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ARTICLE

Hypoallergenic and Physicochemical Properties of the A2 β-Casein Fraction of Goat Milk

Tae-Hwan Jung[1], Hyo-Jeong Hwang[2], Sung-Seob Yun[3], Won-Jae Lee[4], Jin-Wook Kim[4], Ji-Yun Ahn[5], Woo-Min Jeon[6], and Kyoung-Sik Han[2,6]*

[1]Department of Health and Bio-Convergence, Sahmyook University, Seoul 01795, Korea
[2]Biomaterials Research Institute, Sahmyook University, Seoul 01795, Korea
[3]R&D Center, Edam Co. Ltd., Chungbuk 38755, Korea
[4]Department of Animal Bioscience and Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 52828, Korea
[5]Division of Nutrition and Metabolism Research, Korea Food Research Institute, Wanju 55365, Korea
[6]Department of Animal Biotechnology and Resource, Sahmyook University, Seoul 01795, Korea

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*Corresponding author
Kyoung-Sik Han
Department of Animal Biotechnology and Resource, Sahmyook University, Seoul 01795, Korea
Tel: +82-2-3399-1765
Fax: +82-2-3399-1762
E-mail: kshan@syu.ac.kr

Abstract

Goat milk has a protein composition similar to that of breast milk and contains abundant nutrients, but its use in functional foods is rather limited in comparison to milk from other sources. The aim of this study was to prepare a goat A2 β-casein fraction with improved digestibility and hypoallergenic properties. We investigated the optimal conditions for the separation of A2 β-casein fraction from goat milk by pH adjustment to pH 4.4 and treating the casein suspension with calcium chloride (0.05 M for 1 h at 25°C). Selective reduction of β-lactoglobulin and α_s-casein was confirmed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis and reverse-phase high-performance liquid chromatography. The hypoallergenic property of A2 β-casein fraction was examined by measuring the release of histamine and tumor necrosis factor alpha from HMC-1 human mast cells exposed to different proteins, including A2 β-casein fraction. There was no significant difference in levels of both indicators between A2 β-casein treatment and the control (no protein treatment). The A2 β-casein fraction is abundant in essential amino acids, especially, branched-chain amino acids (leucine, valine, and isoleucine). The physicochemical properties of A2 β-casein fraction, including protein solubility and viscosity, are similar to those of bovine whole casein which is widely used as a protein source in various foods. Therefore, the goat A2 β-casein fraction may be useful as a food material with good digestibility and hypoallergenic properties for infants, the elderly, and people with metabolic disorders.

Keywords goat A2 β-casein, α_s-casein, β-lactoglobulin, hypoallergenic property, physicochemical property

Introduction

The composition of goat milk protein is similar to that of breast milk and its casein fraction is mostly comprised of β-casein, followed by α_s-casein (α_{s1}- and α_{s2}-casein) and κ-casein (Bernacka, 2011). Milk protein primarily contains two types of β-casein: A1 and A2 (Jianqun *et al*., 2016). Interestingly, β-casein in goat milk exists mainly as the A2 type (Cieślińska *et al*., 2012) and it does not produce β-casomorphin-7 (BCM-7), generated during the process of milk digestion, which

may be related with various disorders, such as gastrointestinal disturbances (Ho *et al*., 2014). It is known that α_{s1}-casein forms hard curds in the stomach, which might cause digestion problems in infants, but its concentration in goat milk is markedly lower than that in other milks (Bernacka, 2011; Yangilar, 2013).

Although goat milk contains β-lactoglobulin (β-lg), which is the primary cause of allergic reactions to this milk (Bossios *et al*., 2011; Cui *et al*., 2015), Jandal (1996) announced that goat milk has hypoallergenic properties.

Along with the nutritional benefits of goat milk protein, goat milk has more medium-chain triglycerides and smaller fat globules than cow milk, resulting in better digestibility (Haenlein, 2004; Park, 1994). These properties of goat milk can be exploited in functional foods for people with metabolic disorders as well as infants and the elderly (Alférez *et al*., 2001).

Research interest in goat milk is steadily increasing, with primary focus on the high digestibility and good hypoallergenic properties of goat milk in comparison to other types of milk (Espejo-Carpio *et al.*, 2016; Tomotake *et al*., 2009). Therefore, the aim of this study was to prepare an A2 β-casein fraction from goat milk in which β-lg and α_s-casein were selectively reduced by pH adjustment and calcium chloride precipitation. We investigated the optimal condition to isolate the A2 β-casein fraction from goat milk and evaluated its nutritional and physicochemical properties. The release of histamine and tumor necrosis factor alpha (TNF-α) from HMC-1 human mast cells exposed to A2 β-casein fraction was measured to evaluate its hypoallergenic properties.

Materials and Methods

Preparation of goat A2 β-casein fraction

Goat milk was obtained from Edam Co. Ltd. (Daejeon, Korea) and skimmed at 5,000 × *g* for 20 min (Labogene 1736R, Lynge, Denmark). After goat whole casein (GWC) was collected by pH adjustment to pH 4.4 using 1 M HCl to remove whey protein, it was dissolved in distilled water and adjusted up to pH 7.0. The optimal condition to selectively reduce the α_s-casein content was investigated using the calcium chloride precipitation method: the GWC suspension was treated with calcium chloride at various concentrations (0.025 to 0.1 M) and for different incubation periods (15 to 60 min) at 25°C. The casein suspension was centrifuged at 10,000 × *g* for 30 min to collect the supernatant, which contains the A2 β-casein fraction and was freeze-dried using a freeze dryer (Ilshin, Korea). The purity of the A2 β-casein fraction were confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and reverse-phase high-performance liquid chromatography (RP-HPLC) analysis.

SDS-PAGE

The A2 β-casein fraction was resolved on a 12.5% acrylamide gel at 20 mA for 1 h using a Mini-Protean® Tetra System and PowerPac™ HV (Bio-Rad, USA), according to the method of Laemmli (1970). The gel was stained for 2 h with a coomassie blue solution (0.3 M coomassie blue G-250, 40% methanol, and 7% glacial acetic acid). Bands were analyzed using the Molecular Imager® GelDoc™ XR plus Imaging system and the Image Lab™ software 5.1 (Bio-Rad, USA).

RP-HPLC

RP-HPLC was performed as described by Bobe *et al*. (1998), with slight modification to the method. Acetonitrile and water were HPLC-grade, and all other reagents were analytical grade. Bovine whole casein (BWC) was purchased from Sigma-Aldrich (USA). Sample buffer (0.1 M Bis-Tris-HCl, 6 M guanidine hydrochloride, 19.5 mM dithiothreitol, and 5.37 mM sodium citrate, pH 6.8) was mixed with the A2 β-casein fraction or BWC, and the mixture was incubated for 1 h at 25°C and then centrifuged at 12,000 × *g* for 10 min using a Micro High Speed Refrigerated Centrifuge VS-15000CNF (Vision Scientific, Korea). The supernatant was filtered using a polyvinylidene difluoride syringe filter (pore size 0.22 μm; Woongki Science, Korea) and injected (20 μL) into the HPLC system (Waters, USA) comprised of a Binary HPLC Pump 1525 (Waters, USA), a sample injector, and an absorbance detector. A silica-based C_{18} RP-HPLC column (250 mm length × 4.6 mm i.d., 5.0 μm; Waters, USA) was used for protein separation with solvents A and B at a flow rate of 1 mL/min. Solvent A and B were composed of 10% and 90% acetonitrile with 0.1% trifluoroacetic acid in HPLC-grade water, respectively. The absorbance was measured at 220 nm using a Photodiode Array Detector 2996 (Waters, USA). The solvent gradient program started at 27% of solvent B and was retained for 5 min after sample injection, followed by increasing proportions of solvent B at 0.5%/min (for 10 min), 0.33%/min (for 3 min), 0.5%/min (for 11 min), 0.25%/min (for 2 min), 0%/min (for 3 min), 0.5%/min (for 2 min), 0.56%/min (for 9 min), and then, the proportion of solvent B was

increased to 100%. Before the next sample was injected, the column was maintained under the initial condition for 10 min.

Analysis of general amino acids

One milligram of A2 β-casein fraction was labeled with phenyl isothiocyanate by the Pico-tag method to determine the general amino acid composition. The labeled sample was mixed in 400 μL of buffer and 10 μL of the mixture was analyzed by RP-HPLC. A Pico-tag column (300 mm length × 3.9 mm, 4.0 μm; Waters, USA) was used with solvents A and B at a flow rate of 1 mL/min. Solvent A consisted of 140 mM sodium acetate with 6% acetonitrile and solvent B comprised 60% acetonitrile in HPLC-grade water. The absorbance was measured at 254 nm using a 2487 UV detector (Waters, USA). The initial concentration of solvent B of 14% was maintained for 9 min after sample injection, followed by increasing percentages of solvent B at 0.5%/s (for 0.2 min), 3.13%/min (for 8.3 min), and 4.5%/s (for 0.2 min), and finally, the proportion of solvent B was decreased to 0%.

Measurement of allergenic properties

The allergenic properties of A2 β-casein fraction were investigated using the method described by Miyazaki *et al*. (2005), with a slight modification. The HMC-1 cells were supplied by the Pathophysiology Laboratory, Sahmyook University (Korea). The HMC-1 cells were grown in Dulbecco's modified Eagle's medium (Cellgro, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and 1% penicillin/streptomycin (Gibco, USA) at 37°C with 5% CO_2 in a humidified atmosphere. The HMC-1 cells (1×10^5 cells/well) were cultured in a 24-well plate overnight, followed by addition of 100 μL of reagents or samples: the control (without protein), 10 μg/mL of compound 48/80 (C48/80; Sigma-Aldrich, USA) as a positive control, and 100 μg/mL of the A2 β-casein fraction, goat whey protein (GWP), ovalbumin (Sigma-Aldrich, USA), or soy protein (Esfood, Korea), respectively. After the cells were incubated for 24 h, the culture supernatant was collected to measure secreted histamine and TNF-α using the histamine ELISA kit (IBL International, Germany) and human TNF-α ELISA Ready-SET-Go® Kit (eBioscience, USA), respectively, according to the manufacturers' protocols. The absorbance was read at 450 nm using a microplate reader (Molecular Devices, USA).

Protein solubility

Protein solubility was measured by the method of Bera and Mukherjee (1989), with slight modification to the method. After 1 g of A2 β-casein fraction or BWC was suspended in 100 mL of distilled water, the pH of the suspensions was adjusted to pH 2, 4, 6, 8, or 10 using 1 M HCl or 1 M NaOH. The suspensions were stirred for 1 h at 25°C and then centrifuged at 12,000 × *g* for 20 min. The protein content in the supernatants was determined by the Quick StartTM Bradford 1×Dye Reagent (Bio-Rad, USA) and the protein solubility was calculated as follows:

protein solubility (%) = (protein content of supernatant at designated pH/total protein content of sample) × 100

Viscosity measurement

The pH of 10% (w/v) solutions of A2 β-casein fraction and BWC was adjusted to pH 2, 4, 6, 8, or 10, and the viscosity of the solutions was measured using a DV1 Viscometer (Brookfield Ametek, USA) at 25°C.

Statistical analysis

The results were expressed as the mean±standard deviation (SD), and differences were analyzed using the SAS/PROC GLM software (SAS version 9.1; SAS Institute Inc., USA). Statistical significance was accepted at $p<0.05$ or $p<0.01$.

Results

Optimal conditions for preparation of the A2 β-casein fraction

The optimal conditions to selectively decrease the β-lg and α_s-casein contents in the final product, the A2 β-casein fraction, were examined. Firstly, whey protein including β-lg was completely removed from goat skim milk using pH adjustment to pH 4.4, followed by calcium chloride precipitation. As shown in Fig. 1, the amount of α_s-casein among three major goat caseins (α_s-casein, β-casein, and κ-casein) was selectively reduced in the A2 β-casein fraction, depending on increasing calcium chloride concentration and incubation time. Degradation of α_s-casein showed no significant difference when calcium chloride was added at a concentration higher than 0.05 M and the mixture was incubated for longer than 1 h at 25°C. The A2 β-casein fraction was the most effectively prepared by treatment with 0.05 M calcium chloride for 1 h at 25°C.

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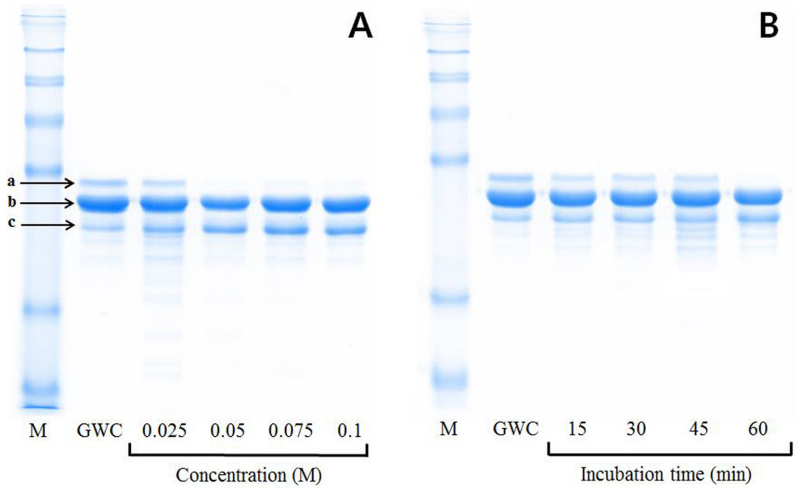

Fig. 1. SDS-PAGE analysis of the A2 β-casein fraction from goat milk. Purity of A2 β-casein fraction according to different calcium chloride concentrations (M) for 1 h at 25°C (A) and for different incubation times (min) with 0.05 M calcium chloride at 25°C (B). a, α_s-casein; b, β-casein; c, κ-casein. M, protein molecular weight marker; GWC, goat whole casein.

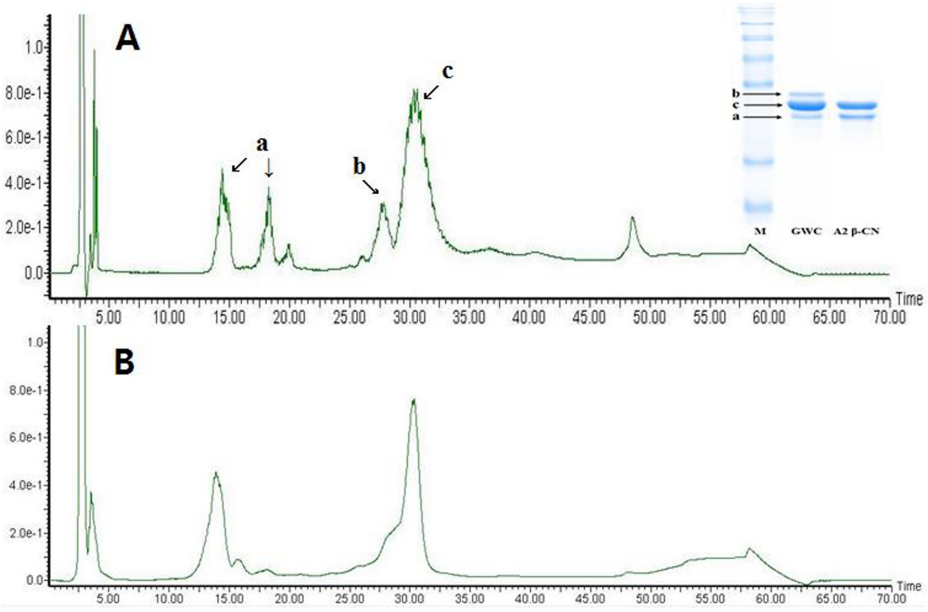

Fig. 2. RP-HPLC analysis of the A2 β-casein fraction from goat milk. A, GWC; B, A2 β-casein fraction. a, κ-casein; b, α_s-casein; c, β-casein. Retention time is shown in min. The inset image shows the SDS-PAGE gel of the A2 β-casein fraction. M, protein molecular weight marker; GWC, goat whole casein; A2 β-CN, goat A2 β-casein fraction.

Separation of the A2 β-casein fraction

Selective degradation of α_s-casein in the A2 β-casein fraction was confirmed by SDS-PAGE and RP-HPLC (Fig. 2). The α_s-casein was effectively reduced in the A2 β-casein fraction compared to GWC. The proportions of α_s-casein and β-casein in A2 β-casein fraction changed from 19.5% to 1.9% and from 52.9% to 63.8%, respectively.

Measurement of histamine and TNF-α released by human mast cells

The HMC-1 cells were cultured with the A2 β-casein fraction or other protein samples (GWP, ovalbumin, soy protein) to measure the released contents of histamine and TNF-α. The C48/80 was included as a positive control. As shown in Fig. 3, addition of C48/80, GWP, ovalbumin, or soy protein significantly (p<0.01) induced the

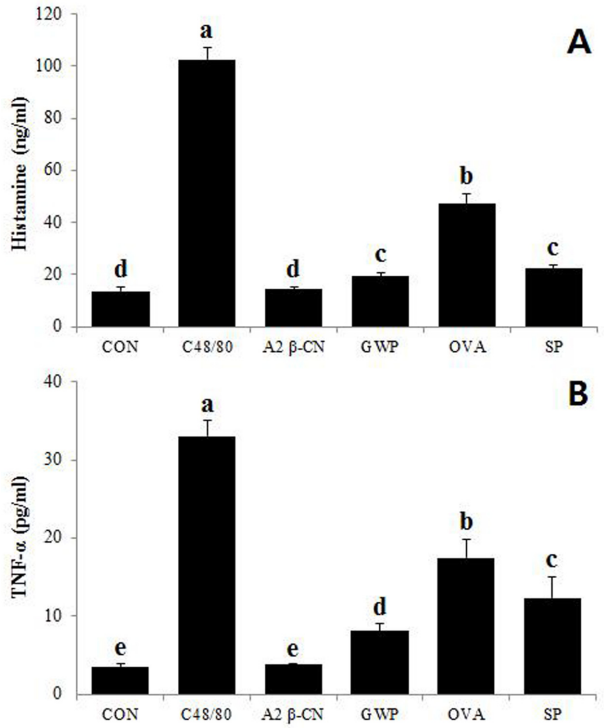

Fig. 3. Measurement of histamine and TNF-α release by human mast cells exposed to different proteins. Data are expressed as the mean±SD. Values with different lowercase letters differ significantly across the samples, $p<0.01$. (A) Histamine; (B) TNF-α. CON, control; C48/80, compound 48/80; A2 β-CN, goat A2 β-casein fraction; GWP, goat whey protein; OVA, ovalbumin; SP, soy protein.

release of histamine or TNF-α in comparison to the control, while addition of the A2 β-casein fraction did not stimulate their release.

General amino acid composition

The percentages of essential and non-essential amino acids in the A2 β-casein fraction were 52.79% and 47.21%, respectively (Table 1). The A2 β-casein fraction was rich in leucine (11.91%) and valine (9.83%) among essential amino acids, and in proline (17.71%) and glutamine (9.83%) among non-essential amino acids.

Physicochemical properties of the A2 β-casein fraction

The protein solubility and viscosity of A2 β-casein fraction and BWC were determined in the pH range of 2 to 10 (Fig. 4). No significant differences were observed at any pH. The protein solubility of the A2 β-casein fraction and BWC was 61.9% and 59.7%, respectively, at pH 2 and decreased to 25.6% and 23.1%, respectively, at pH 4.

Table 1. General amino acid composition of the A2 β-casein fraction from goat milk

Amino acid	Composition (% of total amino acid)
Essential	
Histidine	4.62
Threonine	6.40
Valine	9.83
Methionine	2.01
Isoleucine	6.48
Leucine	11.91
Phenylalanine	5.81
Tryptophan	0.00
Lysine	5.73
Total essential amino acid	52.79
Non-Essential	
Cystein	0.30
Asparagine	1.34
Glutamine	9.83
Serine	5.88
Glycine	1.79
Arginine	3.43
Alanine	3.80
Proline	17.71
Tyrosine	3.13
Total non-essential amino acids	47.21

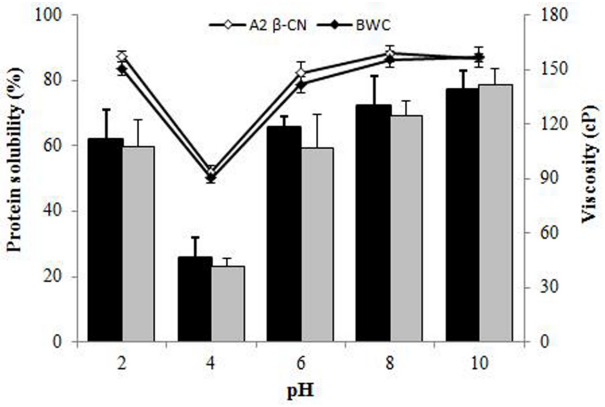

Fig. 4. Protein solubility and viscosity of the A2 β-casein fraction and BWC according to pH. Data are expressed as the mean±SD. Bars show the protein solubility of A2 β-casein fraction (black) and BWC (grey). Lines show the viscosity of both proteins. BWC, bovine whole casein; A2 β-CN, goat A2 β-casein fraction.

Their solubility gradually increased up to 77.3% and 78.6% above pH 4, respectively. The viscosity of the A2 β-casein fraction and BWC was the lowest at pH 4.0 and increased from 93.7 cP to 158.7 cP and 90.3 cP to 157.0 cP, respectively, at alkaline pH.

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Discussion

In general, β-casein exists as A1 or A2 type in milk, and both types differ in amino acid sequence. The A1 type contains histidine at position 67 of the β-casein amino acid sequence, whereas A2 type has a proline at this position (Farrell *et al*., 2004). A few studies have reported that BCM-7 (f60-66 of β-casein: Tyr-Pro-Phe-Pro-Gly-Pro-Ile) which is generated by proteolytic digestion of A1 type β-casein, might cause various disorders, such as gastrointestinal problems (Ho *et al*., 2014). However, goat β-casein is mainly composed of A2 type, and thus, the A2 β-casein fraction prepared in this study may be considered as a safe food material.

Milk α_s-casein is comprised of α_{s1}- and α_{s2}-casein. The goat milk contains mainly α_{s2}-casein and a small amount of α_{s1}-casein (Park *et al*., 2007). During the digestion process, α_{s1}-casein may interfere with digestion by being curdled in the stomach (Tomotake *et al*., 2006). In contrast, α_{s2}-casein hardly forms solid curds. Espejo-Carpio *et al.* (2016) reported that goat milk has hypoallergenic properties despite the fact that it contains β-lg which may cause allergic reactions. For these reasons, goat milk is believed to be more digestible and has lower allergenicity than other types of milk.

In this study, to maximize the utilization of goat milk protein, we investigated optimal conditions to selectively reduce the amounts of both α_s-casein and β-lg in goat milk protein. After casein was separated from whey to remove β-lg by pH adjustment, calcium chloride precipitation method was used to selectively reduce α_s-casein in GWC. The degradation of α_s-casein in GWC was the most effective when the GWC suspension was treated with 0.05 M calcium chloride at 25°C for 1 h, as verified by SDS-PAGE and RP-HPLC analyses.

Previously, Murphy and Fox (1991) isolated a β-casein-enriched fraction from milk by adjusting temperature and twice ultrafiltration, which might be time-consuming and costly for commercial application. However, the procedure developed in the current study is more efficient than other methods.

Allergic reaction to the A2 β-casein fraction was investigated by measuring the release of histamine and TNF-α from HMC-1 cells. In general, allergens activate Th2 lymphocytes through Langerhans cells, resulting in production of immunoglobulin E (IgE) (Novak and Bieber, 2005). The IgE binds to high-affinity IgE receptor on mast cells, causing the release of histamine and TNF-α (Je *et al*., 2013). While the secretion of both inflammatory indicators significantly increased when the cells were treated with C48/80, GWP, ovalbumin, or soy protein, the A2 β-casein fraction did not stimulate their release in comparison to the control. Allergic reaction induced by GWP was supposed to be due to the existence of β-lg. Cordle (2004) and Zhang *et al*. (2015) reported that ovalbumin and soy protein also induce the release of histamine and TNF-α.

The A2 β-casein fraction contains a substantial amount of essential amino acids (52.79%). In particular, it is abundant in leucine (11.91%), valine (9.83%), and isoleucine (6.48%). These amino acids are known as branched-chain amino acids, which improve not only insulin resistance and hypoalbuminemia (Kawaguchi *et al*., 2009; Muto *et al*., 2005) but also protein synthesis and recovery of patients (Wang *et al*., 2003).

The physiochemical properties, including protein solubility and viscosity, of the A2 β-casein fraction were investigated in comparison with those of BWC, which is widely used as a protein material in various foods. The solubility of A2 β-casein fraction and BWC was the lowest at pH 4, the near isoelectric point of casein, and it gradually increased both below and above pH 4, similar to the results reported by Gani *et al.* (2015). The viscosity of both samples showed a similar tendency and was considered to be affected by the net charge of casein depending on the pH. Moreover, emulsifying activity index and emulsifying stability index of each sample changed according to variation in protein solubility (data not shown), similar to the results reported by Raikos *et al*. (2014), and Lee *et al*. (2006).

Conclusions

An A2 β-casein fraction with effectively reduced α_s-casein and β-lg contents was prepared from goat milk by adjusting the pH and treating GWC suspension with 0.05 M calcium chloride for 1 h at 25°C; this was confirmed by SDS-PAGE and RP-HPLC. The A2 β-casein fraction did not stimulate histamine and TNF-α release from HMC-1 cells, and it has abundant essential amino acids, including branched-chain amino acids (leucine, valine, and isoleucine), which can improve nutrition and health. Moreover, the A2 β-casein fraction can be considered as a substitute for BWC (widely used as a protein source for various foods) because the physicochemical properties of the A2 β-casein fraction were proven to be similar to those of BWC. These results indicate that the A2 β-casein

fraction not only has improved digestibility and hypoallergenic properties, but also may have potential as a functional food material.

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