Presence of *O*-Glycosidic Linkage through Serine Residue in κ -Casein Component from Bovine Mature Milk

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Summary This paper describes the glycosylation sites of κ -casein component P-5 from bovine mature milk. A short glycopeptide was prepared from κ -casein component P-5 containing two carbohydrate chains by pronase P digestion, followed by gel filtration and ion exchange chromatographies. The glycopeptide obtained corresponded to residues 128–141 (Gly-Glu-Pro-Thr-Ser-Thr-Pro-Thr-Thr-Glu-Ala-Val-Glu-Ser) of κ -casein A from the results of analyses with chemical and enzymatic procedures. The effect of alkaline borohydride treatment indicated the presence of serine as well as threonine as the binding site of carbohydrate moieties. From the facts of Edman degradation and carboxypeptidase P hydrolysis of glycopeptide treated with alkali, the carbohydrate moieties were considered to be attached to threonine residue No. 133 and serine residue No. 141.

Key Words κ -casein, mature milk, *O*-glycosidic linkage, serine, threonine, galactosamine, carbohydrate, attachment site, glycoprotein

Bovine κ -casein is a heterogeneous protein (1–3), and the heterogeneity is due only to carbohydrate moiety (4). The structure of the carbohydrate portion has been studied by many scientists (5–7). The carbohydrate sequence of the main sugar portion from bovine κ -casein is indicated to be *N*-acetylneuraminyl-galactosyl-*N*-acetylgalactosamine, and the occurrence of other carbohydrate structures in κ -casein is also reported (7). In any case, every carbohydrate chain reported had one molecule of galactosamine. The increase of absorbance at 241 nm and the release of carbohydrate moiety were observed when the glycopeptide was exposed to dilute alkaline solution (5, 8, 9). These reports indicate that the sugar chain of κ -casein was linked *O*-glycosidically to the peptide chain. However, the amino acid residue

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bound to the sugar moiety has not been decided. In our previous paper (10), the attachment sites of carbohydrate moieties were reported with κ -casein component P-6 containing the most carbohydrates. In the case of κ -casein component P-6, the carbohydrate moieties were attached to threonine residues *O*-glycosidically. The existence of *O*-glycosidic linkage through serine residue was, however, suggested from the preliminary experiment of alkali treatment with whole κ -casein prior to the conclusion described above. In this paper, the binding sites of sugars in κ -casein component P-5, which has two molecules each of sialic acid and galactosamine, are examined and the *O*-glycosidic linkage between serine residue as well as threonine residue and the carbohydrate chain is characterized.

EXPERIMENTAL

Materials. κ -Casein component P-5 was prepared from whole acid casein from a single Holstein cow's milk (4). Pronase P was a product of Kaken Chemical Co. (Tokyo). Carboxypeptidase P purified from *Penicillium janthinellum* was obtained from the Protein Research Foundation (Osaka). Precoated thin layer plate (silica gel 60 F₂₅₄) was from Merck (Darmstadt, West Germany). PTH-amino acids were purchased from Sigma Chemical Company (St. Louis, U.S.A.).

Preparation of glycopeptide from κ -casein component P-5. A pure glycopeptide was prepared following the procedures shown in Scheme 1. Aliquots were analyzed manually with phenol-sulfuric acid (11) and with ninhydrin reagent after alkaline hydrolysis (12). Four hundred milligrams of κ -casein component P-5 were digested by 4 mg of pronase P in 20 ml of 20 mM Tris-HCl buffer, pH 8.0, at 37°C for 24 hr. The digestion was stopped by the adjustment of pH to 2.2 with 1 N HCl. All reaction mixture was applied on a Dowex 50WX2 column (2.2 × 40 cm) equilibrated with 0.2 M sodium citrate-HCl buffer, pH 2.2. Elution was carried out with 120 ml each

Scheme 1.

of 0.2 M sodium citrate-HCl buffer, pH 2.2, and 0.2 M sodium citrate-HCl buffer, pH 3.25. The phenol-sulfuric acid reaction positive fraction (5-PD) was lyophilized and applied on a Bio-Gel P-2 column (2×150 cm) for gel filtration and desalination. A major fraction of gel filtration chromatography was lyophilized and chromatographed on a Dowex 50WX2 column (2.2×40 cm) with a linear gradient (0.05 to 0.5 M) of pyridine-acetic acid buffer, pH 5.5.

Other methods. Chemical analysis, alkaline borohydride treatment and the determination of N- and C-terminal amino acid sequences of glycopeptide were carried out as described by Doi *et al.* (13). Alkaline borohydride treatment was performed in the dark with shaking at 37°C in order to heighten the yield of reduced amino acids. The reaction mixture contained norleucine as the internal standard.

RESULTS

Preparation of glycopeptide from κ -casein component P-5

It is important to prepare pure glycopeptide in order to evaluate the attach-

Fig. 1. Dowex 50WX2 chromatogram of κ -casein component P-5 digested by pronase P. Four hundred milligrams of κ -casein component P-5 were dissolved in 20 ml of 20 mM Tris-HCl buffer, pH 8.0, and 4 mg of pronase P was added to the solution. Digestion was carried out at 37°C for 24 hr. Reaction mixture was applied on a Dowex 50WX2 column (2.2×40 cm) equilibrated with 0.2 M sodium citrate-HCl buffer, pH 2.2, after adjustment of pH to 2.2. A flow rate was maintained at 60 ml per hour. One tube contained 4 ml.

Fig. 2. Bio-Gel P-2 gel filtration profile of fraction 5-PD. Fraction 5-PD was lyophilized and dissolved in a minimum volume of water. The solution was applied on a Bio-Gel P-2 column (2×150 cm). Elution was carried out with distilled water. A flow rate was maintained at 12 ml per hour. One tube contained 3 ml.

Fig. 3. Ion exchange chromatogram on Dowex 50WX2 of fraction 5-B. Fraction 5-B was lyophilized and dissolved in a minimum volume of water. The solution was applied on a Dowex 50WX2 column (2.2×40 cm) equilibrated with the starting buffer after the adjustment of pH to 5.5. Elution was carried out with a linear gradient (0.05 to 0.25 M) of pyridine-acetic acid buffer. A flow rate was maintained at 50 ml per hour. One fraction was 3 ml. —, absorbance at 490 nm; -----, absorbance at 570 nm.

ment site of the carbohydrate portion. Figure 1 shows the Dowex 50WX2 chromatogram of κ -casein component P-5 digested by pronase P. One major peak eluted with 0.2 M sodium citrate-HCl buffer, pH 2.2, was detected by phenol-sulfuric acid reaction. This fraction (5-PD) was pooled and lyophilized. Gel filtration of fraction 5-PD was performed on a Bio-Gel P-2 column. The Bio-Gel P-2 gel filtration profile is shown in Fig. 2. The main fraction 5-B (underlined in Fig. 2) was applied on a Dowex 50WX2 column equilibrated with a volatile buffer for purification. Only one main peak, 5-GP, was detected by phenol-sulfuric acid

reaction, while alkaline ninhydrin reaction indicated the existence of plural peaks (Fig. 3). The phenol-sulfuric acid reaction positive peak coincided in position with the main peak of ninhydrin reaction positive peak. Ten milligrams glycopeptide 5-GP were obtained after lyophilization.

Chemical composition

Amino acid and amino sugar analyses of glycopeptide 5-GP were carried out (Table 1). The amino acid composition was in good agreement with that of the residues 128–141 of κ -casein reported by Mercier *et al.* (14). This glycopeptide contained 4 molecules of threonine, 2 molecules of serine and 2 molecules of galactosamine. The number of galactosamine residue suggests the occurrence of 2 carbohydrate chains in glycopeptide 5-GP, considering results obtained by many workers (5, 6, 15). *N*-Glycosidic linkage does not exist in this glycopeptide because it does not contain any aspartic acid.

Table 1. Chemical composition of glycopeptide 5-GP from κ -casein component P-5. The number of residues was calculated based on one residue of glycine per molecule.

Constituent	Residue/mol	Integer
Thr	4.31	4
Ser	2.09	2
Glu	2.82	3
Pro	2.02	2
Gly	1	1
Ala	1.03	1
Val	0.86	1
GalNH ₂	1.74	2

Fig. 4. Effect of alkaline borohydride treatment on glycopeptide 5-GP. Experiment was carried out as described by Doi *et al.* (13). ●, Thr; □, Ala; △, Ser; ○, ABA.

Alkaline borohydride treatment of glycopeptide 5-GP

A carbohydrate chain bound *O*-glycosidically was released from a peptide chain under an alkaline condition, and serine and threonine were converted to alanine and α -aminobutyric acid, respectively, by reduction of corresponding keto acids (16). Glycopeptide 5-GP was incubated in 0.1N NaOH/1 M NaBH₄ at 37°C for several hours with shaking in the dark. Shaking in the dark has been found to be effective to heighten the yield of the reduced amino acid from serine and threonine beyond that reported in our previous paper (10). The effect of alkaline borohydride treatment is shown in Fig. 4. The numbers of threonine and serine decreased from 4.31 and 2.02 residues per molecule to 2.6 and 1.2 residues, respectively, after treatment for 31 hr. On the other hand, 0.98 residue of α -aminobutyric acid was produced and the number of alanine residue increased from 1.03 to 1.8 residues per molecule. These results indicate that glycopeptide 5-GP had 2 carbohydrate chains bound *O*-glycosidically to each residue of threonine and serine.

Terminal amino acid sequences of glycopeptide 5-GP

Terminal amino acid sequences were examined with glycopeptide 5-GP from which sugar moiety had been removed. Figure 5 shows the Sephadex G-10 gel filtration profile of glycopeptide 5-GP treated by alkali under reducing condition. Detection was carried out with alkaline ninhydrin reaction and absorbance at

Fig. 5. Purification on Sephadex G-10 of 5-GP treated by alkali under reducing condition. Fraction 5-GP treated with 0.1 N NaOH/1 M NaBH₄ at 37°C for 31 hr was applied on a Sephadex G-10 column (2.2 × 70 cm) after the adjustment of pH to 5.0 by the slow addition of 4 N acetic acid. A flow rate was maintained at 10 ml per hour. One tube contained 3.2 ml.

Fig. 6. Release of amino acids by the treatment with carboxypeptidase P from carbohydrate-free 5-GP. Experiment was carried out as described by Doi *et al.* (13). □, Ala; ▲, Glu; ■, Val; ●, Thr.

220 nm. It is expected that serine and threonine bound carbohydrate moiety would be detected as alanine and α -aminobutyric acid, respectively, by terminal sequence analyses. Edman degradation and hydrolysis by carboxypeptidase P of carbohydrate-free 5-GP were carried out.

i) *Edman degradation of carbohydrate-free 5-GP*

Edman degradation is the most available procedure in determining the N-terminus of protein. Only glycine was detected in the first step of this procedure. It was established that the N-terminal amino acid sequence of carbohydrate-free 5-GP was Gly-Glu-Pro-Thr-Ser-ABA-Pro-, where ABA is α -aminobutyric acid. This sequence agrees with residues 128–134 of κ -casein, and indicates that threonine residue No. 133 is linked to the carbohydrate chain O-glycosidically.

ii) *Carboxypeptidase P treatment of carbohydrate-free 5-GP*

Carboxypeptidase P treatment of carbohydrate-free 5-GP was carried out to obtain information on the C-terminal amino acid sequence. Figure 6 shows the result. Amino acids were released from carbohydrate-free 5-GP by carboxypeptidase P in the order of alanine, glutamic acid, valine and threonine. This fact suggests that the C-terminal amino acid sequence was -Val-Glu-Ala. If glycopeptide 5-GP comes from residues 128–114 of κ -casein A, it has one molecule of alanine residue No. 138, and does not contain the sequence of -Val-Glu-Ala. Considering that alanine residue of C-terminal amino acid of carbohydrate-free 5-GP was converted from serine of glycopeptide 5-GP by alkaline borohydride treatment, the result of carboxypeptidase P treatment is not in conflict with the primary structure of residues 139–141 of κ -casein.

DISCUSSION

Carbohydrate moieties are attached to the peptide chain through various

threonine and serine residues and the increase of α -aminobutyric acid and alanine residues. These facts show that two sugar moieties attached to each residue of threonine and serine. Threonine residue No. 131 and serine residue No. 132 were not the attachment site of the carbohydrate portion because they were detected by Edman degradation. The detection of α -aminobutyric acid instead of threonine residue No. 133 by Edman degradation indicates that this position was linked to carbohydrate moiety. The C-terminus of carbohydrate-free 5-GP was established as -Val-Glu-Ala by carboxypeptidase P treatment (Fig. 6). Alanine residue No. 138 must exist in glycopeptide 5-GP, which contained only one molecule of alanine. It was considered from the amino acid sequence of κ -casein A established by Mercier *et al.* (14) that alanine of C-terminal amino acid was converted from serine residue No. 141 by alkaline borohydride treatment. From these results, it was concluded that carbohydrate moieties of κ -casein component P-5 attached to threonine residue No. 133 and serine residue No. 141 of κ -casein.

Short glycopeptides have been prepared from κ -casein, and the attachment site of sugar portions was examined by the mild alkali treatment (8, 10, 18, 19). However, the glycopeptide obtained may be different from glycopeptide 5-GP in this paper, considering the amino acid composition and sequence indicated. Therefore, O-glycosidic linkage through serine residue may not be characterized.

One of the secondary structures of protein is a β -turn. A β -turn consists of four consecutive amino acid residues in a region where the polypeptide chain folds back on itself by nearly 180° (20). In all cases, the glycosylation sites of glycoproteins are situated in a β -turn (21, 22). Aubert and Loucheux-Lefebvre determined the secondary structure around the 28 asparagine residues linked glycans N-glycosidically (23). In 19 out of 28 cases, the asparagine residue is localized on a β -turn. On the other hand, in the case of O-glycosidic linkage, 9 residues of threonine or serine linked glycans belong to a β -turn.

κ -Casein does not contain N-glycosidic linkage. The glycosylation sites of κ -casein from normal milk and colostrum reported by us (10, 13) also belong to a β -turn, from the secondary structure (24) predicted according to the rule of Chou and Fasman.

In this work, it is considered that threonine residue No. 133 and serine residue No. 141 are the glycosylation sites of κ -casein component P-5 from bovine mature milk. Threonine residue No. 133 is situated in a β -turn. Serine residue No. 141 of κ -casein component P-5 may be the first example that serine residue not belonging to a β -turn is glycosylated.

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