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## Thermal and Carbon Dioxide Inactivation of Alkaline Phosphatase in Buffer and Milk

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### Summary

The effects of temperature and CO<sub>2</sub> treatment on the inactivation of alkaline phosphatase (ALP) were studied. The thermal stability of ALP was found to be significantly ( $P < 0.05$ ) different in glycine/NaOH buffer, pasteurized milk and raw milk. ALP was completely inactivated in the buffer at 60, 70 and 80 °C but approximately 12 % of activity was present at 50 °C after 55 min of treatment. The time required for complete inactivation of the enzyme in the buffer was reduced from 50 to 4 min as temperature increased from 60 to 80 °C. Complete inactivation of the enzyme in pasteurized milk was achieved at 70 and 80 °C but 28 and 15 % of ALP activity was still present at 50 and 60 °C after 120 min of treatment. Inactivation time for raw milk was reduced nearly 18-fold by increasing temperature from 50 to 70 °C. ALP in the buffer exposed to CO<sub>2</sub> (under atmospheric pressure) treatment at different temperatures showed a decrease in enzyme activity. Inactivation was found to be higher as the temperature increased from 20 to 50 °C. At the end of a 30-min treatment, residual ALP activity was found to be 84 and 19 % at 20 and 50 °C, respectively. Faster drop in pH and enzyme activity occurred within 5 min. The change in pH and enzyme activity dependant on CO<sub>2</sub> treatment was not observed in raw milk mainly due to strong buffering capacity of milk.

*Key words:* alkaline phosphatase, inactivation, milk, thermal stability

### Introduction

Alkaline phosphatase (ALP, orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) is an enzyme naturally present in blood and milk of all mammals. As ALP is slightly more heat resistant than most pathogenic bacteria, the measurement of its destruction confirms proper pasteurization (1). ALP activity in pasteurized milk generally indicates inadequate pasteurization, and the presence of ALP activity may be due to the contamination of pasteurized milk with raw milk or post-process bacterial contamination (2).

In general raw milk is heat treated to increase the shelf life and guarantee its microbiological safety by re-

ducing pathogenic and spoilage microorganisms besides native enzymes in milk. Alkaline phosphatase,  $\gamma$ -glutamyl transferase, lactoperoxidase and leucine arylamidase are native enzymes in milk. These enzymes have thermal resistance greater than that of the most heat resistant nonspore-forming pathogens commonly found in milk (3). Pasteurization is a relatively mild heat treatment resulting in products with a limited shelf life at refrigerated conditions (4,5). On the other hand, sterilization and ultra-heat treatment (UHT) are normally used for products designed to be stable at ambient temperature (6,7). The efficiency of pasteurization of dairy products

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is commonly assessed by determining the residual activity of ALP, either by a colourimetric (8–10) or fluorometric (11,12) assay. It has been reported that no reactivation of the ALP was observed throughout these determinations (1). Indeed, under normal pasteurization conditions (72 °C/15 s or longer time at lower temperature), ALP is slightly more thermostable than *Mycobacterium tuberculosis*, the most heat resistant non-sporogenic pathogenic microorganism in raw milk, and hence the absence of ALP activity is a sign of adequate pasteurization (13).

Consumer demand for fresh-like food products with minimal degradation of nutritional and organoleptic properties has stimulated new treatments or combined processes in food industry (14). Recently, an attempt has been made to apply high hydrostatic pressure for preservation purposes and as a method for changing the physical and functional properties of food systems (15, 16). Effects of high pressure treatments on enzymes may be related to reversible or irreversible changes in the protein structure (17,18). However, the loss of catalytic activity can differ depending on the type of enzyme, nature of substrates, temperature and length of processing. Owaga *et al.* (19) reported that neither pectin-esterase nor peroxidase activity from mandarin juice were completely inactivated after pressurization from 100 to 400 MPa. Similar behaviour was observed for polyphenol oxidase activity, in which case the degree of enzyme inactivation varied depending on the type of fruit and vegetable products studied.

Alternatively, an atmosphere modified with carbon dioxide (CO<sub>2</sub>) has been used to influence many enzyme activities such as the inactivation of polyphenol oxidase in order to prevent discolouration in fruits and vegetables as well as  $\alpha$ -amylase, glucose oxidase, lipase and catalase, which notably affect food quality (20). Although pressure inactivation of these enzymes with high barostability required treatments for more than 30 min with 900 MPa at 45 °C (21), CO<sub>2</sub> treatment under atmospheric pressure successfully inactivated these enzymes (22).

This study was undertaken to investigate the thermal stability of ALP in buffer, pasteurized milk and raw milk. The application of CO<sub>2</sub> under atmospheric pressure was also applied to ALP in buffer and raw milk in order to compare it with thermal treatment.

## Materials and Methods

### Materials

Alkaline phosphatase (EC 3.1.3.1) from bovine intestinal mucosa (Type 1-S, 15 units/mg solid) and *p*-nitrophenyl phosphate dicyclohexyl ammonium salt were obtained from Sigma Chemical Co. (St. Louis, MO). One unit of this enzyme hydrolyses 1.0  $\mu$ mol of *p*-nitrophenyl phosphate per min at pH=9.8 and 37 °C. According to the manufacturer the pH optimum is 9.8. All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany).

### Enzyme dilution buffer

Enzyme dilution buffer was prepared by mixing 0.5 mL of 1 M MgCl<sub>2</sub> and 0.5 mL of 0.1 M ZnCl<sub>2</sub> with 500

mL of glycine/NaOH buffer (25 mM), according to Mori *et al.* (23). The volume of 50 mL of glycerol was dissolved and the pH was adjusted to 9.6.

### Working substrate solution

The solution was prepared by mixing 120 mL of 3.9 mM *p*-nitrophenyl phosphate solution (prepared in 25 mM glycine/NaOH buffer) with 1.25 mL of 1.0 M MgCl<sub>2</sub> and diluted with distilled water to 145 mL and pH was adjusted to 9.6.

### Milk and enzyme solution

Fresh whole raw milk was obtained from a local dairy farm and it was divided into 100-mL portions. One portion was pasteurized at 72 °C for 2 min and they were all stored in refrigerator (4 °C) throughout the study without any chemical preservatives. ALP enzyme solution (0.15 mg/mL) was prepared in enzyme dilution buffer. The same amount of the enzyme was added into pasteurized milk to obtain the same concentration as in milk. No ALP activity was detected at the end of storage period. In a preliminary test a standard curve was drawn revealing up to which enzyme concentration there was a linear relationship with the measured activity.

### Activity assay for alkaline phosphatase

The activity of ALP was determined spectrophotometrically (Beckman model 24 double-beam with a Beckman recorder model 24–25 AC) at 405 nm using *p*-nitrophenyl phosphate as a substrate (24). The temperature of the cell compartment was maintained at 37 °C with Haake circulator KT 33. Working substrate solution (2.9 mL) was placed into a spectrophotometer cuvette (light path: 1 cm). Then 0.05 mL of enzyme dilution buffer and 0.05 mL of the sample (raw milk, ALP in buffer or in pasteurized milk) were added. Enzyme activities were measured in duplicate. Instead of the sample, the blank solution contained the same amount of enzyme dilution buffer. The reactivation of ALP was also controlled in the samples throughout the experimental run but it was not detected. The change in absorbance was measured and  $\Delta A/\text{min}$  was calculated based on the linear range of the curve. One unit was defined as the activity which hydrolyses 1  $\mu$ mol of *p*-nitrophenyl phosphate per min at 37 °C and pH=9.6. One unit of ALP activity per mL was calculated using the following formula:

$$\text{Units/mL} = (\Delta A_{405}/\text{min}) (V_R) / (\epsilon_{405}) (V_E) \quad /1/$$

where:  $\Delta A_{405}$  = change in absorbance at 405 nm,  $V_R$  = reaction volume (3.0 mL),  $(\epsilon_{405}) = 18.2 \text{ L cm}^{-1} \text{ mmol}^{-1}$ , the millimolar absorption coefficient of *p*-nitrophenol at 405 nm,  $V_E$  = sample volume (0.05 mL). The residual activity of the enzymes was calculated using the following formula:

$$\text{Residual activity (\%)} = \frac{\text{Activity (U/mL) of the treated enzyme solution}}{\text{Activity (U/mL) of the untreated enzyme solution}} \times 100 \quad /2/$$

### Thermal inactivation of ALP

Glass tubes containing 5 mL of sample were immersed in a thermostatic water bath with sensitivity of  $\pm 1$  °C (Gerhardt Type SV 2–4, Germany) set to constant temperatures between 50 and 80 °C. At different pre-set time intervals, 0.1 mL of sample was transferred into a clean test tube of 1.0 mm thickness and kept in ice bath (1 min) for rapid cooling, hereby stopping thermal inactivation. Depending on the temperature used, thermal treatment was applied for 1 to 120 min. The ALP activity was determined for the treated samples by the formula given above. In a preliminary experiment, it was verified that no reactivation of the enzyme occurred during this time period.

### CO<sub>2</sub> treatment

A sample of 15 mL (ALP enzyme solution prepared in glycine buffer with pH=9.6 or raw milk) was loaded in 50-mL polymethylpentene tubes and incubated at 20, 30, 40 and 50 °C in a water bath. Following the equilibration of the sample to working temperature, CO<sub>2</sub> (with a minimum purity of 99.99 %) was bubbled through the solution at a flow rate of 200 mL/min for 30 min at atmospheric pressure. An aliquot (200  $\mu$ L) of the enzyme solution was removed at zero time, 30 s, and 5, 10, 20 and 30 min. The samples of 200  $\mu$ L were put in 1.5-mL microcentrifuge tubes and immediately cooled by immersing the tube in an ice bath. Following equilibration to ambient temperature, the remaining activity was determined. The pH changes in the samples during the CO<sub>2</sub> treatment were monitored using a digital pH meter (Jenway model 3010, UK) equipped with a microelectrode. All the results are the average values of at least duplicate measurements.

### Statistical analysis

Statistical analysis (ANOVA) was carried out by using a computer program, Statgraphics (STSC, Rockville, MD). Anova test was performed for all experimental runs to determine the significance at the 95 % confidence interval.

## Results and Discussion

The thermal stability of the ALP enzyme was examined by measuring the residual activity after heat treatment for various periods. Fig. 1 represents the reduction in the activity of ALP in glycine buffer treated at different temperatures. There are significant ( $P < 0.05$ ) differences in the activities of ALP depending on the temperature applied. ALP was completely inactivated at 60, 70 and 80 °C as a result of thermal treatment for 50, 20 and 4 min, respectively. Complete inactivation was not observed at 50 °C and approximately 12 % of activity was present even after 55 min.

The time required for inactivation of ALP in pasteurized milk was significantly ( $P < 0.05$ ) longer than in buffer although the same concentration of enzyme was applied in both cases (Fig. 2). Complete inactivation of ALP in pasteurized milk was achieved in treatments at 70 and 80 °C for 40 and 10 min, respectively. At 50 and 60 °C, residual ALP activity after 120 min was 28 and

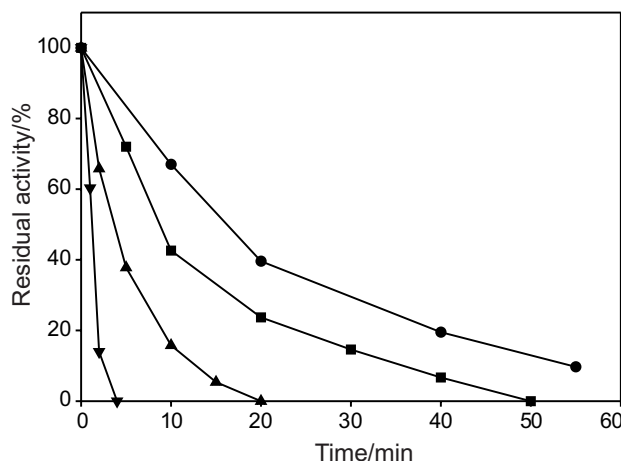


Fig. 1. Effect of temperature on alkaline phosphatase activity in buffer. (●) 50 °C; (■) 60 °C; (▲) 70 °C; (▼) 80 °C.

Reaction conditions: The enzyme solutions (0.15 mg/mL in buffer) were heat treated at indicated temperatures for various periods (0–55 min) in 25 mM glycine/NaOH buffer, pH=9.6, 1 M MgCl<sub>2</sub>, 0.1 M ZnCl<sub>2</sub> and glycerol. The residual enzyme activities were measured at 37 °C in accordance with the method described in the Materials and Methods section

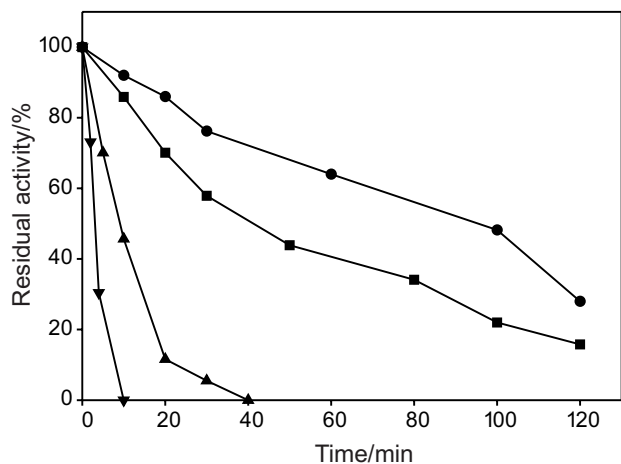
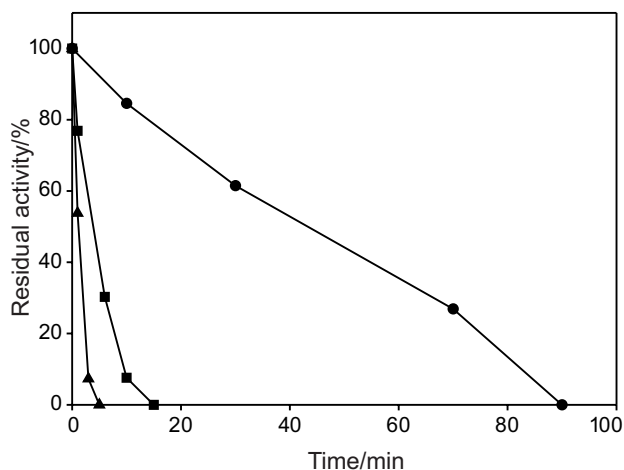


Fig. 2. Effect of temperature on alkaline phosphatase activity in pasteurized milk. (●) 50 °C; (■) 60 °C; (▲) 70 °C; (▼) 80 °C.

Reaction conditions: The enzyme solutions (0.15 mg/mL in pasteurized milk) were heat treated at indicated temperatures for various periods (0–120 min). The residual enzyme activities were measured at 37 °C in accordance with the method described in the Materials and Methods section

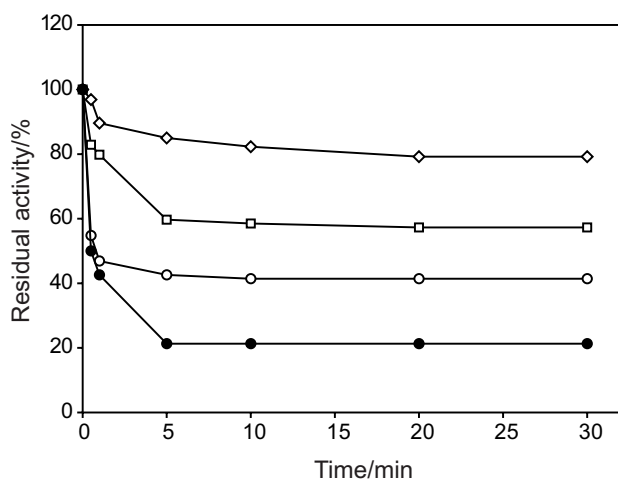
15 %, respectively. Milk had a protective effect on the activity of ALP and the composition of pasteurized milk prevented the complete inactivation of ALP, which was completely inactivated under the same conditions in buffer.

Decrease in ALP activity in raw milk depending on thermal treatment at three different temperatures was shown in Fig. 3. ALP concentration of 0.15 mg/mL was selected from a standard curve of concentration *vs.* activity. This concentration was higher than that of ALP naturally present in milk (0.025 mg/mL). Since our main aim was to compare ALP stability in buffer and pasteur-



**Fig. 3.** Effect of temperature on alkaline phosphatase activity in raw milk. (●) 50 °C; (■) 60 °C; (▲) 70 °C.

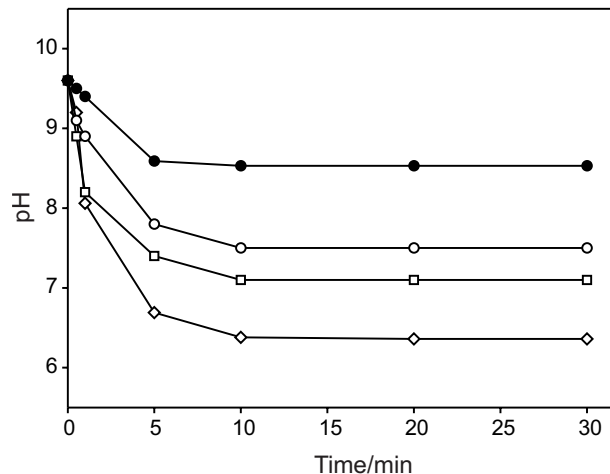
Reaction conditions: Raw milk containing 0.15 mg/mL of enzyme was heat treated at indicated temperatures for various periods (0–90 min). The residual enzyme activities were measured at 37 °C in accordance with the method described in the Materials and Methods section



**Fig. 4.** Effect of carbon dioxide on alkaline phosphatase activity in buffer. (◇) 20 °C; (□) 30 °C; (○) 40 °C; (●) 50 °C.

Reaction conditions: The enzyme solutions (0.15 mg/mL in buffer) were subjected to combined effects of CO<sub>2</sub> and heat treatment at indicated temperatures for various periods (0–30 min) in 25 mM glycine/NaOH buffer, pH=9.6, 1 M MgCl<sub>2</sub>, 0.1 M ZnCl<sub>2</sub> and glycerol. The residual enzyme activities were measured at 37 °C in accordance with the method described in the Materials and Methods section

ized milk, the concentration of ALP in these fluids was kept the same. There were significant differences ( $P < 0.05$ ) in ALP activity when the temperature was increased from 50 to 70 °C, while in the treatment in raw milk the complete inactivation was achieved at 50 °C for 90 min. However, the increase in temperature from 50 to 60 and 70 °C reduced the inactivation time nearly 6- and 18-fold, respectively. It is not surprising that the inactivation time is shorter in the case of raw milk than in buffer when inactivation profiles of ALP in buffer and raw milk were compared at the same temperatures. The



**Fig. 5.** Effect of carbon dioxide on the change of pH in buffer. (◇) 20 °C; (□) 30 °C; (○) 40 °C; (●) 50 °C. The same reaction conditions as in Fig. 4

main reason is that the enzyme residual activity in raw milk is 6-fold lower than that measured in buffer.

The combined effect of CO<sub>2</sub> and temperature on the ALP activity in buffer and in raw milk was studied. Fig. 4 shows the reduction in ALP activity in buffer by this treatment. As it can be seen, ALP solution exposed to CO<sub>2</sub> treatment lost its catalytic activity dramatically and significantly ( $P < 0.05$ ). At initial pH=9.6, the remained enzyme activity after CO<sub>2</sub> treatment for 30 min was found to be 84, 55, 39 and 19 % at 20, 30, 40 and 50 °C, respectively. A sharp decrease in enzyme activity occurred within 5 min and then continued to decrease slowly. Without CO<sub>2</sub> treatment, the same amount of inactivation of ALP in buffer was obtained after a heat treatment of 40 min at 50 °C. Carbon dioxide treatment may affect the conformational structure of the enzyme. Related studies suggest that the sorption of CO<sub>2</sub> might cause decomposition of secondary structure and result in the loss of the enzyme catalytic function (25).

ALP sample subjected to CO<sub>2</sub> treatment at different temperatures showed similar profiles in time related pH changes (Fig. 5). Change in pH was significantly ( $P < 0.05$ ) greater at low temperatures. From the same initial pH of 9.6, the pH of enzyme solution decreased for approximately 3.4 units at 20 °C but only for 1.0 unit at 50 °C. This was an expected result if high solubility of CO<sub>2</sub> at low temperatures is taken into consideration. Similar to the loss of activity, pH decreased sharply within 5 min and then continued to decrease slowly for up to 30 min. Changes in pH in a short time would be expected to affect the existence of salt bridges, which reinforce the tertiary structure of proteins (25). It has been reported that a pH drop of 0.7 units was not adequate for the inactivation of pectinesterase in orange juice by supercritical carbon dioxide (26). pH must decrease for 1.4 units for substantial pectinesterase inactivation (27). When the results of this study were compared with these findings, a pH drop greater than 1.0 unit seemed to be adequate for the inactivation of ALP in the applied system.

The combined influence of temperature and CO<sub>2</sub> treatment was also applied in raw milk, but no signifi-

cant change in pH was observed because of the strong buffering capacity of milk (data not shown). Milk is a buffer solution and contains a large number of substances which can act either as weak acids or as weak bases. As a result, the pH of milk is very stable. This buffering effect is a result of the electrical properties of substances such as proteins, phosphates, carbon dioxide and citrates (28). Since reduction in enzyme activity in CO<sub>2</sub> treatment mainly depends on the change in pH in a short time, no significant ( $P < 0.05$ ) change in ALP activity in raw milk was recorded.

Alkaline phosphatase in buffer could be inactivated by CO<sub>2</sub> treatment at atmospheric pressure. Carbon dioxide is accepted as an innocuous ingredient of foods and beverages, applied mainly for the preservation of foodstuffs (29). It may exert its influence on cells by affecting the rate at which particular reactions proceed *via* induction or repression of cytoplasmic enzyme synthesis. Any combination of CO<sub>2</sub>, nitrogen and oxygen may be used in modified atmosphere packaging to sustain visual appearance and/or to extend shelf life of foods (30).

CO<sub>2</sub> treatment under atmospheric pressure may be an alternative to pressure treatments (hydrostatic or CO<sub>2</sub>) in which extremely high pressures are required for the inactivation of enzymes and microorganisms. This method can be used as a potential non-thermal technique mainly applicable to liquid food systems with no buffering capacity.

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## Inaktivacija alkalne fosfataze toplinom i ugljičnim dioksidom u puferu i mlijeku

### Sažetak

Ispitivan je utjecaj temperature i CO<sub>2</sub> na inaktivaciju alkalne fosfataze (ALP). Utvrđeno je da je toplinska stabilnost ALP-a bitno različita ( $P < 0,05$ ) u puferu glicin/NaOH, pastereziranom i svježem mlijeku. ALP je bio potpuno inaktiviran u puferu pri 60, 70 i 80 °C a pri 50 °C nakon 55 minuta ostalo je približno 12 % aktivnosti. Vrijeme potrebno za potpunu inaktivaciju enzima u puferu skraćeno je od 50 na 4 min ako se temperatura povisila

od 60 na 80 °C. U pasteriziranom mlijeku potpuna inaktivacija enzima postignuta je pri 70 i 80 °C, dok je pri 50 i 60 °C, nakon 120 minuta, zaostalo još 28 odnosno 15 % ALP-aktivnosti. Vrijeme inaktivacije u svježem mlijeku skraćeno je približno 18 puta pri povećanju temperature od 50 na 70 °C. ALP u puferu, izložen utjecaju CO<sub>2</sub> (pod atmosferskim tlakom), pokazivao je pri različitim temperaturama smanjenu enzimsku aktivnost. Inaktivacija je bila veća kada se temperatura povećavala od 20 na 50 °C. Nakon 30-minutne obrade, pri 20 i 50 °C, zaostala ALP-aktivnost iznosila je 84 odnosno 19 %. Nakon 5 minuta opažen je brzi pad pH-vrijednosti i enzimske aktivnosti. Promjena pH i enzimske aktivnosti, ovisno o utjecaju CO<sub>2</sub>, nije opažena u svježem mlijeku uglavnom zbog toga što ono ima jaku sposobnost puferiranja.