Determination of the percentage of α-lactalbumin and β-lactoglobulin of total milk protein in raw and heat treated skim milk

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Raw skimmed milk was pasteurized at 73 and 85°C for 15 s, high pasteurized at 125°C for 15 s (ESL) and UHT treated directly at 150°C for 1-2 s and indirectly at 138°C for 15 s. Kjeldahl methods were used for the determination of total, non-casein and non protein nitrogen fractions. By increasing heat treatment of the milk, the apparent casein nitrogen goes up because of precipitation of denaturated whey proteins in the casein fraction. Capillary electrophoresis and a Lab-on-a-chip method were used for the determination of the percentage of of α-lactalbumin + β-lactoglobulin of total milk protein in raw, pasteurized and UHT treated milk. In raw skimmed milk the sum of α-lactalbumin and β-lactoglobulin was 12.9 and 12.5 % of true protein for capillary electrophoresis and Lab-on-a-chip, respectively. In heat treated milk the sum of α-lactalbumin and β-lactoglobulin was in the range of 12.4 – 13.0 and 12.8 – 14.1 % of true protein for capillary electrophoresis and Lab-on-a-chip, respectively.

Bestimmung des relativen Anteils α-Lactalbumin und β-Lactoglobulin vom totalen Milchprotein in roher und wärmebehandelter Magermilch

Rohe Magermilch wurde bei 73 und 85°C während 15 s pasteurisiert, bei 125°C/15 s hochpasteurisiert (ESL) und bei 150°C/ 1-2 s direkt UHT-behandelt und bei 138°C/15 s indirekt UHT-behandelt. Kjeldahl Methoden wurden für die Bestimmung des Gesamt-, Nichtprotein- und Nichtcasein-Stickstoff eingesetzt. Mit zunehmender Wärmebehandlung der Milch nahm der scheinbare Casein-Stickstoff durch die zunehmende Denaturierung der Molkenproteine in der Caseinfraktion zu. Kapillarelektrophorese und eine Lab-on-a-chip-Methode wurden für die Bestimmung des relativen Anteils an α-Lactalbumin und β-Lactoglobulin vom Gesamtmilchprotein in roher, pasteurisierter und UHT behandelter Milch verwendet. In roher Magermilch betrug die Summe α-Lactalbumin und β-Lactoglobulin mittels Kapillarelektrophorese 12.9, mittels Lab-on-a-chip 12.5 % vom wahren Proteingehalt. In hitzebehandelter Magermilch betrug die Summe von α-Lactalbumin und β-Lactoglobulin mittels Kapillarelektrophorese 12.4 – 13.0, mittels Lab-on-a-chip 12.8 – 14.1 % vom wahren Proteingehalt.

24 Milk protein (percentage of lactalbumin/lactoglobulin) **24 Milcheiweiß** (Laktalbumin-/Laktoglogulin-Anteile)

1. Introduction

Almost all dairy products are manufactured from heated milk for specific reasons involving microbial stability and safety, shelf life extensions or technological aspects related to product functionality or quality. Pasteurization, UHT treatment and sterilization have an effect on the whey protein system as it contains the most heat sensitive milk proteins.

The major whey protein is β-lactoglobulin (BLG), comprising about 50 % of the total serum protein in bovine milk. This globular protein contains two disulfide bonds and one free cysteine (1). At temperatures slightly above 40°C BLG undergoes reversible conformational changes. The thiol group plays a crucial role in the heat-induced aggregation of BLG by acting as an initiator of thiol/disulfide exchange reactions (2).

Alpha-lactalbumin (ALA) is the second most important whey protein, constituting about 20 % of serum protein. ALA participates in lactose synthesis where it acts as a cofactor for lactose synthetase, ALA contains four intramolecular disulfide bonds. Despite its propensity to heat denaturation, ALA is quite resistant to heat coagulation. Denaturation is up to 90 % reversible after heating from 20 to 110°C (3), what explains the high apparent thermal stability of ALA when denaturation is measured by loss of protein solubility.

Bovine serum albumin (BSA) represents about 10 % of serum protein. It appears to be identical to the protein found in bovine blood and may enter the milk via leakage in the mammary gland. BSA contains 17 intramolecular disulfide bridges and one free thiol group. The molecule does not contain long-distance disulfide bonds and is therefore relatively flexible.

The immunglobulin (Ig) fraction, about 10% of serum protein, is a complex mixture of large glycoproteins with molecular weights ranging from 150 to 900 kDa.

The proteose-peptone fraction (PP), about 10 % of serum protein, mainly consists of degradation products from β-caseins. The casein derived proteose-peptones are formed by the action of indigenous plasmin. Proteose-peptones are soluble at pH 4.6 but are not precipitated by heat treatment.

Beta-lactoglobulin, α-lactalbumin, bovine serum albumin and immunglobulins unfold at temperatures above 65°C and react with κ-casein to form heat-induced protein aggregates (4, 5).

Denaturation of globular proteins is, typically, accompanied by several physical, chemical and physicochemical changes. These changes affect the functional and sensory properties of milk and milk products.

Thermal unfolding of globular proteins tends to enhance intermolecular interactions, in particular hydrophobic interactions resulting from the exposure of hydrophobic patches on the surface of the denaturated proteins (6, 7). This reaction leads to a loss of solublility of the protein aggregates. These protein aggregates precipitate with the caseins at pH 4.6. The precipitated coagulum can be centrifuged and the supernatant con-

tains the native whey proteins. Kjeldahl methods are traditionally used for the determination of total protein, casein and non-protein nitrogen in milk.

In heat treated milk, casein nitrogen calculated from Kjeldahl will be estimated systematically too high because of the intermolecular reactions of whey proteins and κ-casein.

The aim of this work was to compare methods, that are able to quantify the total amount of whey proteins in relation to casein in heat treated milk. Capillary electrophoresis with UV detection and a new Lab-on-a-chip method were compared.

2. Materials and methods

2.1 Milk samples

Raw skimmed milk from a local dairy (Uettligen, CH) was pasteurized at 73, 85 and 125°C (ESL) for 15 s, the UHT treatment was performed directly at 150°C for 1-2 s and indirectly at 138°C for 15 s on the ALP pilot plant installation.

2.2 NISECAS milk powder

NISECAS 0, 15, 20, 25 and 100 reference milk powder with 1.6, 16,4, 21.3, 26.2 and 100 g whey protein in 100 g of true protein were purchased from NIZO food research (Ede, NL).

2.3 Chemical methods

Total solids were determined with an oven method (8). The determination of the freezing point was performed with a thermistor cryoscope method (9). Lactose and lactulose were determined with an enzymatic test kit of Boehringer (10). After acid hydrolysis, furosine were separated on a furosine dedicated HPLC column (Socochim SA, CH) and determined by measuring the absorption at 280 nm (11). Total, casein and non protein nitrogen were determined according to Kjeldahl (12, 13).

After precipitation of the caseins, the acid-soluble β-lactoglobulin was quantified with the reference HPLC method (14).

2.4 Capillary electrophoresis

The separation of milk proteins (caseins and whey proteins) was performed in a capillary by applying a strong electrical field (+ 25 kV). The separation buffer contained 6 M urea to prevent the reformation of casein micelles during electrophoresis. All analysis were made on an Agilent Capillary Electrophoresis system 3^D CE with positive polarity. The run time of the method was approximately 70 min including capillary preconditioning. An uncoated capillary 50 cm x 50 µm from Agilent (Basel, Switzerland) was used in combination with 6 M urea in a citrate buffer at pH 3.0 (15). The average standard deviation of the relative whey protein content is 0.50 % (calculated from 6 double determinations).

2.5 Lab-on-a-chip method

The Lab-on-a-chip technology enables sample handling, electrophoresis and chromatographic separation, staining and detection on single integrated systems. Compared to gel electrophoresis, the Lab-on-a-chip system has the following advantages: ease of use, speed of analysis, low sample and reagent consumption and high reproducibility due to standardization and automation.

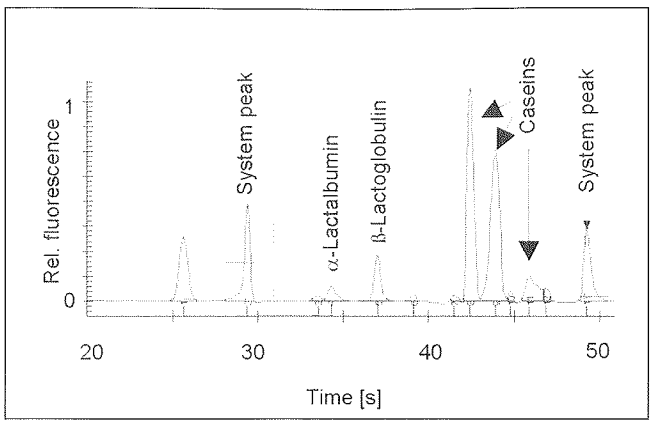

Fig. 1: Separation of milk proteins from raw skim milk on the Agilent 2100 Bioanalyzer system

The separation of whey proteins and caseins were performed on an Agilent 2100 Bioanalyzer system with a Protein 50 Chip. This chip enables the separation of proteins in a range from 5 to 50 kDa. Within this range the caseins, β-lactoglobulin and α-lactalbumin can easily be separated. Bovine serum albumin has a higher molecular weight (66 kDa), migrates after the last system peak, and cannot be quantified. Ten samples can be analyzed simultaneously on one chip within a few minutes. The average standard deviation of the relative whey protein content is 1.0 % (calculated from 6 triple determinations). A separation of milk proteins on the Bioanalyzer system is shown in Fig. 1.

Unfortunately it was not possible to work with the Protein 200 Chip as α-lactalbumin migrated together with a system peak and could therefore not be integrated.

3. Results and discussion

Total nitrogen, non protein nitrogen, total solids, lactose and the freezing point are quite constant for raw, pasteurised and UHT treated milk samples (Table 1). The non casein nitrogen fraction decreases by 0.20 – 0.23 g/kg in milk pasteurized at 85 and 125°C and direct treated UHT milk compared to raw milk. In indirect heated UHT milk, NCN is decreased by 0.66 g/kg. In heat treated milk, non casein nitrogen is systematically lower than in non-treated milk because of the intermolecular reactions of whey proteins and κ-casein and precipitation of the products at pH 4.6. Indirect UHT treatment shows most intense denaturation of whey proteins.

Lactulose content was below 25 mg/kg in raw, pasteurised, ESL and direct UHT treated milk, in indirect heated UHT milk, the content was 504 mg/kg. The furosine level was in the range of 120 to 131 mg/kg protein in raw, pasteurised, ESL and direct UHT treated milk. The furosine content in indirect heated UHT milk raised to 984 mg/kg protein. The lactulose and furosine content were in the normal range for heat treated milk.

The soluble whey protein fraction (NCN - NPN) was decreased by 21–24 % in pasteurized milk 85°C, ESL milk and direct treated UHT milk. In indirect treated UHT milk it was decreased by 69 % compared to the soluble fractions of raw milk.

Table 1: Chemical parameters of raw and heat treated skim milk

Milk sample	NCN (g/kg)	NPN (g/kg)	TN (g/kg)	Freezing point (°C)	TS (g/kg)	Lactose (mmol/kg)	Lactulose (mg/kg)	Furosine (mg/kg protein)
Raw	1.27	0.32	5.32	-0.5279	90.4	139.3	15	123
Past 73	1.24	0.32	5.30	-0.5224	90.4	140.2	17	121
Past 85	1.04	0.32	5.31	-0.5228	90.5	139.9	13	123
ESL 125	1.06	0.32	5.31	-0.5226	91.2	140.2	16	120
UHT direct	1.07	0.32	5.33	-0.5236	91.0	142.4	24	131
UHT indirect	0.61	0.32	5.29	-0.5220	90.9	138.8	504	984

ESL = extended shelf life; TN = total nitrogen; NCN = non casein nitrogen = TN- casein nitrogen; NPN = Non protein nitrogen; TS = total solids

Table 2: Whey protein content in NISECAS powder

NISECAS	Whey protein content (% of true protein) NIZO ref. value*	Capillary electrophoresis	Lab-on-a-chip
0	1.6	0	0
15	16.4	17.0	13.1
20	21.3	21.5	20.9
25	26.2	27.1	23.7
100	100	100	100

*Analyzed with SDS capillary gel electrophoresis

Table 3: Whey protein content in raw and heat treated skim milk

Milk sample	Acid soluble β-lactoglobulin (g/L)	WP (Kjeldahl) (% of TP)	ALA+BLG CE (% of TP)	ALA+BLG Lab-on-a-chip (% of TP)
Raw	4.07	19.0	12.9	12.5
Past 73	3.99	18.4	12.5	14.1
Past 85	2.99	14.4	12.8	13.4
ESL 125	3.13	14.7	13.0	13.9
UHT dir.	3.22	14.9	12.4	13.4
UHTindir.	0.12	5.8	12.9	12.8

ESL = extended shelf life; UHT i = UHT indirect; UHT d = UHT direct; TN = TP = true protein (total nitrogen - non protein nitrogen); WP = whey protein fraction (non casein nitrogen - non protein nitrogen); ALA = α-lactalbumin; BLG = β-lactoglobulin

NISECAS milk powders were used to check the quantification with capillary electrophoresis (CE) and with the Lab-on-a-chip system. The deviation from the reference value was typically between 1 and 3 % (Table 2).

The acid soluble β-lactoglobulin was determined with the official HPLC method(14). The β-lactoglobulin content was 4.07 g/L in raw milk and 3.99 g/L in milk pasteurised at 73°C. In milk pasteurized at 85°C, ESL milk, and direct treated UHT milk the concentration ranged from 2.99 to 3.22 g/L. In indirect treated UHT milk, the remaining soluble β-lactoglobulin was 0.12 g/L.

The calculated amount of soluble whey proteins with the Kjeldahl method (NCN-NPN) in the raw milk was 19.0 % of true protein (TP) (casein 81.0 % of TP). Upon increasing heat treatment (pasteurisation, ESL, UHT direct and indirect) the soluble whey protein fraction decreased to 5.8 % of TP in UHT indirect treated milk. In indirect treated UHT milk, the estimated „casein content" with the Kjeldahl method therefore is 94.2 % of true protein (Table 3). α-lactalbumin and β-lactoglobulin contribute in average to 70 % of the soluble whey protein fraction in raw milk. The estimated α-lactalbumin + β-lactoglobulin content by Kieldahl method is therefore 13.3 % of TP.

Using capillary electrophoresis the sum of α-lactalbumin and β-lactoglobulin in raw and heat treated milk were in the range of 12.4 to 13.0 % of TP, independently of the applied heat treatment. Using Lab-on-a-chip method these values ranged between 12.5 and 14.1 % of TP and no influence of the heat treatments could be observed.

The sum of α-lactalbumin and β-lactoglobulin in raw and heat treated milk with capillary electrophoresis 12.8 ± 0.2 % of TP and the Lab-on-a-chip method 13.3 ± 0.6 % of TP are in good agreement with the estimated Kieldahl value 13.3 % of TP.

4. Conclusions

Capillary electrophoresis and the Lab-on-a-chip method are able to measure the percentage of α-lactalbumin and β-lactoglobulin of total protein in raw and heat treated milk. Total (native and denaturated) α-lactalbumin and β-lactoglobulin can be quantified even in indirect heated UHT milk. Both methods are able to separate α-lactalbumin from β-lactoglobulin and could therefore also be used to detect alteration of the natural proportion of the two whey proteins resulting from new technological processes.

The determination of the percentage of α-lactalbumin and β-lactoglobulin with the Lab-on-a-chip method is favorable, because of the faster protein separation and the lower acquisition costs compared to capillary electrophoresis.

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Detection of β-casein in infant formulas and bovine milk

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Cow's milk has been reported to contain a factor provoking an auto-immune response that destroys β-pancreatic cells, and that may lead to insulin-dependent (type I) diabetes mellitus. Beta-casein might play an important roll on this immune-response. In the present study, β-casein was extracted and analyzed in samples of infant formulas and cow's milk. The protein fractions, obtained through the urea fractionation procedure were determined for their amino acid profiles. They were also examined by using electrophoresis at acidic pH or on gels with sodium dodecyl sulfate. Results indicated presence of β-casein in all samples of infant formulas and bovine milk. However, it was not possible to identify the genetic variants of this protein. Identification of genetic variants may be important to determine if the β-casein present in baby formulas is associated with type I diabetes. This might help in the future to suggest a new guideline for infant feeding practices.

Nachweis von β-Casein in Säuglingsnahrung und Kuhmilch

Kuhmilch soll einen Faktor enthalten, der eine Autoimmunreaktion hervorruft, die β-Pankreaszellen zerstört und die zum Insulin-abhängigen (Typ I) Diabetes mellitus führen kann. β-Casein kann bei dieser Immunreaktion eine wichtige Rolle spielen. Es wurde β-Casein aus Proben von Säuglingsnahrung und Kuhmilch extrahiert und analysiert. Die mit Hilfe des Harnstoff-Fraktionierungsverfahrens gewonnenen Proteinfraktionen wurden auf ihr Aminosäurenprofil untersucht. Durch Elektrophorese wurden die Fraktionen auch bei saurem pH-Wert mit Natriumdodecylsulfat analysiert. Die Ergebnisse zeigten das Vorhandensein von β-Casein in allen Säuglingsnahrungs- und Kuhmilchproben. Jedoch war es nicht möglich, die genetischen Varianten dieses Proteins zu identifizieren. Die Identifizierung der genetischen Varianten könnte wichtig sein um festzustellen, ob das in Säuglingsnahrung vorhandene β-Casein mit dem Typ I-Diabetes in Beziehung steht. Dies könnte in Zukunft helfen, neue Richtlinien für die Säuglingsernährung zu empfehlen.

24 β-**Casein** (detection in infant formula and bovine milk)

24 β-**Casein** (Nachweis in Säuglingsnahrung und Kuhmilch

1. Introduction

Bovine milk contains proteins with high nutritional value, among which casein is the major protein accounting for nearly 80% of total protein. However, it has been reported that infants who consume cow's milk at an early age (e.g. < 4 months) may develop autoimmunity leading to insulin-dependent (type I) diabetes mellitus, while those who are breast-fed or do not consume cow's milk at an early age do not develop immune response to cow's milk proteins (1-6). Beta-casein accounting for approximately 35% of total bovine casein is suggested to be one of the proteins related to the cause of abnormal immune response (2, 7). There is, however, little information available concerning the incidence of type I diabetes in individuals fed infant formulas or chemical quantitative analysis of β-casein in infant formulas. This study was, therefore, undertaken to detect β-casein in commercial samples of infant formula by extracting and analyzing this protein.

2. Materials and methods

2.1 Materials

Samples of milk based infant formula were obtained from local stores in Xalapa, Mexico. Samples I and II are products recommended for individuals between the ages of 0 to 6 months, while sample III is suitable for those between 6 to 12 months of age. Samples of cow's milk powder and pasteurized milk were also obtained from local stores in Xalapa, while raw milk samples were obtained from a local store in Edmonton, Canada. Before extraction of β-casein, all powdered samples were re-hydrated with deionized water according to the manufacturer's guidelines. A β-casein standard was obtained from Sigma-Aldrich Canada Ltd., Mississauga, Ontario, Canada.

2.2 Extraction of β-casein

The method described by SWAISGOOD (8) was used to extract β-casein from samples of infant formulas and