E-ISSN: 2602-2834

FOOD and HEALTH

ORIGINAL ARTICLE/ORİJİNAL ÇALIŞMA

FULL PAPER TAM MAKALE

COMPARISON OF DIFFERENT METHODS FOR BETA LACTOGLOBULIN ISOLATION

Ezgi Demir Özer[1], Zübeyde Öner[2]

[1] Niğde Ömer Halisdemir University, Faculty of Engineering, Department of Food Engineering, Niğde, Turkey

[2] Süleyman Demirel University, Faculty of Engineering, Department of Food Engineering, Isparta, Turkey

Received: 09.02.2017

Accepted: 02.07.2017

Published online: 06.11.2017

Corresponding author:

Ezgi Demir Özer, Niğde Ömer Halisdemir University, Faculty of Engineering, Department of Food Engineering, 51240, Niğde, Turkey

E-mail: ezgidemirozer@gmail.com

Abstract:

The aim of this study was to introduce a simple, high efficient and less expensive method for isolation of β-Lg from whey. Anion exchange chromatography, pepsin enzyme treatment and ultrafiltration tecniques were preferred for isolation process to compare differences. In addition to, centrifuge ultrafiltration techniques were using for the first time for isolation of β-Lg from whey. Physicochemical analysis of whey samples indicated that protein and β-Lg content in whey samples changed from 0.07 to 0.8% and 0.24 to 0.29 g/L, respectively. Treatment with the use of pepsin enzyme, anion exchanges chromatography and ultrafiltration techniques, resulted to β-Lg of 1.43, 6.56 and 43.59 folds respectively. Our results showed that ultrafiltration techniques are rapid and efficient that allows high protein yield and has advantages over other methods since it preserves the native structure of β-Lg. Additionally, when the enzymatic hydrolysis was used together with ultrafiltration technique, it was found efficient and pure than the enzymatic hydrolysis together with dialyse membrane. Also this study concluded that pepsin enzyme treatment and anion exchange chromatography are economic methods but they are not efficient enough and very time consuming. However isolation efficiency can be increased the use of isolation methods together.

Keywords: Beta lactoglobulin, Whey, Ultrafiltration, Anion exchange chromatography, Pepsin enzyme

Introduction

Whey is obtained as a by-product during cheese making and it has recognized as a valuable food ingredient with important nutritional and functional properties in the last decades. However, because it's low concentration of milk constituents (5-6% dry matter), whey has commonly considered waste or animal feed by providing amino acids required by the young animal (Aich et al., 2015). It consists of lactose, protein, minerals and organic acids (Morr & Ha, 1993). Whey proteins which are a diverse mixture of true proteins, peptides and non-protein (NPN) components, is a defined as the components that are soluble at pH 4.6 in their native form (Fox, 2003). Furthermore, whey is an important source of beta lactoglobulin (β-Lg), alfa lactoalbumin (α-La), bovine serum albumin (BSA) and immunoglobulins (Ig) (Yerlikaya, Kınık, & Akbulut, 2010). The most abundant whey protein is β-lactoglobulin (β-Lg), which consists of approximately 50-60% of whey proteins and 12% of the total proteins in milk (Outinen, 2010).

β-Lg is a small, soluble and globular protein that is a dimer at the normal pH of bovine milk with a molecular weight of 36 kDa and is a single chain polypeptide of 18 kDa comprising of 162 amino acid residues (Aich et al.,2015).Essential amino acids such as threonine, valine, isoleucine, leucine, tryptophan and lysine are composed % 45.68 of total amino acid composition of β-Lg (Young, 1994). β-Lg is a rich source of cysteine which is an essential amino acid for the synthesis of glutathione (Karagözlü & Bayarer, 2004). Five genetic variants of bovine and four genetic variants ovine β-Lg of which the phenotypes A and B are most predominant have been discovered. β-Lg exists in the solution as a dimer, with an effective molecular mass of about 36.6 kDa at the normal pH of milk (6.5 - 6.7) (Hernández-Ledesma, Recio, & Amigo, 2008). Furthermore, β-Lg is an important source of biologically active peptides. These peptides are inactive with the sequence of the precursor protein. But they can be released through 'in vivo' or 'in vitro' enzymatic proteolysis. These peptides also play important roles in the human health such as antihypertensive, antioxidant and in antimicrobial activities. Its opioid-like features gives it the ability to decrease body-cholesterol levels (Hernández-Ledesma et al., 2008).

β-Lg is involved with the transfer of passive immunity and the binding of retinol and fatty acids (De Wit, 1998; Yerlikaya et al., 2010). They have been shown to have inhibitory activity against angiotensin converting enzyme (ACE) when deriving various peptides of β-Lg derived from proteolytic digestion. β-lg can be used as an ingredient in the formulation of modern foods and beverages because of its high nutritional and functional value (Chatterton, Smithers, Roupas, & Brodkorb, 2006). Recently, researchers have shown interests in the bioactivities of β-Lg peptides. These peptides are inactive within the sequence of parent protein, and become activated once released during gastrointestinal digestion or during food processing. Bioactive peptides may act as regulatory compounds with hormone-like activity when they are released in the body (Hernández-Ledesma et al., 2008). As well as known its high value as a food ingredient and its technofunctional properties, it can be a significant health risk in patients allergic to milk (Stojadinovic et al., 2012). Also, there has been an increased interest in recent years in ways to isolation and purification of β-lg at laboratory and industrial-scale.

Some studies have been made to isolate this protein because of its superior nutritional and functional properties. Some methods have been used for isolation, such as the salting-out procedure, selective solubility in the presence of 3% w/w trichloroacetic acid (TCA), separation by ion-exchange chromatography, utilizing the differences in thermal stability in acidic conditions. (Bhattacharjee, Bhattacharjee, & Datta, 2006; Cheang & Zydney, 2003; Kinekawa & Kitabatake, 1996).

The aim of the present study was to investigate the isolation of β-lg from whey by using ultrafiltration process, pepsin enzyme treatment and anion exchange chromatography. In addition, the centrifugal ultrafiltration technique was used for the first time for β-lg isolation from whey in this study. These techniques compare the isolated β-lg retained purity degree, yield of isolate and native properties in terms of the different isolation techniques.

Materials and Methods

Fresh whey which was obtained from white cheese manufacturing process using pasteurized bovine milk was provide from the local dairy producer for each of three replications on separate production days. Three isolation methods which are ultrafiltration techniques, pepsin enzyme hy-

drolysis and anion exchange column chromatography were used for isolation of β-Lg. Ultrafiltration techniques were carried out on flat membrane ultrafiltration (Vivaflow 200 PES, Sartorius, Germany) and centrifugal ultrafiltration units (Vivaspin 15R, Sartorius, Germany). Anion exchange chromatography technique was performed by anion-exchange spin column (Vivapure Q mini H column, Sartorius, Germany) and 2-(Diethylamino)ethyl-Sephadex (DEAE) A-50 (Sigma Aldrich, USA). Porcine pepsin enzyme (0.8 IU/mg; Merck, USA) was used for pepsin enzyme treatment.

Physicochemical Analysis

Physicochemical properties of whey which are acidity (SH, pH), fat, protein, dry matter contents and β-Lg contents were determined (Lieske, Konrad, & Faber, 1997). Before the isolation process of β-Lg from whey, all whey samples centrifuged at 15333 x g, 4ºC for 15 min as a pretreatment procedure to remove fat and particulates (Lozano, Giraldo, & Romero, 2008). β-Lg content of whey in the beginning of isolation process and differences between protein content and effectiveness of isolation techniques were measured by using high-performance liquid chromatography (HPLC) (Shimadzu LC-20AT, Japan) which was performed on a Zorbax 300SB-C18 4.6 x 250mm (Agilent, USA) with a diode array detector.

Isolation of β-Lg by Ultrafiltration Techniques

Ultrafiltration methods were performed with flat membrane ultrafiltration and centrifugal ultrafiltration units. Flat membrane systems were Vivaflow 200 Poly(ether sulfones) (PES) (Sartorius Sedium). β-Lg of whey protein concentrate was pre-treated by fractionation of protein using two-stage ultrafiltration (UF) with 30 kDa and 10 kDa molecular weight cut-off (MWCO) (Bhattacharjee et al., 2006). After pre-treatment, whey samples were heated at 42ºC to improve the effectiveness of separation. This temperature was chosen to prevent the present proteins from being denatured. Whey samples were filtered on 30 kDa MWCO ultrafiltration systems and separated into retentate and filtrate (Filtrate-I). Then, these filtrate and retentate were filtered on 10 kDa MWCO ultrafiltration systems (Filtrate-II and retentate, respectively). Separation process with ultrafiltration systems was carried out under 2 bar pressure.

Centrifugal ultrafiltration units (Vivaspin 15R) were performed at 4000 x *g*, 15ºC for 30 min (Sigma 3K30). The amount and purity of β-Lg isolates were analyzed at 214 nm by HPLC. Samples were taken from the last concentrate isolates for the determination of protein profiles by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) (Laemmli, 1970).

Isolation of β-Lg by Anion Exchange Chromatography

The method used by Lozano et al. (2008) were modified and used for the isolation of β-Lg by anion exchange chromatography. The pH of free particulates was adjusted to 3.0 using concentrated phosphoric acid (85%, H_3PO_4, Sigma, Germany). Then, precipitation was performed with 50% ammonium sulfate at 4°C to obtain highly enriched fraction of β-Lg and precipate was obtained by centrifugation at 15330xg, 4ºC for 15 min. The obtained precipitate was dissolved in phosphate buffered saline (PBS) solution at the pH of 3.0. Precipitations were obtained from 50% salt. Then, samples were dialyzed using a 12-14 kDa cut off Spectra/Pors membrane against PBS solution pH 7.2. After dialysis, salt was added to 70%. The resultant precipitate was centrifuged at same centrifugation conditions and obtained supernatant which contained high β-lactoglobulin was dialyzed under the same conditions. Finally, obtained product was lyophilized and stored for anion-exchange chromatography.

Anion-exchange chromatography was performed on a column packed with DEAE Sephadex A-50. A 50 milliliters sample, which was reconstituted in distilled water to a final concentration of 1 g/ 10mL, was added to the column at a flow rate of 0.5mL/min. 18 fractions of β-Lg were collected using 15 mL volumes of 0-0.9 M graduated NaCl. Amount of β-Lg fractions were determined with spectrophotometric analyses (Lieske et al., 1997; Lozano et al., 2008). The amount and purity of β-Lg isolates were determined by HPLC. Samples were taken from the last concentrate isolates for the determination of protein profiles by SDS-Page (Laemmli, 1970).

Isolation of β-Lg by Pepsin Enzyme Treatment

Whey protein is sensitive to pepsin enzyme except β-Lg. Therefore, pepsin enzyme can be used for the isolation of β-Lg from whey (Kinekawa & Kitabatake, 1996). β-Lg was purified from whey by combining pepsin treatment and filtration system. After the pretreatment and preheating (37°C) process, porcine pepsin added to whey (15/100) and the mixture than incubated at pH 2, 37°C for

60 min. The protein fraction was collected by ammonium sulphate (75%) precipitation. After precipitation, the fraction was dialyzed against water using a 20 kDa pore size on the dialyse membrane or filtrated using a 30 kDa pore size on the UF membrane. The amount and purity of β-Lg isolates were determined by HPLC. Samples were taken from the last concentrate isolates for the determination of protein profiles by SDS-Page.

Characterization of Isolated β-Lg by HPLC

The amount and purity of β-Lg were determined by HPLC, as described by Elgar et al. (2000). The column was operated at room temperature (RT) and at a flow-rate of 1 ml/min. The column was equilibrated in 80% of solvent A (0.1%, v/v, TFA in Milli-Q water) and after a sample injection a 1-min isocratic period was applied, followed by a series of linear gradients, to 100% of solvent B (0.09%, v/v, TFA, 90%, v/v) as follows: 1 - 6 min. (20 - 40%), 6 - 16 min (40 - 45%), 16 - 19 min (45 - 50%), 19 - 20 min. (50%), 20 - 23 min (50–70%), and 23 - 24 min. (70 - 100%) . Shimadzu HPLC LC-20AT and DAD detector were used. Isolated samples were calculated via HPLC calibration curves which were made by using β-Lg standards.

Determination of Protein Profiles of Isolated β-Lg

Protein profiles of isolated β-Lg was determined by the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method were preferred (Laemmli, 1970). A Bio-Rad mini gel electrophoresis device equipped with a power supply (Power Pac 3000 - Bio-Rad Laboratories Ltd.,Hemel Hemstead, UK) was used to perform SDS page of whey samples and whey isolates. Discontinuous electrophoresis was conducted with 12% stacking gel and 5% running gel. Samples were dissolved in a sample buffer solution (50 mM Tris-HCl, 10% glycerol, 2% sodium dodecyl sulphate, 1.5% β-mercaptoethanol and 0.1% bromphenol blue, pH 6.8) and boiled at 100°C for 5 min. Gel was stained with Coomassie blue R-250 in methanol-acetic acid-water (4.5:1:4.5, v/v/v) and the excess dye was removed with the same ratio of methanol-acetic acid-water without dye and monitorised using a gel-imaging system (Biolab Uvitec BTS-20M) connected to computer. Sigma SDS7 was used as protein marker. The molecular weight of each protein band was matched to known standard proteins (Şanlidere Aloğlu, 2013).

Statistical Methods

The entire experiments were replicated three times. The statistical evaluation of the results was performed using the SPSS 18.0.0 (SPSS Inc., Chicago, USA). The generated data were analyzed by analysis of variance (ANOVA). Differences among mean values were established using the Tukey's HSD test ($p<0.05$).

Results and Discussion

Gross composition of bovine whey was as follows (mean±standard variation); 95.70% ±0.11 water, 4.93% ±0.95 total solids, 0.21% ±0.01 lactic acid, 0.77% ±0.02 protein, 0.47% ±0.12 fat and 0.43% ±0.12 ash. Chemical composition of whey used in this study was in accordance with previous studies (Lieske et al., 1997; Tunçtürk, Andiç, & Ocak, 2010). pH of whey was 5.51 ±0.11 and β-Lg content was 0.38 ±0.04 g/L. Some researchers have indicated that heat treatment could be effective on content of β-Lg. Because the heat treatment when is above 70ºC, β-Lg undergoes an irreversible polymerization (Reddy, Kella, & Kinsella, 1988). Similarly, Outinen (2010) has reported that high temperature heat treatment of cheese milk significantly decreased β-Lg content of whey (Outinen, 2010). Furthermore some researcher have reported that pretreatment of milk such as homogenization and pressure can be affect chemical properties of whey (Jang & Swaisgood, 1990).

Isolated β-lg by flat and centrifugal ultrafiltration technique are presented in table 1. The high molecular weight proteins such as BSA, immunoglobulin and lactoferrin were removed by 30 kDa ultrafiltration system. Bhattacharjee et al. (2006) have reported similar finding.

Table 1. β-lg concentration for flat (A) and centrifugal (B) ultrafiltration system

	β-lg Concentration (mg/mL)	
	Flat Ultrafiltration	Centrifugal Ultrafiltration
Whey	0.38[a]	0.38[a]
Filtrate-I	14.31[a]	7.94[b]
Filtrate-II	15.78[a]	9.18[b]
Retant	0.08[a]	0.04[a]

a,b (→) Different letters within a row are significantly different.

The result of the peak area calculations showed that centrifugal ultrafiltration units have low concentration of β-lg than flat system. Because the centrifugal ultrafiltration units used for the final sample concentration, the serum proteins were

separated from β-lg by centrifugal ultrafiltration units.

Enzymatic hydrolysis can be used for isolation and purification of milk proteins. Caseins are highly digestible by proteases compared to whey protein and may be selectively digested by proteases converting them to low-molecular-weight fractions that can be sieved out through membrane filters (Guo, Fox, Flynn, & Kindstedt, 1995). Whey proteins are hydrolyzed by pepsin enzyme, except β-Lg. Because β-Lg is fairly resistant to digestion due to their globular structure and have different susceptibility to digestion by pepsin. The purification of β-Lg from contaminating proteins which are α-La and BSA was based on the selective hydrolysis by pepsin at 37 °C and pH 2. β-lactoglobulin is resistant to peptic digestion, but the enzymic hydrolysis of contaminating proteins, α-lactalbumin and traces of serum albumin occurs in these conditions (Sannier, Bordenave, & Piot, 2000). Therefore, the enzymatic hydrolysis technique was used together with ultrafiltration technique in order to remove α-lactalbumin and traces of serum albumin.

When the enzymatic hydrolysis was used together with ultrafiltration technique, it was found efficient (1.43 fold) and pure than the enzymatic hydrolysis together with dialyse membrane (1.32 fold).

The last isolation method was anion exchange chromatography via vivapure Q Mini H column. After anion exchange chromatography, eighteen fractions were collected at the end of column as the elution occurs and all collected fractions analysed by HPLC. According to HPLC analysis, it was determined that the third fraction has α-La and β-Lg. At a later stage, α-La and β-Lg contained in third fraction was separated from each other by using the Vivapure Q Mini H column to obtain pure β-Lg (Figure 1).

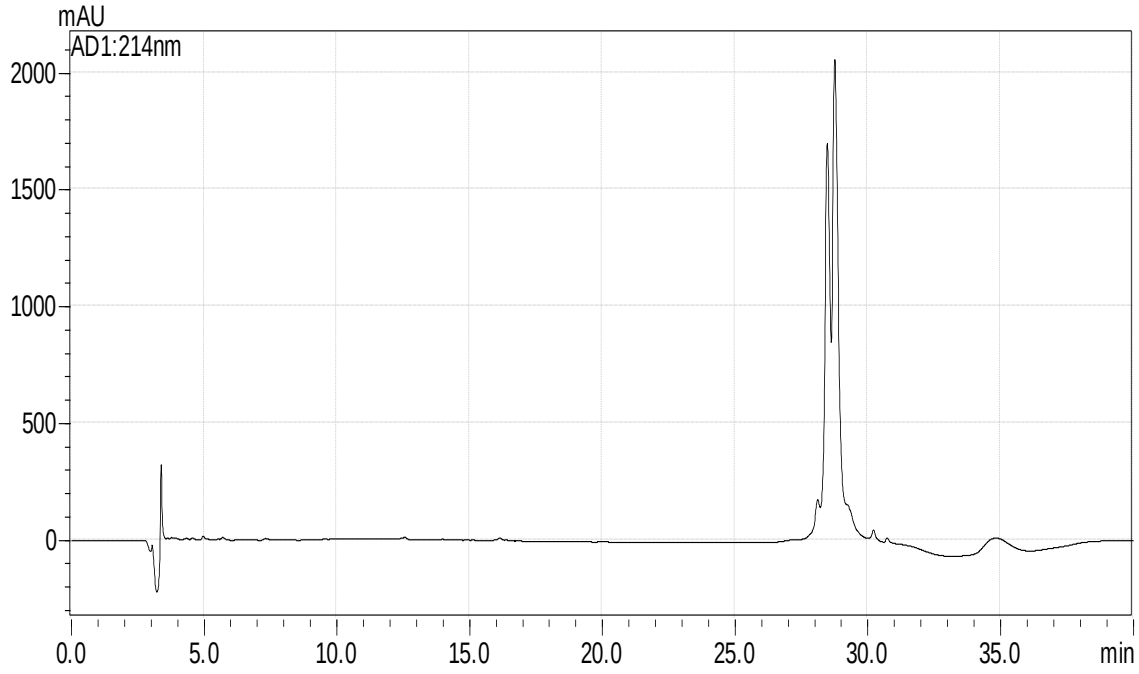
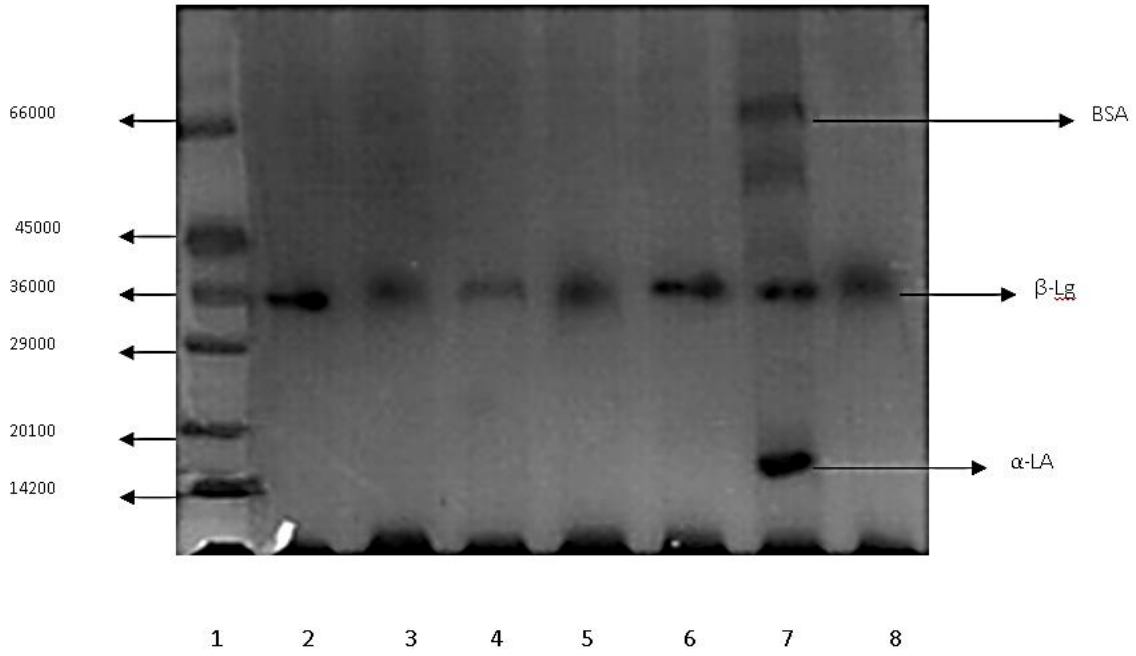


Figure 1. Chromotogram using Vivapure Q Mini H column

Consequently β-Lg isolated was obtained 1.43, 6.56 and 43.59 fold respectively by pepsin enzyme, anion exchange chromatography treatment and ultrafiltration techniques (Table 2). Standard curves of β-Lg were obtained by using LC Solution Software which is analysis program of HPLC. β-Lg amount of all process samples were calculated using these curves. We can say ultrafiltration technique is more effective (43.59 fold) than other isolation techniques. Cheang and Zydney (2003, 2004) were able to obtain 100-fold purification and greater than 90% recovery of β-Lg from a binary mixture with α-La by ultrafiltration techniques. Recently isolation of α-La from sweet whey was achieved through a novel approach involving membrane filtrations and a triptych treatment (Bottomley, 1991). Bottomley (1991) described a two-stage membrane process for obtaining concentrates enriched in whey protein. The separation principle is differences in molecular weight of proteins and a likely challenge is lack of adequate selectivity for the separation of β-Lg and α-La due to the very similar molecular weight of these proteins (Cheang & Zydney, 2003; Fox, 2003; Muller, Chaufer, Merin, & Daufin, 2003). Ye and others were obtained 1260 mg α-La, 1290 mg β-Lg B and 2280 mg β-Lg A from 1 liter rennet whey using ion exchange chromatography (Ye, Yoshida, & Ng, 2000).

All isolate and whey samples are analyzed by SDS-PAGE (Figure 2). The second band has a biggest area as a calculation. Optic densitometers of SDS-PAGE band is calculated by 1.45 ImageJ (Table 3).



1: Marker, 2: Retante which was obtained filtrate 1 used as a sample for 10000MWCO, 3: Filtrate obtained after 30000 MWCO (Filtrate 1.), 4: After dialysis step is in Application of Pepsin enzyme, 5: After ultrafiltration step is in application of Pepsin enzyme, 6: The fraction of anion exchange chromatography, 7: Whey sample, 8: Standard of β-Lg.

Figure 2. SDS Page Bands

Table 2. β-Lg purification rate results after HPLC analyses (g/L-p<0,05)

	β-lg Concentration in Final Volume (g/L)	Fold Purificai-ton
Whey	0.389 ±0.015	1
Pepsin enzyme treatment with ultrafiltion	0.557 ±0.04[c]	1.43
Anion exchange chromatography	2.552 ±0.025[b]	6.56
Ultrafiltration	16.958 ±1.275[a]	43.59

Table 3. Optic densitometers results of SDS PAGE

Techniques	Area	%
Marker	270	8.38
Retante which was obtained filtrate 1 used as a sample for 10000MWCO	545	16.92
Filtrate obtained after 30000 MWCO (Filtrate I.)	384	11.92
The Pepsin enzyme treatment combined with dialysis	341	10.59
The Pepsin enzyme treatment combined with ultrafiltration technique	418	12.98
The fraction of anion exchange chromatography	504	15.65
Whey sample	325	10.09
Standard of β-Lg	434	13.47
Total area	3221	

We can obtain denser β-Lg all techniques, as shown in Figure 2. But ultrafiltration techniques have good yield and purity according to HPLC profile analysis results (Table 2). Our results showed that ultrafiltration techniques are rapid and efficient that allows high protein yield and has advantages over other methods since it preserves the native structure of beta lactoglobulin. Also our ultrafiltration techniques could remove BSA, however the whey sample had BSA band. Our result is agreement with Bhattacharjee et al. (2006). They showed that proteins which have high molecular weight like BSA, immunoglobulin and lactoferrin were removed on first step (30 kDa).

Conclusion

This study concluded that pepsin enzyme treatment and anion exchange chromatography are not efficient enough and very time consuming although these are economic methods. However, ultrafiltration techniques have provided higher purity and yield for isolation of β-Lg. Furthermore, β-Lg doesn't denaturate and contains any contaminants such as other proteins or salts by ultrafiltration techniques. This research suggests that ultrafiltration techniques could be applied on high purity and yield of β-Lg isolation. Therefore further investigation is needed to large scale isolation process.

Acknowledgements

This work was sponsored by Scientific Research Projects, with Coordination from Süleyman Demirel University. The project number is 2727-YL–11.

References

Aich, R., Batabyal, S. & Joardar, S.N. (2015). Isolation and purification of beta-lactoglobulin from cow milk. *Veterinary World*, 8, 621-624.

Bhattacharjee, S., Bhattacharjee, C. & Datta, S. (2006). Studies on the fractionation of β-lactoglobulin from casein whey using ultrafiltration and ion-exchange membrane chromatography. *Journal of Membrane Science*, *275*(1), 141-150.

Bottomley, R. (1991). Process for obtaining concentrates having a high α-lactalbumin content from whey. US Patent, Patent number: 5,008, 376.

Chatterton, D.E., Smithers, G., Roupas, P. & Brodkorb, A. (2006). Bioactivity of β-lactoglobulin and α-lactalbumin—Technological implications for processing. *International Dairy Journal*, 16(11), 1229-1240.

Cheang, B., & Zydney, A. L. (2003). Separation of α-lactalbumin and β-lactoglobulin using membrane ultrafiltration. *Biotechnology and Bioengineering*, 83(2), 201-209.

Cheang, B., & Zydney, A. L. (2004). A two-stage ultrafiltration process for fractionation of whey protein isolate. *Journal of Membrane Science*, 231(1), 159-167.

De Wit, J. (1998). Nutritional and functional characteristics of whey proteins in food products. *Journal of Dairy Science*, 81(3), 597-608.

Elgar, D.F., Norris, C.S., Ayers, J.S., Pritchard, M., Otter, D.E., & Palmano, K.P. (2000). Simultaneous separation and quantitation of the major bovine whey proteins including proteose peptone and caseinomacropeptide by reversed-phase high-performance liquid chromatography on polystyrene–divinylbenzene. *Journal of Chromatography A*, 878(2), 183-196.

Fox, P. (2003). Milk proteins: general and historical aspects *Advanced Dairy Chemistry—1 Proteins* (pp. 1-48): Springer. ISBN 978-1-4419-8602-3

Guo, M., Fox, P., Flynn, A., & Kindstedt, P. (1995). Susceptibility of β-Lactoglobulin and Sodium Caseinate to Proteolysis by Pepsin and Trypsin. *Journal of Dairy Science*, 78(11), 2336-2344.

Hernández-Ledesma, B., Recio, I., & Amigo, L. (2008). β-Lactoglobulin as source of bioactive peptides. *Amino Acids*, 35(2), 257-265.

Jang, H.D., & Swaisgood, H.E. (1990). Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. *Journal of Dairy Science*, 73(4), 900-904.

Karagözlü, C., & Bayarer, M. (2004). Peyniraltı suyu proteinlerinin fonksiyonel özellikleri ve sağlık üzerine etkileri. *Ege Üniv. Ziraat Fakültesi Dergisi,* 41, 197-207.

Kinekawa, Y.-I., & Kitabatake, N. (1996). Purification of β-Lactoglobulin from Whey Protein Concentrate by Pepsin Treatment. *Journal of Dairy Science*, 79(3), 350-356.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(5259), 680-685.

Lieske, B., Konrad, G., & Faber, W. (1997). A new spectrophotometric assay for native β-lactoglobulin in raw and processed bovine milk. *International Dairy Journal*, 7(12), 805-812.

Lozano, J.M., Giraldo, G.I., & Romero, C. M. (2008). An improved method for isolation of β-lactoglobulin. *International Dairy Journal*, 18(1), 55-63.

Morr, C., & Ha, E. (1993). Whey protein concentrates and isolates: processing and functional properties. *Critical Reviews in Food Science & Nutrition*, 33(6), 431-476.

Muller, A., Chaufer, B., Merin, U., & Daufin, G. (2003). Purification of alpha-lactalbumin from a prepurified acid whey: Ultrafiltration or precipitation. *Le Lait*, 83(6), 439-451.

Outinen, M. (2010). Effect of pre-treatment of cheese milk on the composition and characteristics of whey and whey products. TKK Dissertation 212, Aalto University, p. 44. ISBN 978-9-5260-3006-7

Reddy, I.M., Kella, N.K., & Kinsella, J.E. (1988). Structural and conformational basis of the resistance of beta-lactoglobulin to peptic and chymotryptic digestion. *Journal of Agricultural and Food Chemistry*, 36(4), 737-741.

Sannier, F., Bordenave, S., & Piot, J. (2000). Purification of goat β-lactoglobulin from whey by an ultrafiltration membrane enzymic reactor. *Journal of Dairy Research*, 67(01), 43-51.

Şanlidere Aloğlu, H. (2013). The effect of various heat treatments on the antioxidant capacity of milk before and after simulated gastrointestinal digestion. *International Journal of Dairy Technology*, 66(2), 170-174.

Stojadinovic, M.,Burazer, L., Ercili-Cura,D., Sancho, A.,Buchert, J.,Velickovica T.C. & Stanic-Vucinica, D. (2012). One-step method for isolation and purification of native β-lactoglobulin. Journal of the Science of Food and Agriculture, 92, 1432-1440

Tunçtürk, Y., Andiç, S., & Ocak, E. (2010). Homojenizasyon ve pastörizasyonun Beyaz Peynir ve Peyniraltı Suyu Bileşimine Etkisi. *Gıda/The Journal of Food*, 35(5), 339-345.

Ye, X., Yoshida, S., & Ng, T. (2000). Isolation of lactoperoxidase, lactoferrin, α-lactalbumin, β-lactoglobulin B and β-lactoglobulin A from bovine rennet whey using ion exchange chromatography. *The International Journal of Biochemistry & Cell Biology*, 32(11), 1143-1150.

Yerlikaya, O., Kınık, Ö., & Akbulut, N. (2010). Peyniraltı Suyunun Fonksiyonel Özellikleri ve Peyniraltı Suyu Kullanılarak Üretilen Yeni Nesil Süt Ürünleri. *Gıda/The Journal of Food, 35*(4), 289-296.

Young, V. R. (1994). Adult amino acid requirements: the case for a major revision in current recommendations. *The Journal of Nutrition*, 124(8 Suppl), 1517S-1523S.