

Elimination of Lactose Interference in the Determination of Available Lysine Using Fluorine-19 Nuclear Magnetic Resonance Spectroscopy¹

J. R. CAVANAUGH
Eastern Regional Research Center
Philadelphia, PA 19118

ABSTRACT

When the ¹⁹F nuclear magnetic resonance method for determining free ε-amino groups of lysine residues in intact proteins is used on mixtures containing lactose, the amount of available lysine determined decreases with increasing lactose concentration. The reaction of lactose with lysine residues appears to be much more strongly promoted in dimethyl-sulfoxide than in aqueous environments as shown by comparable experiments in water. If the method is modified to remove lactose by dialysis prior to the reaction with S-ethyl trifluorothioacetate, the number of available ε-amino groups determined equals that of the pure proteins. The modified method as tested with nonfat dry milk gives excellent agreement with results obtained from a Kakade-Liener method.

INTRODUCTION

The determination of protein nutritional quality, in particular, loss of available lysine, is an important problem both in food processing as well as food storage. For example, the long-term storage of large quantities of nonfat dry milk currently being carried out under US Government auspices could have an adverse effect on available lysine levels. Good reliable methods for determining available lysine in food products are therefore needed. Although a number of relatively fast chemical methods have been developed (3) and applied to a variety of food systems including vegetable protein (9) and milk products (5, 13, 14),

methods exploring new techniques are very desirable. The ¹⁹F nuclear magnetic resonance (NMR) method previously developed in our laboratories for determining free ε-amino groups of lysine in proteins (12) gave very good results for a selection of pure proteins. The method was based on the trifluoroacetylation of the unsubstituted ε-amino groups and their subsequent quantitation using ¹⁹F NMR spectroscopy. However, when we attempted to extend the method to protein samples containing lactose, we found that lactose interfered with the trifluoroacetylation reaction and gave spurious results. We then investigated the conditions under which lactose interfered and explored a dialysis procedure to remove the lactose prior to the trifluoroacetylation reaction. The modified method was tested with protein and lactose mixtures and was compared with results obtained with a modified Kakade and Liener method (2, 4) on nonfat dry milk samples.

MATERIALS AND METHODS

β-Lactoglobulin was the 3× crystallized and lyophilized product from Sigma Chemical Company (St. Louis, MO) and was used without further purification. α-Lactalbumin was Grade II from Sigma. Because disc gel electrophoresis showed that these material were essentially pure, they were used as is. The other chemicals were the highest purity obtainable from commercial sources and were not further purified. The lactose was α-D-lactose monohydrate obtained from Eastman Kodak Company (Rochester, NY). The whey samples were a gift from V. H. Holsinger of this laboratory.

The ¹⁹F NMR spectra were obtained on a Bruker WH-90 Fourier-transform spectrometer operating at 84.66 MHz. Ten to twenty-five transients were accumulated for each spectrum with a 10 to 12-s delay between pulses. The relative intensities were obtained either from the

Received October 29, 1986.

Accepted December 28, 1987.

¹ Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

weight of the appropriate Fourier-transform resonance peaks or directly from the recorded integral of the spectrum. Both methods gave comparable accuracy and precision. The relative intensities were taken as the average of at least two separate data accumulations.

The incubation experiments with α -lactalbumin-lactose and β -lactoglobulin-lactose mixtures in dimethyl sulfoxide (DMSO) were performed as follows. The protein and lactose mixtures (protein weight of 10 to 35 mg) were dissolved in 500 to 750 μ l DMSO and incubated at room temperature, 40°C, or 60°C, for 24 h to ensure completeness of reaction. The more concentrated β -lactoglobulin samples were placed in a sonicating water bath for .5 to 1 h prior to the incubation in order to prevent gelling of the mixture. After the 24 h incubation, an appropriate amount of a concentrated S-ethyl trifluorothioacetate solution in d_6 -DMSO was added to the sample so that the resulting mixture contained approximately 1 ml of a 2% by weight solution of the thiol ester in DMSO. The ^{19}F NMR spectrum was taken and the available lysine was calculated as the weight percent of free lysine groups (unreacted ϵ -amino groups) in the protein as by Ramirez et al. (12).

In incubation experiments with β -lactoglobulin/lactose mixtures in water, the protein and lactose mixtures were dissolved in .05 M phosphate buffer (pH 7.2) with the protein concentration kept at 1%. The solutions were then incubated at room temperature, 40 or 60°C for 24 h. The samples were then dialyzed against water in the cold (4°C) for 24 h, the dry protein recovered by lyophilization, and the recovered protein reacted with S-ethyl trifluorothioacetate in DMSO (12).

The procedure followed with the α -lactalbumin and lactose mixtures and with nonfat dry milk samples was the same as above with the elimination of the incubation step; that is, the dialysis step was started immediately on preparing the buffered protein solution. In some experiments with the dry milk, samples for dialysis were prepared by adding 10 parts water to 1 part dry sample (as is reconstituted fat-free milk). These samples were dialyzed as above (4°C for 24 h) lyophilized, and the resulting dry uniform samples stored at room temperature.

The whey samples were subjected to 100% humidity at room temperature ($\sim 22^\circ\text{C}$) for 48 h. Samples were then heated to 70°C for 4 or 24 h. The resulting materials were then analyzed according to the procedures used for the nonfat dry milk samples.

The modified Kakade and Liener method developed by Dellamonica et al. (2) and used by Greenberg et al. (4) for the analysis of nonfat dry milk was based on dialysis of the samples prior to treatment with the 2, 4, 6-trinitrobenzene sulfonic acid reagent as in the Kakade and Liener method (7).

RESULTS AND DISCUSSION

When the method (12) with DMSO as solvent was extended to protein samples containing lactose, the number of trifluoroacetylated lysine residues determined was highly variable and seemed to depend on the amount of lactose present and the protein reaction time. Therefore, a set of experiments with protein and lactose mixtures was carried out to determine the effects of lactose under controlled temperature and reaction time. The results are shown in Figures 1 and 2 for β -lactoglobulin and α -lactalbumin, respectively.

Both figures show that the amount of available lysine determined (free ϵ -amino groups) decreased with increasing lactose

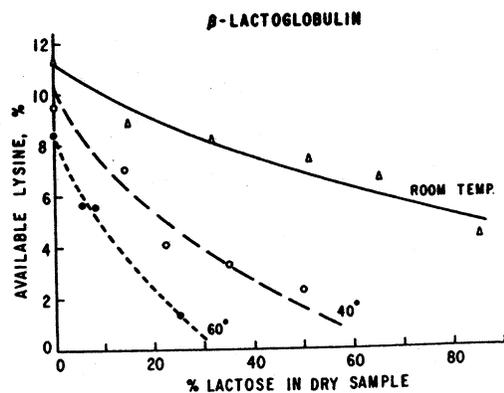


Figure 1. Available lysine determined for β -lactoglobulin and lactose mixtures incubated in dimethylsulfoxide solution at several temperatures. The curves are hand drawn.

AVAILABLE LYSINE DETERMINATION

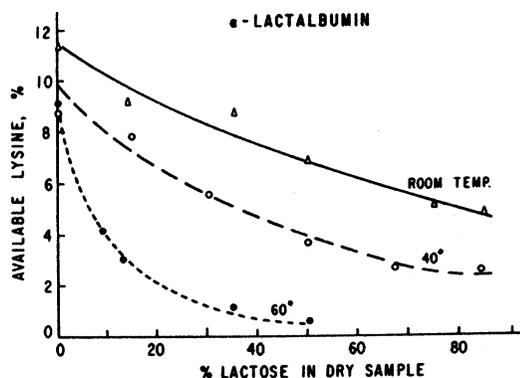


Figure 2. Available lysine determined for α -lactalbumin/lactose mixtures incubated in dimethylsulfoxide solution at several temperatures. The curves shown are hand drawn.

in the mixture. This effect was highly sensitive to incubation temperature; the fall off in the available lysine rapidly increased at the higher temperatures.

These results at first appear to be similar to the effects seen in aqueous environments (8, 11). However, the incubation conditions in the DMSO system are relatively mild in contrast to those in the milk systems under which Maillard type reactions are known to occur (10). In fact, if protein and lactose mixtures in an aqueous environment are incubated at the specified temperatures, a very different result is obtained as shown in the following. Mixtures of lactose and protein were taken up in buffer solution and incubated as described in Materials and

Methods. After lactose was removed by dialysis, the protein was recovered by lyophilization and tested for the number of free ϵ -amino groups of lysine (12). The data for β -lactoglobulin and lactose mixtures (Table 1) were evaluated by an analysis of variance and showed no significant ($P = .05$) evidence of a systematic variation in the amount of available lysine determined either as a function of lactose concentration or incubation temperature. Even though the variability of the results does increase with increasing lactose, it appears that the number of free ϵ -amino groups remains the same throughout and is approximately equal to the value for the pure protein (6). Therefore, DMSO as solvent represents a very different system that strongly promotes the reaction between lactose and proteins. In any event, in order to use the ^{19}F method for determining available lysine in protein-lactose mixtures, lactose must be removed prior to the procedure. Dialysis followed by lyophilization to recover the separated protein, was a convenient method but a variety of procedures, such as column chromatography and precipitation methods for separating the lactose, can be envisioned.

Applying the method to a series of α -lactalbumin/lactose mixtures, we obtained the results shown in Table 2. Using an analysis of variance, the means for the 3 lactose to protein ratios showed no significant different at the 5% level.

The procedure was compared to a modified Kakade and Liener method (2, 4) for deter-

TABLE 1. Determination of available lysine of α -lactose, β -lactoglobulin, mixtures incubated in aqueous solutions (means and standard errors).¹

Incubation temperature	Lysine found		
	Lactose:protein ratio		
	1:1	3:1	10:1
	(wt%)		
Room	10.7 .3	9.8 .8	10.9 .4
40°C	10.3 .6	11.3 1.5	8.7 1.8
60°C	10.7 .9	13.1 .6	9.3 1.1
Average for all temperatures	10.6 .7	11.2 1.7	9.4 1.5

¹ Value for the pure protein is 11.4 weight percent (6) or 15 residues per molecule (1).

TABLE 2. Determination (means and standard errors) of available lysine of α -lactose and α -lactalbumin mixtures following dialysis with incubation.¹

Lactose:protein ratio	Lysine	n ²
	(wt %)	
1:1	9.8 2.0	7
3:1	10.0 1.2	13
10:1	10.4 1.5	6

¹ Value for the pure protein is 11.5% (6) or 12 residues per molecule (1).

² Number of determinations.

mining available lysine with several commercial nonfat dry milk samples and the results are shown in Table 3. An analysis of the data based on Student's *t* test showed no significant difference between the two methods.

Finally, the method was applied to whey samples that had been humidified at 100% humidity at room temperature and then heated at 70°C. The results (in Table 4) show the expected decrease in available lysine with increasing heating times.

Consequently, the ¹⁹F NMR method for determining available lysine can be usefully

TABLE 3. Comparison of available lysine determined for nonfat dry milk samples (means and standard errors).

Sample number	Lysine	
	This method	Modified Kakade-Leiner method ¹
	(wt %)	
1	6.8	7.01
	1.1	.35
2	6.7	6.97
	1.1	.35
3	6.8	6.85
	1.4	.34

¹ References (2, 4).

TABLE 4. Determination of available lysine of whey samples (means and standard errors).

Heating time ¹	Lysine
	(wt%)
0	7.2 1.0
4 h	6.6 1.3
24 h	3.7 1.3

¹ Samples were heated at 70°C for times shown.

extended to protein samples containing reducing sugars. Although the dialysis and lyophilization steps practiced here do increase the time required for analysis, other speedier procedures for separating the lactose could be devised and substituted. The greatest drawback to this method appears to be the variability in results. The standard deviation among replicates often exceeds 1% in the lysine determined. The ease of the method, however, offers distinct advantages when this kind of uncertainty can be tolerated.

ACKNOWLEDGMENTS

We thank R. Greenberg and H. J. Dower for supplying us with nonfat dry milk samples and for the results on the amount of available lysine determined according to the modified Kakade and Liener method.

REFERENCES

- Dayhoff, M. D. 1972. Atlas of protein sequence and structure. Vol. 5. Natl. Biomed. Res. Found., Silver Spring, MD.
- Dellamonica, E. S., E. O. Strolle, and P. E. McDowell. 1976. A modified method for determining available lysine in protein recovered from heat treated potato juice. *Anal. Biochem.* 73:274.
- Erbersdobler, H. F., and T. R. Anderson. 1983. Determination of available lysine by various procedures in Maillard type products. The Maillard reaction in foods and nutrition. *Am. Chem. Soc. Symp. Ser.* 215:419.
- Greenberg, R., H. J. Dower, and J. H. Woychik. 1977. An improved trinitrobenzene sulfonic acid procedure for the determination of available lysine in nonfat dry milk. *Abstr. of papers of The 173rd Natl. Mtg. Am. Chem. Soc., New Orleans, LA.*

AVAILABLE LYSINE DETERMINATION

- paper 72. Publ. Am. Chem. Soc., Washington, DC.
- 5 Holguin, M., and S. Kakai. 1980. Accuracy and specificity of the dinitrobenzene sulfonate methods for available lysine in proteins. *J. Food Sci.* 45: 1218.
 - 6 Jenness, R., and S. Patton. 1959. Page 125 in *Principles of Dairy Chemistry*. John Wiley and Sons, New York, NY.
 - 7 Kakade, M. L., and I. E. Liener. 1969. Determination of available lysine in proteins. *Anal. Biochem.* 27:273.
 - 8 Mottu, F., and J. Mauron. 1967. The differential determination of lysine in heated milk. II. Comparisons of the *in vitro* methods with the biological evaluation. *J. Sci. Food Agric.* 18:57.
 - 9 Obi, I. U. 1982. Application of the 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) method for determination of available lysine in maize seed. *Agric. Biol. Chem.* 46:15.
 - 10 Patton, S. 1955. Browning and associated changes in milk and its products: a review. *J. Dairy Sci.* 38:457.
 - 11 Posati, L. P., V. H. Holsinger, E. D. DeVilbiss, and M. J. Pallansch. 1972. Factors affecting the determination of available lysine in whey with 2,4,6-trinitrobenzene sulfonic acid. *J. Dairy Sci.* 55: 1660.
 - 12 Ramirez, J. E., J. R. Cavanaugh, K. S. Schweizer and P. D. Hoagland. 1975. The determination of free ϵ -amino groups of lysine in proteins using ^{19}F NMR spectroscopy. *Anal. Biochem.* 63:130.
 - 13 Tomarelli, R. M., R. J. Yuhas, A. Fisher, and J. R. Weaber. 1985. An HPLC method for the determination of reactive (available) lysine in milk and infant formulas. *J. Agric. Food Chem.* 33:316.
 - 14 Womach, M., and V. H. Holsinger. 1979. Protein quality of stored dry skim milk with standard and lysine-limiting diets. *J. Dairy Sci.* 62:855.