Fluorometric Analysis of Alkaline Phosphatase in Fluid Dairy Products

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ABSTRACT

A new quantitative assay has been developed for measuring residual alkaline phosphatase (ALP) activity in a wide variety of dairy products including whole milk, low fat and skim milks, chocolate milk, and creams. ALP in the test sample hydrolyzes a nonfluorescent substrate, Fluorophos®, to a highly fluorescent product. Product formation is monitored continuously during a short incubation period and enzyme activity is calculated from the rate of fluorescence increase. Total test time is 3 min. Reaction rates are linear up to 0.5% raw milk (equivalent to 5 μg phenol/ml/15 min) with a detection limit of 0.006% raw milk. Within and between run precision of the fluorometric method was assessed by repeated analysis of a pasteurized milk sample containing added mixed herd raw milk. The within run (N=10) mean was 190.4 μL/L, standard deviation (SD) 3.2, and a coefficient of variance (CV) of 1.7%. The procedure provides a rapid, sensitive, precise, and easy-to-use ALP assay, applicable to a wide variety of dairy products.

Currently, all approved quantitative alkaline phosphatase (ALP, EC 3.1.3.1.) assays for use with fluid dairy products employ one of three colorimetric substrates. The Scharer (10) and Cornell (6) methods use phenyl phosphate as in the original Kay and Graham procedure (4). The Aschaffenburg and Mullen procedure uses p-nitrophenyl phosphate (3), and the Rutgers' assay uses phenolphthalein monophosphate (5). Quantitative methods using phenyl phosphate substrate measure enzymatically liberated phenol by coupling it with a color reagent and forming an indophenol. The blue indophenol is removed from interfering turbidity caused by the dairy product in one of two ways. First, the American Public Health Association (APHA) (9) and the Association of Official Analytical Chemists (AOAC) (11) have approved methods using butanol extraction followed by spectrophotometric measurement. Second, both the AOAC and the International Dairy Federation (IDF) have approved methods in which the indophenol is read after protein precipitation with barium and zinc salts (1-2). In the quantitative Rutger's assay, liberated phenolphthalein is dialyzed into deionized water during a 3 h incubation period followed by spectrophotometric reading of the dialysate (11). The IDF has also approved a p-nitrophenyl phosphate assay in which the liberated p-nitrophenol is read after protein precipitation following 2 h incubation (2). These quantitative methods, despite their complexity and long incubation times, have served the dairy industry well and have aided in monitoring milk quality throughout the world for over 50 years. The purpose of this study is to present data on a fluorometric method for ALP testing in dairy products. This method is based on a specially designed aromatic monophosphoric ester substrate called Fluorophos® which, when acted upon by ALP, is converted to a highly fluorescent product, Fluoroyellow®. This fluorometric quantitative assay is the first dairy ALP test which permits the continuous and direct measurement of the released reaction product from a self-indicating substrate. The use of this fluorometric substrate eliminates the interferences and nonspecificity encountered in colorimetric assays and avoids the need for dialysis, butanol extraction, or protein precipitation.

MATERIALS AND METHODS

Samples

Pool samples were prepared by adding fresh mixed-herd raw milk to portions of pasteurized homogenized commercial dairy products. Between run and within run precision was assessed with frozen pooled samples which were allowed to thaw at room temperature and mixed thoroughly before analysis.

Equipment

Spectrophotometric readings were made in a Perkin Elmer Model 554 dual beam spectrophotometer (Perkin Elmer Corp., Norwalk, CT 06859) using 1 cm square polystyrene micro cuvettes (Elkay Products, Shrewsbury, MA 01545). Fluorescence measurements were made in a filter fluorometer with an electronically thermostated cuvette compartment at 38 ± 0.1°C (Advanced Instruments, Inc., Needham Heights, MA 02194).

Excitation was 436 nm and emission at 560 nm. Fluorescence output was monitored on a flat bed strip chart recorder, Model 500 (Linear Instruments, Inc., Reno, NV 89510) and through an analog to digital converter into a programmable calculator with built-in thermal printer (Sharp Electronics Corp., Mahwah, NJ 07430). Cuvetters were 12 x 75 mm round nonfluorescent glass (AI).
Reagents

Reagents for the APHA (9) and AOAC (11) standard quantitative spectrophotometric methods were prepared from phenyl phosphate, sodium carbonate, sodium bicarbonate, copper sulfate, and CQC (2,6-dichloroquinone-4-chloroimide) (Sigma Chemical Co., St. Louis, MO 63178). Spectrophotometric grade methanol and normal buty1 alcohol were obtained from Mallinkrodt, St. Louis, MO 63134. Phenol standards were prepared from redistilled molecular biology grade phenol (Boehringer Mannheim Biochemicals, Indianapolis, IN 46250). Diethanolamine (DEA) was from Sigma; Fluorophos substrate and Fluoroyellow were from Advanced Instruments. All other reagents were reagent grade or better.

APHA and AOAC method

ALP in fluid dairy products was measured essentially according to the APHA (9) method 17.5 and AOAC (11) method 16.121 quantitative spectrophotometric procedures. Phenol calibration curves were run with each batch of unknowns.

Fluorophos method

Three milliliters of working Fluorophos (FP) substrate were dispensed into 12 x 75 mm glass cuvettes and pre-incubated at 38°C for 5 min. Working substrate was found to be stable for 8 h at 38°C and for three weeks at 4°C. A 0.1 ml sample of the well mixed dairy product was added to 3.0 ml of the pre-warmed substrate, mixed by inversion, and placed into the fluorometer. The kinetic increase in fluorescence was monitored for 2 to 3 min and the average increase in fluorescence per min was obtained. Calibration was performed with Fluoroyellow (FY) in DEA buffer and the ng of product formed per min in the unknowns converted to mU/L ALP activity (see Enzyme Units).

RESULTS AND DISCUSSION

Method optimization and performance of the fluorometric ALP assay were assessed using various pool milk samples prepared from fresh mixed-herd raw milk added to whole regular milk or other pasteurized fluid dairy products. Pooled milk samples were found to be stable for at least one month when stored frozen at -10°C.

Effect of pH on milk ALP activity

Milk ALP Activity was optimum at pH 10.0 in DEA buffer (Fig. 1). This is similar to the pH of 10.1 used in the Rutgers' ALP method which employs another amino alcohol buffer, 2-amino-2-methyl-1-propanol. The pH optimum of bovine milk ALP has been shown to be higher in ethanolamine buffers compared to carbonate (12). The following dairy products caused no change in the pH of the substrate when added at 0.1 ml per 3.0 ml: whole milk, chocolate milk (1% fat), cream (half and half), heavy cream, buttermilk (cultured), evaporated milk, sour cream, and skim milk.

Effect of buffer concentration on milk ALP activity

Concentrations of DEA above 2.4 M showed a tendency to be inhibitory (Fig. 2); therefore, DEA between 2.0 and 2.3 M is considered optimum.

Effect of substrate concentration on milk ALP activity

The relationship between activity and Fluorophos substrate concentration is shown in Fig. 3. Lineweaver-Burk transformation of the data gave a V max of 1.47 x 10 2 and a K m of 3.06 x 10 4.

Time course changes in the reaction

The reaction rate was linear from 1 min to at least 30 min, using pooled milk samples containing 0.2% (v/v) and

Effect of interfering substances

Various pasteurized dairy milk products were spiked with 0.1% (v/v) mixed herd raw milk and assayed according to methods except that no substrate was added to the DEA buffer. No interfering substances (causing a measurable increase in the fluorescence reading), up to 10 min
in the substrate was found to cause no increase in the activity of a 0.1% (v/v) raw milk pool in whole milk. Bovine milk ALP has been shown to be inhibited by magnesium ion above 10 mM/L (7). Although magnesium is generally required for optimum ALP activity, the endogenous levels of the ion in milk may be sufficient for adequate activation.

Figure 3. Milk ALP activity vs. substrate concentration. Fluorophos substrate was added to DEA buffer 2.4 M, pH 10.0, from 0.1 to 3.5 mM/L and tested as in Fig. 1.

Figure 4. Milk ALP activity vs. time. Time course change in the reaction measured as in Fig. 1, with a low (△) and high (○) level pool.

 assay time, were noted with chocolate milk, cream (half and half), skim milk, cream, and whole milk. Nonpasteurized 100% mixed herd raw milk was also assayed and found to cause no nonenzymatic increase in the fluorometric reading. In all cases, the increase in fluorescence for 10 min corresponded to a baseline drift equivalent to less than 0.005 ng FY/min.

Linearity with sample volume
The assay is linear with increasing sample volumes as shown by adding from 10 to 200 μl of a pooled milk sample to 3.0 ml working substrate (Fig. 5).

Detection limit
The detection limit is the concentration of milk ALP which gives a reading twice the SD of the reagent blank. As shown in Fig. 6, the practical detection limit for the assay is 0.006% (v/v) raw milk.

Effect of magnesium ion
Magnesium from 0.5 to 2.2 mM/L final concentration

Figure 5. Milk ALP activity vs. sample volume. Mixed herd raw milk was added to whole milk, and 10 to 200 μl of this pool were added to 3.0 ml substrate and assayed as in Fig. 1.

Figure 6. Detection limit. Mixed herd raw milk was added to heated (95°C for 2 min) whole milk from 0.1% to 0.003% (v/v) and each pool was assayed as in Fig. 1. Each point is the mean and SD of three readings.

Figure 7. ALP activity linear response. Linearity of response with concentrations of raw milk in various fluid dairy products.
Linearity of the assay response

Pooled samples of mixed herd raw milk in whole milk, egg nog, and chocolate milk were prepared at 0.04 to 0.5% (v/v) and assayed in triplicate (Fig. 7). The assay is linear to 0.5% raw milk as shown. The decreased response obtained with chocolate milk (Fig. 7) appears to be due to two phenomena. First, the flavoring agents in chocolate milk (and to a certain extent in egg nog) quench the observed fluorescence. A second factor is that unknown compound(s) in chocolate milk are known to be inhibitory to milk ALP activity and these results are consistent with what has been reported (9).

Calibration curves

Calibration was performed by adding known concentrations of FY (the product of the enzyme action on FP substrate) to DEA buffer. First, saline was added to the substrate at 0.1 ml in place of sample and a linear response was obtained from 10 to 100 ng FY (Fig. 8). Each point is the average of duplicate samples. Figure 8 shows the decreased fluorescence yield obtained when FY is added to various sample matrices. Chocolate milk causes about a 40% decrease in the measured fluorescence due primarily to quenching of the fluorescence by endogenous compounds in the milk. Calibration curves in saline and regular whole milk, however, are identical to well over 60 ng. No effect on the linearity of the calibration curve was seen with raw milk added to chocolate milk or egg nog. Each dairy milk product was assayed according to the FP Method using a calibration procedure which included the addition of a portion (0.1 ml) of milk product being tested to the calibrators which compensated for any quenching in fluorescence caused by endogenous flavoring or coloring agents in the finished dairy product.

Correlation with the Scharer method

The Scharer rapid colorimetric phosphatase method was run on a selected number of pool samples according to AOAC method 16.121 (11). Pool samples run in duplicate by the AOAC and Fluorophos fluorometric method gave a correlation of 0.995 with a least square regression of Y = 0.003x + 0.299 (Y = Scharer) (Fig. 9).

Precision

Within-run and between-run precision of the Fluorophos ALP method was assessed by repeated analysis of a pooled sample. The results obtained were: within run (N=10) 190.4 mU/L mean, 3.2 SD and a CV of 1.7%; between run (N=20), 188.8 mU/L mean, 4.8 SD, and a CV of 2.6%.

Enzyme activity units

The traditional units of ALP activity in dairy products have been established based on the liberation of phenol from phenyl phosphate or a similar colorimetric substrate. Samples which generate 1 |g phenol/ml/15 min, for example, are considered to contain at least 0.1% (v/v) raw milk (8). The units for the Fluorophos ALP assay are International Units of enzyme activity which are based on micromoles of substrate transformed/min/liter of sample and are converted into the corresponding mU/L enzyme activity using a molecular weight for FY of 290.

Activity vs. temperature

Table 1 shows the results for the Fluorophos assay in mU/L vs the temperature of pasteurization. Skim milk samples were removed from a commercial pasteurization unit at various temperatures and assayed in triplicate.
samples were removed at 16 sec from a high temperature short time production pasteurizer at the temperatures indicated and assayed in triplicate for ALP activity by the Fluorophos test method.

This procedure provides a rapid and easy to use method for low levels of ALP in a wide variety of dairy products. The correlation with current methods is good. Using a single working reagent and 3 min read time, a detection limit of 0.006% raw milk is achieved.

ACKNOWLEDGMENTS

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REFERENCES


Heaton et al., cont. from p. 599

TABLE 3. Sensory preference ratings on a hedonic scale (9 = excellent; 1 = very poor) for Starkrimson apples stored at 26°C for 13 weeks.

<table>
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<th>Texture</th>
<th>Flavor</th>
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</table>

Values in the same column within the same parameter (maturity or packaging) not followed by the same letter are significantly different (P<0.05).

Includes values for unwrapped and wrapped apples.

Includes values for fully red and less mature apples.

REFERENCES