Journal of Industrial Microbiology, 10 (1992) 191–194

Published by Elsevier

SIM 00443

Properties of the deglycosylated β-fructofuranosidase *P*-2 from *Aureobasidium* sp. ATCC 20524

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(Received 30 April 1992; revision received 12 June 1992; accepted 29 June 1992)

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

SUMMARY

The carbohydrate moiety of β-fructofuranosidase *P*-2 from *Aureobasidium* sp. ATCC 20524 was largely removed by exposure of the enzyme to endo-β-*N*-acetylglucosaminidase F; the total carbohydrate content of the enzyme was decreased from 53% (w/w) to 15% (w/w). The stability of the deglycosylated enzyme at pH 4 to 7 and 40 to 50 °C was decreased and the K_m value for sucrose was increased from 0.65 to 1.43 M. The deglycosylated enzyme was more sensitive to proteases such as pronase E and subtilisin than the native enzyme. It is concluded that the carbohydrate moiety of β-fructofuranosidase *P*-2 contributes to the stability of the enzyme as well as its affinity for sucrose.

INTRODUCTION

Certain enzymes have been deglycosylated by enzymatic reactions to investigate the significance of their carbohydrate moieties. Deglycosylation of porcine pancreatic ribonuclease indicated the involvement of the carbohydrate moiety in the stabilization of surface conformation [8]. Deglycosylated yeast invertase was more sensitive to proteases [1]. The carbohydrate moiety of Taka-amylase A is not an essential participant in the catalytic activity [6]. The thermal stability and tolerance to proteolysis of β-*N*-acetylhexosaminidase was decreased after deglycosylation [9]. The deglycosylation of glucoamylase [7] and phospholipase B [5] has also been reported. There are, however, few reports on the deglycosylation of sugar-transferring enzymes. In a previous paper, we reported on the deglycosylation of β-fructofuranosidase *P*-1 from *Aureobasidium* sp., which has high fructosyl-transferring activity, and noted the importance of the carbohydrate moiety for stabilization of the enzyme [4]. We also reported that the strain produces β-fructofuranosidase *P*-2, which is another enzyme with a high sugar content, 53% (w/w) [3]. There is, however, no report on the deglycosylation of a sugar-transferring enzyme which has a high sugar content in comparison with the properties of other enzymes from the same source after deglycosylation.

In the present paper, we describe the properties of deglycosylated β-fructofuranosidase *P*-2, normally containing a high sugar content, produced by *Aureobasidium* sp. [3], as compared with those of the deglycosylated β-fructofuranosidase *P*-1, which has been described previously. The objective is to clarify the role of the carbohydrate moiety of the *Aureobasidium* enzymes that are capable of a fructosyl-transferring reaction at high activity.

MATERIALS AND METHODS

Microorganism and cultivation

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

Purification of P-2 enzyme

P-2 enzyme was solubilised from dry cells by Kitalase (Wako) in McIlvain buffer (pH 5) at 40 °C for 2 h and then centrifuged [3]. *P*-2 enzyme was purified to homogeneity by fractionations involving ethanol, $(CH_3COO)_2Ca$ and $(NH_4)_2SO_4$ and DEAE-Cellulofine A-800 and Sephadex

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G-200 column chromatography as described previously [3].

Endo-β-N-acetylglucosaminidase treatment

Purified *P*-2 enzyme (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 *P*-2 enzyme was estimated by Sephadex G-200 gel filtration as described previously [3].

Protease digestion

Digestion of deglycosylated and native *P*-2 enzyme 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 *P*-2 enzyme) and 1 mg protease (pronase E 4.4 U and 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.

Enzymatic activity assay

The enzymatic activity was assayed in a mixture consisting of 0.1 ml of enzyme solution (deglycosylated or native *P*-2 enzyme), 0.4 ml of 75% sucrose, 0.5 ml of 0.15 M McIlvain buffer (pH 5) at 50 °C for 20 min using 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.

RESULTS AND DISCUSSION

Endo-β-N-acetylglucosaminidase treated P-2 enzyme

The molecular weight of deglycosylated *P*-2 enzyme was determined by the Sephadex G-200 gel filtration method. The molecular weight of native *P*-2 enzyme (346000), with a carbohydrate content of 53% (w/w) [3], was decreased to 216000. The carbohydrate content was 15% (w/w) after deglycosylation. The decrease in molecular weight reflects the removal of carbohydrate from native *P*-2 enzyme by endo-β-*N*-acetylglucosaminidase F.

Enzymatic profiles of deglycosylated P-2 enzyme

The effect of pH on the activity of deglycosylated *P*-2 enzyme is shown in Fig. 1. The optimum pH was slightly more alkaline than that of native *P*-2 enzyme after deglycosylation. At pH 4, deglycosylated *P*-2 enzyme showed only 23% activity of the activity at optimum pH, while native *P*-2 enzyme retained 75% [3].

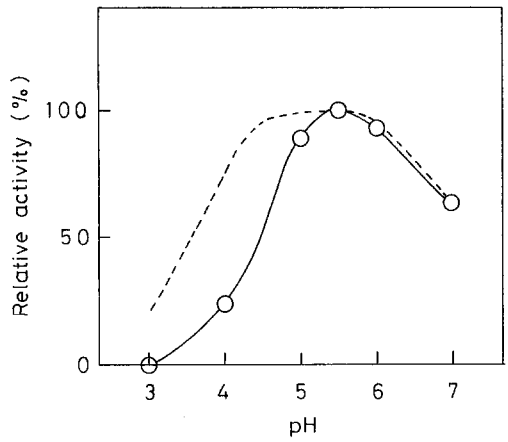

Fig. 1. Effect of pH on the activity of deglycosylated β-fructofuranosidase *P*-2. Symbols: deglycosylated β-fructofuranosidase *P*-2 (○); native β-fructofuranosidase *P*-2 (– – –).

The effect of pH on the stability of deglycosylated *P*-2 enzyme is shown in Fig. 2. The stability of *P*-2 enzyme at pH 4 to 7 was decreased by deglycosylation. At pH 4, deglycosylated *P*-2 enzyme retained only 34% of the activity at optimum pH, while native *P*-2 enzyme retained 99% [3].

Remarkable effects of pH on the properties of the deglycosylated *P*-2 enzyme were observed, especially at acidic pHs and at pH 10.

The effect of temperature on the activity of deglycosylated *P*-2 enzyme is shown in Fig. 3. The optimum temperature was slightly lower than that of native *P*-2 enzyme

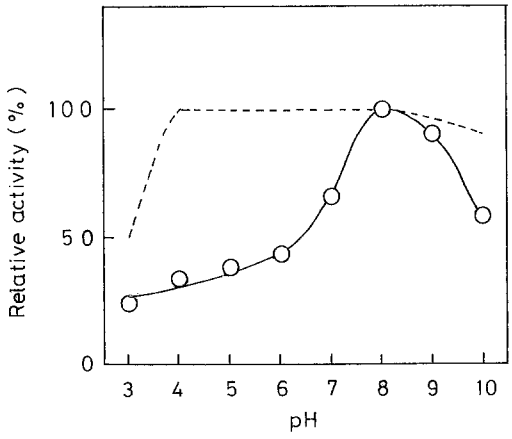

Fig. 2. Effect of pH on the stability of deglycosylated β-fructofuranosidase *P*-2. Symbols: deglycosylated β-fructofuranosidase *P*-2 (○); native β-fructofuranosidase *P*-2 (– – –). The activities were measured after 3 h incubation at each pH (pH 3 to 8, McIlvain buffer; pH 8 to 10, Michaelis buffer).

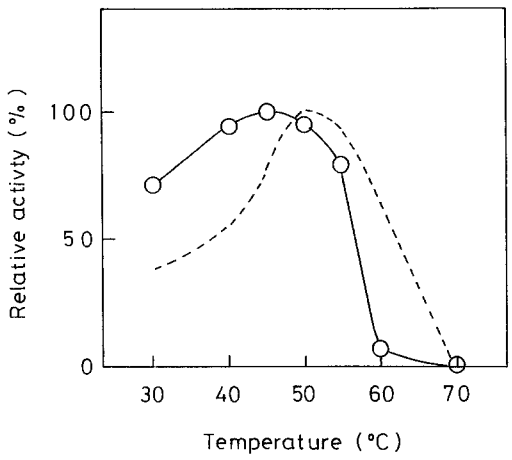

Fig. 3. Effect of temperatures on the activity of deglycosylated β-fructofuranosidase *P*-2. Symbols: deglycosylated β-fructofuranosidase *P*-2 (○); native β-fructofuranosidase *P*-2 (– – –).

after deglycosylation. At 60 °C, the deglycosylation of *P*-2 enzyme showed only 7% of the activity at optimum temperature, while native *P*-2 enzyme retained 63%.

Thermal stability of the deglycosylated *P*-2 enzyme is shown in Fig. 4. Thermal stability of native *P*-2 enzyme at 40 to 50 °C was decreased by deglycosylation. At 50 °C, the deglycosylated *P*-2 enzyme retained only 7% of its original activity, while native *P*-2 enzyme retained 98% after 15 min. The decrease in thermal stability by deglycosylation was observed with glucoamylase [7] and phospholipase B [5], but not yeast invertase [9]. The above results suggest that the carbohydrate moiety contributes to the thermal stability of *P*-2 enzyme.

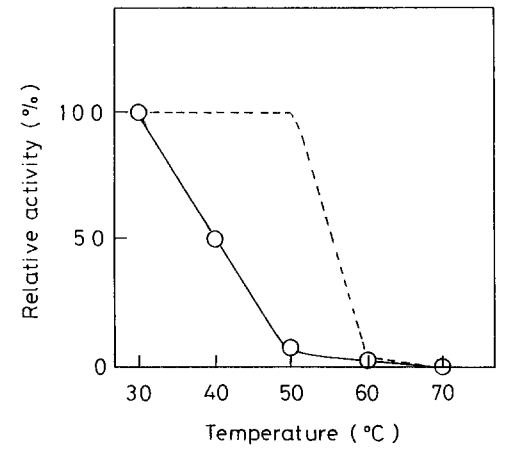

Fig. 4. Effect of temperature on the stability of deglycosylated β-fructofuranosidase *P*-2. Symbols: deglycosylated β-fructofuranosidase *P*-2 (○); native β-fructofuranosidase *P*-2 (– – –). The activities were measured after 15 min incubation at each temperature.

The effect of deglycosylation on the decrease of pH and temperature stabilities of *P*-2 was more significant than for the *P*-1 enzyme [4].

A Lineweaver-Burk plot of deglycosylated *P*-2 enzyme showed that the K_m value for sucrose of deglycosylated *P*-2 enzyme was 1.43 M, in contrast to 0.65 M for the native *P*-2 enzyme [3]. The K_m value of *P*-2 enzyme was increased by deglycosylation, while that of *P*-1 enzyme was not changed [4]. In contrast, the K_m value of carbohydrate-depleted phospholipase B was lower than that of the native enzyme [5]. The above result suggests that the carbohydrate moiety of *P*-2 enzyme is affects with the affinity of the enzyme for sucrose.

Protease digestion of P-2 enzyme

Deglycosylated and native *P*-2 enzymes were digested by proteases such as pronase E and subtilisin (Fig. 5). Deglycosylated and native *P*-2 enzymes retained 27 and 69% of their original activity after pronase E digestion for 90 min, respectively (Fig. 5A). After subtilisin digestion, deglycosylated and native *P*-2 enzymes retained 41 and 76% of their original activity, respectively (Fig. 5B). Deglycosylated *P*-2 enzyme was more susceptible to these proteases than the native enzyme, as in the case of the *P*-1 enzyme [4]. From the above results, it is concluded that the carbohydrate moiety plays a role in protecting the enzyme from proteolysis, as in the case 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-fructofuranosidases from *Aureobasidium* sp. is important for stabilization of the enzymes. The affinity to sucrose, however, of the *P*-2 enzyme was decreased by deglycosylation in contrast to the case of the *P*-1 enzyme.

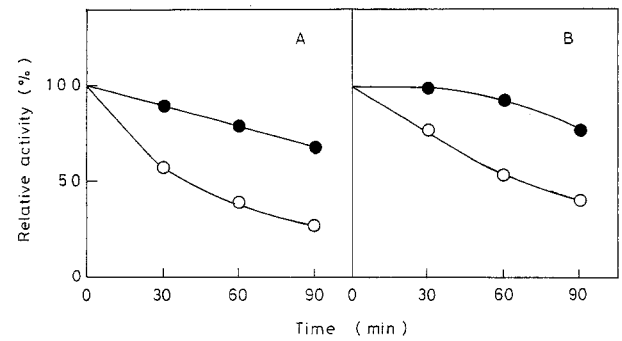

Fig. 5. Time course of protease digestion (A, pronase E; B, subtilisin) of deglycosylated and native β-fructofuranosidase *P*-2. Symbols: deglycosylated β-fructofuranosidase *P*-2 (○); native β-fructofuranosidase *P*-2 (●).

REFERENCES

1 Chu, C.K., R.B. Trimble and F. Maley. 1978. The effect of carbohydrate depletion on the properties of yeast external invertase. J. Biol. Chem. 253: 8691–8693.

2 Hayashi, S., M. Nonoguchi, K. Imada and H. Ueno. 1990. Production of a fructosyl-transferring enzyme by *Aureobasidium* sp. ATCC 20524. J. Indust. Microbiol. 5: 395–400.

3 Hayashi, S., M. Nonoguchi, Y. Takasaki, H. Ueno and K. Imada. 1991. Purification and properties of β-fructofuranosidase from *Aureobasidium* sp. ATCC 20524. J. Indust. Microbiol. 7: 251–256.

4 Hayashi, S., M. Nonoguchi, Y. Shimokawa, Y. Takasaki and K. Imada. 1992. Effect of deglycosylation on the properties of β-fructofuranosidase *P*-1 from *Aureobasidium* sp. ATCC 20524. J. Indust. Microbiol. 9: 251–255.

5 Murayama, M., H. Kadowaki, Y. Watanabe and Y. Tamai. 1990. Role of the carbohydrate moiety in phospholipase B from *Torulaspora delbruckii*. Agric. Biol. Chem. 54: 599–603.

6 Takahashi, N., H. Toda, H. Nishibe and K. Yamamoto. 1982. Isolation and characterization of Taka-amylase A apoprotein deglycosylated by digestion with almond glycopeptidase immobilized on Sepharose. Biochim. Biophys. Acta 707: 236–242.

7 Takegawa, K., M. Inami, K. Yamamoto, H. Kumagai, T. Tochikura, B. Mikami and Y. Morita. 1988. Elucidation of the role of sugar chains in glucoamylase using endo-β-*N*-acetylglucosaminidase from *Flavobacterium* sp. Biochim. Biophys. Acta 995: 187–193.

8 Wang, F.-F.C. and C.H.W. Hirs. 1977. Influence of the heterosaccharides in porcine pancreatic ribonuclease on the conformation and stability of the protein. J. Biol. Chem. 252: 8358–8364.

9 Yamamoto, K., K. Takegawa, H. Kumagai and T. Tochikura. 1987. Effect of protease digestion on the activity of sugar-depleted enzymes prepared with endo-β-*N*-acetylglucosaminidase from *Furabobacterium* sp. Agric. Biol. Chem. 51: 1481–1487.