

## Standardization of method for lactoperoxidase assay in milk

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**Abstract** — The spectrophotometric method used to measure lactoperoxidase activity in milk was standardized. The assay system consisting of ABTS [2,2'-azinobis-(3-ethyl benzthiazoline-6-sulphonic acid)] as chromogenic substrate gave a linear initial rate of reaction up to 700  $\mu\text{g}\cdot\text{L}^{-1}$  lactoperoxidase concentration, with maximum activity at pH 6.0. The lactoperoxidase activity was higher in buffalo milk than cow milk and the corresponding concentration of the enzyme was 31 and 24  $\text{mg}\cdot\text{L}^{-1}$ , respectively. A loss of 10–15 % of peroxidase activity occurred on the preparation of rennet whey or acid whey. © Inra/Elsevier, Paris

**lactoperoxidase assay / cow milk / buffalo milk / rennet whey / acid whey**

**Résumé** — **Standardisation de la méthode de dosage de la lactopéroxydase dans le lait.** La méthode spectrophotométrique de dosage de l'activité de la lactopéroxydase dans le lait a été standardisée. La méthode, comprenant de l'ABTS [acide 2,2' azinobis - (3-ethyl benzthiazoline 6-sulfonique)] comme substrat chromogénique, donnait un taux initial de réaction linéaire jusqu'à une concentration en lactopéroxydase de 700  $\mu\text{g}\cdot\text{L}^{-1}$ , avec un maximum d'activité à pH 6,0. L'activité de la lactopéroxydase dans le lait de bufflesse était supérieure à celle du lait de vache et les concentrations de l'enzyme étaient respectivement de 31 et 24  $\text{mg}\cdot\text{L}^{-1}$ . Une perte d'activité de la peroxydase de 10–15 % intervenait lors de la préparation de lactosérum présure ou de lactosérum acide. © Inra/Elsevier, Paris

**lactopéroxydase / lait de vache / lait de bufflesse / lactosérum présure / lactosérum acide**

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## 1. INTRODUCTION

Lactoperoxidase (EC 1.11.1.7) is an enzyme found in mammalian milk, saliva and tears. It is present in milk of different species in varying concentrations [12]. Lactoperoxidase acts in a system together with thiocyanate and hydrogen peroxide by catalysing the peroxidation of thiocyanate to putative antimicrobial hypothiocyanite ion [1]. The antimicrobial property of lactoperoxidase gives it a potential application in the preservation of raw milk under ambient conditions [8]. Methods available for the estimation of peroxidase activity are based on different donors such as *p*-phenylenediamine [2], pyrogallol and guaiacol [4, 10], ABTS [2,2'-azinobis-(3-ethyl benzthiazoline-6-sulphonic acid)] [9, 10, 14] and are, therefore, difficult to compare. The complex chemistry of peroxidation reactions requires that experimental conditions be carefully designed and rigorously controlled in order to obtain linear initial rates that are directly proportional to peroxidase concentrations. In the kinetic studies of lactoperoxidase catalysed reactions, Bardsley [3] specified the concentration  $1 \text{ mmol}\cdot\text{L}^{-1}$  ABTS,  $0.1 \text{ mmol}\cdot\text{L}^{-1}$  hydrogen peroxide and pH 5.6 as reaction conditions in assay, giving highest initial rate and minimal suicidal inhibition over a period of 2 min. However, lactoperoxidase has been shown to be surface active [8]. At low concentration there is considerable reduction of enzyme activity in fluid phase because of adsorption of the enzyme on the container surface, which can be prevented by including gelatin in assay [10]. However, there is a practical difficulty in data acquisition because it requires a great deal of equipment. Therefore, a simple and convenient assay system was standardized using ABTS as chromogenic substrate by spectrophotometer. The distribution and level of lactoperoxidase in cow and buffalo milk was determined using this method.

## 2. MATERIALS AND METHODS

### 2.1. Reagents

#### 2.1.1. ABTS solution ( $1 \text{ mmol}\cdot\text{L}^{-1}$ )

Fifty-five mg of ABTS (Sigma) was dissolved in phosphate buffer ( $0.1 \text{ mol}\cdot\text{L}^{-1}$ , pH 6.0) and made up the volume to 100 mL.

#### 2.1.2. Hydrogen peroxide solution ( $0.1 \text{ mmol}\cdot\text{L}^{-1}$ )

From the commercial hydrogen peroxide solution 30 % v/v (Merck) a stock solution of  $100 \text{ mmol}\cdot\text{L}^{-1}$  was prepared by titrating against standard potassium permanganate solution. The stock solution was diluted to  $3.2 \text{ mmol}\cdot\text{L}^{-1}$  concentration immediately before use.

#### 2.1.3. Peroxidase enzyme

Lactoperoxidase from buffalo milk was purified to a purity index  $A_{412}/A_{280} = 0.94$  [6]. It was dissolved to concentrations varying from 10 to  $800 \mu\text{g}\cdot\text{L}^{-1}$  in phosphate buffer saline containing 0.1 % gelatin (PBSG) ( $0.1 \text{ mol}\cdot\text{L}^{-1}$ , pH 7.0).

### 2.2. Peroxidase assay

The ABTS solution (3.0 mL) and peroxidase sample (0.1 mL) were added together in a cuvette. The reaction was initiated by the addition of 0.1 mL of hydrogen peroxide solution and immediately the measurement of absorbance started at 412 nm as a function of time for 2 min at 10-s intervals using Spectronic 21 D spectrophotometer (Milton Roy). Measurements were carried out against the reagent blank containing ABTS and enzyme solution only.

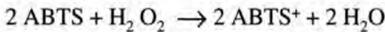
#### 2.2.1. Unit of activity

One unit of activity (U) is defined as the amount of enzyme that catalyses the oxidation of  $1 \mu\text{mol}$  of ABTS per min at  $20^\circ\text{C}$ , in  $0.1 \text{ mol}\cdot\text{L}^{-1}$  phosphate buffer pH 6.0, using a concentration of  $1 \text{ mmol}\cdot\text{L}^{-1}$  ABTS and  $0.1 \text{ mmol}\cdot\text{L}^{-1}$  hydrogen peroxide in the reaction mixture. Taking the molar extinction coefficient of oxidized ABTS at 412 nm as  $32\,400 \text{ mol}\cdot\text{L}^{-1}\cdot\text{cm}^{-1}$  [15], both at pH 6.0 and 4.4, the activity expressed in  $\text{U}\cdot\text{mL}^{-1}$  was calculated according to Putter and Becker [11].

Change in absorbance at 412 nm  $\times$  assay volume, V (mL)

Extinction coefficient ( $1 \text{ mmol}^{-1} \text{ cm}^{-1}$ )  $\times$  sample volume, v (mL)  $\times$  time, t (min)

The enzymatic reaction of lactoperoxidase with ABTS as substrate takes place as follows:



since the degradation of 1 mol of hydrogen peroxide yields 2 mol of oxidized ABTS. Therefore, the stoichiometric coefficient  $v_1 = 2$  was taken in the denominator of the above relation. Using the above volumes, it becomes

$$\frac{\Delta A_{412} \times 3.2 \times 1}{32.4 \times 0.1 \times 1 \times 2} = 0.4938 \times \Delta A_{412} / \text{min Units of lactoperoxidase/mL of sample}$$

### 2.3. Milk samples

Pooled milk samples were collected from Murrah buffaloes and crossbred cows maintained at the National Dairy Research Institute (Karnal, India). The samples were skimmed using Alfa Laval cream separator.

#### 2.3.1. Whey samples

The rennet whey was prepared from skim milk by adding Meito rennet at the rate of  $20 \text{ mg} \cdot \text{L}^{-1}$  and incubating at  $30^\circ \text{C}$  for 30 min. After setting, the curd was cut and the whey was filtered using a Whatman No. 1 filter paper. For acid whey, the skim milk was diluted 1:1 by distilled water and acidified to pH 4.6 using 2 N HCl. The clear whey was obtained by filtration through Whatman No. 1 filter paper.

#### 2.3.2. Peroxidase activity

Ten samples each from cow and buffalo whole milk, skim milk, rennet whey and acid whey were analyzed for peroxidase activity after dilution (1:250) with PBSG ( $0.1 \text{ mol} \cdot \text{L}^{-1}$ , pH 7.0) both at assay pH 6.0 and 4.4.

## 3. RESULTS AND DISCUSSION

### 3.1. Calibration curve for peroxidase assay

The reaction rate was a linear function of enzyme concentration from  $10\text{--}700 \text{ } \mu\text{g} \cdot \text{L}^{-1}$

(figure 1) with the corresponding constant reaction rate varying from 0.007 to 0.35 up to 2 min. Thus, it was inferred that for estimation of lactoperoxidase activity in milk or whey, the samples should be diluted using PBSG to bring the observed reaction rate in this range.

### 3.2. Effect of pH on assay

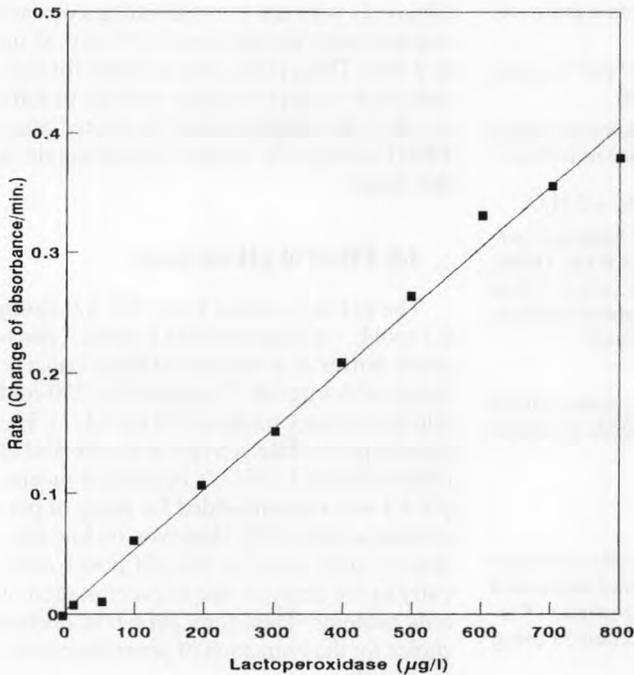
The pH was varied from 4.0–8.0 using  $0.1 \text{ mol} \cdot \text{L}^{-1}$  acetate buffer/ $0.1 \text{ mol} \cdot \text{L}^{-1}$  phosphate buffer at a lactoperoxidase concentration of  $0.4 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$  (prepared by 250-fold dilution of stock solution  $100 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ ). The highest peroxidase activity was observed at pH 6.0 (figure 1, table I). In cervical mucus, pH 4.4 was recommended for assay of peroxidase activity [15]. However, on low dilution of milk samples this pH poses difficulty in the analysis due to precipitation of milk proteins. Therefore, pH 6.0 is a better choice for the estimation of peroxidase activity in milk.

### 3.3. Effect of thiocyanate on assay

The average content of thiocyanate in milk samples analyzed for peroxidase activity was  $0.1 \text{ mmol} \cdot \text{L}^{-1}$ . It has been reported that  $0.1 \text{ mmol} \cdot \text{L}^{-1}$  thiocyanate in milk can result in 10 % reduction in the observed rate [9]. Since the milk samples were diluted 250-fold for peroxidase assay, the corresponding thiocyanate content was reduced to  $0.4 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$ . To determine the effect of thiocyanate at this level,  $0.1 \text{ mmol} \cdot \text{L}^{-1}$  thiocyanate was added to  $100 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$  lactoperoxidase solution. It was diluted to 250-fold using PBSG ( $0.1 \text{ mol} \cdot \text{L}^{-1}$ , pH 7.0) and analyzed for peroxidase activity. The reduction in the observed rate was below 0.05 %. Hence, there was only a negligible effect of thiocyanate on the assay system.

### 3.4. Distribution and level of lactoperoxidase in milk

On separation of milk the peroxidase activity was found to increase slightly both



**Figure 1.** Standard curve for lactoperoxidase assay.

**Figure 1.** Courbe standard de détermination de la lactopéroxydase.

**Table I.** Distribution of lactoperoxidase in milk.

**Tableau I.** Distribution de la lactopéroxydase dans le lait.

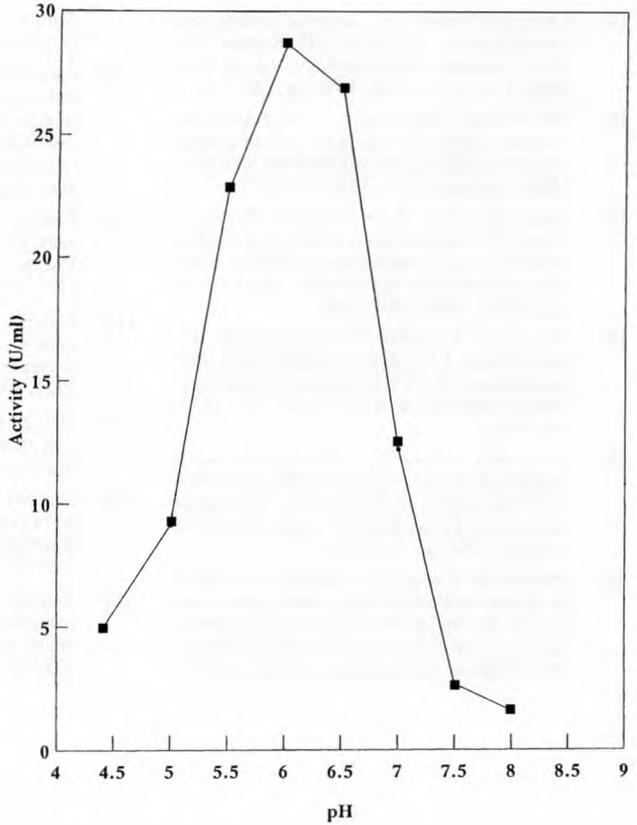
Species	Assay pH	Peroxidase activity (U·mL <sup>-1</sup> )*			
		Whole milk	Skim milk	Rennet whey	Acid whey
Buffalo	4.4	1.371 ± 0.262	1.495 ± 0.129	1.252 ± 0.152	1.182 ± 0.427
	6.0	7.315 ± 0.134	7.720 ± 0.109	6.913 ± 0.188	6.786 ± 0.114
Cow	4.4	1.263 ± 0.227	1.355 ± 0.160	1.114 ± 0.245	1.095 ± 0.163
	6.0	5.724 ± 0.274	5.973 ± 0.147	5.251 ± 0.196	5.135 ± 0.180

\* Mean of ten observations ± standard error.

\* moyenne et écart type de dix observations.

in cow and buffalo milk samples (table I). Furthermore, on preparation of acid whey and rennet whey, about 10–15 % loss of activity was observed. The increase in peroxidase activity on skimming might be due to an effective increase in concentration of lactoperoxidase corroborating that the

enzyme is mainly present in the serum phase [13]. The peroxidase activity was observed to be higher in buffalo milk than cow milk both at pH 6.0 and 4.4, which is in contrast to an earlier report [5] showing higher peroxidase activity in cow milk (1.2 U·mL<sup>-1</sup>) than buffalo milk (0.9 U·mL<sup>-1</sup>) at pH 4.4.



**Figure 2.** Effect of pH on lactoperoxidase assay.

**Figure 2.** Effet du pH sur l'activité de la lactopéroxydase.

In cow milk the peroxidase activity observed at pH 4.4 was similar [12].

Based on the reaction rate of lactoperoxidase in buffalo milk and cow milk, its concentration was determined to be 31 and 24 mg·L<sup>-1</sup>, respectively. However, the level of lactoperoxidase in cow milk has been reported to be slightly higher (30 mg·L<sup>-1</sup>) [7].

#### 4. CONCLUSION

The assay conditions employed in the method for peroxidase estimation gave a linearity of initial reaction rate of up to 2 min with a negligible effect of thiocyanate

and the peroxidase activity was around five-fold higher at pH 6.0 than at pH 4.4 both in cow and buffalo milk and whey samples.

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