Immunodetection of lactosylated proteins as a useful tool to determine heat treatment in milk samples

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This paper reports the optimisation of a competitive immunoassay (ELISA) to detect lactosylated proteins in milk samples. The assay employs monoclonal antibodies for lactosylated proteins produced in our laboratory and requires no pre-treatment of the samples other than a dilution step. Monoclonal antibodies were fully characterised in terms of selectivity and cross-reactivity with structurally related molecules and used in a competitive assay format with lactosylated standard proteins (lactosylated ovalbumin). The detection limit for lactosylated ovalbumin was 0.015 μg ml⁻¹ and the working range was from 0.010 to 40 μg ml⁻¹. The data obtained indicate that the ELISA developed is applicable to diluted milk samples and is able to distinguish between milk samples that have undergone different heat treatments (UHT and pasteurised milk).

Introduction

Heat treatment of milk samples during pasteurisation processes leads to the non enzymatic lactosylation of free amino groups of proteins due to the Maillard reaction. The addition of lactose to lysine ε- amino groups forms N-substituted-1-amino-deoxy-2-ketoses, known as Amadori compounds, subsequently leading to unavailable amino acid sugar reaction products. The blocked lysine will not be susceptible to further enzyme attack, becoming nutritionally unavailable. Since lysine is an essential amino acid, it is not synthesised by humans, and it must be present in the diet. Hence, the main consequence of these heating processes is the modification of the nutritional values of milk.

The two main heat induced markers used as indicators of the severity of heat treatment are furosine and lactulose. Furosine, namely ε-N-[(2-furomethyl)-l]-lysine, is an amino acid obtained by acid hydrolysis of glycosylated proteins and is mainly detected by reversed-phase HPLC or ion-exchange chromatography; although lately capillary electrophoresis has also been reported. Hydrolysis of lactulosyllysine, the lysine–lactose adduct formed in MR, yields three different products, lysine (~ 50%), furosine (~ 30–40%) and pyridosine (~ 10–20%), but the conversion factor for the calculation of the content of the Amadori product is uncertain since the proportions of the hydrolysis products are variable. Microwave hydrolysis of proteins has been applied to furosine determination, shortening the time required for the whole procedure. The furosine method has the advantage of being sensitive at very low levels of blocked lysine, but the overall procedure, consisting of 24 h hydrolysis and chromatographic determination, is cumbersome and time consuming, requiring expensive equipment and skilled operators.

Lactulose (4-O-β-galactopyranosyl-β-fructofuranose) is formed by isomerisation of lactose (4-O-β-galactopyranosyl-β-glucopyranose) during the heat treatment of milk, and has been proposed by the International Dairy Federation (IDF) and by the European Commission (EC) as an analytical index to distinguish UHT milk from sterilised milk. The detection of lactulose is mainly based on gas chromatography, liquid chromatography, spectrophotometric methods and enzymatic methods with either spectrophotometric or amperometric detection. Our group has developed several methods for the determination of lactulose in milk samples, aimed at producing much simpler and shorter protocols that do not require expensive equipment. Immunological methods for the measurement of the extent of protein lactosylation have been developed based on the well known antigenic properties of lactose. Several groups have reported the production of polyclonal antibodies for lactosylated proteins using different immunogens such as lactosylated proteins and peptides, but to date no-one has been able to set up a simple ELISA procedure to distinguish among different classes of milk. All of the immunoassays proposed so far require long pre-treatment of the samples, such as protein precipitation and further purification.

The aim of this paper is to report the characterisation of monoclonal antibodies for lactosylated proteins and their use in an immunoassay (enzyme-linked immunosorbent assay (ELISA)) for the detection of lactosylated proteins in milk samples. Two commercially available classes of milk (UHT and pasteurised) treated with different heating procedures were tested in order to assess the applicability of the monoclonal antibodies. This assay does not need any pre-treatment of the milk samples other than a dilution step and seems suitable for the measurement of the extent of protein lactosylation in milk.

Experimental

Reagents and materials

All proteins, carbohydrates, chemicals for buffers and gels were obtained from Sigma (St Louis, MO, USA). Anti-mouse HRP conjugated Ab was purchased from Bio-Rad Laboratories (Richmond, CA, USA). ELISA plates were Maxisorp from Nunc (Roskilde, Denmark). Protein A for antibody purification was Protein A Sepharose 4 Fast Flow (Amersham Pharmacia

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Biotec, Uppsala, Sweden). Culture media for hybridoma cells was GIBCO BRL (Life Technologies, UK) and HAT medium was obtained from Flow Laboratories (USA). Skimmed milk was purchased directly in shops; all the brands tested are commercially available in Rome, Italy.

**Apparatus**

A Model 550 microplate reader (Bio-Rad) was used to read the absorbance on ELISA plates at 490 nm. A model 238 Uvicord S II instrument from LKB (Bromma, Sweden) at 280 nm was used for the detection of proteins during the collection of antibodies in the purification process.

**Protein lactosylation**

Lactosylated proteins were prepared according to the procedure described by Matsuda et al. with some modifications. Ovalbumin (Ova), casein (Cas), ß-lactoglobulin (LG) and hemocyanin keyhole limpet (KLH) were lactosylated and used as standards for the antisera characterisation and milk tests and for animal immunisation. The same procedure was used for lactosylation and the modified protein was injected into the animals as antigen for the production of antibodies. The lactose solutions were adjusted to pH 8.0 with dilute NaOH and freeze-dried. The dried samples were kept at 50°C until use.

**Immunisation procedures**

Ten mice (female Balb/c) were immunised by intraperitoneal injection of lactosylated KLH (0.1 mg ml⁻¹, 1 ml per mouse) emulsified with complete Freund’s adjuvant (CFA, Sigma). Injections were repeated several times every 4 weeks with phosphate-buffered saline (PBS)–lactosylated KLH (1 mg ml⁻¹). Blood was collected and centrifuged and serum was stored at −20°C until use.

**Production of monoclonal antibodies (mAb)**

Somatic hybridisation was performed by incubating for 1 min at 37°C 10⁸ immune spleen cells with 10⁷ mouse myeloma cells (NSO) in presence of 0.5 ml of 15% v/v dimethyl sulfoxide and 41% w/v polyethylene glycol (PEG). A 0.5 ml volume of 25% w/v PEG was then added. Three minutes later, fused cells were suspended slowly in 48 ml of complete culture medium (CCM) supplemented with 20% fetal bovine serum (FBS) (GIBCO) and plated on to 96-well tissue culture plates (0.2 ml per well). HAT medium (0.15 ml per well) was added on days 2 and 8, after removing 0.15 ml of spent medium. Cells from positive wells were cloned twice to 0.1 and 0.3 cells per well by limiting dilution in 96-well plates screened for the presence of antibody able to bind l-Ova or l-LG by direct ELISA (see later). Selected clones were grown in 15 ml flasks and the supernatants were used as the source of mAbs.

**Antibody purification**

Antibodies were purified by affinity chromatography on a 10 ml protein A column (Protein A Sepharose 4 Fast Flow, 5 ml gel), the whole system consisting of the chromatographic column attached to a UV reader (λ = 280 nm) and a chart recorder. The column was subjected to sequential pre-washing with the following solutions: PBS (pH 7.4), 0.7% NaCl, 1 M NH₄SO₄ (pH 9), 3.5 M MgCl₂ and 1 M NH₄SO₄ (pH 9). The hybridoma supernatant diluted in NH₄SO₄ (1 ml of supernatant in 9 ml of NH₄SO₄) was allowed to run overnight with a continuous flow system. The next day the antibodies were eluted with MgCl₂ and the protein fraction was collected. Antibodies were dialysed in de-ionised water overnight and their titre was determined spectrophotometrically.

**Antibody characterisation**

The binding specificity of the sugar-specific antibody for the lactosylated proteins was measured by competitive and non-competitive ELISA. Both polyclonal and monoclonal antibody screening were performed by coating the plates with all of the lactosylated protein standards (1 µg ml⁻¹ in 50 mmol l⁻¹ carbonate buffer (pH 9) overnight at 4°C (100 µl per well)). The plate was washed three times with PBS, blocked for 1 h at 37°C with 0.5% gelatine in PBS (200 µl per well) and then washed three times with PBS containing 0.05% Tween 20 (polyoxyethylene sorbitan monolaureate) (Sigma) (PBST).

In direct ELISA, serial dilutions of serum or hybridoma cell-culture supernatant or purified antibodies (100 µl per well) were incubated at 4°C for 2 h. The wells were then washed three times (PBST), and further incubated for 1 h at 4°C with 0.1 ml of horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad). After washing (PBS), 0.1 ml per well of o-phenylenediamine in 0.1 M citrate–phosphate buffer (pH 4.5) containing 1 mM H₂O₂ was added (20 min at room temperature). The enzymatic reaction was stopped with 2 M HCl and the absorbance was read on an ELISA plate reader at 495 nm.

Competition curves were obtained mixing 50 µl of sample (as stated) with 50 µl of antisera or hybridoma cell-culture supernatant or purified antibodies at the appropriate dilution in the wells and incubated for 1 h at 4°C. The plates were then processed exactly as before. Western immunoblot on lactosylated and native proteins with purified antibodies was carried out to confirm antibody specificity.

**Competitive ELISA in milk samples**

Pasteurised and UHT milk samples were tested in competitive ELISA and the total milk protein concentration was determined spectrophotometrically. Serial milk dilutions from 1:500 (~60 µg ml⁻¹, considering that the average protein concentration is 3.1%) to 1:500000 (~0.06 µg ml⁻¹) in PBS were incubated with the appropriate mAb dilution (1 µg ml⁻¹) and added to the plate (incubation for 1 h at 4°C). The ELISA plates were then processed as before. The data obtained from each competition curve were plotted and fitted using SigmaPlot software (SPSS) and a regression analysis on the linear portion of the sigmoidal curves was performed. The slopes obtained for the regression analysis were used as an index of protein lactosylation.

Each experiment was performed in triplicate and the mean of each value was used for curve fitting.

**Lactulose determination in milk samples**

Lactulose was determined according to the procedure described by Amine et al. using a spectrophotometric assay.
Results and discussion

Protein lactosylation

Lactosylated and native proteins were subjected to 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to compare the difference in mobility and to evaluate the molecular weight of lactosylated samples. As expected, lactosylated proteins had a lower mobility than the native proteins because of the higher molecular weight. The molecular weights of Ova and L-Ova were calculated to be 45 000 and 48 000, respectively, LG and L-LG 25 000 and 30 000, respectively, and Cas and L-Cas 58 000 and 62 000, respectively; these are comparable to the molecular weights reported by Matsuda et al.21

Characterisation of the antibodies

Selectivity for lactosylated proteins. Polyclonal (pAb) and monoclonal antibody (mAb) selectivity for lactosylated proteins was tested in direct ELISA, using L-Ova, L-Cas and L-LG as antigens. Both pAbs and mAbs exhibited high selectivity for the lactosylated standard proteins as shown in Fig. 1(a) and 1(b). The antibodies recognise all the lactosylated proteins with different affinity; this can be explained by the difference in the number and position of the epitopes (lactosylation sites) for each of the proteic molecules. The results clearly indicate that the antibodies are specific for the lactose-lysine adduct present on each of these proteins, regardless of the structure of the protein itself. Western immunoblot on both lactosylated and native proteins confirmed these data (results not shown).

As expected, mAb and pAb present different responses to lactosylated proteins; this is more evident by the competition curves reported in Fig. 2. The main difference that should be pointed out is in selectivity (Fig. 1) and sensitivity (Fig. 2; different working ranges for mAb and pAb). The positive hybridoma clone (mAb) was selected for the assay because it gives major advantages for the standardisation of the procedure. In fact, the analyst is able to operate with a reagent (mAbs) exhibiting constant characteristics in terms of selectivity, affinity and cross-reactivity with any structure related molecule. Moreover, no further immunisation (or sacrifice) is needed, nor re-optimisation of the assay conditions owing to the large variability in the immunogenic responses of different animals.

The affinity constants for mAbs were determined from the competition curves with lactosylated standards by the method of Friguet et al.32 [Fig. 2(b)]. The values obtained were $10^{-8.5} \times 10^{-9}$ for L-Ova, $5 \times 10^{-8.8} \times 10^{-8}$ for L-Cas and $10^{-9.5} \times 10^{-8}$ for L-LG.

The working range and the detection limit for L-Ova were also determined from the competition curve in Fig. 2(b). The detection limit, defined as the concentration of lactosylated standard protein equivalent to three standard deviations at A0 (no competition) was 0.015 mg ml$^{-1}$. The working range for L-Ova, defined as the standard protein concentration range between 90 and 10% of the maximum signal ($A_0$)33 was 0.010–40 mg ml$^{-1}$. Since mAbs had the lowest affinity for l-
immunoassays for lactosylated proteins.21,25 be the major interfering compound in previously developed sugars in milk (~5% w/w) and because it has been reported to standard. Lactose was tested because is the main source of was investigated using lactosylated ovalbumin as a protein structurally related glycosylated proteins and carbohydrates. The cross-reactivity of mAb with some carbohydrates. Cross-reactivity with glycosylated-OVA adducts and carbohydrates. The cross-reactivity of mAb with some structurally related glycosylated proteins and carbohydrates was investigated using lactosylated ovalbumin as a protein standard. Lactose was tested because is the main source of sugars in milk (~5% w/w) and because it has been reported to be the major interfering compound in previously developed immunoassays for lactosylated proteins.21,25 Glucose and galactose as the product of lactose hydrolysis and lactulose which is formed during milk heat treatment, although at low concentration compared with lactose (below 50 ppm in pasteurised milk and ~300 ppm in UHT), were also tested. Other cross-reactivity tests were run with the glycated adducts of Ova with glucose, galactose and lactulose prepared as described in the Experimental section. Cross-reactivity (CR%) was expressed as the ratio of the concentration of free l-Ova that binds to 50% of mAb used for the competition (x) to the concentration of the competitor that binds the same amount of antibody (y) (CR% = x/y).34 The results in Table 1 show that none of these molecules is able to interfere significantly with the detection of lactosylated proteins. Galactose and the galactose–ovalbumin adduct exhibited the highest cross-reactivity, suggesting that the galactose residue is probably recognised by the antibodies. Their effect is negligible considering both their CR% (15 and 10%, respectively) and the very low concentration of galactose in milk. The latter consideration applies also to lactulose. The selectivity of the mAbs versus lactose was very high. However, considering the concentration in milk samples, it was calculated that a minimum dilution of 1:100 was required in order to have a negligible effect on the assay.

Measurement in milk samples

Preliminary experiments performed on milk samples kept at 37 °C for up to 4 d demonstrated that it was possible to follow the increase in the extent of lactosylation by a direct ELISA using polyclonal antibodies.35 A higher absorbance value was registered for the heated milk samples during a 4 d period of time versus the same samples kept at 4 °C.

The purpose of this work was to produce antibodies and optimise an ELISA competitive assay able to work in milk samples with no pre-treatment of the sample other than dilution. The extent of lactosylation was evaluated by running the competitive assay for different dilutions of milk and plotting the absorbance against the total amount of proteins of the sample calculated as reported in the Experimental section.28 The total milk protein concentration range chosen for ELISA was from ~120 to ~0.06 µg ml⁻¹ (corresponding to a dilution range of 1:250 to 1:500 000). Typical competition curves for pasteurised and UHT milk are shown in Fig. 3.

Our main goal was to determine whether this immunoassay was able to distinguish between commercial classes of milk. Five pasteurised and five UHT commercial milk brands were analysed for this purpose. The increase in the severity of heat treatment in milk pasteurisation processes enhances the number of epitopes (lactosylation sites) on the milk protein. This influences the shape of the ELISA competition curves, the two main differences being the difference in the absorbance between the highest and lowest competition values and the slope of the linear portion of the sigmoidal curves. As expected, UHT milk presents a more negative slope because of the larger amount of lactosylated protein (see Fig. 3). The difference in absorbance is larger compared with fresh milk samples for the same reason, the greater amount of antigen able to bind a larger amount of mAb used in the assay.

The ELISA lactosylation index chosen to compare the results obtained for all of the samples was the slope of the competition curves between 60 and 3 µg ml⁻¹ total protein concentration (corresponding to 1:500 to 1:10 000 dilutions, respectively). Four different dilutions in triplicate were considered for the regression analysis and only r² ≥ 0.990 was considered acceptable. The intra- and inter-day reproducibility of the slope were evaluated on sample No. 8 (six replicates for five days); The RSD was 3% intra-day and 8% inter-day. Data obtained for milk samples are reported in Table 2.

The lactulose content of the samples was also measured with a spectrophotometric procedure. Lactulose was not detectable in pasteurised milk samples since this method has a detection limit of 10 mg l⁻¹.20 The results obtained for UHT milk show the

<table>
<thead>
<tr>
<th>Competitor</th>
<th>y/µg ml⁻¹</th>
<th>CR%</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-Ova</td>
<td>0.88</td>
<td>100.0</td>
</tr>
<tr>
<td>Ovalbumin–glucose</td>
<td>174.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Ovalbumin–galactose</td>
<td>8.64</td>
<td>10.2</td>
</tr>
<tr>
<td>Ovalbumin–lactulose</td>
<td>1534</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Galactose</td>
<td>180.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Lactose</td>
<td>5.9</td>
<td>15</td>
</tr>
<tr>
<td>Lactulose</td>
<td>44</td>
<td>2</td>
</tr>
<tr>
<td>Galactose</td>
<td>17.2</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Monoclonal antibody supernatant cross-reactivity (CR%) with glycosylated proteins and free carbohydrates. CR% = y/x × 100; x = µg ml⁻¹ l-Ova that corresponds to 50% A/Ao, y = µg ml⁻¹ competitor that correspond to 50% A/Ao.

<table>
<thead>
<tr>
<th>Pasteurised milk sample No.</th>
<th>Slope</th>
<th>Lactulose/ mg l⁻¹</th>
<th>UHT milk sample No.</th>
<th>Slope</th>
<th>Lactulose/ mg l⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.21</td>
<td>6</td>
<td>2</td>
<td>0.35</td>
<td>236</td>
</tr>
<tr>
<td>2</td>
<td>0.19</td>
<td>7</td>
<td>3</td>
<td>0.33</td>
<td>220</td>
</tr>
<tr>
<td>3</td>
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<td>8</td>
<td>4</td>
<td>0.34</td>
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<tr>
<td>4</td>
<td>0.23</td>
<td>9</td>
<td>5</td>
<td>0.48</td>
<td>352</td>
</tr>
</tbody>
</table>

Table 2 Lactosylated protein and lactulose assay in pasteurised and UHT milk samples. The slope of the regression analysis of the ELISA assay (sample dilution from 1:500 to 1:10 000) is reported for lactosylated proteins.
same trend as for the lactosylated proteins: the higher the amount of lactulose, the more negative is the slope of the ELISA. To prove that the difference between the two classes of milk reported in Table 2 was significant, a t-test on the sample means was performed. The probability that this difference is relevant was > 99% at the 95% confidence level, meaning that this difference is statistically relevant.

Conclusions

Monoclonal antibodies for lactosylated proteins were produced, characterised and used in a competitive assay format. The data obtained indicate that the ELISA developed is applicable to diluted milk samples and is able to distinguish between milk samples that have undergone different heat treatments (UHT and pasteurised milk).

Acknowledgement

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