Rapid methods for assessing efficiency of heat treatment of milk

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Revised: 5 January 2012 / Accepted: 24 January 2012 / Published online: 23 February 2012

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Abstract Two methods based on the activity of γ -glutamyl transpeptidase and lactoperoxidase are developed for assessing the efficiency of heat treatment of milk at 80 °C for 15 s. Heat treatment of milk at 80 °C (15 s) completely inactivates both milk enzymes. Under the selected assay conditions, enzyme activities are related with intensity of pink colour of product. In contrast to raw (unheated) milk which gives pink colour under the test conditions, heated milk (80 °C, 15 s) remains either white or gives pink colour with significantly reduced intensity. It is recommended to always use raw milk as positive control for the enzyme assay. Principles of colour formation in enzymatic reactions are discussed.

Keywords Milk · Heating · Pasteurization · γ -glutamyl transpeptidase · Lactoperoxidase

Introduction

Heat treatment is essential for hygienic safety and for extension of shelf-life of milk and milk products. Due to their varying thermal resistance, some milk enzymes can be used as indicators of the severity or effectiveness of heat treatment of milk and milk products (Andrews et al. 1987; Stepaniak 2004;

Yoshitomi 2004; Sharma et al. 2009; Balkishan et al. 2010). Absence of alkaline phosphatase (ALP) activity in pasteurized milk is an accepted indicator for evaluation of efficiency of pasteurization (72 °C, 16 s) during milk processing (IDF 1991; Rampling et al. 2004). Measurement of ALP activity requires incubation of milk or milk products with chromogenic (*p*-nitrophenyl phosphate), fluorogenic (benzothiazole orthophosphoric monoester) or other substrates such as disodium phenylphosphate (IDF 1999; ISO 2006). Apart from milk, ALP test has also been used as marker for ensuring proper heat treatment of milk products such as cream, ice-cream mix, milk powder, cheese etc. (IDF 1999; ISO 2006; Rampling et al. 2004; Rocco 2004; Yoshitomi 2004). Further, ALP has been reported to be reactivated during storage of pasteurized milk and cream (Linden 1986; Karmas and Kleyn 1990; Farkye and Imafidon 1995; Fox and Kelly 2006) and thus leading to false positive results. The other milk indigenous enzymes which have been identified as potential indicators of heat treatment of milk are γ -glutamyl transpeptidase (GGTP) and lactoperoxidase (LPO) (Anjos et al. 1998; Lombardi et al. 2000; Blel et al. 2002; Marin et al. 2003; Sharma et al. 2009). These two enzymes are inactivated at 80 °C in liquid milk and at 90 °C in cream and ice-cream mix (Sharma et al. 2009; Balkishan et al. 2010). Rapid and colour based methods are required for ascertaining heat treatment at temperature higher than 72 °C applied to milk. The present paper describes two such methods based on the activities of GGTP and LPO.

Material and methods

Pooled raw cow and buffalo milk samples were collected from Institute's cattle yard in pre-cleaned and sterilized amber-

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Table 1 Formation of pink colour in milk heated to various temperatures when assessed by γ -glutamyl transpeptidase (GGTP) and lactoperoxidase (LPO) tests ($n=5$)

Milk	Enzyme based test	Raw milk	Boiled milk ^a	Temperature			
				65 °C	70 °C	75 °C	80 °C
Buffalo	GGTP	+	–	+	+	+	–
	LPO	+	–	+	+	+	–
Cow	GGTP	+	–	+	+	+	–
	LPO	+	–	+	+	+	–

^a 10 ml milk in a test tube kept in boiling water-bath for 5 min

+ Indicates the appearance of distinct pink colour; – Indicates absence of pink colour and the solution remains white

coloured glass bottles. Milks from at least five different animals were pooled. These samples were brought to the laboratory and stored at 5 °C till analysis. L- γ -Glutamyl-*p*-nitroanilide, naphthylethylenediamine (NEDA), ammonium sulfamate, and aminoantipyrine were procured from Sigma-Aldrich USA. Glycylglycine, tris, acetic acid, sodium nitrite, phenol and hydrogen peroxide were procured from Merck (Germany).

Heat treatment of milk Glass tubes (5 ml, size 10×70 mm) containing 3 ml milk sample were placed in a precision water-bath maintained at 65, 70, 75 and 80 °C. The temperature of milk was continuously monitored. After 15 s of reaching the desired temperature, tubes were removed and placed in ice-cold water. Milk taken in separate tubes was kept in boiling water bath for 10 min and then cooled in an ice water bath.

Test for heat treatment based on GGTP activity The assay was done as described by McKellar et al. (1991) which is given below.

Preparation of substrate solution: Glycylglycine (1.3212 g) was dissolved in 100 ml 0.1 M Tris-HCl

(pH 9.0). Then, 131 mg L- γ -glutamyl- *p*-nitroanilide was added. Contents were warmed and stirred. The substrate was prepared fresh and was either colourless or light pale yellow.

Method: Fifty microliters of diluted milk (10 times with distilled water) was added to 0.5 ml substrate solution in a test tube. The tube was incubated at 37 °C for 10 min. To this 1.0 ml of 10% acetic acid (v/v) was added and mixed. Then, 1.5 ml each of 0.1% (w/v) sodium nitrite, 1.0% (w/v) ammonium sulfamate, and 0.052% (w/v) NEDA were added and contents mixed and incubated for 3 min at room temperature after each addition. Finally color of contents in the test tube was noted.

Test for heat treatment based on LPO activity The assay was done as described by Allain et al. (1974) and Torabi et al. (2007) which is given below.

Preparation of aminoantipyrine and phenol mix: Aminoantipyrine (0.0203 g) and phenol (0.056 g) were dissolved in 100 ml of 0.1 M Tris-HCl buffer (pH 7.7). The contents were mixed. The prepared aminoantipyrine and phenol mix solution is stored at 4 °C.

Fig. 1 γ -Glutamyl transpeptidase (GGTP) assay tubes. Colour indication due to GGTP activity in milks samples subjected to heat treatment. 1. Raw cow milk; 2. 70 °C/15 s; 3. 75 °C/15 s; 4. 80 °C/15 s

Fig. 2 Lactoperoxidase (LPO) assay tubes. Colour indication due to LPO activity in milks samples subjected to heat treatment. 1. Raw cow milk; 2. 70 °C/15 s; 3. 75 °C/15 s; 4. 80 °C/15 s

Fig. 3 Reaction chemistry of γ -glutamyl transpeptidase test

Method: Two hundred microliters of milk sample was added to 2 ml of aminoantipyrine and phenol mix solution. The contents were vortexed briefly followed by the addition of 20 μl of hydrogen peroxide solution (30%). The contents were mixed, incubated at 37 $^\circ\text{C}$ for 10 min and colour of the solution was noted.

Fig. 4 Reaction chemistry of lactoperoxidase test

Results and discussion

The developed rapid methods for assessing heat treatment of milk are based on the detection of activity of GGTP and LPO in milk samples. When activities of GGTP and LPO in raw milk (unheated) and heated milk samples (65 $^\circ\text{C}$, 70 $^\circ\text{C}$, 75 $^\circ\text{C}$ and 80 $^\circ\text{C}$ for 15 s) were measured qualitatively by observing formation of pink colour at the end of described method, it was observed that raw milk from cow or buffalo exhibited activities of these enzymes (Table 1). In milk (cow or buffalo) samples heated at 65 $^\circ\text{C}$, 70 $^\circ\text{C}$ and 75 $^\circ\text{C}$, there was substantial remaining activities of enzyme leading to pink colour formation (Table 1, Figs. 1 and 2). However, in the milk samples heated at 80 $^\circ\text{C}$ or boiled, both the enzymes were completely inactivated and pink colour products did not appear at the end of reaction (Table 1, Figs. 1

and 2). Therefore, absence of pink colour is an indication that both the enzymes (GGTP and LPO) are completely inactivated and milk samples have undergone heat treatment at $\geq 80^\circ\text{C}$.

The described methods for measurement of GGTP and LPO activities are simple. The developed pink colour in milk samples heated at 65°C or 70°C or 75°C can be easily compared with milk samples heated at 80°C (Figs. 1 and 2). The observed colour can be used for distinguishing milk samples subject to heating at 80°C or above with samples undergone lower heat treatments. The test results for GGTP and LPO are available in 19 and 10 min respectively. The developed methods for the detection of activity of GGTP and LPO are based on the following principles:

GGTP transfers glutamyl residues from L- γ -glutamyl-*p*-nitroanilide to glycylglycine and *p*-nitroaniline is formed. *p*-Nitroaniline is diazotized by reaction of sodium nitrite under acidic conditions. Ammonium sulfamate reacts with excess (unreacted) nitrite. NEDA then reacts with diazo compound and azo dye is formed (Fig. 3). The colour contrast of azo dye (pink) is far better in comparison to yellow colour of *p*-nitroaniline when colour developed in heated (less than 80°C) milk samples was compared with raw milk (Fig. 1). The pink colour formed in the reaction was observed visually.

For LPO test, the enzyme lactoperoxidase, acts on hydrogen peroxide which leads to the formation of a coupling compound between phenol and 4-aminoantipyrine (Allain et al. 1974) leading to formation of pink coloured quinoneimine dye (Fig. 4). The amount of pink dye is therefore related to LPO activity.

Pasteurization of raw milk is being applied to extend shelf-life of product and destroy pathogens (Cerf 1986; IDF 1991). Measurement of ALP activity is accepted indicator of proper pasteurization ($72^\circ\text{C}/16\text{ s}$) in dairy products for more than 65 years (Harding and Garry 2005). Recent reports indicate that, *Mycobacterium avium* ssp. *paratuberculosis*, an emerging pathogen in raw milk, is not always destroyed when milk is heated at 72°C for 16 s (Hammer et al. 2002; McDonald et al. 2005; Grant 2006; Brandt et al. 2011) and therefore, re-look for the heat treatment conditions (time-temperature combination) for ensuring killing of this pathogen. Accordingly search of other indicator enzymes as an index of proper pasteurization assumes considerable interest. The methods based on the inactivation of the activities of GGTP and LPO may be used as indicators of heat treatment of milk to 80°C , which has been reported to destroy *M. avium* ssp. *paratuberculosis* completely, and thus can ensure the safety of milk (Rademaker et al. 2007). Earlier studies (Sharma et al. 2009) have indicated no reactivation of these two enzymes and thus both the methods can be adapted by the dairy industry for monitoring the heating of milk around 80°C .

Conclusion

Rapid indicative methods measuring activities of the enzyme viz., GGTP and LPO can be used for ascertaining heat treatment (80°C , 15 s) subjected to milk during processing. The developed methods are simple and a distinction between milk samples heated at 80°C (15 s) can be easily made with those samples subjected to lower heat treatments (75°C or lower). The developed methods can be used in a quality control laboratory.

Acknowledgement This work was supported by APCESS Funds, Indian Council of Agricultural Research, India and authors sincerely acknowledge the financial support.

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