Deglycosylation of Hen Ovalbumin in Native Form by Endo- β -N-Acetylglucosaminidase

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The properties of endo- β -N-acetylglucosaminidase isolated from a *Flavobacterium* sp. were examined with native hen ovalbumin as a substrate for the first step in preparing a large amount of the deglycosylated glycoprotein with the native structure. This enzyme cleaved the oligosaccharide chain in native ovalbumin not only for the A₁ ovalbumin containing two phosphoryl residues per molecule, but also for the A₂ and A₃ ovalbumins, containing one and no phosphoryl residues, respectively. With polyacrylamide gel electrophoresis, glycosylated ovalbumin of the native form was separated from the deglycosylated variety, which was then transferred to a vinylidene fluoride membrane and stained with concanavalin A and peroxidase to confirm the deglycosylation. The apparent *K_m* value was about 2.67 mM at pH 6.0 when native ovalbumin was used as the substrate, the optimum pH for the enzyme reaction with native ovalbumin being around 5.0. This enzyme was not inhibited by its end product.

The oligosaccharide side chains of glycoproteins are important in biological recognition for the circulatory clearance of serum protein,¹⁾ the compartmentalization of lysosomal protein,²⁾ the specificity involved in legume-symbiont interaction,³⁾ in allergen recognition,⁴⁾ and also in the functional properties of food proteins, because these side chains interact with water molecules specifically and are involved in maintaining the protein structure, which seems to influence the water-holding capacity of food proteins. To investigate these roles of the oligosaccharide side chains of glycoproteins, including enzyme and food proteins, neoglycoprotein has been used.⁵⁾ With neoglycoprotein, it has been shown that the critical role of oligosaccharides in glycoproteins is to take part in binding the glycoproteins with hepatocytes,^{6,7)} in the uptake of glycoprotein by macrophage,⁸⁾ and in the resistance to inactivation by proteolysis of enzymes.⁹⁾

Neoglycoprotein prepared by chemical mod-

ification techniques can change the functional properties of food proteins by the introduction of oligosaccharides into the proteins. The solubility of β -lactoglobulin attached with melibiose was increased in the acidic pH region.¹⁰⁾ Waniska and Kinsella^{11,12)} have reported an alteration of the functional properties of β -lactoglobulin by the attachment of oligosaccharides to food protein. However, the preparation of neoglycoprotein by chemical modification may be accompanied by an alteration of the protein structure somewhere other than in the oligosaccharide side chain. Therefore, a milder technique is required to study the role of the oligosaccharide side chain of glycoprotein alone. For this purpose, removal of the oligosaccharide side chain by an enzyme would be useful. However, for extensive work on the effect of such side chains in the functional properties of food proteins, a large quantity of deglycosylated glycoprotein must be prepared without changes in the protein conformation.

A variety of endoglycosidases, including the endo- β -*N*-acetylglucosaminidases from *Streptomyces griseus*¹³⁾ and *Diplococcus pneumoniae*,¹⁴⁾ have been purified and some are already commercially available; but it is not easy to obtain a large amount of deglycosylated protein with these enzymes, because their purification is difficult. Recently, a new endo- β -*N*-acetylglucosaminidase (endo- β -GlcNAcase) was isolated from a *Flavobacterium* sp. and purified with a simple procedure in a high yield.¹⁵⁾ It was suggested that deglycosylation of glucoamylase resulted in the decreased heat stability of such enzymes,¹⁶⁾ and endo- β -GlcNAcase from this *Flavobacterium* sp. liberated the oligosaccharide side chain of ovalbumin in native form,¹⁷⁾ which could not be hydrolyzed with endo- β -GlcNAcase from *Streptomyces griseus*.¹³⁾ Therefore, this enzyme from the *Flavobacterium* sp. is the only one known that gives the native deglycosylated ovalbumin in quantity. The properties of this enzyme when a native protein is used as a substrate are not known, and this study was done to identify these properties for the first step in preparing a large amount of deglycosylated protein with this enzyme.

MATERIALS AND METHODS

Chemicals and materials. Endo- β -GlcNAcase (EC 3.2.1.96) from a *Flavobacterium* sp. was a gift from Seitetsu Kagaku Co. Ltd. (Osaka, Japan). Hen ovalbumin was purified from fresh egg white by repeated crystallizations, as described elsewhere.¹⁸⁾ Ovalbumin concentrations were calculated from the absorbance at 280 nm based on the value of $E_{1\%}^{1\text{cm}} = 7.12$.¹⁹⁾ Acid phosphatase (EC 3.1.3.2) from wheat germ was obtained from Fluka AG (Buchs, Switzerland). The assay for acid phosphatase, the dephosphorylation of ovalbumin by acid phosphatase, and the fractionation of A₁, A₂, and A₃ ovalbumin were as described in a previous paper.²⁰⁾ Concanavalin A (type IV) and peroxidase (type II) were obtained from Sigma Chemical Co. (St. Louis, MO.). 4-Chloro-1-naphthol was obtained from E. Merck (FRG). Gelatin and α -methyl-D-mannoside were from Nakarai Chemicals Ltd. (Kyoto, Japan) and Tween 20 was from Kao-Atlas Co., Ltd. (Tokyo, Japan). Other chemicals of reagent grade were purchased from Nakarai or Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Assay for the endo- β -GlcNAcase reaction. The activity of the enzyme sample in the 10 mM potassium phosphate buffer (pH 7.0) being supplied was 15 units/ml. One unit is defined as the activity that releases 1.0 μ mol of dansyl (5-dimethylaminonaphthalene-1-sulfonyl) asparaginy *N*-acetylglucosamine (DNS-Asn-GlcNAc) in 1 min at 37°C and pH 6.0 with DNS-ovalbumin glycopeptide as a substrate.¹⁵⁾ Purified ovalbumin and A₁, A₂, and A₃ ovalbumins were used as substrates for the enzyme reaction. A typical reaction mixture contained 10 μ l of ovalbumin (6 mg/ml), 4 μ l of endo- β -GlcNAcase (15 units/ml), and 16 μ l of 142 mM sodium acetate buffer at pH 5.0 containing both 0.2 mM EDTA and 0.04% NaN₃. The reaction temperature was 37°C, and the reaction was stopped by cooling the mixture with liquid nitrogen. The reaction mixture was immediately electrophoresed after thawing. Polyacrylamide gel electrophoresis (PAGE) was done by the procedure of Ornstein²¹⁾ and Davis.²²⁾ After electrophoresis, the protein bands were stained by the method of Blakesley and Boezi.²³⁾ To avoid variations in the intensity of the colored bands in different experiments, the solution of Coomassie Brilliant Blue G250 was freshly prepared from the powder each time. The color intensity of each band was measured on a Shimadzu dual-wavelength TLC scanner (CS-910) equipped with a Shimadzu recorder (U-225 MCS) at 600 nm against 750 nm as a reference. From the peak area, the ratio of the product to the substrate plus product was calculated. This ratio per unit reaction time was used as a measure of the enzyme activity.

Affinity blotting (affinoblotting) with concanavalin A-peroxidase. The electrophoretically separated proteins were transfer-blotted with a Horizeblot (AE-6670, Atto Co., Tokyo) as described by Faye and Chrispeels,²⁴⁾ except that we used vinylidene fluoride sheets (Clear Blot Membrane-P, type AE-6660, Atto Co., Tokyo) instead of nitrocellulose sheets. First, a clear blot membrane was immersed in methanol for 20 sec. Absorption paper, which closely resembled filter paper, and the membrane were soaked in a transfer medium (12.5 mM Tris-HCl buffer, pH 7.4, 96 mM glycine and 20% methanol) for at least 30 min. A polyacrylamide gel after electrophoresis was placed on the vinylidene fluoride sheet and these were sandwiched with absorbent papers. A small amount of the transfer buffer was added to them so that they did not become dry during the electrophoretic transfer, which was carried out at 95 mA for 2 hr at room temperature. After transfer, the sheet was immersed in a fixing solution (acetic acid:isopropanol:water = 10:25:65) for about 10 min, and then rinsed with water five times and finally with Tris-buffered saline (TBS; 500 mM NaCl and 20 mM Tris-HCl, pH 7.4). To block the background staining, the sheet was left overnight in TBS containing 3% gelatin at 4°C. The treated sheet was dipped in TBS containing 1% gelatin, 1 mM MnCl₂, 1 mM CaCl₂ and 25 μ g/ml concanavalin A with gentle stirring for 1 hr. In this step, the protein band

that had been transferred to the sheet was bound with concanavalin A. After a quick rinse with TBS, the sheet was washed four times in TBS containing 1% gelatin, 0.1% Tween 20, 1 mM MnCl_2 and 1 mM CaCl_2 for 1 hr to remove the free concanavalin A remaining, and then washed with TBS to remove the Tween 20. Then, the sheet was incubated with TBS containing 1% gelatin, 1 mM MnCl_2 , 1 mM CaCl_2 and 50 $\mu\text{g}/\text{ml}$ peroxidase with gentle shaking for 1 hr to attach the peroxidase to the concanavalin A that was bound to the protein on the sheet, and the excess peroxidase was washed in the same way as that for washing away the concanavalin A. The protein bands attached to concanavalin A and peroxidase were stained next. The sheet was put into 50 ml of TBS containing 30 μg of 4-chloro-1-naphtol and 30 μl of H_2O_2 , both of which had been prepared just before use. After 15 min of staining, the stained sheet was washed several times with H_2O , blotted dry, and immediately photographed. Controls were treated in exactly the same way, except for the inclusion of 200 mM α -methyl-D-mannoside in the buffers used for binding, washing and staining.

Enzyme reaction during dialysis of the reaction mixture against the buffer. To remove the oligosaccharide occurring with the reaction, the reaction was carried out while dialyzing the reaction mixture. The reaction mixture contained 15 μl of ovalbumin (178.9 mg/ml) with 0.02% NaN_3 , 5 μl of endo- β -GlcNAcase (15 units/ml) and 4 μl of 0.3 M sodium acetate buffer at pH 5.06. The reaction was run for 18 hr in the sample wells of a microdialyzer (MEGA system Microdialyzer, Health Products Inc.) at 37°C, with dialysis against 0.3 M sodium acetate buffer at pH 5.06.

RESULTS

Course of the enzyme reaction for ovalbumin

The purified ovalbumin gave three bands by PAGE with different mobilities, corresponding to the A_1 , A_2 and A_3 ovalbumin, with two, one and no phosphoryl residues in each molecule, respectively. Each ovalbumin fraction purified was treated with endo- β -GlcNAcase. Figure 1 shows PAGE results of ovalbumins A_1 to A_3 after enzyme treatment for 6, 18 and 36 hr, and Fig. 2 shows the affinoblotting of the polyacrylamide gel electrophoresed. A new band with higher mobility than those of the ovalbumin species appeared after the enzyme reaction, and no other bands were found by PAGE. However, affinoblotting did not give a band with the same mobility, but a band with the same mobility as that of the native species.

FIG. 1. Enzyme Activity of Endo- β -N-acetylglucosaminidase toward Native Ovalbumins A_1 , A_2 and A_3 Followed by Polyacrylamide Gel Electrophoresis (PAGE). Native Ovalbumins A_1 , A_2 and A_3 treated with endo- β -N-acetylglucosaminidase were separated by PAGE. Staining was with Coomassie Brilliant Blue R250, the reaction mixture containing 30 μl of ovalbumin (20 mg/ml), 20 μl of endo- β -N-acetylglucosaminidase (15 units/ml), and 10 μl of 0.6 M sodium acetate buffer (pH 5.0) containing EDTA (6 mM) and NaN_3 (0.12%). The total volume of the reaction mixture (60 μl) was adjusted by the addition of distilled water, and the reaction was run at 37°C. Reaction times are indicated at the bottom of each lane, and 2.0 μg of ovalbumin was added after enzyme treatment for PAGE.

FIG. 2. Visualization of Concanavalin A-Binding Ovalbumin by the Concanavalin A/Peroxidase Method. Ovalbumins A_1 , A_2 and A_3 treated with endo- β -N-acetylglucosaminidase were separated by PAGE (Fig. 1) and transferred to a vinylidene fluoride membrane sheet, which was incubated first with concanavalin A and then with peroxidase. The number at the bottom of each lane indicates the enzyme reaction time. For PAGE, 0.5 μg of ovalbumin was added after the enzyme treatment.

This suggests that these new bands from PAGE were deglycosylated ovalbumin.

Figure 3 shows the increase in the intensity

FIG. 3. Course of the Endo- β -N-acetylglucosaminidase Reaction with A₁, A₂ or A₃ Ovalbumin as the Substrate.

●, A₁; ■, A₂; ▲, A₃. At each reaction time, the reaction mixture was fractionated by PAGE and the color intensity of each band was measured. The extent of the reaction is expressed by the ratio of the color intensity of a new band, which corresponded to deglycosylated ovalbumin, to the total color intensity, which corresponded to the original A₁ ovalbumin plus deglycosylated ovalbumin, thus representing the enzyme activity. The composition of the reaction mixture and the reaction conditions were the same as those in the legend to Fig. 1.

of the new band measured by a chromatoscanner. As the reaction time increased, the intensity of the new band with high mobility increased for all three of ovalbumin; no difference in the availability as a substrate for this enzyme was found in the ovalbumin species.

Apparent K_m and V_{max} values with native A₁ ovalbumin and the pH profile of the enzyme

Figure 4 shows the course of the enzyme reaction over 18 hr, the reaction being linear with enzyme concentration; the amounts of deglycosylated ovalbumin produced after 6 and 18 hr of the reaction were plotted against the enzyme concentration. Deglycosylated A₁ ovalbumin was formed in proportion to the amount of enzyme added, indicating that the reaction proceeded quantitatively. Using this assay method, the enzyme activity was measured with various concentrations of ovalbumin.

Figure 5 showed the enzyme activities at various substrate concentrations of A₁ ovalbumin. From an extrapolation of the Lineweaver-Burk plot in the inset to Figure 5, the apparent K_m and V_{max} values were esti-

FIG. 4. Course of the Enzyme Reaction and Dependence of the Enzyme Reaction on the Concentration of the Enzyme.

(A): Courses of the endo- β -N-acetylglucosaminidase reaction were plotted against the reaction time with different enzyme concentrations (15, 30, 45, 60 and 75 munits/reaction mixture).

(B): The amounts of deglycosylated ovalbumin produced (the extent of reaction) after 6 and 18 hr of reaction were plotted against the enzyme concentration. The reaction mixture contained 15 μ l of A₁ ovalbumin solution (178.9 mg/ml) with 0.02% NaN₃, 1–5 μ l of endo- β -N-acetylglucosaminidase (15 units/ml of 10 mM potassium phosphate, pH 7.0) and 4 μ l of 0.6 M potassium phosphate buffer, pH 6.0, containing 0.12% NaN₃ and 0.6 mM EDTA. The total volume of the reaction mixture was brought to 24 μ l by the addition of distilled water. At different times, 5 μ l of the reaction mixture was sampled and analyzed by polyacrylamide gel electrophoresis. The extent of the reaction (%) was calculated as in the legend to Fig. 2.

mated as 2.67 mM and 0.0726 μ g/min, respectively. In the region of a high concentration of the substrate, the line was straight, suggesting that there was no product inhibition.

Sodium phosphate buffer (pH 6.0) was used throughout the experiments, because the optimum pH for this enzyme has been reported to be about 6 when DNS-glycosylated peptide was used;¹⁵⁾ however, no information about the pH profile of the enzyme reaction when protein was used as the substrate has been reported. Figure 6 shows that the pH optimum was at around 5, but from 4 to 6, the enzyme activity did not change greatly.

FIG. 5. Plot of the Reaction Rate against Substrate Concentration.

Enzyme activity is expressed as the concentration of the deglycosylated ovalbumin produced after 18 hr of reaction. The reaction mixture was the same as that in the legend to Fig. 4, except for the concentration of the A_1 ovalbumin. The inset figure is a reciprocal plot.

FIG. 6. Effects of pH on Enzyme Activity.

The reaction mixture was the same as that in the legend to Fig. 4 except for the buffer used. ●, sodium acetate buffer; ▲, sodium phosphate buffer. The enzyme activity at each pH value is expressed as the extent of reaction (%) after 18 hr of progress. Details of the extent of the reaction are shown in the legend to Fig. 2.

Effect of dialysis during the reaction

No change in the reaction rate was observed when the enzyme reaction was carried out with dialysis to remove the glycosyl residue produced. Therefore, the possibility could be excluded of product inhibition by the oligosaccharide appearing.

DISCUSSION

When native ovalbumin was used as a sub-

strate, the reaction rate decreased with time, and some of the ovalbumin could not be deglycosylated by the enzyme. These results are similar to those reported by Tongpim *et al.*²⁵⁾ and Yamamoto *et al.*¹⁵⁾ To prepare a large quantity of deglycosylated protein, the efficiency of this enzyme reaction was important. As ovalbumin exists in three forms (A_1 , A_2 , and A_3), if the substrate specificity of the enzyme was different for each form, one or more might be less suitable as a substrate for this enzyme. However, no difference was found. Product inhibition by the oligosaccharide liberated was possible, but this possibility was excluded. As the concentration of the substrate decreased during the enzyme reaction, the reaction rate decreased. This was shown by measuring the K_m value; the value of 2.67 mM found in this study corresponded to a 12% (w/v) ovalbumin solution, much higher than the initial concentration of substrate usually used for enzyme assays in this and other studies. The decrease in enzyme activity resulted in a decrease in the substrate concentration during the enzyme reaction. Since the enzyme activities for A_2 and A_3 ovalbumin were similar to that for A_1 ovalbumin as

shown in Fig. 3, the K_m values of this enzyme for A_2 and A_3 ovalbumin seem to be close to the K_m value for A_1 ovalbumin; 2.67 mM.

Yamamoto *et al.*¹⁵⁾ reported that the apparent K_m value for DNS-glycopeptides was 0.40 mM. This value is about one-sixth of the K_m value for native A_1 ovalbumin, that was estimated in this study. However, considering the protein-protein interaction (substrate-enzyme) and steric hindrance, this difference was not large and can be regarded as a similar value to that of the DNS-glycopeptides of low molecular weight. This suggests that the oligosaccharide side chain of the ovalbumin including an amino acid sequence linked to the oligosaccharide might be exposed to the outside of the molecule to be available for this enzyme, and not in the ovalbumin core.

The oligosaccharide species linked to the ovalbumin molecule was heterogeneous; some (about 11%) were not substrates for the endo- β -GlcNAcase (data not shown). Therefore, the concentration of available substrate was lower by 11% than that of the A_1 ovalbumin used here, indicating that the K_m value for the available molecular species in the A_1 ovalbumin used as a substrate in this study should be slightly lower than 2.67 mM.

This study shows that a large amount of deglycosylation can proceed with this enzyme if a suitable substrate concentration is chosen. The physicochemical and functional properties of the deglycosylated ovalbumin are now being studied.

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REFERENCES

- 1) E. F. Neufeld and G. Ashwell, "The Biochemistry of Glycoproteins and Proteoglycans," ed. by W. J.

- Lennarz, Plenum Press, New York, 1980, pp. 241~266.
- 2) A. Kaplan, D. T. Achord and W. S. Sly, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 2026 (1977).
- 3) E. L. Schmidt, *Annu. Rev. Microbiol.*, **33**, 355.
- 4) S. Kaminogawa, A. Enomoto, J. Kurisaki and K. Yamauchi, *J. Biochem.*, **98**, 1027 (1985).
- 5) C. P. Stowell and Y. C. Lee, *Carbohydr. Chem. Biochem.*, **37**, 225 (1980).
- 6) C. P. Stowell, R. T. Lee and Y. C. Lee, *Biochemistry*, **19**, 4904 (1980).
- 7) D. T. Connolly, R. R. Townsend, K. Kawaguchi, M. K. Hobish, W. R. Bell and Y. C. Lee, *Biochem. J.*, **214**, 421 (1983).
- 8) V. L. Shepherd, Y. C. Lee, P. H. Schlesinger and P. D. Stahl, *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 1019 (1981).
- 9) B. P. Wasserman and H. O. Hultin, *J. Food Biochem.*, **6**, 87 (1982).
- 10) N. Kitabatake, J.-L. Cuq and J. C. Cheftel, *J. Agric. Food Chem.*, **33**, 125 (1985).
- 11) R. Waniska and J. E. Kinsella, *Int. J. Pept. Protein Res.*, **23**, 467 (1984).
- 12) R. Waniska and J. E. Kinsella, *Int. J. Pept. Protein Res.*, **23**, 573 (1984).
- 13) A. L. Tarentino and F. Maley, *J. Biol. Chem.*, **249**, 811 (1974).
- 14) T. Muramatsu, N. Koide and K. Maeyama, *J. Biochem.*, **83**, 363 (1978).
- 15) K. Yamamoto, S. Kadowaki, K. Takegawa, H. Kumagai and T. Tochikura, *Agric. Biol. Chem.*, **50**, 421 (1986).
- 16) K. Yamamoto, K. Takegawa, H. Kumagai and T. Tochikura, *Agric. Biol. Chem.*, **51**, 1481 (1987).
- 17) K. Yamamoto, K. Takegawa, F. Jianqiang, H. Kumagai and T. Tochikura, *J. Ferment. Technol.*, **64**, 397 (1986).
- 18) H. Hatta, N. Kitabatake and E. Doi, *Agric. Biol. Chem.*, **51**, 771 (1987).
- 19) A. Glazer, H. A. MacKenzie and R. G. Wake, *Biochim. Biophys. Acta*, **69**, 240 (1963).
- 20) N. Kitabatake, A. Ishida and E. Doi, *Agric. Biol. Chem.*, **52**, 967 (1988).
- 21) L. Ornstein, *Ann. N.Y. Acad. Sci.*, **121**, 321 (1964).
- 22) B. J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).
- 23) R. W. Blakesley and J. A. Boezi, *Anal. Biochem.*, **82**, 580 (1977).
- 24) L. Faye and M. J. Chrispeels, *Anal. Biochem.*, **149**, 218 (1985).
- 25) S. Tongpim, K. Yamamoto, K. Takegawa, H. Kumagai and T. Tochikura, *Ann. Int. Center. Coop. Res. Biotech.*, **9**, 221 (1986).