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Analysis of the Maillard reaction products of β-lactoglobulin and lactose in skimmed milk powder by capillary electrophoresis and electrospray mass spectrometry

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Abstract

When analysed by capillary electrophoresis, certain skimmed milk powders are seen to exhibit additional peaks migrating after the whey protein β-lactoglobulin. Using a model reaction between β-lactoglobulin and lactose, and studying the reaction products using electrospray mass spectrometry, it is demonstrated that these protein peaks are almost certainly due to a Maillard reaction between lactose and the ε-amino group of lysine. This results in the formation of a series of lactulose–protein conjugates exhibiting throughout molecular mass increments of 324, which is sufficient to allow their separation by capillary electrophoresis. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The analysis of the proteins of bovine milk is an important area within food science and there are numerous publications outlining their separation and quantitation. The two main protein classes in milk are the caseins (80%) and serum proteins (20%). The former comprise four gene products; α_{S1}, α_{S2}, β, and κ-casein, whilst the latter comprise bovine serum albumin, α-lactalbumin and β-lactoglobulin [1]. Due to the presence of several genetic variants e.g., β-casein A1, A2, A3 and β-lactoglobulin A and B, the separation problems in the analysis are quite challenging. In addition, since most of the casein is bound within a micellar structure [2], preparation and analysis procedures must ensure that this is disrupted, and that protein aggregation is prevented. Faced with these problems, a range of methods utilising slab gel electrophoresis [3], isoelectric focusing [4], and liquid chromatography [5,6] have been developed over the years, and are routinely employed.

Recently, Olieman et al. [7] introduced a method for the separation of milk proteins based on capillary electrophoresis (CE) that was both rapid, and in contrast to previous methods, allowed a high efficiency separation of the individual proteins and their variants. Subsequently, this approach has been used to differentiate goat, sheep and cows milk [8], to determine the level of denatured serum proteins following heat treatment of milk [9], and to estimate the level of renneted whey solids [10].

Within our laboratory we have been using the CE methodology for several years for a number of

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applications. During the course of this work, a number of additional peaks have been observed for skimmed milk powder (SMP) obtained from a variety of sources. Two recent publications [11,12] have demonstrated that β-lactoglobulin can be multiply glycosylated under relatively mild conditions. This paper examines the additional peaks found in SMP to determine if glycosylation during the early stages of the Maillard reaction is indeed responsible for their formation.

2. Experimental

2.1. Equipment

Electrophoresis was undertaken using a HP^{3D} CE system (Hewlett-Packard, Waldbronn, Germany) fitted with a 50 μm I.D. CElect P150 capillary (Supelco, Bellefonte, PA, USA). Buffer pH was adjusted with an EA940 Ionalyser (Orion, Forrest Row, East Sussex, UK), and buffers were filtered through a 0.2-μm Minisart membrane (Sartorius, Gottingen, Germany). Electrospray mass spectrometry was conducted with a Quattro 1 triple quadrupole mass spectrometer (Micromass, Manchester, UK) connected to a SFC-500 Micro Flow Pump (Isco, Lincoln, NE, USA). Sample was injected via a Rheodyne Model 5020 valve (Jones Chromatography, Hengoed, Mid Glamorgan, UK) using a 10-μl loop.

2.2. Reagents

All reagents were of the highest available grade. Trisodium citrate dihydrate, citric acid monohydrate, dithiothreitol (DTT) and urea were from Sigma (Poole, Dorset, UK). Hydroxymethyl cellulose (HMEC) was purchased from Serva (Heidelberg, Germany), and acetonitrile and acetic acid from Sherman Chemicals (Sandy, UK).

Protein standards α-lactalbumin and β-lactoglobulin A and B were obtained from Sigma. The water was Milli-Q (Millipore, Watford, Herts., UK) with resistivity >18 MΩ.

2.3. Electrophoresis

The running buffer was 10 m*M* trisodium citrate dihydrate prepared from 147 mg of the solid, 25 mg HMEC and 1.6 g citric acid monohydrate dissolved in 37.5 ml of 8 *M* urea in a 50-ml volumetric flask. This was subsequently adjusted to pH 3 with solid citric acid and diluted to volume with water. All buffers and samples were filtered through a 0.2-μm membrane prior to use.

New capillaries were cut to 64.5 cm and operated with a 56 cm effective length. They were equilibrated with 1 *M* sodium hydroxide for 30 min, 0.1 *M* sodium hydroxide for 30 min, water for 10 min and finally running buffer for 3 h. Samples were injected hydrodynamically at 50 m bar for 5 s. The capillary was flushed at 1000 m bar for 2 min with water and 4 min with running buffer between runs. The applied voltage was 25 kV with detection at 214 nm. The column temperature was maintained at 45°C throughout.

2.4. Electrospray mass spectrometry

Samples were introduced into the mass spectrometer in a 10 μl/min flow of acetonitrile–water—acetic acid (49.5:49.5:1). The solvent was micro-filtered and helium sparged before use. Manual injection of sample solutions was carried out via a Rheodyne valve fitted with a 10-μl loop. Fused-silica capillary tubing (25 μm I.D.) was coupled directly into the megaflow electrospray interface of the mass spectrometer. The electrospray conditions used a nitrogen bath gas flow of 300 l/h, and a nebulising gas of 15 l/h. The electrospray capillary potential was 3.5 kV and the source was at 80°C. Positive ion mode was used, acquiring continuum data, m/z 100 to 2000, with a cone voltage set at 30 V. The detector was at 650 V. Typical data processing parameters: continuum spectra were combined, smoothed (double Savitsky-Golay, 1.00 Da width), centroided (1.0 Da width, top, 80%) and transformed.

2.5. Model reaction between β-lactoglobulin and lactose

An aqueous solution containing 2 mg/ml of β-lactoglobulin A, 4 mg/ml β-lactoglobulin B, and 60

mg/ml lactose was heated at 50°C for 120 h and aliquots removed at given time points. A sample containing β-lactoglobulin without lactose was also subjected to the same regime as a control. All aliquots were dialysed against Milli-Q water to remove any excess lactose and salts, and then freeze–dried.

For analysis by CE, a dissolution buffer of 5 m*M* citrate was prepared by dissolving 147 mg of tri-sodium citrate dihydrate and 100 mg DTT in 75 ml of 8 *M* urea. This was subsequently diluted to 100 ml and used to prepare solutions of 1 mg/ml of the freeze–dried material. For analysis by electrospray mass spectrometry, the material was dissolved in acetonitrile–water–acetic acid (49.5:49.5:1).

3. Results and discussion

3.1. Secondary peaks in skimmed milk powder

An electropherogram of fresh skimmed milk is shown in Fig. 1 (top). β-lactoglobulin exists as two major variants [13] which differ in their primary amino acid sequence at positions 64 and 118. These can be partially separated under the conditions used, which leads to a characteristic "doublet" for the main peak. Although the electropherogram of skimmed milk powder varies from source to source, it invariably exhibits a β-lactoglobulin peak with a broad tail [Fig. 1 (bottom)]. Closer examination (Fig. 2) reveals that this is comprised of a number of discrete "secondary" peaks and as these appear in a doublet pattern, it suggests that they too are derived from β-lactoglobulin. Since the electropherogram is run in the presence of DTT, the secondary peaks can not be due to aggregated β-lactoglobulin linked via disulphide bridges. However, the slower migration time does imply higher-molecular-mass species (larger hydrodynamic volume), or one that is more negatively charged, or a combination of the two.

A likely explanation for the peaks is a chemical modification during the SMP manufacturing process, which normally entails spray drying. This is likely to involve lactose which is present at around 50% (w/w). In examining the literature, three significant chemical reactions are known to occur involving protein and lactose; the conversion of lactose to lactulose [14], the formation of lysinoalanine crosslinks [15], and the Maillard reaction [16]. The formation of lactulose cannot provide an explanation for the secondary peaks, and whilst lysinoalanine crosslinks between β-lactoglobulin polypeptide chains would certainly provide the required higher molecular weight species, it is reported to occur at only very low levels in milk and milk products [17]. The Maillard reaction occurs between a reducing sugar and an amine. For milk systems the source of the former is lactose, with the free ε-amino group of lysine the prime candidate for the latter. The initial reaction results in the formation of a lactulosyl-amino group (Fig. 3), but this subsequently degrades and a whole host of further reactions ensue leading to both low and high molecular mass material. Finally, the complex paths of the Maillard reaction terminate in brown nitrogenous polymers and co-polymers termed "melanoidins". The initial stages of the Maillard reaction will, in forming the lactulosyl-amino group, produce higher-molecular-mass species, which apparently form the peaks migrating after β-lactoglobulin.

3.2. Model reaction between lactose and β-lactoglobulin

The electropherograms in Fig. 4 shows the effect of reacting lactose and β-lactoglobulin for 24, 72 and 120 h at 50°C. Clearly, secondary peaks are observed, with the 120-h reaction demonstrating a series of five doublets, all apparently characteristic of the A and B forms of the protein. For comparison, a β-lactoglobulin control heated at 50°C for 120 h, is also shown.

To determine the identity of the material produced during the reaction some of the product was removed after 30 h, dialysed, freeze–dried and subjected to electrospray mass spectrometry. Fig. 5 shows the transformed mass spectrum for the β-lactoglobulin starting material, which as expected produces two major ions representing forms A and B. The theoretical mass difference between these is 86 Da, in good agreement with observed values of 18 274 and 18 360 Da. The data for the 30-h reaction product is shown in Fig. 6. In addition to the unmodified protein, there is a series of doublets with molecular mass of 18 598 and 18 683, 18 922 and 19 008 and

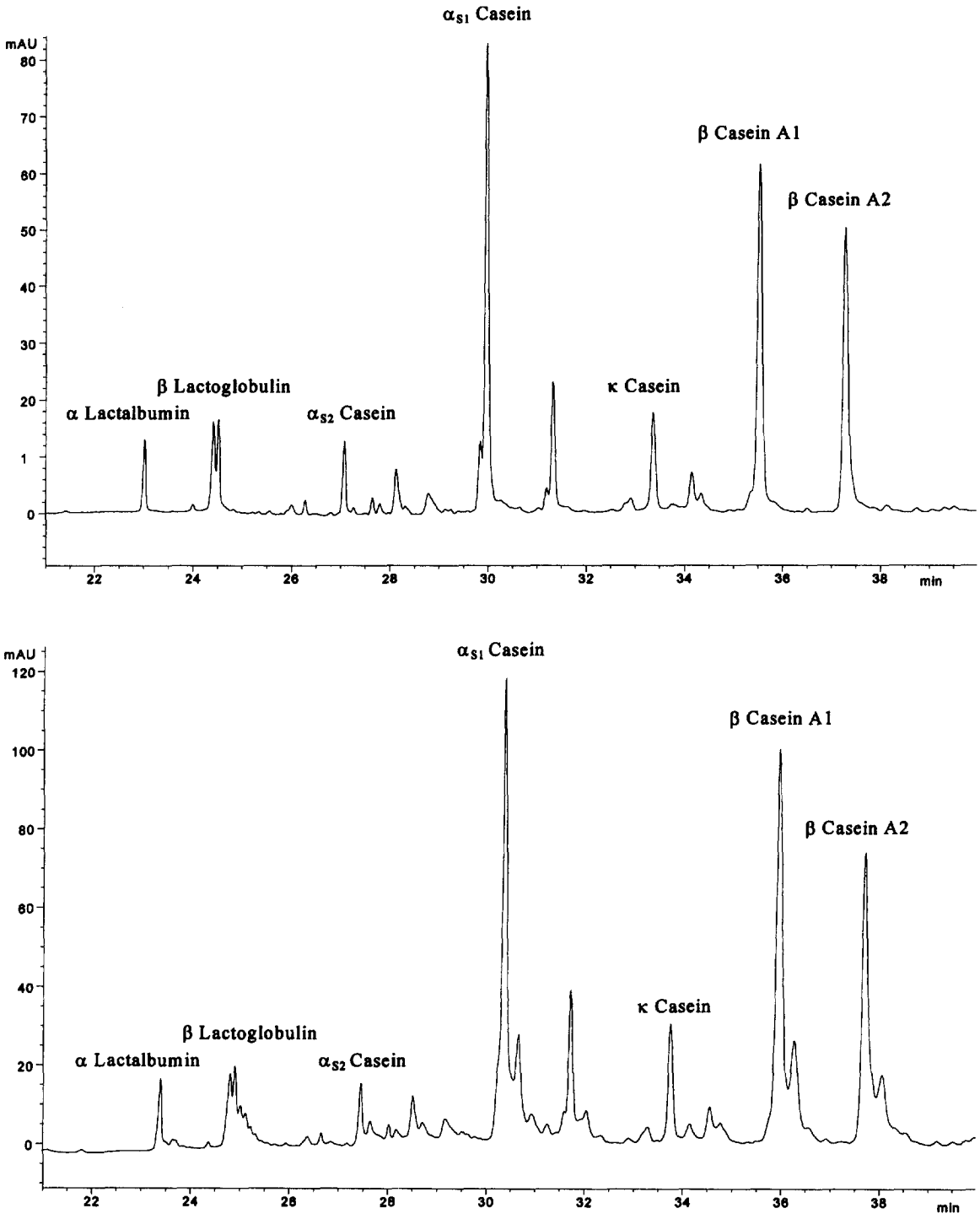

Fig. 1. (top) Electropherogram of fresh skimmed milk. (bottom) electropherogram of skimmed milk powder. Conditions: capillary, CElect P150 column, 64.5 cm (effective length 56 cm)×50 μm I.D.; running buffer, 10 m*M* trisodium citrate in 6 *M* urea adjusted to pH 3 with citric acid and containing 0.5 g/l HMEC; voltage, 25 kV; temperature, 45°C; detection, 214 nm.

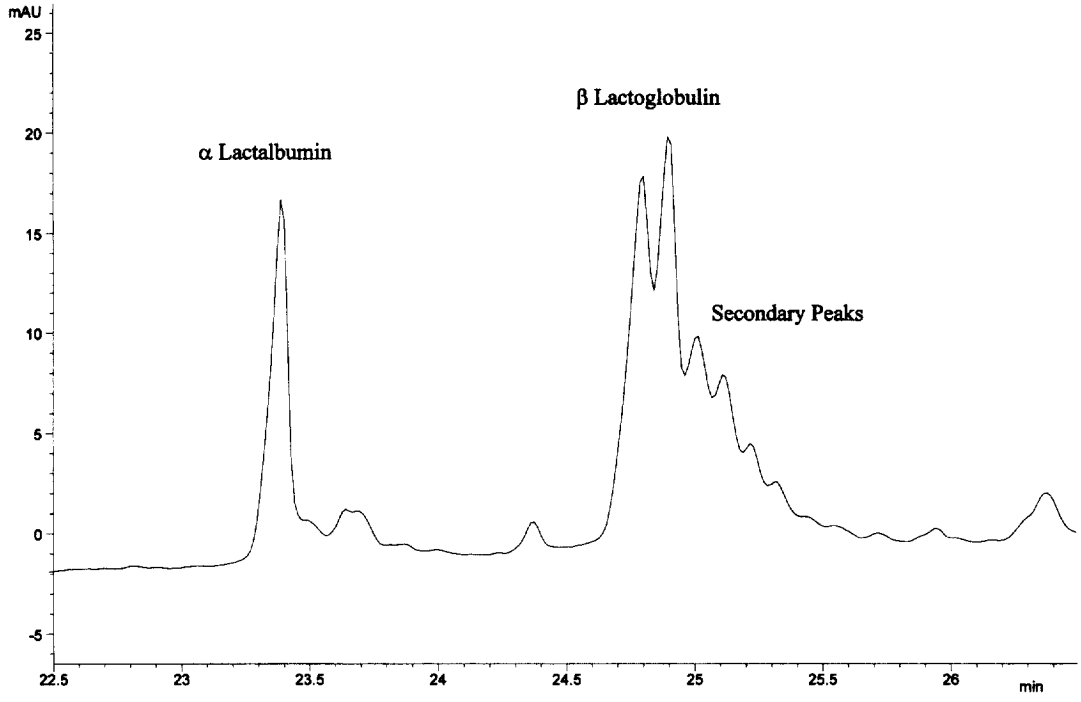

Fig. 2. Secondary peaks migrating after β-lactoglobulin. Conditions as in Fig. 1.

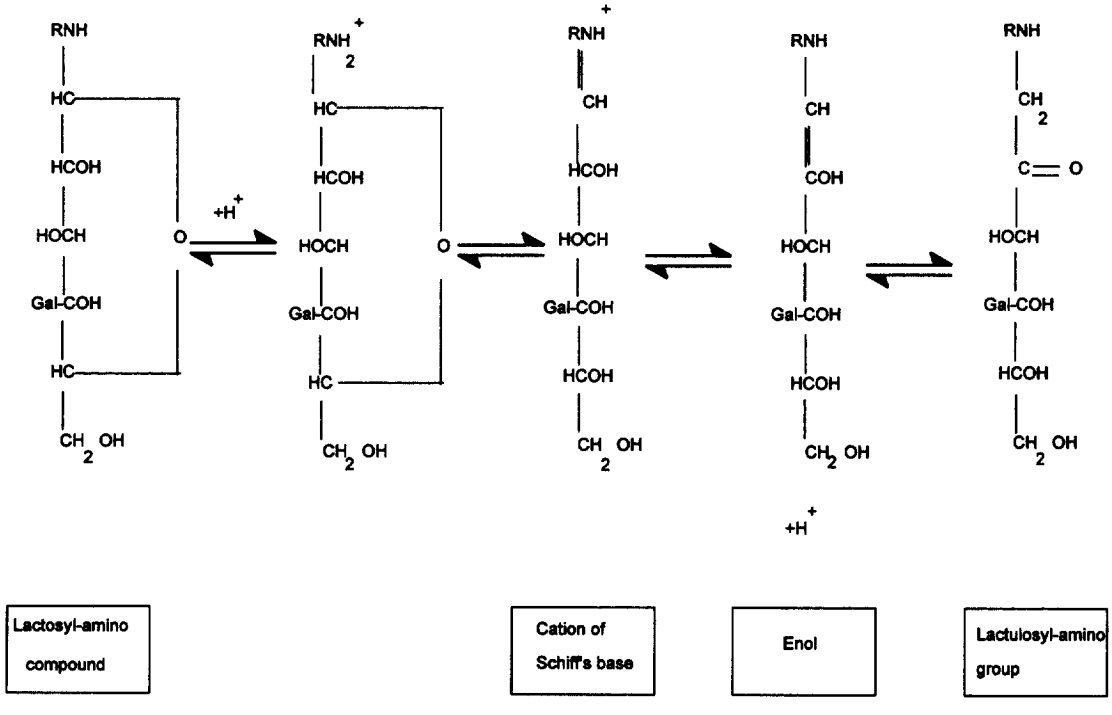

Fig. 3. Amadori rearrangement and formation of lactulosyllysine.

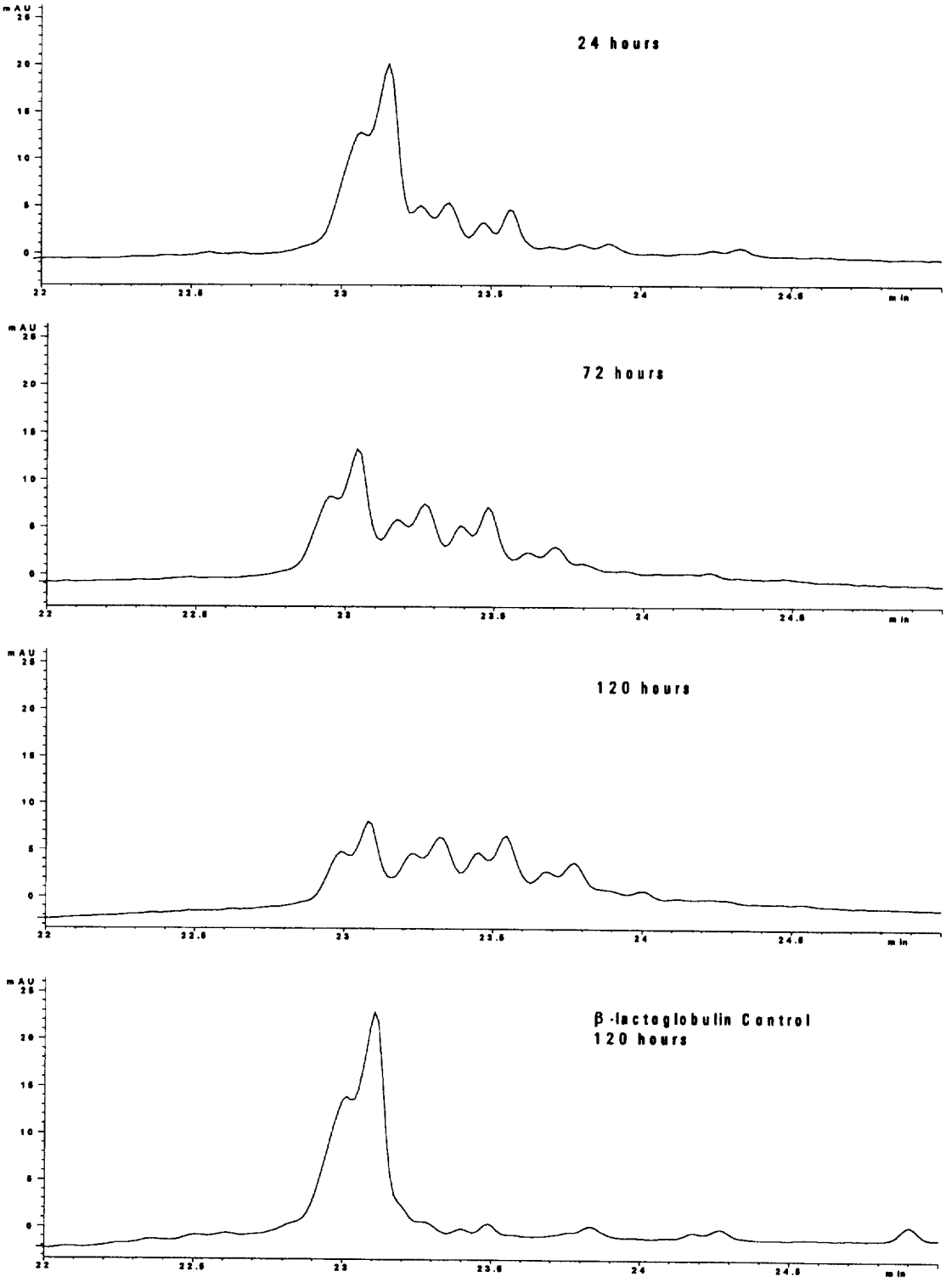

Fig. 4. Reaction of β-lactoglobulin and lactose at 50°C. Conditions as in Fig. 1.

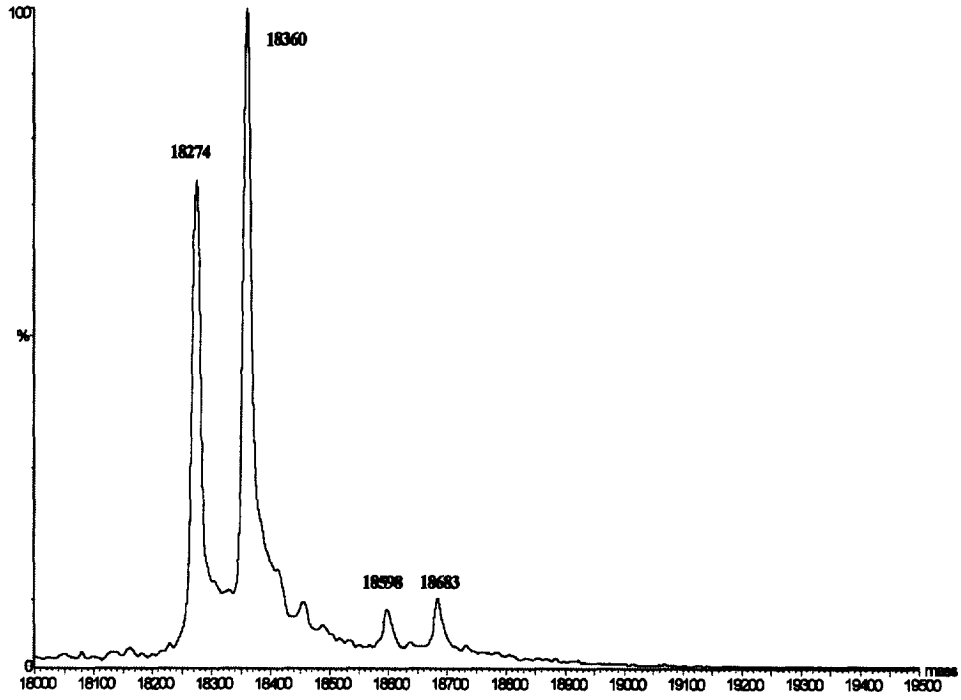

Fig. 5. Transformed electrospray mass spectrum of β-lactoglobulin.

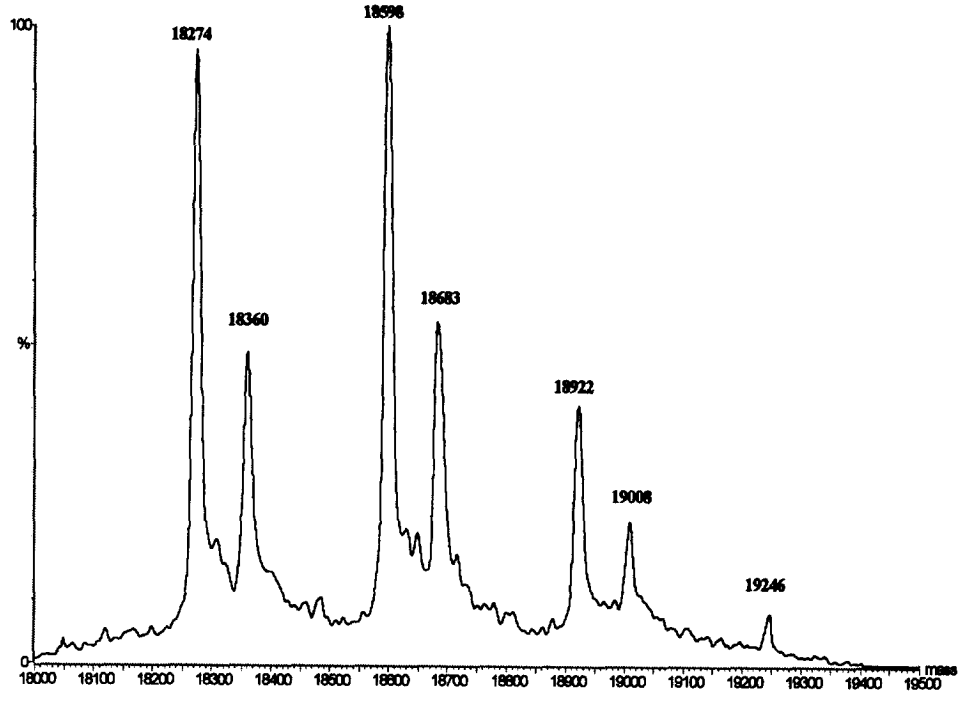

Fig. 6. Transformed electrospray mass spectrum of β-lactoglobulin/lactose reaction product.

19 246 and 19 332. Assuming that each of these pairs represent the formation of one, two, and three lactulosyllysine adducts respectively, then the theoretical mass increments should be 324 Da (i.e., addition of lactose and the elimination of water; (342−18=324). This is in excellent agreement with what is observed, and would appear to confirm that the Maillard reaction is indeed the source of these secondary peaks.

4. Conclusions

In studying skimmed milk powder, it has been demonstrated that there are significant chemical differences between SMP and fresh skimmed milk, and that these can be conveniently observed via CE. Hence, secondary peaks which migrate after β-lactoglobulin are undoubtedly due to the initial stages of the Maillard reaction, which results in the formation of lactulosyllysine as a consequence of the reaction between lactose and protein lysine groups. Since there are some 15 lysine groups in β-lactoglobulin, a series of such adducts are theoretically possible, although lysine steric availability together with subsequent lactulosyllysine degradation, will limit the number observed. These results are in complete agreement with those of Maubois et al. [12] and Burr et al. [11] who used electrospray mass spectrometry to demonstrate a series of ions with a 324 Da increment, although neither sets of authors reported the electrophoretic or chromatographic separation of the adducts.

In speculating as to the point at which the Maillard reaction occurs within the SMP, it is assumed that it most probably occurs during spray drying. However, Andrews and Cheesman [18] have shown that the Maillard reaction can occur even under refrigerated conditions. It is therefore possible that the peaks are formed at least in part, during ambient storage of SMP. It is interesting to note that Burr et al. [11] speculate that in their study 30 min at 40°C is sufficient to cause glycosylation, but that physiological conditions within the udder of the cow may even promote the reaction. Since we have never observed secondary peaks in fresh pasteurised milk we believe this can be discounted. Further work to clarify the situation for stored SMP is however, in progress.

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