ELSEVIER

PII: S0308-8146(96)00204-X

Food Chemistry, Vol. 58, No. 1–2, pp. 53–58, 1997

0308-8146/97 $17.00+0.00

An immunological approach to monitor protein lactosylation of heated food model systems

V. Fogliano,* S. M. Monti, A. Ritieni, C. Marchisano, G. Peluso & G. Randazzo

Dipartimento di Scienza degli Alimenti, Università di Napoli 'Federico II' Via Università 100, 80055, Portici (NA), Italy

(Received 26 January 1996; revised version received 7 May 1996; accepted 7 May 1996)

Poly-L-lysine, a high molecular weight synthetic compound with a large number of free amino groups, was glycated with lactose through the Maillard reaction (MR). Polyclonal mouse and rabbit antibodies (Abs) were obtained and characterised by competitive ELISA (enzyme-linked immuno assay), immunoblotting and cytofluorimetric assays. Results demonstrated that the Abs react specifically with lactosylated food proteins, i.e. bovine serum albumin (BSA) and casein and do not cross react with the underivatised proteins. The immunoreactivity between Abs and lactose-BSA adducts was strongly inhibited by N-ϵ-deoxy-lactulosyl-L-lysine, indicating that this typical MR product is recognised specifically by our Abs. These reagents can be a useful tool to monitor the MR reaction between carbohydrates and lysine, which occurs during the thermal treatment of food.

INTRODUCTION

Heating treatments are responsible for many reactions involving sugars and amino acids, peptides or proteins. In particular, the Maillard reaction (MR), which mainly involves the ϵ-amino group of lysine and reducing sugars, plays an important role in the food industry (Baltes, 1986; Ames, 1990). Monitoring MR products (MRPs) is valuable for investigating the effect of the thermal treatment applied during food processing. Over the last 15 years, different methods have been developed to monitor MRPs (Hurrel & Carpenter, 1981; Erbersdobler & Anderson, 1983). In recent papers Shaw & Crabbe (1994), followed non-enzymic glycation in a BSA-glucose model system using nitroblue tetrazolium, while Hirch *et al.* (1995), evaluated the extent of the MR by measuring the triazine derivatives of some dicarbonyl intermediates arising from the degradation of Amadori compounds. The most successful method for the detection of non-enzymic protein glycation was developed by Resmini *et al.* (1990), who used ion-pair chromatography to detect furosine (N-ϵ-2-furoylmethyl-L-lysine), resulting from the acid hydrolysis of MRPs.

The work presented here proposes the identification of protein glycation, which takes place during heat treatments of relevance to food, using an immunological method. Lactose was chosen as the reducing sugar because of its well known antigenic properties (Matsuda *et al.*, 1985). The aim was to probe the extent of the MR directly and to raise antibodies capable of recognising the lactulosyl–lysine (N-ϵ-deoxy-lactulosyl-L-lysine) epitope, which is a stable intermediate produced at an early stage of the reaction between lactose and the ϵ-NH_2 group of lysine (Amadori compound).

In order to obtain a high concentration of the lactulosyl–lysine antigenic determinant, poly-L-lysine (a synthetic polymer) was used as the carrier (Tam, 1988; Hayase *et al.*, 1989). The working hypothesis was that the Abs raised against the lactosylated polymer would recognise the lactulosyl–lysine epitopes of the heated food model systems.

In this paper, the antisera obtained by immunization of mice and rabbit were characterised. Their ability to recognise lactosylated proteins was tested on BSA-lactose and casein–lactose model systems by competitive ELISA, immunoblotting and cytofluorimetric assays.

MATERIALS AND METHODS

Chemicals

Electrophoresis reagents and apparatus, horseradish peroxidase conjugated secondary antibodies and Western blotting apparatus were obtained from Bio–Rad. Nunc Maxi-Sorp ELISA plates were from Intermed. All other products of analytical grade were purchased from

*To whom correspondence should be addressed.

Fluka. Polyvinylidene difluoride (PVDF) membranes were from Millipore.

Latex particles of 3 μm diameter were obtained from Polyscience; Freund's complete adjuvant (CFA), fluorescent antibodies, tetramethyl benzidine and horse serum were purchased from Sigma. NMR spectra of N-ϵ-deoxylactulosyl-lysine were performed on a Bruker 600.13 MHz instrument.

Synthesis of N-ϵ-deoxylactulosyl-L-lysine

N-ϵ-deoxylactulosyl-L-lysine was prepared according to Njoroge *et al.* (1988). N-α-t-BOC-lysine (2.5 g) and 25 g of D-lactose in 700 ml of methanol were heated under reflux for 4 h. The N-α-t-BOC-N-ϵ-deoxylactulosyl-L-lysine was isolated by ion-exchange chromatography on a Dowex 50 W-X4 column, followed by an AG-X11 ion-retardation column (Bio–Rad). The BOC group was removed after incubation with 70% TFA for 1 h at room temperature. The chemical structure of N-α-t-BOC-N-ϵ-deoxylactulosyl-L-lysine was confirmed by ^{1}H and ^{13}C NMR spectroscopy.

Antigen preparation

Poly-L-lysine 30–70 kDa (40 mg) was dissolved in 1.6 ml 0.5 M sodium phosphate buffer at pH 7.5 containing 70 mM lactose. The reaction was carried out at 50 °C for 1, 5, 15, 40, 60 and 75 h. Samples were extensively dialyzed at 4 °C against deionized water and lyophilized. The amount of bound lactose was evaluated directly by the method of Dubois (Dubois *et al.*, 1956). The free amino group content was determined by the OPA-NAC method (Medina Hernandez & Garcia Alvarez-Coque, 1992). The sample obtained after 40 h reflux was used for the immunization experiments.

Immunization procedures

Rabbit

A 5 month old New Zealand female rabbit was immunized by injection of 0.5 mg of antigen emulsified with CFA. The rabbit received two booster injections 4 weeks after the first injection. Blood was collected 20 days after the last injection by ear puncture. The serum was collected and stored at −20 °C.

Mice

Four mice (female Balb/c) were immunized by intraperitonal injection of 10 or 100 mg of lactosylated poly-L-lysine in 500 μl CFA. The mice received two booster injections (10 or 100 mg) of antigen mixed with 500 μl of CFA 2 and 3 weeks after the first injection. Blood was collected 4 and 8 weeks after the first injection by puncture of the ophthalmic retro-orbital venous plexus. The serum was separated and stored at −20 °C.

***In vitro* lactosylation of BSA and casein**

BSA (100 mg) was purified by anion-exchange chromatography using a Macroprep high Q resin from Bio–Rad. Purified protein (50 mg) was dissolved in 2 ml of 0.5 M sodium phosphate buffer pH 7.5 containing 70 mM lactose and incubated at 50 °C for 48 h.

Casein was precipitated from commercial defatted cow milk samples by addition of acetic acid and resuspended in 9 M urea according to Aschaffenburg & Dewry (1959). Glycation of 50 mg was carried out as for BSA. After derivatization, solutions were extensively dialyzed and subjected to Dubois analysis and 12% SDS PAGE (Laëmmli, 1970), to verify protein lactosylation.

Antisera characterization

Indirect and competitive ELISA were performed as described (Harlow & Lane, 1988), using horse serum 10% in 0.15 M phosphate buffered saline pH 7.2 (PBS) as blocking agent, peroxidase conjugated antibodies as secondary reagent and tetramethylbenzidine as substrate.

Dot blot assays were performed on nitrocellulose paper according to Harlow & Lane (1988). Rabbit and mouse Abs were used at dilutions of 1:200 and 1:100, respectively. The reaction was revealed by peroxidase conjugated anti-rabbit or mouse IgG and 4-chloro-1-naphtol as substrate. The reaction was stopped with 12.5% sulfuric acid.

Immunoblotting was performed on PVDF sheets according to Towbin *et al.* (1979). PVDF membranes were incubated in 3% gelatin in tris buffered saline pH 8.0 (TBS) for blocking. After washing, the membranes were incubated overnight with 1:200 rabbit Abs with gentle agitation. Immunodecoration was achieved as above.

Flow cytofluorimetry

About 10^7 latex particles were suspended in 1 ml of 0.1 M borate buffer pH 8.5 containing either 10 or 100 mg of poly-L-lysine, BSA or casein, both lactosylated and unlactosylated. Particles were incubated for 24 h, washed twice with PBS and incubated (1 h) with 1 ml of 1% BSA in PBS. Particles were incubated again (24 h) with mouse Abs (diluted from 10^{-1} to 10^{-4}), washed twice with PBS and finally incubated with rabbit anti mouse IgG labelled with fluorescein. Flow cytometry was performed on a FaCSort instrument (Becton–Dickinson). Marker settings were the same throughout the experiments. The number of events acquired was 10 000 per sample. A control, where the mouse Abs was omitted, was set up for each sample.

RESULTS AND DISCUSSION

Antigen selection and preparation

The aim of this study was to obtain antibodies which were able to recognise, with high specificity and sensitivity, the epitopes formed by the MR in heated food model systems. For this purpose we used poly-L-lysine as the carrier and lactose as the reducing sugar. Poly-L-lysine was chosen since it is not present in any food ingredients; it has a large number of amino groups available for reaction, hence the potentially large number of lactulosyl–lysine epitopes which could result from its glycation.

The time course of the glycation reaction between poly-L-lysine and lactose was monitored. Results reported in Fig. 1 showed that the amount of carbohydrate bound to the polymer did not increase after 40 h, but available free amino groups continued to decrease. It is suggested that a protein-like intramolecular cross-linking occurred between the intermediate reaction products and free amino groups of the polymer, resulting in the formation of a large molecular weight aggregate (Matsuda *et al.*, 1991). The increased absorption of the reaction mixture at 420 nm confirmed this hypothesis (data not shown).

Considering that 0.1 mg of lactose per mg of poly-L-lysine was measured (see Fig. 1), it was estimated that one residue out of 28 ϵ-NH_2 groups was derivatized. After 40 h of reaction, the solution was still clear; therefore, this sample was used for mice and rabbit immunization.

Model systems preparation

Two typical milk proteins, BSA and a mixed casein fraction, were partially purified and derivatized by heating at 50 °C for 48 h in the presence of 70 mM lactose. After extensive dialysis, protein-bound lactose was quantified by the Dubois assay. For BSA, 80 μg of sugar per mg of proteins was detected and it was calculated that about 15 moles of lactose were bound to each mole of BSA. Protein glycation was also monitored by SDS polyacrylamide gel electrophoresis. A decreased mobility of the lactosylated proteins in comparison to the native proteins indicated that glycation had taken place (Fig. 2, Panel A). In addition the apparent molecular mass shift on SDS-PAGE (about 5 kDa) agreed with the amount of bound-lactose as calculated by the Dubois method. However, the rate of casein glycation was lower and it was estimated to be 25 μg of lactose per mg of protein.

Thus, two model systems (based upon the use of two different lactosylated proteins, BSA and casein) were available to permit Abs to recognise lactosylated epitopes formed by the MR involving lysine residues of proteins.

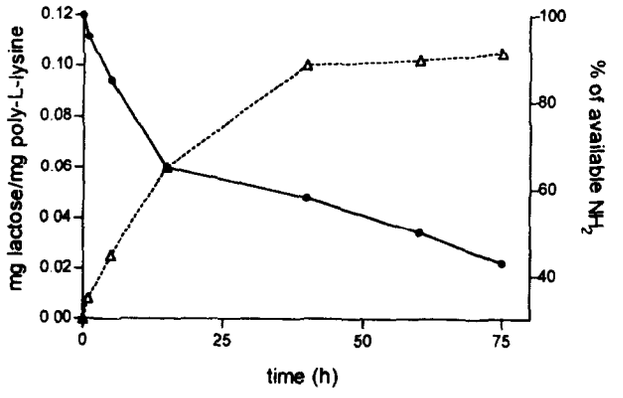

Fig. 1. Time course of poly-L-lysine lactosylation reaction. •: % of available NH_2 monitored by the OPA-NAC reagent (Medina Hernandez & Garcia Alvarez-Coque, 1992). △: mg of bound-lactose/mg of poly-L-lysine monitored by the method of Dubois (Dubois *et al.*, 1956).

Characterization of rabbit antiserum

Dot blot analysis clearly demonstrated that, as expected, rabbit Abs recognised the lactosylated poly-L-lysine but not the underivatized carrier (data not shown). Furthermore, a direct ELISA assay where plates were coated with BSA and casein, both in the native and the lactosylated form, showed that only the lactosylated molecules were recognised by the Abs.

The binding properties of the Abs were confirmed by immunoblotting analysis of the lactose-BSA model system (Fig. 2, Panel B). Immunostaining with Abs resulted in the development of a distinct band for the lactose-BSA (Panel B, lane 1), although no stained band was detected in the lane of native BSA (Panel B, lane 2).

As the Abs did not cross-react with native food proteins, they were further characterized by the ELISA

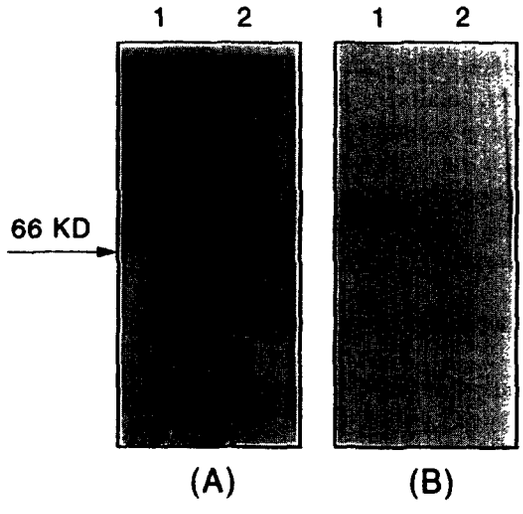

Fig. 2. SDS-PAGE and immunoblotting analysis for the rabbit Abs binding to the lactose-BSA Maillard model system. A sheet of gel was stained with Coomassie Brilliant Blue (A) and the PVDF membrane was immunostained with rabbits Abs (B). Native BSA was also analysed for comparison: (1) lactose-BSA; (2) BSA native.

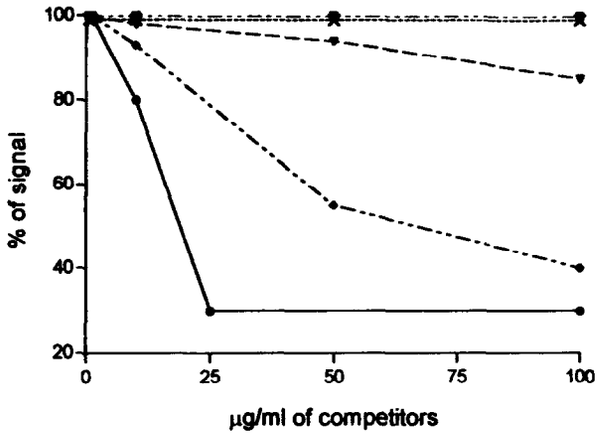

Fig. 3. Competitive ELISA using 1–100 μg of lactosylated BSA as coating agent. Concentrations between 1 and 100 μg/ml of the following competitors were added together with the anti lactosil poly-L-lysine rabbit antiserum: ■ glucose; x galactose; ▼ lactose; ◆ lactulosyl-lysine; ● lactosyl poly-L-lysine.

competitive inhibition assay (Fig. 3) carried out with lactosyl-poly-L-lysine, lactulosyl-lysine, free lactose, glucose and galactose, in order to identify the recognised epitopes.

Lactosylated poly-L-lysine (25 μg/ml) and lactulosyl-lysine (100 μg/ml) inhibited Abs binding to the lactosylated BSA (1-100 μg/well) by 70 and 60%, respectively. Glucose and galactose, as well as underivatized BSA, did not inhibit the reaction at all, while lactose (100 μg/ml) inhibited only 15% of the Abs binding. These results indicate that the Amadori compound N-ε-deoxy-lactulosyl-L-lysine, is the major epitope recognized on the lactosylated proteins and is most easily bound by the Abs.

These results are in partial agreement with those reported by Matsuda *et al.* (1992) using monoclonal Abs against lactose-protein Maillard adducts raised from mice. They showed that lactulose, which is a structural analogue of the Amadori product between lactose and ε-amino group of lysine, was the best competitor in the ELISA using free sugars. However, we did not observe the strong recognition between lactose and galactose and the polyclonal Abs, as reported by Matsuda *et al.* (1985).

Characterization of mice antiserum

Preliminary dot blot analysis showed that Abs recognize lactosylated poly-L-lysine better than poly-L-lysine itself (data not shown). Due to the low titre of Abs, ELISA was not very informative. Thus, the antisera were characterized by flow cytofluorimetry. This technique is very sensitive and allows quantitative measurements at rates of up to 5000 events per second (Watson, 1991). However, these measurements can be made only on a population of single cells. To mimic a cell population, the antigens were adsorbed onto latex particles. In this way soluble antigens could be analysed by flow cytometry.

The first set of experiments fixed the optimal dilutions of the various reagents. Using 10^6 latex particles per tube, 1 ml of antigen at 10 or 100 μg/ml of 0.1 M borate buffer at pH 8.5 to sensitise the latex particles, 50 μl of mouse Abs at the dilution of 1:1000, and 50 μl of fluorescein labelled rabbit anti mouse at the dilution of 1:400, clear cut differences were displayed between native and derivatized proteins. The amount (%) of the signals due to the recognition of the Abs is related to the separation of the two Gaussian curves. The greater the separation, the higher the specific signal. No separation was detected when coating the latex particles with native proteins, thus confirming that the mice Abs did not recognise underivatized proteins. However, the percentages of particles giving signals for the control tubes were 85, 48 and 28% when using the lactosylated forms of poly-L-lysine, BSA and casein, respectively (Fig. 4). Quantitative data obtained by flow cytometry confirmed those obtained for the glycation level of the two model systems (lactosylated BSA and casein).

Results coming from the characterization by means of different techniques of different antisera, obtained from mouse and rabbit, were in full agreement, suggesting that the adopted procedure gave a reproducible immunological response.

CONCLUSION

In this paper we have shown that the synthetic polymer, poly-L-lysine, has immunogenic properties, both in mice and rabbits. The large number of available NH_2 groups allows the formation of hapten-like molecules (lactulosyl-lysine). It is worth noting that using this polymer as the carrier, non-specific cross-reactions between antisera and food proteins were avoided. In fact, although protein-bound lactose is a good antigenic determinant (Matsuda *et al.*, 1985), antisera obtained in our laboratory by immunization with BSA-lactose and HSA-lactose mainly recognised the carrier protein (Monti *et al.*, 1994).

From the characterization of the antisera the following conclusions can be drawn:

1. Antibodies obtained against lactosylated poly-L-lysine recognize, with high specificity, the glycated epitopes formed in heated food model systems.
2. The carbohydrate moiety of lactose-proteins Maillard adducts is an antigenic determinant which plays an important role in Abs binding.
3. N-ε-deoxy-lactulosyl-L-lysine is a stable and dominant intermediate produced at an early stage of the MR, confirming previous studies (Finot & Mauron, 1972; Kato *et al.*, 1989; Matsuda *et al.*, 1992) and it has strong immunogenic properties.

The possibility of using these antisera to monitor milk thermal treatments is currently being investigated.

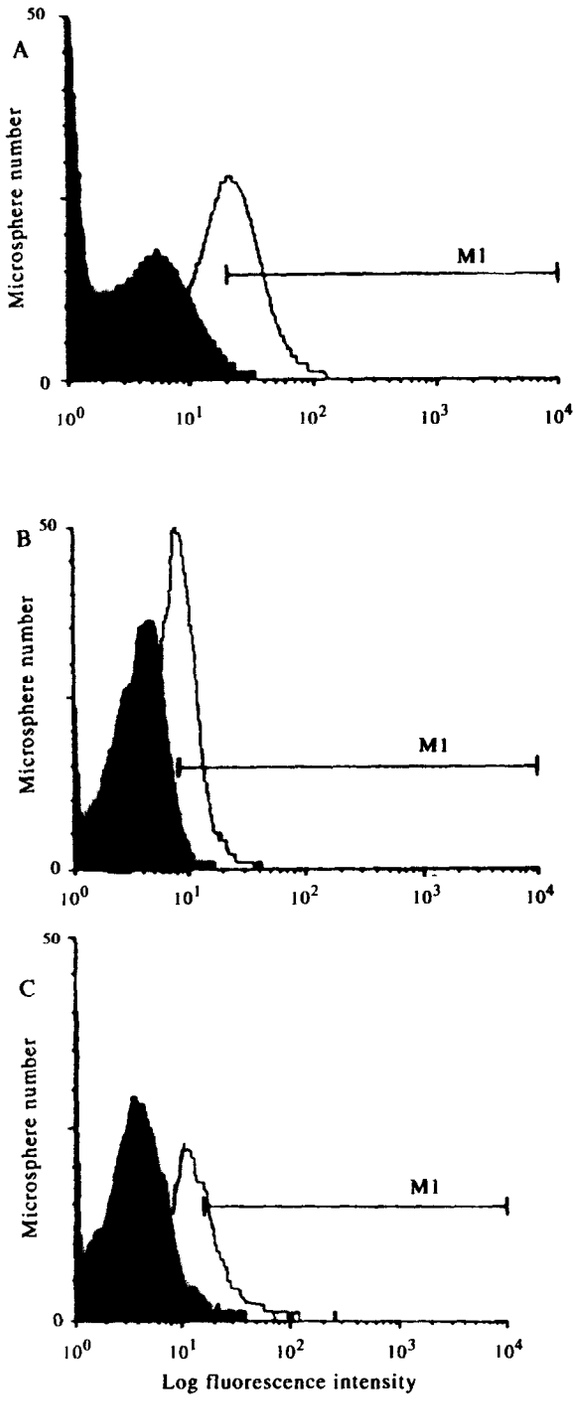

Fig. 4. Cytofluorimetric histogram obtained with anti lactosylated poly-L-lysine mice antiserum. Panel A, lactosylated poly-l-lysine. Panel B, lactosylated BSA. Panel C, lactosylated casein. White area is proportional to the antiserum response; staining of a control experiment (black area) is shown for comparison.

ACKNOWLEDGEMENTS

We thank Professor D. Iannelli and Dr R. Pizzano for helpful discussions and R. De Matte' for performing mice immunizations. We are indebted to the NMR service of Montelibretti CNR Area for recording the spectra.

REFERENCES

Ames, J. M. (1990). Control of the Maillard reaction in food systems. *Trends Fd Sci. Technol.*, **1**, 150–154.

Aschaffenburg, R. & Dewry, J. (1959). New procedure for routine determination of various non-casein proteins of milk. *15th Int. Dairy Congr. Lond.*, **3**, 1631–1637.

Baltes, W. (1986). Reactions of the Maillard-type in foods. *Food Chem.*, **20**, 213–214.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analyt. Chem.*, **28**, 350–356.

Erbersdobler, H. F. & Anderson, T. R. (1983). Determination of available lysine by various procedures in Maillard-type products. In *The Maillard Reactions in Foods and Nutrition*, eds G. R. Waller & M. S. Feather, American Chemical Society, Washington D.C., p. 419.

Finot, P. A. & Mauron, J. (1972). Le blocage de la lysine par la reaction de Maillard. II Proprietes chimiques des derives N-(desoxy-1-D-fructosyl-1) et N-(desoxy-1-D-lactulosyl-1) de la lysine. *Helv. Chim. Acta*, **55**, 1153–1164.

Harlow, E. & Lane, D. (1988). *Antibodies: a Laboratory manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Hayase, F., Nagaraj, R. H., Miyata, S., Nyoroge, F. G. & Monnier, V. M. (1989). Aging of proteins: immunological detection of a glucose-derived pyrrole formed during Maillard reaction *in vivo*. *J. Biol. Chem.*, **263**, 3758–3764.

Hirch, J., Mossine, V. V. & Feather, S. M. (1995). The detection of some dicarbonyl intermediates arising from the degradation of Amadori compounds (the Maillard Reaction). *Carbohy. Res.*, **273**, 171–177.

Hurrel, R. F. & Carpenter, K. J. (1981). The estimation of available lysine in foodstuffs after Maillard reaction. *Prog. Food Nutr. Sci.*, **5**, 159–176.

Kato, Y., Matsuda, T., Kato, N. & Nakamura, R. (1989). Maillard reaction of disaccharides with protein: suppressive effect of nonreducing end pyranoside groups on browning and protein polymerization. *J. Agric. Fd Chem.*, **37**, 1077–1081.

Laëmmli, U. K. (1970). Cleavage of structural protein during the assembly of the head of bacteriofage T4. *Nature*, **227**, 680–685.

Matsuda, T., Kato, Y., Watanabe, K. & Nakamura, R. (1985). Immunochemical properties of proteins glycosylated through Maillard reaction: β-lactoglobulin-lactose and ovalbumin-glucose systems. *J. Fd Sci.*, **50**, 618–621.

Matsuda, T., Kato, Y. & Nakamura, R. (1991). Lysine loss and polymerization of bovine β-lactoglobulin by amino carbonyl reaction with lactulose (4-O-β-D-galactopyranosyl-D-fructose). *J. Agric. Fd Chem.*, **39**, 1201–1204.

Matsuda, T., Ishiguro, H., Ohkubo, I., Sasaki, M. & Nakamura, R. (1992). Carbohydrate binding specificity of monoclonal antibodies raised against lactose-protein Maillard adducts. *J. Biochem.*, **111**, 383–387.

Medina Hernandez, M. J. & Garcia Alvarez-Coque, M. C. (1992). Available lysine in protein, assay using o-phthalaldeyde/N-acetyl-L-cysteine spectrophotometric method. *J. Fd Sci.*, **57**, 503–505.

Monti, S. M., Fogliano, V., Ritieni, A., Pizzano, R. & Randazzo, G. (1994). Maillard reaction monitoring using immunometrical techniques. *It. J. Biochem.*, **43**, 151–152.

Njoroge, F. G., Fernandes, A. A. & Monnier, V. M. (1988). Mechanism of formation of the putative advanced glycosylation end product and protein cross link 2-(2-furoyl)-4 (5)-2-furanyl) ^{1}H-imidazole. *J. Biol. Chem.*, **263**, 10646–10652.

Resmini, P., Pellegrino, L. & Battelli, G. (1990). Accurate quantification of furosine in milk and dairy products by a direct HPLC method. *Ital. J. Fd Sci.*, **3**, 173–183.

Shaw, S. M. & Crabbe, J. C. (1994). Monitoring the progress of non-enzymic glycation *in vitro*. *Int. J. Protein Res.*, **44**, 594–602.

Tam, J. P. (1988). Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc. Nat. Acad. Sci. (U.S.A.)*, **85**, 5409–5413.

Towbin, H., Stahelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci., (U.S.A.)*, **76**, 4340–4354.

Watson, J. (1991). *Introduction to Flow Cytometry*. Cambridge University Press, Cambridge.