Optimisation of Indirect Competitive ELISAs of α-, β-, and κ-caseins for the Recognition of Thermal and Proteolytic Treatment of Milk and Milk Products

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Abstract

VÍTKOVÁ M., RAUCH P., FUKAL L. (2002): **Optimisation of indirect competitive ELISAs of** α-, β-, **and** κ-caseins **for the recognition of thermal and proteolytic treatment of milk and milk products**. Czech J. Food Sci., **20**: 53–62.

Polyclonal antibodies were raised against six immunogens (three native and three thermally treated casein fractions: α+β-casein, κ-casein and whole casein). Using these antibodies the procedures of an indirect competitive enzyme immunoassay (ELISA) were constructed, optimised and characterised for determination of each immunogen. It was found that ELISA of caseins is very specific without any interferences of whey proteins and with proportionally low cross-interactions between caseins. Detection limits for α-, β-, and κ-caseins and whole casein were 110, 49, 58 and 505 ng/ml, respectively. The coefficient of variation was lower than 12% in intra-assay and lower than 9% in inter-assay. The developed ELISA format was used to study changes in casein immunoreactivity during heat-treatment and proteolytic hydrolysis. Heating below 100°C did not change the milk immunoreactivity but heating above 100°C caused its significant changes. Depending on type of proteolytic treatment (with enzyme preparation Pancreatin or with microbial cultures of *Lactobacillus helveticus* and *Lactococcus*) a decrease and an increase in casein immunoreactivity were observed. While Pancreatin reduced the casein immunoreactivity substantially (5–1000 times in dependence on the casein type), the more gentle proteolysis by bacteria caused not only its reduction (even 100 times at κ-casein) but also its increase (1.5 times at α-casein).

Keywords: α-casein; β-casein; κ-casein; casein hydrolysates; antibody; immunoassay; ELISA

The quality attributes of dairy products, such as texture, sensory characteristics and flavour, are strongly influenced by the milk protein fraction. Thermal and proteolytic modification of milk proteins can have both positive and negative effects on the quality of processed milks and dairy products. Milk protein fraction consists of two phases, colloidal aggregates known as casein micelles (consisting of α-, β-, and κ-caseins), which are surrounded by more soluble, essentially globular whey proteins. The caseins have comparatively few secondary and tertiary structures, but on heating they undergo changes in the way they associate within the micelles and with denatured whey proteins (CREAMER & MATHESON 1980; NOH & RICHARDSON 1989). On heating milk above 60°C the whey proteins increasingly lose their globular confor-mation, become associated with casein micelles through disulphide or hydrophobic interactions, and precipitate together with the caseins on acidification to pH 4.6 (HILL 1989; DALGLEISH 1990; CORREDIG & DALGLEISH 1999). Kappa-casein plays a key role in stabilising the micelles, cleavage of which allows them to coagulate (DALGLEISH *et al*. 1989).

Thermal modification of milk proteins occurs during various heat treatments (that are employed primarily for milk preservation). The level of this type of casein structure modification can influence the quality and shelf-life of UHT-milks (PICARD *et al*. 1996); it is used to classify milk powders as the degree of denaturation that occurs on drying and affects performance in dairy processes such as cheese-making (MARSHALL 1986; CALVO *et al*. 1995);

it is important for efficient reduction of antigenicity in the manufacture of hypoallergenic milk baby foods (LEARY 1992). Efficient control of thermal processing is important because of prevention of over-heating that may adversely affect milk proteins.

Proteolytic modification results from the action of endogenous milk proteases, proteases secreted by contaminating bacteria or by addition of lactic bacteria or proteases during processing. This type of modification of caseins can impair the shelf-life of UHT-milk (LOPEZ-FANDINO *et al.* 1993); it can lead to the accumulation of bitter flavour peptides in cheese (CROW *et al.* 1995) and milk protein hydrolysates (PEDERSEN 1994); it must be optimised to mimic the *in vivo* digestion of milk proteins in the manufacture of value-added hypoallergenic milk products (SIEMENSMA *et al.* 1993); it is crucial for development of desired texture and flavour during cheese ripening and yoghurt fermentation (VISSER 1993).

In view of the impact that heat and proteolytically induced changes of caseins have on the final quality of processed milks and dairy products, there is a need to understand the nature of these changes at a molecular level, and to develop effective means of monitoring and controlling these changes during production. Because of the complexities of working with a food system a great deal of research on the biochemical effects of thermal denaturation and proteolytic degradation of milk proteins has been performed using model systems. To date researchers have extensively used electrophoretic (ADDEO *et al.* 1995; CARRETERO *et al.* 1994; GUZMAN-GONZALES *et al.* 2000;) and chromatographic procedures (GROEN *et al.* 1994; BOBE *et al.* 1998; BORDIN *et al.* 2001) to quantify caseins and their peptide parts, in addition to investigating proteolytic degradation of these proteins in dairy products or determining variations in milk protein composition. Some authors also tried to use these techniques to detecting heat-induced changes in caseins (LAW *et al.* 1994; HURLEY *et al.* 1993). However, such methodology cannot give much useful information on changes in protein conformation caused by thermal processing, it requires a high degree of technical expertise and expensive equipment and can be time consuming to perform. These factors make these existing methodologies unsuitable for use in quality assurance testing at the factory level.

An obvious alternative technique, gaining in popularity for quality assurance in the food analysis, is immunoassay (FUKAL 1995). It has many advantages in terms of speed, ease of use, it requires little equipment, does not involve handling toxic chemicals, can be formatted either for high sample throughput or single analyses and is increasingly receiving industrial and regulatory approval. Antibodies also represent a much simpler and cheaper alternative to spectroscopic methods for analysing conformational changes in proteins that are important for predicting their functional quality. Although immunoassays,

particularly ELISAs, have been developed for the analysis of milk and milk products with a view to monitoring adulteration with cows' milk, the techniques using antibodies against caseins (RODRÍGUEZ *et al.* 1993; ANGUITA *et al.* 1995, 1997; RICHTER *et al.* 1997), against short peptide structure from α-casein (ROLLAND *et al.* 1993) or against caseinomacropeptide from κ-casein (PICARD *et al.* 1994; BITRI *et al.* 1993), identification and quantification of caseins after chromatographic (ANGUITA *et al.* 1996) or electrophoretic separation (immunoblot) (HURLEY *et al.* 1993; ADDEO *et al.* 1995) have not been focused on the issues of quality. Recently, we have investigated the use of anti-whey protein immunoprobes for this purpose. Antibodies have been produced that can detect thermal changes in α-lactalbumin and β-lactoglobulin (KARASOVÁ *et al.* 1999; ČURDA *et al.* 1997), thus demonstrating the feasibility of applying antibody methods to the analyses of thermally and proteolytically induced changes in dairy proteins. In addition these methods can be used to investigate foods and not just model systems.

In this report, using polyclonal rabbit antibodies against casein and casein fractions we optimised and used ELISAs to determine changes in the immunoreactivity of caseins after thermal or proteolytic treatment of milk and casein concentrates.

MATERIAL AND METHODS

Material

Standards of α-, β-, and κ-caseins, and *o*-phenylenediamine dihydrochloride (OPD) were from Sigma Chemical Company (St. Louis, USA). Tween 20 and gelatin were obtained from Fluka Chemie AG (Switzerland). The "second antibody" labelled with enzyme (the conjugate of swine anti-rabbit IgG and peroxidase (SwAR/Px)) was from SEVAPHARMA (Prague, Czech Republic). Seven types of microtitration plates were purchased from these producers: Costar 9018 (Corning Costar, USA), Clini and Clini-EB (Labsystem OY, Finland), Maxisorp and Polysorp (Nunc A/S, Denmark), Gama (Gama, Czech Republic), Fondo celleta (Kartell, Italy).

Isolation of Caseins for Immunisation

The unheated and pasteurised milk (72°C, 45 s) were acidified to pH 4.6 using 1N HCl. The insoluble casein fractions were separated by centrifugation (5000 G, 20 min).

The precipitated casein fractions were suspended in distilled water, solubilised by adjusting the pH value to 7.5 (1M NaOH) and re-precipitated by acidification using 1N HCl. This procedure was repeated 3 times (centrifugation 20 000 G, 5 min). The caseins in soluble form were dialysed against water and lyophilised. The solid caseins were dissolved in minimal volume of 6.6M urea, pH was adjusted to 1.3 with sulphuric acid. Precipitation of the

mixture of α- and β- caseins was achieved by diluting the concentrated urea solution to 2.2M. The κ-casein, in supernatant after centrifugation (20 000 G, 5 min), was concentrated by precipitation with ammonium sulphate (132 g per l), resuspended in distilled water and immediately centrifuged for 1 min. This procedure was repeated 5 times. The sediment was dissolved in 3.3M urea (0.01M imidazole, 6mM 2-mercaptoethanol, pH 7.5) and applied directly to MQ column. Elution was performed using a linear gradient of NaCl. Fractions containing κ-casein were collected, dialysed against distilled water and lyophilised.

The mixture of precipitated α- and β-caseins, after removal of the essential amount of κ-casein, was suspended in 3.3M urea (0.01M imidazole, 6mM 2-mercaptoethanol, pH 7.5) and pH was adjusted to 7.5 (1M NaOH). Dissolved α- and β-caseins were separated from a small amount of other proteins by using a linear gradient of NaCl on MQ column. Fractions containing α- and β-caseins were collected, dialysed and lyophilised.

The purity of the isolated caseins was controlled by SDS-PAGE electrophoresis in 15% gels.

Production of Rabbit Polyclonal Antisera against Native and Pasteurised Caseins

Native casein fractions (α- and β-casein fraction, κ-casein, whole casein) and the same proteins but pasteurised were used as immunogens. Polyclonal antisera were raised against each of these proteins in New Zealand White rabbits using the following procedure:

Solution (200 µl) of 250 µg of protein in PBS-buffer was emulsified in 200 µl of complete Freund's adjuvant. This emulsion was administered to the animal subcutaneously in the rear legs. After 21 days a booster injection was given in the same way, but 250 µg of protein was emulsified in adjuvant containing aluminium hydroxide (160 µl of PBS-buffer, 160 µl of Al-span Oil I and 480 µl of Al-Span Oil II). This injection was repeated twice at intervals of 21 days. On day 90, the final injection was given with 500 µg of protein emulsified as described for the booster injections.

Seven days after the final injection the animals were bled. The IgG fraction from each antiserum was isolated by affinity chromatography on protein A (Prosep A column). Lactose was added as a cryoprotective agent at a ratio 1:1 (w:w) and fractions were lyophilised.

Indirect Competitive ELISA Procedure

The main steps of this procedure are shown in Fig. 1. The polystyrene microplates were filled with caseins diluted in carbonate-bicarbonate buffer, pH 9.6 (0.1 ml/well) and incubated overnight at 4°C for absorption. The coated plates were washed four times with 0.01M PBS containing 0.05% Tween 20 (PBS-Tw) to remove unbound antigens. Aliquots (50 µl) of the antigen solution in PBS-Tw containing 1% gelatin (casein standard solution in the range 0.5 ng–500 µg per ml, or diluted milk or casein samples) and 50 µl of antibody solution in the same buffer were added to the wells.

After incubation for 1 hour at 37°C and washing four times with PBS-Tw, the conjugate of "second antibody" (swine anti-rabbit IgG) with horseradish peroxidase diluted 1:2000 in PBS-Tw containing 1% gelatin, was added (100 µl). Plates were incubated for 1 h at 37°C and washed four times with PBS-Tw. 100 µl of solution of peroxidase substrate and chromogenic compound (50 mg of *o*-phenylenediamine in 100 ml of 0.1M citrate/phosphate buffer pH 5.0 containing 0.03% hydrogen peroxide) were added into each well. After incubation at 37°C for 15 min the enzyme reaction was stopped by addition of 50 µl of 2M H_2SO_4, and absorbance was measured at 492 nm by a Lab-system Multiscan MCC/340. The absorbance was inversely proportional to the amount of antigen in solution.

Standard Curve and Calculation of Results

Calibration sigmoidal curves were fitted using the computer programme Microsoft Excel with a four-parameter regression function:

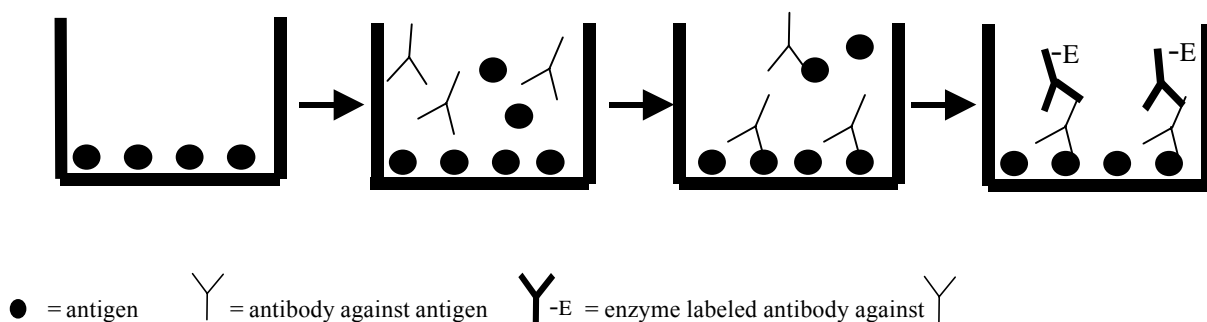$$A = C + \frac{D - C}{1 + \exp(-2 \times (\alpha + \beta \times x))} \tag{1}$$

= antigen   = antibody against antigen   -E = enzyme labeled antibody against

Fig 1. Scheme of indirect competitive ELISA

where: C = level of bottom asymptote

D = level of top asymptote

a = curve characteristic of the position of linear non-asymptote part from the view of antigen concentration coordinates

b = curve characteristic of the slope of the linear non-asymptote part

x = decadic logarithm of antigen concentration

A = absorbance at 492 nm

The sigmoidal curve was constructed not only for casein standards but also for all samples tested for immunoreactivity. Various dilutions of samples in the wide range were used in ELISA for these purposes.

From the measured absorbances the corresponding $c_{50\%}$ was calculated using the equations:

$$\log(c_{50\%}) = \frac{\dfrac{\ln \dfrac{(D - A_{50\%})}{(A_{50\%} - C)}}{(-2)} - \alpha}{\beta} \qquad (2)$$

where: $A_{50\%} = \dfrac{(C + D)}{2}$

Cross-reactions were calculated as a percentage using the equation:

$$CR(\%) = \frac{(c_{50\%})_1}{(c_{50\%})_2} \times 100 \qquad (3)$$

where: $(c_{50\%})_1$ = concentration of corresponding immunogen at $A_{50\%}$

$(c_{50\%})_2$ = concentration of cross-reacting antigen at $A_{50\%}$

Immunoreactivity was calculated as a percentage in the same way:

$$IR(\%) = \frac{(c_{50\%})_S}{(c_{50\%})_T} \times 100 \qquad (4)$$

where: $(c_{50\%})_S$ = concentration of standard (reference) sample at $A_{50\%}$

$(c_{50\%})_T$ = concentration of treated sample or compared sample at $A_{50\%}$

The detection limit was calculated as the casein concentration corresponding to the absorbance $A_{85\%}$ calculated as

$$A_{85\%} = C + 0.85 \, (D - C) \qquad (5)$$

Testing of Sorptive Quality of Microtitration Plates

This procedure was carried out in the same way as ELISA described above but without the competition step. Seven types of plates were coated with casein solutions in the wide range of concentrations (4 ng–4 μg of casein in 1 ml). The specific antibody was added into coated wells and

after incubation and washing, the "second antibody" labelled with peroxidase was added, followed by the measurement of peroxidase activity immunochemically bound to the solid phase.

Heat Treatment of Milk

Fresh raw milk was heated at temperatures 60, 70, 80, 90, 100, 120 and 140°C (in water bath or in oil bath using the special pressure resistant tubes with cover) for holding time 1 min, immediately cooled and immunochemically analysed.

Microbial Hydrolysates

Two casein hydrolysates were produced by means of two microbial cultures (*Lactobacillus helveticus* 5 Viesby, Lactococcus II). *Lactobacillus helveticus* 5 Viesby was revitalised in sterile milk (110–121°C, 15 min), 24 hrs, 37°C. After that time 2% of culture was inoculated into pasteurised milk (95°C, 30 min), 24 hrs, 37°C. Final inoculation was 2% into pasteurised milk, 24 hrs and 37°C.

The lyophilised culture of Lactococcus II (mix) was revitalised by a similar procedure, but the temperature of incubation was 23°C.

Casein fraction was separated from pasteurised milk after 24 hours cultivation (pH of milk = 3.5) by centrifugation (5000 G, 20 min). The pellet containing caseins, separated from whey, was resuspended in water and refined by centrifugation (5000 G, 5 min). The final pellet was dissolved in water by adjusting pH to 7.2 (1M NaOH) and lyophilised.

Pancreatin Hydrolysates

The basic material for preparation was cow milk from which a part of whey proteins was removed by membraneless osmosis. The casein concentrate was enzymatically hydrolysed, and subsequently fortified and dried.

The casein concentrate was prepared as follows: the fat content of milk was reduced by separation on a dairy separator to 0.2–0.05% at 40°C. The milk was pasteurised at 72°C for 15 s and cooled down. It was let stand at the laboratory temperature for 2 h at least. A 7% solution of apple pectin in distilled water heated to 40°C was prepared and stirred to get a homogeneous solution. The solution was added to milk heated to 40°C at concentration 10%. This fortified milk was let stand at a laboratory temperature for about one hour and subsequently it was placed in static conditions at 8°C. The separation of the casein concentrate was finished within 8 to 18 hrs. The upper part, casein-free fraction, was separated from the sediment by carefully pouring away. The casein concentrate was diluted with distilled water to protein content 6% prior to hydrolysis and pasteurised at 65°C for 30 min.

The casein concentrate was hydrolysed by Pancreatin USP enzyme (Merck, Darmstadt, Germany) at concentration 0.025 g of enzyme per 1 g of protein in casein concen-

trate at 50°C; pH was controlled in the range 7.2–8.3. Finally, the enzyme was inactivated by heating at 80°C for 20 min after hydrolysis.

Four samples were prepared in this way: nonhydrolysed casein concentrate and casein concentrates hydrolysed for 1, 3, and 5 hours.

RESULTS AND DISCUSSION

The indirect competitive enzyme immunoassay (ELISA) has been optimised for α-, β-, and κ-casein and for whole casein. Sensitivity and specificity of this procedure are influenced by many factors, such are sorptive quality of microtitration plate, antigen concentration in a solution used for the coating of wells on microtitration plate, affinity and specificity of antibody and its concentration in the system.

Choice of the Type of Microtitration Plate

The sorptive quality of seven types of microtitration plates from different producers was tested. All plates enabled only uncovalent interactions with proteins. The solution of α-casein was used for coating in the concentration range of 4 ng/ml–4 μg/ml. Fig. 2 shows expressive

differences in sorption not only for plates from different producers but also for different types of plate from the same producer. A suitable plate chosen with regard to its price and sorptive quality was plate "COSTAR 9018".

Calibration Curves and Cross-reactivity

Suitable concentrations of antigen in a coating solution and corresponding antibody dilution were found for all mentioned types of caseins using checkboard titration (KARASOVÁ *et al.* 1999). These experiments were made using the described ELISA procedure but without competition with antigen in the solution. The measured values represented the level of top asymptote (letter D of equation 1). Useful combination of values was chosen from these experiments so that it accorded with absorbance value between 1.0–1.5.

A pair of concentration values that gave the best parameters of calibration curves was determined for each tested casein and corresponding antibody. The calibration curve that was obtained for κ-casein determination is represented in Fig. 3. Calibration curves of the other caseins are similar and their characteristics are listed in Table 1. The estimated coefficient of variation was lower than 12% in intra-assay and lower than 9% in inter-assay for the casein standard solution in buffer.
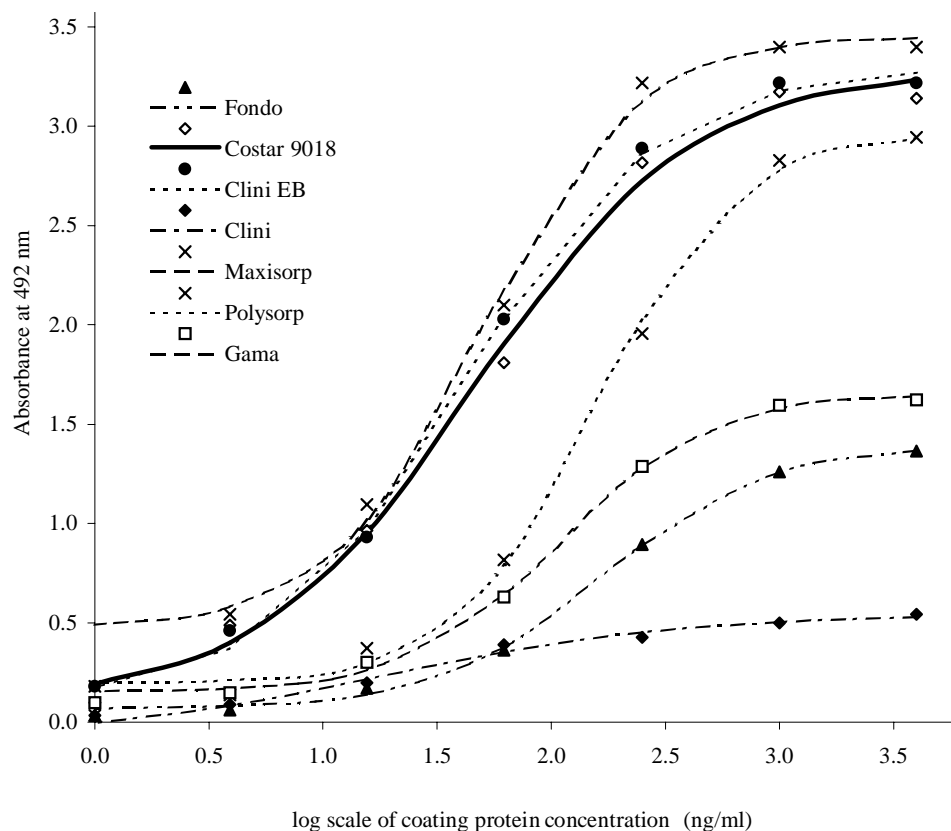

Fig. 2. The effect of the micro-titration plate type and α-casein concentration in a coating solution on the intensity of α-casein sorption to the microtitration plate wall. The antibody against α+β-caseins was diluted at 1:10 000

Table 1. Parameters of ELISA calibration curves for α-, β-, and κ-casein and whole casein using antibodies against α+β-caseins, against κ-casein and against whole casein. All antibodies were raised against unheated and pasteurised caseins. Coefficient of variation for $c_{50\%}$ and for detection limit is lower than 9%

Antibody against	Antigen for coating and competition	Coating concentration (μg/ml)	Antibody dilution	$c_{50\%}$ (ng/ml)	Detection limit (ng/ml)
α+β-casein	α	0.1	1:10 000	2 500	110
	β	0.1	1:10 000	420	49
α+β-casein pasteurised	α	4	1:5 000	6 350	1 080
	β	4	1:5 000	9 640	1 670
κ-casein	κ	1	1:10 000	560	58
κ-casein pasteurised	κ	1	1:5 000	800	67
Whole casein	whole casein	1	1:10 000	4 300	505
Whole casein pasteurised	whole casein	4	1:2 500	4 550	240

The specificity of the raised polyclonal antibodies was checked by determining the possible cross-reactivity against the other casein fractions and whey proteins (Table 2).

As the values in Table 2 show, interactions with whey proteins can be considered as insignificant. Of course, there exists certain similarity in the structure of caseins, especially α- and κ-casein, which have about 20% cross-reactivity between each other. Cross-reactions of antibodies against α+β-caseins (pasteurised) with κ-casein were very strong. It is not easy to explain this fact. Perhaps, the rabbit used to raise antibodies could be sensitised with dietary casein in the past before our immunisation.
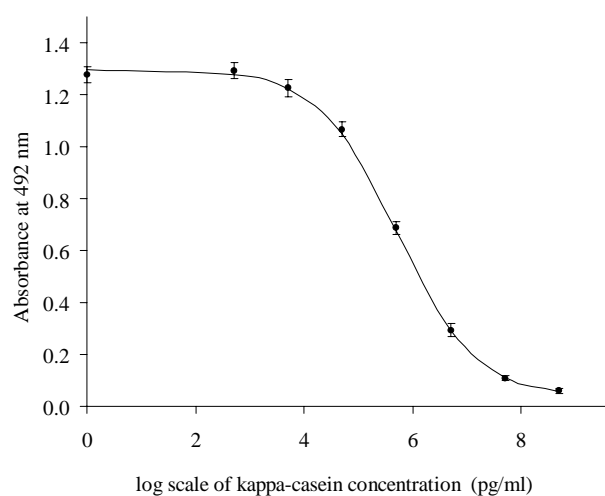

Fig. 3. Typical calibration curve of competitive ELISA for the determination of κ-casein. Points represent the mean values measured in 4 ELISA plates where 12 replicates were assessed for each κ-casein concentration

Immunoreactivity of Heat Treated Milk

The optimised competitive ELISA was used to study changes in the immunoreactivity of caseins in raw milk after heat treatment. At temperatures below 100°C no significant changes in casein immunoreactivity were established for any of the above-mentioned antibodies. The increase in temperature above 95°C causes not only the decrease in immunoreactivity (Figs. 4 and 5). The explanation of immunoreactivity increase is not easy. It is necessary to remember the complexity of the used system. We used polyclonal antibodies with a broad population of immunoglobulins that had different affinities to an undefined number of epitopes in casein molecules. The number of actually available epitopes is variable because during the heat treatment the inner molecule epitopes are manifested on the surface of the molecule. Conformational (in space formed) epitopes also change their shape and immunoreactivity. The change in measured immunoreactivity reflected the sum of all these structural changes in the casein molecule. The changes are not the same using antibodies against native caseins in comparison with antibodies against heat-treated caseins (compare Figs. 4 and 5). Moreover, the heat denaturation intensity of caseins is surely influenced by the duration of heat treatment. These facts prompt us to investigate the possibility of immunochemical distinguishing between pasteurised, sterilized and UHT milks in subsequent experiments. In comparison with electrophoretic and chromatographic methods, our ELISAs seem to be more suitable for damage detection in the casein molecule during heating. Using alkaline PAGE and ion-exchange FPLC, LAW *et al*. (1994) found only slight changes in caseins from milk heated for 1 and 5 min at temperatures 110–140°C. A decrease or an increase in the amount of protein in α-, β-, and κ-regions were consistent with changes in the charges of

Table 2. Cross-reactions (relative immunoreactivities) of antibodies against casein fractions with various proteins from cows' milk estimated by ELISA

Antigen in coating solution	Antibody against	Cross reaction (%) of protein						
		α-casein	β-casein	κ-casein	whole casein	LA	LG	BSA
α-casein	α+β-casein	100	1.9	22.2	45.5	<0.01	<0.01	<0.01
	α+β-casein pasteurised	100	5.6	115.0	80.5	<0.01	<0.01	<0.01
β-casein	α+β-casein	6.9	100	1.6	34.1	<0.01	<0.01	<0.01
	α+β-casein pasteurised	27.5	100	180	70.3	<0.01	<0.01	<0.01
κ-casein	κ-casein	19.4	4.4	100	21	<0.01	<0.01	<0.01
	κ-casein pasteurised	15.4	1.6	100	17.4	<0.01	<0.01	<0.01
Whole casein	whole casein	98.3	18.4	81.5	100	<0.01	<0.01	<0.01
	whole casein pasteurised	145.5	18.5	149.8	100	<0.01	<0.01	<0.01

LA = α-lactalbumin, LG = β-lactoglobulin, BSA = bovine serum albumin

amino acid residues for heating only, not for protein denaturation or proteolysis.

Immunoreactivity of Casein Hydrolysates

Protein hydrolysis is considered as one of the key chemical reactions in the dairy industry. It occurs in the preparation of cheese curd, cheese ripening, production of fermented milks, preparation of whey protein hydrolysates and casein, and during milk storage. The hydrolytic degradation of milk proteins is mostly a positive process occurring in technical procedures aimed at improving organoleptic properties, reducing the allergenic potential (SPUERGIN *et al.* 1997).

Hydrolysis of milk proteins can be accomplished by means of suitable proteolytic enzymes in suitable reac-
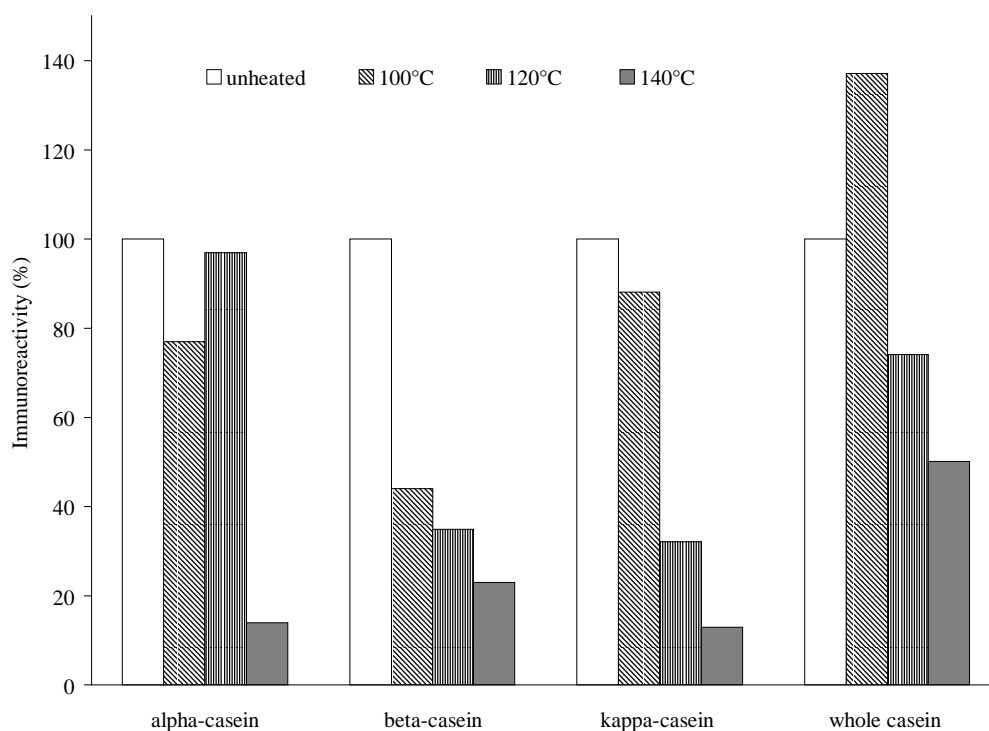

Fig. 4. Effect of heat treatment at different temperatures on the immunoreactivity of milk with antibodies against native caseins. (Type of casein described on the *x*-coordinate expresses the coating protein in ELISA; the specificity of used antibodies is corresponding with coating protein)
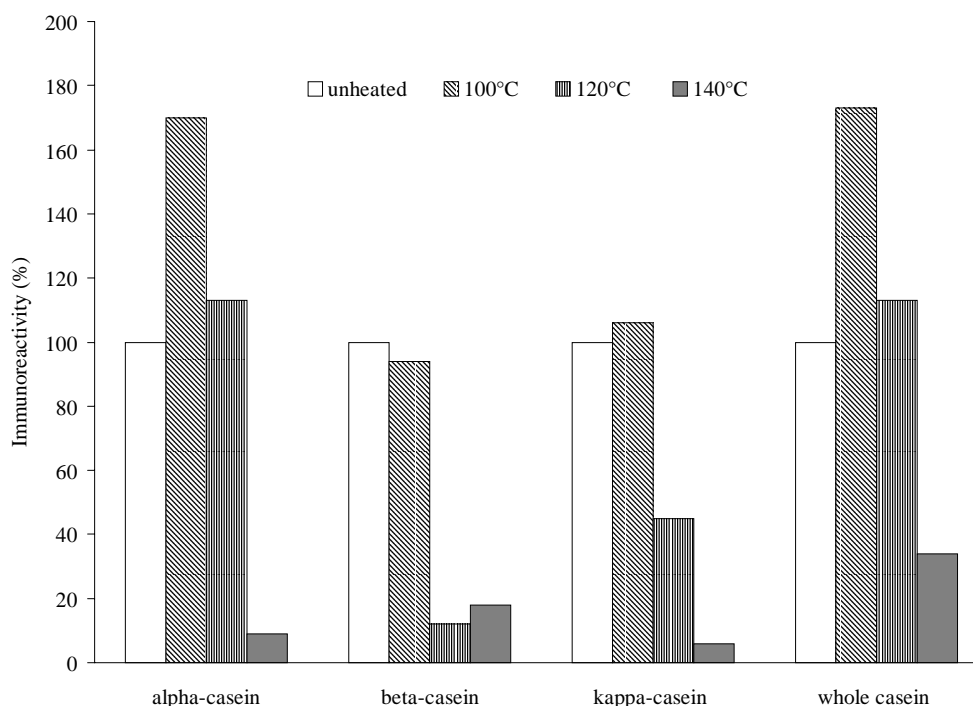
Fig. 5. Effect of heat treatment at different temperatures on the immunoreactivity of caseins with antibodies against heat-treated caseins. (Type of casein described on the *x*-coordinate expresses the coating protein in ELISA; the specificity of used antibodies is corresponding with coating protein)

tion conditions, or by application of acids and higher temperature. The enzymatic hydrolysis appears preferable for several reasons: it is inert to other components of milk, especially to lactose, it does not give rise to undesirable by-products of Maillard reactions, the course of the reaction can be better controlled. Milk proteins can be hydrolysed enzymatically by adding specific enzyme preparations or by using microbial cultures with higher proteolytic activity, or by combination of both.

Samples of two groups of milk products were prepared. The enzyme proteolytic preparation was used for hydrolysis in the first group (preparation of baby milk food with

reduced allergenic potential) while microbial cultures were used in the second one (fermented milk products).

Both samples were measured by indirect competitive ELISA to determinate casein hydrolysis. The levels of immunoreactivity listed in Table 3 show that pancreatin hydrolysed all caseins significantly, and the highest immunoreactivity decrease was observed in connection with κ-casein. Only κ-casein was degraded when we used microbial cultures (the decrease of immunoreactivity to 1.2%) while immunoreactivity increased for the other caseins. This fact can be explained easily: because this κ-casein is located on the surface of the casein micelle, determinant

Table 3. Relative immunoreactivities of casein hydrolysates estimated by ELISA

Casein hydrolysed by	Type of the analysed casein hydrolysate	Immunoreactivity value (%) of casein hydrolysate found by various couples of coating casein/and antibody (anti-casein)			
		α-casein/anti α+β-casein	β-casein/anti α+β-casein	κ-casein/anti κ-casein	whole casein/anti whole casein
Pancreatin	nonhydrolysed	100	100	100	100
	1h hydrolysis	17.6	15.7	1.2	27.8
	3h hydrolysis	5.7	1.7	0.2	23.1
	5h hydrolysis	3.4	0.5	< 0.01	17.9
Lactobacillus helveticus	nonhydrolysed	100	100	100	100
	hydrolysed	85.6	113.3	50.7	135.4
Lactococcus	nonhydrolysed	100	100	100	100
	hydrolysed	140.1	134.6	1.2	163.7

groups of other caseins are disclosed during its degradation.

It is a well-known fact that polyclonal antibodies compared with monoclonal ones are a better tool to evaluate the residual antigenic activity in hypoallergenic infant formulas (PLEBANI *et al.* 1997). We will use developed ELISAs for these purposes in subsequent experiments.

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Souhrn

VÍTKOVÁ M., RAUCH P., FUKAL L. (2002): **Optimalizace nepřímých kompetitivních ELISA metod stanovení α-, β- a κ-kaseinu ke sledování termického a proteolytického ošetření mléka a mléčných produktů**. Czech J. Food Sci., **20**: 53–62.

Byly připraveny polyklonální protilátky proti šesti imunogenům (třem nativním a třem termicky ošetřeným kaseinovým frakcím: α+β-kaseinu, κ-kaseinu a celkovému kaseinu). Za použití těchto protilátek byla sestavena, optimalizována a charakterizována metoda nepřímé kompetitivní enzymové imunoanalýzy (ELISA) pro stanovení všech uvedených kaseinů. Všechny sestavené ELISA metody byly velice specifické. Nebyly zjištěny žádné interference se syrovátkovými proteiny a prokázané křížové reakce mezi jednotlivými kaseiny byly přiměřeně nízké. Detekční limity (v ng/ml) byly pro α-kasein 110, β-kasein 49, κ-kasein 58 a celkový kasein 505 ng/ml. Koeficient variace byl pro stanovení v rámci jedné mikrotitrační destičky < 12 %, pro stanovení mezi více destičkami < 9 %. Vyvinuté ELISA metody byly použity ke sledování změn v imunoreaktivitě kaseinu během tepelného ošetření a proteolýzy. Zahříváním mléka při teplotách pod 100 °C nedocházelo ke změně imunoreaktivity, ale záhřevy nad 100 °C způsobily její významné změny. V závislosti na druhu proteolýzy (mikroorganismy *Lactobacillus helveticus* a *Lactococcus* nebo enzymový preparát Pankreatin) bylo zjištěno snížení i zvýšení imunoreaktivity. Zatímco Pankreatin způsoboval u kaseinového koncentrátu pouze její výrazné snížení (5krát až 1000krát podle typu kaseinu), jemnější proteolýza pomocí mikroorganismů způsobovala jak její snížení (až 100krát u κ-kaseinu), tak i zvýšení (1,5krát u α-kaseinu).

Klíčová slova: α-kasein; β-kasein; κ-kasein; kaseinové hydrolyzáty; protilátky; imunoanalýza; ELISA

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