Original paper

ELECTROPHORETIC DISTINCTION OF THE ORIGIN IN DIFFERENT DAIRY PRODUCTS AND MILK SAMPLES

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Abstract

Caseins, lacto-albumin, and lacto-globulin are major milk proteins. These globular proteins could be significant indicators of the milk and dairy products origin. Knowing that caseins, lacto-albumins and lacto-globulins vary in molecular weight and concentration in different types of milk, this fluctuation can be used for determination of milk origin. The aim of this study was to develop an appropriate method for distinction of milk proteins from different origin. Twelve samples of milk, white cheese, yellow cheese and whey cheese from cow, sheep and goat were obtained and studied. The protein separation was made using SDS-PAGE. SDS is an anionic detergent that breaks all inter and intramolecular bonds and leaves the polypeptide subunits of proteins in forms that can be separated on the basis of their molecular weight. Polyacrylamide gels, used as support medium, restrain larger molecules from migrating as fast as smaller molecules. In order to optimize the conditions of the experiment, some of the parameters were modified (polyacrilamide concentration from 10-15% according to the molecules size, duration of electrophoresis, quantity of applied material, sonification treatment of the different samples). Bovine milk proteins standards were used for the determination of the proteins. The results have shown differences, as well as other fractions that can be used for identification of the origin. In yellow and white cheese the differences among the samples from different origins appear in lacto-albumin fractions and some digested fractions below the caseins. The main differences in whey cheese samples were identified in casein fractions. The milk samples showed differences in upper fractions, probably serum albumins that remained in the milk samples.

Key words: *dairy products, milk proteins, origin, SDS-PAGE*

Introduction

The detection of milk species is important in cheese producing branch, especially in those made from one pure species and with protected designation of origin (PDO) (Bottero at al., 2002). Different analytical approaches have been applied for identification purposes, such as: immunological, chromatographic, PCR mass spectrometry and electrophoresis (Zachar at al.,

2011). Usually, capillary electrophoresis and isoelectric focusing were used as reference methods according to the Commission Regulation EC No 273/2008). On the other hand, zonal electrophoresis could be a good choice because of the low expenses and availability of the technique for wider range of laboratories, especially for internal control. Furthermore, the results from SDS PAGE can serve as a basis for 2D electrophoresis and eventual development of antibodies for ELISA testing of adulteration.

Electrophoresis is a commonly used technique in many scientific fields, where charged molecules are separated by using an electrical field. Molecules have different migration rates depending on their total charge, size and conformation. The separation of particles during electrophoresis depends on the following factors: the sample, the electric field, medium and buffer (Palashevski and al., 2001).

The mobility or rate of migration, of a molecule increases by increased applied voltage and increased net molecule charge. Conversely, the mobility of a molecule decreases with increased molecular friction, or resistance to flow through the viscous medium, caused by molecular size and shape, total actual movement of the molecules increases with increased time (AES, 2003).

Most electrophoretic systems use an equal and constant voltage on all of the cross-sectional areas of different matrices employed in the electrophoretic separation. These electric fields are best defined in terms of volts per linear centimeter. However, according to the Ohm's law voltage is function of current and resistance. The resistance of the system is important because it will determine the amount of heat generated during electrophoresis. The "smiling" pattern often seen on slab gel electrophoresis is the result of non-uniform heating of the gel ((Bottero at al., 2002).

Because many biological compounds have charges and ionisable groups, electrophoresis is frequently utilized in biochemical research, as a tool for separation of the complex biological molecules such as proteins, nucleic acids, peptides (Gersten, 1996).

For separation of proteins, polyacrylamide electrophoresis (PAGE) is commonly used. Sodium dodecyl sulfate (SDS) - an anionic detergent is applied to protein sample to linearize proteins and to impart a negative charge to linearized proteins, and proteins are separated only by length of their polypeptide chains (Palashevski at al., 2001).

Gels are formed as acrylamide monomer polymerizes into long chains that are linked together by big molecules (N,N'-methylenebisacrylamide). In order that polymerization can start, ammonium persulfate (APS) and N,N,N',N'- tetramethylethylenediamine (TEMED) are added to form the free radicals. TEMED accelerates the decomposition of persulfate molecules into sulfate free radicals and these, in turn, initiate the polymerization. This study reports optimization of the conditions for identification of the origin of milk and dairy products with SDS-PAGE (AES, 2003).

Milk is a complex biological emulsion produced by the mammals whose composition makes it an important source of nutrients and a protector of the immune system. The major milk components are water, lactose, fats, proteins, minerals, vitamins, etc. (Barlowska at al., 2007). The total protein content of milk is composed of different specific proteins. The primary group of milk proteins are the caseins (distinct molecules but similar in structure) which are highly digestible in the intestine and represent high quality source of amino acids (Jensen, 1995). All other proteins found in milk are grouped together under the name of whey molecules. The

major whey proteins are β-lactoglobulin and α-lactoalbumin. Other whey proteins are the immunoglobulins and serum albumin (Hurley at al., 2010).

Authentication of milk and milk derived products is important because fraudulent incorporation of non-declared kind of milk during technological processing can later cause problems for reasons related to intolerance or allergy, ethical objections and legal requirements (Zuchar at al., 2011).

Materials and methods

As an initial material we used cow, sheep and goat milk, and derived whey cheese, white cheese and yellow cheese. Bovine α-casein, β casein, κ-casein and α-lactoalbumin were used as standards.

Sample preparation. The samples were prepared using 200 mg of each dairy product separately, immersed in 0.4 mL of distilled water. Then, the samples were homogenized using vortex for 10 min at 2500 min^{-1}. Afterwards, the samples were treated with ultrasonic homogenizer 3 minutes at 4 watts, and finally centrifuged for 15 minutes at 5000g. The separated supernatant in amount of 100 µL was further mixed with 200 µL reducing buffer 2x. Then these mixtures were heated 3 min, at 99°C, for denaturing of the proteins. The milk was prepared using 10 µL 2x reducing buffer which is added to 10 µL milk. Then the mixture was heated for 3 min, at 99°C and the samples stored in a freezer.

Gel preparation. The gel used for electrophoresis was divided into an upper stacking gel with lower concentration and lower resolving gel with smaller pores. The stacking gel has a role to deposit the proteins at the top of the resolving gel as a narrow band. In the electrophoretic set the two gels can be cast. For preparation of two resolving gels, the needed reagents are listed in Table 1 (Macdonald, 2011).

For preparation of 3% stacking gel we used 4.8 mL dH_2O, 1.8 mL 4X Upper TRIS, 0.9 mL protogel, 15 µL TEMED, and 60 µL APS, and for preparation of SDS- PAGE reagents, the needed components are given in Table 2 (Jensen, 1995).

The electrophoresis was done under the following conditions: voltage of 100 -150 V, duration of 1h – 2 h, staining of 1.5 hour, and overnight distaining. The analysis of the gels was done with Gene Tools software using G-box device.

Table 1. *SDS-PAGE Gel Formulae for resolving gel*

Reagents(mL)	12.5% gel	15% gel
dH_2O	6.6	4.8
4X Lower TRIS	5.1	5.1
Protogel	8.7	10
TEMED	30µL	30µL
10% APS	80µL	80µL

Table 2. *SDS-PAGE reagents*

4x Lower TRIS pH 8.8		**4x Upper TRIS pH 6.8**		**Running buffer(10X)**	
Tris BASE	18.17 g	Tris BASE	6.06 g	Tris BASE	60 g
10% SDS	4.0 mL	10% SDS	4.0 mL	Glycine	288 g
q.s. with H_2O to	100 mL	q.s. with H_2O to	100 mL	SDS (add last)	20 g
pH to 8.8 before adding SDS		pH to 6.8 before adding SDS		q.s. with H_2O to	2.0 L
Reducing buffer (2X)		**Coomassie Brilliant Blue R-250 gel stain**			
10% SDS	3.0 mL	Coomassie Brilliant Blue R-250			400 mg
Upper Tris(4x)	1.25 mL	Methanol			400 mL
b-mercaptoetanol	500µ L	Glacial acetic acid			400 mL
Glycerol	1.0 mL	q.s. with dH2O to 1L			
Bromphenol Blue	Pinch	**Destain**	**Protogel**		
q.s. with H2O to	10.0 mL	10% acetic acid	30% acrylamide	0.8% bis	

Results and discussion

The analysis that can be used for identification of the milk and dairy products origin is based on the electrophoretic determination of protein profile. Milk, white cheese, yellow cheese and whey cheese samples from cow, sheep and goat were studied in order to determine the optimal conditions and factors that affect this method and to prove the distinction between different samples.

During the experiment, some of the parameters were adjusted, in order to acquire proper conditions for good resolution of protein fractions from milk and dairy products samples. The protein standards fractions together with milk samples are shown in Figure 1, where the electrophoresis lasted 50 minutes on 150 V, 1.5 hour staining and overnight distaining.

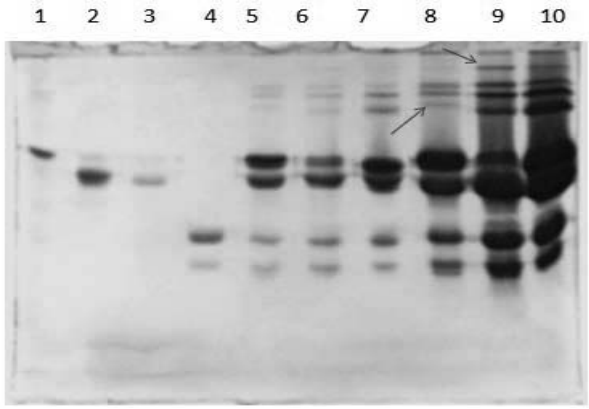

Figure 1. *12.5% SDS-PAGE: 1. α-casein, 2. β-casein, 3. κ-casein, 4. α-lactoalbumin, 5. Sheep milk, 6. Cow milk, 7. Goat milk, 8. Sheep milk (5x), 9.Cow milk (5x), 10. Goat milk (5x)*

The current technique related to control of the milk origin is isoelectric focusing of g caseins after plasminolysis knowing that whey proteins are thermally unstable, especially during the processing and cheese producing (10). In order to avoid complicate preparation procedures, we tried to distinguish different milk and diary product samples using common homogenizing approach.

The casein fractions are well resoluted in the samples from lanes 5, 6 and 7 and applied in the amount of 1.5 μL. In the lanes 8, 9 and 10, because of a higher quantity of the applied material (10 μL), the separation of casein fractions is not very clear. However, good separation is acquired in the upper fractions what was the intention when applying 10 μL. There is a possibility that these upper fractions are the remains of serum albumins in milk. Furthermore, these fractions distinguish from one another in band that is present in the milk from sheep, but not in the other milk samples, and it is with higher molecular weight. There is also another specific band that appears only in the cow milk sample. Every lane on the gel ends nearly at the half of the gel length, so it can be concluded that the duration of the electrophoresis was short in this case and should be extended.

In order to obtain better protein profile, the concentration of the gel was increased up to 15% (Figure 2), and the conditions were also modified: 130V for 1hour and 50 minutes, 1.5 h staining and overnight distaining.

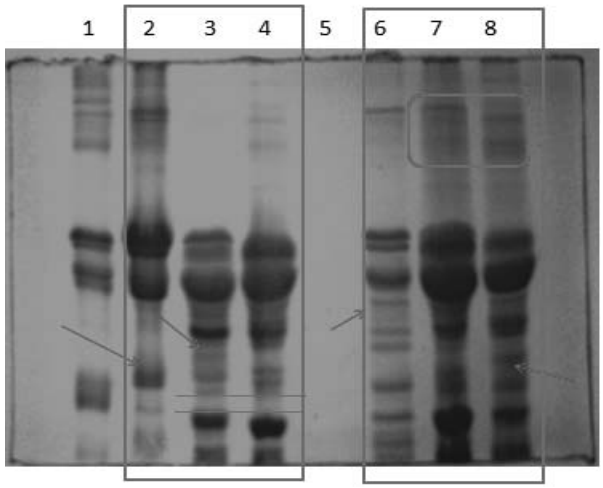

Figure 2. *15% SDS-PAGE: 1. Milk standards-mix, 2. Cow yellow cheese, 3. Goat yellow cheese, 4. Sheep yellow cheese, 5./6. Cow white cheese, 7. Goat white cheese, 8. Sheep white cheese*

Because of the increased concentration of the acrylamide in the gel and lower voltage applied, more protein fractions are visible. Differences among the yellow cheese samples are identified in lacto-albumin fractions. Every lacto-albumin fraction from lanes 2, 3 and 4 differs in the rate of migration, which means that they all have different molecular weight. There is another fraction that appears only in the yellow cheese sample from goat and it is located under the casein fractions. As for the white cheese, the distinction can be made by some digested fractions below the caseins. There is a band located under the casein fraction, present only in

the sheep white cheese sample, and another band from the cow white cheese sample, also specific only for this kind of white cheese.

Figure 3 shows also 15% SDS PAGE, where the differences among protein fractions from yellow cheese are even more visible. The electrophoresis was run 2 hours on 100V.

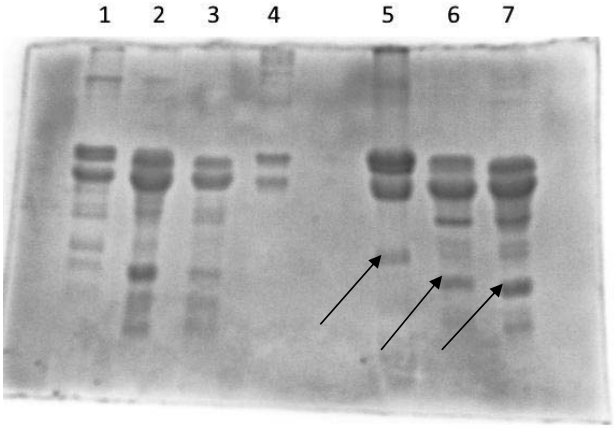

Figure 3. *15% SDS-PAGE: 1. Cow white cheese, 2. Goat white cheese, 3. Sheep white cheese, 4. Milk standards - mix, 5. Cow yellow cheese, 6. Goat yellow cheese, 7. Sheep yellow cheese*

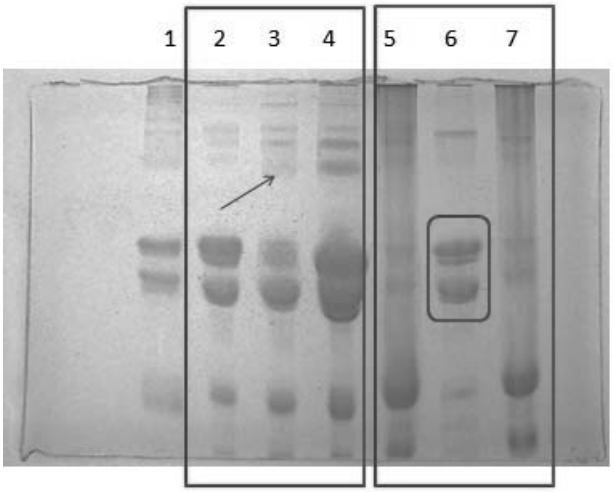

Figure 4. *15% SDS-PAGE: 1. Milk standard - mix, 2. Cow milk, 3. Goat milk, 4. Sheep milk, 5. Cow whey cheese, 6. Goat whey cheese, 7. Sheep whey cheese*

Differences among the yellow cheese samples are more visible in Figure 3. Three different bands, each in one sample can be clearly noticed (marked with arrows in the figure).

Moreover, below the casein fractions of cow yellow cheese sample a band does not appear, unlike the other two samples in lanes 6 and 7.

The whey cheese samples were analyzed (Figure 4) using less quantity on 15% gel, under the following conditions: 130V, 1 hour and 50 min.

Figure 4 again shows the whey cheese samples. The same differences can be identified as in the first figure, only here, because of the lower amount applied, they are more visible. The samples in lanes 5 and 7 appear to be smeared, probably due to a lipoprotein complex that was not disrupted during the homogenization. With another analysis of the milk samples, the differences in the upper fractions were confirmed.

Conclusion

Using SDS PAGE as a technique for distinguishing the origin in different dairy products and milk samples is suitable. Best results are obtained with 15% concentration polyacrylamide gel. Longer duration of electrophoresis (that comes with lower applied voltage) aids in better resolution. The samples should not undergo centrifugation, neither filtration, because some of the proteins stay in the precipitate.

This approach allows its use as an internal checking of milk and dairy products and can eventually result in finding some specific protein fraction in different samples which can be used for development of appropriate antibodies for ELISA testing.

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