The following abstract is taken from an article written by Richard M. Rocco, Ph.D., a consultant to the food and clinical diagnostic markets. Dr. Rocco developed the Fluorophos® ALP Test, which has become a world standard for the rapid confirmation of pasteurization via alkaline phosphatase monitoring in fluid, flavored, bovine, sheep, and goat milks and milk-based products.

Abstract:
The article simplistically explains what alkaline phosphatase (ALP) is and how it behaves when milk or milk-based products are pasteurized. Occasionally, low-fat products may exhibit reactivation, but it is more prevalent in high-fat products, such as: butter, cheese, and cream that have been thermally abused, causing the ALP enzyme to become reactivated. Having a zero-time result helps in this situation to ascertain whether the product was "legally pasteurized" or not prior to reactivation. The Magnesium (Mg) method to test for reactivated ALP is explained and results are interpreted. Finally, bovine and microbial ALP, their structure, survival, and interpretation of results are explained. This paper is a useful document for all laboratories interested in the pasteurization of milk and milk-based products.

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Milk Alkaline Phosphatase; the Effects of Heat Treatment.
The concept of reactivated milk alkaline phosphatase (ALP) is best understood in relation to the current understanding of what happens to milk ALP during pasteurization. Milk ALP is a dimer composed of two equal length polypeptide chains with a combined total molecular weight of about 180,000. The two 90,000 molecular weight chains are attached to each other along their length with sulfur bond bridges much like the steps of a ladder hold together the sides of the ladder. The three dimensional structure of the ALP dimer is complex and involves twists and folds. Think of a ladder twisted around itself like a pretzel. The application of heat to this ALP dimmer, which occurs during pasteurization causes the ladder to unfold. In the heated form, ALP resembles an unfolded ladder. In dairy chemistry, ALP which has been unfolded is called "inactivated" ALP.

Unfolding of ALP
Unfolded ALP is called inactive because the protein in this state is not capable of hydrolyzing any known substrate. Only intact, folded ALP ladders can hydrolyze ortho- phosphoric esters from phenolic and other type substrates. It was Kay and Graham in England in 1931 that discovered that the unfolding of ALP in milk and dairy products is time and temperature dependent and follows a linear time course. From their original work they established the use of the ALP activity level as a predictor of the time and/or temperature to which the milk had been heated. Measurement of this "residual" ALP activity therefore, is simply a measure of the percentage or amount of ALP that has not yet undergone unfolding. Needless to say, residual ALP activity will also reflect if any raw milk has leaked into the product.

The total amount of ALP in a raw unheated milk sample will vary widely due to a host of factors. If we assume a value of about 400,000 mU/L for a raw milk sample and a value of 50 mU/L after proper pasteurization, then 399,950 mU/L have been unfolded or inactivated. The Fluorophos ALP method will measure only the residual 50 mU/L remaining, despite the fact that the unfolded ALP is still present in all dairy samples.

Definition of Reactivation

In 1953 in England, Wright (Wright, R.C. & Tramer. J 1953, "Reactivation of milk phosphatase following heat treatment" Dairy Res. 20, 177-188) described the “reactivation” of unfolded heat treated ALP. Under conditions defined by Wright, unfolded ALP could be made to refold and therefore become catalytically active. Two conditions have been shown over the years to cause unfolded ALP to reactivate. First is storage temperature, generally 30 deg C or above. Secondly, the addition of high concentrations of magnesium will reactivate unfolded ALP. All milks contain a sufficient amount of magnesium, which is needed in order to measure ALP activity. The presence of magnesium is so important that all clinical diagnostics companies that produce test kits for measuring ALP in biological fluids include magnesium in their reagents to insure full ALP activity in the assay.

Reactivated ALP and Regulatory Issues

Reactivation from a regulatory perspective can cause a problem. For example, a sample of milk that has been pasteurized is found to contain 50 mU/L of residual ALP activity prior to shipment from a plant. If reactivation due to improper storage occurs, then the repeat ALP value after storage may show a value of 1,000 mU/L or more. This product meets “legal” requirements because the original ALP value of 50 mU/L indicated proper pasteurization, however, reactivation has occurred indicating that the repeat 1,000 mU/L is a “false” positive. At worse, only improper storage can be suggested.

Magnesium Method for Testing for Reactivated ALP

Historical experience starting with Wright’s work in the early 1950’s indicates that a simple lab test could determine if reactivation has occurred in a stored sample, whether a result of 1,000 mU/L indicates residual ALP due to improper pasteurization, or if ingress of raw milk during production has occurred. Incubation of the milk sample with high concentrations of magnesium for 1 hour at 34 deg C will promote reactivation. In the current AOAC and other approved methods for detecting reactivated ALP, the sample being tested is incubated with magnesium acetate (1 hour @ 34°C) followed by a x6 dilution. Using the example above of 1,000 mU/L interpretation is as follows:

Original Sample	=	1,000 mU/L
Original Sample after Mg and dilution x6	=	< 1,000 mU/L

The Original Sample contained residual non-pasteurized milk, dilution of the sample x6 reduced the original sample by 6 or more as would be expected.

Original Sample after Mg and dilution x6	=	> 2,000 Plus mU/L

The Original Sample contained reactivated ALP. This sample contains reactivated ALP because dilution x 6 did not reduce the original value but in fact increased the original value by two or more times.

The interpretation using the above magnesium test for reactivation is based on a number of assumptions. Samples that contain ALP capable of being reactivated by improper storage will when incubated with magnesium. As a result, they will show a marked increase in ALP activity over the original starting value

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despite the fact that the sample was diluted down by a x6 factor. Samples containing ALP incapable of being reactivated will show a 6 times or greater decrease in ALP activity after dilution or incubation with magnesium. In simplified terms, if the milk sample is diluted x6 then the original result will fall by x6 or more or increase by a factor of x4 or more unless reactivation is occurring.

It is important to understand that the use of magnesium and incubation for 1 hour at 34° C is based on work done in the 1950's and although useful, it has yet to be re-examined using today's understanding of enzyme chemistry, protein structure, and unfolding properties.

Reactivation of ALP in High Fat Products

An additional question arises as to why high fat products are more prone to contain reactivated ALP compared to low fat products. For example cream, butter and cheese are more likely to contain reactivated ALP compared to skim or low fat products. Also, products that have undergone ultra-high temperature short time (HTST) pasteurization, frequently display reactivation. Reasons for this are complex and not well understood, but one can speculate however as to the reasons.

It has been shown by numerous researchers that ALP in all milks is found free in solution and tightly bound to the milk fat globule's outer membrane. It is assumed that ALP when tightly bound to this outer membrane, is slightly more protected from complete unfolding compared to ALP free in solution. Although both free and bound ALP is inactivated by heat, complete irreversible unfolding may only occur with free ALP in solution. It is therefore the ALP bound to fat that may be protected from complete unfolding and is most easily refolded with 30°C or higher temperature storage or the use of magnesium in the laboratory.

Use of Antibodies to Measure ALP

Alternative approaches to the measurement of reactivated ALP have been investigated for many years, but none have proven useful. Lately, antibodies have been proposed for this purpose. However, the problems associated with the use of antibodies are daunting but technically possible. The first task, which has yet to be accomplished, is to make an antibody that binds ("recognizes") only the native and not unfolded, heated ALP molecule. For example, any fluid dairy product containing 25 mU/L of ALP, has without question up to 300,000 mU/L or more of unfolded denatured ALP. The antibody must not cross react with any of these dominate forms of the molecule or false positives will occur. In addition, re-folded ALP, namely reactivated ALP will also be measured by the antibody. As discussed above, reactivation is defined by magnesium treatment and it is not clear how an antibody could be used to circumvent this approach.

Microbial ALP

The presence of microbial ALP further complicates the story. ALP derived from bacteria is similar to bovine milk ALP in its structure. It is made up of a dimer composed of two equal length polypeptide chains connected like the steps of a ladder and folded onto itself. Unlike bovine milk ALP however, its total size is smaller with an average molecular weight of about 90,000 compared to the 180,000 for milk ALP. All current methods for measuring ALP will also measure microbial ALP and cannot distinguish between the two forms of ALP. Microbial ALP however appears to exist in two different "types." Type I will unfold and inactivate when exposed to pasteurization temperatures and Type II will not. Type II appears to be resistant to pasteurization unfolding or inactivation. Neither Type I or II has been shown to undergo reactivation with magnesium or storage temperature. Interpretation of milk samples with either Type I or Type II microbial ALP will look like this:

ALP Present	Post-Pasteurization Initial Results	Re-Test after Lab Pasteurization	Results

Bovine Milk	25 mU/L	25 mU/L	Pass
Bovine Milk	700 mU/L	25 mU/L	Fail, raw milk ingress or improper pasteurization
Bovine Milk Type I Micro	25 mU/L	25 mU/L	Pass, original pasteurization inactivated both milk and Microbial ALP.
Bovine Milk Type II Micro	700 mU/L	700 mU/L	Pass, Microbial Type II present
Bovine Milk Type II Micro	700 mU/L	300 mU/L	Pass, but some amount of Type II may be present.

It can readily be seen from the last example that mixtures of raw milk and Type II microbial ALP in the post-production product are very difficult to interpret.

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