HIGH-PRESSURE LIQUID CHROMATOGRAPHIC METHOD FOR EVALUATING FREE LYSINE STABILITY IN LYSINE-FORTIFIED WHEAT-FLOUR PRODUCTS[1]

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ABSTRACT   Cereal Chem. 55(4): 481-488

A rapid high-pressure liquid chromatographic method was developed to quantify free lysine in lysine-fortified foods. Free lysine was extracted from fortified wheat flour or fortified wheat-flour products and reacted with dansyl chloride. Didansyl lysine was separated by reverse-phase high-pressure liquid chromatography and detected by monitoring absorbance at 254 nm. Added lysine in wheat flour heated at 100°C for 2 hr. With 10% added glucose and 10% added water, only 19.1% of the added lysine was recovered after 2 hr at 100°C.

Doughs made with fortified flour and various carbohydrates were baked at 190°C for 15 min. Recoveries of lysine were 85.5% with cellulose, 70.6% with sucrose, and 20.9% with high-fructose corn syrup. Yeast bread made with flour fortified with 0.5% lysine hydrochloride gave lysine recoveries of 23% from the crust portion of the bread and 97% from the interior portion, with an overall recovery of 85% from the loaf. Breads made with sucrose or high-fructose corn syrup showed no difference in free lysine stability.

Because wheat proteins are low in lysine, fortification of wheat-based foods with free lysine has been proposed as one way of improving the nutritional value of the protein. Numerous investigators (1—5) have demonstrated the nutritional improvements that addition of lysine to white bread and other wheat products effect. Reactions of lysine with reducing sugars in the Maillard browning reaction can lead to losses in lysine availability during heat treatment. The stability of added lysine in cereal products is a concern, because the amount of lysine that survives heat processing may be considerably less than the amount added before heating. The destruction of free lysine during baking of lysine-fortified bread has been reported in the range of 10—30% (6—9). Mathews *et al.* (9) demonstrated a 4% loss of added lysine during baking of chapatties, while Shoup and Henry (10) noted lysine losses in excess of 50% during the commercial cooking of bulgur. The type of baking treatment (11) and the type and amount of sugar present during heating (12) have recently been shown to affect the destruction of protein lysine.

Methods used to determine lysine losses during baking of fortified bread include growth studies in rats (7,8), microbiological assays (1,7), and amino acid analysis by ion-exchange chromatography (9). The methods can be time-consuming and expensive and may not be suitable for routine analysis involving a large number of samples. Ferrel *et al.* (13) proposed a colorimetric method for determining lysine in fortified wheat, but this was found to be inadequate for fortified bulgur (10). Finley *et al.* (14) proposed a colorimetric procedure for fortified wheat and bulgur, but the method has not been used to evaluate the influence of formulation or varying heat treatment on added lysine stability. Bayer *et al.* (15) recently introduced a rapid amino acid procedure involving

high-pressure liquid chromatography (HPLC). The objective of this research was to evaluate the HPLC amino acid procedure as a method of measuring free lysine in lysine-fortified wheat products and to investigate the influence of sugar type on the stability of free lysine in products made with lysine-fortified flour.

MATERIALS AND METHODS

Fortification

Lysine-fortified flour was prepared by blending wheat flour (12.3% protein) with 0.1−1.0% lysine monohydrochloride (Sigma Chemical Company, St. Louis, MO) in a twin shell dry blender (Patterson-Kelley Co., East Stroudsburg, PA). The lysine was premixed with a small amount of flour before being added to the blender. The fortified flour was blended for 20 min. All fortification levels and analysis information discussed in this article were calculated as the hydrochloride salt of lysine (lysine HCl).

Heating of Fortified Flours

The stability of added lysine was evaluated in wheat flour and wheat flour with added water and glucose that were heated at 100°C. Five-gram samples of 0.5% lysine HCl-fortified flour were placed in laminated foil retort pouches that were sealed under vacuum. Water was added to another batch of the flour in a ratio of eight parts flour to one part water. This material was blended in an Oster blender for 10 min and placed in pouches. A third mixture was prepared in a similar manner with eight parts fortified flour to one part glucose. A fourth mixture was prepared with an 8:1:1 ratio of fortified flour/glucose/water. Samples with added water were allowed to equilibrate for 1 hr prior to heating. The pouches representing different formulations were heated in a 100°C oven for varied lengths of time up to 2 hr. After heating, duplicate samples from each pouch were analyzed for free lysine and compared with unheated control samples.

Heated Dough Systems

The influence of carbohydrate source on the stability of free lysine was studied in a dough system undergoing extensive browning. Twenty grams of flour fortified with 0.5% lysine HCl was mixed with either 2.0 g of microcrystalline cellulose (Avicel, FMC Corp., New York) or sucrose, or 2.85 g of high-fructose corn syrup (HFCS). The HFCS contained 70.5% solids made up of 42% fructose and 50% glucose. Eleven milliliters of water was added to the flour and sugar, and the samples were mixed thoroughly. When HFCS was used, the amount of water was decreased to account for water added with the syrup. The doughs were placed on aluminum foil, flattened to approximately 2 mm, and perforated to prevent gas retention. The samples were placed in a 190°C oven for 15 min. After cooling, the samples were ground in an Oster blender and analyzed for moisture and free lysine. Two doughs with each type of carbohydrate were prepared; each dough was analyzed in duplicate. Moisture analysis was done by drying at 130°C for 1 hr (16). Lysine retention was calculated on a solids basis compared with unheated fortified flour.

In a second set of experiments, doughs made with sucrose or HFCS were heated at 190°C and removed from the oven at 2-min intervals for periods of up

to 16 min. After heat treatment, samples were air dried, ground, and analyzed in duplicate for moisture and free lysine.

Lysine-Fortified Bread

Yeast bread was prepared with HFCS or sucrose as the sugar ingredient. Each loaf contained 500 g of wheat flour fortified with 0.5% lysine HCl, 290 ml of water, 50 g of sugar, 15 g of yeast, and 5 g of salt. After mixing the ingredients by hand, the dough was kneaded and placed in an 8.5 × 4.5 × 2.6-in. bread pan. The pans were placed in a proofing cabinet at 32° C for approximately 60 min and then baked at 190° C for 35 min. After baking, the loaves were sliced, air dried,
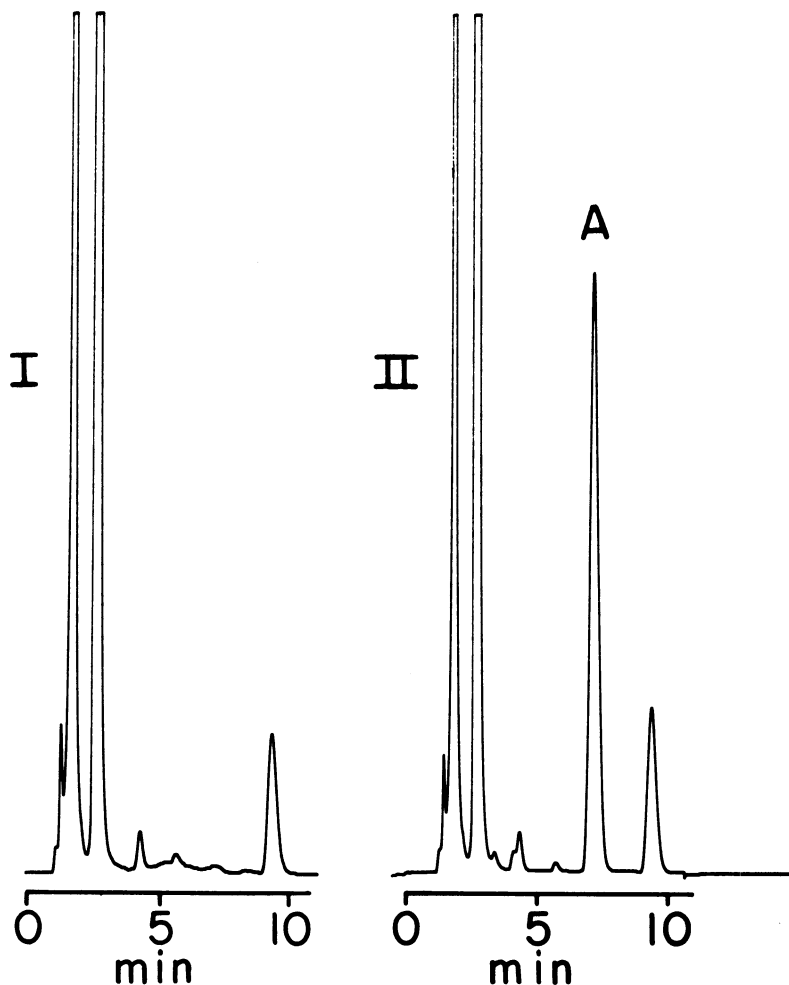

Fig. 1. Chromatogram I is unfortified wheat flour and II, wheat flour fortified with 0.5% lysine HCl. Conditions are as described in **Materials and Methods** with a recorder sensitivity of 0.124 AUFS. Peak A corresponds to standard didansyl lysine.

and ground to a fine particle size with an Oster blender. Duplicate samples from each loaf were used for moisture and lysine analysis. Four loaves were made with each type of sugar.

In a second yeast bread experiment, bread made with sucrose or HFCS was prepared as described above except the crust was separated from the crumb prior to analysis. The crust portion comprised approximately 10% of the loaf on a dry basis. Four loaves with each type of sugar were prepared. Statistical differences between breads made with the two types of sugar were evaluated with a t-test at a P of 0.05.

Lysine Extraction

Two grams of flour was weighed into a 50-ml beaker, and 25—30 ml of 12% trichloroacetic acid (TCA) was added to the sample. The sample was stirred on a magnetic stirrer for 15 min, quantitatively transferred to a 40-ml plastic centrifuge tube, and centrifuged at 1,500 rpm for 10 min. The pellet was washed twice with 10 ml of 12% TCA. The three supernatants were combined, and the pH was adjusted to approximately 9 with 5 N NaOH. The sample was transferred to a 100-ml volumetric flask and made to volume with 0.1 M borate buffer, pH 9.0. Dough and bread samples were extracted by mixing a 2-g sample with 25 ml of water on a Sorvall Omni Mixer for 2.5 min at top speed. Ten milliliters of 40% TCA was added, and the sample centrifuged and handled in the same manner as the flour samples.

Dansyl Reaction

The dansylation procedure was adapted from that of Bayer *et al.* (15) and Gray (17). One-half milliliter of sample was placed in a 2-dr vial, along with 0.5 ml of 0.1 M borate buffer. One-half milliliter of freshly prepared 10 mM dansyl chloride (Sigma Chemical Company) in acetone was added, and the vial contents were mixed and incubated at 40°C for 40 min in the dark. The sample was filtered through a 0.2-μ membrane filter, and 5 μl was used for chromatographic analysis.

HPLC

The didansyl derivative of lysine was separated and quantified using an HPLC unit from Waters Associates Inc., Milford, MA. The system consisted of a model 6000A pump, U6K injector, and 440 absorbance detector sensitive at a

TABLE I
Free-Lysine Analysis of Fortified Flours

Lysine HCl Added (mg/g Flour)	Number of Analyses	Lysine HCl Determined (mg/g Flour)	SD	Coefficient of Variation (%)
1.00	6	1.03	0.03	2.9
2.50	6	2.46	0.12	4.9
5.00	10	5.07	0.16	3.2
10.00	6	10.08	0.26	2.6

wavelength of 254 nm. Detector output was recorded on a Hewlett-Packard 3380A recorder integrator. The column was 4 mm × 30 cm packed with μBondapak C_{18} (Waters Associates Inc.), and the solvent, a 2:5 ratio of acetonitrile to $0.01\,M$ disodium phosphate buffer (pH 7.0) pumped at a flow rate of 1.5 ml/min. Under these conditions, didansyl lysine eluted after approximately 8 min. For quantitation, a standard lysine solution of 0.1 mg/ml was used in place of the sample in the dansylation reaction. Duplicate lysine standards were used with each set of samples. Didansyl lysine (Sigma Chemical Company) also was used as a standard.

RESULTS AND DISCUSSION

The reaction of lysine with dansyl chloride allows the formation of a lysine derivative that absorbs light strongly at 254 nm. Using standard lysine solutions, the reaction was found to be 99% complete under the conditions described. Didansyl lysine is readily separated from dansyl derivatives of other amino acids and absorbing compounds associated with the dansyl chloride reagent. A typical chromatogram from lysine-fortified flour compared with unfortified flour is shown in Fig. 1. The HPLC method measures only the lysine that has free α- and
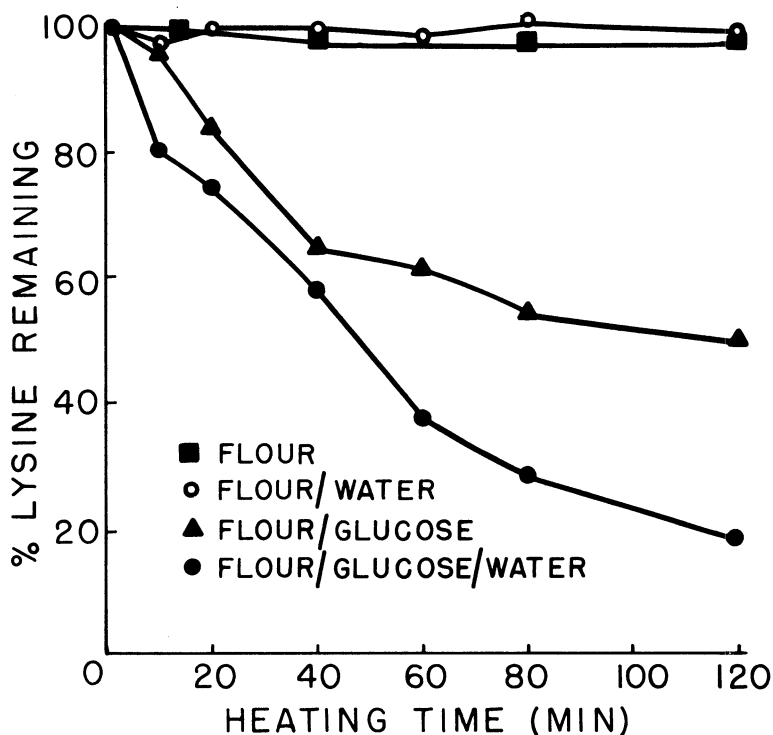

Fig. 2. Effect of heating at 100°C on retention of added lysine in wheat flour and wheat flour with added water and glucose. Mixtures were formulated with the following ratios: flour/water, 8:1; flour/glucose, 8:1; flour/glucose/water, 8:1:1.

ε-amino groups. This may be particularly important in heat-processed products in which one or both of the amino groups may be bound to carbonyl compounds and not be in a nutritionally usable form. Another advantage of the dansyl procedure for free lysine is that the sample is not subjected to acid hydrolysis conditions. Procedures that involve an acid hydrolysis step may measure total rather than available lysine.

As shown in Table I, the recovery of lysine from lysine-fortified flours is good, and the reproducibility is demonstrated by a coefficient of variation of about 3%. When the starting sample size was increased to 3 g of flour and the size of the HPLC injection increased to 30 μl, the method had sufficient sensitivity to measure free lysine levels as low as 0.10 mg/g flour. The amount of free lysine in the unfortified flour used in this study was 0.12 mg of lysine/g of flour. The free lysine determination in fortified flour reflects both the native and added free lysine.

The stability of lysine in fortified wheat flour and flour mixtures heated at 100°C is shown in Fig. 2. The level of lysine remaining in fortified flour after 2 hr indicated little destruction. When water was added prior to heating, bringing the moisture of the flour up to 21.5%, the recovery of added lysine after 2 hr was 99.6%. Apparently the reducing sugar content of the flour was not sufficient to cause Maillard browning and lysine loss under these conditions. The effect of a
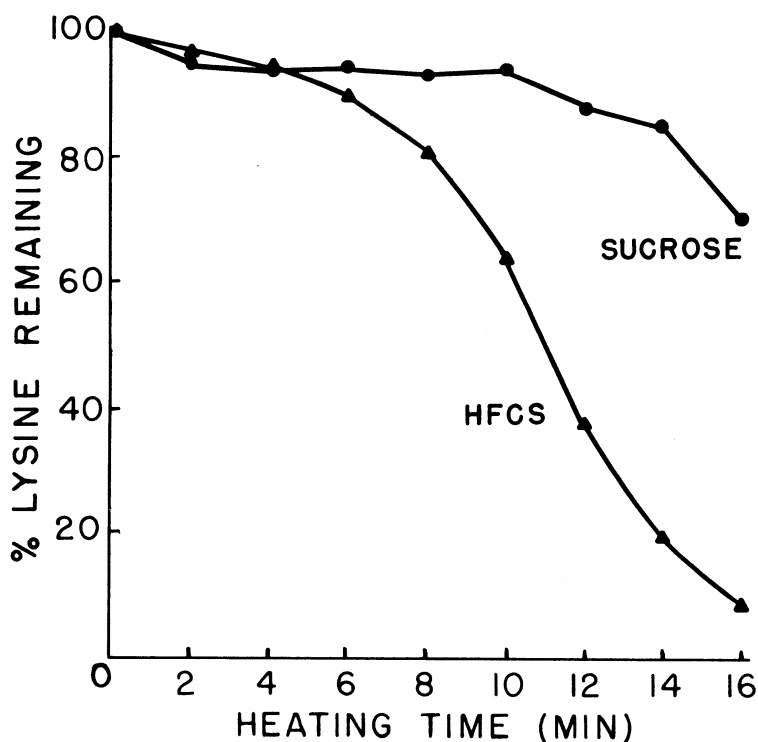

Fig. 3. Effect of heating at 190°C on loss of added lysine in doughs made with either sucrose or high-fructose corn syrup (HFCS).

reducing sugar on the stability of lysine in flour was determined by adding glucose to fortified flour and to fortified flour with added water. Added glucose caused substantial losses of lysine with about 50% destruction after 2 hr. Glucose and 10% added water showed even greater lysine loss, with the recovery being only 20% after 2 hr. This demonstrates the instability of free lysine in heated flour systems containing glucose. In the samples containing glucose, loss of free lysine paralleled development of visual browning.

The influence of sugar type on the stability of added lysine was determined using flour-sugar mixtures made into doughs and heated at 190° C. Doughs were formulated with either cellulose, sucrose, or HFCS at a level of 10% of the flour. After baking for 15 min, the retention of added lysine was 85.5, 70.6, and 20.9%, respectively, indicating more instability of added lysine in the dough containing reducing sugars. With this type of formulation and heating, however, lysine losses were also encountered with sucrose and cellulose. Landes and Miller (12) recently found substantial differences in rat growth when the animals were on diets that were formulated with either sucrose or glucose prior to baking. They suggested that differences in nutritional value were due to increased destruction of protein lysine in those diets formulated with glucose. In the dough system used in the present study, the type of sugar apparently was a major factor influencing the stability of free lysine.

The effect of heating time on doughs formulated with either sucrose or HFCS is shown in Fig. 3. In this experiment, the advantage of sucrose over HFCS in maintaining free lysine is again demonstrated. The HFCS samples show much greater losses of lysine at the longer heating times.

The stability of added lysine was evaluated in yeast bread made with sucrose or HFCS. The results of the lysine stability study are shown in Table II. When the entire loaf was treated as one sample, HFCS showed 85.4% lysine retention, and sucrose 84.6% retention; these were not significantly different at a P of 0.05. Bread is an obvious product in which lysine fortification would be used and in which stability of the added lysine during the baking process is important. The losses of free lysine during the breadbaking process have been well documented (6–9), although the extent of reported destruction varies. The overall lysine retention found in the present study is similar to the findings of other workers using different methods to determine lysine stability.

More extensive lysine destruction would be expected in the crust than in the crumb because of browning reactions. Rosenberg and Rohdenburg (6) analyzed the interior of a loaf of bread and indicated that the free lysine in the interior was essentially unchanged with baking. The results in Table II support that finding in that the recovery of free lysine in the interior was above 95%, with no significant

TABLE II
Percentage of Added Lysine Remaining in Yeast Bread After Baking

	Sucrose		High-Fructose Corn Syrup	
	Average	Range	Average	Range
Entire loaf	84.6	80.6–93.6	85.4	81.1–88.7
Crumb	95.7	91.4–101.6	98.0	91.8–104.0
Crust	23.2	20.2–26.2	22.6	19.6–26.0

difference noted between the two types of sugars. The lysine retention in the crust was about 23%, with no difference found with the type of sugar. The yeast bread can be contrasted to the unleavened dough system in which sucrose samples showed a better retention of lysine than did HFCS samples. The different results in the two products may be due to fermentation or differences in product size and shape that would affect the extent of the browning reaction.

Acknowledgment

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