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Improved purification of β-lactoglobulin from acid whey by means of ceramic hydroxyapatite chromatography with sodium fluoride as a displacer

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Abstract

The successful separation of β-lactoglobulin from other bovine whey proteins was performed by ceramic hydroxyapatite chromatography with a fluoride ion gradient in phosphate buffer as displacement agent. The method was applied to acid whey originating from milk of healthy as well as of mastitic cows. β-Lactoglobulin was completely eluted in one peak at a fluoride concentration of about 0.6 mol/l. The purity of β-lactoglobulin in this fraction was at least 96% if whey from healthy milk was processed. Co-eluted contaminants are traces of immunoglobulin G, serum albumin and lactoferrin. In case of mastitic whey the proportion of β-lactoglobulin is diminished as the amounts of immunglobulin G, serum albumin and lactoferrin are increased within this fraction. Size exclusion chromatography on Superdex 75 pg effectively removed contaminants resulting in a purity for β-lactoglobulin from normal whey of approximately 99%. The yield of β-lactoglobulin from physiological whey was 50–55% referring to the fraction highly enriched with β-lactoglobulin by hydroxyapatite chromatography. In case of mastitic milk the higher amounts of contaminants were also removed successfully by size exclusion chromatography.

Keywords: Ceramic hydroxyapatite chromatography; β-Lactoglobulin; Bovine whey proteins

1. Introduction

In cow's milk, β-lactoglobulin accounts for about 50% of the whey protein and for about 10% of the total milk protein [1]. In ruminant milk, at least it exists as a dimer with a molecular mass of 36,400 Da [2,3]. Different methods for separating β-lactoglobulin from other bovine whey proteins were reported as early as 1957 [4]. Traditionally, β-lactoglobulin is prepared by fractional precipitation with ammonium sulfate at a low pH or by lowering of pH of whey with or without heating in order to cause precipitation of all whey proteins other than β-lactoglobulin [5]. Modern chromatographic methods have allowed β-lactoglobulin to be isolated by size exclusion [6], preparative ion-exchange [7,8] or by bioaffinity chromatography [9,10]. To our knowledge, there are only two reports on the use of hydroxyapatite displacement chromatography for separating whey proteins [11,12]. Reported displacers are either phosphate [11] or acidic organics [12] which form stronger complexes with calcium than do carboxyls of the absorbed proteins. β-Lactoglobulin eluted by phosphate appears to be contaminated by substances of similar size and additionally by higher molecular weight species [11]. Acidic organics on the other hand separated β-lactoglobulin more efficiently from other whey proteins in anion-exchange displacement chromatography than by hydroxyapatite displacement chromatography [12]. In the course of developing an isolation procedure for the enzyme prostaglandin D synthase [13] from inflamed mammary glands secretions, proteins of similar size, enclosing β-lactoglobulin, had to be removed. We made use of the reported atypical elution behavior of this acidic protein being displaced by fluoride ions from hydroxyapatite [14]. Due to the high enrichment of β-lactoglobulin in the last eluting peak we applied this

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strategy also to the whey from physiological mammary glands with the aim to find a further method for the purification of this protein. Additionally, the behavior of both kinds of whey on hydroxyapatite stationary phases should be compared in the experiments mainly with respect to their protein composition.

2. Experimental

2.1. Reagents and materials

Protein standards (LMW, 14–97 kDa) were purchased from Amersham Biosciences, Freiburg, Germany. Bovine gamma globulin was from Cappel Labortories, Downingtown, PA, USA; β-lactoglobulin A, α-lactalbumin and calmodulin were from Sigma–Aldrich Chemie, Munich, Germany; lactoferrin was purchased from Fluka Chemie, Buchs, Switzerland. Macro-Prep Ceramic hydroxyapatite (Type I, 80 μm) was obtained from BioRad Laboratories, Munich, Germany. All other chemicals were of analytical grade rate.

2.2. Sample preparation

Whey protein fractions were prepared according to [11]. Milk fat separation was done by centrifugation (4000 × *g*, 4 °C, 20 min). Casein was precipitated by acidification of skim milk to pH 4.5 with 4 M acetic acid and removed by centrifugation (20,000 × *g*, 4 °C, 30 min). The supernatant was dialysed in tubings (cut-off: <10 kDa) overnight at 4 °C against phosphate buffer (10 mM, pH 7.2). Undissolved material was removed by centrifugation. The dissolved whey proteins were lyophilized and then stored at −20 °C. Protein determination was performed according to Bradford [15] using a protein assay (BioRad, Hercules, CA, USA).

2.3. Chromatography

The HPLC-system consisted of the Biologic Chromatography System (BioRad Laboratories, Hercules, CA, USA). A Bio-Scale column (12 × 88 mm) was filled with ceramic hydroxyapatite (type I, 80 μm), both from BioRad Laboratories, Munich, Germany. The mobile phase was composed of phosphate buffer (0.01 M, pH 6.25; buffer A) and sodium fluoride (0.75 M) in buffer A (buffer B). Five hundred microlitres of buffer A containing 20–30 mg protein in freeze–dried whey material were injected into the HPLC system. Protein displacement was performed in a stepwise as well as in a linear gradient manner using buffer A and buffer B under the following conditions: flow rate 2.0 ml/min, fraction size 1.5 ml, detection wavelength 280 nm at 4 °C in the cold room.

Fractions of the last eluting peak (D) were combined, dialysed against 0.005 M phosphate buffer, pH 7.0, and lyophilized.

Size exclusion chromatography was performed on a Superdex 75 prepgrade (pg) column (100 × 1.6 cm) obtained from Amersham Biosciences, Freiburg, Germany. Elution was isocratic with sodium phosphate buffer (0.05 M, pH 7.2, 0.3 M NaCl). An amount of 5–10 mg protein from the lyophilized preparation was injected in 500 μl of this buffer into the Superdex separation system controlled by the Bio-Rad chromatography system under the following conditions: flow rate 0.8 ml/min, fraction size 1.0 ml, 4 °C, detection wavelength 280 nm.

2.4. Electrophoresis

The electrophoretic equipment consisted of a dual (mini)vertical electrophoresis unit (Novex, Minicell) purchased from Invitrogen GmbH, Karlsruhe, Germany. Gels were NuPAGE Bis–Tris-gels, 10%, 10 wells. Samples were taken up in NuPAGE LDS Sample Buffer with NuPAGE Reducing-Agent. Running buffer was MES Running Buffer with NuPAGE Running Buffer Antioxidant. Electrophoresis conditions were 200 V, 110 mA/gel at room temperature. Gels were silver-stained using the SilverQuest Silver Staining. All reagents for electrophoresis were obtained from Invitrogen GmbH, Karlsruhe, Germany.

3. Results and discussion

In the present investigation, the chromatographic isolation of β-lactoglobulin was comparatively performed on whey proteins in bovine milk originating from healthy udders and from inflamed mammary gland quarters, respectively.

In Fig. 1, the elution profile of whey proteins from physiological milk is shown following chromatography on ceramic hydroxyapatite. The fraction eluted at the end of the NaF-gradient (fraction D) contains the entire amount of β-lactoglobulin within whey obtained from physiological as well as from mastitic milk (Fig. 2). The β-lactoglobulin containing fractions were analyzed by size exclusion chromatography after having been dialyzed and lyophilized. Next to the main peak consisting of dimeric β-lactoglobulin some minor contaminants within two fractions were observed (Fig. 3). The hydroxyapatite peak D of physiological whey contains more than 96% of β-lactoglobulin as derived from protein determination in size exlusion chromatography fractions. The proteins contaminating this fraction amount between 2 and 3%. These minor proteins are lactoferrin, serum albumin and traces of immunoglobulin G (Fig. 6). β-Lactoglobulin consists of two genetically determined variants of similar molecular weight reported to have a different motility in native but not in SDS-PAGE (Figs. 2 and 6).

Among whey proteins several phosphorylated species are known to be similar sized like β-lactoglobulin or are degradation products of caseins. “Stains-all” staining with the cationic carbocyanine dye [16,17] was performed

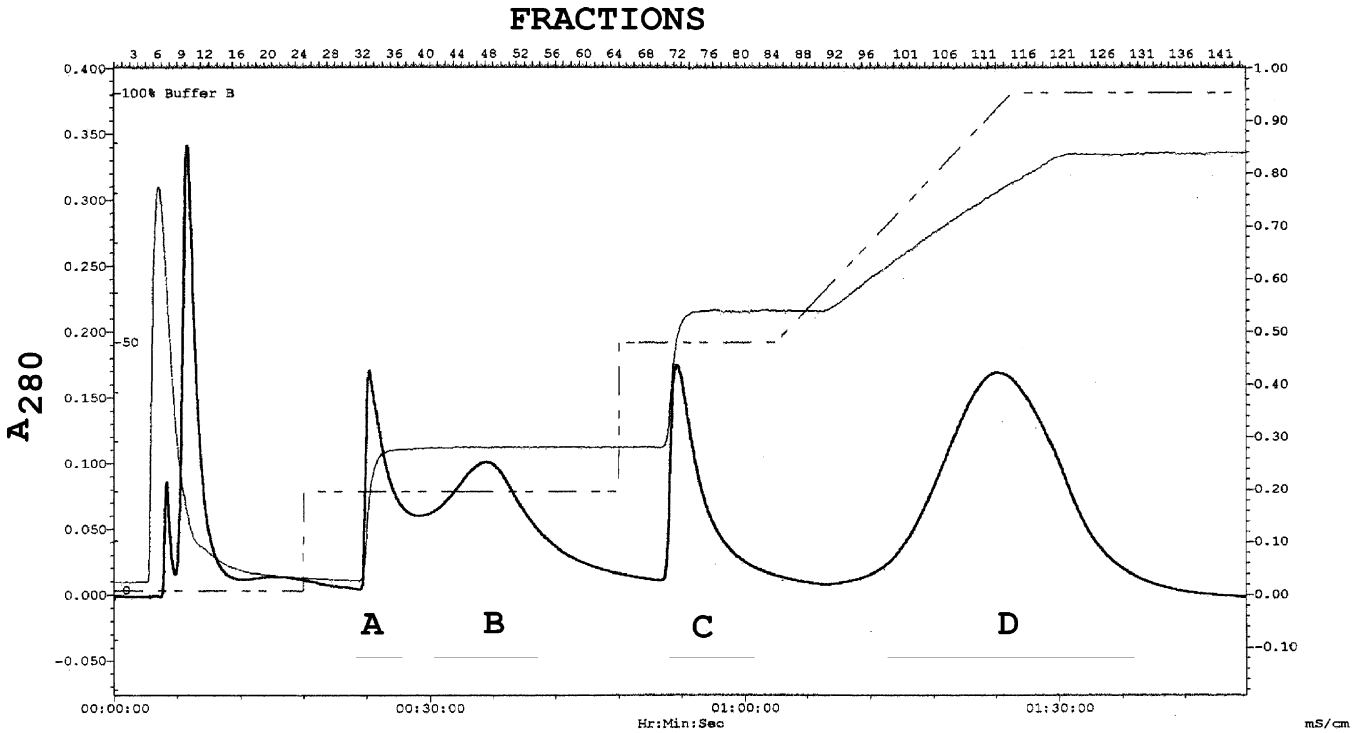

Fig. 1. Displacement of whey proteins from physiological milk on ceramic hydroxyapatite. Carrier, 0.010 M sodium phosphate buffer, pH 6.25 (buffer A); displacer, sodium fluoride in buffer A. Dashed line, predetermined NaF-gradient; solid line, conductivity (mS/cm) of the eluate; UV absorbance at 280 nm.

for the detection of such molecules within the isolated β-lactoglobulin fractions. However, no blue or purple staining specific for such phosphorylated proteins could be detected within the respective fractions (not shown).

When analyzing milk from inflamed mammary gland quarters the peak containing β-lactoglobulin (D) appears to be eluted at a slightly higher fluoride concentration in comparison to whey from physiological milk (Fig. 4). In the following size exclusion chromatography it is shown that in contrast to whey from physiological milk two prominent peaks are eluting in front of β-lactoglobulin (Fig. 5). According to SDS-PAGE, the respective hydroxyapatite peak D contains mainly lactoferrin apart from smaller amounts of serum albumin and immunoglobulin G (Fig. 6). Due to the high amount of aromatic amino acids (>9%) in lactoferrin the absorption of peak B appears relatively high in comparison to that of β-lactoglobulin. In contrast to β-lactoglobulin, which is an acidic protein with a theoretical p*I* of 4.83 (Swiss-Prot, PO 2754), lactoferrin is a basic protein with a theoretical p*I* of 8.67 (Swiss-Prot, P 24627). Their comparable elution behavior in the performed hydroxyapatite chromatography is therefore outstanding with regard to other representatives of the respective polarity class.

The calculated mass fractions of β-lactoglobulin in the hydroxyapatite peak and in the respective peak after size exclusion chromatography are given in Table 1. The yield of β-lactoglobulin from whey of inflamed mammary glands

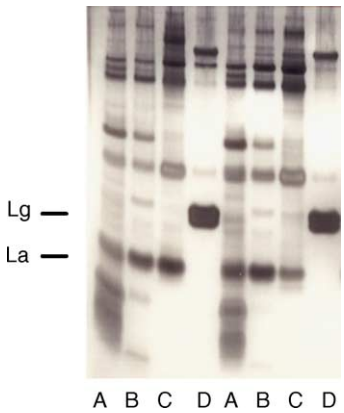

Fig. 2. SDS-PAGE (reducing) of whey and whey fractions after hydroxyapatite chromatography. Lanes correspond to the fractions in Fig. 4. Two independent separations are shown. Lg, β-lactoglobulin; La, α-lactalbumin.

Table 1
Protein yield (%) of chromatographic fractions

	Whey (normal)	Whey (mastitic)
Hydroxyapatite fraction D	49.2 ± 3.3 ($n = 3$)	34.5 ± 7.1 ($n = 3$)
Superdex fraction C	25.6 ± 2.7 ($n = 3$)	6.4 ± 1.2 ($n = 3$)
Yield of β-lactoglobulin referring to fraction D	50–55	18–20

The amount of protein was determined according to Bradford [15] in source material (whey), in fraction D before and after lyophilisation as well as in all fractions resulting from size exclusion chromatography. The indicated yield for fraction D and C refers to whey protein. With respect to fraction D, containing highly enriched β-lactoglobulin, the yield at the end of the purification procedure amounts 50–55% (normal whey) and 18–20% (mastitic whey), respectively.

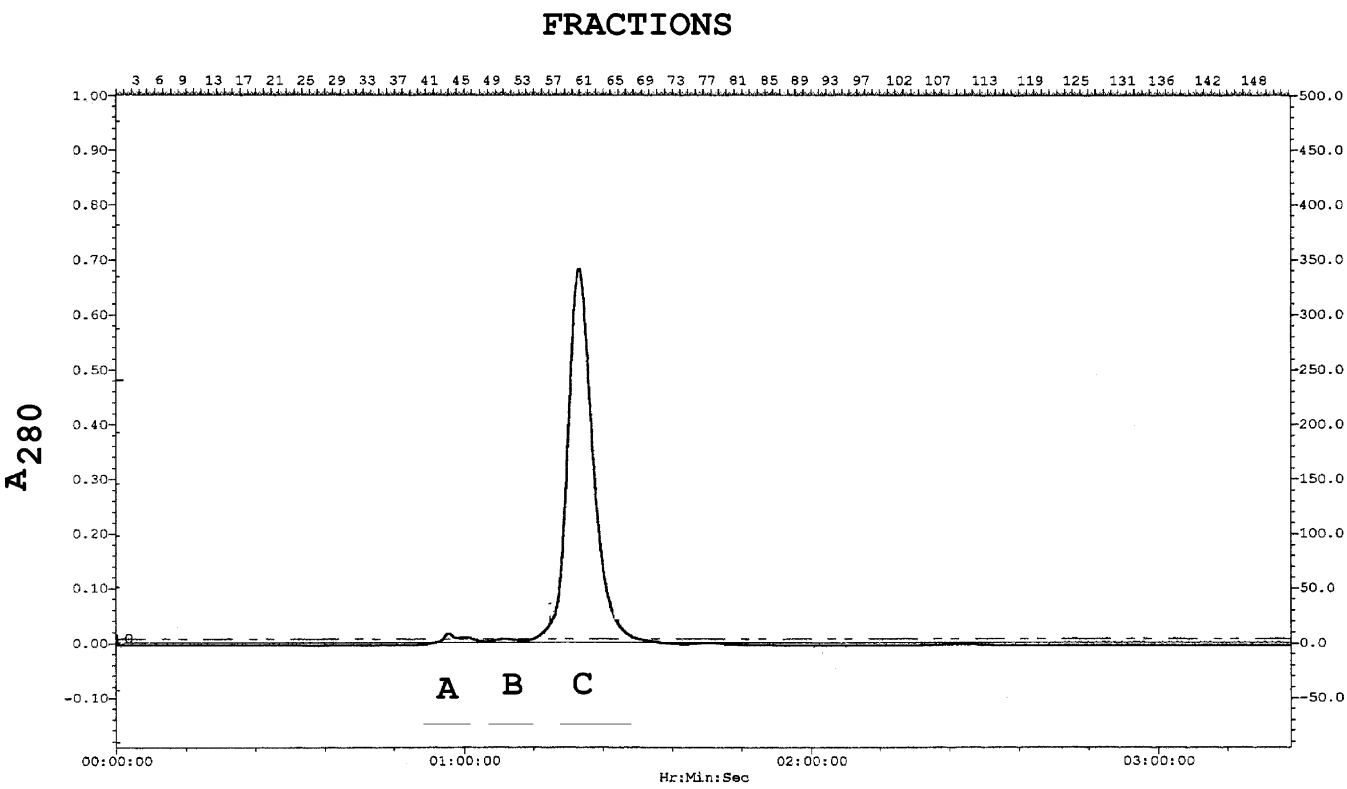

Fig. 3. Elution profile of fraction D of the ceramic hydroxyapatite chromatogram (Fig. 1, physiological whey) obtained on Superdex 75 pg. Elution with 0.30 M NaCl in 0.050 M sodium phosphate buffer, pH 7.2; UV absorbance at 280 nm.

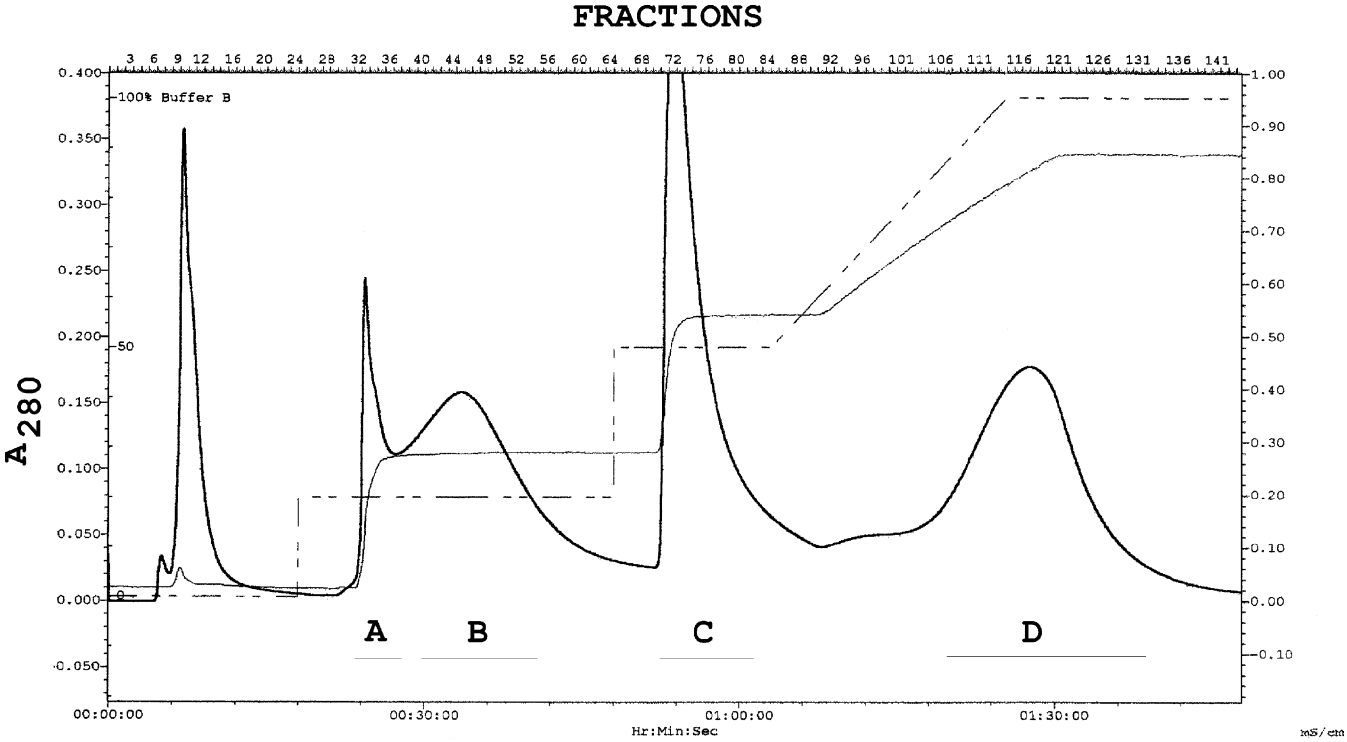

Fig. 4. Displacement of whey proteins from mastitic milk on ceramic hydroxyapatite. Conditions as described in Fig. 1.

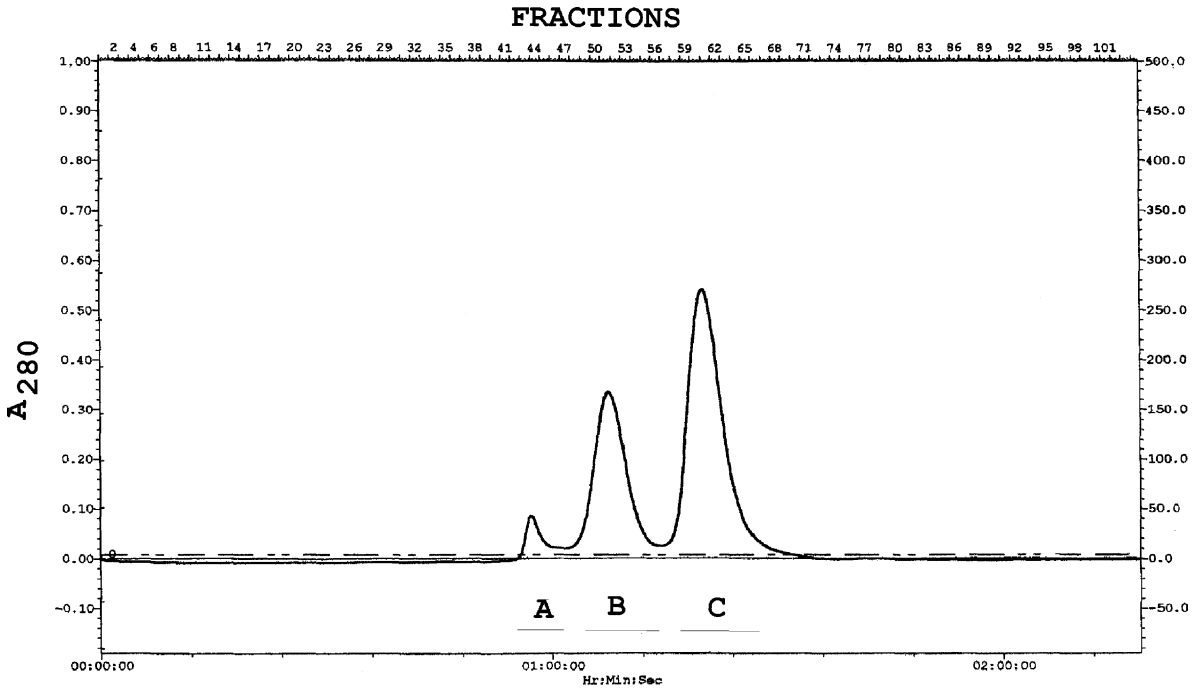

Fig. 5. Elution profile of fraction D of the ceramic hydroxyapatite chromatogram (Fig. 4, mastitic whey) obtained on Superdex 75 pg.

is much lower than in physiological whey. Considering the fact that in the present investigation all β-lactoglobulin of whey is displaced within the high concentration range of the NaF gradient, the resulting yield of the highly purified β-lactoglobulin after size exclusion chromatography amounts from 50 to 55% referring to fraction D containing highly enriched β-lactoglobulin and obtained by hydroxyapatite chromatography. The purity of β-lactoglobulin was improved up at this stage to 99%. The so far reported investigations using hydroxyapatite, however, give no details about yield and purity of β-lactoglobulin [11,12].

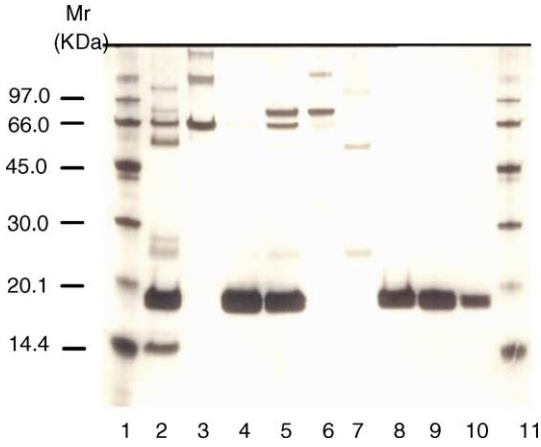

Fig. 6. SDS-PAGE (reducing) of whey and whey fractions after hydroxyapatite chromatography and size exclusion chromatography on Superdex 75 pg. Lanes: (1) low molecular weight standards; (2) acid whey; (3) bovine serum albumin; (4) fraction D of physiological whey (Fig. 1); (5) fraction D of mastitic whey (Fig. 4); (6) lactoferrin; (7) bovine immunoglobulin G; (8) fraction C of Fig. 3; (9) top fraction of peak C in lane 8; (10) commercial β-lactoglobulin A; (11) low molecular weight standards.

A scale-up method using affinity separation of β-lactoglobulin with all-*trans*-retinal immobilized on calcium biosilicate in fluidized bed columns gained a purification efficiency of greater than 95%. This time consuming multistep procedure with phosphate buffers of varying ionic strength yielded a mass fraction of 45–50% for β-lactoglobulin [18]. The purity of β-lactoglobulin desorbed from a bioaffinity column of all-*trans*-retinal immobilized on Celite varied between 73 and 94.5% depending on elution conditions. The highest purity was obtained if α-lactalbumin was totally desorbed before the β-lactoglobulin was displaced by higher concentrated phosphate buffer of pH 7.0 [9].

Final size exclusion chromatography is considered to be indispensable for receiving highly purified β-lactoglobulin regardless of the preceding step of purification [19]. The purity of β-lactoglobulin received from DEAE-Sepharose CL6B was improved by this way from 95.2 to 98.5% with a yield of 80%, which appears significantly higher compared to other reports [20,21].

Inflammatory events within the mammary gland increase the physiological amount of total milk protein by “leakage” via the paracellular route. Bovine serum albumin (BSA) is one of these proteins. It is reported to be eluated from hydroxyapatite at a fluoride concentration of about 0.13 M [14]. Its co-elution with the β-lactoglobulin/lactoferrin peak during hydroxyapatite chromatography may be due to the association presumably with one of these two proteins. Both, BSA and lactoferrin, having a similar molecular weight are not separated by size exclusion chromatography. However, in comparison to lactoferrin, bovine serum albumin

represented a minor component in the fraction eluted from Superdex 75 pg as shown by SDS-PAGE (Fig. 6). In mastitic milk, the concentration of lactoferrin increases manifold due to high amounts of lactoferrin released from activated leukocytes [22]. The small peak eluting first (A) in gel permeation chromatography contains immunoglobulin G as suggested from SDS-PAGE (Fig. 6).

The results show, that ceramic hydroxyapatite chromatography with sodium fluoride as a displacement agent can be used as an one-step-procedure on a laboratory scale to enrich β-lactoglobulin from either milk quality. With larger ceramic hydroxyapatite columns the yield of β-lactoglobulin could be increased. Highly purified β-lactoglobulin can then be prepared by a succeeding size exclusion chromatography.

References

[1] L.K. Creamer, L. Sawyer, in: H. Roginski, J.W. Fuquay, P.F. Fox (Eds.), Encyclopedia of Dairy Sciences, Academic Press, Amsterdam, 2003, p. 1932.
[2] R.M. Whitney, in: N.P. Wong (Ed.), Fundamentals of Dairy Chemistry, AVI Books, New York, 1988, p. 81.
[3] L. Sawyer, G. Kontoopidis, Biochim. Biophys. Acta 1482 (2000) 136.
[4] R. Aschaffenburg, J. Drewry, Biochem. J. 65 (1957) 273.
[5] R.J. Pearce, Aust. J. Dairy Technol. 38 (1983) 144.
[6] X. Felipe, A.J.R. Law, J. Dairy Res. 64 (1997) 459.
[7] R. Hahn, P.M. Schulz, C. Schaupp, A. Jungbauer, J. Chromatogr. A 795 (1998) 277.
[8] S.J. Gerberding, C.H. Byers, J. Chromatogr. A 808 (1998) 141.
[9] R.A. Heddleson, J.C. Allen, Q. Wang, H. Swaisgoog, J. Agric. Food Chem. 45 (1997) 2369.
[10] H.K. Vyas, J.M. Izco, R. Jiménez-Flores, J. Dairy Sci. 85 (2002) 1639.
[11] R. Rossano, A. D'Elia, P. Riccio, Protein Exp. Purif. 21 (2001) 165.
[12] S. Vogt, R. Freitag, J. Chromatogr. A 760 (1997) 125.
[13] R. Baeker, S. Haebel, K. Schlatterer, B. Schlatterer, Prostaglandins Other Lipid Mediat. 67 (2002) 75.
[14] M.J. Gorbunoff, Anal. Biochem. 136 (1984) 425.
[15] M.H. Bradford, Anal. Biochem. 205 (1976) 22.
[16] K.P. Campbell, D.H. MacLennan, A.O. Jorgensen, J. Biol. Chem. 258 (1983) 11267.
[17] J.M. Myers, A. Veis, B. Sabsay, A.P. Wheeler, Anal. Biochem. 240 (1996) 656.
[18] H.K. Vyas, J.M. Izco, R. Jiménez-Flores, J. Dairy Sci. 85 (2002) 1639.
[19] H.H.J. de Jongh, T. Gröneveld, J. de Groot, J. Dairy Sci. 84 (2001) 562.
[20] K.K. Fox, V.H. Holsinger, L.P. Posati, M.J. Pallansch, J. Dairy Sci. 50 (1967) 1363.
[21] S. Yoshida, J. Dairy Sci. 73 (1990) 2292.
[22] L. Sanchez, P. Aranda, M. Perez, M. Calvo, Biol. Chem. Hoppe-Seyler 369 (1988) 1005.