Occurrence of Superoxide Dismutase in Bovine Milk

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Bovine milk contains several oxidoreductases including peroxidase, catalase, aldehyde oxidase and xanthine oxidase. Among them xanthine and aldehyde oxidases produce superoxide anion radicals (O_2^-) during the oxidation of the respective substrates by molecular oxygen.^{1,2)} Superoxide is a reactive species of oxygen and, further, generates hydrogen peroxide and highly reactive hydroxyl radical ($HO\cdot$) and excited singlet molecular oxygen (1O_2) through the following reactions;

Therefore, the removal of O_2^- prevents a deteriorative effect of these activated oxygen and milk is expected to contain superoxide dismutase catalyzing the dismutation of O_2^- (reaction 1). In this paper an evidence is presented for the occurrence of Cu, Zn-superoxide dismutase in bovine skim milk at about 10^{-8} M.

Unpasteurized whole milk was obtained from a local ranch and separated into skim milk and cream by centrifugation at 6000 g for 10 min. Cream fraction was suspended in 10 mM potassium phosphate, pH 7.8, and the both fractions were dialyzed against 10 mM potassium phosphate, pH 7.8, for 24 hr. The results are presented on the basis of the volume of whole milk.

Reduction of cytochrome *c* in turbid milk was followed from an absorbance change at 550 nm using a reference at 540 nm employing a Hitachi 356 dual wavelength spectrophotometer. Reduction of nitroblue tetrazolium by O_2^- generated with the FMN-light system³⁾ was followed using a Shimadzu UV-200 spectrophotometer modified so that an increase of the absorbance at 604 nm due to formazane could be monitored continuously while the sample was being irradiated by blue-light from the side. Xanthine oxidase was obtained from Boehringer and horse heart cytochrome *c* (Type III) and beef liver catalase were from Sigma.

The addition of xanthine to a mixture of cream and cytochrome *c* induced the reduction of the cytochrome (Fig. 1). This reduction was inhibited by crystalline spinach Cu, Zn-⁴⁾ and also by *Chromatium* Fe-supero-

FIG. 1. Reduction of Cytochrome *c* by Xanthine and Cream and Its Inhibition by Superoxide Dismutase and Skim Milk.

The reaction mixture (1 ml) contained 50 mM potassium phosphate, pH 7.8, 10 μ M cytochrome *c*, 0.1 mM xanthine, 1600 units of catalase and cream corresponding to 75 μ l of whole milk. In addition, SOD: 0.4 μ M spinach superoxide dismutase was added. Where indicated, 1 mM NaCN was added. Volume indicates those of skim milk. Skim milk did not contain any xanthine oxidase activity.

FIG. 2. Effect of Cream and Skim Milk on the Reduction of Nitroblue Tetrazolium Mediated by O_2^- Produced by Illuminated Flavin.

The reaction mixture contained 50 mM tricine-KOH, pH 8.5, 25 μ M FMN, 25 μ M nitroblue tetrazolium in a total volume of 2 ml. Where indicated, the reaction mixture contained skim milk or cream corresponding to 125 μ l of whole milk and 2 mM cyanide.

xide dismutases.²⁾ In the case of Cu, Zn-superoxide dismutase the inhibition was released by cyanide. These observations indicate the mediation of cytochrome *c*-reduction by O_2^- produced during the univalent reduction of molecular oxygen by xanthine oxidase in cream. Skim milk also inhibited the cytochrome-reduction induced by cream, however, boiled skim milk did not affect the reaction, suggesting the occurrence of superoxide dismutase in skim milk. The addition of catalase was required to prevent the re-oxidation of cytochrome *c* by lactoperoxidase in skim milk and H_2O_2 formed through the action of superoxide dismutase.

Further confirmation for the occurrence of superoxide dismutase in milk is the inhibition of the O_2^- -induced reduction of nitroblue tetrazolium. O_2^- was generated by illuminated flavin. Skim milk inhibited the reduction, however, the cream fraction showed little effect (Fig. 2). Thus, superoxide dismutase is absent in cream and found only in skim milk. The enzymatic activity was recovered in the pale yellow supernatant after the removal of almost all of casein by the addition of ammonium sulfate at 50% saturation. The inhibition by skim milk was released by cyanide. Three kinds of superoxide dismutase have been found with respect to the metal in the enzyme. Among them only the Cu, Zn-containing enzyme is sensitive to cyanide.^{1,2,5)} The present results suggest the occurrence of Cu, Zn-superoxide dismutase and the absence of the cyanide-insensitive Fe- or Mn-containing enzyme in skim milk.

Superoxide dismutase in skim milk was assayed from the inhibition of cytochrome *c*-reduction with O_2^-

generated with the xanthine-xanthine oxidase system as described previously.⁵⁾ To depress the oxidation of cytochrome *c* by lactoperoxidase catalase was added to the assay mixture (1600 units per cuvette) and small amounts of skim milk corresponding to 0.2 to 0.8 unit of superoxide dismutase were used. The enzymatic unit was estimated using an equation described previously.⁵⁾ In this range the enzymatic unit ($V/v-1$) was proportional to skim milk. Superoxide dismutase was found at about 5 units per ml of skim milk. The concentration of superoxide dismutase in bovine milk is estimated to be about 10^{-8} M, assuming that Cu, Zn-superoxide dismutase in skim milk have the same specific activity and molecular weight as those of superoxide dismutase in bovine erythrocyte (erythrocuprein).

Cu, Zn-superoxide dismutase is a resistant enzyme to denaturing stresses including heating. In fact commercial pasteurized milk retained the enzymatic activity at similar level to the unpasteurized milk. Physiological function of this enzyme in bovine milk is totally unknown, however, the removal of O_2^- by this enzyme may play a role for preservation of milk.

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