Volume 316, number 2, 157–160 FEBS 11988 January 1993

Effect of modification of carbohydrate component on properties of glucoamylase

K.N. Neustroev[a], A.M. Golubev[a], L.M. Firsov[a], F.M. Ibatullin[a], I.I. Protasevich[b] and A.A. Makarov[b]

[a]*Petersburg Nuclear Physics Institute, Gatchina, St. Petersburg, Russian Federation* and [b]*Institute of Molecular Biology, Moscow, Russian Federation*

Received 18 November 1992

In this study, we investigated enzymatic deglycosylation of glucoamylase from *Aspergillus awamori* X 100/D27, a glycoprotein which has two *N*-linked and about forty short mannose-bearing *O*-linked sugars per molecule. *O*-Linked sugars were modified by treatment with α-mannosidase and *N*-linked sugars were removed using endo-β-*N*-acetylglucosaminidase F. Analysis of conformational changes following deglycosylation suggests that *O*-linked sugars essentially contribute to the stabilization of glucoamylase domains. Modification of the carbohydrate component by adding 1-deoxymannojirimycin to the culture medium induced inhibition of α-mannosidases involved in the processing, leading to a more complete glycosylation and, consequently, to a higher stability of the enzyme.

Glucoamylase; Glycoprotein; Deglycosylation

1. INTRODUCTION

Glucoamylase (EC 3.2.1.3) catalyses the hydrolysis of α-1–4, α-1–6 glycosidic bonds in starch and maltooligosaccharides. In culture filtrate, glucoamylase is commonly found in two forms. The minor form results from the major form by the action of the fungus' own acid proteinase which liberates the C-terminal fragment from the polypeptide chain [1]. Preliminary data suggest that glucoamylase from *Aspergillus awamori* X 100/D 27, like glycoamylase from *Aspergillus niger* and *Aspergillus oryzae*, contains 18–20% total sugars [2]. Carbohydrate structures of some glucoamylases have been well defined [3,4] but little is known about the role of glycosylation. To investigate this problem, we used glucoamylase which carries *N*- and *O*-linked sugars and is therefore a convenient model to individually examine both types of glycosylation. Effects of glycosylation on conformation and properties of the two forms of glucoamylase were also studied.

2. EXPERIMENTAL

α-Mannosidase from jack beans, glucose oxidase, peroxidase, maltose, pronase E from Sigma (USA), [^{3}H]$NaBH_4$ (1,600 mCi/mM) (Izotop, Russia), 1-deoxymannojirimycin (1-DM), Endo-β-*N*-acetylglucosaminidase F from *Flavobacterium meningoserpticum* (Endo F) (Boehringer-Mannheim, Austria) were used in this study. The major and minor forms of glucoamylase and acid proteinase were isolated according to [1], α-galactosidase and α-glucosidase from *Asp. awamori* X 100/D27 were purified as in [5]. The enzymatic activity of glucoamylase was assayed by the glucose oxidase method [6]. Conversion of the glucoamylase major form into the minor one was determined as described by [1].

Deglycosylation of glucoamylase by treatment with α-mannosidase was carried out in accordance with [7]. Deglycosylation by α-galactosidase or α-glucosidase was performed using 0.02 M acetate buffer, pH 4.5, 40°C, and 2–3 U α-galactosidase or α-glucosidase per 1 mg of protein. Deglycosylation by Endo F was done in 0.02 M Na acetate buffer, pH 5.2, 28°C, 24 h, using 1.4–1.5 U Endo F per 500–600 μg of glucoamylase. Treatment of glucoamylase with pronase E was carried out in 0.02 M Tris-HCl buffer, pH 7.5, 37°C, 48 h. Pronase content was 2% of that of glucoamylase.

The native protein was subjected to ^{13}C NMR analysis according to [3]. The sample contained 200 mg/ml of protein and the number of data accumulations was $2–3 \cdot 10^4$. Monosaccharides were determined as alditol acetates by GLC and mass spectrometry (MS) after hydrolysis with 1 M HCl at 100°C for 5 h [6]. Sugars liberated by β-elimination [2] gave sugar alditols which were methylated by the Hakomori procedure [8] and then subjected to GLC and MS on a Finnigan MAT 90 fitted with an OV 17 column [4]. Changes in the carbohydrate component were analysed by GLC and MS as outlined above. The quantity of *N*-linked sugars was determined as follows: the protein was treated with Endo F and the amount of liberated sugars was measured after they were reduced with [^{3}H]$NaBH_4$ according to [2]. Calorimetric measurements were done on a differential adiabatic microcalorimeter DASM-1M at a scan rate of 1 K/min, at the protein concentrations of 0.5–2.0 mg/ml in 0.05 M Na acetate buffer.

3. RESULTS AND DISCUSSION

The major form of glucoamylase pretreated with Endo F was used to determine the structure of *O*-linked sugars. The ratio between neutral hexoses was: mannose/glucose/galactose 21:1:2. ^{13}C NMR spectroscopic and GLC/MS measurements gave the carbohydrate structure represented in Table I. Similar carbohydrate structure of *O*-linked sugars has also been reported for glucoamylases from *Asp. niger* and *Asp. oryzae* [3,4].

Correspondence address: K.N. Neustroev, Dept. of Molecular and Radiation Biophysics, Petersburg Nuclear Physics Institute, Gatchina, 188 350 St. Petersburg, Russian Federation. Fax: (7) (812) 217 01 34.

The minor form bears only 4 *O*-linked mannoses. Treatment of the major and minor native forms of glucoamylase with Endo F leads to liberation of two *N*-linked sugars from a polypeptide chain. Similarly, only two carbohydrate chains are removed by Endo F treatment of the pronase hydrolysate of glucoamylase. This means that there are two such chains per molecule and that the native protein is completely deglycosylated. α-Mannosidase treatment of the native glucoamylase results in the removal of 24–26% of total mannose (Table I). A higher mannosidase concentration or longer incubation fail to induce a greater extent of deglycosylation. Complete deglycosylation, however, occurs only when glucoamylase pronase hydrolysate is treated with α-mannosidase together with α-galactosidase and α-glucosidase. It is noteworthy that proteolytic degradation causes the formation of oligopeptides with molecular weights of no more than 3,500 Da (checked by analytical gel filtration on a TSK GS 2000 column). Complete cleavage of *O*-linked sugars by α-glycosidases provides additional evidence that all bonds have an α-configuration. The α-mannosidase-modified protein is less stable to pH or heat than the native protein. The Endo F-treated protein also loses pH and heat stability, but to a lesser extent. Neither the native protein nor the α-mannosidase-deglycosylated glucoamylase show changes in the enzymatic activity. Thus, the K_m values for maltose in the native and in the α-mannosidase- or Endo F-treated proteins coincide and are equal to 0.2 mM. There are no differences in the V_{max} values either.

Deglycosylation-induced conformational changes were estimated by calorimetric measurements which were done for the major, minor and α-mannosidase-deglycosylated major forms of glucoamylase. Prior to this, all forms were treated with Endo F. The thermodynamic parameters of heat denaturation, such as T_d (denaturation temperature), ΔH_{cal} (calorimetric denaturation enthalpy) and ΔH_{eff} (van 't Hoff's effective denaturation enthalpy) were calculated from experimental calorimetric curves [9]. ΔH_{cal} was determined by measuring the area under the heat absorption peak and using a calibration coefficient, ΔH_{eff} was calculated from the equation:

$$\Delta H_{eff} = 4RT_d^2 \Delta C_p^{max}/Q_d$$

where ΔC_p^{max} is the maximal change of the protein heat capacity at T_d and Q_d is the area under the heat absorption peak expressed in energy units. As can be seen from Fig. 1, melting of both protein forms occurs within the same temperature range. Thermal stability of the protein, devoid of the C-terminal fragment with no less than 90% *O*-sugars, remains unchanged. Comparison of calorimetric curves shows slight differences in the profiles preceding the T_d. However, values for heat denaturation effects calculated per unit of the protein molecular weight coincide (Table II). The relation $r = \Delta H_{cal}/\Delta H_{eff}$ used as a criterion for melting cooperativity [10,11] allows a quantitative estimation of the relatively independently melting cooperative regions, or energy domains, in the protein molecule. For the major form of glucoamylase, $r = 4.1$, indicating that there are four such domains in the protein. In the minor form, $r = 4.3$, i.e. the value remains almost unchanged after removal of the C-terminal fragment. These results suggest that the minor form has the same number of domains. However, this is not the case with the α-mannosidase-modified major form. As shown by a quantitative analysis of calorimetric curves (Table II), ΔH_{cal} for deglycosylated and non-deglycosylated glucoamylases are 308±22 kcal/mol and 4.25±3.08 kcal/mol, respectively. Such a prominent alteration of denaturation enthalpy following a slight change in denaturation temperature clearly points to a contribution of sugars to the formation of spatial structure. Obviously, deglycosylation results in loosen-

Table I

Structure of *O*-linked sugars of glucoamylase major form

Residues	Number of residues per enzyme molecule			
	Native	Deglycosylated with α-mannosidase	1-Deoxymannojirimycin	
			60 μg/ml	10 μg/ml
D-Mannose	19	37	6	12
2-*O*-D-mannopyranosyl-D-mannopyranose	21	3	34	27
D-Mannopyranosyl-(1-6)-[D-glucopyranosyl (1-3)]-mannopyranose	3	1	3	3
D-Mannopyranosyl-(1-6)[D-galactopyranosyl (1-3)]-mannopyranose	3	3	3	3
D-Mannopyranosyl-(1-6)-mannopyranose	–	2	–	–

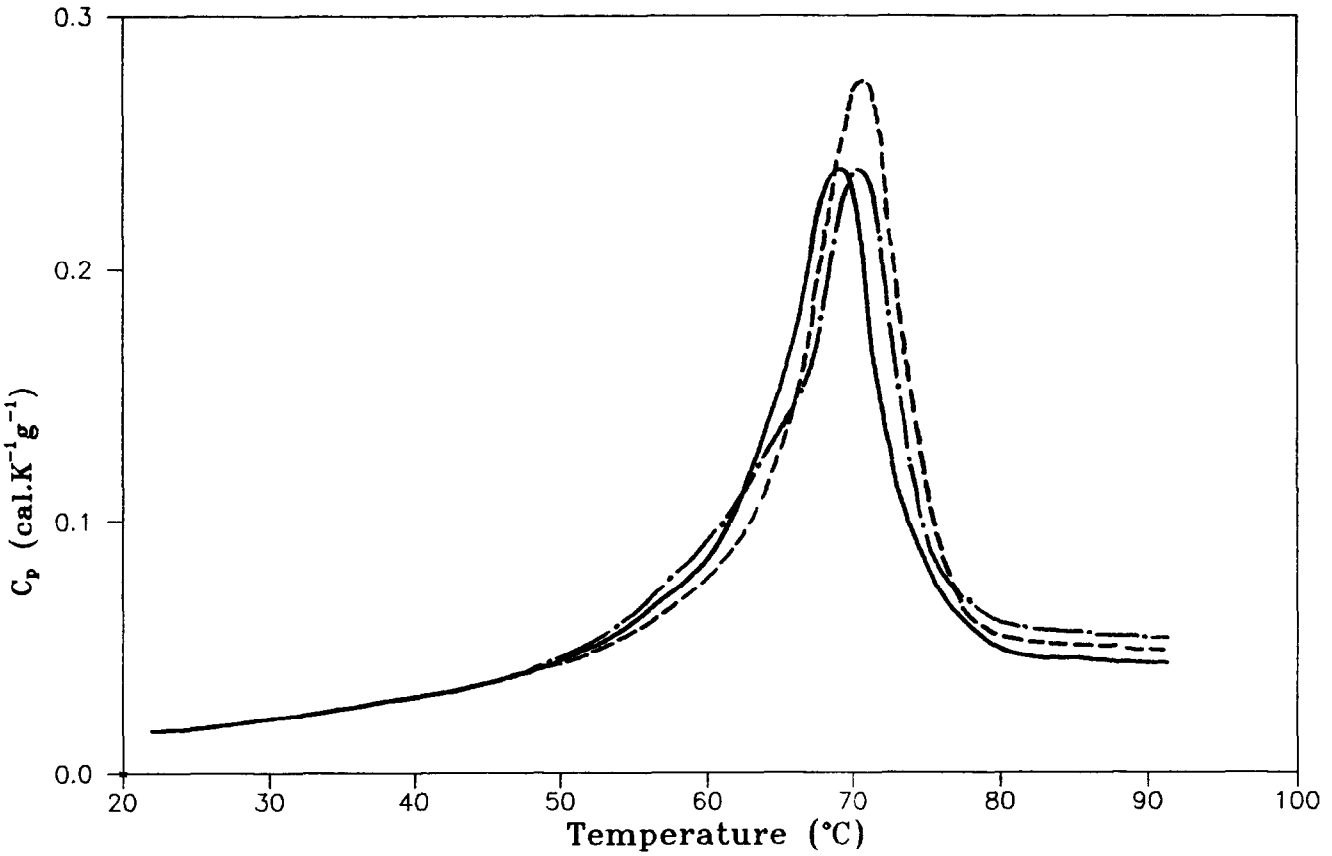

Fig. 1. Temperature dependence of glucoamylase heat capacity. (---) major form; (-•-) minor form; (——) deglycosylated major form.

ing or in breaks of the intramolecular bond either along the whole molecule or in its local part. In order to elucidate which of the two possibilities holds, the cooperativity criterion was used. As seen from Table II, the r value for the modified form is 3.3. Accordingly, there are at least three domains present in the deglycosylated protein. It is noteworthy that ΔH_{eff} correlating with the average sizes of the cooperative domains of the molecule [10] is altered insignificantly. Consequently, the deglycosylated major form contains energy domains whose size is unchanged but the number is reduced by one unit. *O*-Glycosylation of the C-terminal fragment leads to the stabilization of domains in the 'main part' of glucoamylase. It should be noted that the native and the Endo F-deglycosylated forms show no changes in heat-denaturation parameters. Altered properties and stability of the enzyme due to deglycosylation seem to be associated with the magnitude of conformational changes of the deglycosylated protein. Thus, a decrease in stability of thyroglobulin after deglycosylation is accompanied by changes in the tertiary structure [12]. However, removal of 30% of sugars from *Asp. niger* glucose oxidase does not affect stability and enzymatic properties. In this case, CD spectrometry does not show conformational changes [13].

In the alternative approach, carbohydrate component was modified by adding to the culture an α-mannosidase inhibitor, I-DM [14]. This inhibitor was chosen because of the possibility that the carbohydrate moiety may be partially deglycosylated (processed) by the fungus' own α-mannosidases. Accordingly, by inhibiting their action, a more highly glycosylated protein

Table II

Calorimetric parameters of heat-denatured glucoamylase

Glucoamylase forms	T_d (°C)	H_{cal} (kcal/mmol)	H_{eff} (kcal/mmol)	r
Major form	70.8 ± 0.8	415 ± 35	101 ± 11	4.1
Minor form	70.2 ± 0.4	343 ± 3	80 ± 6	4.3
Major form deglycosylated with α-mannosidase	67.9 ± 0.4	302 ± 22	92 ± 6	3.3

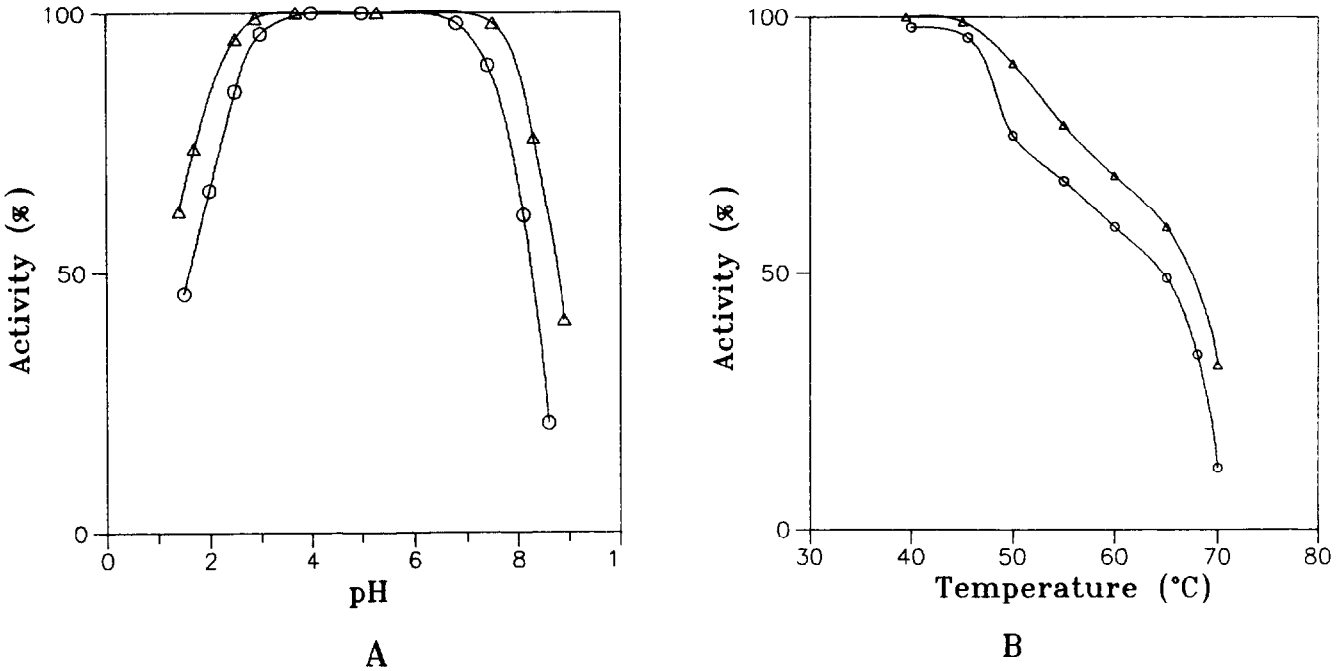

Fig. 2. pH stability (A) and heat stability (B) of native glucoamylase; glucoamylase produced with 1-DM; pH stability was measured by incubation in 0.1 M HCl-Na acetate or TRHC HCl buffer at 37°C for 24 h, at several pH's.

would be obtained. Indeed, I-DM worked as expected: the number of dimers among *O*-sugars increased. The rate of glycosylation was found to increase with a higher concentration of the inhibitor (Table I). It is of interest that this alteration of the carbohydrate structure significantly improved thermal and pH stabilities of the enzyme (Fig. 2A,B). The major form secreted in the presence of 1-DM is not converted into the minor one by the action of acid proteinase. In nature, such conversion is necessary for degradation of maltooligosaccharides [1] and is effected through the optimal level of glycosylation.

REFERENCES

[1] Neustroev, K.N. and Firsov, L.M. (1990) Biokhimiya 55, 776–785.
[2] Savelyev, A.N., Yakovleva, M.F. and Firsov, L.M. (1984) Biokhimiya 49, 1754–1761.
[3] Carter, R.D., Hu, S. and Dill, K. (1987) Int. J. Biol. Macromol. 9, 269–272.
[4] Gunnarson, A., Svensson, B., Nilson, B. and Svensson, S. (1984) Eur. J. Biochem. 145, 463–467.
[5] Neustroev, K., Krylov, A., Abroskina, O., Firsov, L., Nasonov, V. and Khorlin, A. (1991) Biokhimiya 56, 447–457.
[6] Lloyd, J.B. and Whelan, W.S. (1969) Anal. Biochem. 30, 467–470.
[7] Golubev, A.M., Neustroev, K.N., Aleshin, A.E. and Firsov, L.M. (1992) J. Mol. Biol 226, 271–272.
[8] Hakomori, S. (1964) J. Biochem. 55, 205–209.
[9] Privalov, P.L. and Rhechinashvili, N.N. (1974) J. Mol. Biol. 86, 665–681.
[10] Privalov, P.L. (1982) Adv. Protein Chem. 35, 1–107.
[11] Privalov, P.L., Mateo, P.L., Rhechinashvili, N.N., Stepanov, V.M. and Revina, L.P. (1981) J. Mol. Biol. 152, 455–459.
[12] Grimaldi, S., Pozzi, D., Verna, R., Lio, S., Giganti, G., De Pizzo, R. and Monaco, F. (1988) BBI 957, 105–110.
[13] Takegawa, K., Fujiwara, K., Iwanara, S., Yamamoto, K. and Tochikura, T. (1989) Can. J. Biochem. Biol. 67, 460–463.
[14] Lebein, A. (1987) Methods Enzymol. 138, 693–694.