Enzymatic Determination of Residual Hydrogen Peroxide in Milk [1, 2]

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Abstract

A method for enzymatically detecting hydrogen peroxide at low levels in milk was developed. It involved the use of horseradish peroxidase with *o*-dianisidine as a chromogenic hydrogen donor. The milk curd was precipitated at pH 4.5 with hydrochloric acid and the whey added to a mixture of peroxidase and chromogen. The amount of color developed (measured at 400 mμ) as a result of the enzymatic reaction gave a measurement of the peroxide. The method detected levels of less than 1 μg/milliliter in milk.

The hydrogen peroxide and catalase treatment of milk for cheese making has received considerable attention in recent years. It has been shown to reduce the microbial load of the milk sufficiently for cheese making. On the other hand, it has been suggested that peroxide may remain in sufficient amounts to be inhibitory to the lactic streptococci used as starter cultures (3, 9, and 11). The starch-iodine method is usually employed to check the treated milk for residual peroxide. Even though a negative test is obtained for the starch iodine method, inhibition of lactic streptococci in such milk has been reported (9 and 11). According to Subramanian and Olson, the lower limit which can be detected by this method in milk is 10 μg/milliliter (9). This indicates the need for a more sensitive test.

There are several methods in addition to the starch-iodine test for detecting or measuring hydrogen peroxide in milk (1, 6, and 8), but none of these is capable of quantitating levels lower than 10 μg/milliliter. According to Munday (8), the Arnold-Mantzel method, involving vanadic acid as indicator, is the most sensitive for milk. This procedure can detect less than 80 ppm hydrogen peroxide in milk. Amin and Olson (1) have developed a spectrophotometric method using titanium tetrachloride which can be used to quantitate peroxide in milk at concentrations of 33-500 μg/milliliter.

Luck (6) mentioned the possible use of peroxidase and benzidine as a qualitative test for the peroxide in milk. Peroxidase with various hydrogen donors has been used to quantitate peroxide in fluids or liquids other than milk (4, 5, 7). The hydrogen donor used in these methods is usually a colorless compound which develops color when oxidized in the reaction:

$$H_2O_2 + AH_2 \text{ (donor)} \xrightarrow{\text{Peroxidase}} H_2O + A \text{ (oxidized donor)}.$$

Such reactions can be measured colorimetrically and the amount of hydrogen peroxide determined by comparing the amount of color developed with a standard curve.

This paper presents data obtained by using horseradish peroxidase to measure residual hydrogen peroxide in milk.

Methods

Reagents. All reagents were prepared with glass-distilled water using acid-cleaned glassware. The hydrogen peroxide (30%) was obtained from Allied Chemical Co., Morrison, N. J., and was diluted to the desired concentration with distilled water or milk just before each assay. Horseradish peroxidase (Nutritional Biochemicals Corp.) was dissolved in distilled water at a concentration of 1 mg/milliliter. The stock peroxidase solution was then filter-sterilized and stored at 5 C until used. A 1:100 dilution of this stock solution was used in the assays. One per cent *o*-dianisidine (Nutritional Biochemicals Corp.) in methanol was used as the chromogen for the hydrogen peroxide-peroxidase reaction.

Preparation of standard curve. Standards containing 1, 2, 3, 4, and 5 μg H_2O_2/milliliter were prepared in 0.01 M acetate buffer (pH 4.5). Ten milliliters of each standard were then diluted with an additional 10 ml of the buffer. The assay was accomplished by adding 5 ml of each diluted peroxide standard to sep-

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arate test tubes containing 1 ml peroxidase (0.01 mg/milliliter) and 0.1 ml *o*-dianisidine solution. The blank consisted of 5 ml 0.01 M acetate buffer plus 1 ml peroxidase and 0.1 ml *o*-dianisidine. The contents of each tube were mixed well and incubated at 23-26 C. After exactly 10 min of incubation, 0.2 ml of 4 N HCl was added to each tube to stop the reaction and stabilize the color. Five minutes after adding HCl the optical density of each standard was measured at 400 mμ in a Spectronic 20 colorimeter. The standard curve was constructed by plotting the optical density readings against micrograms of hydrogen peroxide.

Hydrogen peroxide determination in milk. Milk samples were adjusted to room temperature before adding the desired amount of H_2O_2. Ten milliliters of the milk sample, or a dilution thereof, were immediately adjusted to pH 4.5 with 0.1 N HCl. If the milk sample were diluted before analysis, 0.01 N HCl was used to adjust the pH. Two milliliters of 0.1 M acetate buffer (pH 4.5) were added and the mixture diluted to 20 ml with distilled water. The samples were then filtered through Whatman no. 42 filter paper to remove the curd. Centrifugation of the samples at 3,400 × *g* for 10 min may also be used to remove curd. To have the filtrate for each sample serve as the blank, 5 ml of the filtrate for each sample were added to a tube containing 1 ml distilled water and 0.1 ml *o*-dianisidine solution, then adding another 5 ml of the filtrate to a tube containing 1 ml peroxidase solution and 0.1 ml *o*-dianisidine. The assay tubes were then incubated 10 min at room temperature (23-26 C), 0.2 ml of 4 N HCl added to each tube, and the optical density determined after an additional 5 min of incubation. This was deemed necessary, to eliminate interference of the milk solids in determining the amount of color developed by the reaction of the H_2O_2, peroxidase, and chromogen. This would be especially true for milk containing a low level of H_2O_2, requiring that it be assayed without dilution.

Results

Standard curve. The stock hydrogen peroxide (30%) was titrated with a standardized solution of sodium thiosulfate to determine the exact concentration. The solution was found to be 30.6% H_2O_2. Based on this concentration, standards were prepared and subjected to the enzymatic analysis. The standard curve thus obtained is shown in Fig. 1. Each point on this curve represents an average of seven assays.

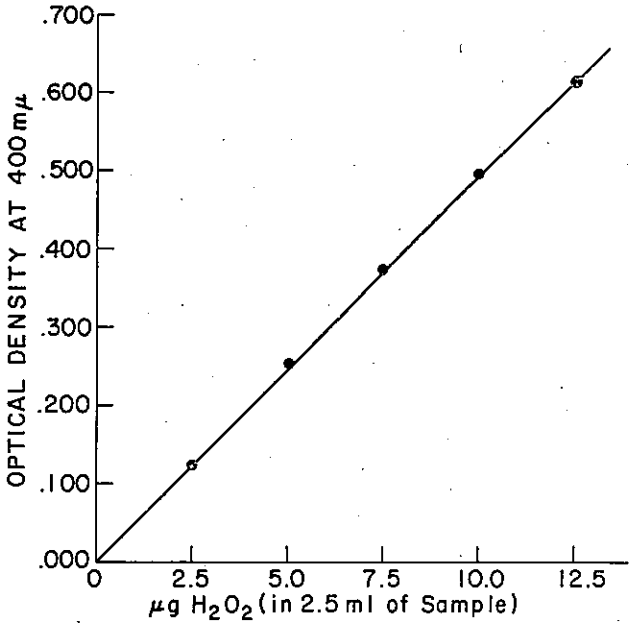

FIG. 1. Standard curve for peroxidase-*o*-dianisidine determination of hydrogen peroxide.

Hydrogen peroxide determination in reconstituted nonfat milk solids. Nonfat milk solids was reconstituted in distilled water to contain 10% solids, steamed (100 C) for 30 min, and stored in the refrigerator until used in an assay. The reconstituted milk was adjusted to room temperature and hydrogen peroxide added to the desired concentrations. Immediately after adding peroxide the samples were mixed well, diluted if necessary, and assayed. Table 1 shows the results from a series of such experiments. The per cent recovery was lowest in the samples to which 1 μg/milliliter was added. The amount of recovery increased as the amount of added peroxide increased. This apparently was due to greater destruction of the lower levels of peroxide by milk components, indicating a higher rate of destruction as the ratio of milk solids to added peroxide increased.

The type of milk, as well as the heat treatment to which it was subjected, influenced the

TABLE 1. Measurement of hydrogen peroxide in reconstituted nonfat milk solids.

Hydrogen peroxide		
Added	Measured[a]	Recovered
(μg/ml)		(%)
1	0.36	36.0
2	0.95	47.5
3	1.47	49.0
4	2.14	53.5
5	2.77	55.5
30	23.48	78.5
300	235.21	78.5

[a] Average of four to 14 replicates.

per cent recovery of added peroxide. This was shown in a series of experiments involving pasteurized homogenized whole milk and raw skimmilk obtained from the North Carolina State University dairy plant. The raw skimmilk was divided into two portions; one portion was used as a raw sample and the other was steamed (100 C) for 30 min. Reconstituted milk (steamed 30 min) was included for comparison. Hydrogen peroxide (30 μg/milliliter) was added to each sample and the assay immediately performed in the usual manner. A summary of results obtained is presented in Table 2. These results are averages of five replicates for each type of milk. Recoveries from reconstituted milk and heated skimmilk were about equal, being lowest for raw skimmilk. Pasteurized whole milk was not as detrimental to the recovery of added hydrogen peroxide as was raw skimmilk.

The hydrogen peroxide content of raw and heated skimmilk from the same lot was adjusted to 300 μg/milliliter and assayed at various time intervals. A portion of the sample was diluted and five replicate aliquots assayed immediately. The original samples were incubated at room temperature and assayed in the same manner after 15 and 30 min. The results are shown graphically in Fig. 2. These curves show that less peroxide was detectable in each sample as the incubation time increased. The hydrogen peroxide in the raw skimmilk dissipated more than that in the heated skimmilk. This was probably due to inactivation of lactoperoxidase or catalase in the milk by the heat treatment to which the heated sample was subjected before adding peroxide.

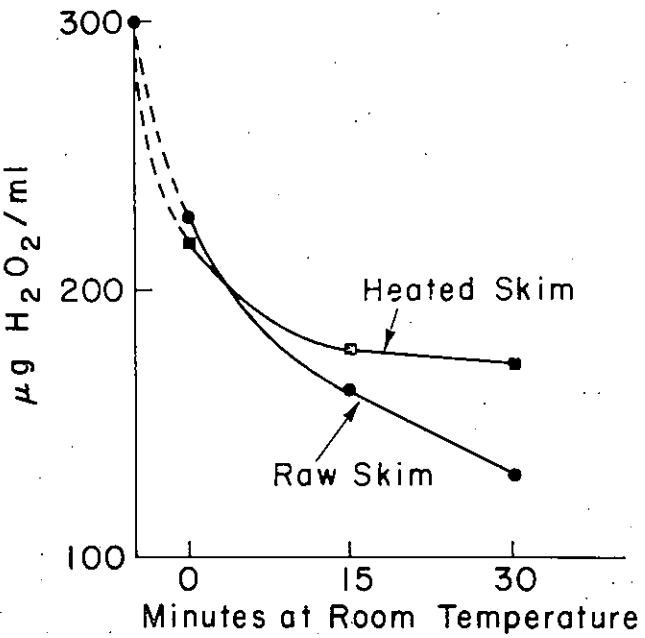

FIG. 2. Decomposition of hydrogen peroxide in raw and heated skimmilk.

Discussion

Horseradish peroxidase has been used to quantitate low levels of peroxide in systems other than milk (4-7). The sensitivity of lactic starter cultures attributed to traces of hydrogen peroxide remaining in hydrogen peroxide–catalase-treated milk (3, 9, 11) indicates the need for a more sensitive test for peroxide, to determine when milk can be used without adversely affecting growth of starter culture. The starch-iodine method has been reported to detect levels of peroxide in milk as low as 10 μg/milliliter; however, it lacks sufficient sensitivity to ensure that milk does not contain levels which may be inhibitory for growth of starter cultures (9). Use of peroxidase in conjunction with *o*-dianisidine appears to provide a method of detecting hydrogen peroxide in milk at levels of 1 μg/milliliter or lower.

Because hydrogen peroxide is subject to decomposition by organic matter, it is impossible to accurately quantitate the total amount which has been added to milk at any given time. Attempts to construct a standard curve using milk resulted in a plot which neither paralleled the curve for standards prepared in water nor passed through the origin. This might indicate decomposition of peroxide in milk before adding peroxidase, as well as different amounts of decomposition at each level of added peroxide. Subsequent experiments revealed that the per cent recovery increased as the amount of added peroxide increased. In determining the hydrogen peroxide concentration in a sample of milk, therefore, it is possible to measure only the residual peroxide and not the amount originally added. The foregoing was the basis for developing the standard curve in water rather than in milk.

TABLE 2. Efficiency of recovery of hydrogen peroxide in various types of milk.

Type of milk	Hydrogen peroxide		
	Added	Measured	Recovered
	——(μg/ml)——		(%)
Reconstituted, NFMS	30	22.5	75
Whole milk	30	16.6	55
Raw skimmilk[a]	30	5.9	20
Heated skimmilk[a]	30	23.3	78

[a] Same lot of skimmilk.

The fact that the standard curve formed a straight line which passed through the experimentally determined points for each standard, as well as the origin of the graph, indicated no detectable destruction of peroxide in the water or buffer before adding the enzyme in the assay. On the other hand, since it is evident that low levels of hydrogen peroxide added to milk are very unstable and decompose rapidly, it would be impossible to prepare a valid standard curve from data in which milk was used to prepare the standards. By preparing standards in water, satisfactory stability of hydrogen peroxide can be maintained during measurement. It must be realized, however, that the peroxide measured in milk is dependent on the content of reactive components as well as the length of time the peroxide has been added.

Our results support those of Amin and Olson (1, 2) wherein the stability of hydrogen peroxide in milk was shown to be dependent to a certain extent on the composition and heat treatment of the milk. Some of the decomposition in raw skimmilk was apparently due to enzymatic action, since heating (100 C for 30 min) the milk before adding hydrogen peroxide resulted in reduced decomposition. The milk fat may have been responsible for the lower recovery in the pasteurized whole milk than in the nonfat milk samples. Our results also indicate that the amount of decomposition in milk tends to reach a point of equilibrium.

Acknowledgments

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