

Stability of β -Galactosidase from *Aspergillus oryzae* and *Kluyveromyces lactis* in Dry Milk Powders

M.S. PALUMBO, P.W. SMITH, E.D. STRANGE, D.L. VAN HEKKEN, M.H. TUNICK, AND V.H. HOLSINGER

ABSTRACT

A milk-based beverage powder with 2% vegetable fat and reduced levels of lactose is needed by some segments of the consumer market as well as military units. To provide such, two commercial β -galactosidases were dry-blended into milk powders made with coconut, cottonseed, canola or sunflower oils and stored at -20 , 4 , 25 , and 45°C . Enzyme activity was measured monthly by reconstituting the powders and measuring freezing point depression after incubation at 32°C (optimum for β -galactosidase from *Kluyveromyces lactis*) and incubation at 50°C with pH adjusted to 5.0 (for activity of *Aspergillus oryzae* enzyme). Both enzymes retained $>95\%$ of original activity at -20 and 4°C . Activity of *K. lactis* enzyme declined moderately at room temperature ($\approx 23^\circ\text{C}$) and rapidly at 45°C . The *A. oryzae* enzyme retained 93.4% of original activity after 6 mo at 45°C .

Key Words: galactosidase, *Kluyveromyces lactis*, *Aspergillus oryzae*, dry milk, vegetable fat

INTRODUCTION

EFFORTS TO INCLUDE NONFAT DRY MILK as a beverage powder in dried field rations have been mostly unsuccessful. Much of it is not consumed, either because the flavor is unacceptable or because of lactose intolerance. A reduced-lactose milk-based beverage powder would provide the nutritional advantages of skim milk and unsaturated vegetable fat when reconstituted. Hydrolysis of lactose to glucose and galactose prior to drying leads to excessive browning and burning from sticking to hot surfaces in the dryer. Another possible approach would be to incorporate β -galactosidase enzymes into the dry fat-containing powder. The stability of the enzymes in dry form under highly variable storage conditions is not known.

β -Galactosidases are produced by a variety of plant, animal, and microbial cells. Commercially available enzymes are derived from yeasts such as *Kluyveromyces lactis* and fungi such as *Aspergillus oryzae* and *A. niger*. The *K. lactis* enzyme has an optimum pH near that of milk and exhibits appreciable activity at 4 – 6°C (Nijpels et al., 1980) making it suitable for addition to milk before consumption. The *A. oryzae* enzyme has a pH optimum of 5.0 and an optimum temperature of 50 – 55°C (Park et al., 1979), making it effective in the acid environment of the stomach.

Rosado et al. (1984) noted that *in vitro* hydrolysis of lactose by addition of β -galactosidase before consumption is not practical when refrigeration facilities are not available. They found *in vivo* hydrolysis with *A. niger* β -galactosidase to be partially effective, however. Barillas and Solomons (1987) demonstrated that β -galactosidase derived from *A. oryzae*, orally administered, was effective in preventing lactose malabsorption in children. Situations in which such milk powders would be used might include field use, where no refrigeration is available, and large-scale feeding operations where overnight refrigeration would be common. In the first situation, the *A. oryzae* enzyme would be effective; in a situation where pre-hydrolysis during overnight

refrigeration was possible, the *K. lactis* enzyme would be more effective. Therefore, both enzymes were incorporated for study.

Our objectives were to establish processing conditions to prepare such beverage powders and determine stability of the *K. lactis* and *A. oryzae* enzymes during storage at temperatures which might be encountered in field use. We also evaluated possible effects of different vegetable oils on enzyme stability.

MATERIALS & METHODS

Enzymes

β -Galactosidase enzymes from *K. lactis* (22,200 Neutral Lactase Units/g) and *A. oryzae* (112,000 Food Chemicals Codex Lactase Units/g) were obtained in powder form from Lactaid Inc., Pleasantville, NJ. The level of addition of the *K. lactis* enzyme (0.858 mg/g powder) was determined by the amount necessary to give 80% hydrolysis in 24 hr at refrigeration temperatures. The amount of *A. oryzae* enzyme used (1.52 mg/g powder) was equivalent to one Lactaid™ caplet per 240 mL serving of milk-based beverage.

Preparation of milk powders

Raw skim milk was pasteurized at 76.7°C for 15 sec and evaporated at 43.3°C to 42–44% solids. Each batch of concentrate was divided into three portions, warmed to 65.6°C , blended with vegetable oil to yield 2% fat when rehydrated to 11% total solids, and homogenized double-stage at 13,800–3450 kPa. The homogenized concentrate was immediately spray-dried at 88.9 – 90.6°C (outlet temperature) to moisture content $< 3\%$. Enzymes were dry-blended into powders (1.76g *K. lactis* enzyme and 3.12g *A. oryzae* enzyme/2050 g milk powder) and immediately sealed into cans (size 211 \times 300, Can Corp. of America, Blandon, PA) under nitrogen. Lactose content was determined enzymatically using the Boehringer-Mannheim lactose/D-galactose test kit (AOAC, 1990).

Three batches of powders were prepared. In the first two batches, the vegetable oils were coconut (supplied by California Oils Corp., Richmond, CA), canola (supplied by Archer Daniels Midland Co., Decatur, IL), and sunflower (Trisun 95, supplied by SVO Enterprises, Eastlake, OH). In the third batch, cottonseed oil (supplied by Lou Ana Foods, Inc., Opelousas, LA) was substituted for canola oil because of flavor concerns.

Analysis of powders

Each powder was analyzed for moisture content by drying under vacuum for 24 hr at 60°C , and for fat content by the Roese-Gottlieb procedure (Marth, 1978). Initial powders were also analyzed for free fat by the method of Tamsma et al. (1959). Lactose content of each powder was determined by the enzymatic-UV method using the Boehringer-Mannheim Lactose/Galactose kit after deproteinization with Carrez solutions (AOAC, 1990).

Storage

Sealed cans from each batch were stored at -20°C , 4°C , room temperature (25°C), and 45°C for 6 mo. Samples were removed and analyzed for enzyme activity initially and at monthly intervals.

Enzyme activity

Preliminary experiments were conducted to determine hydrolysis conditions which would permit estimation of the activity of each enzyme. For these experiments, a 9% solution of nonfat dry milk was prepared and divided into three 240-mL aliquots. To one of these was added 14.1

Authors Smith, Strange, Van Hekken, Tunick, and Holsinger are with the USDA-ARS, Eastern Regional Research Center, 600 East Mermaid Lane, Philadelphia, PA 19118. Author Palumbo's present address: Delaware Valley College, 700 E. Butler Ave., Doylestown, PA 19118.

Table 1—Hydrolysis of lactose by enzymes incorporated into nonfat dry milk reconstituted to 9% total solids after 2 hr incubation at 50°C, pH 5.0, or 32°C, pH 6.5, or after 24 hr at 4°C^a

Enzyme	Percent lactose hydrolysis		
	50°C, pH 5 2 hr	32°C, pH 6.5 2 hr	4°C, pH 6.5 24 hr
<i>K. lactis</i> (0.059 mg/mL)	0.3	55.6	65.2
<i>A. oryzae</i> (0.112 mg/mL)	87.6	29.6	25.9
Both enzymes same conc as above	89.7	61.5	64.5

^a Average of three trials.

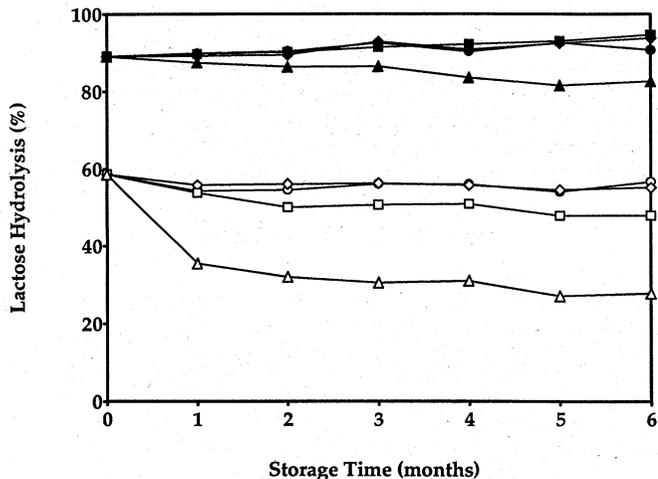


Fig. 1—Effect of storage of milk powders containing *K. lactis* and *A. oryzae* β -galactosidases on lactose hydrolysis in rehydrated products (average of all oils). \blacktriangle 45°C storage, hydrolysis at 50°C, pH 5.0; \triangle 45°C storage, hydrolysis at 32°C; \blacksquare 25°C storage, hydrolysis at 50°C, pH 5.0; \square 25°C storage, hydrolysis at 32°C; \blacktriangledown 4°C storage, hydrolysis at 50°C, pH 5.0; \triangledown 4°C storage, hydrolysis at 32°C; \bullet -20°C storage, hydrolysis at 50°C, pH 5.0; \circ -20°C storage, hydrolysis at 32°C.

mg *K. lactis* enzyme; to a second aliquot, 26.8 mg *A. oryzae* enzyme was added; and to a third aliquot both enzymes were added in the amounts stated. Each of these three samples was divided into three parts: one portion was refrigerated for 24 hr, one portion was incubated at 32°C, while the other was acidified to pH 5.0 and incubated at 50°C. Freezing points were determined initially and after 1 and 2 hr, or after 24 hr for the refrigerated sample, using a Cryette A Automatic Cryoscope (Precision Systems, Inc., Natick, MA). Freezing point depression (FPD) was calculated and converted to percent lactose hydrolysis (described below).

For the storage study, enzyme activity was determined by a similar procedure. For each powder at each time, 22g powder were blended with 178g cold deionized water. The sample was then divided. For one part the pH was adjusted to 5.0 with 2 N HCl; the other part was unadjusted. Aliquots of each portion were used for zero time measurement. The remainder of each portion was divided into test tubes and placed in water baths for incubation. The pH 5 samples were incubated at 50°C for measurement of *A. oryzae* enzyme activity. Unadjusted samples (pH 6.5) were incubated at 32°C for estimation of *K. lactis* activity. Samples were removed after 2 hr and placed in an ice bath. One portion of the pH 6.5 sample was held for 24 hr under refrigeration. Freezing points were determined in triplicate on each sample. Freezing point depression was calculated as the difference between the average freezing point of the incubated sample and the corresponding zero-time sample.

Calculation of percent hydrolysis

FPD data were converted to lactose hydrolyzed (g/100 mL) by dividing by a factor representing the FPD for each 1% lactose hydrolyzed. This factor was determined for each hydrolysis condition (32°C at pH 6.5 and 50°C at pH 5.0) as follows. Representative samples of powder were hydrolyzed as indicated; samples were removed every 30 min, and freezing points determined. A weighed aliquot was diluted and deproteinized immediately to stop the reaction and prepare the sample for

measurement of galactose by the enzymatic-UV method described. Hydrolyzed lactose (g/100 mL or percent) was calculated from galactose concentration. The ratio of FPD to hydrolyzed lactose was calculated for each time; these values were averaged for each hydrolysis condition. Percent hydrolysis was calculated from amount of lactose hydrolyzed and total lactose as measured enzymatically in each powder batch.

Statistical analysis

The SAS Software System (SAS Institute, Inc., 1987) was used to evaluate effects of storage temperature and time on enzyme activity in each powder using a general linear model. A Bonferroni T test was used to determine significance of differences between means.

RESULTS & DISCUSSION

ACTIVITY OF β -GALACTOSIDASES has been measured spectrophotometrically using o-nitrophenyl- β -D-galactopyranoside (ONPG) as substrate (Dickson et al., 1979). Also, using lactose as substrate one could measure glucose or galactose produced (Richmond et al., 1981; Kleyn and Trout, 1984). It can also be measured by the freezing point depression cryoscopically in milk or whey (Nijpels et al., 1980; Baer et al., 1980; Chen et al., 1981). Measurement of freezing point depression was chosen as the most suitable for our study because of its speed and accuracy.

The freezing point method was evaluated for use in both neutral and acid products by Baer et al. (1980) and Chen et al. (1981). The accuracy of the method depends on the accuracy of freezing point determinations and the accuracy of lactose/galactose determinations (Chen et al., 1981). We found freezing point measurements were highly reproducible. The enzymatic/UV determinations of lactose and galactose were more variable. However, any error in this procedure should be reflected as a consistent error in the conversion of FPD to percent hydrolysis and would not change relative conclusions regarding enzyme stability.

Results of lactose hydrolysis by the enzymes under conditions for assay were compared (Table 1). Hydrolysis for 24 hr at 4°C was comparable to hydrolysis for 2 hr at 32°C; the latter conditions were reported as a measure of activity of *K. lactis* enzyme. Activity of *A. oryzae* enzyme was not completely eliminated at 32°C or 4°C, but it was sufficiently reduced to permit evaluation of *K. lactis* enzyme activity. When hydrolysis was carried out at 50°C, which at pH 5.0 were optimum conditions for the *A. oryzae* enzyme, activity of the *K. lactis* enzyme was eliminated. These conditions were assumed to provide a measure of the activity of *A. oryzae* enzyme; (they do not, of course, represent true physiological conditions).

Average composition of the milk powders was 19.90% total fat, 39.9% lactose, and 2.76% moisture. Preliminary studies to establish processing conditions involved homogenizing vegetable oils into skim milk before and after condensing. Free fat as a percent of total fat was determined on these powders as a measure of the degree of emulsification achieved. Values of 0.88 to 1.32% indicated excellent emulsification of the added vegetable fats. No difference attributable to sequence of condensation and homogenization was observed. When reconstituted to 11% total solids, the powders produced a beverage containing 2% fat and 9% nonfat milk solids.

The effect of storage temperature on enzyme activity could be seen by comparing percent lactose hydrolysis in all reconstituted powders (Fig. 1). At 32°C, unadjusted pH, levels of hydrolysis >30% must be due to *K. lactis* enzyme activity. Levels of activity < 30% may be due to either enzyme. While incubation at 32°C at the natural pH of milk did not completely eliminate activity of *A. oryzae* enzyme, simple calculation indicates that no *K. lactis* enzyme activity would remain after 6 mo at 45°C. *A. oryzae* enzyme alone could hydrolyze 29.6% of the lactose in 2 hr at 32°C (Table 1); after 6 mo storage at 45°C, this enzyme retained 93.4% of its original activity as measured at 50°C pH 5.0 (Table 2). Multiplying 29.6% by 0.934 gives 27.6% hydrolysis which could be expected as a result of *A.*

Table 2—Retention of original (0 month storage) enzymatic activity after 6 mo storage^a

Storage (°C)	Percent of original activity	
	32°C, pH 6.5 ^b	50°C, pH 5.0 ^c
-20	98.4	102.6
4	95.3	105.9
25	83.5	106.0
45	49.4	93.4

^a Average of all powders.

^b Values over 50% reflect activity of *K. lactis* enzyme.

^c Conditions for *A. oryzae* enzyme.

Table 3—Lactose hydrolysis (%) in reconstituted milk powders containing different oils after 6 mo storage; hydrolysis at 32°C, pH 6.5, for *K. lactis* activity

Storage °C	Lactose hydrolysis (%) after 2 hr at 32°C ^a			
	Coconut	Canola	Sunflower	Cottonseed
-20	57.8	58.4	55.1	55.4
4	55.1	56.3	54.7	53.6
25	48.2	48.8	46.9	48.4
45	26.9	27.7	27.2	31.6
no storage	57.8	61.1	59.2	52.9

^a Averages of all replicates.

Table 4—Lactose hydrolysis (%) in reconstituted milk powders containing different oils after 6 mo storage; hydrolysis at 50°C, pH 5.0, for *A. oryzae* activity

Storage (°C)	Lactose hydrolysis (%) after 2 hr, 50°C, pH 5.0 ^a			
	Coconut	Canola	Sunflower	Cottonseed
-20	89.5	96.4	89.8	88.6
4	95.4	94.5	91.2	94.7
25	95.4	97.2	93.8	89.8
45	82.4	83.0	81.7	84.5
no storage	88.8	89.5	89.7	86.9

^a Averages of all replicates.

oryzae enzyme activity. This is approximately equal to the average percent hydrolysis observed in the powders stored 6 mo at 45°C and hydrolyzed at 32°C (Fig. 1).

Comparison of 32°C with 50°C, pH 5.0, indicated that the *A. oryzae* enzyme was considerably more stable at higher storage temperatures than the *K. lactis* enzyme (Fig. 1). Analysis of variance showed that storage time, temperature, and the interaction of time and temperature significantly affected both assay conditions (32°C/pH 6.5 and 50°C/pH 5.0). Under refrigerated or frozen storage, both enzymes retained ≥ 95% percent of original activity after 6 mo (Table 2). No significant difference occurred between these storage temperatures for either enzyme under any assay conditions used. Most of the deactivation of the *K. lactis* enzyme at 45°C occurred in the first month; loss of activity at room temperature became significant at 3 mo. Activity of the *A. oryzae* enzyme decreased significantly at 5 mo when stored at 45°C. Room temperature storage caused no significant loss after 6 mo. An apparent slight upward trend in activity of *A. oryzae* enzyme was seen (Table 2) as more than 100% retention of activity. This probably was a result of experimental error in pH adjustment or temperature control during the assay, although every effort was made to carefully control such variables.

Tables 3 and 4 show the effect of oil type on enzyme stability when stored for six months at different temperatures. Comparison of the effect of oil type on enzyme stability during storage at 45°C, where differences were greatest, showed relatively minor differences. Differences among coconut, canola, and sunflower oil-containing powders were not significant, but the interaction of oil with storage temperature was significant for the 50°C/pH 5.0 assay. Cottonseed oil was not included in the analysis of variance because only one batch was prepared.

CONCLUSIONS

THE *K. LACTIS* ENZYME would retain appreciable activity if milk powders were stored under nitrogen at 20°C or less, but would lose activity rapidly when stored at 45°C. The *A. oryzae* enzyme would tolerate considerably more temperature abuse when stored under nitrogen. No loss of activity of that enzyme was observed after 6 mo at 25°C, and powders stored at 45°C retained an average 89% of the *A. oryzae* enzyme activity of the same powder stored at 4°C. Therefore incorporation of the *A. oryzae* enzyme can be recommended, but addition of *K. lactis* enzyme would be justified only if the powders could be kept cool.

REFERENCES

- AOAC 1990. *Official Methods of Analysis*, 14th ed., Association of Official Analytical Chemists, Arlington, VA.
- Baer, R.J., Frank, J.F., and Loewenstein, M. 1980. Freezing point measurement of lactose hydrolysis in acid whey and lactose solutions. *J. Assoc. Off. Anal. Chem.* 63: 587.
- Barillas, C. and Solomons, N.W. 1987. Effective reduction of lactose maldigestion in preschool children by direct addition of β-galactosidases to milk at mealtime. *Pediatrics* 79: 766.
- Chen, S.-L.Y., Frank, J.F., and Loewenstein, M. 1981. Estimation of lactose hydrolysis by freezing point measurement in milk and whey substrates treated with lactases from various microorganisms. *J. Assoc. Off. Anal. Chem.* 64: 1414.
- Dickson, R.C., Dickson, L.R., and Markin, J.S. 1979. Purification and properties of an inducible β-galactosidase isolated from the yeast *Kluyveromyces lactis*. *J. Bacteriology* 137: 51.
- Kleyn, D.H. and Trout, J.R. 1984. Enzymatic-ultraviolet method for measuring lactose in milk: collaborative study. *J. Assoc. Off. Anal. Chem.* 67: 637.
- Marth, E.H. (Ed.) 1978. *Standard Methods for the Examination of Dairy Products*, 14th ed., Amer. Public Health Assoc., Washington, DC.
- Nijpels, H.H., Evers, P.H., Novak, G., and Ramet, J.P. 1980. Application of cryoscopy for the measurement of enzymatic hydrolysis of lactose. *J. Food Sci.* 45: 1684.
- Park, Y.K., DeSanti, M.S.S., and Pastore, G.M. 1979. Production and characterization of β-galactosidase from *Aspergillus oryzae*. *J. Food Sci.* 44: 100.
- Richmond, M.L., Gray, J.I., and Stine, C.M. 1981. β-Galactosidase: Review of recent research related to technological application, nutrition concerns, and immobilization. *J. Dairy Sci.* 64: 1759.
- Rosado, J.L., Solomons, N.W., Lisker, R., and Bourges, H. 1984. Enzyme replacement therapy for primary adult lactase deficiency: Effective reduction of lactose malabsorption and milk intolerance by direct addition of β-galactosidase to milk at mealtime. *Gastroenterology* 87: 1072.
- SAS Institute Inc. 1987. *SAS/STAT Guide for Personal Computer, Version Edition*. SAS Institute Inc., Cary, NC.
- Tamsma, A., Edmondson, L.F., and Vettel, H.E. 1959. Free fat in foam-dried whole milk. *J. Dairy Sci.* 42: 240.

Use of brand or firm name does not imply endorsement by the U.S. Dept. of Agriculture over others of a similar nature not mentioned. We thank Richard Konstance for assistance with manufacture of milk powders and John Phillips, North Atlantic Area statistician, for assistance with statistical analysis.