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Mobile Phase Composition for Resolving Whey Proteins in Reversed-Phase High Performance Liquid Chromatography

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Abstract−Reversed-phase high-performance liquid chromatography was successfully developed for the simultaneous and rapid separation for the main whey proteins, α-Lactalbumin and β-Lactoglobulin. This method consisted of a linear gradient of the two mobile phases of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile. The total run time for this separation was approximately 30 min, and α-Lactalbumin was eluted followed by β-Lactoglobulin. The injection volume was fixed at 20 µl and the flow rate was 1 ml 1/min. The optimum mobile phase composition and gradient conditions to separate α-Lactalbumin and β-Lactoglobulin (A+B) were experimentally obtained at the 15 µm particle with a pore size of 300 Å on the linear-gradient mode.

Key words: Column Liquid Chromatography, Reversed-Phase Separation, Whey Proteins, α-Lactalbumin, β-Lactoglobulin A and B

INTRODUCTION

The separation and determination of biopolymers (peptides, proteins, etc.) are frequently achieved by reversed-phase high performance liquid chromatography (RP-HPLC), although other HPLC methods such as ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), size-exclusion chromatography (SEC) and different types of affinity chromatography (AC) have also been used [Torre et al., 1996]. With the development of recombination DNA techniques, HPLC has become an important tool in both quality control and process control in the production of recombinant proteins of pharmacological interest. This has contributed significantly to the development of RP-HPLC methods that could be used to carry out very rapid analyses for proteins with minimum complexity in both instrumentation and operating conditions [Nadler et al., 1994]. Given the low diffusivity of biopolymers, the need to achieve fast separations of proteins by RP-HPLC has led to the development of different types of packing materials such as non-porous microparticles (dp<5 µm) or wide-porous materials [Kalghatgi et al., 1988; Lloyd et al., 1990], that solve the mass transfer problems in the stationary phase, allowing good separations within a reasonable time. The particles used in RP-HPLC have a porous structure composed of 300 Å. There are different factors that may cause an allergy against animal whey proteins (constituted mainly by α-Lactalbumin and β-Lactoglobulin). Mostly (85%), a genetic factor is responsible for this disease, but there are others such as an excessive intake of milk or inadequate working conditions [Garcia et al., 1998]. World-wide an increasing amount of whey protein, available due to increasing cheese production and the development of ultrafiltration methods to concentrate these proteins in their native form, has stimulated intensive research on expanding the utilization of whey protein [Madsen et al., 1997]. The whey proteins are used to replace other proteins or to improve the functional properties of baby food, luncheon meat, soft and milk-based drinks, ice cream, bakery, and convenience products [Kim et al., 2003].

The purpose of this study was to investigate the optimum mobile phase condition to separate the whey proteins of α-Lactalbumin and β-Lactoglobulin on gradient modes. In an analytical column (3.9×300 mm), the experiments were performed at the particle size of 15 µm with large pore sizes of 300 Å. The mobile phase was a binary system of water with 0.1% of TFA and acetonitrile with 0.1% of TFA.

EXPERIMENTAL

1. Chemicals

The chemical composition of whey protein is shown in Table 1. The whey powder (from Bovine Milk) used in this experiment was purchased from Sigma Co. Samples were prepared by diluting the appropriate amount of whey powder in water. The concentration of the samples remained at approximately 200,000 ppm. The standard chemicals of α-Lactalbumin (TypeIII: deplete, form Bovine Milk Approx. 85%), β-Lactoglobulin (form Bovine Milk Approx. 90%) were purchased from Sigma Co. Standard protein solutions were freshly prepared in water. Standard solutions were made for each protein of 10 mg dissolved into 1 ml with water. That water was filtered with HA-0.5 µm membranes (Division of Millipore, Waters Co.) and deionized prior to use. The extra-pure grade solvent of acetonitrile (ACN) was purchased from Duksan Pure Chem-

Table 1. Molecular masses and isoelectric points for whey proteins

Protein	Molecular mass	pI
α-Lactalbumin	14,000	4.2-4.5
β-Lactoglobulin	18,300	5.35-5.49
Albumin (BSA)	69,000	5.13

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icals Company (Incheon Korea). Trifluoroacetic acid (TFA) was purchased from Sigma Co. The mobile phases used were as follows: buffer A, 0.1% TFA in water and buffer B, 0.1% TFA in acetonitrile.

2. Pretreatment Step

An example of a multi-component protein mixture containing proteins of commercial value is dairy whey. In general, whey is a dilute liquid composed of lactose, a variety of proteins, minerals, vitamins and fat. Whey is a liquid mixture composed of a variety of these chemical compounds but unfortunately low in concentration. Pretreatment was necessary because of the various molecular weights. Among others method, the most basic tool is molecular size filtration. In particular, ultrafiltration (UF) is the most widely used commercial method of whey fractionation offering considerable diversity in manufacturing a wide range of whey protein concentrates. UF process is employed when the molecular mass cutoff point for solutes from proteins is 500 or greater [Gerverding et al., 1998]. The primary reason for the success of UF is that membranes have been developed that can tolerate the cleaning compounds and temperatures utilized for sanitation [Choi et al., 2002]. Whey protein 10 g was dissolved in deionized water of 50 ml. The solution was passed through Amicon 8400 (USA, Operation condition-max 75 psi) as an ultrafiltration kit. Molecular weights of α-Lactalbumin and β-Lactoglobulin ranged between 14,000 and 18,000; therefore, the UF membrane of cellulose discs 10,000 or 30,000 molecular weight cut off (MWCO) was utilized to extract lactose.

3. High-Performance Liquid Chromatography

The analytical HPLC system in this experiment was a Waters Model 600S liquid chromatography (Waters Associates, Milford, MA, U.S.A.) equipped with the Waters 515 Multi-solvent Delivery System with 486 Tunable Absorbance analytical Detector, and injector (20 µl sample loop) from Rheodyne. The data acquisition system was Chromate (Ver. 3.0, Interface Eng., Korea) installed in a PC. The wavelength was fixed at 280 nm and the experiment was performed at room temperature. The analytical column was a stainless steel μ-Bondapak^{TM} C_{18}. The size of analytical column was 3.9× 300 mm, and it was packed in-house with RP-C_{18} (15 µm, Merck Co.) by high-pressure pumping with solvent.

RESULTS AND DISCUSSION

The whey proteins of α-Lactalbumin and β-Lactoglobulin from bovine milk were separated by RP-HPLC at room temperature using a silica-based, wide pore C_{18} (300 Å) column and a linear binary gradient of acetonitrile/water/0.1% TFA. However, it was observed that because of the small structural differences between α-Lactalbumin and β-Lactoglobulin, a desirable resolution of these proteins at the baseline was difficult to achieve.

The several purification steps in Fig. 1 were applied to obtain preparatively the pure α-Lactalbumin and β-Lactoglobulin. Therefore, the aim of the method development was to achieve sufficient resolution of the two whey proteins. Several gradients of water-acetonitrile containing TFA (0.1%) were used. Fig. 2 shows the comparison of separation of the two standard chemicals of α-Lactalbumin and β-Lactoglobulin (dotted line) and the real whey proteins (solid line) after the ultrafiltration (MWCO 30,000). The gradient condition consisted of a two-step linear binary gradient: buffer A/buffer B=70/30-35/65 (vol%), gradient time of 15 min. The amount of the proteins in whey was so small that the part of #1 in Fig. 2. was collected several times, and the samples were concentrated by

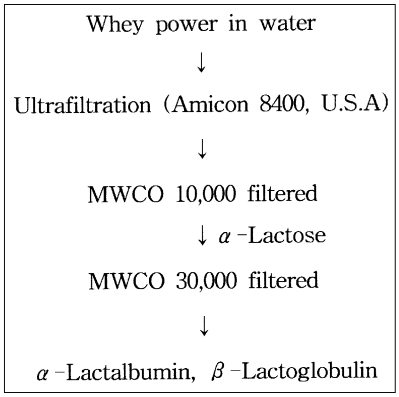

Fig. 1. Ultrafiltration of whey power.

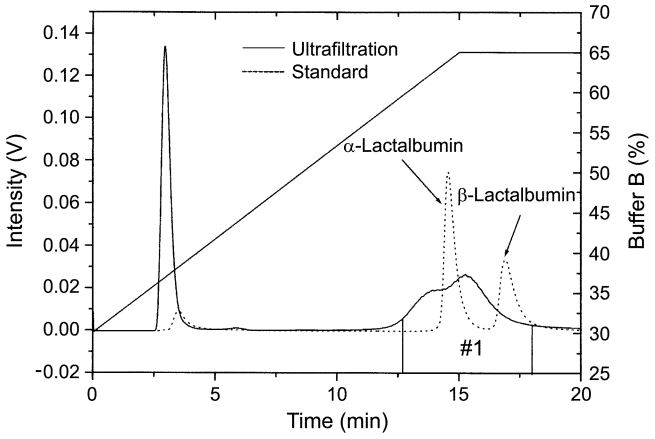

Fig. 2. Comparison of the two standard chemicals of á α-Lactalbumin and β-Lactoglobulin and whey proteins after ultrafiltration (MWCO 30,000) by RP-HPLC (Buffer A/Buffer B=70/30-35/65 vol%, gradient time of 15 min).

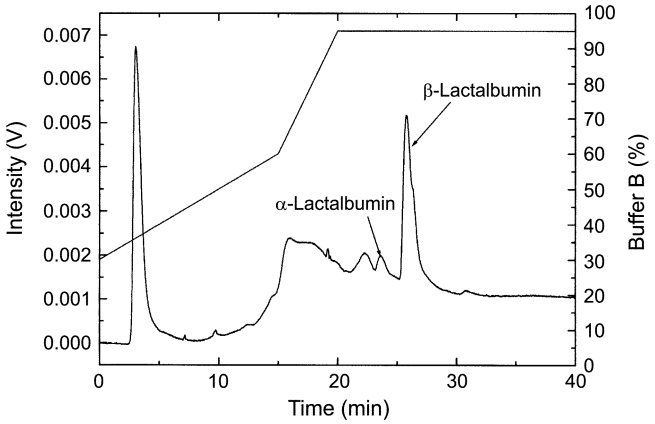

Fig. 3. Chromatogram of whey proteins after ultrafiltration (MWCO 30,000) from the fraction number #1 in Fig. 2 by RP-HPLC (Buffer A/Buffer B=70/30-40/60 vol%, gradient time of 15 min, Buffer A/Buffer B=40/60-5/95 vol%, gradient time of 20 min).

a rotary evaporator. They were analyzed by the same C_{18} column (300 Å and 15 μm), and the chromatogram is shown in Fig. 3. The two whey proteins of α-Lactalbumin and β-Lactoglobulin were resolved. The mobile phase composition was the two linear-gradient conditions of buffer A/buffer B, 70/30-40/60 (0-15 min) and 40/60-5/95 (15-20 min) vol%. Actually two β-Lactoglobulins, A and B existed, but they were coeluted as a single peak in the mobile phase composition. To separate the two β-Lactoglobulin A and B, another mobile phase composition was tried, and it was composed of the two linear-gradient conditions of buffer A/buffer B, 70/30-35/75 (0-10 min) and 35/75-10/90 (10-15 min) vol%. The initial gradient composition was the same as in Fig. 3, but the increase rate of buffer B was greater in Fig. 4. The partially resolved part of region #2 between 10 min and 15 min was collected. The retention times of α-Lactalbumin and β-Lactoglobulin were about 10 minutes shorter than in Fig. 3. To improve the resolution, the part of #2 in Fig. 4 was collected and concentrated. The HPLC-analysis is shown in Fig. 5, where β-Lactoglobulin was resolved into the two components, A and B with the mobile phase composition of the three linear-gradient compositions of buffer A/ buffer B, 70/30-45/55 (0-10 min), 45/55-20/80 (10-15 min), 20/80-5/95 (15-20 min) vol%.

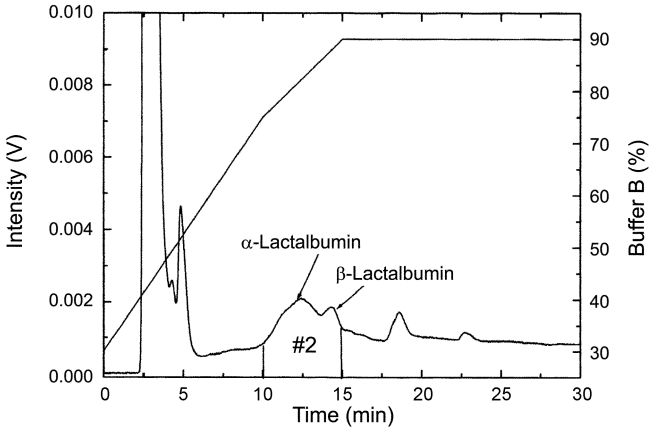

Fig. 4. Chromatogram of whey proteins after ultrafiltration (MWCO 30,000) by RP-HPLC (Buffer A/Buffer B=70/30-35/75 vol%, gradient time of 10 min, Buffer A/Buffer B=35/75-10/90 vol%, gradient time of 15 min).

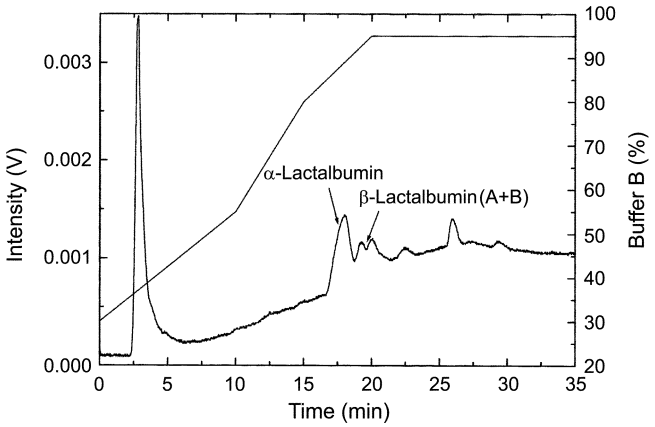

Fig. 5. Chromatogram of whey proteins after ultrafiltration (MWCO 30,000) from the fraction number #2 in Fig. 3 by RP-HPLC (Buffer A/Buffer B=70/30-45/55 vol%, gradient time of 10 min, Buffer A/Buffer B=45/55-20/80 vol%, gradient time of 15 min, Buffer A/Buffer B=20/80-5/95 vol%, gradient time of 20 min).

By the optimized gradient conditions, whey proteins in bovine were successfully separated. The RP-HPLC method in this work could be of great interest in quality control to resolve the whey proteins.

CONCLUSIONS

The RP-HPLC method was presented for the separation of α-Lactalbumin and β-Lactoglobulin. The optimum experimental conditions of the mobile phase compositions and gradient conditions were experimentally determined to separate α-Lactalbumin and β-Lactoglobulin A, B contained in whey. UF step was employed, because the molecular mass cutoff point of lactose was much smaller than that of whey proteins. The elution order of the whey proteins was α-Lactalbumin and β-Lactoglobulin. Only the adjustment of mobile phase conditions enabled the resolution of β-Lactoglobulin into A and B. The described RP-HPLC method may be applicable to wide application in the routine analysis of whey, fractionated whey streams and whey protein powders produced by dairy industries.

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