Enzymatic Hydrolysis of Maltosyl and Glucosaminyl Derivatives of β-Lactoglobulin

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The in vitro digestibilities of maltosyl and glucosaminyl derivatives of β-lactoglobulin (M-β-LG and G-β-LG, respectively) were measured by using trypsin and α-chymotrypsin. The rate and extent of proteolysis of β-lactoglobulin were affected by the extent of modification, by the type of carbohydrate residue coupled to β-LG, and by the type of proteolytic enzyme. The rate of hydrolysis increased as the number of residues modified increased. The rates of hydrolysis of M-β-LG derivatives were higher than those of G-β-LG derivatives. This resulted because the tertiary conformation of M-β-LG derivatives was more expanded than that of the modified G-β-LG derivatives. The rates and extents of hydrolysis of M-β-LG and G-β-LG were greater with α-chymotrypsin than with trypsin because the α-chymotrypsin hydrolyzing sites were increasingly exposed to the surface of the protein as modification increased.

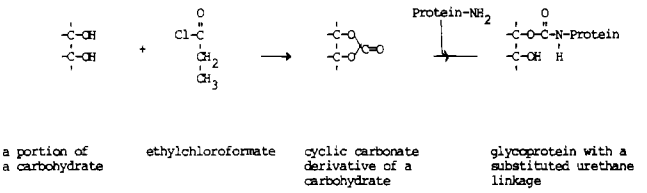
Studies to determine the digestibility and biological value of the chemically modified proteins should be conducted concurrently with research to enhance the functional properties of the proteins. A number of researchers have reported decreased digestibility of chemically modified proteins (Puigserver et al., 1979a,b; Galembeck et al., 1977; Shetty and Kinsella, 1982).

Glycosylation of β-lactoglobulin (β-LG) caused significant changes in its chemical and physicochemical properties, e.g., increased viscosity, increased exposure of aromatic amino acid residues to ultraviolet radiation, and decreased α-helical structure of the glycosylated derivatives of β-LG (Waniska and Kinsella, 1984a,b). Since research on the structure and function of proteins should include some measure of the proteins digestibility, the in vitro digestibilities of the glycosylated derivatives of β-LG were studied. The rate and extent of enzymatic hydrolysis of maltosyl- and glucosaminyl-β-lactoglobulin derivatives (M-β-Lg and G-β-LG, respectively) were determined by using trypsin and α-chymotrypsin.

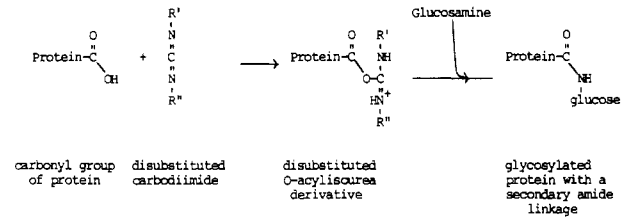
MATERIALS AND METHODS

Analytical Methods. Bovine β-lactoglobulin (crystallized and lyophilized; Sigma Chemical Co., St. Louis, MO) was dialyzed against distilled water containing 0.01% sodium azide and then lyophilized before use. Maltosyl-β-lactoglobulin (M-β-LG) derivatives were prepared as reported by Waniska and Kinsella (1984a). The M-β-LG derivatives were prepared by reacting the cyclic carbonate derivative of maltose with β-LG according the method of Doane et al. (1967) (Scheme I). Maltose cyclic carbonate was prepared by adding ethyl chloroformate to a solution of maltose and triethylamine in dimethyl sulfoxide. After purification, the activated sugar was reacted with the amino groups of β-LG (16 amino groups per protein) in 0.02 N sodium phosphate buffer (pH 8.0) for 15 h at 22 °C with constant stirring. The derivatized protein was then dialyzed 3 times against 0.10 M sodium chloride and 0.01% sodium azide, and then the protein was dialyzed 3 times against 0.010 M sodium phosphate (pH 6.3) containing 0.01% sodium azide. This was done to ensure the complete removal of the noncovalently bound carbohydrates. The protein was used immediately or stored at 4 °C until used. The M-β-LG derivatives had 1.3, 2.2, 3.5, 5.7, 9.1, and 14.5 amino acid residues glycosylated with a total of 1.3, 2.6, 5.7, 9.9, 18.8, and 32.0 maltose groups, respectively (Waniska and Kinsella, 1984a). Note: The number of maltose groups per amino acid residue modified was greater than or equal to one. Some activated maltose derivatives were covalently linked to another maltose derivatives, via a cyclic carbonate linkage, during activation or during the glyosylation reaction.

Scheme I. Simplified Sequence of Reactions in the Cyclic Carbonate Method Required To Activate Carbohydrates and To Couple the Cyclic Carbonate Derivatives to the Amino Groups of Protein



Scheme II. Simplified Sequence of Reactions in the Carbodiimide Method Required To Activate the Carboxyl Groups of Protein and To Couple Glucosamine (2-Amino-2-deoxy-D-glucose) to the Activated Carboxyl Derivative



The glucosaminyl-β-lactoglobulin (G-β-LG) derivatives were prepared by the carbodiimide method of Hoare and Koshland (1967) as described by Waniska and Kinsella (1984a) (Scheme II). Various amounts of water-soluble carbodiimide were utilized to activate the carboxyl groups of β-LG (25 free carboxyl groups per protein) in the presence of the amino sugar glucosamine. The conditions of the reaction were pH 4.75 at 4 °C for 15 h. The G-β-LG derivatives had 3.6, 6.3, 11.8, 16.0, and 21.9 groups modified (Waniska and Kinsella, 1984a). These derivatives had 3.2, 4.2, 8.8, 12.8, and 16.6 carboxyl groups glycossylated with one glucosamine residue per carboxyl group (Waniska and Kinsella, 1984a). Note: Not all modified amino acid residues were glycosylated. Only carboxyl residues yield stable derivatives with the carbodiimide method (Hoare and Koshland, 1967).

Protein concentration was determined by the biuret method (Means and Feeney, 1971) using β-LG as a standard. The concentration of amino groups of the hydrolyzed proteins was determined by the trinitrobenzenesulfonic acid (TNBS) method (Fields, 1972).

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Enzymatic Methods. Trypsin (Type 1; 10000 units/mg of protein) and α-chymotrypsin (Type II; 40–50 units/mg of protein) (Sigma Chemical Co., St. Louis, MO) were used to hydrolyze solutions of 0.30% protein in 0.10 M sodium phosphate (pH 7.6) according to the method of Lee et al. (1979). A molar ratio of 1:750 (enzyme:protein) was used to determine the initial rate of protein hydrolysis as determined by the TNBS method of Fields (1972). The relative rate of enzyme hydrolysis (percent) was calculated from the rate of hydrolysis of the glycosylated derivative divided by the rate of hydrolysis of native β-LG.

The extent of proteolysis after 18 h was determined by using a larger ratio of enzyme to protein (1:250). The enzymes and conditions of reaction were the same as above but the hydrolysis was conducted for 18 h. The relative extent of enzyme hydrolysis (percent) was calculated from the concentration of amino groups of the hydrolyzed proteins after 18 h.

RESULTS AND DISCUSSION

Modification of β-LG with carbohydrate residues increased the mass and altered the physicochemical properties of β-LG (Waniska and Kinsella, 1984a,b). Electrophoretic analysis of the modified proteins indicated a heterogeneous population of glycosylated derivatives (Waniska and Kinsella, 1984a). The relative viscosities of these derivatives increased as the molecular weights increased (Waniska and Kinsella, 1984b). The microenvironments of the aromatic amino acid residues of β-LG became more polar, i.e., these residues were increasingly exposed to the surface of the protein, as the extent of modification increased (Waniska and Kinsella, 1984b). The hydrophobicities of M-β-LG derivatives decreased as the extent of modification increased, while the hydrophobicities of G-β-LG derivatives were relatively unchanged. The ordered secondary structures of the extensively modified derivatives of β-LG were partially unfolded. The carbohydrates covalently lined to β-LG altered many physicochemical properties because the glycosylated proteins showed increased hydrophilic interactions and decreased ionic and hydrophobic interactions (Waniska, 1982; Waniska and Kinsella, 1984b).

Initial Rate of Proteolytic Hydrolysis of Glycosylated Derivatives of β-Lactoglobulin. The apparent digestibility of glycosylated derivatives of β-LG was estimated by the initial rate of hydrolysis using trypsin and α-chymotrypsin. The rate of proteolysis provided some information about the binding affinities of the chemically modified amino acid residues for the enzymes and/or of the apparent concentration of amino acid residues exposed on the surface of the modified proteins (Mihalyi, 1972; Hsu et al., 1977). Thus, the rate of hydrolysis provided an estimate of the digestibility (one measure of biological value), the binding affinity of the substrate for the enzyme, and the alterations in the conformation of β-LG caused by glycosylation.

The relative rates of hydrolysis of M-β-LG derivatives using either enzyme were either unchanged or increased (Figure 1). The number of sites available for trypsin hydrolysis decreased after chemical modification of lysyl residues of β-LG, and the conformation of β-LG, e.g., secondary, tertiary, and quaternary conformations, was altered by the additional maltose residues (Waniska, 1982). The number of polar, hydrophilic groups, e.g., carbohydrate residues, increased dramatically with the extent of modification, whereas the hydrophobicity of M-β-LG derivatives decreased (Waniska and Kinsella, 1984b). Thus, changes in the proportion of forces stabilizing the protein structure, i.e., ionic, hydrophobic, and hydrophilic interactions, altered the chemical and physical properties of β-LG. Glycosylation of β-LG caused the exposure of basic amino acid residues on the surface of the protein either from the central region or from disassociation of its quaternary structure. This resulted in an increased rate of enzymatic hydrolysis.

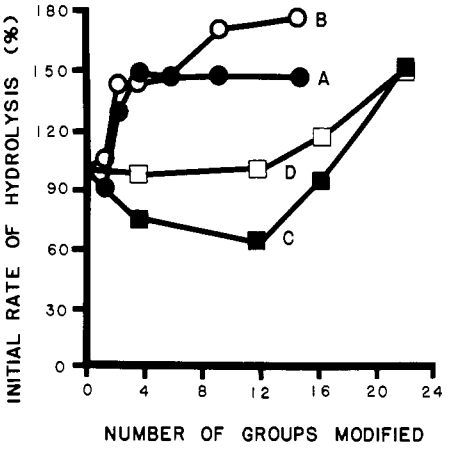

Figure 1. Relative rate of proteolysis of the glycosylated derivatives of β-lactoglobulin as affected by the extent of modification with maltose or glucosamine. The symbols correspond to maltosyl-β-lactoglobulin derivatives (● and ○) hydrolyzed by trypsin (A) and α-chymotrypsin (B), respectively, and to glucosaminyl-β-lactoglobulin derivatives (■ and □) hydrolyzed by trypsin (C) and α-chymotrypsin (D), respectively. Conditions of hydrolysis are described in the text.

In other studies, the initial rates of hydrolysis of alkylated caseins by α-chymotrypsin were lower than that of unmodified casein (Lee et al., 1978, 1979; Galembeck et al., 1977; Chiba et al., 1976). Lee et al. (1979) explained the decrease in the initial rates of hydrolysis in terms of steric hindrance of the added residues, product inhibition, and/or formation of nonproductive enzyme complexes. In contrast, Matoba and Doi (1979) reported a higher initial rate of hydrolysis of succinylated casein by α-chymotrypsin. They proposed that the electrostatic repulsion of the succinyl residues enhanced the unordered conformation of casein, exposed some of the buried aromatic amino acid residues, and thereby increased the rate of hydrolysis.

The relative rates of hydrolysis of G-β-LG samples with less than 12 groups modified were lower than or similar to the control while those of the extensively modified derivatives were greater than the controls (Figure 1). The glucosamine residues of the less modified derivatives probably interfered with the binding of the basic amino acid residues to trypsin, via steric hindrance, since the results of physicochemical analyses of these derivatives revealed little change in the secondary structures (Waniska and Kinsella, 1984b). The extensively modified derivative of G-β-LG had altered secondary, tertiary, and quaternary conformations, which increased the number of basic and aromatic amino acid residues exposed to the surface of the protein (and rates of proteolytic hydrolysis) (Waniska and Kinsella, 1984b). This resulted because the distribution of forces stabilizing the original protein structure was changed. The electronegativity of β-LG decreased while the hydrophilicity of β-LG increased as the extent of glycosylation increased and the hydrophobicity of β-LG decreased when modified with 16.6 glucosamine residues (Waniska and Kinsella, 1984a,b).

The initial rates or proteolysis of chemically acylated caseins, i.e., alanyl-, tryptophyl-, glycyl-, methionyl-, and aspartylcasein, using α-chymotrypsin were progressively reduced as more amino acid residues were attached (Puigserver et al., 1979a,b). The rates of hydrolysis of

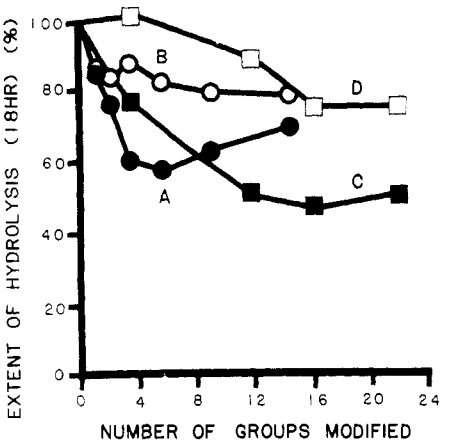

Figure 2. Relative extent of proteolysis of the glycosylated derivatives of β-lactoglobulin after 18 h as affected by the extent of modification with maltose or glucosamine. The symbols correspond to maltosyl-β-lactogloculin derivatives (● and ○) hydrolyzed by trypsin (A) and α-chymotrypsin (B), respectively, and to glycosaminyl-β-lactoglobulin derivatives (■ and □) hydrolyzed by trypsin (C) and α-chymotrypsin (D), respectively. Conditions of hydrolysis are described in the text.

acylated caseins using bovine pancreatin or rat bile pancreatic juice were lower than that of the control, but the rates were higher than those observed with α-chymotrypsin. Puigserver et al. (1979b) attributed the decreased rates of hydrolysis to the large size of the modifying groups, the negative charge of some amino acid residues, and conformational changes in the modified proteins.

Thus, extensive chemical glycosylation of β-LG with maltose or glucosamine yielded derivatives that were hydrolyzed more rapidly by trypsin or α-chymotrypsin, i.e., improved in vitro digestibility. Apparently, the loss of some organized secondary structure and more unordered structure of β-LG exposed more substrates for enzymatic hydrolysis.

Extent of Proteolytic Hydrolysis of Glycosylated Derivatives of β-Lactoglobulin. The digestibility of the glycosylated derivatives of β-LG was also estimated by the extent of hydrolysis after 18 h by using trypsin and α-chymotrypsin. The extents of hydrolysis of the glycosylated derivatives of β-LG using either enzyme were less than that of the control (Figure 2). In addition, the extents of hydrolysis of M-β-LG and G-β-LG derivatives by trypsin were lower than those obtained by using α-chymotrypsin. Apparently, the carbohydrate residues attached to β-LG decreased the proteolytic efficiency and/or the glycosylated peptides resulting from the hydrolysis of these glycorproteins inhibited proteolysis.

The hydrolysis by trypsin of M-β-LG derivatives with more than eight groups modified was more extensive than expected. Hough et al. (1960) reported that substituted urethane bonds can be chemically hydrolyzed in basic solutions. Since a slightly basic solution was used for proteolysis, the slight increase in the extent of hydrolysis of M-β-LG derivatives from 5.7 to 14.5 groups modified probably resulted in part from the exposure of amino groups that were covalently linked to maltosyl residues.

Galembeck et al. (1977) reported that the extents of hydrolysis of several chemically modified proteins, i.e., methylcasein, ethylcasein, methyl-bovine serum albumin (BSA), acetyl-BSA, and carbanoyl-BSA, using α-chymotrypsin were decreased because the hydrolysis products of modified proteins inhibited further proteolysis. Peptides from an α-chymotrypsin hydrolysate of an acetylated and reductively alkylated derivative of BSA effectively reduced the rate and extent of hydrolysis of native BSA by α-chymotrypsin. Lee et al. (1979) also reported that the extents of hydrolysis of gluycosyl-, fructosyl-, or lactosylcaseins using α-chymotrypsin were decreased. Proteolysis of caseins alkylated with hydrophobic residues was inhibited by hydrolysis products (Sen et al., 1981). Thus, the glycopeptides from the hydrolysis of the glycosylated β-LG derivatives probably inhibited trypsin and α-chymotrypsin in this study, i.e., hydrolysis products of glycosylated β-LG reduced its in vitro digestibility.

These studies reveal that the amino acid residue modified and the nature of the substituent group affect the proteolytic digestibility of proteins.

Abbreviations Used: β-LG, β-lactoglobulin; M-β-LG, maltosyl-β-lactoglobulin; G-β-LG, glucosaminyl-β-lactoglobulin; BSA, bovine serum albumin; TNBS, trinitrobenzenesulfonic acid.

Registry No. Trypsin, 9002-07-7; α-chymotrypsin, 9004-07-3.

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