity of enzyme responsible for the transfer of 1 μmol of fructose in 1 min.

Chemicals. All chemicals were of the highest grade commercially available.

ESULTS AND DISCUSSION

ndo-β-N-acetylglucosaminidase treated β-fructofurano-dase P-1

The molecular weight of deglycosylated β-fructoranosidase *P*-1 was determined by the Sephadex G-200 l filtration method. As shown in Fig. 1, the molecular eight of native β-fructofuranosidase *P*-1 (318000), with carbohydrate content of 30% (w/w) [3], was decreased 214000 Da. The decrease in molecular weight reflects e removal of carbohydrate from native β-fructofura-osidase *P*-1 by endo-β-*N*-acetylglucosaminidase F. No igar was detected in the fraction of deglycosylated β-uctofuranosidase *P*-1. The above results suggest that ost of the carbohydrate moiety of β-fructofuranosidase *P*-1 was released by endo-β-*N*-acetylglucosaminidase F.

SDS-PAGE of deglycosylated and native β-fructofuranosidase *P*-1 is shown in Fig. 2. It shows a decrease in molecular weight due to the removal of sugar chains and a main protein band, a subunit of 94000, appeared in SDS-PAGE after deglycosylation. This result suggests that the carbohydrate moiety of β-fructofuranosidase *P*-1 was involved in subunits linkage.

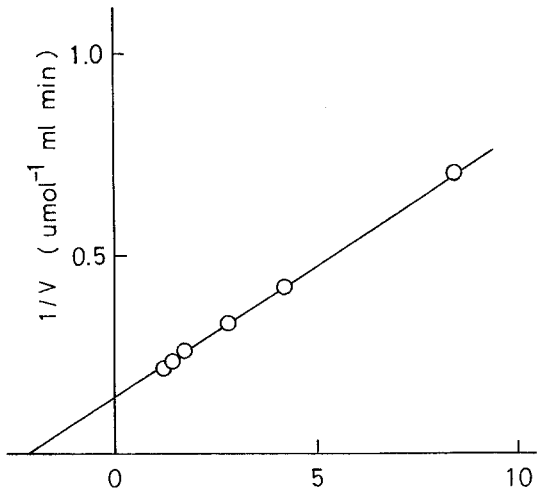

Fig. 3. Lineweaver-Burk plot of deglycosylated β-fructofuranosidase *P*-1 for the determination of K_m and V_{max} for sucrose.

Kinetic properties of the deglycosylated β-fructofuranosidase P-1

The enzymatic activity of deglycosylated β-fructofuranosidase *P*-1 was compared with that of the native enzyme. A Lineweaver-Burk plot of deglycosylated β-fructofuranosidase *P*-1 is shown in Fig. 3. The K_m and V_{max} values for sucrose of deglycosylated β-fructofuranosidase *P*-1 were 0.44 M and 6.84 μmol/ml per min, respectively. These values were similar to those of native β-fructofuranosidase *P*-1 (0.47 M and 6.84 μmol/ml per min) [3]. In contrast, the K_m value of carbohydrate-depleted phospholipase B was lower than that of the native enzyme [5]. The above results suggest that the carbohydrate moiety of β-fructofuranosidase *P*-1 is not essential in catalysis as in the case of Taka-amylase A [6].

Effects of pH and temperature on the properties of deglycosylated β-fructofuranosidase P-1

The effect of pH on the activity of deglycosylated β-fructofuranosidase *P*-1 is shown in Fig. 4. The optimum pH was slightly more alkaline than the that of native β-fructofuranosidase *P*-1 after deglycosylation. At pH 4, deglycosylated β-fructofuranosidase *P*-1 showed only 9% activity of the activity at optimum pH while native β-fructofuranosidase *P*-1 retained 71% [3].

The effect of pH on the stability of deglycosylated β-fructofuranosidase *P*-1 is shown in Fig. 5. The stability of β-fructofuranosidase *P*-1 at pH 4–5 was decreased by deglycosylation. Deglycosylated β-fructofuranosidase *P*-1 retained only 24% of the activity at optimum pH while native β-fructofuranosidase *P*-1 retained 92% [3].

Remarkable effects of pH on the properties of deglycosylated β-fructofuranosidase *P*-1 were observed especially at acidic pH.

The effect of temperature on the activity of deglycosylated β-fructofuranosidase *P*-1 is shown in Fig. 6. The optimum temperature was slightly lower than that of native β-fructofuranosidase *P*-1 after deglycosylation.

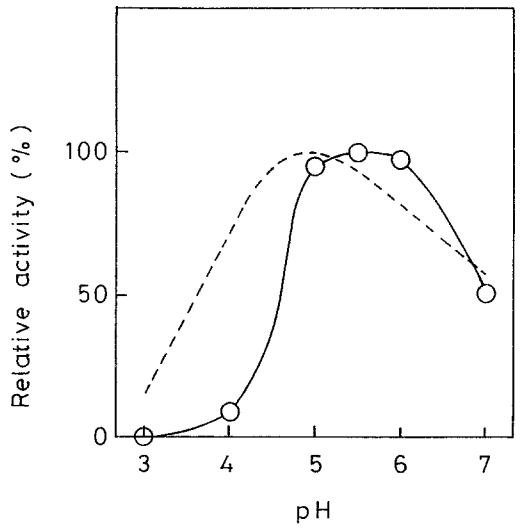

Fig. 4. Effect of pH on the activity of deglycosylated β-fructofuranosidase *P*-1. (○) deglycosylated β-fructofuranosidase *P*-1; (---) native β-fructofuranosidase *P*-1.

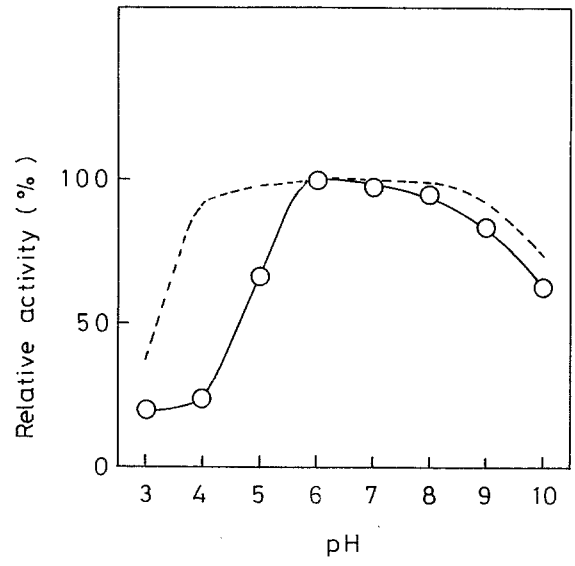

Fig. 5. Effect of pH on the stability of deglycosylated β-fructofuranosidase *P*-1. The activities were measured after 3 h incubation at each pH (pH 3–8, McIlvain buffer; pH 8–10, Michaelis buffer) to determine stability of β-fructofuranosidase *P*-1. (○) deglycosylated β-fructofuranosidase *P*-1; (---) native β-fructofuranosidase *P*-1.

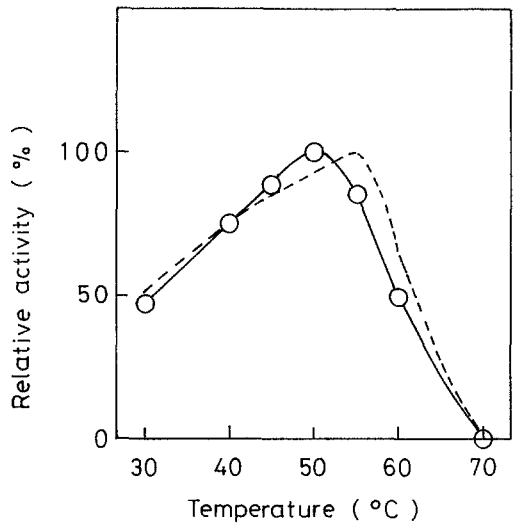

Fig. 6. Effect of temperature on the activity of deglycosylated β-fructofuranosidase *P*-1. (○) deglycosylated β-fructofuranosidase *P*-1; (– – –) native β-fructofuranosidase *P*-1.

Thermal stability at 50 °C of the deglycosylated and native β-fructofuranosidase *P*-1 are shown in Fig. 7. Thermal stability of native β-fructofuranosidase *P*-1 was decreased by deglycosylation. The deglycosylated β-fructofuranosidase *P*-1 retained only 20% of its original activity while native β-fructofuranosidase *P*-1 retained 49% after 60 min. The decrease in thermal stability by deglycosylation was observed with glucoamylase [7] and phospholipase B [5] but not yeast invertase [9]. The above

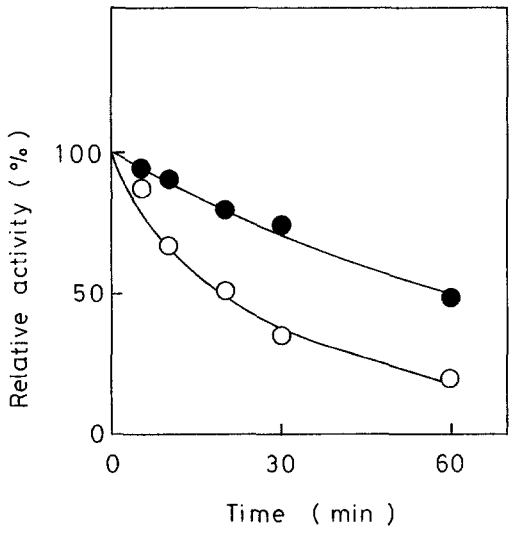

Fig. 7. Thermal stability of deglycosylated β-fructofuranosidase *P*-1 at 50 °C. (○) deglycosylated β-fructofuranosidase *P*-1; (●) native β-fructofuranosidase *P*-1.

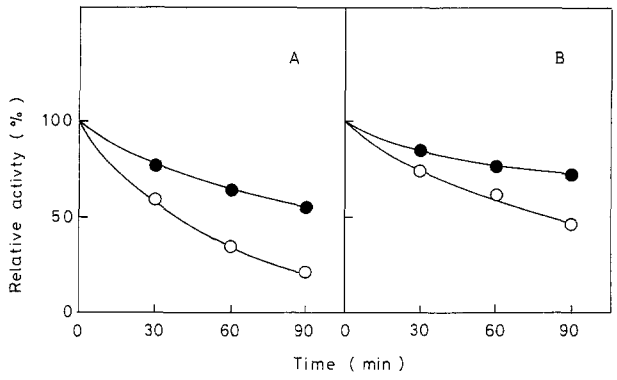

Fig. 8. Time course of protease digestion ((A) pronase E; (B) subtilisin) of deglycosylated and native β-fructofuranosidase *P*-1. (○ deglycosylated β-fructofuranosidase *P*-1; (●) native β-fructofuranosidase *P*-1.

results suggest that the carbohydrate moiety contributes to the thermal stability of β-fructofuranosidase *P*-1.

Protease digestion of β-*fructofuranosidase P-1*

Deglycosylated and native β-fructofuranosidase *P*-1 were digested by proteases such as pronase E and subtilisin (Fig. 8). Deglycosylated and native β-fructofuranosidase *P*-1 retained 22% and 56% of their original activity after pronase E digestion for 90 min, respectively. (Fig. 8A). After subtilisin digestion, deglycosylated and native β-fructofuranosidase *P*-1 retained 48% and 73% of their original activity, respectively (Fig. 8B). Deglycosylated β-fructofuranosidase *P*-1 was more susceptible to these proteases than the native enzyme. From the above results, it is concluded that the carbohydrate moiety plays a role in protecting the enzyme from proteolysis as in the cases of many other enzymes such as porcine pancreatic ribonuclease [8], yeast invertase [9], β-*N*-acetylhexosaminidase [9] and glucoamylase [7]. It is concluded that the carbohydrate moiety of β-fructofuranosidase *P*-1 is important for stabilization of the enzyme but not for catalytic activity in these experiments.

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