Effects of the Length of Polysaccharide Chains on the Functional Properties of the Maillard-Type Lysozyme−Polysaccharide Conjugate

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The effect of the length of polysaccharide chains on the functional properties of Maillard-type protein−polysaccharide conjugates was investigated using the lysozyme as a model protein, various sizes of galactomannan as a polysaccharide, and xyloglucan as an oligosaccharide. The emulsifying properties of the lysozyme−galactomannan conjugates increased in proportion to the length of polysaccharide chains. On the other hand, the heat stability of the lysozyme−galactomannan conjugates greatly increased in the range of 3.5−24 kDa galactomannan, regardless of its molecular mass. In addition, the effects of the binding number of polysaccharide to lysozyme on the emulsifying properties of the lysozyme−galactomannan conjugates were further investigated using 1 and 2 mol of the galactomannan (24 kDa)-attached lysozyme. The 2 mol of the polysaccharide-attached lysozyme showed better emulsifying properties than 1 mol of the polysaccharide-attached protein. The residue most sensitive to the Maillard reaction in a lysozyme was identified as the 97-lysine using the lysozyme−xyloglucan conjugate.

Keywords: *Lysozyme; Maillard-type protein−polysaccharide conjugate; galactomannan; xyloglucan*

INTRODUCTION

Many researchers have attempted to convert food proteins into useful proteins with better functional properties by chemical and enzymatic modification. However, only a few chemical approaches to proteins were available to food applications because of the safety issue. We have proposed that protein− polysaccharide conjugates prepared by a naturally occurring reaction without any chemicals are useful as a new functional biopolymer having excellent emulsifying, antioxidant, and antimicrobial effects for food applications (Kato et al., 1990; Nakamura et al., 1991, 1992). First we reported that the safe ovalbumin−dextran conjugate can be prepared by covalent binding between the ϵ-amino groups in the protein and the reducing-end carbonyl group in the polysaccharide through a controlled Maillard reaction in the dry state without using any chemical reagents (Kato et al., 1990). Interestingly, the emulsifying properties of this conjugate were much better than those of commercial emulsifiers even under acidic pH or in high-salt conditions. In addition, other protein conjugates with polysaccharides also showed a significant improvement in functional properties such as emulsifying properties, solubility, antioxidative effect, and antimicrobial activity. For instance, insoluble wheat gluten was solubilized and its functional properties improved by Pronase treatment followed by dextran conjugation (Kato et al., 1991). In the case of casein, the conjugation of protein−polysaccharide was quickly done within 24 h. On the other hand, it takes a long time to form protein−polysaccharide conjugates in the case of folded or rigid proteins, and the emulsifying properties of this conjugate were compared to those of commercial emulsifiers and found to be much better in acidic and high-salt conditions (Kato et al., 1992). Further, Nakamura et al. (1991) reported that the lysozyme−dextran conjugate had bifunctional properties and excellent emulsifying properties with antimicrobial effects against both Gram-positive and -negative bacteria.

Thus, various hybrid proteins with polysaccharides such as dextran or galactomannan commonly revealed excellent functional properties. On the other hand, the conjugation of proteins with small carbohydrate molecules such as glucose or lactose under controlled dry-heating resulted in insoluble aggregates having poor surface properties (Kato et al., 1990). Therefore, to improve the surface properties of the proteins, conjugation with a polysaccharide is desirable for industrial applications. However, it remains to be solved how long a polysaccharide chain is required to improve the functional properties of the protein and how many molecules of the polysaccharide should be attached to the proteins. Therefore, this paper describes the effects of the length and binding number of the carbohydrate chains on the functional properties of protein−polysaccharide conjugates using lysozyme as the model protein. Galactomannan and xyloglucan, derived from guar gum and tamarind seed, respectively, are used as the model polysaccharide and oligosaccharide, because these are well-characterized and favorable polysaccharides that are utilized as a thicker binder and stabilizing agent in food applications. Guar gum (galactomannan) is composed of a (1→4)-β-D-mannan backbone substituted with a side chain of α-D-galactose linked (1→6) to mannan residues. In addition, tamarind seed (xyloglucan) contains a relatively similar polysaccharide chain framework composed of a (1→4)-β-D-glucan backbone substituted with a side chain of α-D-xylose and β-D-galactosyl-(1→2)-α-D-xylose linked (1→6) to glucose residues.

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MATERIALS AND METHODS

Materials. Galactomannan (GM, mannase hydrolysate of guar gum) was supplied by Taiyo Chemicals Co. Xyloglucan (XG, hydrolysate of tamarind gum, average molecular weight (MW) of 1400) was supplied from Dainihon Pharmaceutical. Lysozyme was crystallized from fresh egg white at pH 10.0 in the presence of 5% sodium chloride and recrystallized five times (Alderton and Fevold, 1946). Sephadex G-50 and CM-Toyopearl 650M were purchased from Pharmacia and Tosoh Co., respectively. Various sizes of dialysis membranes for a molecular weight cutoff (MWCO) from 3500 to 12 000−14 000 were obtained from Sanko Junyaku Co. for 12 000−14 000 and from Spectrum Medical Industries for 6000−8000 and 3500. All other chemicals were of analytical grade.

Preparation of Galactomannan with Various Molecular Sizes by Dialysis. Galactomannan solution (5%, w/v) was dialyzed against deionized water at 4 °C for 48 h using dialysis tubing with a molecular weight cutoff (MWCO) of 12 000−14 000. The inner dialyzate was collected and lyophilized. The molecular weight was determined to be 24 000 using a low-angle laser light scattering technique combined with HPLC (Takagi and Hizukuri, 1984). The outer dialysate was collected and lyophilized, dissolved in some distilled water, and further fractionated using a dialysis membrane with a MWCO of 6000−8000. The outer and inner dialysates were separately collected and lyophilyzed. The former was used as a galactomannan (6000−12 000), and the latter was further fractionated using a dialysis membrane with a MWCO of 3500. The inner dialysate was collected, lyophilized, and used as a galactomannan 3500−6000.

Preparation of Lysozyme Conjugates with GMs and XG. The lysozyme and various sizes (3500−6000, 6000−12 000, 24 000) of GMs were dissolved in distilled water each in a mole ratio of 1:4 and then lyophilized. Each powder mixture was incubated at 60 °C for 2 weeks at a relative humidity of 78.9% in a desiccator containing a saturated KBr solution in the bottom. The lysozyme−XG conjugate was prepared in the mole ratio of 1:8 for 24-h incubation in a controlled dry state, as described above. Lysozyme−saccharide conjugates were separated from unreactive lysozyme and polysaccharides by gel filtration on a columns of Sephadex G-50 and CM-Toyopearl 650M as described below.

Gel Filtration of Lysozyme Conjugates on a Sephadex G-50 Column. Lysozyme−polysaccharide conjugates were separated by gel filtration on a Sephadex G-50 column (2 × 82 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.0) at a flow rate of 20 mL/h. The protein content in each fraction was detected by measuring the absorbance at 280 nm, and the carbohydrate was determined by measuring the absorbance at 490 nm after color development with the phenol−sulfuric acid reaction. The void volume fraction containing the lysozyme−polysaccharide conjugates was collected, dialyzed against deionized water at 4 °C for 48 h, and lyophilized.

Separation of Conjugates by Cation-Exchange Chromatography on CM-Toyopearl 650M Column. For further purification, the lysozyme conjugates with saccharide were loaded on a CM-Toyopearl 650M column equilibrated with 20 mM sodium phosphate buffer (pH 7.0) at a flow rate of 30 mL/h. Elutions were done stepwise with the same buffer containing 0.1, 0.2, and 0.3 M NaCl concentration. The protein and carbohydrate contents were determined according to the same methods described above. Each peak was collected, dialyzed against deionized water at 4 °C for 48 h, and lyophilized.

Electrophoresis in SDS Slab Polyacrylamide Gel. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to the method of Laemmli (1970) using a 15% acrylamide separating gel and a 5% stacking gel containing 0.1% SDS. Samples (20 mL, 0.1%) were prepared in a 0.01 M Tris−glycine buffer at pH 8.8 containing 1% SDS. Electrophoresis was carried out at a constant current of 15 mA on a sheet for 2 h in a Tris−glycine buffer containing 0.1% SDS. The gel sheets were stained for protein with Coomassie brilliant blue G-250.

Measurement of Emulsifying Properties. The emulsifying properties were determined according to the modified method of Pearce and Kinsella (1978). To form an emulsion, 1.0 mL of corn oil and 3.0 mL of a sample solution (0.1%) in $^1/_{15}$ M sodium phosphate buffer at pH 7.4 were homogenized in a Polytron PT 10-35 homogenizer (Kinematica Co., Switzerland) at 12 000 rpm for 1 min. One hundred microliters of the emulsion was taken from the bottom of a test tube at different times (0, 1, 2, 3, 5, and 10 min) and diluted with 5 mL of a 0.1% SDS solution. The turbidity of the diluted emulsion was then determined at 500 nm. The emulsifying activity was determined from the turbidity measured immediately after the emulsion had formed (0 min). The emulsion stability was estimated by measuring the half-time of the turbidity measured immediately after the emulsion had formed.

Measurement of Amino Groups. The free amino group content in the lysozyme conjugates with polysaccharides was determined according to the methods of Haynes et al. (1967) using trinitrobenzenesulfonate, a specific reagent for amino groups.

Measurement of Heat Stability. The apparent heat stability was estimated by measuring the developed turbidity when a 0.074% protein concentration solution of native lysozyme and lysozyme−saccharide conjugates was heated to 95 °C at a rate of 1 °C/min from 50 °C in $^1/_{15}$ M sodium phosphate buffer (pH 7.4). After a given temperature, the heated sample was immediately put into a cuvette and the turbidity was measured at 500 nm. The temperature interval of the measurements was 1 °C near the melting point (T_m), while it was 5 °C otherwise.

Peptide Mapping of Purified Lysozyme−Xyloglucan Conjugate. To determine the active lysine residues on lysozyme, peptide mapping of the lysozyme−XG conjugate separated by cation-exchange chromatography was performed. The trypsin digestion was carried out by using the suspension of reduced *S*-2-aminoethylated native and conjugated lysozyme as described by Okazaki et al. (1985). The enzymatic digestion was allowed to proceed for 120 min. The analysis of the tryptic hydrolysate was carried out by reversed-phase high-performance chromatography. A TSK gel ODS-120T column (4 × 200 mm, Tosoh, Japan) was attached to a Hitachi 655-A-11 liquid chromatograph equipped with a Hitachi 655-A LC detector. The column was equilibrated with 1% acetonitrile containing 0.1% concentrated HCl and eluted with a gradient of 40 mL of 40% acetonitrile containing 0.1% concentrated HCl at a flow rate of 0.4 mL/min for 200 min. Peptide elution was monitored by absorbance of the effluent at 210 nm.

RESULTS AND DISCUSSION

Effect of the Length of Polysaccharide Chains on the Emulsifying Properties of Lysozyme−Polysaccharide Conjugates. The effect of the length of the polysaccharide chains on the emulsifying properties of the lysozyme−GM conjugates is shown in Figure 1. The emulsifying activity and emulsion stability calculated from the figure are shown in Table 1. The emulsifying properties of the lysozyme−GM conjugates increased in proportion to the length of the polysaccharide chains. The emulsion stability of the lysozyme−GM (3500−6000) conjugate was very low. This result suggests that the conjugation of protein with GM having a molecular size of more than 6000−12 000 is essential for the improvement of the emulsifying properties. To further elucidate the effect of the oligosaccharide chain on the emulsifying properties of the protein−saccharide conjugate, XG was used in the experiment. XG is a well-documented oligosaccharide composed of hepta-, octa-, and nonasaccharides at a weight ratio of 10.4, 33.3, and 53.2%, respectively. The emulsifying properties of the lysozyme−XG conjugate were almost the same as those of the lysozyme−GM (3500−6000) conjugate. Thus, it was confirmed that the lysozyme−oligosaccharide conjugate does not improve the emulsifying properties. The

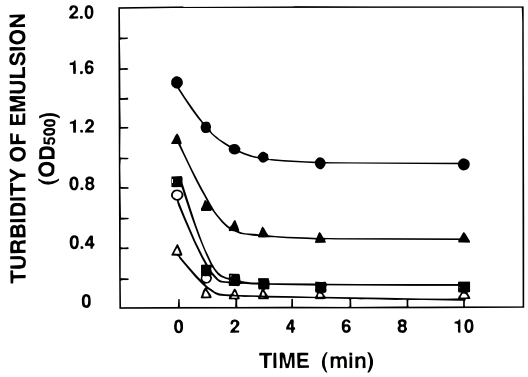

Figure 1. Emulsifying properties of lysozyme−polysaccharide conjugates prepared with various sizes of GM and XG: △, native lysozyme; ●, lysozyme−GM (24 000) conjugate; ▲, lysozyme−GM (6000−12 000) conjugate; ■, lysozyme−GM (3500−6000) conjugate; ○, lysozyme−xyloglucan (1400) conjugate. Data are from a representative experiment repeated three times with similar results.

Table 1. Emulsifying Activity and Emulsion Stability of Lysozyme−Polysaccharide Conjugates

conjugate	emulsifying activity (OD_{500})	emulsion stability (min)
native lysozyme	0.374	<1
lysozyme−GM (3500−6000)	0.837	<1
lysozyme−GM (6000−12 000)	1.112	1.7
lysozyme−GM (24 000)	1.479	>25
lysozyme−XG (1400)	0.740	<1

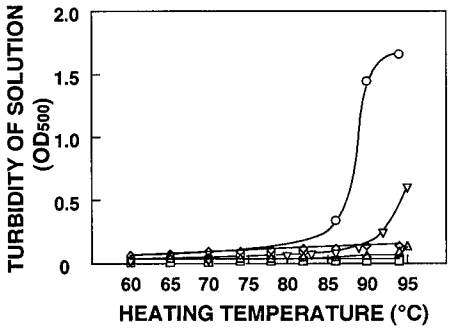

Figure 2. Heat stability of lysozyme−GM and lysozyme−XG conjugates: ○, native lysozyme; ▽, lysozyme−XG (1400) conjugate; □, lysozyme−GM (24 000) conjugate; ◇, lysozyme−GM (6000−12 000) conjugate; △, lysozyme−GM (3500−6000).

role of polysaccharide in the stabilization of emulsion of protein−polysaccharide conjugates is considered as follows. The hydrophobic residues in protein molecule are anchored in the oil droplets during emulsion formation. The polysaccharide orients to the aqueous layer after the emulsion preparation and accelerates the formation of a thick steric stabilizing layer around the emulsion, thereby inhibiting the coalescence of oil droplets. Thus, it was elucidated that the dramatic improvement of lysozyme is brought about by the attachment of polysaccharides but not oligosaccharides.

Heat Stability of Lysozyme−GM Conjugates. The effect of the polysaccharide chains on the heat stability of the lysozyme−polysaccharide conjugates is shown in Figure 2. The apparent heat stability of the native and conjugated lysozymes was examined at a pH of 7.4 from 60 to 95 °C. The turbidity of the native lysozyme gradually increased with a transition point at 88 °C and reached the maximum turbidity of 1.6 (OD_{500}) at 95 °C. On the other hand, the lysozyme−GM

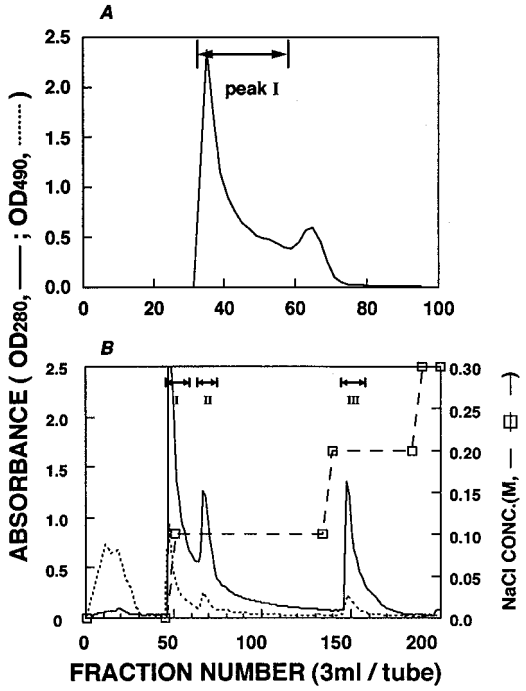

Figure 3. Elution patterns on a column of Sephadex G-50 (A) and CM-Toyopearl 650M (B) of lysozyme−GM (24 000) conjugate. Peak I (horizontal arrow in panel A) was collected and then applied to CM-Toyopearl column. Peaks I, II, and III were collected in the fraction numbers indicated in each horizontal arrows in panel B, respectively, and used for the experiment.

conjugates showed no aggregation up to 95 °C, regardless of the molecular size of the attached GM. This result suggests that the lysozyme is converted into the heat-stable form by the conjugation with several kilodaltons of GM. To elucidate the critical size of the saccharide chain that stabilizes the protein−saccharide conjugates, XG (1400) was used in the experiment. As shown in Figure 2, the lysozyme−XG conjugate was not as heat-stable as the lysozyme−polysaccharide conjugates, although it was more stable than the native lysozyme. Therefore, several kilodaltons of saccharide may be necessary for the lysozyme to stabilize against heating. This suggests that the polysaccharide attached to lysozyme may stabilize the protein molecule according to the manner in which the protein unfolded during heating is sterically protected against aggregation by polysaccharide.

Further Purification and Characterization of Lysozyme−GM Conjugates Using Cation-Exchange Chromatography. To further elucidate the effect of the Maillard-type polyglycosylation of protein on the emulsifying properties, the conjugates were further purified by cation-exchange chromatography. The elution patterns of the lysozyme−GM (24 000) are shown in Figure 3. Panel A shows the elution pattern on a Sephadex G-50 column, and panel B shows the elution pattern on CM-Toyopearl. After gel filtration on a Sephadex G-50 column, the void volume fraction was collected and then applied to a CM-Toyopearl column. The conjugates were stepwise eluted with the same buffers containing 0.1, 0.2, and 0.3 M NaCl. Two peaks (peaks I and II) were eluted with 0.1 M NaCl concentration, and another peak (peak III) was eluted with 0.2 M NaCl. These peaks are considered to be separated by the difference in the number of the positive charges of the lysozyme−GM conjugates and the size of the

Table 2. Residual Free Amino Groups and Carbohydrate Content in Peaks I–III Separated by CM-Toyopearl Column Chromatography of Lysozyme–GM Conjugate

	no. of free amino groups	carbohydrate content[a]
native lysozyme	7	0
peak I	4.7	2
peak II	6.1	1
peak III	5.9	0.6

[a] Indicated as a mole ratio to protein.

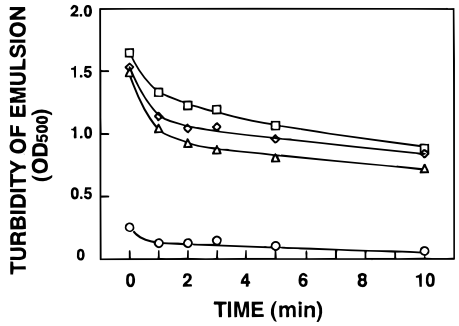

Figure 4. Emulsifying properties of peaks I–III in lysozyme–GM conjugate separated by a CM-Toyopearl column: ○, native lysozyme; □, peak I; ◇, peak II; △, peak III. Data are from a representative experiment repeated three times with similar results.

attached saccharide, because the Maillard-type protein–polysaccharide conjugates are formed between the free amino groups in the proteins and the reducing end carbonyl groups in the polysaccharides. Therefore, peaks I–III seem to elute in proportion to the number of attached polysaccharide chains through the amino groups in the lysozyme. Table 2 shows the number of free amino groups and the molar ratio of carbohydrate to protein in peaks I–III. Taking into account these data and the elution position on a cation-exchange chromatography, peak I must be 2 mol of the GM-attached conjugate and peaks II and III may be 1 mol of the GM-attached conjugates. Since the elution position of the lysozyme–GM conjugates may also be affected by the size of the attached carbohydrate chain, the difference in the elution of peaks II and III may be due to the size of the attached polysaccharide. The carbohydrate content of peak II was higher than that of peak III, suggesting that the length of the polysaccharide chain in peak II is longer than that in peak III.

Emulsifying Properties of Peaks I–III. The emulsifying properties of each peak are shown in Figure 4. Two polysaccharide-attached lysozymes (peak I) showed better emulsifying properties than the one polysaccharide-attached protein (peak II). The smaller carbohydrate chain-attached lysozyme (peak III) showed lower emulsifying properties than peaks I and II. We reported that the lysozyme has two reactive amino groups (N-terminal and 97-lysine) for the Maillard reaction (Nakamura et al., 1994). Therefore, it seems likely that peak I is glycosylated both at the N-terminal- and 97-positions, and peaks II and III are glycosylated at either the N-terminal- or 97-position. It is interesting to elucidate which lysyl residue is the sensitive site for the Maillard reaction. Thus, the reactive lysyl residue was detected as follows.

Identification of the Most Sensitive Site for the Maillard Reaction in the Lysozyme. To identify the most sensitive lysyl residue for the Maillard reaction,

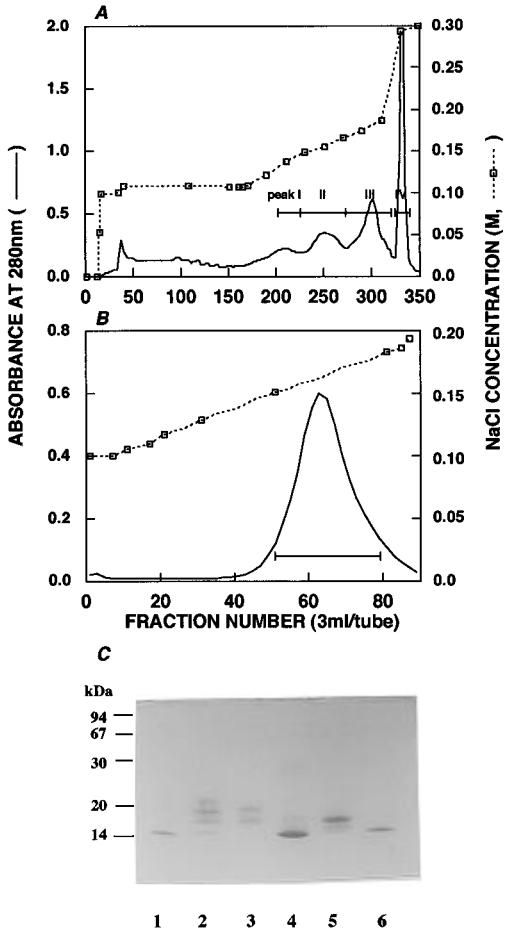

Figure 5. Elution patterns of 1 mol XG-attached lysozyme by a CM-Toyopearl column chromatography (A) and rechromatography (B) and SDS–PAGE patterns (C) of each fraction. Peaks I–IV in panel A were collected in the fraction number indicated in figure and then applied to SDS–PAGE. Peak III was applied to rechromatography on a CM-Toyopearl column. Lanes 1 and 6, native lysozyme; lane 2, peak I in panel A; lane 3, peak II in panel A; lane 4, peak IV in panel A; lane 5, rechromatographed peak III (panel B).

the 1 mol XG-attached lysozyme was purified as shown in Figure 5. Since XG is an oligosaccharide having a small molecular weight distribution range (hepta-, octa-, and nonamer), it is easy to purify and identify the 1 mol saccharide-attached lysozyme. This is the reason the lysozyme–XG conjugate was used for identifying the most sensitive lysyl residue for the Maillard reaction in the lysozyme. The conjugate was applied to a column of CM-Toyopearl (panel A). The four peaks were separated, and then each peak was rechromatographed on a CM-Toyopearl column. The rechromatographic pattern of peak III is shown in panel B. The SDS–PAGE patterns of the each purified peak are shown in panel C. Taking into account the molecular size of XG, the peaks I (lane 2), II (lane 3), III (lane 5), and IV (lane 4) seem to bind 3, 2, 1, and 0 mol of XG to lysozyme, respectively. Thus, peak III was confirmed to be the 1 mol XG-attached lysozyme. By using this fraction, the most sensitive site to Maillard reaction was determined from the peptide map as described below. Figure 6 shows the reversed-phase high-performance liquid chromatography elution patterns of tryptic peptides from native lysozyme (panel A) and lysozyme–XG conjugate (panel B) purified by CM-Toyopearl. The tryptic hydrolysate of the lysozyme–XG conjugate significantly reduced the peak of the segment 97–112 (T12+13).

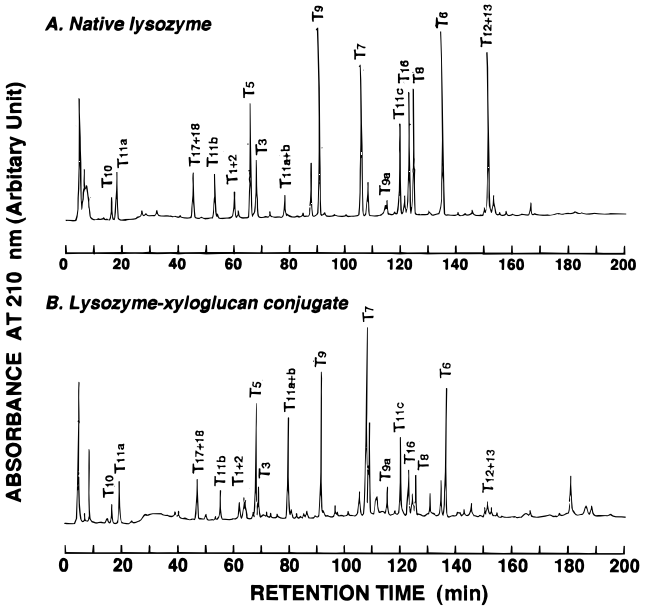

Figure 6. Reversed-phase HPLC elution patterns of tryptic digests of native lysozyme (A) and purified lysozyme−XG conjugate (B). The nomenclature of peptide fragments (T) is referred to Canfield (1963).

These results indicate that among the seven amino groups, Lys-97 in the lysozyme is the most susceptible to an amino−carbonyl reaction in a dry state. Another peak of segment 1−5 (T1+2) was slightly reduced, suggesting the N-terminal lysyl α- or ϵ-amino groups may subsequently react with XG through the Maillard reaction. The fact that Lys-97 in the lysozyme is the most reactive nucleophile suggests that the same site (position 97) is also preferentially involved in the formation of the lysozyme−GM conjugate.

In conclusion, the length of the polysaccharide and the binding number to a protein greatly affect the functional properties of Maillard-type lysozyme−polysaccharide conjugates. The emulsifying properties of the conjugates were greatly increased in proportion to the length of the polysaccharide, while the heat stability of the conjugates was enhanced regardless of the molecular size of the saccharide. The 2 mol of polysaccharide-attached lysozyme showed better emulsifying properties than the 1 mol of polysaccharide-attached protein. The residue most sensitive for the Maillard reaction in the lysozyme was identified as the 97-lysine, and the subsequent reactive site was the N-terminal lysine.

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