Nutritional characteristics of a neoglycoprotein, casein modified covalently by glucose

Yukio Furuichi, Tetsuo Oogida, Chinami Mitsui, Mitsuyoshi Matsuno, and Takao Takahashi

Laboratory of Nutritional Chemistry, Faculty of Bioresources, Mie University, Tsu, Japan

Glucose was combined covalently with the ε-amino groups of lysyl residues of bovine casein in the presence of sodium cyanoborohydride as a reducing reagent by reductive alkylation, forming stable secondary amine linkages. Solubility characteristics and nutritional values of the neoglycoprotein were examined. The degree of modification (%) of the glucosylated casein was 82.5. Solubility of the modified casein was increased by the attachment of glucose. The modification did not disturb the digestion of casein by pepsin or trypsin. Rat feeding experiments using 10% protein diets demonstrated that the protein efficiency ratio (PER) of the modified casein was 0.35 ± 0.33 compared with 2.99 ± 0.29 for the unmodified casein. When the modified casein was supplemented with L-lysine to equal the level of total lysine of unmodified casein, the PER value was increased to 2.21 ± 0.29. Nitrogen balance experiments showed that the modified casein was digested completely. On the other hand, biological value and net protein utilization of the modified protein were shown to be considerably lower than those of the unmodified casein.

Keywords: Neoglycoprotein; bovine casein; nutritional values; modification; glucosylation

Introduction

A variety of chemical and enzymatic methods of improving the functional and nutritional characteristics of food proteins have been developed.[1,2] Lee et al.[3] prepared the modified caseins by reductive alkylation with the intention of minimizing the deteriorative reactions, such as the Maillard-type reaction involving lysyl residues in proteins and reducing sugars during food processing and storage. These investigators found that a preparation of partially (50%) methylated casein could support the normal growth of rats and suggested that the limited methylation of proteins may be useful for the protection of lysyl residues against deteriorative reactions.[3] Similar favorable effects are expected when ε-NH_2 groups of the lysyl residues in proteins are modified by reducing sugars. However, it is most important to determine whether any nutritional changes are brought about by the modification process.

A variety of methods for the covalent attachment of carbohydrates to protein molecules have been developed.[4,5] Among these, reductive alkylation seems to be a relatively simple one. In the present paper, we describe the nutritional characteristics of bovine casein covalently modified by glucose (glucosylated casein) using reductive alkylation in the presence of sodium cyanoborohydride.

Materials and methods

Materials

Vitamin-free casein was obtained from Oriental Yeast Co., Ltd., Tokyo, Japan. α-D-Glucose was obtained from a commercial source. Unless otherwise mentioned, all the chemicals used were of reagent grade.

Preparation of glucosylated casein

Glucosylated casein was prepared according to Lee et al.[6] Casein (300 g) was dispersed in 3 l of 0.2 M potassium phosphate buffer, pH 8.0, containing 5% dioxane

Address reprint requests to Dr. Yukio Furuichi, Laboratory of Nutritional Chemistry, Faculty of Bioresources, Mie University, Tsu 514, Japan.
Received September 22, 1989; accepted November 20, 1989.

at 40°C. Glucose, 150 g, and sodium cyanoborohydride (Aldrich Chemical Co.), 40 g,were added to the casein solution. The solution was stirred for 120 hours at 37°C. After NaCl was added to give a final concentration of 0.1 M, the solution was dialyzed against 0.1 M NaCl solution, then deionized water. The modified casein was precipitated by adjusting pH of the solution to 4.5 and was lyophilized. The dry preparation was ground finely with a mortar and used for chemical analysis and for preparing the experimental diets.

Analytical procedures

Five-milligram samples of proteins were hydrolyzed in 5 ml of 6 N-HCl in sealed tubes at 110°C for 24 hours. Amino acid composition of the proteins was determined according to Spackman et al.[7] with an amino acid analyzer (Hitachi HPLC System 655A, Tokyo, Japan). The extent of glycosylation was estimated by determining the amount of unreacted lysine residues and subtracting the amount of reacted residues.

pH-Dependent solubility

Modified and unmodified caseins were dispersed in distilled water (0.1%, wt/wt) by vigorous mixing. The pH of the solutions was adjusted from 2.0 to 9.0 with HCl or NaOH solution of high normality to limit dilution. Each solution was filtered by a membrane filter, and the optical density of the filtrates was measured at 280 nm.

Nutritional evaluation

In vitro digestibility by pepsin and trypsin. Five hundred milligrams of modified or unmodified casein was dispersed in 30 ml of water, and the pH of the solution was adjusted to 2.00 with HCl. Enzymatic digestion was performed at 37°C by 25 mg of pepsin (Sigma, St. Louis, MO, USA; preparation from porcine stomach mucosa). After sequential times, 5-ml aliquots were withdrawn and equal volumes of 20% trichloroacetic acid were added to precipitate the undigestible proteins. Precipitates were removed by centrifugation (12,000 rpm, 10 minutes) and optical densities of the clear supernatants were measured at 280 nm. Tryptic digestion was performed in 30 ml of 0.1 M phosphate buffer, pH 7.60, at 40°C using 25 mg of trypsin (Sigma, preparation from bovine pancreas, Type III) and 300 mg of the proteins. Optical densities of the supernatants were measured by the same method used for the pepsin digestion.

Animal feeding experiment. Weanling male rats of the Wistar strain (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan), weighing 40 to 50 g, were used. The rats were housed individually in metabolism cages suitable for collecting feces and urine in an air-conditioned room (room temperature, 22 ± 2°C; lights on, 08:00 to 20:00). After the adaptation period of 7 days, the experimental animals were divided, on the basis of body weight, into three groups of five rats each and were fed the experimental diets ad libitum. The dietary compositions are listed in *Table 1*. Each protein source was incorporated into the diet to provide 10% of protein (N × 6.25). Body weights and food consumption were recorded daily. The protein efficiency ratio (PER) was determined according to the method previously described,[9] except that the 4-week experimental period was changed to 2 weeks. During the middle 4 days (5th to 9th day) of the experimental period, all feces and urine were collected separately and frozen for subsequent analysis of nitrogen content. True digestibility (TD) and biological value (BV) were determined by the nitrogen balance method of Mitchell.[10] Net protein utilization (NPU) was calculated as TD × BV/100. The nitrogen content of feed, feces, and urine was determined by the Kjeldahl method.[11]

Table 1 Compositions of the experimental diets (%)

	Unmodified casein	Glucosylated casein	Glucosylated casein + lysine
Protein source	12.5	12.5	13.1
L-Lysine HCl	—	—	0.6
Soybean oil	5.0	5.0	5.0
Salt mixture[8]	4.0	4.0	4.0
Vitamin mixture[8]	1.0	1.0	1.0
Cellulose powder	2.0	2.0	2.0
Corn starch	75.5	75.5	74.3

Statistical analysis

Statistical significance of the difference between diet groups was analyzed by the Student's *t* test.[12]

Results and discussion

Chemical composition

Table 2 shows the approximate composition of the unmodified (control) casein determined according to a method previously described.[11] *Table 3* represents the amino acid compositions of modified and unmodified caseins. The lysine content was decreased remarkably (11.2 → 1.57 g/100 g protein) by the modification. Modification degree (%) of the glucosylated casein was calculated to be 82.5 according to the reduction of lysine content on amino acid analysis. This result is consistent with that obtained by Lee et al.[6] The secondary amine linkage formed between a carbonyl group of glucose and an ε-amino group of lysyl residue by reductive alkylation is stable to acid-catalyzed pro-

Table 2 Approximate composition of casein (%)

Moisture	9.15
Crude protein (N × 6.25)	84.5
Crude fat	0.03
Ash	1.52
Crude fiber	not detected

Table 3 Amino acid compositions of unmodified and glucosylated caseins (g/100 g protein)

Amino acid	Unmodified casein	Glucosylated casein
Asp	9.98	9.20
Thr	6.20	5.91
Ser	10.3	10.6
Glu	30.9	29.0
Pro	28.9	23.5
Gly	2.62	2.47
Ala	4.59	4.21
Val	8.76	7.88
Met	3.63	4.01
Ile	6.90	6.29
Leu	12.8	12.2
Tyr	7.79	8.47
Phe	7.29	6.03
Lys	11.2	1.57
His	4.21	3.64
Arg	5.03	4.21
Trp[a]	1.93	1.89

[a] Tryptophan was determined colorimetrically.[13]

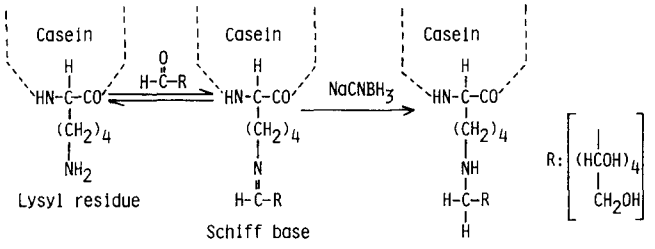

Figure 1 Reductive alkylation of bovine casein by glucose in the presence of sodium cyanoborohydride

tein hydrolysis conditions.[14] *Figure 1* represents the scheme of reductive alkylation. The content of other amino acids of the control casein did not decrease through the modification process.

pH-Dependent solubility properties

In this experiment, the effects brought about by the modification of the solubility characteristics were examined. *Figure 2* shows the pH-dependent solubility curves of unmodified and modified caseins. Both types of caseins were found to have minimum solubility at their isoelectric points (pH = 4.6). Higher solubility was observed with the modified casein at acidic and basic pHs from the isoelectric point. The higher solubility of the modified casein seems to be due to the hydrophilic property of carbohydrate moieties. Recently, Courthaudon et al.[15] examined the solubility properties of glucosylated bovine milk casein and showed a higher solubility (60%) in the range of isoelectric point although the degree of modification (35%) was remarkably lower than ours (82.5%).

Digestibility by enzymes

As shown in *Figure 3*, the glycosylated casein was digested more efficiently than the unmodified casein

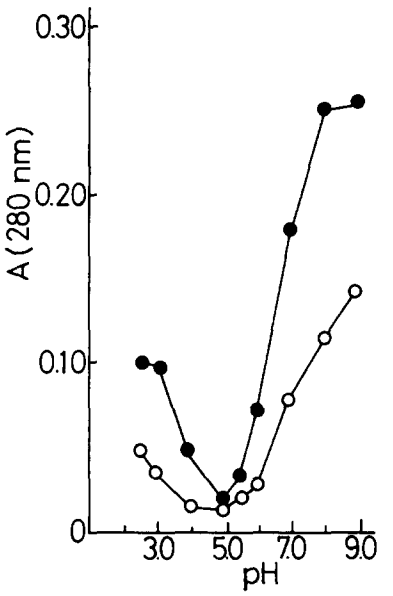

Figure 2 pH-Dependent solubility curves. ○—○, Unmodified casein; ●—●, glucosylated casein

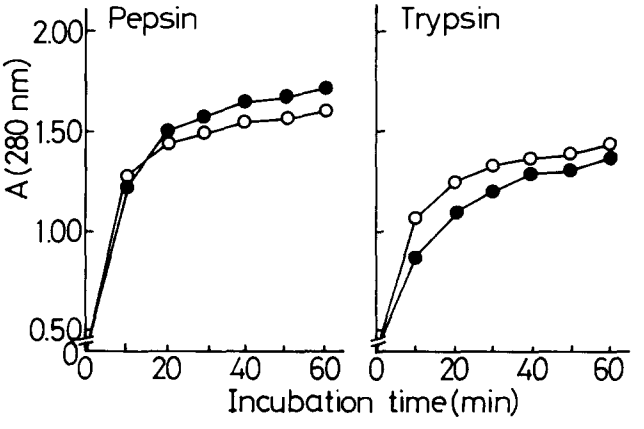

Figure 3 Time course of casein digestion by pepsin and trypsin. ○—○, Unmodified casein; ●—●, glucosylated casein

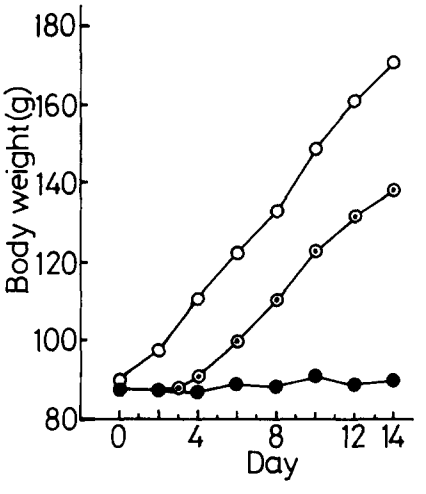

Figure 4 Growth curves of rats. ○—○, Unmodified casein; ●—●, glucosylated casein; ⊙—⊙, glucosylated casein supplemented by lysine

Table 4 Nutritive values of glucosylated casein in comparison with those of unmodified casein

Diet	True digestibility	Biological value	Net protein utilization	Protein efficiency ratios	Body weight (g) Initial	Body weight (g) Final	Food intake (g/2 weeks)
Unmodified casein	99.6 ± 1.7[a]	79.1 ± 7.7[a]	78.9 ± 8.2[a]	2.99 ± 0.29[a]	90	171	262
Glucosylated casein	105.1 ± 4.0[a]	55.0 ± 4.8[b]	57.9 ± 6.7[b]	0.35 ± 0.33[b]	87	90	146
Glucosylated casein + L-lysine	104.4 ± 4.3[a]	82.1 ± 3.1[a,c]	85.6 ± 2.9[a,c]	2.21 ± 0.29[c]	89	138	197

All groups, n = 5. Values in the same vertical column not sharing common superscript letters are significantly different ($P < 0.05$).

by pepsin. On the contrary, it was less digestible than the unmodified casein by trypsin. These reverse phenomena cannot be explained. However, the differences of digestibility between unmodified and modified casein by pepsin or trypsin were small. Therefore, it may be concluded that the modification of lysine residues by glucose does not bring about any adverse effects on the casein digestibility in vitro. Lee et al.[6] reported tht the glucosylation of casein depressed the digestibility by α-chymotrypsin.

Animal Experiment

Figure 4 shows the growth responses of rats on the experimental diets. Although the normal growth curve was observed with the unmodified casein diet group, the rats fed the modified casein diet did not have a gain in body weight. In the lysine-supplemented modified casein diet group, however, a small transitory reduced growth rate was observed during the initial 3 days. Slight diarrhea was observed in the glucosylated casein diet group. This result suggests that the low nutritional value of the modified casein is mainly due to the decreased availability of lysine. Lee et al.[6] observed severe growth depression in rats fed with the glucose-modified casein prepared by the same method as used in the present study. They also reported that the addition of lysine to the diet containing glucose-modified casein alleviated the growth-depressing effects.

The data for body weight gain, food consumption, PERs, and other nutritional values are summarized in *Table 4*. Although the rats on the modified casein diet consumed less food than those on the unmodified casein diet, lysine supplementation increased the food consumption. An extremely lower PER value was found with the modified casein diet group; the reasons for this are probably the reduced food intake (because of its low palatability) and the shortage of available lysine. Supplementation of the modified casein with L-lysine hydrochloride to compensate for the amount glucosidated gave a PER value corresponding to 74% of that of the unmodified casein. As shown by the TD values given in *Table 4*, the modified casein was almost completely digested in vivo. Both BV and NPU are considerably lower with the modified casein compared with the unmodified casein. However, supplementation of the modified casein with lysine improved both BV and NPU significantly.

These results indicate that the modified casein has extremely lower nutritional values due to the shortage of available lysine, although the in vivo digestibility of the protein is comparable to that of the unmodified casein. The lower nutritional value of the modified casein shows that the linkage formed between glucose and lysyl residue is not broken by an enzyme. However, Lee et al.[6] reported that a methyl group attached to lysyl residue by reductive alkylation could be split by an enzyme, methylase, in the body after absorption as a methylated lysine.

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