PHOSPHATASE REACTIVATION IN DAIRY PRODUCTS[1]

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Equipment is available to the dairy industry for pasteurizing milk at temperatures much higher than those employed with conventional methods. Under certain conditions, ultra high temperature pasteurization results in reactivation of the enzyme phosphatase. Reactivation is influenced by several factors including temperature of heating, holding time, fat content of product, etc.

The phosphatase test has been of considerable value to regulatory officials and dairy plant laboratories as a means of detecting irregularities in the pasteurization of dairy products, particularly milk. With certain products, such as cream and butter, it has been recognized that the test has certain limitations. As early as 1940, Fay and Barber (*2*) noted that cream pasteurized under commercial conditions or under controlled laboratory conditions, and which had a negative phosphatase test immediately after pasteurization, might develop a positive test within one or more days. No evidence was found which indicated that the change from a negative to a positive reaction was due to bacteria and the authors thought the change involved reversible coagulation of the enzyme. Brown and Elliker (*1*) stated that the phosphatase test determined the adequacy of heat treatment when cream was pasteurized by the vat method; however, pasteurization by the flash method gave variations in the phosphatase reaction. In many cases the phosphatase test on flash-pasteurized cream was negative or slightly positive immediately after heating, and the phosphatase value increased with storage. Shadwick and Parker (*4*) found that a large percentage of butter samples made from pasteurized cream and giving a negative phosphatase test gave a positive test after holding at 70°F. for 8 days.

Rather recently, several investigators have noted irregularities in the phosphatase test when applied to milk heated to higher temperatures than the conventional pasteurization exposures. Wright and Tramer (*6*) noted that "Uperized" milk (canned sterile milk) developed a positive phosphatase reaction after storage even though the samples remained sterile. The reactivated phosphatase was similar to milk phosphatase. Raw milk which was heated rapidly to temperatures above 185°F. developed a positive phosphatase reaction when the milk was stored at 64.4 to 98.6°F.; optimum temperature for reactivation was 86°F. Reactivation did not occur in milk heated at 161°F. for 15 seconds, or at 145°F. for 30 minutes. These investigators (*7*) also noted that a decrease in the bacteriological quality of milk, the absence of air, and the presence of reducing conditions, increased reactivation of phosphatase. Reactivation did not occur when milk was heated sufficiently to denature the soluble proteins completely. In additional experiments (*8*), several samples of milk from farms were pasteurized using an exposure of 161°F. for 23 to 24 seconds. Two of the samples developed a positive phosphatase reaction after storage and it was shown that the phosphatase was not of bacterial origin. Bulk

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raw milk from tanks did not exhibit reactivation when given a smiliar heat treatment. Metallic ions were considered to play a part in phosphatase activity and in reactivation (9). Either Mg^{++} or Zu^{++}, or both, seemed to play a part in reactivation.

Fram (3) heated skim milk, milk, and cream in a tubular heater using an exposure of 165 to 240°F. for 16 seconds. Also, portions were pasteurized at 145°F. for 30 minutes. Immediately after pasteurization, and when stored at 40°F., all products showed negative phosphatase tests. When the samples were stored at 88°F., phosphatase reactivation was noted in all products heated to high temperatures but not at the low temperature. The minimum temperature above which reactivation occurred, and the time of storage at 88°F. required for reactivation, was a function of the fat content. Bacteriological studies indicated that reactivated phosphatase was not of bacterial origin.

Since the methods employed by Wright and Tramer (6) for measuring phosphatase activity were different than those commonly used in this country, and the test used by Fram (3) was the Scharer rapid qualitative test, it appeared desirable to conduct additional studies on phosphatase reactivation at high temperatures using a quantitative method. The effect of temperature of heating, time of heating, storage temperature, storage time, and fat content on phosphatase reactivation were studied.

PROCEDURES

The milk used in these experiments was bulk milk, representing a mixture from several patrons. When desired, the milk was separated by means of a cold-milk separator, or cream was removed from milk after standing in a refrigerator at 40°F.

Heating of samples was carried out in sealed capillary tubes (1 x 100 mm.). The tubes were heated in a glycerol bath allowing 4 seconds "come-up" time. "Come-up" time was determined by the melting point method, employing chemicals whose melting points were within a suitable range of the desired exposure.

Phosphatase determinations were made by the method of Sanders and Sager (5). The contents of several capillaries were pooled in order to obtain the quantity of milk recommeded in this method. Color intensity was measured with a Coleman Spectrophotometer at a wavelength of 610 mμ. Results are reported as micrograms of phenol liberated per 1 ml. of milk.

Bacterial counts were made by the plate method using plate count agar, and incubation temperature ranging from 25° to 45°C., depending upon the information desired.

RESULTS

Temperature reactivation of phosphatase in skim milk

Raw skim milk obtained by the cold separation of mixed herd milk was heated to temperatures ranging from 167 to 284°F., and held at these temperatures for 5, 10 and 15 seconds. Phosphatase activity was determined immediately after heating and also after holding at 41°F. for 24 hr. and at 86°F. for 24 and 48 hr. The samples held were preserved with chloroform (5).

Data presented in Table 1 show that no phosphatase activity was observed in skim milk given an exposure of 190.4°F. for 5, 10, or 15 seconds, even after holding at 86°F. for 48 hrs. Trials also were conducted at temperatures of 167°, 176°, and 185°F. with holding times of 5, 10, and 15 seconds; the data in these trails are not reported since no phosphatase activity was observed. The first indication of phosphatase reactivation occurred with an exposure of 197.6°F. for 10 or 15 seconds and holding at 86°F. No reactivation was observed in any of the samples held at 41°F. With skim milk, an increase in the extent of reactivation was noted as the temperature was increased over the range of 197.6° to 284.0°F. Since phenol values higher than 4μg. per ml. indicate under-

TABLE 1 — EFFECT OF HEATING SKIM MILK TO VARIOUS TEMPERATURES ON PHOSPHATASE ACTIVITY AFTER STORAGE AT 41 AND 86°F.

Temperature of heating (°F.)	Holding time (seconds)	Phosphatase activity (micrograms phenol liberated from substrate) after storage for			
		0 hrs.	24 hrs. at 41°F.	24 hrs. at 86°F.	48 hrs. at 86°F.
190.4	5	0	0	0	0
	10	0	0	0	0
	15	0	0	0	0
197.6	5	0	0	0	0
	10	0	0	2	1
	15	0	0	2	2
204.8	5	0	0	3	2
	10	0	0	4	4
	15	0	0	4	3
212.0	5	0	0	3	4
	10	0	0	4	4
	15	0	0	4	4
248.0	5	0	0	5	3
	10	0	0	4	4
	15	0	0	5	D
284.0	5	0	0	5	6
	10	0	0	6	6
	15	0	0	6	7

TABLE 2 — EFFECT OF HEATING WHOLE MILK TO VARIOUS TEMPERATURES ON PHOSPHATASE ACTIVITY AFTER STORAGE AT 41 AND 86°F.

Temperature of heating (°F.)	Holding time (seconds)	Phosphatase activity (micrograms phenol liberated from substrate) after storage for			
		0 hrs.	24 hrs. at 41°F.	24 hrs. at 86°F.	48 hrs. at 86°F.
176	5	0	0	0	0
	10	0	0	0	0
	15	0	0	0	0
185	5	0	0	4	4
	10	0	0	0	0
	15	0	0	0	4
194	5	0	0	3	1
	10	0	0	6	6
	15	0	0	8	7
212	5	0	0	12	18
	10	0	0	38	38
	15	0	0	40	42
230	5	0	0	39	36
	10	0	0	43	44
	15	0	0	43	43
248	10	0	0	43	42
	15	0	0	41	42
268	10	0	0	25	25
	15	0	0	23	25

TABLE 3 — EFFECT OF HEATING HALF (MILK) AND HALF (CREAM) CONTAINING 10% FAT TO VARIOUS TEMPERATURES ON PHOSPHATASE ACTIVITY AFTER STORAGE AT 41 AND 86°F.

Temperature of heating (°F.)	Holding time (seconds)	Phosphatase activity (micrograms phenol liberated from substrate) after storage for			
		0 hrs.	24 hrs. at 41°F.	24 hrs. at 86°F.	48 hrs. at 86°F.
176	10	0	0	0	0
	15	0	0	0	0
185	10	0	0	0	0
	15	0	0	2	2
194	10	0	0	13	12
	15	0	0	10	10
203	10	0	0	9	13
	15	0	2	10	12
212	10	0	0	—	22
	15	0	0	—	28
230	10	0	0	20	22
	15	0	0	24	24
248	10	0	0	12	16
	15	0	2	23	22
268	10	0	0	6	7
	15	0	0	8	10
284	15	0	0	5	11

pasteurization with the Sanders and Sager method (5), samples exposed to 248°F. for 5 or 15 seconds and held 24 hr. at 86°F., and also samples exposed to 284°F. for 5, 10, or 15 seconds and held 24 or 48 hr. at 86°F. would be classed as underpasteurized.

Temperature reactivation of phosphatase in whole milk

Mixed raw milk from several producers was used in these trails. The fat content of the milk was 3.9 per cent. Table 2 indicates that no reactivation occurred in milk heated at 176°F. for 5, 10, or 15 seconds. At 185°F., some reactivation was observed but it was not sufficient to class the samples as underpasteurized. An exposure of 194°F. for 10 or 15 seconds, with holding at 86°F., resulted in sufficient reactivation to class the samples as underpasteurized. An increase in the extent of reactivation was observed over the temperature range of 185° to 230°F., when the samples were stored at 86°F. Temperatures higher than 230°F. gave less reactivation; an appreciable decrease was noted in the trails at 268°F. As with skim milk, no phosphatase activity was observed in samples immediately after heating or after storage at 41°F. for 24 hrs.

Temperature reactivation of phosphatase in Half and Half

A mixture of raw milk and raw cream containing 10 per cent fat was used to obtain the data presented in Table 3. No. reactivation of phosphatase occurred in the mixture when the temperature employed was 176°F. Very slight reactivation resulted when the exposure was 185°F. for 15 seconds. Sufficient reactivation was obtained with an exposure of 194°F. for 10 or 15 seconds and holding at 86°F. for 24 or 48 hr. to class the product as underpasteurized. All exposures within the range of 194° to 284°F. gave appreciable reactivation. However, a noticable decrease in phosphatase activity was observed at 268° and 284°F. A very slight amount of reactivation was noted in two samples held at 41°F. for 24 hrs. (203°F. for 15 seconds, and 248°F. for 15 seconds) but not sufficient to class them as under pasteurized.

Temperature reactivation of phosphatase in cream

Raw cream containing 20 per cent fat was used for obtaining the data given in Table 4. The lowest

TABLE 4 — EFFECT OF HEATING CREAM (20% FAT) TO VARIOUS TEMPERATURES ON PHOSPHATASE ACTIVITY AFTER STORAGE AT 41 AND 86°F.

Temperature of heating (°F.)	Holding time (seconds)	Phosphatase activity (micrograms phenol liberated from substrate) after storage for			
		0 hrs.	24 hrs. at 41°F.	24 hrs. at 86°F.	48 hrs. at 86°F.
167	10	0	0	2	10
	15	0	0	0	13
176	10	0	0	7	48
	15	0	2	7	50
194	10	0	3	14	52
	15	0	3	16	60
212	10	0	2	8	100
	15	0	4	16	100
230	10	0	0	12	126
	15	0	4	16	120
248	10	0	0	8	100
	15	0	2	15	100
268	10	0	0	16	94
	15	0	1	12	100
284	10	0	0	11	79
	15	0	0	10	80

exposure employed (167°F. for 10 seconds) showed some enzyme reactivation when the sample was held at 41°F. for 48 hrs., and sufficient reactivation to class the sample as underpasteurized when held for 48 hrs. at 86°F. Phosphatase activity increased progressively over the temperature range of 167° to 230°F. Above 230°F. a decrease in enzyme activity was noted and the decrease was particulary noticeable at 284°F. No phosphatase activity was noted in any of the cream samples immediately after pasteurization. Slight activity could be detected in many of the samples after holding for 24 hrs. at 41°F. All of the samples heated in the range of 176° to 284°F. with an exposure time of 10 or 15 seconds and held for 48 hours at 41°F. showed sufficient phosphatase activity to class them as underpasteurized.

Additional observations on reactivated phosphatase

An experiment was designed to compare the rate of hydrolysis of disodium phenyl phosphate by normal milk phosphatase and reactivated phosphatase. A sample of cream containing reactivated phosphatase liberated 33 μg. phenol per ml. A sample of raw milk was diluted with boiled milk so that it also would liberate 33 μg. of phenol per ml. Several 1-ml. aliquots of each sample were tested by the Sanders–Sager test, varying only the contact time with the substrate. Curves representing the results of this experiment were prepared but they are not included since they indicated that the rates of hydrolysis for both the normal and reactivated enzyme were the same.

Another comparison was made of the rate of formation of indophenol by phenol, phenol liberated from disodium phenyl phosphate by normal milk phosphatase, and phenol liberated from disodium phenyl phosphate by reactivated milk phosphatase. Appropriate samples adjusted to the same phenol content showed no difference in the rate of formation of indophenol.

When milk samples were heated in the laboratory to 145°F. and held for 30 minutes, it was not possible to reactivate phosphatase by additional exposures at high temperatures known to produce reactivation. Samples containing reactivated phosphatase showed no phosphatase activity when given an exposure of 145°F. for 30 minutes, nor could the phosphatase be reactivated again by an exposure to a high temperature known to produce reactivation.

Phosphatase activity of sterile cream

Four samples of commercial cream labeled "sterilized" were tested for phosphatase. All of the samples contained appreciable phosphatase as they liberated 25, or more, micrograms of phenol when tested by the Sanders–Sager method. The cream samples were plated using plate count agar and the plates were incubated at 25°, 32° and 45°C. No bacterial colonies appeared on the plates during incubation for 5 days. Laboratory pasteurization of these samples using an exposure of 145°F. for 30 minutes destroyed the phosphatase activity.

DISCUSSION

During the past several years, equipment has been available to the dairy industry for heating milk and other dairy products to temperatures higher than the conventional high-temperature short-time method (161°F. for 15 seconds). At least one such apparatus has been approved by regulatory officials for pasteurizing milk and cream. The experiments conducted for this study were designed to determine whether the phosphatase test, which has been used for detecting irregularities in the pasteurization of milk, cream, and other dairy products, could be applied to milk and cream heated to higher temperatures and held for the same, or less time, than the regular method.

The enzyme phosphatase is associated with the fat globule membrane but is not soluble in the fat. Consequently, skim milk contains less of the enzyme than whole milk or cream. With skim milk, the first evidence of reactivation occurred at 197.6°F., whereas with milk the temperature was 185°F. and with cream (20% fat) it was 167°F., or less. Therefore, there is a definite relation between reactivation temperature and fat content of the product.

Skim milk showed an increase in the amount of reactivation as the temperature was increased over the range of 190.4° to 284°F. Perhaps even more reactivation would have occurred at a higher temperature but it was not possible to attain temperatures higher than 284°F. by the method of heating employed. With milk and cream, reactivation of phosphatase increased with temperature only up to a certain point and then a decrease was noted; the temperature for maximum reactivation was about 230°F. for both products.

Regulatory officials generally collect one sample of milk or cream that is used for bacteriological analysis, the phosphatase test, and chemical analysis It is recommended that samples be cooled promptly and held at 32 to 40°F. (5). Such a procedure limits reactivation of phosphatase. Data presented on skim milk, milk, or cream show that when products were held at 41°F. for 24 hrs., insufficient reactivation

occurred to class the samples as underpasteurized. However, some samples of cream held at 41°F. for 24 hrs. liberated 4μg. of phenol in the phosphatase test; this is the maximum permitted in classifying the samples as properly pasteurized.

In some laboratories large numbers of samples are tested for phosphatase activity at one time. If the small sample used is measured into a warm test tube and held in a warm room for some time before testing appreciable reactivation may occur, particularly with cream, and a properly pasteurized sample may be classed as underpasteurized. Regulatory officials and plant personnel should recognize that reactivation of phosphatase may occur in dairy products heated to higher-than-normal temperatures, and that such reactivation can be limited by cooling to a low temperature after the heat treatment. Also, the low temperature must be maintained until the sample is tested for phosphatase.

SUMMARY

Samples of raw skim milk, milk, and cream were heated in capillary tubes at various temperatures over a range of 167° to 284°F. with holding times up to 15 seconds. After heat treatment the samples were stored at 41° and 86°F. in the presence of chloroform. Phosphatase determinations were made on all samples before heat treatment, immediately after heat treatment, and after storage.

Immediately after heat treatment, phosphatase tests on all products were negative. After storage at 41°F. for 24 hrs., no phosphatase activity was detected in skim milk or whole milk but a slight amount was noted in half and half (10% fat) and cream (20% fat). However, there was not sufficient activity to class the samples as underpasteurized. Cream (20% fat) heated at 176 to 284°F. and held at 41°F. for 48 hrs. showed sufficient phosphatase activity to be classed as underpasteurized.

Samples heated to high temperatures and held at 86°F. showed considerably more enzyme reactivation than samples held at 41°F. After storage at 86°F. for 24 hrs., phosphatase activity was observed in skim milk exposed to 190.4°F. or higher, in whole milk and half-and-half exposed to 185°F. or higher, and in cream exposed to 167°F. or higher. With all products held at 86°F. there was sufficient phosphatase activity to class them as underpasteurized, but the temperature necessary to bring about such reactivation varied with fat content of the product.

Samples of milk which gave a negative phosphatase test after an exposure of 145°F. for 30 minutes did not give a positive test after subsequent heating to high temperatures and storage at temperatures known to give reactivation of phosphatase in raw milk. Samples of milk containing reactivated phosphatase did not show phosphatase activity when given an exposure to 145°F. for 30 minutes; subsquent heating to high temperatures also failed to activate the enzyme.

Four samples of cream labeled "sterilized" showed appreciable phosphatase activity. Bacteriological analyses of the samples indicated that they were sterile.

ADDENDUM

A technical note entitled, "Phosphatase Reactivation in High-Temperature, Short-Time Pasteurized Cream" (J. Dairy Sci. **40**: 1649. 1957) was published by Harvey Fram after this work has been completed and prepared for publication. The data presented in this article confirms the work of Fram and provides additional information on the subject.

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