Electroassisted Glycosylation of Bovine Casein: An Alternative to the Use of Reducing Chemicals in N-Alkylation of Proteins

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A new method of glycosylation of proteins is proposed. It uses a potential-controlled cathode as reducing agent instead of the usual borohydride. The method was applied to whole casein and ordinary carbohydrates (ribose, galactose, fructose). It led to modification rates bordering 30% in 24 h, comparable to those obtained by chemical reduction using dimethylamine borane.

INTRODUCTION

One of the main objectives of the chemical modification of proteins is to improve their functional properties for wider use in the food industry (Feeney, 1977; Feeney et al., 1982). Many examples of this improvement have already been described; phosphorylation and succinylation increase the solubility of casein in acidic medium (Matheis et al., 1983; Girerd et al., 1984); solubility, viscosity of the solutions, surface properties, and heat stability of milk proteins are also enhanced by glycosylation (Lee et al., 1979; Sen et al., 1981; Canton and Mulvihill, 1983; Kitabatake et al., 1985; Colas et al., 1988; Courthaudon et al., 1989).

Among the methods available for chemical modification of proteins, direct reductive coupling of oligosaccharides to the ϵ-amino groups of lysyl residues has been widely used. Figure 1 shows the universally accepted general mechanism of this reaction (Borch et al., 1971). The first elemental step is, as in the well-known nonenzymatic browning of food, the self-occurring nucleophilic attack of the amino groups on the carbonyl moiety of the osidic reagent. It gives an iminium ion as an intermediate which is in equilibrium both with the starting reagents and with the corresponding imine. In the so-called reductive alkylation procedure, the transient iminium cation (Borch et al., 1971) or imine is usually reduced into the corresponding amine by a hydride donor as described by Means and Feeney (1968).

Many reducing agents have been proposed for this purpose, but borohydrides are particularly suitable for their ability to selectively reduce the imine even in the presence of a great excess of carbohydrate (Borch, 1971).

Up to now, the use of chemically modified proteins as food additive was hampered by the toxic character of most of the necessary chemical reagents. The aim of this paper is to propose electrochemical reduction as an unprecedented alternative to the usual chemicals in the reductive glycosylation of proteins. Whole bovine casein, which contains from 9 to 24 lysyl groups per molecule, has been selected as a model protein for this study. A preliminary estimation of the efficiency of the electrochemical method has been obtained by the determination of the degree of modification and comparison with the results of glycosylation using dimethylamine borane as a reducing agent.

MATERIALS AND METHODS

Materials and Reagents. Carbohydrates and dimethylamine borane were purchased from Aldrich; 2,4,6-trinitrobenzenesulfonic acid was from Fluka. Bovine casein was prepared in the laboratory by isoelectric precipitation, washed three times with distilled water, and then dissolved at pH 7. This process was repeated three times, and then the solution was freeze-dried.

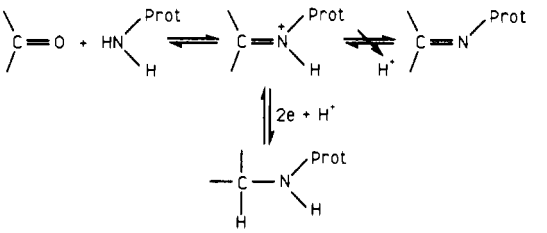

Figure 1. General mechanism for the reductive coupling of a carbonyl compound to a protein.

Chemical Glycosylation of Casein. Glycosylation of casein was performed with an excess of carbohydrate according to a modification of the classical methods (Lee et al., 1979; Canton and Mulvihill, 1983). Instead of sodium borohydride, borane dimethylamine complex was used as a reducing agent according to the methods of Lane (1973), Geoghegan et al. (1981), Cabacungan et al. (1982), and Wong et al. (1984). The molar ratios carbohydrate/protein and reducing agent/lysyl residues were, respectively, 64:1 and 5.2:1. Casein (4.5 g, 0.195 mmol) and 12.5 mmol of carbohydrate were dissolved in 100 mL of a buffer solution (pH 9.15) prepared according to the method of Britton and Robinson (1931); 0.71 g (12.17 mmol) of borane dimethylamine complex was then added, and the resulting mixture was maintained at 38 °C and stirred for 24 h.

Electroassisted Modification of Bovine Casein, Mercury Electrode. To a mixture of casein (0.897 g, 0.039 mmol) in 15 mL of Britton and Robinson buffer (1931) (pH 9.15) is added 2.5 mmol of carbohydrate in 5.0 mL of water. Sodium azide (0.2%) was added, and the resulting mixture was poured into an electrolytic cell. The reference electrode was a saturated calomel electrode separated from the solution by a sintered glass disk. The auxiliary electrode was a platinum grid, and a mercury pool (area = 8 cm^2) was used as a working electrode. The mixture was deoxygenated, blanketed with a slow current of nitrogen, and continuously stirred with the help of a magnetic stirrer. To prevent evaporation of water from the reacting mixture, the protecting gas was saturated by preliminary bubbling in the buffer solution heated at 38 °C. The cell was then connected to a Tacussel Model ASA 4-HT potentiostat equipped with a Tacussel IG 4 integrator and maintained at 38 °C. The appropriate potential was applied for a period of 1–5 days.

Electroassisted Modification of Bovine Casein, Carbon Electrode. The experiment was identical to the procedure above except for the nature of the electrolytic cell. The modified cell consisted of a glassy carbon beaker (Carbone Lorraine, V25 glassy carbon) which contained the solution and served as a working electrode. The working surface of this electrode was 15 cm^2.

Determination of the Degree of Modification (TNBS Method). The degree of alkylation was obtained by the deter-

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mination of the amount of unreacted primary ε-amino groups of lysyl residues by 2,4,6-trinitrobenzenesulfonic acid (TNBS) using the procedure described by Habeeb (1966), Mokrasch (1967), Kakade and Liener (1969), Fields (1979), and Adler-Nissen (1979). The absorbance of a solution of trinitrophenyl-substituted glycosylated casein was compared at 420 nm to that of a similarly processed equimolar solution of control casein.

Determination of the Degree of Modification (Oxidation Method). To verify the results of the method described above, the determination of the degree of modification was performed according to a procedure based upon periodate oxidation of the protein-N-bonded osidic residues. The solution of modified casein was dialyzed against three changes of a 0.1 M NaCl aqueous solution and against three changes of distilled water and then oxidized following the procedure described by Gallop et al. (1981).

Theoretical Electrochemical Degree of Modification. It is known that the complete reduction of an imino group or an iminium ion into the corresponding amine needs two electrons (Reed and Wightman, 1984). If the electrochemical reduction is assumed to be completely specific (i.e., the unreacted carbohydrate is not reduced), the moles of N-glycosylated amino groups (y) is related to the quantity of electricity (Q) by

$$y = 0.5Q/96500$$

If x refers to the moles of primary amino groups in the starting solution, the degree of modification is then given by $100y/x$. Comparison of the values of the theoretical degree of modification with the results of the TNBS method indicates the degree of chemospecificity of the electrochemical reduction.

Amino Acid Analyses. The mixture after electrochemical reaction was dialyzed one time against 0.1 M NaCl solution and three times against distilled water and then freeze-dried. Unmodified and treated casein samples were hydrolyzed with 6 M HCl in vacuo at 110 °C for 22 h. The resulting amino acids were separated and quantified on a Beckman amino acid analyzer (Model 119CL).

RESULTS AND DISCUSSION

Chemical Modification of Casein. To compare dimethylamine borane to the usual sodium cyanoborohydride reducing agent, a preliminary galactosylation of the whole casein was performed using the same concentration (90 g/L) and parameters as Courthaudon et al. (1989). The reaction rate at 38 °C is very similar for both hydrides, leading in 5 days to a degree of modification bordering 80%. Chemical modification at a lower concentration (45 g/L) using the same parameters (38 °C, 24 h) as for electroassisted reaction led to the following degrees of modifications: D-galactose, 58%; D-glucose, 54%; D-fructose, 27%; D-ribose, 38%. The two aldohexoses give similar degrees of modification, but the yield is much lower when starting from D-fructose as reported by Lee et al. (1979) and Courthaudon et al. (1989) for glycosylation using cyanoborohydride as a reducing agent. D-Ribose is known to give in solution a relatively high amount of the open aldehydic form (Cantor and Peniston, 1940). Since the degree of modification obtained from this carbohydrate is somewhat low, it suggests that the rate of the overall process is not directly related to this amount.

Mercury Cathode Assisted Modification of Casein. The challenge was to reduce selectively the imine even in the presence of an excess of starting carbohydrate. The half-wave polarographic potential of acetaldehydimine is −1.4 V (Schwabe, 1957), less negative than the potential of the corresponding acetaldehyde (−1.87 V), showing that, in this case, the imine is more easily reducible than the starting aldehyde. In the case of oses, the half-wave potential of the imine/glucosylamine is unknown, and the polarographic reduction of aldoses and ketoses themselves is not easy to observe. In usual polarographic conditions they do not give a well-defined reduction wave, but, for some of them, a wave corresponding to a kinetically controlled process can be observed near 40 °C and pH 7.75 (Cantor and Peniston, 1940; Delahaye and Strassner, 1952). A similar temperature was chosen for our experiments, and the applied potential was close to −1.65 V, which is the $E_{1/2}$ value of galactose (Delahaye and Strassner, 1952; Overend et al., 1961). The influence of the applied potential on the rate of galactosylation was studied using two different concentrations of casein, 90 and 45 g/L. The results obtained with the more concentrated solution were not significant since we observed the filling in of the glass frit separating the anodic from the cathodic compartment of the electrochemical cell. This observation could be explained by the electrophoretic migration of negatively charged (pH 9.15), poorly soluble casein micelles toward the positive platinum counter electrode. The process obtained from the less concentrated solution was reproducible, allowing a study of the influence of the applied potential. The results of this study are shown in Figure 2. For a cathodic potential of −1.5 V the galactosylation rate is somewhat slow, leading in 20 h to approximately 23% of modification. Increasing the negative character of the reducing electrode enhances the degree of modification. However, exceeding the $E_{1/2}$ value of the sugar reduction is not advantageous, as shown by the curve relative to −1.7 V. Moreover, for this last potential, the value of the electrochemical theoretical degree of modification calculated from the quantity of electricity is very high compared to the value found by the TNBS method. It shows that the more negative the applied potential is, the more the unwanted cathodic reduction of the osidic reagent takes place.

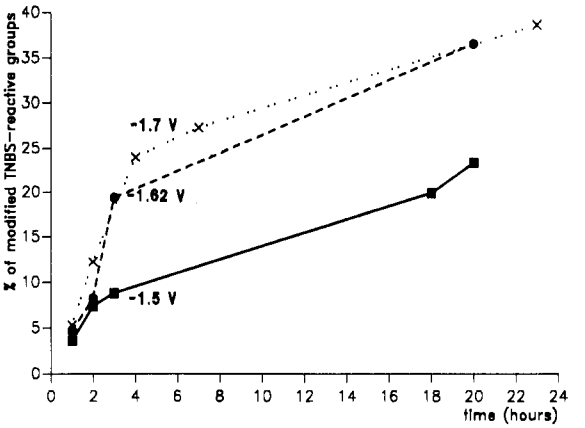

Figure 2. Influence of the applied potential (volts SCE) on the rate of electroassisted coupling of galactose and casein (mercury pool, 38 °C, 45 g/L, pH 9.15). The degree of modification was estimated according to the TNBS method.

An experiment performed at −1.62 V during 120 h led to a degree of glycosylation of 55%, which is somewhat lower than the value obtained in the same duration by Courthaudon et al. (1989) using sodium cyanoborohydride as reducing reagent.

The degree of modification reported in Figure 2 for the experiment at −1.62 V was compared to the results obtained from the periodic oxidation method (Gallop et al., 1981). The two methods gave similar results: TNBS, periodic oxidation 8.2, 9.1; 19.4, 20.0; 36.5, 38.2.

A test experiment using the same conditions including the presence of mercury as above but without applied reduction potential was performed to estimate the occurrence of the natural coupling by glycosylation–Amadori transposition. This experiment led to a negligible degree of modification, definitely showing the need of a reducing agent.

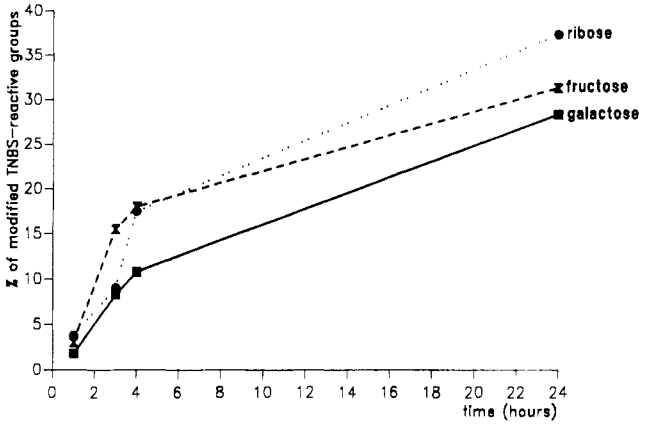

Figure 3. Rate of electroassisted coupling between carbohydrates and casein (carbon electrode, 38 °C, 45 g/L, pH 9.15, E_{SCE} = −1.62 V). The degree of modification was estimated according to the TNBS method.

Table I. Degrees (Percent) of Galactosylation of Casein on Mercury or Carbon Electrodes

	mercury		carbon	
	TNBS	theor	TNBS	theor
ribose			37	86
galactose	36	70	31	90
fructose			28	80

Carbon Cathode Assisted Glycosylation of Casein. As the cathodic reduction of D-galactose can be observed on a dropping mercury electrode, we checked the reduction of this carbohydrate on a glassy carbon disk electrode (diameter = 3 mm) by voltammetry. As we were unable to observe any reduction wave under these conditions, the potential of the carbon electrode in preparative experiments was tentatively adjusted to −1.62 V (SCE), which corresponds to the optimal value for a mercury pool electrode. This potential was shown to be suitable for ribose, galactose, and fructose (Figure 3), leading to similar rates of modification for the three carbohydrates. The degree of modification observed for fructose (31%) is interesting but somewhat surprising when compared to the weak modification obtained in chemical fructosylation with dimethylamine borane (see above) and with sodium cyanoborohydride as described by Courthaudon et al. (1989). Ribose led to the highest modification observed for the four studied sugars. Owing to the low modification obtained by the chemical method, this result was completely unexpected.

A comparison of the degrees of galactolysation obtained in 24 h with the two different electrodes is shown in Table I. The carbon electrode appears to be somewhat less efficient. The difference probably originates either from the potential of the carbon electrode, the value of which was not optimized, or from the difference in physical state of the two electrodes. In contrast to the unchanging surface of the solid carbon electrode, the liquid surface of the mercury electrode appears to be more prone to continuous renewing by mechanical stirring.

The theoretical degrees of modification calculated from the consumed electricity amount (Table I) are somewhat higher than the corresponding values obtained with the TNBS method. As for this calculation it is assumed that the reduction of the transient imine is completely specific; the difference clearly shows that, in our hands, the chemospecificity of the reduction is not complete. However, as the reported values do not exceed 3-fold the value obtained from TNBS, and taking into account the high molar excess of sugar used in this experiment, we can conclude that the transient imine/glycosylamine is more easily reduced than the starting monosaccharide.

As the negative electrode may be prone to reduce disulfide bonds, complete hydrolysis and subsequent amino acid determination were performed. Comparison of the chromatograms issued from ordinary and treated casein shows that the amount of cystine residues is slightly lowered in modified casein. Reduction of cystine residues of casein may be an important feature of the method. The amount of lysine is largely depressed, showing that, as expected from the results of the TNBS method, the lysyl groups of the protein are an important target of the modification.

Conclusion. This study shows that the electrochemically assisted glycosylation of casein is an interesting alternative to the usual methods with borohydridic reducing agents. Although the procedure is not completely optimized, the degrees of modification reported here are not far from the corresponding values obtained via classical chemicals. Moreover, the method avoids the somewhat toxic borohydrides and can afford products usable in food technology. Some improvements are presently under investigation, and further results, dealing with casein and other proteins, will be published in a forthcoming paper.

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Registry No. Ribose, 50-69-1; galactose, 59-23-4; fructose, 57-48-7.