Effect of Elevated Temperature Storage on the Digestible Reactive Lysine Content of Unhydrolyzed- and Hydrolyzed-Lactose Milk-Based Products

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ABSTRACT

The study aimed to evaluate the effects of storage at elevated temperatures on reactive Lys content and true ileal reactive Lys digestibility in a skim milk powder and a hydrolyzed-lactose skim milk powder. A validated bioassay based on guanidination of food and digesta samples was applied. Semisynthetic diets containing the milk powders as the sole sources of protein were formulated and fed to growing rats. Chromic oxide was included in each diet as an indigestible marker. Digesta were collected posteuthanasia and analyzed along with the diets for reactive Lys (homoarginine), and true ileal reactive Lys digestibility was calculated after correction for endogenous Lys loss. For the skim milk powder, there was no decrease in reactive Lys digestibility (Lys availability) when the powder was stored at 30 and 35°C for 18 mo. In contrast, when stored at 40°C for 12 mo, a small but statistically significant (6%) decrease was observed. For the hydrolyzed-lactose product, a 22% decrease in Lys availability was observed after storage at 35°C for 18 mo, and a 17% decrease was observed when stored at 40°C for only 6 mo. Digestible reactive (available) Lys content decreased by more than 20% for the skim milk powder stored at 30 and 35°C for 18 mo and 40% when stored at 40°C for 12 mo. Furthermore, available Lys decreased in the hydrolyzed-lactose skim milk powder by 41% when stored at 30°C for only 18 mo and 34 and 65% when stored at 35 and 40°C, respectively, for 6 mo. Elevated temperatures and prolonged storage periods negatively influenced the available Lys contents of both milk powders. The decrease in available Lys content and Lys availability was greater for the hydrolyzed-lactose skim milk powder compared with the normal skim milk powder, after storage at elevated temperatures.

Key words: lysine, milk, availability, storage

INTRODUCTION

Lysine is often the first-limiting indispensable AA in diets for humans, underlining the nutritional importance of milk proteins, which are significant dietary sources of Lys. In milk products that undergo heat processing or during storage, however, the ϵ -amino group of Lys can react with compounds in milk, particularly milk sugars, to produce nutritionally unavailable derivatives (Hurrell and Carpenter, 1981; Moughan, 2003). Some of these derivatives are acid labile and revert back to Lys during the acid hydrolysis step of conventional AA analysis, leading to inaccuracy in the determination of the Lys and digestible Lys contents of processed milk products (Rutherford and Moughan, 2005). There are a number of methods that can be used to accurately determine the chemically reactive Lys content of processed protein sources, including the fluoro-2,4-dinitrobenzene (FNBS), 2,4,6-trinitrobenzenesulfonic acid (TNBS), and guanidination methods (Hurrell and Carpenter, 1974). However, these techniques do not take into account the incomplete absorption from the small intestine that can occur with a damaged protein. A method has been developed (Moughan and Rutherford, 1996) that accurately determines digestible reactive (available) Lys in processed foods (Rutherford et al., 1997). The assay utilizes the guanidination reaction, specific to the ϵ -amino group of Lys (Klee and Richards, 1957; Kassell and Chow, 1966; Moughan and Rutherford, 1996; Catrein et al., 2005; Yamaguchi et al., 2005; Zhang et al., 2006), to determine the amounts of reactive Lys in both diet and digesta for an animal fed that test diet. Reactive Lys digestibility is then calculated as the difference between dietary reactive Lys intake and ileal reactive Lys output. The assay has been applied to selected milk-based products (Rutherford and Moughan, 2005) to a variably heated skim milk powder (Rutherford and Moughan, 1997) and to a range of cereal breakfast foods (Rutherford et al., 2006). The aim of the present study was to apply the assay to determine the effect of long-term storage at elevated temperatures (30 to 40°C) on digestible reactive Lys content in an unhydrolyzed and hydrolyzed-lactose skim milk powder. The study

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Table 1. Conditions used to test the effect of storage duration and air temperature on digestible reactive Lys contents in a skim milk powder and a hydrolyzed-lactose skim milk powder

Storage time (mo)	Storage temperature (°C)					
	Skim milk powder			Hydrolyzed-lactose skim milk powder		
	30	35	40	30	35	40
0	X ¹	X	X	X	X	X
1.5			X			X
3	X	X	X	X	X	X
4.5						X
6	X	X	X	X	X	X
9	X	X	X	X	X	
12	X	X	X	X	X	
15					X	
18	X	X		X	X	

¹An X indicates that a sample of either skim milk powder or hydrolyzed-lactose skim milk powder underwent storage at the corresponding time and temperature.

sought to determine the degree of protein damage and indicate shelf life for products stored under temperatures encountered in warmer world climates.

MATERIALS AND METHODS

Materials

O-Methylisourea was obtained from Sigma Chemicals (St. Louis, MO) and barium hydroxide octahydrate from BDH Laboratory Supplies (Poole, UK). Two milk-based protein products were obtained locally; these included a commercial skim milk powder and a commercial hydrolyzed-lactose skim milk powder. The protein content of the milk powders was determined on a Leco analyzer using the Dumas method (IDF, 1993), and CP was calculated as the total N content multiplied by 6.38. The skim milk powder and the hydrolyzed-lactose skim milk powder contained 37.5 and 38.2% protein, respectively. Laboratory rats were obtained from the Small Animal Production Unit, Massey University, Palmerston North, New Zealand.

Storage Conditions

The skim milk powder and the corresponding hydrolyzed-lactose skim milk powder were stored in a temperature-controlled room at 30, 35, and 40°C in 500-g lots in vacuum-sealed sample bags for up to 18 mo. The storage regimen is shown in Table 1.

Digestibility Study

One hundred and sixty male Sprague-Dawley rats of approximately 150 g of BW were housed individually in stainless steel wire-bottomed cages in a room maintained at 22 ± 2°C, with a 12-h light-dark cycle. Thirty-two semisynthetic wheat starch-based test diets were

formulated to each contain 100 g/kg of CP. The ingredient compositions of the experimental diets are shown in Table 2. Chromic oxide was included (0.5%) in each diet as an indigestible marker. All diets met the nutritional requirements of the growing rat with the exception of protein (NRC, 1995). The animals were randomly allocated to the dietary treatments such that there were 5 animals per treatment, and they were fed their respective diets for a 14-d experimental period. On each day, each rat received its diet as 9 meals given hourly (0830 to 1630 h); each meal time consisted of a 10-min period in which the rats had unrestricted access to their diet. Water was available at all times. On the final day of the study, from 5.5 to 7 h after the start of feeding, the rats were asphyxiated using CO₂ gas and then decapitated. The 20 cm of ileum immediately anterior to the ileo-

Table 2. Ingredient compositions¹ (g/kg of air dry weight) of the experimental diets

Item	Skim milk powder ²	Hydrolyzed-lactose skim milk powder ³
Wheat starch	433	428
Soybean oil	50	50
Cellulose	50	50
Sucrose	100	100
Vitamin premix ⁴	50	50
Mineral premix ⁴	50	50
Skim milk powder	262	—
Hydrolyzed-lactose milk powder	—	267
Chromic oxide	5	5

¹All diets were formulated to contain equal CP contents.

²All the skim milk powder-based diets for samples stored at the varying times and temperatures were prepared using this formulation.

³All the hydrolyzed-lactose skim milk powder-based diets for samples stored at the varying times and temperatures were prepared using this formulation.

⁴Vitamin-mineral mix was formulated to meet the requirements of the growing rat for vitamins and minerals in the final diets (NRC, 1995).

cecal junction was dissected out, carefully washed with distilled deionized water, and dried on absorbent paper. The digesta were gently flushed from the ileum section with distilled deionized water and freeze-dried ready for chemical analysis.

Ethics approval for the animal trial was obtained from the Animal Ethics Committee, Massey University, Palmerston North, New Zealand.

Chemical Analysis

Reactive Lys contents were determined in duplicate 5-mg milk powder and digesta samples and quadruplicate 5-mg diet samples after incubation for 1, 7, and 7 d, respectively, in 0.6 M *O*-methylisourea, pH 10.6 (pH 11.0 for the digesta samples), at 21°C in a shaking water bath, with the reagent:Lys ratio being greater than 1,000, according to the procedure of Moughan and Rutherford (1996). The 0.6 M *O*-methylisourea solution was prepared as described by Moughan and Rutherford (1996). After incubation, the samples were dehydrated using a Speedvac concentrator (Savant Instruments Inc, Farmingdale, NY) and analyzed for homoarginine using a Waters ion-exchange HPLC system, utilizing postcolumn ninhydrin derivatization and detection using absorbance at 570 nm, after hydrolysis in 6 M glass-distilled HCl containing 0.1% phenol for 24 h at $110 \pm 2^\circ\text{C}$ in evacuated sealed tubes. The weight of reactive Lys was calculated from the determined molar quantity of homoarginine and the molecular weight for free Lys.

The Cr contents of the diet and ileal digesta samples were determined in duplicate on a GBC 902 AA absorption-emission spectrophotometer (GBC Scientific NZ Ltd., Auckland, New Zealand) following the method of Costigan and Ellis (1987).

Data Analysis

Ileal reactive Lys flows at the terminal ileum were calculated using the following equation (units are $\mu\text{g/g}$ of DMI): ileal reactive Lys flow = ileal reactive Lys $\times$ diet Cr/ileal Cr.

True ileal reactive Lys digestibility was calculated using the following equation (units are $\mu\text{g/g}$ of DMI): True ileal reactive Lys digestibility (%) = [dietary reactive Lys intake – (ileal reactive Lys flow – endogenous Lys flow)]/dietary reactive Lys intake $\times$ 100. The Lys flow was based on the endogenous AA flows for the growing rat reported by Rutherford and Moughan (1998).

The reactive Lys digestibility and digestible reactive Lys data were analyzed, using an analysis of covariance. The covariate was fitted as an exponential form. Temperature and milk powder type were fixed factors, and time was the covariate. Fixed factor effects were incorporated

into the model by means of the dummy variable technique (Zar, 1984). SigmaPlot (Jandel Scientific, San Raphael, CA) software was used for model fitting.

RESULTS

The Effect of Storage on the Reactive Lys Content of a Skim Milk Powder and a Hydrolyzed-Lactose Skim Milk Powder

The 2 milk powders (skim milk powder and hydrolyzed-lactose skim milk powder) were stored at 30, 35, and 40°C from 0 to 18 mo. The reactive Lys content of the milk powders was determined periodically throughout this storage period, and the results are shown in Table 3. For the skim milk powder stored at 30 and 35°C, the reactive Lys content decreased linearly ($R^2 = 0.93$ and 0.94 for the skim milk powder stored at 30 and 35°C, respectively) from 33 g/kg initially to approximately 26 g/kg after 18 mo of storage. The reactive Lys content of the skim milk powder stored at 40°C decreased linearly ($R^2 = 0.94$) from 33 to 21 g/kg after 12 mo of storage.

For the hydrolyzed-lactose skim milk powder stored at 30°C, the reactive Lys content decreased linearly ($R^2 = 0.99$) from 28 g/kg initially to 18 g/kg after 18 mo of storage, whereas over the same storage period, the reactive Lys content in the hydrolyzed-lactose milk powder stored at 35°C decreased linearly ($R^2 = 0.90$) from 28 to 13 g/kg. When this hydrolyzed-lactose milk powder was stored at 40°C, the reactive Lys content decreased linearly ($R^2 = 0.91$) from 28 to 12 g/kg after only 6 mo of storage.

True Ileal Reactive Lys Digestibility of a Skim Milk Powder and a Hydrolyzed-Lactose Skim Milk Powder Before and After Storage

The determined mean true ileal reactive Lys digestibilities for the 2 products determined before and after storage are shown in Table 4. The reactive Lys digestibility of both the skim milk powder and hydrolyzed-lactose skim milk powder before storage was 100%. There was a statistically significant effect of time and temperature on the true ileal reactive Lys digestibility ($P < 0.001$ and 0.05 , respectively), in which digestibility decreased with increasing storage temperature and storage time. There was a significant interaction ($P < 0.001$) between milk powder type and time ($P < 0.001$) and temperature ($P < 0.001$) in which digestibility decreased to a greater degree in the hydrolyzed-lactose skim milk powder than the normal skim milk powder as storage temperature and time increased. There was also a significant interaction ($P < 0.001$) between time and temperature.

Table 3. Mean ($\pm$ SE) reactive Lys content¹ (g/kg of air-dry weight) for a skim milk powder and a hydrolyzed-lactose skim milk powder stored at 30, 35, and 40°C for varying time periods

Storage period (mo)	Storage temperature (°C)					
	Skim milk powder			Hydrolyzed-lactose skim milk powder		
	30	35	40	30	35	40
0	33.0 (0.06)	33.0 (0.06)	33.0 (0.06)	28.0 (1.8)	28.0 (1.8)	28.0 (1.8)
1.5			28.3 (1.18)			19.6 (0.30)
3	31.3 (1.38)	31.3 (2.14)	28.1 (0.70)	27.0 (0.10)	22.7 (0.82)	16.8 (1.05)
4.5						13.3 (1.06)
6	29.8 (0.01)	30.1 (0.13)	26.1 (0.07)	24.0 (0.04)	18.9 (0.65)	11.7 (0.40)
9	29.7 (0.19)	28.8 (0.14)	22.1 (5.03)	22.4 (0.10)	18.2 (0.08)	
12	26.4 (1.99)	25.7 (0.21)	21.0 (0.10)	21.2 (0.43)	17.0 (0.34)	
15					15.9 (0.10)	
18	26.1 (1.47)	25.6 (0.15)		17.9 (0.64)	12.6 (0.00)	

¹Mean values based on duplicate determinations.

True Ileal Digestible Reactive (Available) Lys Content of a Skim Milk Powder and a Hydrolyzed-Lactose Skim Milk Powder Before and After Storage

True ileal digestible reactive Lys contents for the skim milk powder and the hydrolyzed-lactose skim milk powder stored at elevated temperatures for up to 18 mo were determined (Table 5). There was a 20 and 23% ($P < 0.001$) decrease in digestible reactive Lys content for the skim milk powder stored for 18 mo at 30 and 35°C, respectively, whereas for the skim milk powder stored at 40°C for 12 mo, there was a 40% decrease ($P < 0.001$) in digestible reactive Lys content. For the hydrolyzed-lactose skim milk powder stored for 18 mo at 30 and 35°C, there was a 41 and 65% ($P < 0.001$) reduction in the digestible reactive Lys content, respectively. After only 6 mo of storage at 40°C, the digestible reactive Lys content of the hydrolyzed-lactose skim milk powder was also decreased by 65%. The hydrolyzed-lactose skim milk powder suffered greater digestible reactive Lys loss with increasing storage time and temperature compared with the normal skim milk powder.

DISCUSSION

Lysine is a nutritionally important AA, and because of this, the accurate assessment of its content and availability in foods is important. Lysine can be chemically modified during the processing or storage of foods to form nutritionally unavailable derivatives (e.g., Maillard products). Because some of these products can interfere with the chemical analysis of Lys, traditional methods for determining available Lys may not always be accurate. Recently, a new assay methodology for accurately determining ileal digestible reactive Lys (available Lys) has been developed (Moughan and Rutherfurd, 1996). This assay was used here to determine the available Lys content of a skim milk powder and a hydrolyzed-lactose skim milk powder after long-term storage at elevated temperatures.

The Effect of Storage on the Reactive Lys Content of a Skim Milk Powder and a Hydrolyzed-Lactose Skim Milk Powder

For the skim milk powder, the reactive Lys content decreased by approximately 22% when stored at 30 and

Table 4. Mean ($n = 5$) true ileal reactive Lys digestibility¹ (%) for a skim milk powder and a hydrolyzed-lactose skim milk powder stored at 30, 35 and 40°C for varying time periods

Storage period (mo)	Storage temperature (°C)					
	Skim milk powder			Hydrolyzed-lactose skim milk powder		
	30	35	40	30	35	40
0	100	100	100	100	100	100
1.5			99			94
3	100	100	99	98	98	93
4.5						96
6	100	100	99	99	98	83
9	101	100	98	98	95	
12	101	100	94	94	85	
15					85	
18	101	98		94	78	

¹Reactive Lys digestibility was determined using a true ileal AA digestibility assay (rat). The guanidination reaction was used to quantitate reactive Lys in the diets and digesta.

Table 5. Mean ($\pm$ SE, $n = 5$) digestible reactive Lys contents¹ (g/kg of air-dry weight) for a skim milk powder and a hydrolyzed-lactose skim milk powder stored at 30, 35 and 40°C for varying time periods

Storage period (mo)	Storage temperature (°C)					
	Skim milk powder			Hydrolyzed-lactose skim milk powder		
	30	35	40	30	35	40
0	32.9 (0.05)	32.9 (0.05)	32.9 (0.05)	28.1 (0.03)	28.1 (0.03)	28.1 (0.03)
1.5			28.0 (0.08)			18.4 (0.13)
3	31.2 (0.06)	31.3 (0.07)	27.9 (0.12)	26.6 (0.11)	22.3 (0.10)	15.5 (0.12)
4.5						12.7 (0.23)
6	29.8 (0.12)	30.3 (0.07)	25.9 (0.39)	23.9 (0.27)	18.5 (0.21)	9.7 (0.58)
9	29.9 (0.01)	28.9 (0.17)	21.5 (0.12)	22.0 (0.32)	17.3 (0.23)	
12	26.5 (0.10)	25.7 (0.21)	19.8 (0.45)	19.8 (0.19)	14.5 (0.37)	
15					13.5 (0.36)	
18	26.3 (0.15)	25.2 (0.26)		16.7 (0.23)	9.9 (0.35)	

¹Digestible reactive Lys was calculated from true ileal reactive Lys digestibility (rat, guanidination analysis), and the reactive lysine content of the respective milk powder, which was also determined using guanidination.

35°C for 18 mo, whereas during storage at 40°C, the reactive Lys content decreased by 36% in only 12 mo. For the hydrolyzed-lactose skim milk powder, the reduction in reactive Lys content during storage at elevated temperatures was greater, with decreases of 36% after 18 mo of storage at 30°C, 55% after 18 mo of storage at 35°C, and 58% after 6 mo of storage at 40°C. In all cases, there was a considerable reduction in reactive Lys content after storage, suggesting extensive Lys damage had occurred to both milk powders after storage at elevated temperatures. Furthermore, and as expected, the reduction in Lys was greater in the milk powders stored at the higher temperatures. It is also interesting that the loss of Lys over time was very nearly linear for both products over all the temperatures tested.

It is of note that the Lys content decreased to a much greater extent and more rapidly in the hydrolyzed-lactose skim milk powder than in the normal skim milk powder. The reducing sugar content of the hydrolyzed-lactose skim milk powder is twice that of the skim milk powder, because lactose (a reducing sugar) is hydrolyzed to glucose and galactose, which are in turn both reducing sugars. It is likely that the higher concentration of reducing sugars in the hydrolyzed-lactose product compared with its unhydrolyzed counterpart explains the difference in Lys damage observed for the 2 milk powders.

True Ileal Reactive Lys Digestibility of a Skim Milk Powder and a Hydrolyzed-Lactose Skim Milk Powder Before and After Storage

The reactive Lys in the fresh skim milk powder was completely digested. Rutherfurd and Moughan (1997) also found the reactive Lys in skim milk powder to be highly digestible (100%). For the skim milk powder stored at 30 and 35°C, there was no reduction in reactive Lys digestibility over the 18-mo storage period. When

stored at 40°C, the reactive Lys digestibility did decrease after 12 mo of storage, but the decrease was not large (6%). In contrast, a similar small decrease (6%) in reactive Lys digestibility was observed for the hydrolyzed-lactose skim milk powder when stored for 12 mo at only 30°C. When stored at 35°C, a similar (5%) decrease was observed after only 9 mo, and after 18 mo of storage, reactive Lys digestibility had decreased by 22%. When stored at 40°C, digestibility had dropped markedly (a 17% decrease) after only 6 mo.

Clearly, when skim milk powder underwent prolonged storage at elevated temperatures, Lys availability decreased, but this reduction was not large. In contrast, for a skim milk powder that had undergone lactose hydrolysis before storage, the decrease in Lys availability was large. The lactose hydrolysis process clearly had a major effect on the digestibility of reactive Lys when the products were stored for long periods of time at elevated temperatures, most likely an effect of greater Lys damage due to the higher concentration of reducing sugars in the hydrolyzed products. The decrease in reactive Lys digestibility may be brought about by a reduction in the effectiveness of trypsin in hydrolyzing the peptide bonds in proximity to the modified Lys residues. This would then further hinder digestion by other intestinal proteases such as chymotrypsin, resulting in the presence of indigestible limit peptides (Moughan et al., 1996). These limit peptides may contain any of the AA present in proteins and will therefore result in a reduction in the digestibility of all AA, not just Lys (Moughan and Rutherfurd, 1996).

True Ileal Digestible Reactive (Available) Lys Content of a Skim Milk Powder and a Hydrolyzed-Lactose Skim Milk Powder Before and After Storage

For the skim milk powder stored at 30 and 35°C, the reduction in the true ileal digestible reactive Lys content

after storage for 18 mo was large and was similar to the reduction observed for the gross reactive Lys content of the skim milk powder, because digestibility was essentially complete for these samples. When the skim milk powder was stored at 40°C, the reduction in digestible reactive Lys was almost double that observed after storage at the lower temperatures after only two-thirds the storage time. For the hydrolyzed-lactose skim milk powder, the reduction in digestible reactive Lys content was very large even at the lower storage temperatures (30 and 35°C), whereas for the samples stored at 40°C, well over half of the digestible reactive Lys was destroyed after only 6 mo of storage.

Clearly, temperature plays a significant role in the stability of Lys in milk powders. Digestible reactive Lys content was 34% higher when the powders were stored at 30°C compared with storage at 40°C for the same length of time (12 mo). For the hydrolyzed-lactose product, this difference was even more dramatic with digestible reactive Lys content, being as much as 146% higher in the product stored for 6 mo at 30°C compared with the same time at 40°C. Other studies have shown similar effects; van Barneveld et al. (1994) found a 73% decrease in the available Lys content of field peas heated from 110 to 165°C when determined using an animal growth assay.

Overall, skim milk powder is a highly digestible, high-quality protein source. However, there was a sizable loss of reactive Lys and digestible reactive Lys in the skim milk powder when stored at elevated temperatures for prolonged periods. Again, the effect was much more dramatic for the hydrolyzed-lactose product. The study demonstrates that at elevated temperatures, such as those experienced in warmer world climates, considerable Lys damage can occur in relatively short periods of time (6 mo) and that these may be high enough to warrant concern from a nutritional viewpoint. The study also demonstrates the utility of the BIOLYSINE assay (Moughan and Rutherford, 1996) in predicting the effects of storage on protein quality and evaluating product shelf life.

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