Calcium-binding Peptides Derived from Tryptic Hydrolysates of Cheese Whey Protein

S. B. Kim and J. W. Lim*

Dairy Science Major, Division of Animal Science and Technology, College of Agriculture and Life Sciences
Gyeongsang National University, Institute of Agriculture and Life Sciences, Jinju, Gyeongnam 660-701, Korea

ABSTRACT : The purpose of this research was to investigate the potential use of cheese whey protein (CWP), a cheese by-product. The physiological activity of calcium-binding peptides in CWP may be used as a food additive that prevents bone disorders. This research also examined the characteristics of calcium-binding peptides. After the CWP was heat treated, it was hydrolyzed by trypsin. Then calcium-binding peptides were separated and purified by ion-exchange chromatography and reverse phase HPLC, respectively. To examine the characteristics of the purified calcium-binding peptides, amino acid composition and amino acid sequence were analyzed. Calcium-binding peptides with a small molecular weight of about 1.4 to 3.4 kDa were identified in the fraction that was flowed out from 0.25 M NaCl step gradient by ion-exchange chromatography of tryptic hydrolysates. The results of the amino acid analysis revealed that glutamic acid in a calcium-binding site took up most part of the amino acids including a quantity of proline, leucine and lysine. The amino acid sequence of calcium-binding peptides showed Phe-Leu-Asp-Asp-Asp-Leu-Thr-Asp and Ile-Leu-Asp-Lys from α-LA and Ile-Pro-Ala-Val-Phe-Lys and Val-Tyr-Val-Glu-Glu-Leu-Lys from β-LG. *(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 10 : 1459-1464)*

Key Words : Cheese Whey Protein, Calcium-binding Peptide, Tryptic Hydrolysate

INTRODUCTION

Milk proteins and milk protein hydrolysis products are an excellent source of calcium because they are capable of the absorption and bioavailability of calcium (Wong et al., 1978; Harwalkar and McMahon, 1993). Recently, with the increasing number of the aged, there is a growing interest in calcium supplementary medicine to treat and prevent bone diseases. Intake of calcium increases the bone density of small children, and calcium also is essential among middle-aged people to prevent osteoporosis (Renner, 1994). Calcium deficiency often causes osteoporosis, hypertension, and large intestine cancer (Avioli, 1984; Lipkin and Newmark, 1985; Spencer and Kramer, 1986; Osborne et al., 1996).

Studies showed that calmodulin and vitamin D-dependent calcium-binding protein are the main source of calcium supply (Wasserman et al., 1978; Wang and Waisman, 1979). However, CN and whey protein (WP) also seem to have calcium-binding sites since they are the main components of milk protein (Stuart et al., 1986; Jeyarajah and Allen, 1994; Bennett et al., 2000).

Recently, casein phosphopeptides (CPP) derived from CN was found to have many phosphoseryl groups that prevent calcium phosphate deposition. CPP results in effective calcium absorption inside intestines (Kitts and Yuan, 1992). Furthermore, WP also has a site that binds calcium with β-LG, α-LA, and lactoferrin (Stuart et al., 1986; Jeyarajah and Allen, 1994; Feng et al., 1995; Kim et al., 2004). Especially, α-LA has a strong calcium-binding sites (Hiraoka et al., 1980; Permyakov et al., 1981).

There has been much research about purifying and separating CPP (Reynolds, 1997; Vegarud et al., 2000). However, there are many phases to pass through to purify and separate them and it is very expensive.

The objectives of this research were to separate and purify calcium-binding peptides from the peptide derived from tryptic hydrolysates of CWP and investigate their characteristics.

MATERIALS AND METHODS

Substrates and enzymes

Fresh Holstein milk was obtained from a Gyeongsang National University livestock farm. Cheddar cheese was manufactured according to the methods of Kosikowski (1982), and CWP separated from the cheese process contained 1.03% protein, 4.26% lactose, 0.7% ash and 93.53% moisture (determined by AOAC method, 1990). Defatting and demineralization of CWP were performed by ultracentrifuge (Supra 25 K, Hanil Sci., Korea) and dialysis (Sigma Co., USA), respectively. CWP was adjusted to pH 8.0 through the addition of 0.5 N NaOH and then heat-treated for 10 min at 100°C.

Trypsin (Bovine pancreas, activity 3.3 Anson units/g protein) was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark).

Tryptic hydrolysis and selective precipitation of calcium-binding peptides

Hydrolysis of heated-CWP was determined by the

* Corresponding Author: Jong-Woo Lim. Tel: +82-55-751-5415, Fax: +82-55-751-5410, E-mail: limjw@nongae.gsnu.ac.kr
Received February 20, 2004; Accepted June 12, 2004

methods described by Adamson and Reynolds (1996). Trypsin was added to the reaction mixture of a enzyme to the substrate (E:S) ratio of 1:100 (wt/wt, protein basis). Hydrolysis was performed at 50°C for 4 h with the pH maintained at 8.0±0.1 by the addition of 0.5 N NaOH using a pH-stat (Metrohm Ltd., Herisan, Switzerland). The enzyme was inactivated by heating for 10 min at 90°C. The calcium-binding peptide was precipitated from the hydrolysate by the addition of 10% (wt/vol) calcium chloride to a 100 mM final concentration and then ethanol to a 50% (vol/vol) final concentration. The suspension was centrifuged (12,000×g) and the supernatant discarded. The precipitate was freeze-dried and stored at -20°C.

Separation of calcium-binding peptides

Separation of calcium-binding peptides in tryptic hydrolysates adopted the method of Rose et al. (1969). DEAE-cellulose (Sigma Co., USA) was equilibrated at 500 mL of 20 mM Tris-HCl buffer (pH 7.8), and the slurry of equilibrated DEAE-cellulose was packed in a column (20×2.5 cm). The tryptic hydrolysates were dissolved in the same buffer (pH 7.8) and loaded on a column and then eluted by a step gradient with the same buffer containing 0.25 M, 0.5 M, 0.75 M and 1 M NaCl. The flow rate was 3 mL/min, fraction volume 15 mL/tube, and elution was monitored at 280 nm. The injection volume was 40 mL, containing about 200 mg of protein. The samples were filtered through 0.5 μm syringe filters prior to application to the column.

Tricine SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

Tricine SDS-PAGE of calcium-binding peptides was performed according to the procedure of Schagger and von Jagow (1987). The 10% acrylamide gels contained 1.0 M Tris-HCl, pH 8.45, and 0.1% SDS. The anode buffer was 200 mM Tris-HCl, pH 8.9; and the cathode buffer was 100 mM Tris-HCl, pH 8.25, 100 mM tricine and 0.1% SDS.

Reverse phase HPLC

Peptides in heated-CWP hydrolysates were analyzed by RP-HPLC on a Nucleosil (Nucleosil C-18 5 Micron, Alltech Associates, Inc., USA) C-18 column (4.6×250 mm), equilibrated with solvent A [0.1% trifluoroacetic acid (TFA) in H_2O] and eluted with a linear gradient to solvent B (0.1% TFA in acetonitrile) for 40 min. Runs were conducted at room temperature using a Dionex HPLC system (ASI 100, Dionex Co., USA) at a flow rate of 1.0 mL/min, and the absorbance of the column elute was monitored at 214 nm. The injection volume was generally 10 μL, and the concentration of peptide material applied was approximately equivalent to 0.5 mg/mL protein. The samples were filtered through a 0.2 μm syringe filters prior to application to the C-18 column.

Preparative HPLC

A large-scale separation of the fractions was performed by preparative HPLC (Delta Prep 4000, Waters Co., USA) using a C-18 column (Cosmosil 15C18-AR II, 28×250 mm) at a flow rate of 150 mL/min. The column was equilibrated with solvent A (0.1% TFA in H_2O) and eluted with a linear gradient to solvent B (0.1% TFA in acetonitrile) for 40 min. The injection volume was 15 mL, and the absorbance was measured at 214 nm.

Calcium-binding ability

The calcium-binding abilities of tryptic hydrolysate fractions were determined by Ion chromatography on an IonPac CS12A column. Runs were conducted at room temperature using a Dionex IC system (DX120, Dionex Co., USA) at a flow rate of 1.0 mL/min and eluted with 18 mM of methane-based sulfuric acid. The injection volume was 25 μL, and six cations (DIONEX P/N 43162) were used as a standard. All samples were filtered through a 0.2 μm syringe filter prior to application to the column. The calcium-binding abilities of tryptic hydrolysate fractions were expressed as mM of calcium bound in mg of protein.

Amino acid analysis

The amino acid analysis was performed by the method of Moore et al. (1958). The purified calcium-binding peptides (exactly 1 mg of protein) were dialyzed exhaustively against distilled water and lyophilized, and then hydrolyzed in 1 mL of 6 N HCl in evacuated tubes at 110°C for 24 h. After being concentrated by speed vacuum concentration (MAXI-DRY PLUS, Heto-Holten A/S., Denmark), the sample was dissolved in 0.2 M sodium citrate loading buffer (pH 2.2), and then filted through a 0.2 μm syringe filters. The analysis of amino acid was carried out on an amino acid analyzer (Biochem 20, Pharmacia, Sweden).

Amino acid sequence

The N-terminal sequence of the purified calcium-binding peptides was identified by sequence analysis with a protein sequencer (962592A, Perkin-Elmer, USA). If the N-terminus of a protein was blocked and could not be sequenced, the protein was subjected to trypsin digestion followed by peptide sequencing (Shaw et al., 1993).

Protein determination

Protein concentration in the tryptic hydrolysates and fractions was determined by the dye-binding method of Bradford (1976). BSA was used as the standard.

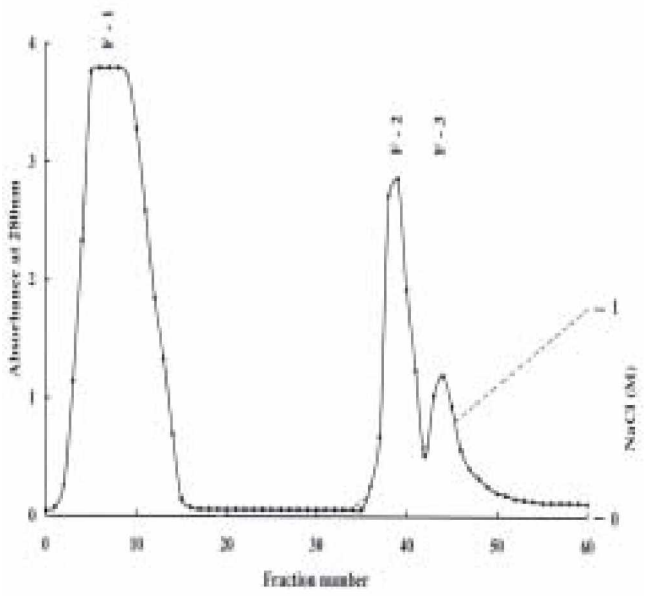

Figure 1. Ion-exchange chromatograms of heated-CWP hydrolysates by trypsin. Legend: F-1 (unbounded fraction), F-2 (0.25 M NaCl step gradient fraction) and F-3 (0.5 M NaCl step gradient fraction).

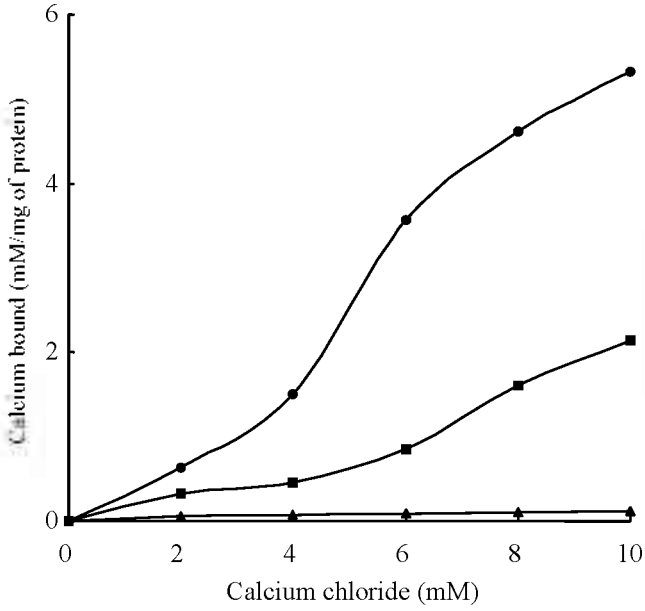

Figure 2. Calcium-binding ability of fractions eluted with 20 mM Tris-HCl buffer containing 0, 0.25 and 0.5 M NaCl from tryptic hydrolysate. Legend: F-1 (▲), F-2 (●) and F-3 (■).

RESULTS AND DISCUSSION

Separation, identification, and purification of calcium-binding peptides

Figure 1 shows the ion-exchange chromatogram of tryptic hydrolysates. The eluted F-1, F-2 and F-3 from 0 M, 0.25 M and 0.5 M NaCl step gradient all had respective peaks.

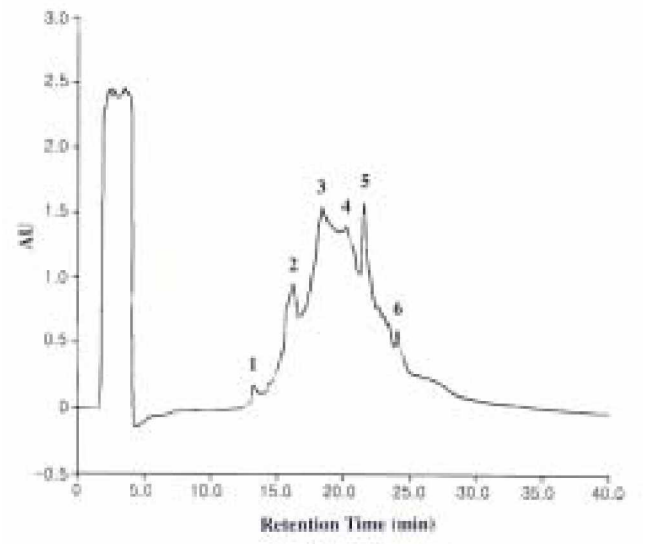

Figure 3. Preparative HPLC chromatogram of tryptic hydrolysate fraction 2 by ion-exchange chromatography.

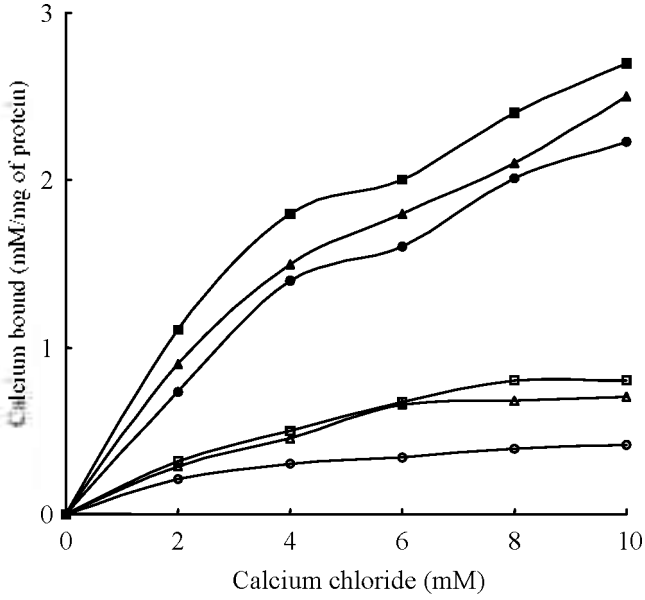

Figure 4. Calcium-binding ability of fractions by preparative HPLC from tryptic hydrolysates. Legend: peak 1 (○), peak 2 (□), peak 3 (●), peak 4 (▲), peak 5 (■) and peak 6 (△).

The diatomic calcium electrolyte of F-1, F-2 and F-3 was measured by ion chromatography tagged with calcium affinity column to confirm calcium-binding abilities as shown in Figure 2. The calcium-binding ability of F-2 increased rapidly beginning at $CaCl_2$ 2 mM. F-3 appeared to increase steadily, and F-1 showed no changes. In this experiment, calcium-binding ability was highest in F-2 from 0.25 M NaCl step gradient. These results are similar to those of Friedlander and Norman (1980) who reported on calcium-binding abilities in fraction from 0.22 M NaCl gradient by ion-exchange chromatography.

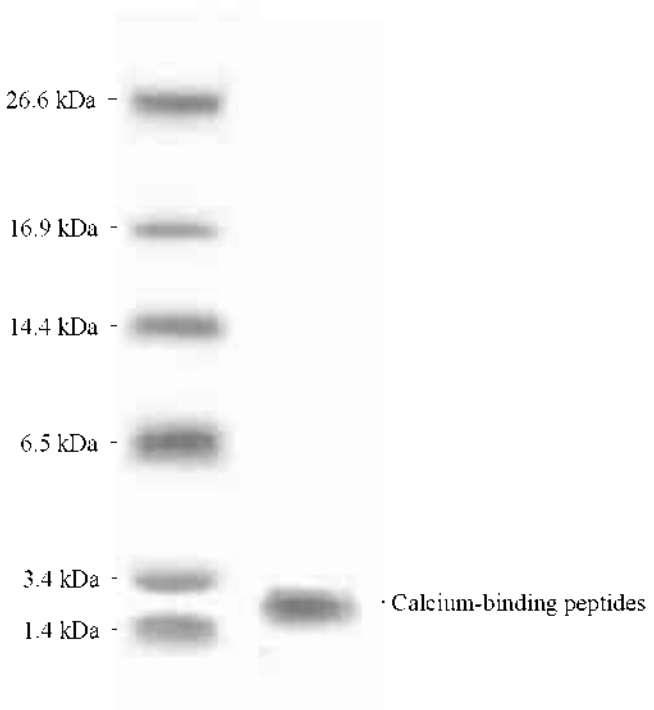

Figure 5. SDS-PAGE patterns of 5 kDa molecular weight cut off (MWCO) treatments in peak (3, 4 and 5) by preparative HPLC. A: Polypeptide standard marker (Bio-Rad, USA); triosephosphate isomerase (26.6 kDa), myoglobin (16.9 kDa), α-lactalbumin (14.4 KDa), aprotinin (6.5 kDa), insulin, β chain, oxidized (3.4 kDa) and bacitracin (1.4 kDa). B: peak 3, 4 and 5 by 5 kDa MWCO.

F-2, with calcium-binding ability purified by RP-HPLC, was fractionated into 6 fractions (Figure 3). After carrying out a large-scale separation, the calcium-binding ability was identified by ion chromatography. As a result, fractions 3, 4, and 5 seemed to have superb calcium-binding ability (Figure 4).

The fractions that have superb calcium-binding abilities were cut off to the size under 5 kDa [using by concentrator (Sartorius, Membrane 5 kDa MWCO PES., USA)]-the size in which physiological activity appears. Figure 5 shows that the molecules have a small molecular weight of about 1.4 to 3.4 kDa.

Amino acid composition of calcium-binding peptides

Table 1 shows the amino acid compositions of calcium-binding peptides. This experimental material CWP had the highest amount of Glu (17.80%) and the lowest amount of Cys (0.68%). Other amino acids were distributed evenly. These results are similar to the reports of Turgeon and Gauthier (1990) and Pintado et al. (1999) on amino acid compositions of CWP. In addition, the amino acid composition of calcium-binding peptides had the most Glu (30.08%) but did not any Thr, Ser and Met. These results are similar to the N-terminal amino acid composition of calcium-binding proteins in bovine intestines, which was researched by Fullmer and Wasserman (1981). Their studies

Table 1. Amino acid composition of CWP and calcium-binding peptides[1]

Amino acids	Cheese whey protein	Calcium-binding peptides[2]
Asp	7.55	4.21
Thr	8.49	ND[3]
Ser	6.53	ND[3]
Glu	17.80	30.08
Pro	8.27	16.25
Gly	3.87	3.85
Ala	9.07	8.37
Val	4.90	0.72
Cys	0.68	0.35
Met	1.66	ND[3]
Ile	4.93	8.65
Leu	10.36	12.92
Tyr	1.73	1.08
Phe	2.81	3.47
Lys	8.29	7.12
His	1.42	1.63
Arg	1.63	1.29
Total mol. ratio	100.00	100.00

[1]Results from acid hydrolysis for 24 h are given in molar ratios (%).
[2]Below the 5 kDa. [3]Not determined.

show that there are no Lys, Met, Trp, His and Arg.

The high percentage of Glu in calcium-binding peptides, in comparison with CWP, would be predicted that because trypsin cannot isolate amino acids with many branches. Glu, Pro and Leu were abundant, and Lys (7.12%) also taked up a high percentage.

Bovine α-LA is known to have strong calcium-binding sites with Asp and Lys as binding residues (Anderson et al., 1997; Veprintsev et al., 1999; Noyelle and van Dael, 2002). The results of this experiment are similar to those in the report of Tamura et al. (1982) who stated that Asp, Glu, Leu and Lys were abundant in the two materials, which showed calcium-binding ability in milk protein. Similarly, Tuan et al. (1978) has also reported on the acidic amino acids (Asp and Glu), basic amino acids (Lys and Arg) being the most abundant in calcium-binding proteins of chick chorioallantoic membrane. In general, proteins having the ability to interact with calcium ion have side chains composed of carbonyl, carboxyl, and alcohol functional groups (e.g., Asp, Asn, Glu, Ser and Thr) (Nemirovskiy and Gross, 2000).

Amino acid sequence of calcium-binding peptides

Calcium-binding peptides appeared to have 6 peaks by RP-HPLC (Figure 6). Each was separately fractionated, and the amino acid sequence was examined starting from the N-terminus (Table 2).

In peak 3, the strong calcium-binding sites with Asp (82, 84, 87), which are originated from α-LA, were detected. Peaks 1, 4, and 6 also appeared to have calcium-binding sites with Lys, Leu and Glu. New sites were found in peaks

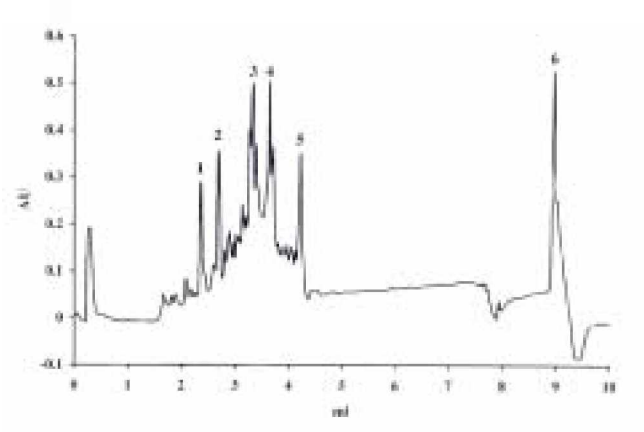

Figure 6. RP-HPLC chromatogram of calcium-binding peptides.

Table 2. Amino acid sequence of calcium-binding peptides

Peak no.[1]	Sequence	Origin
1	Ile-Pro-Ala-Val-Phe-Lys	78-83 (residual β-LG)
2	ND[2]	
3	Phe-Leu-Asp-Asp-Asp-Leu-Thr-Asp...	80-87...(residual α-LA)
4	Ile-Leu-Asp-Lys	95-98 (residual α-LA)
5	ND[2]	
6	Val-Tyr-Val-Glu-Glu-Leu-Lys	41-47 (residual β-LG)

[1] Peak number referred to Figure 6. [2] Not determined.

1 and 6 that have calcium-binding abilities, which were originated from β-LG. This shows the possibilities that β-LG may also have calcium-binding sites.

CONCLUSIONS

This research has revealed the characteristics of calcium-binding peptides originated from tryptic hydrolysates in cheese whey protein after separating and purifying them. Our results show that calcium-binding peptides are applicable in therapeutic formula concerning osteoporosis. They are also good for food additives that utilize cheese by-product to enhance calcium supply within the human body.

REFERENCES

Adamson, N. J. and E. C. Reynolds. 1996. Characterization of casein phosphopeptides prepared using alcalase. Determination of enzyme specificity. Enzyme Microb. Tech. 19:202-207.

Anderson, P. J., C. L. Brooks and L. J. Berliner. 1997. Functional identification of calcium binding residues in bovine α-lactalbumin. Biochemistry. 36:11648-11654.

AOAC. 1990. Official Methods of Analysis. 15th edn. Association of Official Analytical Chemists, Arlington, Verginia.

Avioli, L. V. 1984. Calcium and osteoporosis. Annu. Rev. Nutr. 4:471-491.

Bennett, T., A. Desmond, M. Harrington, D. McDonagh, R. FitzGerald, A. Flynn and K. D. Cashman. 2000. The effect of high intakes of casein and casein phosphopeptide on calcium absorption in the rat. Br. J. Nutr. 83:673-680.

Bradford, M. M. 1976. A rapid and sensitive methods for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Feng, M., L. van der Does and A. Bantjes. 1995. Preparation of apo-lactoferrin with a very low iron saturation. J. Dairy Sci. 78:2352-2357.

Friedlander, E. J. and A. W. Norman. 1980. Purification of chick intestinal calcium binding protein. Methods Enzymol. 67:504-508.

Fullmer, C. S. and R. H. Wasserman. 1981. The amino acid sequence of bovine intestinal calcium-binding protein. J. Biol. Chem. 256:5669-5674.

Harwalkar, V. R. and D. J. McMahon. 1993. Symposium: Biological and food functional characterizations of milk protein hydrolysis products. J. Dairy Sci. 76:300.

Hiraoka, Y., T. Segawa, K. Kuwajima, S. Sugai and N. Murai. 1980. α-Lactalbumin: A calcium metalloprotein. Biochem. Biophys. Res. Commun. 95:1098-1104.

Jeyarajah, S. and J. C. Allen. 1994. Calcium binding and salt-induced structural changes of native and preheated β-lactoglobulin. J. Agric. Food Chem. 42:80-85.

Kim, S. B., H. S. Shin and J. W. Lim. 2004. Separation of calcium-binding protein derived from enzymatic hydrolysates of cheese whey protein. Asian-Aust. J. Anim. Sci. 17:712-718.

Kitts, D. D. and Y. V. Yuan. 1992. Caseinophosphopeptides and calcium bioavailability. Trends Food Sci. Tech. 3:31-35.

Kosikowski, F. 1982. Cheese and fermented milk foods. 2nd ed. Edward Brothers Inc., Michigan, USA.

Lipkin, M. and H. Newmark. 1985. Effect of added dietary calcium on colonic epithelial-cell proliferation in subjects at high risk for familial colonic cancer. New England J. Med. 313:1381-1384.

Moore, S., D. H. Spackman and W. H. Stein. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Fed. Proc. 17:1107-1115.

Nemirovskiy, O. V. and M. L. Gross. 2000. Intrinsic Ca^{2+} affinities of peptides: Application of the kinetic method to analogs of calcium-binding site III of rabbit skeletal troponin C. J. Am. Soc. Mass Spectrom. 11:770-779.

Noyelle, K. and H. van Dael. 2002. Kinetics of conformational changes induced by the binding of various metal ions to bovine α-lactalbumin. J. Inorg. Biochem. 88:69-76.

Osborne, C. G., R. B. Mc Tyre, J. Dudek, K. E. Roche, R. Scheuplein, B. Silverstein, M. S. Weinberg and A. A. Salkeld. 1996. Evidence for the relationship of calcium to blood pressure. Nutr. Rev. 54:365-381.

Permyakov, E. A., V. V. Yarmolenko, L. P. Kalinichenko, L. A. Morozova and E. A. Burstein. 1981. Calcium binding to alpha-lactalbumin: Structural rearrangement and association constant evaluation by means of intrinsic protein fluorescence changes. Biochem. Biophys. Res. Commun. 100:191-197.

Pintado, M. E., A. E. Pintado and F. X. Malcata. 1999. Controlled whey protein hydrolysis using two alternative protease. J. Food Eng. 42:1-13.

Renner, E. 1994. Dairy calcium, bone metabolism and prevention of osteoporosis. J. Dairy Sci. 77:3498-3505.

Reynolds, E. C. 1997. Remineralization of enamel subsurface

lesions by casein phosphopeptide-stabilized calcium phosphate solutions. J. Dent. Res. 76:1587-1595.

Rose, D., D. T. Davies and M. Yaguchi. 1969. Quantitative determination of the major components of casein mixture by column chromatography on DEAE-cellulose. J. Dairy Sci. 52:8-11.

Schagger, H. and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166:368-379.

Shaw, D., M. Messer, A. M. Scrivener, K. R. Nicholas and M. Griffiths. 1993. Isolation, partial characterization and amino acid sequence of α-lactalbumin from platypus (*Ornithorynchus anatinus*) milk. Biochem. Biophys. Acta. 1161:177-186.

Spencer, H. and L. Kramer. 1986. NIH consensus conference: osteoporosis. Factors contributing to osteoporosis. J. Nutr. 116:316-319.

Stuart, D. I., K. R. Acharya, N. P. C. Walker, S. G. Smith, M. Lewis and D. C. Phillips. 1986. α-lactalbumin possesses a novel calcium binding loop. Nature (Load). 324:84-87.

Tamura, M., T. Oku and N. Hosoya. 1982. Calcium-binding proteins in bovine milk: Calcium-binding properties and amino acid composition. J. Nutr. Sci. Vitaminol (Tokyo). 28:533-541.

Tuan, R. S., W. A. Scott and Z. A. Cohn. 1978. Purification and characterization of calcium-binding protein from chick chorioallantoic membrane. J. Biol. Chem. 253:1011-1016.

Turgeon, S. L. and S. F. Gauthier. 1990. Whey peptide fractions obtained with a two-step ultrafiltration process: Production and characterization. J. Food Sci. 55:106-110.

Vegarud, G. E., T. Langsrud and C. Svenning. 2000. Mineral-binding milk proteins and peptides; occurrence, biochemical and technological characteristics. Br. J. Nutr. 84:S91-98.

Veprintsev, D. B., M. Narayan, S. E. Permyakov, V. N. Uversky, C. L. Brooks, A. M. Cherskaya, E. A. Permyakov and L. J. Berliner. 1999. Fine tuning the N-terminus of a calcium binding protein: α-lactalbumin. Proteins. 37:65-72.

Wang, J. H. and D. M. Waisman. 1979. Calmodulin and its role in the second-messenger system. Curr. Top. Cell. Regul. 15:47-107.

Wasserman, R. H., C. S. Fullmer and A. N. Taylor. 1978. The vitamin D-dependent calcium-binding proteins. In: Vitamin D (Ed. D. E. M. Lawson). Academic Press Inc., NY, USA. 133-166.

Wong, N. P., D. E. LaCroix and F. E. McDonough. 1978. Minerals in whey and whey fractions. J. Dairy Sci. 61:1700-1703.