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Short communication

Use of amino acid analysis for estimating the individual concentrations of proteins in mixtures

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Abstract

The concentrations of five individual proteins in a mixture were determined from one amino acid analysis of the mixture by solving for each protein using simultaneous equations. Dried casein and whey were separated into five individual protein components using reversed-phase HPLC. Individual proteins were collected and analyzed for amino acid composition. These data were used as standards. Mixtures of purified proteins were analyzed for total amino acid composition and the concentrations of individual proteins in the mixtures were determined by solving simultaneous equations based on the amino acid analysis composition of the standards.

Keywords: Amino acid anaylsis; Mixtures; Protein concentrations

1. Introduction

The proteins in milk can be divided into two main groups based on solubility at pH 4.6 and 20°C. Proteins remaining in solution at pH 4.6 are termed whey proteins and those precipitating at pH 4.6 are known as caseins. In bovine milk, the casein fraction accounts for approximately 80% of the total milk protein and is comprised of four main proteins. The caseins (CNs) are present at 27 to 34 g/L in milk at the approximate ratio of 4:1:4:1 corresponding to α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN. The whey fraction, approximately 20% of the total protein, contains only two proteins found in relatively high concentrations, α-lactalbumin (α-LB) and β-lactoglobulin (β-LG) (reviewed in [1]).

Measuring the individual caseins and whey proteins in milk is difficult because most accurate procedures are lengthy and quick methods are often inaccurate. Time-consuming methods involve the separation of casein from whey and measuring the fractions separately either for total protein or for individual proteins. Faster methods often involve total milk protein measurement with the casein considered a constant fraction.

AOAC official methods for the determination of total nitrogen, nonprotein nitrogen and protein nitrogen in milk use the Kjeldahl method for total nitrogen determination. The total nitrogen is multiplied by a conversion factor, 6.38, to express the data as percent protein [2]. Other methods for total protein determination are dye binding and mid-infrared spectroscopy [3]. While these methods are rapid, they assume the casein fraction is constant since the total protein in milk samples is determined. These techniques can not determine the percent whey

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protein in milk samples, which is necessary if the adulteration of milk with whey is suspected.

Measuring the concentrations of individual proteins in a mixture frequently involves procedures such as column chromatography or sodium dodecyl sulfate–polyacrylamide gel electrophoresis or SDS–PAGE accompanied by densitometric scanning. Current analytical methods suitable to measure the concentration of individual milk proteins in samples are based on either chromatography or electrophoresis (reviewed in [4]). To detect the adulteration of milk with whey requires the quantitation of the individual milk proteins. Analytical chromatography methods using various supports (ion-exchange, reverse phase, and hydrophobic interaction) have been developed to separate the milk proteins. Under some conditions, such as reversed-phase, it is possible to obtain partial separation of the individual milk proteins. To date, the complete separation of the individual milk proteins with chromatography requires prior separation of the whey and casein fractions [4].

Dairy manufacturers would like to know the casein content of milk, yet this is not easily distinguished from the other milk proteins [5]. Cheese yield formulas and milk pricing systems are based on the casein and fat content of milk but there is no rapid, accurate procedure to determine the level of casein [6]. All proteins differ in the sequence and numbers of each amino acid, a characteristic that can be used to measure the amounts of individual proteins in a mixture. This paper describes a method to estimate the concentrations of the major milk proteins using amino acid analysis.

2. Experimental

2.1. Preparation of casein and whey proteins

Casein was prepared from whole, fresh milk from Utah State University Dairy Products Laboratory (Logan, UT, USA). Milk was skimmed by centrifuging at 5000 *g* for 30 min at 5°C. Casein was precipitated by acidifying skim milk to pH 4.6 with concentrated HCl. Casein was washed twice with distilled water and adjusted back to neutral pH with NaOH before freeze drying. This sample of crude casein was used in all experiments. Original supernatant was also freeze dried and used as crude whey protein.

2.2. HPLC separation

HPLC was carried out using a Beckman HPLC system consisting of two model 110A pumps, a 420 controller, a 340 organizer, a 164 variable-wavelength detector and a 427 integrator (Palo Alto, CA, USA). All chromatography samples and buffers were made with HPLC water, filtered through a 0.2 μm hydrophilic filter (Millipore, Bedford, MA, USA), and degassed under vacuum. All salts for buffer solutions were from Sigma (St. Louis, MO, USA) and acetonitrile was from Fisher Scientific (Pittsburgh, PA, USA). The reversed-phase column, C_3 was from Beckman (Palo Alto, CA, USA) and had dimensions of 75×4.6 mm, 5 μm particle diameter and a 300 A pore size. Protein samples were dissolved in initial HPLC buffer solutions for a final concentration of 1 mg/ml, of which 50 μ1 was injected into the HPLC and separated as described below at 1 mL/min at ambient temperature. Proteins were detected using a wavelength of 280 nm and peak areas of standards were used to calculate the concentrations of sample milk proteins.

For casein separation, dried crude casein and purchased α_s-, β-, and κ-CN standards (Sigma), were dissolved in 70% 5 *M* urea in solution A, 10% mercaptoethanol, and 10% acetonitrile. Solution A consisted of 0.15 *M* sodium chloride and 0 1% (v/v) triethylamine, pH 2.5. Buffer B consisted of 40% acetonitrile in water. The separation gradient began with 40% solution A and 60% solution B and continued linearly to 100% solution B.

Dried whey protein and commercial α-LB and β-LG standards (Sigma) were dissolved in 5 *M* urea in solution A. Proteins were separated by a method similar to Pearce [7] on a C_3 column by gradient using solution A, 0.15 *M* sodium chloride, pH 2.4, and solution B, 100% acetonitrile. The gradient began with 95% solution A and 5% solution B and continued linearly to 100% solution B.

2.3. Amino acid analysis

Casein and whey proteins collected from the HPLC were dried under nitrogen gas. Dried protein fractions were transferred to hydrolysis vials with distilled water and dried again. Protein fractions were collected and dried individually from each HPLC run. Some fractions were combined in one vial prior to amino acid analysis.

Hydrochloric acid (6 *M*) was added to sample vials for a final concentration of 5 mg of protein per mL of HCl. Hydrolysis vials were placed in an ultrasonic cleaner and flushed with nitrogen gas before sealing under vacuum. Samples were placed in a heating block and heated for 4 h at 145±2°C. After hydrolysis, samples were removed from the heating block and allowed to cool before filtering through a 0.2 μm filter. Samples were dried with nitrogen gas and dissolved in Beckman sample dilution buffer before analyzing for amino acids on a Beckman 6300 Automated Amino Acid Analyzer. Areas of amino acid standards were used to calculate quantity of each amino acid in samples with the amino acid composition of each protein the average of four analysis.

2.4. Calculations

The coefficient (or standard) matrix consisted of the amino acid composition of the five HPLC purified milk proteins. This matrix is arranged in the matrix format A=(m×n) yielding a (14×5) coefficient matrix. The solution matrix consisted of the amino acid analysis values of a mixture of proteins in the matrix format B=(14×1). In order to estimate the concentrations of the five individual proteins in the mixture, the matrices were transformed using the equation $A^TAX=A^TB$. This resulted in a coefficient matrix with a dimension of (5×5) and a solution matrix with dimensions of (5×1). A simultaneous equation program written for a Tektronix 4052 computer (Salt Lake City, UT, USA) was used to store the standard matrix for continual use, to transform the matrices, and to solve for each major milk protein. The program solves for α_s-CN, β-CN, κ-CN, α-LB, and β-LG in a mixture using simultaneous equations and the amino acid composition of the mixture.

3. Results and discussion

3.1. Protein separation

A chromatogram of casein standards is shown in Fig. 1a. Major peaks 1, 2, and 3 correspond to κ-, α_s-, and β-CN. A chromatogram of sample casein is shown in Fig. 1b. Retention times of major peaks correspond to κ-, α_s-, and β-CN standards. Commercial α_s-CN is a mixture of both α_{s1}- and α_{s2}-CN. These two proteins were co-purified with this reversed-phase separation method and are termed α_s-CN. κ-Casein is heterogenous with respect to the level of glycosylation that may have resulted in the two protein peaks in Fig. 1a.

Fig. 2a is a chromatogram of whey protein standards and Fig. 2b is a chromatogram of the whey sample. Peak 1 is α-LB and peaks 2 and 3 are β-LG. β-Lactoglobulin variants A and B are found in USA milk, therefore, we believe we separated both var-

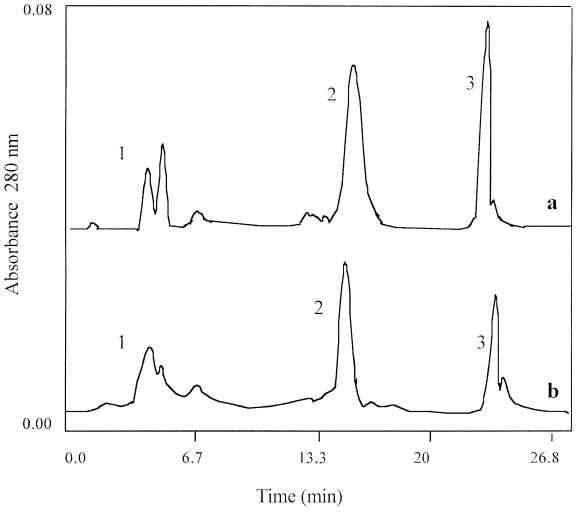

Fig. 1. Characteristic elution profiles of casein standards and crude casein. Conditions: C_3 (75×4.6 mm) column, 0.15 *M* sodium chloride–triethylamine pH 2.5 and 40% acetonitrile mobile phase. Ambient temperature, flow-rate 1 mL/min, 50 μg of protein loaded. Peaks: 1=κ-CN, 2=α_s-CN and 3=β-CN. (a) Commercial casein standards: (b) crude casein.

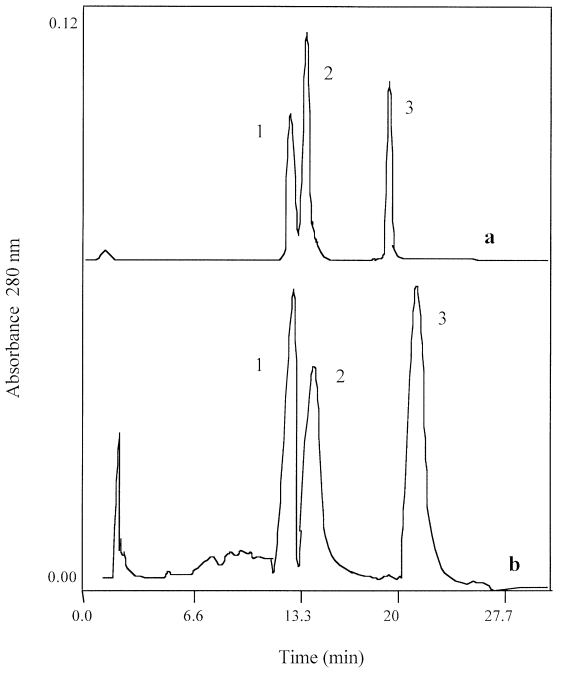

Fig. 2. Characteristic elution profiles of whey protein standards and crude whey protein. Conditions: C_3 (75×4.6 mm) column, 0.15 *M* sodium chloride pH 2.5 find acetonitrile mobile phase. Ambient temperature, flow-rate 1 mL/min, 50 μg of protein loaded. Peaks: 1=α-LB, and 2 and 3=β-LG. (a) Commercial whey standards: (b) crude whey.

iants of β-LG. The milk proteins in chromatogram b were collected and analyzed individually and after combining for amino acid composition.

3.2. Individual milk protein determinations

Table 1 shows differences in amino acid composition among the HPLC-purified milk proteins. The sulfur-containing amino acids, cysteine and tryptophan, which are partially or totally destroyed by acid hydrolysis, were not measured to simplify the procedure. The amino acid composition of each of the milk proteins correlates with the protein sequence and amino acid composition previously reported [8]. Acid hydrolysis time and temperature influence the yield of amino acids, which is protein dependent. In this case, we used a standard acid:protein ratio and a 4 h hydrolysis time. As shown by the standard deviations in Table 1, our results contained little variability.

Each milk protein has a characteristic amino acid composition that was used to quantify each of the proteins in a mixture. As shown in Table 1, the caseins contain more proline and less leucine than the whey proteins. Amino acid compositions of each milk protein were entered into the matrix program resulting in the coefficient matrix. The amino acid

Table 1
Amino acid composition of HPLC purified milk proteins[a]

Amino acid	Protein (g amino acid/100 g protein)				
	α_S-CN	β-CN	κ-CN	β-LG	α-LB
Asx[b]	8.28±0.06	5.03±0.09	7.27±0.06	10.20±0.11	18.10±0.07
Glx[c]	22.78±0.05	21.65±0.22	20.25±0.11	18.23±0.06	13.06±0.01
Ser	4.94±0.06	4.26±0.11	4.35±0.03	2.87±0.04	3.64±0.02
Gly	2.46±0.01	1.25±0.04	1.13±0.03	1.17±0.01	2.62±0.04
His	3.37±0.11	3.25±0.19	2.77±0.09	1.67±0.02	3.12±0.12
Arg	4.39±0.03	3.10±0.07	4.43±0.03	2.98±0.08	1.63±0.03
Thr	2.60±0.02	3.83±0.05	5.29±0.06	4.39±0.15	5.17±0.04
Ala	2.85±0.07	1.72±0.08	4.10±0.02	5.44±0.11	1.72±0.07
Pro	8.04±0.17	14.55±0.06	10.52±0.11	4.94±0.03	2.72±0.06
Tyr	6.54±0.06	3.19±0.03	5.98±0.03	3.82±0.05	5.06±0.08
Val	4.74±0.05	7.65±0.08	5.92±0.08	5.19±0.04	4.41±0.02
Ile	5.19±0.06	4.64±0.08	6.47±0.03	5.70±0.01	6.53±0.04
Leu	9.30±0.10	10.66±0.01	7.18±0.11	13.94±0.03	11.26±0.09
Phe	5.55±0.06	5.72±0.04	4.34±0.06	3.45±0.11	4.53±0.03

[a] HPLC purified milk proteins were hydrolyzed in 6 *M* HC1 before amino acid analysis on a Beckman 6300 amino acid analyzer. Values are means±standard error. Each protein was ana1yzed four times for amino acid composition.

[b] Asx is the combination of aspartic acid and asparagine.

[c] Glx is the combination of glutamic acid and glutamine.

composition of a mixture of the five proteins was also entered into the program as the solution matrix and the concentrations of each protein in five different mixtures was determined. This analysis can be done with fewer amino acids but including additional amino acids in the standard matrix improves the accuracy of the procedure.

The proteins in cow's milk contain multiple variants of each of the dominant proteins. There are six genetic variants of β-LG, although only two genetic variants (A and B) are present in commercial milks in the USA. α-LB contains two genetic variants (A and B), but only B is found in Western cattle. The caseins also contain genetic variants. α_{s1}-CN B is the most common of the five variants of α_{s1}-CN: κ-CN B is the most common of the two κ-CN variants, β-CN A^2 is the most common of the seven variants of β-CN, and α_{s2}-CN A is the only variant of this protein, but it does exhibit variability in the extend of phosphorylation (reviewed in [9]).

Table 2 compares the concentration of each protein by amino acid analysis to the values calculated by HPLC analysis. Different mixtures contained varying concentrations of milk proteins. In this example, the casein content can be determined by adding the concentrations of α_s-CN, β-CN, κ-CN; the total protein was approximately the sum of the caseins and whey proteins. Each protein determination was the result of one analysis, and error is associated with calculating the amount of protein using both amino acid analysis and HPLC. Therefore, the differences in protein values listed in Table 2 are a combination of both errors.

Table 2
Comparing concentrations of proteins in five different mixtures by HPLC and amino acid analysis (AAA). Values are grams per 100 grams of protein

	Protein	HPLC calculated	AAA calculated
Mixture 1	α_s-CN	46.6	44.3
	β-CN	19.1	21.9
	κ-CN	10.6	9.5
	β-LG	12.0	13.1
	α-LB	11.7	11.1
Mixture 2	α_s-CN	36.3	38.4
	β-CN	22.1	21.7
	κ-CN	11.1	9.1
	α-LB	19.9	18.8
Mixture 3	α_s-CN	20.8	21.5
	β-CN	18.9	19.4
	κ-CN	20.6	18.7
	β-LG	19.2	18.6
	α-LB	21.0	21.5
Mixture 4	α_s-CN	31.1	36.9
	β-CN	19.7	21.3
	κ-CN	10.3	8.9
	β-LG	13.3	15.0
	α-LB	19.7	17.4
Mixture 5	α_s-CN	49.5	48.8
	β-CN	21.0	23.3
	κ-CN	8.9	7.2
	β-LG	11.4	12.1
	α-LB	9.3	8.6

Although there are multiple variants of the milk proteins, there typically exists only one or two variants in Western milks. The milk used in this study, and milk in general entering dairy processing plants, are combined from regional milk producers, therefore the variants of each of the proteins are consistent, within a dairy manufacturing region. In order to use this same technique to analyze for the individual milk proteins in different regions of the USA of the World, the standard or coefficient matrix must be generated. Therefore, the standard matrix is unique for a given region and represents the protein variants found in that region.

It may be possible to use this method on other mixtures of proteins; milk proteins were used here as an example. This method requires the initial separation of each protein from the mixture for amino acid analysis. The amino acid composition of the separated proteins can then be used repeatedly to determine the amount of each protein in a mixture. The standard matrix must represent the sample being analyzed, including concentrations of individual protein variants. This method makes it possible to monitor the concentrations of individual proteins in a mixture without repeatedly separating the mixture into its individual components.

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