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Detection of bovine milk adulterated with cheese whey by Western blot immunoassay

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Milk processor industries and distributors have problems of milk adulteration with cheese whey. The most frequently used method to detect cheese whey is the identification of a glycomacropeptide (GMP) which is present only in cheese whey but not in milk. At present, methods to detect GMP require major work and time, presenting problems of sensitivity and accuracy at low concentrations. We developed a novel Western blot analysis for GMP detection, using anti-GMP polyclonal antibodies. This assay is easy, rapid, sensitive and specific and can detect 0.001% (w/v) GMP, 0.5% (v/v) liquid cheese whey and 0.001% w/v dehydrated cheese whey. Furthermore, it can be used successfully to detect GMP in various food matrices containing cheese whey.

Keywords: glycomacropeptide; Western blot; polyclonal antibodies; cheese whey; milk adulteration

Introduction

Milk has a high nutritional and biological value. Milk processor industries and distributors have problems with adulteration of liquid or dehydrated milk by adding cheese whey. This adulteration is economically attractive because the cheese whey cost is four to five times lower than milk and does not adversely affect the sensory perception of the product by consumers. However, it has nutritional, economic and legal implications (Alcázar, Rosas, Jaramillo, & Peña, 2000) and is the reason for the low milk yield in its processing.

There are different methods to detect the presence and in some cases the amount of cheese whey as an adulterant in milk. They include the determination of the ratio of casein present in the whey by the quantification of non-caseic nitrogen; the quantification of sulphhydryl groups per gram of protein; the measurement of the increase in the ammonium levels in milk using a potentiometer; and the quantification of the complex cystein-cistin through polarography (Alcázar et al., 2000). These methods have low sensitivity and a significant margin of error. Moreover, they are expensive and require major work (Greenberg & Dower, 1986; Meisel & Carstens, 1989).

Other methods detect a specific peptide called glycomacropeptide (GMP) (Alcázar et al., 2000). When milk is treated with chymosin during cheesemaking, milk protein

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(k-casein) is hydrolysed into two peptides. The larger peptide, called para-k-casein, (residues 1–105) remains with the curd, but the smaller one, the GMP (residues 106–169) is located in the whey (Brody, 2000). GMP is a glycosylated soluble peptide with a molecular weight of 6.8 kDa without the glycoside portion (Furlanetti & Prata, 2003). In solution, GMP aggregates to form polymers by interaction of different GMP monomers. The size of the aggregate is affected by the pH of the solution (Brody, 2000; Mikkelsen, Rasmussen, Olsen, Barkholt, & Frøkiær, 2006).

Detection of GMP is reliable as this peptide is a specific component of cheese whey and it should be absent in non-adulterated milk (Benitez, Ponce, & Noa, 2001; Mikkelsen et al., 2006). Presently, the determination of GMP is undertaken by gravimetric methods (Galindo, Valbuena, & Rojas, 2006), high performance liquid chromatography resolution (Leonil & Molle, 1991) or acrylamide gel electrophoresis with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS) (SDS-PAGE) (Alcázar et al., 2000; Galindo et al., 2006). These assays require major work and time and have problems of sensitivity and accuracy at low concentrations.

Therefore, it is necessary to develop an easy, rapid, sensitive and specific method to detect GMP as a milk adulteration index with cheese whey. Immunological techniques are of great sensitivity and specificity, based mainly on the high specificity of the antibodies to an antigen. Antigens can be detected and measured in samples without prior purification, concentration, extraction or special treatments.

The aim of this study was to develop a Western blot system for the detection of GMP in adulterated milk with cheese whey. The method can also identify GMP in different food matrices.

Materials and methods

Immunisation

Seven female New Zealand rabbits (6 weeks old) were used for immunisation. Animals were injected subcutaneously with 1 mg of GMP (Arla Foods Amba, Viby, Denmark) emulsified with an equal volume of complete Freund's adjuvant (Sigma Chemical Corporation, St. Louis, USA). Four subcutaneous boost injections followed at 4-week intervals with the same dose in incomplete Freund's adjuvant. Two weeks after each injection, rabbits were bled from the ear marginal vein and the antiserum was characterised by Western blot.

Purification of anti-GMP antibody

The polyclonal antiserum was treated with an equal volume of saturated ammonium sulphate solution, mixed gently at 22°C and centrifuged at 3000 *g* for 30 min. The precipitate was resuspended in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 100 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 7.4) and desalted by ultrafiltration (Amicon 8050, Millipore Corporation, Bedford, USA) versus PBS at 4°C using a 10 kDa molecular weight cut-off membrane (Millipore Corporation, Bedford, USA). The antibody solution was mixed with two volumes of 60 mM sodium acetate buffer. Subsequently, 7.5% (v/v) of caprylic acid was slowly added (dropwise) with constant stirring. The solution was centrifuged at 5000 *g* for 10 min and the supernatant was desalted and concentrated by ultrafiltration versus PBS until pH 7.4, with a 10 kDa molecular weight cut-off membrane.

The GMP (5 mg/ml) was covalently coupled to NHS-activated (Sephacrose) HP columns (GE Healthcare Bio-Sciences Corporation, Piscataway, USA) according to the manufacturer's instructions, and then the antibody solution was passed through the GMP-coupled column to bind GMP-specific antibodies. Bound antibody was eluted in 100 mM glycine, 0.5 M NaCl, pH 2.7 and neutralised with 1 M Tris HCl pH 9.0. Fractions were tested for binding activity using Western blot analysis and the total protein was determined by the Bradford assay. The IgG purity was tested by 10% SDS-PAGE under reducing conditions and gel silver staining (Crestler, Tulman, & Figueroa, 2001). Antibody fractions were pooled, desalted and concentrated by ultrafiltration versus 0.1 M sodium phosphate buffer, pH 7.2 to a final concentration of 0.5 mg/ml. The pure antibody solution was stored in aliquots at -80°C until use.

Western blot

The Bio-Rad Miniprotean III System (Bio-Rad, Hercules, USA) was used for electrophoretic separation of protein sample by 13.5% SDS-PAGE (Laemmli, 1970). Separation was performed under reducing conditions for 1.5 h at 80 V in running buffer (0.025 M Trizma[®] base, 0.25 M glycine, 3.7 mM, pH 8.3).

Western blot was developed using One-Step[™] Western Kit (GenScript Corporation, Piscataway, USA), according to the manufacturer's instructions. Briefly, proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane WestClear[™] for 2 h at 100 mA with transfer buffer (0.024 M Trizma[®] base, 0.186 M glycine, 5.2 M methanol). Membranes were incubated for 5 min in the pretreat solution. After washing the membrane it was incubated with the Western blot solution containing the anti-GMP antibody (dilution 1:1000) for 40 minutes at room temperature with constant shaking. The membrane was washed and the blot was developed with ChromoSensor[™] One-Solution 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate.

Milk and food sample processing

25 ml of non-adulterated milk, milk with different amounts of GMP or whey, yogurt, dietary supplement and margarine were analysed. Liquid samples were mixed with a TCA solution to a final concentration of 8%, in order to precipitate κ -casein (Benitez et al., 2001) which cross-react with GMP. A second trichloroacetic acid (TCA) treatment to a final concentration of 14% was developed with the supernatant to precipitate GMP (Benitez et al., 2001; Galindo et al., 2006). The precipitate was dissolved in 20 μl of 75 mM Tris HCl, pH 8.0 and the samples were analysed by immuno-blot. Margarine (2 g) was previously dissolved in chloroform-methanol (2:1) to remove lipids. Later GMP was solubilised by adding water (Nakano, Ikawa, & Ozimek, 2007), recovered with TCA treatment to a final concentration of 14%, dissolved in 20 μl of 75 mM Tris HCl, pH 8.0 and analysed by immuno-blot. Dietary supplement, yogurt and margarine contained whey as a component as it was specified in the product.

Results

Confirmation of the antibody specificity

Figure 1 shows that polyclonal anti-GMP antibody detects three bands of 45, 20.1 and 14 kDa in liquid cheese whey samples. Bands of 20.1 and 14 kDa correspond to that

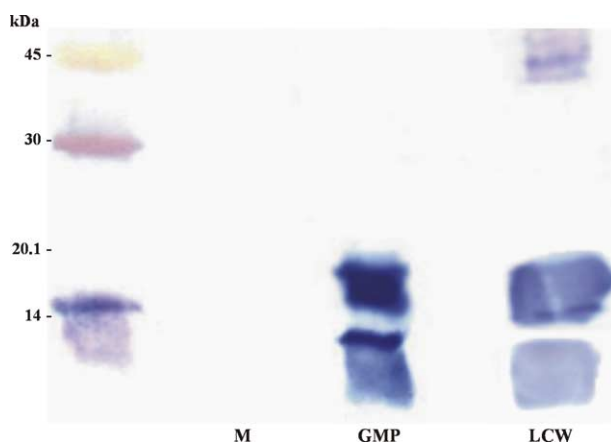


Figure 1. Specificity of rabbit antibodies to GMP. M: 10 μ l of processed milk without whey. GMP: 10 μ g of pure GMP. LCW: 10 of liquid cheese whey. Rabbit polyclonal anti-GMP antibody was used 1:1000.

observed in pure GMP samples, the original immunogen. Bands correspond to GMP in different aggregation state. No bands appear in samples of non-adulterated milk, indicating no cross-reaction with other milk components.

Limit of detection and duration of the assay

Western blot with anti-GMP antibody detected 0.1 μ g of pure GMP (Figure 2A) and a concentration of up to 0.001% of pure GMP (w/v) added to milk (Figure 2B). When GMP was added to milk and later samples were processed to remove κ -casein, there was no modification in the visualised bands to GMP (Figure 2B). Band of 20.1 kDa was always more intense than one of 14 kDa and when the concentration of GMP was reduced the last disappears.

To identify directly cheese whey in milk, samples of milk adulterated with liquid or dehydrated cheese whey were analysed (Figure 3). Incubation of the membranes with polyclonal anti-GMP antibody showed bands of 45, 20.1 and 14 kDa, corresponding to GMP identification in whey. GMP aggregates of 20.1 kDa were more abundant than those of 45 and 14 kDa. When whey concentration was low we visualised only GMP forming polymers of 20.1 kDa of molecular weight. Non-adulterated milk and pure GMP were used as negative and positive controls, respectively. The lowest limit of cheese whey detected in milk was of up to 0.5% of liquid (Figure 3A) and of up to 0.001% of dehydrated (Figure 3B) cheese whey.

The procedure is 4.5 hours long, including TCA treatment of the milk.

Detection of GMP in commercial foods

Polyclonal anti-GMP antibodies were used to identify by immuno-blot analysis GMP in commercial foods with whey (Figure 4). In margarine three bands of molecular weights of 45, 20.1 and 14 kDa were identified. These bands were the same as those obtained when whey was analysed. In dietary supplement and yogurt, only GMP aggregates of 20.1 kDa were mainly detected because of the lower whey amount.

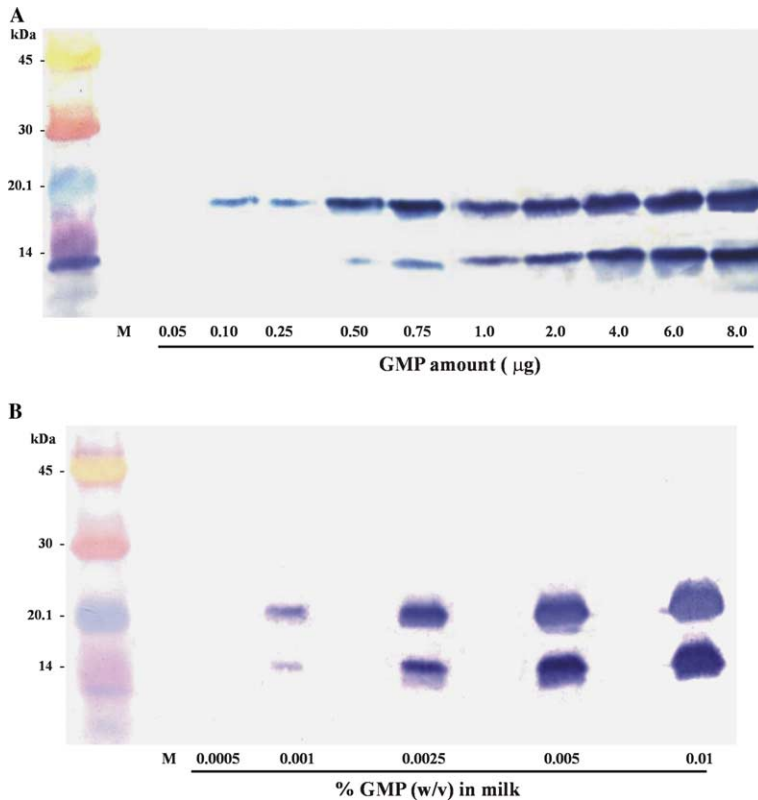


Figure 2. Immuno-blot analysis of GMP using purified polyclonal anti-GMP antibody. (A) Increasing amounts of pure GMP; (B) milk with increasing concentrations of GMP; antibody was used 1:1000; M: 10 µl of processed milk without whey (negative control).

Discussion

In the present study, we developed an easy, rapid, sensitive and specific method to detect GMP as an index of milk adulteration with cheese whey. It was an immuno-blot assay which successfully detected GMP in adulterated milk with liquid or dehydrated cheese whey. This method was developed using specific polyclonal anti-GMP antibodies which recognised two bands of 20.1 and 14 kDa for pure GMP. When cheese whey was analysed, one more band of 45 kDa of molecular weight was visualised. GMP as a monomer has a molecular weight of 6.8 kDa, without considering the glycoside portion (Brody, 2000; Galindo et al., 2006). However, it was also demonstrated that this peptide forms aggregates, originating dimers, trimers, tetramers, etc (Galindo et al., 2006). This fact explains the identification of GMP with bands of different molecular weights. The mechanism of aggregation is not known, nor is it clear why the GMP is not dissociated itself in the presence of SDS. It has been proposed that SDS joins to the peptide portion of the GMP molecule, but the free glycosylated chains from different molecules of GMP interact by hydrogen bridges, resulting GMP polymers with higher molecular weights (Galindo et al., 2006). In our work GMP aggregates of 20.1 kDa are more abundant than those of 14 or 45 kDa, in pure GMP as well as in whey.

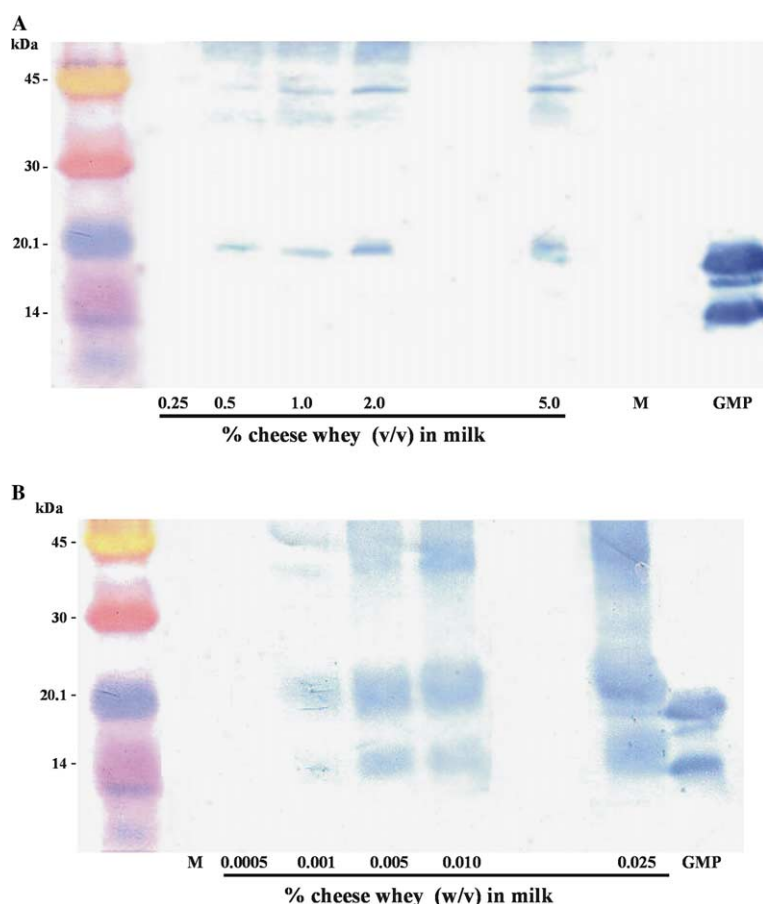


Figure 3. Immuno-blot analysis of milk samples adulterated with increasing concentrations of liquid (A) or dehydrated (B) cheese whey, using purified polyclonal anti-GMP antibody diluted 1:1000; M: 10 µl of processed milk without whey (negative control); GMP: 10 µg of pure GMP (positive control).

The development of a Western blot method using polyclonal antibodies instead of monoclonal antibodies, is justified since different food processing techniques can affect their proteins or peptides, for example, by destroying specific epitopes. By using polyclonal antibodies, the probability of detecting peptides or proteins which are altered or denaturalised is higher, as the antibodies recognise several different epitopes. It is accepted that the use of polyclonal antibodies is favourable for food analysis (Holden, Fæste, & Egaas, 2005).

The specificity of the anti-GMP antibody was tested in non-adulterated milk, which was treated with TCA to eliminate k-casein. GMP derives from k-casein, so it is necessary to work with milk devoid of k-casein to avoid cross-reactivity. There was no positive reaction of the anti-GMP polyclonal antibody with milk samples, indicating that GMP does not cross-react with other milk components, thus there is no possibility of positive false results. This fact increases the veracity of the test to determine adulteration of milk with cheese whey.

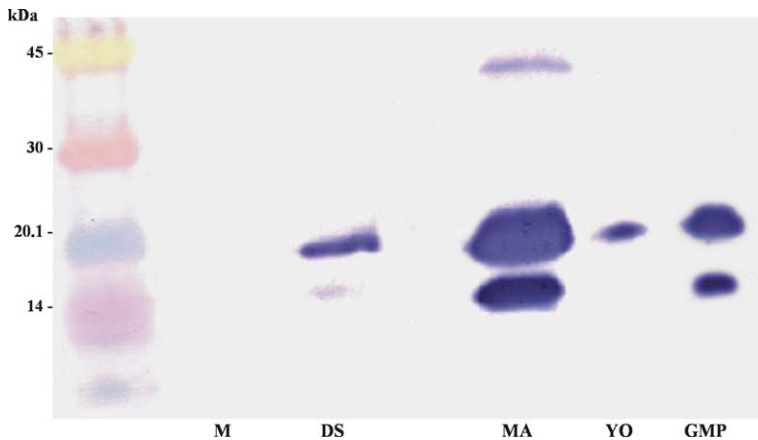


Figure 4. Immuno-blot analysis of different commercial foods with cheese whey using purified polyclonal anti-GMP antibody diluted 1:1000. M: 10 μ l of processed milk without whey (negative control); DS: 10 μ l of processed dietary supplement; MA: 5 μ l of processed margarine; YO: 10 μ l of processed yogurt; GMP: 10 μ g of pure GMP (positive control).

The sensitivity of the Western blot immunoassay to detect whey was higher than reported with other methods. By reverse-phase HPLC 0.8% (w/v) dehydrated whey (Olieman & Van Riel, 1989) was detected while we obtained a detection limit of 0.001% (w/v) of dehydrated whey. Using HPLC, 1% (v/v) of liquid whey in milk was identified (Galindo et al., 2006). To perform this analysis it was necessary to purify previously GMP and this requires at least 6 hours. With the Western blot assay we detect up to 0.5% (v/v) of liquid whey in milk within 4.5 h. In addition, the use of HPLC equipment is more complex and expensive than the method developed by us. Therefore, immuno-blot to detect GMP by polyclonal anti-GMP antibody proved to be an easier, faster, more sensitive and less expensive method.

Another advantage of the immuno-blot method is its specificity to detect GMP, while in other methods such as HPLC and SDS-PAGE the exact identification of GMP is not assured, because of peptides of the same molecular weight as GMP may be present in milk by action of psychrophile microorganisms (Alcázar et al., 2000). This approach can produce false positive results in the adulterated milk analysis by HPLC or SDS-PAGE.

Western blot analysis successfully detects GMP in commercial foods containing whey, as dietary supplement, yogurt and margarine. Actually, GMP is used as a nutraceutical food by its nutritional and biological properties such as: bifid bacteria stimulating factor, source of sialic acid, antiviral activity and modulator of gastric secretion (Brody, 2000). Digestions of GMP give rise to other bioactive peptides that possess anti-thrombotic activity (Chabance et al., 1995). At present, newcomer foods contain GMP, so Western blot developed in our work could be used to identify the presence of this peptide in a quality control.

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