Alkaline Phosphatase Activity in *Penicillium roqueforti* and in Blue-Veined Cheeses

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ABSTRACT

The *Penicillium roqueforti* mold exhibits alkaline phosphatase activity. Therefore, blue mold-ripened cheeses made from properly pasteurized milk test positive for phosphatase. Because microbial phosphatases are considered to be more resistant to heat than is milk phosphatase, a statutory control test recommends the repasteurization of cheese at 66°C for 30 min to inactivate selectively the native milk enzyme. However, because of thermal lability of *Penicillium roqueforti* phosphatase, this control test leads to confusion of the fungal enzyme with native milk alkaline phosphatase and does not confirm whether the milk used to make cheese has been pasteurized.
(**Key words**: *Penicillium roqueforti*, alkaline phosphatase, testing, pasteurization)

Abbreviation key: **AP** = alkaline phosphatase.

INTRODUCTION

Because of the potential danger of food poisoning from cheese made from unpasteurized milk, many public health bodies require that cheese be made exclusively from pasteurized milk. The alkaline phosphatase (**AP**) test has been generally accepted as the statutory test for confirmation of pasteurization treatment. Indeed, the *Official Methods of Analysis* of AOAC (1) lists two procedures for residual phosphatase in cheese: a qualitative method using phenolphthalein monophosphate, and the quantitative method of Sanders and Sager (17, 18). The quantitative procedure is rather complex for routine use and even more so for occasional use in quality control laboratories. *Standard Methods for the Examination of Dairy Products* (4), among other options, endorses also the Scharer rapid phosphatase test as a simplified phosphatase test for cheeses.

Until recently, the phosphatase test was thought to be able to detect cheese made from unpasteurized milk (6), regardless of the age of the cheese, providing that proper controls were performed. For example, the AP activity detected in salted white cheeses in Spain that was apparently unrelated to the use of raw milk (2) was explained either by reactivation of milk enzyme after heat treatment (20) or by the production of phosphatase by cheese microflora (9). It has been generally accepted that AP production in dairy products by microorganisms can be discriminated from native AP in milk on the basis of the higher heat resistance of the former; a control test was devised that consisted of repasteurization of a cheese sample at 62.8 or 66°C (depending on the fat content of the cheese) for 30 min (4). This postulate was questioned when some Mexican-style soft cheeses were reported to contain both heat-labile and heat-stable microbial AP (12, 14, 15, 16). The initial procedure for detecting microbial AP instructed that the cheese sample be extracted in an aqueous solution of 7.5% *n*-butanol; *n*-butanol was thought to loosen the tertiary structure of the enzyme molecule, and, when the extract was repasteurized, the microbial phosphatase could be inactivated more easily and consequently mistaken as being milk phosphatase (15). A subsequent investigation (12) of the factors that affect methods of differentiating milk and microbial AP noted that the NaCl present in finished cheese has a heat-denaturing effect that increases the lability of the microbial enzyme. In general, AP activity in cheese was closely related to the absolute fat content, type of cheese, and ripening stage (11).

The present study investigated the ability of *Penicillium roqueforti* mold to produce an AP enzyme and the effect of this enzyme on the analytical assay used to test for use of pasteurized milk in the manufacture of blue-veined cheeses.

MATERIALS AND METHODS

Cheeses were purchased at retail outlets or obtained from the processing line of a local dairy. Cheeses were tested before the expiration date. The AP activity was routinely assayed by the Scharer rapid phosphatase test (Applied Research Institute, Perth Amboy, NJ) (4) as follows: 5 g of cheese and 2 ml of diluted (1:1, wt/vol) carbonate buffer (0.44 *M* Na_2CO_3 + 0.44 *M* $NaHCO_3$) were macerated with a

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glass rod. After addition of 18 ml of 8.3% neutralized *n*-butyl alcohol, mixing on a Vortex stirrer (number 4 setting for 30 s), and standing for 5 min, the slurry was filtered through paper. For testing, a 0.5-ml portion of filtrate was mixed with 0.1 ml of magnesium acetate solution [8.82 g of $Mg(CH_3COO)_2 \cdot 4H_2O$ in 100 ml of water]. To this mixture, 5 ml of double-strength buffered substrate were added. The content was mixed and then incubated at 40 ± 1°C for 15 min. To this solution, six drops of CQC reagent were added and reincubated for an additional 5 min. Subsequently, the solution was cooled in an ice-water bath, and the indophenol blue color was extracted in 3 ml of neutralized cold *n*-butanol. The concentration of phenol in the test sample was determined by comparing the absorbance at 650 nm wavelength to a calibration curve. Absorption spectra were obtained with a spectrophotometer (Spectronic 1201; Milton Roy Co.). A value of >1 μg of phenol equivalent/0.25 g of cheese (or 1 ml of cheese extract) was indicative of improper pasteurization. One positive and one negative control (4) were performed for each series of samples.

For negative and microbial phosphatase controls, slurries composed of 5 g of cheese macerated in 2 ml of diluted (1:1, wt/vol) carbonate buffer were heated in a boiling water bath to 95°C for 1 min and at 66°C for 30 min, respectively [standard methods (4) prescribe heating of an undiluted 5-g sample of cheese for microbial phosphatase control, but we digressed from this recommendation for more homogeneous heating conditions]. Subsequently, the slurries were cooled in an ice-water bath and supplemented with 18 ml of 8.3% neutralized *n*-butyl alcohol.

Penicillium roqueforti mold (Chr. Hansen's Lab, Copenhagen, Denmark) was grown as described (19). Mycela were harvested from culture medium by filtration, washed with distilled water, resuspended in veronol buffer 0.05 *M*, pH 8.0, and homogenized under CO_2 refrigeration. After centrifugation at 25,000 × *g* for 30 min at 5°C, the supernatant was separated, concentrated by ultrafiltration to 75 mg of DM (30 mg of protein)/ml, and used as enzymatic extract.

RESULTS AND DISCUSSION

For safety, the increasing international trade of delicate foods demands the occasional investigation of the sanitary history of these foods. For dairy products, a common question is whether properly pasteurized milk was employed in their manufacture, particularly if legislation, as in Israel, prohibits completely the use of raw milk even for aged cheeses. Therefore, we screened a variety of cheeses of local and foreign origin for the presence of AP activity by the Scharer rapid phosphatase test. Most cheeses surveyed tested negative (<1 μg of phenol equivalent/0.25 g of cheese), including fresh cheeses such as cottage; hard, sliceable cheeses such as Danbo, Edam, and Gouda; and pasta filata-type cheese, such as Halloumi and Mozarrella. Conversely, samples of different brands of blue-veined cheeses, such as Danish Blue, French Roquefort, Italian Gorgonzola, and Israeli Gallyl, showed positive results. To clarify the origin of this effect, blue-veined cheese was manufactured in a local dairy plant under controlled conditions from properly pasteurized milk (75°C for 15 s). The proximate composition of the cheese was 62% DM, 33% fat, 25% protein, and 3.5% salt; pH was 6.3. The cheese was monitored for AP during 3.5 mo of storage. Thus, 2-wk-old cheeses, still with hardly any development of blue mold, showed positive results of 3.5 to 4.5 μg of phenol equivalent/0.25 g of cheese; cheese samples from the same production batch and aged for >3 mo tested positive with >9 μg of phenol equivalent/0.25 g of cheese. Because the intensity of the positive reaction was affected by the period of cheese storage, the residual AP activity was probably due to a microbial enzyme.

Microbial phosphatases are considered to be more heat resistant than milk AP, and, therefore, heating a sample of cheese at 66°C for 30 min is expected to inactivate the residual native milk enzyme and have little effect on microbial phosphatase activity (4). However, all of the blue-veined cheese samples described showed almost complete inactivation of AP activity (1 μg of phenol equivalent/0.25 g of cheese) as a result of this treatment. Thus, the positive results of the Scharer test could not, legally, be attributed to microbial AP and could not therefore be reported as false positive, although the cheese was manufactured from properly pasteurized milk. These results support previous observations that phosphatases in cheeses ripened with blue mold were inactivated by heat faster than was native milk phosphatase (12). The blue-veined cheese is a salty cheese (in the present case, 3.5% NaCl), and the high salt content was suspected to destabilize microbial AP (12). For butter tested for the presence of microbial AP, however, salt not only did not contribute to the heat inactivation of the enzyme (13), but also played a role in the reactivation or in release of residual AP from a natural protective structure (10).

High heat lability of a *P. roqueforti* phosphatase was a more attractive explanation. In fact, precedents of heat-sensitive AP at pasteurization conditions have been reported (3, 5, 7, 8). Thus, microbial phospha-

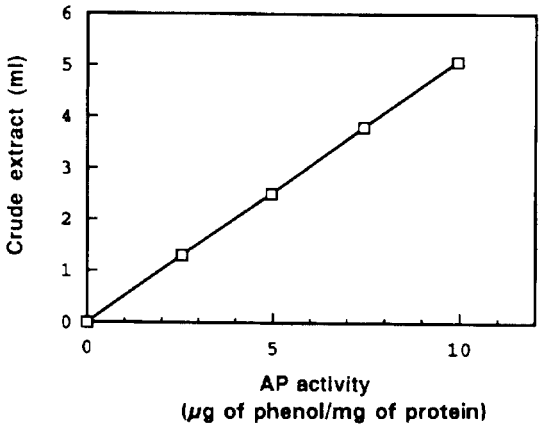

Figure 1. Alkaline phosphatase (AP) activity in extract from *Penicillium roqueforti*.

TABLE 1. The alkaline phosphatase (AP) activity of *Penicillium roqueforti* phosphatase after thermal treatment in buffer and cheese extract solutions.

Thermal treatment of the sample	AP Activity[1]	
	In buffer	In cheese extract[2]
No heating	5.1	5.1
40°C for 30 min	4.3	3.9
50°C for 30 min	2.9	2.7
62.8°C for 30 min	1.5	1.0
66°C for 30 min	1.0	1.0
95°C for 1 min	<1	<1

[1]The tests were performed in triplicate, and the results are mean values (±0.1). Activity is expressed as micrograms of phenol equivalent per milligram of extract protein.

[2]The cheese extract was heated at 90°C for 1.5 min and cooled at ambient temperature before addition of *P. roqueforti* phosphatase.

tases, such as those from *Bacillus anthracis*, *Bacillus cereus*, *Bacillus megaterium* (3), *Micrococcus sodonensis* (7), *Saccharomyces cerevisiae* (8), and *Penicillium caseicolum* (5), have been reported to be thermally labile.

To confirm that in the present case the pasteurization-sensitive AP activity was produced by *P. roqueforti*, the mold was propagated in a semisynthetic medium and examined for AP activity. This activity was linearly related to the protein concentration in the fungal extract (Figure 1). The thermal stability of the enzyme was determined by heating the enzymatic extract at different temperatures for 30 min in carbonate-bicarbonate buffer and in an aqueous cheese extract obtained after maceration of 5 g of cheese in 20 ml of water before analysis by the Scharer test. The results (Table 1) c learly indicated that *P. roqueforti* phosphatase was inactivated by heating at 66°C for 30 min. The slightly higher thermal susceptibility of the enzyme in the presence of cheese extract than in buffer solution might be due to a destabilization effect of NaCl or to another unidentified cheese constituent.

The present results indicate that, when the history of the blue-veined cheese is not ascertainable, the AP test cannot routinely be applied to assess the lack of pasteurization of cheese milk. This result is in contradiction to the inclusion of blue-mold cheeses, independent of the extent of curing, in the list of statutory criteria of pasteurization by AP test (1). We also failed to trace an explanation for the postulated threshold for proper pasteurization of ≤3 μg of phenol equivalent/0.25 g of blue cheese (1), because *Standard Methods for the Examination of Dairy Products* (4) suggests a lower value of ≤1 μg of phenol equivalent/0.25 g for all types of cheeses, without explicit reference to blue-mold cheese. In fact, the absolute figures for the amount of phenol equivalent measured in the AP test, as conventionally reported (including in the present paper), are inaccurate. According to standard procedures (4), a graph for quantitation of the test results is constructed based on phenol standards consisting of 5-ml solutions containing phenol in the range from 0.5 to 5 μg versus absorbance. On the graph, these values are literally doubled to 1 to 10 μg of phenol equivalent per 5 ml of standard solution to provide direct readings for phenol equivalent per 1 ml of liquid sample assayed, because 0.5 ml of liquid sample is actually tested. The fact that the true concentrations of phenol equivalent standards are only 0.2 to 2 μg/ml is apparently ignored, and, erroneously, the concentration of phenol equivalent per milliliter of sample is derived by comparison with concentration of standards per 5 ml. Therefore, the correct threshold values of phenol equivalent per milliliter of test sample (or 0.25 g of cheese) for proper pasteurization should be only one-fifth of those presently postulated.

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ADDED IN PROOF

Another example that also questions the accepted ability of the phosphatase test to detect cheese made of nonpasteurized milk was recently found. Thus, Grana-type hard cheese made of nonpasteurized milk yielded consistently negative phosphatase tests. Seemingly, the specific manufacturing conditions of this type of cheese curds (cooked at about 55°C for approximately 1 h in whey of increased acidity and long aging time) deactivate native alkaline phosphatase. Incidentally, the *n*-butanol extract had a yellow color (λ_{max} = 443 nm and an absorption shoulder at 325 nm) of unknown origin. Although homogenized pasteurised milks have been reported to yield a slight yellow *n*-butanol solution because of the more complete extraction of the milk fat, control extraction tests run on Grana cheese, without one of the phosphatase reagents do not support this explanation.