

A Rapid Method for the Determination of Whey Protein Denaturation

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ABSTRACT

The use of reversed-phase HPLC for the determination of whey protein denaturation was investigated. Denatured whey proteins and caseins were isolated from undenatured whey proteins by isoelectric precipitation at pH 4.6. Whey protein denaturation was determined by comparing reversed-phase HPLC protein profiles of isolates for heat-treated and unheated NDM from pooled herd, Holstein cows. In general, protein profiles for heat-treated skim milk indicated that whey protein denaturation began at about 40°C, became more rapid at 70°C, and was 95% complete at 85°C. Undenatured whey protein was also quantified as whey protein nitrogen based on their absorbance and nitrogen content compared with known whey protein standards or by augmenting the same samples with a known amount of lysozyme. Whey protein nitrogen values obtained by the reversed-phase HPLC method were in good agreement with those obtained by the modified Kjeldahl nitrogen procedure for three NDM powders prepared from skim milk preheated to 63, 74, and 85°C before spray drying, and by commercial low and high heat NDM standards. Isolation of undenatured whey proteins and their separation by reversed-phase HPLC takes approximately 1.5 h.

(Key words: reversed-phase high performance liquid chromatography, whey protein denaturation, whey protein nitrogen)

Abbreviation key: α -LA = α -lactalbumin, AUFS = absorbance units full scale, β -LG = β -

lactoglobulin, BSA = bovine serum albumen, KN = Kjeldahl nitrogen, RP = reversed-phase, SPA = standard peak areas, TFA = trifluoroacetic acid, WPN = whey protein nitrogen.

INTRODUCTION

Skim milk is used in the food industry for the manufacture of baked goods, dairy products, and processed foods. During processing of skim milk and milk products, various heat treatments are used that can denature whey proteins. Preheat treatment of skim milk before spray drying is used widely as a means of improving water absorption and functional properties of NDM powders. The degree of whey protein denaturation depends on the intensity and duration of the heat treatment. There is, therefore, a need for a rapid method for assessing the extent of heat denaturation in milk protein products. Methods frequently used to assess the extent of protein denaturation include physical measurements of aggregation, Kjeldahl nitrogen (KN) determination, calorimetry (9), electrophoresis (3), and immunological properties (4). Most of these methods are either tedious and time consuming or exhibit poor reliability and accuracy (6). Recently, fast anion exchange chromatography has been shown to be more effective for determining whey protein denaturation in heat-treated milks (9) and gel permeation HPLC for the heat classification of skim milk powders (7, 12). This paper describes reversed-phase (RP) HPLC as a method for determining the extent of whey protein denaturation in heat-treated, reconstituted NDM powders.

MATERIALS AND METHODS

Milk Proteins and Protein Standard

Purified κ -, α -, and β -caseins were isolated from milk according to the procedure of Basch

et al. (3). α -Lactalbumin (α -LA), β -lactoglobulin (β -LG), bovine serum albumin (BSA), and lysozyme (95% protein, 17.5 nitrogen) were purchased from Sigma Chemical Co. (St. Louis, MO). Low and high heat standard reference NDM samples were supplied by the Western Commodities Scientific Support Division (CSSD) Laboratory, Agriculture Marketing Service, USDA and the American Dairy Products Institute (Chicago, IL).

Heat Treatment of Milk Samples

Pooled Holstein herd milk obtained from a local dairy was skimmed at 38°C. The skim milk was lyophilized immediately or heated at 63, 74 and 85°C for 30 min before spray drying to yield low, medium, and high heat NDM powders according to a previously published procedure (10). Whey protein denaturation profiles were obtained by heating reconstituted skim milk (1 g/10 ml) in a water bath at various temperatures for 30 min.

Isolation of Undenatured Whey Protein

Caseins and denatured whey proteins were precipitated from skim milk and reconstituted NDM samples by adjusting the pH to 4.6 with *N* HCl. The samples were held for 30 min, then either centrifuged for 30 min at 1000 \times *g* or filtered through S & S Number 605 filter paper. The supernatant or filtrate containing the undenatured whey proteins was used for chromatography.

Whey Protein Nitrogen

The whey protein nitrogen (WPN) content in the soluble fraction of reconstituted NDM was determined using the KN method (2, 11) or a modification of the KN technique in conjunction with the Harland-Ashworth salt precipitation method (5, 8).

Chromatography

Separation of milk proteins was performed by RP-HPLC at room temperature with a Spectra-Physics (San Jose, CA) model 8700XR pumping system, model 8750 injection system, model 4270 data system, and a model 450 Waters (Milford, MA) spectrophotometer, detector gain .1 AUFS (absorbance units full

scale). The column used was .46 \times 25-cm, C-4 reversed-phase, 10- μ particle size equipped with guard column containing the same packing material, VYDAC (Hesperia, CA). The mobile phase was solvent A: .1% trifluoroacetic acid (TFA) and solvent B: acetonitrile, .1% TFA. Flow was .8 ml/min. The supernatant or filtrate was diluted with an equal volume of solvent A, then passed through a .2 μ M filter, and 20 μ L was injected onto the column. Percentage of denaturation was calculated from the sum of the standardized peak areas (SPA) or peak heights for the heated whey proteins BSA, α -LA, β -LG B, and A and compared with those of the control (c) (heated at 38°C for skimming) (9):

$$\% \text{ Denaturation} = (\Sigma_{\text{SPA}_c} - \Sigma_{\text{SPA}_h}) / (\Sigma_{\text{SPA}_c})$$

Whey protein nitrogen in preheated NDM samples was determined using RP-HPLC by comparing whey protein peaks in the soluble fraction from the Kjeldahl determination or the diluted supernatant to known protein standards of known nitrogen content or by augmenting a .95-ml sample with .05 ml of lysozyme (2 mg/ml) immediately before injection into the column. Standard curves comparing absorbance at 280 nm to the concentration of protein standards; BSA, α -LA, and β -LG-B and β -LG-A, were linear at the same concentration range and chromatographic conditions at which the milk samples were run. The nitrogen content and extinction coefficient used $E_{281.5}^{1\%}$ for lysozyme was 17.5% (wt/wt) and 26.4, respectively.

Gas Electrophoresis

Polyacrylamide gel electrophoresis of lysozyme and milk proteins was carried out on a Phast System (Pharmacia, Piscataway, NJ) using a 8 to 25% gradient acrylamide gel as described by Parris et al. (10).

RESULTS AND DISCUSSION

Isolation of Undenatured Whey Protein

Methods for the evaluation of the extent of whey protein denaturation are based on various procedures to precipitate caseins and denatured whey proteins. Ultracentrifugation was not

suitable because β -casein and protein complexes formed due to heat treatment were not separated from undenatured whey proteins (unpublished). Precipitation with saturated NaCl or 2.5% TCA resulted in lower yields of undenatured whey protein with an enrichment of β -LG for the latter (7). We found that isoelectric precipitation of reconstituted milk powders with 1N HCl, followed by centrifugation or filtration (as described in Material and Methods) yielded a supernatant free of caseins except for a small amount of β -casein fragments (f106-209) and (f108-209). These fragments elute with heavy chain IgG and were identified by SDS-PAGE. The elution profile of the filtrate isolated from the reconstituted skim milk sample (heated to 38°C to facilitate skimming) contains BSA, α -LA, IgG (containing β -casein fragments) and genetic variants β -LG-B, β -LG-A (Figure 1A). Subsequent heating of the skim milk at 74 and 85°C for 30 min significantly reduced the amount of undenatured protein (Figures 1B and 1C). Optimization of conditions for the isolation of undenatured whey proteins by centrifugation or filtration showed no significant difference in chromatographic profile. However, filtration was preferred because of ease and convenience.

Determination of Whey Protein Denaturation

Percentage whey protein denaturation for BSA, α -LA, and β -LG-B and β -LG-A, was calculated as described using Equation [1]. Immunoglobulin G was not included in the calculation because it was contaminated with β -casein fragments. In general, whey protein denaturation, as a result of heating skim milk, begins at 40°C and proceeds slowly until a minimum denaturation point is reached at 65°C. Denaturation then proceeds more rapidly until about 95% of the whey proteins are denatured at 85°C (Figure 2). This denaturation pattern varies somewhat between milk batches and is probably dependent on milk protein composition since differences in the denaturation minimum were observed between batches of milk samples but not within the same batch. Examination of the supernatant of heat-treated skim milk after ultracentrifugation indicated that the denaturation minimum coincides with the formation of the whey-casein complex. The minimum is due to a stronger detector signal and

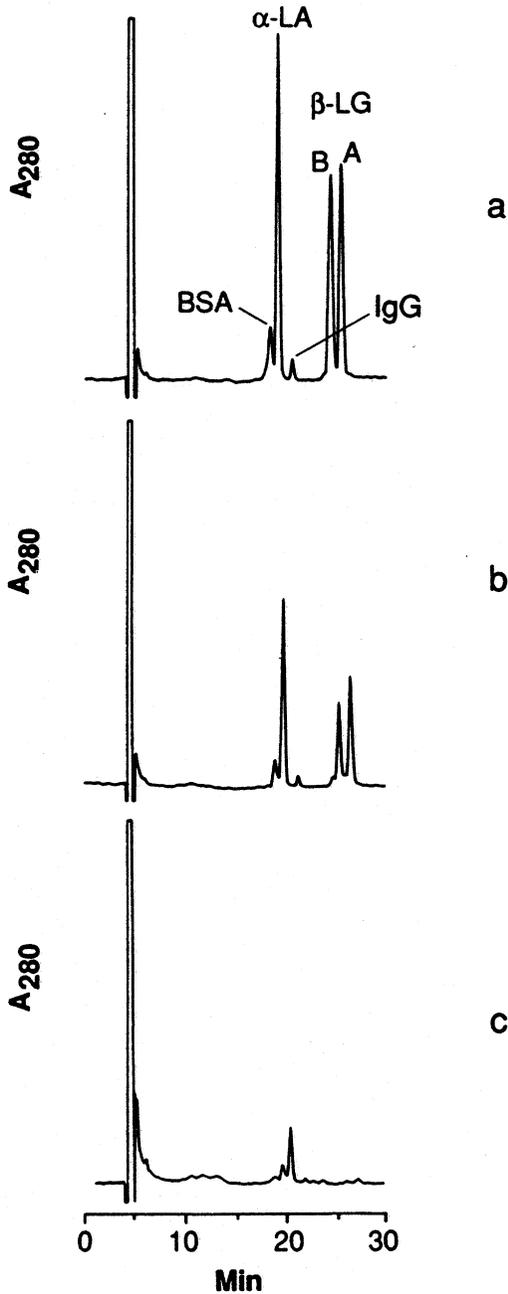


Figure 1. Reversed-phase HPLC elution profiles of heated skim milk. Gradient 30% to 45%B, 30 min. Heat treatment and attenuation: a) 38°C and 8 \times ; b) 74°C and 8 \times ; c) 85°C and 2 \times . A₂₈₀ = Absorbance at 280 nm, α -LA = α -lactalbumin, β -LG = β -lactoglobulin.

TABLE 1. Whey protein nitrogen in preheat-treated NDM samples by reversed-phase HPLC (RP-HPLC) and Kjeldahl nitrogen (KN).

NDM Powder	Methods			
	RP-HPLC ^{1,2,3}		KN ^{2,3}	Modified KN ²
	Standards	Augmented		
Low (standard)	6.7	6.4	16.1	7.4
High (standard)	0	0	5.1	.4
Low ⁴	7.8	7.7	13.4	7.4
Medium ⁴	4.3	4.2	11.0	3.7
High ⁴	.1	.1	3.7	5

¹Soluble fraction from KN determination was used for RP-HPLC method.

²Whey protein nitrogen in NDM, milligrams per gram.

³Average of three determinations.

⁴Prepared at the Eastern Regional Research Center.

probably results from conformational changes in the whey proteins with greater exposure of aromatic amino acid residues. The minimum always precedes complex formation and warrants further investigation.

Whey Protein Nitrogen

In order to compare RP-HPLC with other methods for measuring whey protein denaturation, it was necessary to quantify undenatured whey proteins in terms of WPN. One of the most widely used methods to determine whey protein denaturation is the measurement of WPN in the soluble fraction using the KN determination (2, 11). By comparing the absorbance of the same fraction used for the KN

determination to whey protein standards of known nitrogen content or by spiking the same fraction with a known amount of lysozyme, WPN can be determined for heat-treated milk samples using the RP-HPLC method. Lysozyme was selected as the protein standard because of its good solubility, its high extinction coefficient, and because it was well resolved from the whey proteins (Figure 3). A comparison between these two methods and the modified KN procedure (5, 8) is given for NDM powders in Table 1. The amount of WPN in NDM standard determined by the KN method was much higher than that reported for the chromatographic method. However, WPN values determined by the modified KN method were in good agreement with the chromato-

TABLE 2. Variance associated with peak area and peak height method of measurement for the reversed-phase HPLC method.

NDM powder	Standards			Augmented		
	\bar{X}^1	SD	CV ²	\bar{X}^1	SD	CV ²
	Peak area					
Low (standard)	7.5	.28	3.7	8.2	.30	3.6
Low	7.2	.19	2.7	7.6	.29	3.8
Medium	3.4	.05	1.4	3.8	.11	3.1
	Peak height					
Low (standard)	6.9	.07	1.0	8.8	.45	5.2
Low	7.2	.16	2.2	8.1	.23	2.8
Medium	3.5	.04	1.1	4.2	.14	3.4

¹Whey protein nitrogen in NDM, milligrams per gram, average of four determinations.

²Coefficient of variation, $100(\delta/\bar{X})$.

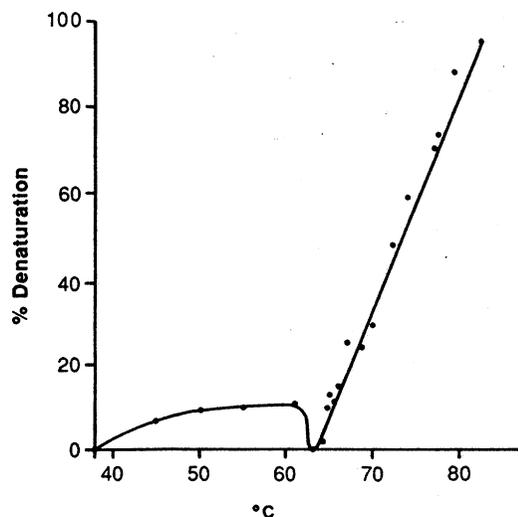


Figure 2. Typical whey protein denaturation profile for heat-treated skim milk.

graphic method calculated from known protein standards or by augmenting the sample. For NDM powders prepared at the Eastern Regional Research Center, KN values were also high and could not be classified properly. Based on the American Dry Milk Institute method (1) the milligrams of WPN content per gram of powder is low heat, > 6.0; medium heat, 1.51 to 5.99; high heat, < 1.5. The RP-HPLC and modified KN methods, therefore, classified the NDM powders properly. It appears that precipitation of casein and denatured whey protein with saturated NaCl is an important step in the modified KN procedure. Examination of the chromatograms for the soluble fraction from the KN method indicated the presence of a significant amount of α -, and β -caseins, which would also be quantified using this method. The soluble fraction for the low and medium heat powder contained up to 14 and 47% caseins, respectively. Because the RP-HPLC method selectively measures only the whey protein peak area or height, it is less affected by the presence of caseins and denatured whey proteins that were not removed by precipitation. Elution profiles of whey proteins from reconstituted NDM sample contained small peaks that elute before BSA and β -LG-B and β -LG-A (Figure 3). These peaks probably contain some heat-denatured whey protein be-

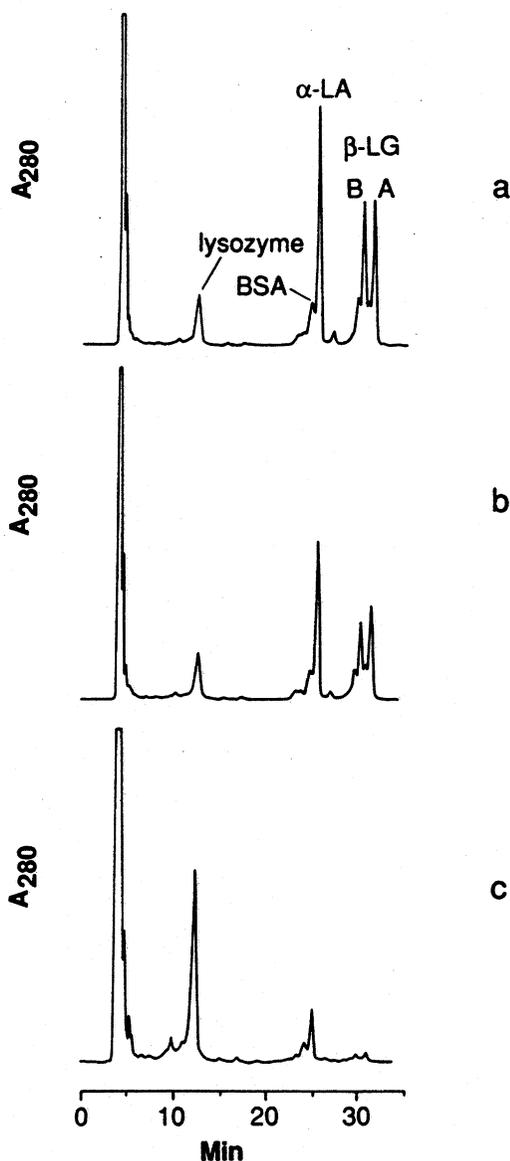


Figure 3. Reversed-phase HPLC elution profiles for reconstituted NDM samples augmented with lysozyme for whey protein nitrogen (WPN) determination. Gradient 30% to 32.5%B, 5 min; hold 5 min; 32.5% to 45%B, 25 min. Preheat treatment and attenuation: a) 63°C and 8 \times ; b) 74°C and 8 \times ; 85°C and 2 \times .

cause they are not present in the control (see Figure 1A) but are present in heat-treated milk (Figure 1B). Variance associated with method of measurement is shown in Table 2. Larger WPN values were obtained by augmenting the

TABLE 3. Whey protein in preheat-treated NDM samples at constant moisture.¹

NDM powder	Reversed-phase HPLC		Modified Kjeldahl nitrogen
	Standards	Augmented	
	WPN (mg/g)		
Low			
ERRC ²	7.2	7.1	7.2
9130L ³	6.7	6.9	7.0
9141L	6.5	6.5	7.1
9142L	7.4	7.6	7.5
Medium			
ERRC	4.2	4.5	4.4
High			
ERRC	.2	.2	.5
9130H	0	0	.4
9143H	0	0	.4
2102H	0	0	.5

¹At 3.16% H₂O. WPN = Whey protein nitrogen.

²Prepared at Eastern Regional Research Center.

³Sample number of powders obtained from Western Commodities Scientific Support Division.

samples than from protein standards. The CV varied between 1.0 and 3.7% using protein standards and between 2.8 and 5.2% for augmented samples. This variation was significantly smaller than that obtained for the KN method, which varied between 10 to 15%. In addition, CV values were smaller for the peak area method of measurement. Whey protein nitrogen values, at constant moisture, are given for four high, one medium, and four low preheat-treated NDM powders (Table 3). Good agreement was obtained using the RP-HPLC and modified KN methods for the low and medium heat powders. However, WPN values for the high heat powders were significantly higher using the modified KN procedure. This could be attributed to residual casein or denatured whey protein, which are measured using this method.

CONCLUSIONS

In conclusion, the potential of RP-HPLC was examined for measuring whey protein denaturation. Reversed phase-HPLC selectively measures undenatured whey protein and results can be expressed as WPN. The method based on comparison of whey proteins to known standards exhibited the least variability. The NDM powders were properly classified according to the American Dry Milk Institute heat treatment

classification ranges and WPN values compared favorably with those obtained using the modified KN procedure.

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