Effects of Ionic Strength on Thermostability of Lactoperoxidase

Kaoru SATO, Shun’ichi DOSAKO, Ichiro NAKAJIMA, and Kazuo IDO

Technical Research Institute, Snow Brand Milk Products Co., Ltd., 1-1-2 Minamidai, Kawagoe, Saitama 350, Japan

Received March 10, 1992

Lactoperoxidase (Lpo) (EC 1.11.1.7) is a basic protein in milk with a molecular mass of approximately 75 kDa. One of its unique biological functions is the bactericidal effect against Gram-negative bacteria in the presence of hydrogen peroxide and thiocyanate or halogens (Lpo system). Many attempts have been made so far to use Lpo as an ingredient of foods for preventing milk and milk products from deteriorating. Although it has been reported that Lpo is stable upon heating, its activity was reduced with increasing temperature and time. Since pasteurization of most foods is by heat treatment, we have to know the residual Lpo activity under pasteurization conditions.

Haschke and Friedhoff have suggested the importance of calcium ion in the structural stability of peroxidase upon heating. Hernandez et al. also demonstrated that the Lpo activity, when heated, increased with increases in calcium concentration. However, effects of other salts, particularly the relationship between Lpo activity and ionic strength, have not been studied. Thus the major objective of this study is to examine the residual Lpo activity at varying ionic strengths and heating conditions.

Rabbit anti-bovine Lpo antibody was prepared by the conventional method, and then purified by ammonium sulfate precipitation and affinity chromatography with protein G-Sepharose FF (Pharmacia).

Anti-Lpo IgG diluted 1000-fold with 0.05 M sodium bicarbonate was adsorbed onto a 96-well microtitrator plate (Immulon 4, Dynatech) overnight at 4°C. The plate was filled with Block Ace (1/4 dilution with deionized water, Dainippon Pharmaceuticals) for 1 h at room temperature. Fifty μl of standard Lpo and samples diluted with Block Ace (1/10 dilution with deionized water) was added to each well, followed by incubation for 1 h at room temperature. After the plate was washed six times with phosphate-buffered saline (PBS) containing 0.02% Tween 20, 100 μl of 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Wako Pure Chemical Industries) solution (0.3 mg/ml ABTS/0.006% hydrogen peroxide/0.05 M citrate buffer, pH 4) was added to develop a green color. After this was left for 30 min at room temperature with gentle stirring, the absorbance at 405 nm was measured with an autoreader (Titertek Multiskan, Flow Lab.). The limit of detection was found to be 50 ng/ml. The Lpo concentration measured with this assay method was parallel to the Lpo activity measured with the guaiacol assay method (data not shown).

Bovine Lpo (Oleofina, Belgium) dissolved in deionized water (0.3 mg/ml) was mixed with an equal volume of test solutions. One ml in a 1.5-ml tube with a stopper was heated in a temperature-controlled water bath. After a given time, the tube was immediately cooled to room temperature in tap water. Specimens were left for 1 h and then stored at -20°C until used.

Since this assay method detects only Lpo with antigenicity, the method can follow the denaturation of Lpo. Figure 1 shows residual Lpo after heating in the absence or presence of 0.1 M sodium chloride. The concentration of Lpo with antigenicity decreased with increases in temperature. In the absence of the salt, the residual Lpo heated at 65°C and then cooled to room temperature was found to be 20%; it decreased to about 1.7% after heating at 70°C. On the other hand, the residual Lpo was much higher in the presence of 0.1 M sodium chloride. Approximately 90% of Lpo was undenatured after heating at 65°C; 39% was after heating at 70°C. Even after heating at temperatures higher than 75°C, 1—1.6% of Lpo remained in the presence of 0.1 M sodium chloride, but nearly complete denaturation occurred in deionized water.

Lpo was stable over the pH range between 4 and 9 but completely denatured at pH 2.6 (Fig. 2). The denaturation at pH 2 was partially reversible, depending on the incubation time. When pH was promptly

![Fig. 1](image1.png)  
**Temperature-dependent Decrease in Residual Lpo.** Lpo was heated at given temperatures for 10 min in the presence of 0.1 M NaCl (C) or in the absence of NaCl (○), and then cooled to room temperature. Residual Lpo was measured with the ratio of heated and unheated Lpo concentrations.

![Fig. 2](image2.png)  
**Fig. 2. Effects of pH and Heating on Stability of Lpo.** Lpo (0.2 mg/ml) was dissolved in citrate (pH 3—6) or Tris (pH 7—9) buffers containing 0.1 M NaCl. Lpo solutions were heated at 65°C for 30 min. ○, unheated; ●, heated.

![Fig. 3](image3.png)  
**Fig. 3. Effects of Ionic Strength on Stability of Lpo upon Heating.** Lpo dissolved in CaCl₂ or NaCl solutions was heated at 65°C or 70°C for 30 min, and then cooled to room temperature. ○, CaCl₂ 65°C; ●, NaCl 65°C; △, CaCl₂ 70°C; ▲, NaCl 70°C; ○, pH of the calcium solution was adjusted to 6.2 with HCl, and then the solution was heated to 65°C.
increased to 7.75% of Lpo retained its antigenicity; however Lpo incubated at pH 2 for 1 h was unable to renature (data not shown), probably due to almost complete destruction of its three-dimensional structure. Although Lpo was stable at pH higher than 4, heated Lpo was stable only at pH range between 5.6 and 7.7.

Lpo was much more stable when heated in the presence of 0.1 M sodium chloride. The thermostability of Lpo in monovalent ion solutions was also observed with sodium nitrite and potassium chloride solutions (data not shown). Hernandez et al. demonstrated the contribution of calcium ion to the thermostability. Figure 3 shows residual Lpo after heating at 65°C and 70°C in sodium or calcium chloride solutions in relation to ionic strength. The residual Lpo increased with increases in calcium concentration with a maximum at the ionic strength of 0.03, which was in concordance with Hernandez et al. At the ionic strength of 3 with calcium chloride Lpo completely lost its antigenicity. Since the pH of calcium chloride at the ionic strength of 3 (molar concentration: 1 M) was approximately 8.5 where Lpo lost the thermostability (Fig. 2), the pH of the 1 M calcium chloride solution was adjusted to 6.2 with dilute hydrochloric acid. Nevertheless the residual Lpo was only about 10%. Since the pH adjustment had little effect on the enhancement of the thermostability, the destabilization seemed not to be attributable to structural alteration due to higher pH. Maximum residual Lpo was also found at the ionic strength of 0.03 with calcium chloride when Lpo was heated at 70°C for 30 min and then cooled to room temperature. The same phenomenon was observed with magnesium (data not shown). Contrarily, the thermostability in the presence of sodium chloride increased in proportion to the salt concentration. At the ionic strength of 1.0 with sodium chloride, the residual Lpo was higher than that in calcium chloride; more than 20% remained at 70°C. We therefore speculate that the mechanism of thermostability enhancement with monovalent cations is different in its mechanism from that of calcium.

References