Influence of Salts on Selective Coagulation of Whey Proteins and their Application in the Isolation of β-Lactoglobulin

Jana HANUŠOVÁ[1], Miroslava MIHULOVÁ[1], Lenka DIBLÍKOVÁ[1] and Ladislav ČURDA[1,2]

[1]Department of Dairy, Fat and Cosmetics, Faculty of Food and Biochemical Technology, Institute of Chemical Technology in Prague, Prague, Czech Republic; [2]MemBrain s.r.o., Stráž pod Ralskem, Czech Republic

Abstract

Hanušová J., Mihulová M., Diblíková L., Čurda L. (2014): **Influence of salts on selective coagulation of whey proteins and their application in the isolation of β-lactoglobulin**. Czech J. Food Sci., **32**: 77–81.

Whey proteins are an important constituent of milk, especially whey from cheese manufacture and have many valuable functional properties such as foaming and emulsifying ability or gel formation. Some whey proteins are sensitive to salt content in a solution. High or low salt content may lead to selective coagulation of these proteins. A part of whey proteins was precipitated by addition of 7% (wt) NaCl and β-lactoglobulin and caseinomacropeptide remained in the supernatant. It was necessary to demineralise the supernatant by electrodialysis for the selective coagulation of caseinomacropeptide from this material. Subsequently, ethanol was added and pH was adjusted. This reduction of the ionic strength and the addition of ethanol induced the selective precipitation of caseinomacropeptide (91.4% from the original amount of CMP). β-lactoglobulin of 91% purity remained in the solution.

Keywords: whey protein concentrate; electrodialysis; precipitation of caseinomacropeptide; separation of proteins; salting out

Whey, the by-product of cheese manufacture, contains proteins which are a valuable source of essential amino acids (Velíšek 1999). The three main whey proteins are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), and caseinomacropeptide (CMP) which constitute 48–58, 13–19, and 12–20% (wt), respectively (Bonnaillie & Tomasula 2008). Thus, β-Lg accounts for about one half of the total amount of proteins and therefore its characteristics prevail over the other ones and significantly define the properties of whey. Due to its amphiphilic character and cysteine content this protein is a good emulsifying, foaming and gelling agent (Ju & Kilara 1998; Moro *et al*. 2013). Above that, it is the biologically active compound which is involved in the retinol metabolism, promotes lipolysis and has antioxidant, antimicrobial, antiviral and anticancer properties (Sawyer *et al*. 1998; Madureira *et al*. 2007). The listed properties of β-Lg lead to an effort aimed at its fast, simple and efficient isolation (Bonnaillie & Tomasula 2008; Santos *et al*. 2012).

Technologies currently used for β-Lg isolation combine methods of ion-exchange chromatography, enzymatic hydrolysis, complex formation, precipitation, electrodialysis (ED) and membrane separation (Casal *et al*. 2006; Cheison *et al*. 2012; Santos *et al*. 2012). They are mostly based on different sensitivity of whey proteins to pH, salt content, and ionic strength as shown in Table 1.

The electrodialysis could be used for β-Lg fractionation of generated bioactive peptides after its hydrolysis (Doyen *et al*. 2013). When combined with precipitation, this method could also have a potential for isolation of β-Lg with high yield and purity. Thus, the aim of this study was to optimise conditions such as pH, NaCl addition, and ionic strength of this technology for β-Lg manufacturing.

Supported by Specific University Research, Project No. MSMT 21/2012 and by the Ministry of Industry and Trade of the Czech Republic, Project No. MPO FR-TI1/470.

Table 1. The effect of pH, salt content and ionic strength on the stability of main whey proteins

Proteins		Conditions	References
Soluble	insoluble		
β-Lg, CMP	α-La, BSA, Ig	citric acid, pH ~ 4.0, 50–55°C	Bramaud *et al.* (1997)
β-Lg	α-La, BSA, Ig	lactic acid, pH 4.0, 55°C	Lucena *et al.* (2006)
β-Lg	α-La, BSA, Ig	sodium hexametaphosphate or sodium citrate, pH 3.9, 35°C	Alomirah and Alli (2004)
β-Lg	α-La, CMP, BSA	Na_2SO_4, pH 2, 20°C	Onwulata *et al.* (2008); Aschafenburg *et al.* (1957)
β-Lg	α-La, BSA, Ig	NaCl (7% wt), pH 2, 20°C	Mailliart and Ribadeau-Dumas (1988)
α-La, β-Lg, BSA	Ig	$MgSO_4$ (saturated), pH ~ 4.0	Fox (2009)

MATERIAL AND METHODS

Material. Commercial whey protein concentrate (Volac, Hertfordshire, UK) was diluted in demineralised water so that the starting whey protein (WPC) solution with protein content 7.5% wt was prepared.

β-Lg isolation by NaCl salting out at low pH. The fractionation technique was based on the methods developed by Mailliart and Ribadeau-Dumas (1988) and Maté and Krochta (1994). The starting WPC solution was adjusted to pH 2 by 10% HCl by wt. Thereafter, different amounts of NaCl were added to reach final concentrations of 1, 3, 5, 7, 9, and 11% wt, respectively. Precipitation of α-La and minor whey proteins was carried out at 20°C for 20 minutes. The precipitate was removed by centrifugation at 23 000 *g*, 20°C, 30 min (Universal 32R; Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). Four parallel β-Lg separations were carried out with each NaCl concentration.

β-Lg isolation by combination of demineralisation and NaCl salting out at low pH. The fractionation technique described above was based on the methods developed by Mailliart and Ribadeau-Dumas (1988) and Maté and Krochta (1994). Addition of 7% NaCl by wt was used for salting out α-La and minor whey proteins. This process was preceded by a lowering of salt content in the starting WPC solutions to 0.936 mS/cm (conductometer pH/cond 340i; WTW GmbH, Weilheim, Germany) using electrodialysis (described in 2.5). β-Lg separation was performed four times.

β-Lg isolation by combination of NaCl salting out, demineralisation and ethanol addition. The fractionation technique was based on the methods developed by Mailliart and Ribadeau-Dumas (1988), Berrocal and Neeser (1993), and Maté and Krochta (1994), and combined three steps: addition of 7% NaCl by wt (described above), demineralisation of supernatant (described in 2.5) to 0.987 mS/cm (conductometer pH/cond 340i; WTW GmbH, Weilheim, Germany) and addition of ethanol (EtOH). The 7, 9 or 11% wt of food ethanol were added into demineralised supernatant to precipitate the remaining CMP and pH was adjusted to 5.5 with 10% HCl by wt. The CMP precipitation was carried out at 20°C for 30 minutes. The obtained precipitate was removed by centrifugation at 23 000 *g*, 20°C, 30 min (Universal 32R; Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). Four parallel β-Lg separations were carried out with each ethanol concentration.

Demineralisation of whey protein solutions. The salt content of the materials for β-Lg isolation described above was reduced by electrodialysis. Demineralisation process was performed by an ED-Z Mini electrodialysis unit (Mega a.s., Prague, Czech Republic) at the temperature range from 18°C to 26°C. The electrode solution was Na_2SO_4 (c = 10 g/l). Salts cumulated in demineralised water which was acidified by HNO_3 to pH 2. The starting WPC solution was demineralised to 0.936 mS/cm, the supernatant after α-La and minor whey protein precipitation to 0.987 mS/cm (conductometer pH/cond 340i; WTW GmbH, Weilheim, Germany). The content of cations was determined before and after demineralisation by capillary electrophoresis. Conductivity was measured before and after demineralisation (conductometer pH/cond 340i; WTW GmbH, Weilheim, Germany) twice for each material.

Determination of cation content. The content of Ca^{2+}, Mg^{2+}, Na^+, K^+ cations was determined before and after demineralisation of the starting WPC solution and supernatant after α-La and minor whey protein salting out (2.4) by capillary electrophoresis Prince CEC 750 (Prince Technologies, Emmen, the Netherlands) with DAD detector (λ = 206 nm) according to Suaréz-Luque *et al.* (2007). Samples were diluted with ultrapure water (Millipore Smart Simplicity UV System; EMD Millipore Corporation, Billerica, USA), adjusted to pH 4 by 1 mol/l CH_3COOH, filtered through 0.22 μm MS® PVDF syringe filter

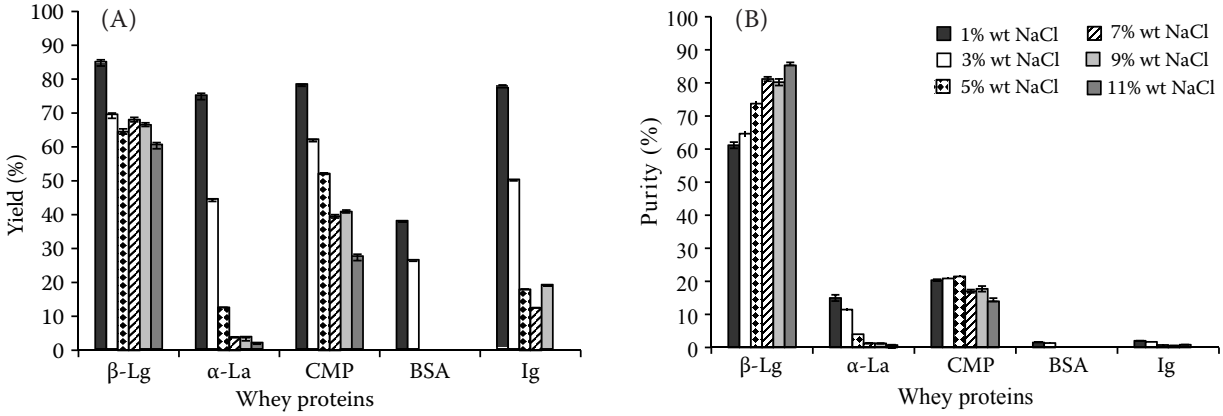

Figure 1. The yield (A) and purity (B) of whey proteins in supernatant after β-Lg isolation by NaCl addition
β-Lg – β-lactoglobulin; α-La – α-lactalbumin; CMP – caseinomacropeptide; BSA – bovine serum albumin; Ig – immunoglobulins; n = 4

(Membrane Solutions, North Bend, USA) and mixed with internal standard (0.02 g/l LiCl) at a 1:1 ratio. The analysis was performed twice for each sample.

Determination of whey protein fractions. The composition of whey proteins in starting WPC solution and precipitation supernatants was determined by RP-HPLC chromatography (Series 1100; Agilent Technologies GmbH, Waldbronn, Germany) with gradient elution [mobile phase A was 0.1% (v) CF_3COOH in ultrapure water, mobile phase B was 0.1% (v) CF_3COOH in a mixture of 80% (v) acetonitrile and 20% (v) ultrapure water] and UV detection (λ = 220 nm; UV/VIS detector by Thermo Separation Products Spectra System UV 2000; Germany) according to the method published by Thomä *et al*. (2006). Samples were filtered through 0.22 µm MS® PVDF syringe filter (Membrane Solutions, North Bend, USA) before analysis. Injection volume of the sample was 20 µl. Whey proteins were separated on a PLRP-S 1000A polymer column (4.6 × 150 mm, 8 µm; Polymer Laboratories Ltd., Shropshire, UK) at 40°C and the flow rate of the mobile phase was 1 ml per minute. The analysis was performed twice for each sample. The yield and purity of isolated proteins were evaluated from obtained results.

Statistical analysis. Statistical analysis was performed using MS Excel 2007 (Microsoft Office Excel 2007; Microsoft Corporation, Redmond, USA). Results are the arithmetic mean of four parallel fractionations. Error bars in the graphs indicate the standard deviation. The outliers were removed from obtained data by Grubbs' test and results were evaluated by ANOVA test on the level of significance $P(\alpha)$ = 0.05 (Vorlíček *et al*.1984).

RESULTS AND DISCUSSION

β-Lg isolation by NaCl salting out at low pH. The addition of different amounts of NaCl resulted in the coagulation of whey proteins in various proportions. The addition of lower amounts of NaCl (1 and 3% wt) did not result in selective coagulation and all proteins were precipitated only partially (Figure 1A). A higher content of added NaCl led to the precipitation of a major part of α-La, bovine serum albumin (BSA) and immunoglobulins and the β-Lg and CMP remained in supernatant. In terms of β-Lg purity, the best result was obtained when 11% NaCl by wt was added (85.3%) but the yield was too low in this case (60.4%). Hence, the addition of 7% NaCl by wt was chosen

Table 2. Cation content and conductivity of materials used for β-Lg isolation (n = 2)

Material	ED[3]	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	Conductivity (mS/cm)
		(g/l)				
WPC	before	0.494 ± 0.034	0.066 ± 0.002	0.158 ± 0.029	0.353 ± 0.021	1.660 ± 0.001
	after	0.460 ± 0.045	0.044 ± 0.001	0.104 ± 0.011	0.072 ± 0.001	0.936 ± 0.001
WPC_{NaCl}	before	0.345 ± 0.013	0.010 ± 0.004	18.701 ± 0.823	0.164 ± 0.006	96.700 ± 0.001
	after	0.256 ± 0.015	0.000 ± 0.000	0.542 ± 0.028	0.000 ± 0.000	0.987 ± 0.001

WPC – starting whey protein solution; WPC_{NaCl} – supernatant from the starting whey protein solution after α-La and minor whey protein precipitation; ED – electrodialysis

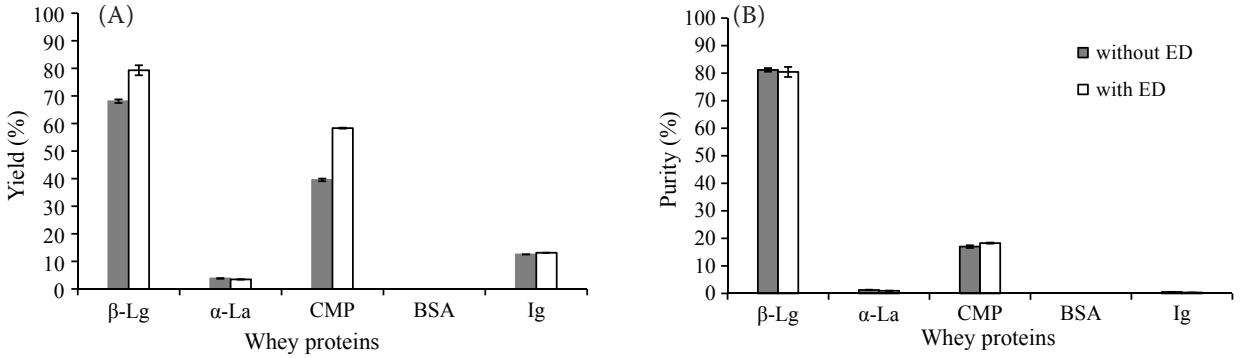

Figure 2. The yield (A) and purity (B) of whey proteins in supernatant after β-Lg isolation by combination of electrodialysis (ED) and NaCl addition (for abbreviations see Figure 1)

for the next procedure (Figure 1B). The yield of β-Lg in supernatant was 68.1% and the purity was 81.2%. Maté and Krochta (1994) reported the β-Lg yield of 65% and the purity 95%. However, they used whey protein isolate without CMP as a starting material.

β-Lg isolation by combination of demineralisation and NaCl salting out at low pH. Demineralisation of the starting WPC solution to the value 0.936 mS/cm decreased the coagulation of β-Lg and CMP after addition of 7% NaCl by wt. Demineralisation was carried out. Concentrations of cations in initial WPC solution and in diluate after ED are given in Table 2. Figure 2A shows that the demineralisation resulted in a higher yield of β-Lg and CMP, which was 79.3 and 58.3%, respectively (Figure 2A). The purity (Figure 2B) of these proteins remained almost unchanged. It is obvious that the demineralisation led to better yields, but the supernatant was still a mixture of two proteins.

β-Lg isolation by combination of NaCl salting out, demineralisation and ethanol addition. This method was based on the precipitation of whey proteins by addition of 7% NaCl by wt and subsequent demineralisation of the obtained supernatant. This reduction of the salt content followed by the addition of ethanol resulted in the selective coagulation of CMP. Demineralisation was primarily used for the reduction of sodium content (Table 2). The addition of ethanol was based on the paper of Berrocal and Neeser (1993). They observed that CMP of 80% purity could be obtained by the addition of ethanol. The addition of 7% ethanol by wt resulted in the highest yield of β-Lg, namely 91.0% (Figure 3A). CMP of 91.4% purity was precipitated with this amount of ethanol and β-Lg of 97.1% purity (Figure 3B) remained in the final product (supernatant).

CONCLUSION

The isolation of β-Lg was performed using three different methods based on NaCl addition, demineralisation of the whey protein solution and ethanol addition and removing of precipitated whey proteins. The highest yield (91.0%) and purity (97.1%) of β-Lg were obtained by the method which combined the addition of 7% NaCl by wt, demineralisation of the supernatant and subsequent addition of 7% ethanol by wt. The proposed method is a very good tool for the isolation of β-Lg from the WPC solution.

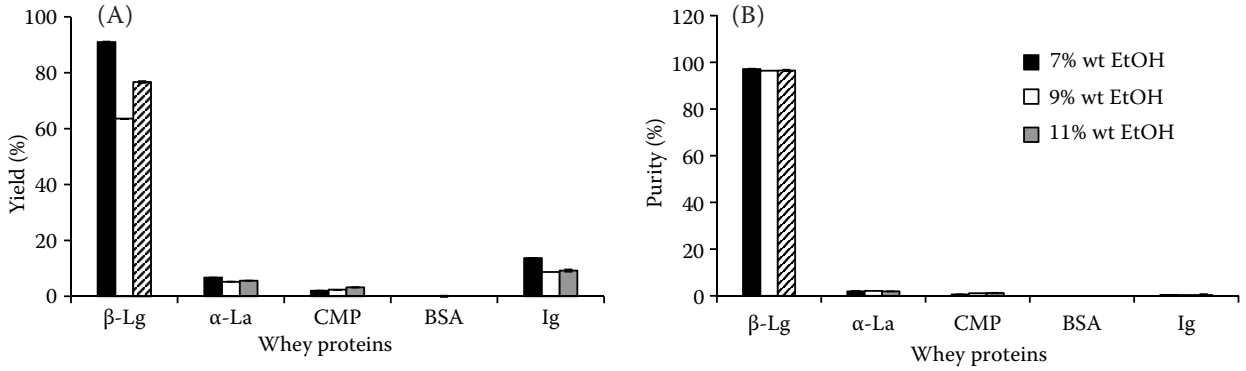

Figure 3. The yield (A) and purity (B) of whey proteins in supernatant after β-Lg isolation by combination of NaCl salting out, demineralisation and ethanol (EtOH) addition (for abbreviations see Figure 1)

References

Alomirah H.F., Alli I. (2004): Separation and characterization of β-lactoglobulin and α-lactalbumin from whey and whey protein preparations. International Dairy Journal, **14**: 411–419.

Aschafenburg R., Drewry J. (1957): Improved method for the preparation of crystalline beta-lactoglobulin and alpha-lactalbumin from cow's milk. Biochemical Journal, **65**: 273–277.

Baumy J.J., Brule G. (1988): Binding of bivalent cations to α-lactalbumin and β-lactoglobulin: effect of pH and ionic strength. Le Lait, **68**: 33–48.

Berrocal R., Neeser J.R. (1993): Production of kappa-caseino-glycomacropeptide. US 5216129.

Bonnaillie L.M., Tomasula P.M. (2008): Whey protein fractionation. In: Onwulata Ch.I., Huth P.J. (eds): Whey Processing, Functionality and Health Benefits. John Wiley & Sons, London: 15–38.

Bramaud c., Aimar p., Daufin g. (1997): Optimisation of a whey protein fractionation process based on the selective precipitation of α-lactalbumin. Le Lait, **77**: 411–423**.**

Casal E., Montilla A., Moreno F.J., Olano A., Corzo N. (2006): Use of chitosan for selective removal of β-lactoglobulin from whey. Journal of Dairy Science, **89**: 1384–1389.

Chatterton D.E.W., Smithers G., Roupas P., Brodkorb A. (2006): Bioactivity of β-lactoglobulin and α-lactalbumin – Technological implications for processing. International Dairy Journal, **16**: 1229–1240.

Cheison S.Ch., Bor E.K., Faraj A.K., Kulozik U. (2012): Selective hydrolysis of α-lactalbumin by acid protease A offers potential for β-lactoglobulin purification in whey proteins. LWT-Food Science and Technology, **49**: 117–122.

Doyen A., Husson E., Bazinet L. (2013): Use of an electrodialytic reactor for the simultaneous β-lactoglobulin enzymatic hydrolysis and fractionation of generated bioactive peptides. Food Chemistry, **136**: 1193–1202.

Dybowska B.E. (2011): Whey protein-stabilized emulsion properties in relation to thermal modification of the continuous phase. Journal of Food Engineering, 104: 81–88.

Fox P.F. (2009): Milk constituets. In: Thompson A., Boland M., Singh H. (eds): Milk Proteins: from Expression to Food. Academic Press, San Diego: 28

Ju Z.Y., Kilara A. (1998): Properties of gel induced by heat, protease, calcium salt and acidulant from calcium ion-aggregated whey protein isolate. Journal of Dairy Science, **81**: 1236–1243.

Kinsella J.E., Whitehead D.M. (1989): Proteins in whey: Chemical, physical and functional properties. Advanced Food Nutrition Research, **33**: 343–438.

Lucena M.E., Alvarez S., Menéndez C., Riera F.A., Alvarez R. (2006): Betalactoglobulin removal from whey protein concentrates production of milk derivates as a base for infant formulas. Separation and Purification Technology, **52**: 310–316.

Madureira A.R., Pereira C.I., Gomes A.M.P., Pintado M.E., Malcata F.X. (2007): Bovine whey proteins – Overview on their main biological properties. Food Research International, **40**: 1197–1211.

Mailliart P., Ribadeau-Dumas B. (1988): Preparation of β-lactoglobulin-free proteins from whey retentate by NaCl salting out at low pH. Journal of Food Science, **53**: 743–745.

Maté J.I., Krochta J.M. (1994): β-Lactoglobulin separation from whey protein isolate on a large scale. Journal of Food Science, **59**: 1111–1113.

Moro A., Báez G.D., Ballerini G.A., Busti P.A., Delorenzi N.J. (2013): Emulsifying and foaming properties of β-lactoglobulin modified by heat treatment. Food Research International, **51**: 1–7.

Onwulata C. (2008): Whey Processing, Functionality and Health Benefits. Wiley-Blackwell, New York.

Santos M.J., Teixeira J.A., Rodrigues L.R. (2012): Fractionation of the major whey proteins and isolation of β-lactoglobulin variants by anion exchange chromatography. Separation and Purification Technology, **90**: 133–139.

Sawyer L., Brownlow S., Polikarpov I., Wu S.-Y. (1998): β-Lactoglobulin: Structural studies, biological clues. International Dairy Journal, **8**: 65–72.

Suárez-Lugue S., Mato I., Huidobro J.F., Simal-Lozano J. (2007): Determination of major metal cations in milk by capillary zone electrophoresis. International Dairy Journal, **17**: 896–901.

Thomä C., Krause I., Kulozik U. (2006): Precipitation behaviour of caseinomacropeptides and their simultaneous determination with whey proteins by RP-HPLC. International Dairy Journal, **16**: 285–293.

Velíšek J. (1999): Chemie potravin 1. OSSIS, Tábor: 43–48.

Vorlíček M., Holický M., Špačková M. (1984): Pravděpodobnost a matematická statistika pro inženýry. České vysoké učení technické v Praze, Praha: 195–202; 208–210.

Received for publication November 11, 2012
Accepted after corrections June 14, 2013

Corresponding author:
Jana Hanušová, MSc., Vysoká škola chemicko-technologická v Praze, Fakulta potravinářské a biochemické technologie, Ústav mléka, tuků a kosmetiky, Technická 3, 166 28 Praha 6, Česká republika; E-mail: jana.hanusova@vscht.cz