

**ALKALINE PHOSPHATASE: SIGNIFICANCE IN DAIRY INDUSTRY**Darshana Prajapati<sup>a</sup>, Kalpna Desai, S.N. Patil, S.V. Chaudhari.

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**Keywords:** Alkaline phosphatase, dephosphorylation**Abstract**

Alkaline phosphatases (ALP, EC 3.1.3.1) is widely present in nature, and are found in many organisms. Alkaline phosphatase is an enzyme which catalyzes the hydrolysis of phosphate monoesters. ALP is located on the luminal surface of the epithelial cells of the ducts, it is released into milk as part of the plasma membrane during the formation of milk fat globules.

**Introduction**

Alkaline phosphatases (ALP, EC 3.1.3.1) is widely present in nature, and are found in many organisms. Alkaline phosphatase is an enzyme which catalyzes the hydrolysis of phosphate monoesters. It is membrane-bound and widely found in liver - 55%; in bone - 45%; in osteoblasts, gut - 5%.

Alkaline phosphatase is 1 of over 60 endogenous enzymes in raw bovine milk. It is classified as hydrolase type enzyme, since it is involved in removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The process of removing the phosphate group is called *dephosphorylation*. As the name suggests, alkaline phosphatases are most effective in an alkaline environment. It is sometimes used synonymously as *basic phosphatase*.

ALP is located on the luminal surface of the epithelial cells of the ducts, it is released into milk as part of the plasma membrane during the formation of milk fat globules. ALP is associated with the fat globule membrane (MFGM) in raw milk (Kosikowski, 1988). Alkaline phosphatase is concentrated in the fat globule membrane. Bovine milk ALP is firmly bound to insoluble particles of a fat lipoprotein and found in the lipid-water interface, milk phosphatase obtained in true solution. ALP is a membrane bound glycoprotein with sialic acid as sugar moiety.

Colostrum milk contains high levels of enzymes and intermediate milk may be due to sudden activation of the milk secretory mechanism. The enzyme has been characterized in human milk. Structural, functional, antigenic, and structural analysis indicates that the milk enzyme is the same protein species as that of adult human liver. Milk enzyme is a mixture of isozymes similar to bone and liver alkaline phosphatase.

**Characteristics of ALP**

Purified bovine ALP have a molecular mass of 187 kDa and isoelectric point ranging from pH 5.4 to 6.0. It has maximum activity in the pH range 9.65 to 10.1 at 37 °C. Milk alkaline phosphatase requires two metals for maximal activity: zinc is essential and magnesium is stimulatory needed. Alkaline phosphatase contains 4.0 g-atoms of zinc per mole of protein.

Mg<sup>2+</sup> ions cannot replace Zn<sup>2+</sup> at the zinc sites to give an active ALP, if Zn<sup>2+</sup> fixed on the magnesium site they becomes an inhibitor. Mg<sup>2+</sup>, Mn<sup>2+</sup>, and Ca<sup>2+</sup> ions enhances ALP activity in milk. Inorganic phosphate is a substrate, a product and an inhibitor of ALP activity. In milk pasteurized at high temperatures for short periods of time (161°F/15s), ALP is inactivated, but it shows reactivation properties during storage.

**Prospects in dairy industry**

Alkaline phosphatase (EC 3.1.3.1) occurs in the milks of all mammals and shows variations in level of enzymes. Technological importance of this enzyme to dairy industry as the test for its presence in pasteurized milk is



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universally used as an index of the efficiency of HTST pasteurization. This is because alkaline phosphatase is always present in raw milk at easily measurable levels and its heat stability profile closely follows that required for adequate pasteurization. ALP hydrolyze most phosphate ester bonds, releasing inorganic phosphate including adenosine monophosphate, glycerophosphate, phosphates of glucose, serine, threonine, and phosphoproteins. ALP also shows reactivation properties during storage, generally bulk HTST milk does not show reactivation of phosphatase activity, however it is often observed in UHT treated milk. This problem is usually overcome by HTST pasteurization after UHT treatment. Overall alkaline phosphatase activity does not have a direct significance in milk or milk products.

Milk contains several phosphatases, the principal ones being alkaline and acid phosphomonoesterases, which are of technological significance, and ribonuclease, which has no known function or significance in milk. The enzyme is readily assayed, and a test procedure based on alkaline phosphatase inactivation was developed as a routine quality control test for the HTST pasteurization of milk.

### Principle



p-nitrophenyl phosphate is hydrolyzed to p-nitrophenol and inorganic phosphate. The rate at which the p-NPP is hydrolyzed, measured at 405 nm, is directly proportional to the alkaline phosphatase activity.

### Alkaline phosphatase activity of normal whole milk

There is a considerable variation in the activity of normal milk. 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.

### Alkaline phosphatase activity of fractions obtained with rennin and pepsin

Treatment conditions			Specific activity (units/mg. N)
pH	Precipitation with	Fraction	
6.5	Rennin	Milk	1.32
		Curd	0.3
		Whey	2.6
6.0	Pepsin	Milk	3.90
		Curd	0.2
		Whey	7.8

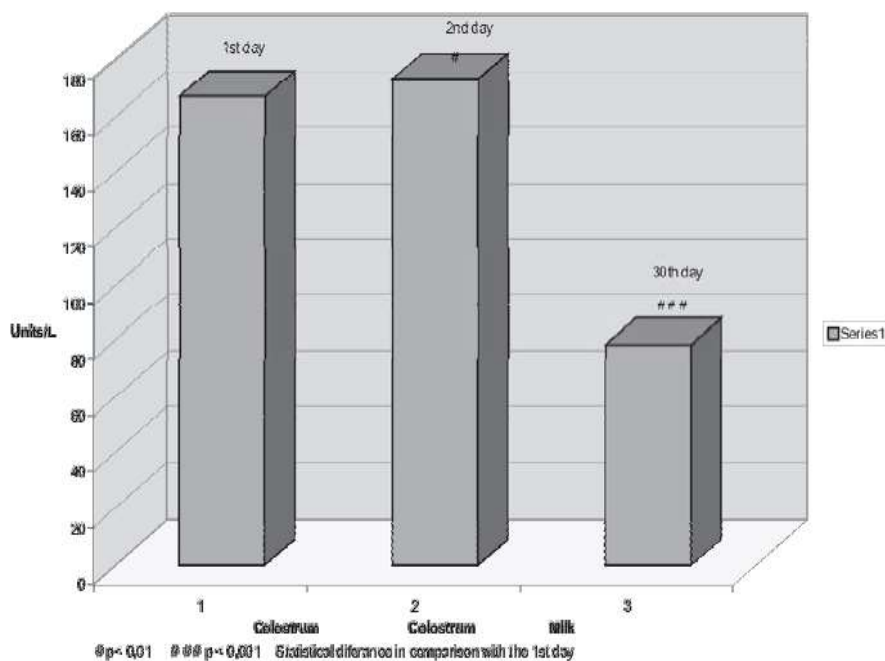
The low specific activity of normal cow's milk (2.5 units/mg. N) indicates that it compares very unfavourably as a source of alkaline phosphatase with animal tissues.

The analysis for alkaline phosphatase (ALP) and inorganic phosphate (P) in human colostrums and milk are presented in Graphs.



Characteristics	Alkaline phosphatase	Acid phosphatase
pH optimum	9-10.5	4
Temperature optimum	37 ° C	65 ° C
Quaternary structure	2 subunits each of 85 KDa	2 subunits each of 55 KDa
Avg. activity of milk	160 IU	72 IU
For colostrum milk	30 IU	14 IU
Inactivating heat treatment	62 ° C/ 30 min	88 ° C / 30 min
Units / ml	0.18-0.27	0.0026-0.0037

Fig.1 Alkaline phosphatase activities in human colostrum and milk



**Significance of ALP**

Alkaline phosphatase used as an index of HTST pasteurization in milk. However, the enzyme may not be the most appropriate for this purpose becomes (a) reactivation of alkaline phosphatase under certain conditions complicates interpretation of the test; (b) the enzyme appears to be fully inactivated by sub pasteurization conditions (70 °C for 16 sec); and (c) the relationship between log10% initially activity and pasteurization equivalent (PE) is less linear than the relationship of lactoperoxidase or β-glutamyl transpeptidase.

Although alkaline phosphatase can dephosphorylate casein under suitable conditions, it has no direct technological significance in milk. Perhaps its pH optimum is too far removed from that of milk, especially acid milk products, although the pH optimum on casein is reported to be 7. It is also inhibited by inorganic phosphate. Proteolysis is a major contributor to the development of flavor and texture of cheese during ripening.



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Most of the small water-soluble peptides in cheese are from the N-terminal half of  $\alpha$ s1- or  $\beta$ -casein; many are phosphorylated but show evidence of phosphatase activity (i.e., they are partially dephosphorylated). In cheese made from pasteurized milk, both indigenous acid phosphatase and bacterial phosphatase are probably responsible for dephosphorylation (which is the more important is not clear), but in raw milk cheese, e.g., Parmigiano Reggiano or Grana Padano, milk alkaline phosphatase appears to be the most important.

### Reactivation of Phosphatase

Phosphatase reactivation was first described by Wright and Tramer in 1953, who observed that UHT-treated milk was phosphatase-negative immediately after processing but became positive on standing; microbial phosphatase was shown not to be responsible. Two conditions have been shown cause unfolded ALP to reactivate: First is storage temperature, generally 30°C or above, Secondly, addition of high concentrations of magnesium will reactivate unfolded ALP. Form of the enzyme which becomes reactivated is membrane bound, factors which influence reactivation are: 1. Availability of free  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions, 2. Heating and storage conditions, 3. Availability of phosphates, 4. pH conditions (Fox et al., 1981).

Bulk HTST milk never show reactivation, although occasional individual cow samples does. HTST pasteurization after UHT treatment usually prevents reactivation which is never observed in container sterilized milk. Reactivation can occur following heating at a temperature as low as 84 °C for milk or 74 °C for cream. The optimum storage temperature for reactivation is 30 °C, at which reactivation is detectable after 6 h and may continue for up to 7 days. The greater reactivation in cream than in milk may be due to protection by fat, but this has not been substantiated. A number of attempts have been made to explain the mechanism of reactivation (Wright and Tramer, 1953).

The role of -SH groups, supplied by denatured whey proteins, is considered to be chelation of heavy metals, which would otherwise bind to -SH groups of the enzyme (also activated on denaturation), thus preventing renaturation. It has been proposed that  $\text{Mg}^{2+}$  and  $\text{Zn}^{2+}$  cause a conformational change in the denatured enzyme, necessary for renaturation. Maximum reactivation occurs in products heated at 104 °C; incubated at 34 °C, adjusted to pH 6.5, and containing 0.064MM $\text{Mg}^{2+}$ ; homogenization of products before heat treatment reduces the extent of reactivation.

Enzymes present in milk in free form and as membrane bound. Free form of enzymes present in skim milk part and which is inactivated by heat treatment but membrane bound denatured enzymes have tendency to reform when suitable temperature is provided. There is evidence that the form of the enzyme which becomes reactivated is membrane bound, and several factors which influence reactivation have been established.  $\text{Mg}^{2+}$  and  $\text{Zn}^{2+}$  strongly promote reactivation;  $\text{Sn}^{2+}$ ,  $\text{Cu}^{2+}$  and EDTA are inhibitory, while  $\text{Fe}^{2+}$  has no effect. Sulfhydryl (SH) groups appear to be essential for reactivation; perhaps this is why phosphatase becomes reactivated in UHT milk but not in HTST milk.

Reactivation of alkaline phosphatase is of considerable practical significance since regulatory tests for pasteurization assume the absence of phosphatase activity. Analytical steps for differentiate between residual and reactivated ALP are based on the premise that ALP will reactivate (4 – 10 fold) in the presence of magnesium salts (Richardson et al., 1964) .so for assay of original ALP and residual ALP is shown in assay part.

### 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. 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.



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### ELISA and immunohistochemistry applications

Thermo Scientific Pierce Alkaline Phosphatase is purified calf-intestinal alkaline phosphatase (AP or alk-phos) enzyme for use in activity assays and conjugation to antibodies for ELISA and immunohistochemistry applications. This purified calf intestinal alkaline phosphatase (CIP) is supplied in Tris buffer and 50% glycerol for use in protein research methods. The main applications for alkaline phosphatase in molecular biology and protein research are to remove 5'-phosphate groups from DNA or as a reporter system for immunoassays such as ELISA.

### Conclusion

Alkaline phosphatase have been studied widely because it has significant effect on milk pasteurization and it is importantly related to the dairy industry. Milk and milk products are related to public health issues and ALP verified pasteurized index shows there is no pathogen. The enzyme can readily be assayed, and a test procedure based on alkaline phosphatase inactivation was developed as a routine quality control test for the HTST pasteurization of milk. Also, alkaline phosphatase enzyme is also important in the human body and thus is finding applications in various bio-chemical and disease detection tests

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