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Generation of hydrogen peroxide by a low molecular weight compound in whey of Holstein dairy cows

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Using an ultrafiltration membrane (molecular cut-off, 3000), low molecular weight compounds in bovine milk were collected (YM-3 filtrate). A hydrogen peroxide (H_2O_2)-like substance was generated in the YM-3 filtrate. This substance was undetected at 0 h, but increased in a time-dependent manner, peaking after 2 h of incubation at 38 °C. After incubating the YM-3 filtrate with catalase and lactoperoxidase, the signal showing the presence of this substance disappeared. The substance was quantified using one chemiluminescence and three colorimetric H_2O_2 detection systems. In all systems, their estimates were within the same range. The amount of substance, as estimated by the chemiluminescence H_2O_2 detection system, was correlated with that estimated by the other three colorimetric systems (r=0·98, 0·95 and 0·87). The substance was eluted at the same position as H_2O_2 by gel filtration on Superdex 30. Thus, the substance had the same characteristics as H_2O_2. An H_2O_2-generating substance in either the YM-3 filtrate or whey had a molecular mass of about 600. In this study, we clarify that bovine milk is capable of generating H_2O_2 by utilizing a low molecular weight compound. Thus, we present a new type of H_2O_2-supplying system in bovine milk.

Keywords: Dairy cow, hydrogen peroxide production, milk whey, chemiluminescence.

It is known that non-specific antibacterial defence systems consisting of lactoperoxidase (LPO), hydrogen peroxide (H_2O_2) and thiocyanate are present in bovine milk (Reiter & Harnulv, 1984; Ekstrand, 1989). In the LPO/H_2O_2/thiocyanate system, LPO with H_2O_2 catalyses the conversion of thiocyanate into antibacterial hypothiocyanite (Björck et al. 1975; Barett et al. 1999). LPO and thiocyanate are present in bovine and ovine milk throughout lactation (Zapico et al. 1991; Althaus et al. 2001; Fonteh et al. 2002). However, for the system to function, H_2O_2 must be present in milk.

A supply of H_2O_2 has been clearly demonstrated in the mouse, as L-amino acid oxidase in milk generates H_2O_2 through the oxidation of free amino acids (Sun et al. 2002). However, the presence of this enzyme in bovine milk has yet to be confirmed. Polymorphonuclear leucocytes release large amounts of H_2O_2 into bovine milk, particularly during phagocytosis (Korhonen & Reiter, 1983; Upadhyay, 1992). H_2O_2 is also generated by lactic acid bacteria present in milk (Björck, 1978). Xanthine oxidase generates H_2O_2 in the presence of hypoxanthine (Björck & Claesson, 1979; Escribano et al. 1988) and bovine milk contains both free and fat globule membrane-bound xanthine oxidase (Brieley & Eisenthal, 1974), while hypoxanthine is also present in bovine milk (Tiemeyer et al. 1984). H_2O_2 can also be produced by specific generation systems, such as the oxidation of ascorbic acid, oxidation of glucose by glucose oxidase and the manganese-dependent aerobic oxidation of reduced pyridine nucleotides by peroxidase (Wolfson & Summer, 1993). As the catalases and peroxidases in milk rapidly consume H_2O_2, it is normally present in very small amounts (Björck et al. 1975; Korhonen & Reiter, 1983). However, relatively high levels of H_2O_2 have been reported in bovine (Schiffman et al. 1992) and ovine milk (Althaus et al. 2001).

Following gel filtration of bovine milk, we found that an H_2O_2-like substance was generated in the small molecular mass fraction. In this study, the substance is identified as H_2O_2. We report here that bovine milk generates H_2O_2 by utilizing a low molecular weight compound.

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Materials and Methods

Milk samples

Foremilk (first 15 ml) was collected from fourteen Holstein cows, 2–7 months into lactation, on the dairy farms at Nihon University and the University of Tokyo. Milk samples were of deliverable grade, and were cooled on ice after milking and stored at −60 °C.

Whey was prepared by centrifugation at 265 000 ***g*** at 4 °C for 2 h according to the findings of Morr & Swenson (1973). Whey was applied to the YM-3 filter unit with a molecular limit of 3000 (Millipore Japan, Tokyo, Japan) and was centrifuged at 14 000 ***g*** at 4 °C for 90 min. Filtrate was collected (YM-3 filtrate), diluted with an equal volume of 100 mM-sodium phosphate (pH 7·0) and incubated at 25 °C for 4 h with 0·5 μg (5 U)/ml bovine liver catalase (Wako Pure Chemicals, Osaka, Japan) or 1 μg (0·08 U)/ml LPO (Sigma, St. Louis MO, USA). Insoluble materials appearing during incubation were removed by centrifugation at 20 000 ***g*** for 5 min.

Determination of H_2O_2

H_2O_2 was determined using one chemiluminescence and three colorimetric detection systems.

System 1: Test sample was mixed with an equal volume of assay reagent. The assay reagent consisted of 1·2 mg/ml TDPO (bis [2-(3, 6, trioxadecanyloxycarbonyl)-4-nitrophenyl] oxalate) and 0·2 mg/ml pyrene in acetonitrile (Wako Pure Chemicals) and was prepared on the day of the experiment (Imai et al. 1986). The luminescence intensity was counted for 10 s immediately after mixing using an Atto AB-2200 luminometer.
System 2: In the presence of LPO, the oxidation of ABTS (2,2′-azinodi-ethylbenzthia-zoline-6-sulphonic acid) (Wako Pure Chemicals) by H_2O_2 was measured at an absorbance of 412 nm using a Shimadzu UV-1600 spectrophotometer. The assay reagent consisted of 1 μg/ml LPO and 1 mM-ABTS in 100 mM-sodium phosphate (pH 6·7). The reaction was started by mixing the test sample with the assay reagent. The final concentration of the test sample was 3% (v/v). Increases in optical density (OD) were recorded. Initial rates of change were linear for 1 min in all samples examined.
System 3: The oxidation of phenol red by H_2O_2 was determined in the presence of horseradish peroxidase (HRPO) (Pick, 1986). HRPO and phenol red (Wako Pure Chemicals) was dissolved in 50 mM-potassium phosphate (pH 7·0). The reaction mixture, consisting of 20 U/ml HRPO, 0·56 mM-phenol red and 10% (v/v) test sample, was incubated at 37 °C for 60 min. The reaction was interrupted by adding a 1/10 volume 1 M-NaOH and the OD was measured at 600 nm.
System 4: H_2O_2 was determined using a colorimetirc H_2O_2 kit supplied by Sigma. Test sample was diluted with three volumes of 70 mM-sodium phosphate (pH 6·0) according to the manufacturer's instructions and the OD was measured at 550 nm with a spectrophotometer.

Each determination was performed in triplicate. A standard calibration curve was constructed for each assay using known amounts of H_2O_2 in water. The amount of H_2O_2 in the test sample was calculated using the standard curve. In this study, the signal obtained without H_2O_2 in water was taken as the background for each detection system.

Gel filtration

Samples (0·5 ml) of whey and YM-3 filtrate were separated at 4 °C on an Amersham Biosciences FPLC with Superdex 30 (HR 16/50) (Uppsala, Sweden) at a flow rate of 1 ml/min, and 1-ml fractions were collected. OD was monitored at 280 nm. The elution buffer consisted of 100 mM-sodium phosphate (pH 7·0). The column was calibrated using vitamin B_{12}, cystine and H_2O_2 (Wako Pure Chemicals). The void volume was determined using blue dextran (Amersham). Details are given in the legend of Fig. 2.

Statistical methods

Results are expressed as means ± SD with the experiment number. Statistical analyses were performed using Student's *t* test, correlation analysis and one way analysis of variance (ANOVA). Differences with *P* values below 0·05 were considered to be statistically significant.

Results

Milk samples used for evaluation

Fourteen individual milk samples were used for evaluation. YM-3 filtrates were incubated at 25 °C for 4 h and then introduced into System 1. As compared with the standard curve, no TDPO-reactive substances were detected at 0 h, but at 4 h the amount increased to between 2·4 and 6·5 nmol/ml, depending on the sample. The mean production was 4·2 ± 1·7 nmol/ml (mean ± SD) indicating that TDPO-reactive substances were newly synthesized during the 4-h incubation. We then attempted to clarify whether the TDPO-reactive substance was H_2O_2.

Measurement of H_2O_2 using System 1

The minimum amounts of H_2O_2 detected by each detection system are shown in Table 1. System 1 had the highest sensitivity, and the dose-response curve was linear from 0·1 to 10 nmol/ml H_2O_2. System 1 also had no background interference in determining H_2O_2 (Fig. 1). In three milk samples, the intra-assay variation was within 6% (each with five determinations) and the inter-assay variation was within 13% (3 repetitions, each with five determinations).

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Table 1. Hydrogen peroxide (H_2O_2) determined using systems 1–4. YM-3 filtrate was incubated at 25 °C for 4 h in the presence and absence of catalase and then H_2O_2 was determined using the detection system indicated.

Values are means ± SD (n=14)

	System 1	*System 2*	*System 3*	*System 4*
H_2O_2 (nmol/ml)†	4·2 ± 1·7	4·3 ± 1·8	4·1 ± 1·9	3·9 ± 1·6
Correlation (r)‡	–	0·98**	0·95**	0·87*
Detection limit (nmol/ml)¶	0·1	1	1	1

† Amount of H_2O_2 had been subtracted from that obtained in the presence of catalase

‡ Compared with data obtained using System 1: *P<0·05, **P<0·01

¶ Detection levels in each system were obtained using standard H_2O_2 solutions

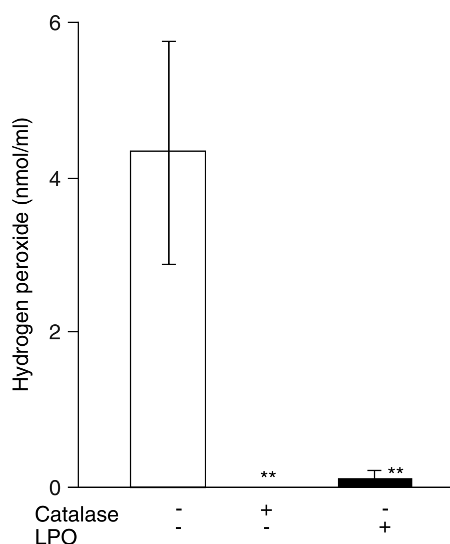

Fig. 1. Effects of catalase and lactoperoxidase. YM-3 filtrate was incubated at 25 °C for 4 h with (+) or without (–) catalase and lactoperoxidase (LPO). The amount of H_2O_2 was determined using System 1. Values are means ± SD (n=14), **P<0·01 compared with no enzyme control.

Identification of H_2O_2

If the TDPO-reactive substance is H_2O_2, the substance will be degraded by incubation with catalase and LPO. The YM-3 filtrate was incubated with catalase and LPO and the amount of TDPO-reactive substance was then determined using System 1 (Fig. 1). The TDPO-reactive substance disappeared during incubation with calalase, returning to background levels in System 1. After 4 h of incubation with LPO, a TDPO-reactive substance decreased significantly (P<0·005). Thus, the TDPO-reactive substance disappeared by incubation with catalase and LPO.

If the TDPO-reactive substance is H_2O_2, it will similarly be detected in the various H_2O_2 detection systems. The YM-3 filtrate was incubated in the presence and absence of catalase, and was then assayed using systems 1–4. Even after incubation with catalase, the OD was occasionally higher than the background OD in systems 3 and 4 (results not shown). The catalase-dependent decrease is shown in Table 1, and was comparable among the four detection systems (F=0·53, P>0·25). The amount estimated using System 4 was slightly lower than that estimated using System 1, but the difference was non-significant.

If the four systems accurately and correctly detect the same substance, the data must be correlated. In this study, the catalase-dependent decrease was used for comparison. Results obtained using System 1 were compared with those obtained using the other three colorimetric systems (Table 1). The correlation coefficient was 0·98 with System 2 (P<0·01), 0·95 with System 3 (P<0·01) and 0·87 with System 4 (P<0·05).

Based on this evidence, the TDPO-reactive substance was confirmed to be H_2O_2.

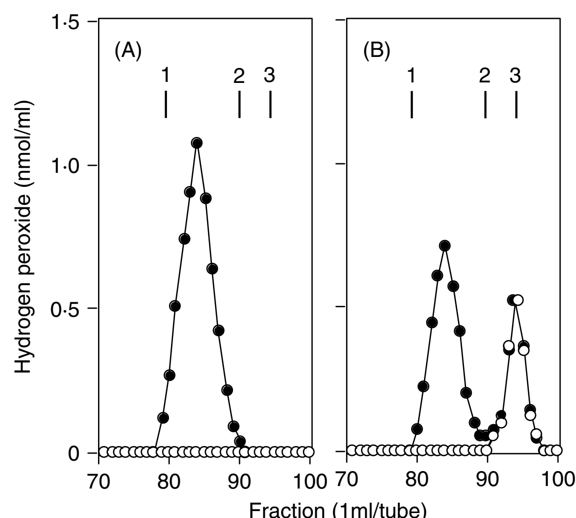

Fig. 2. Gel filtration of whey and YM-3 filtrate. The amount of H_2O_2 was determined using System 1. The positions of molecular mass markers (vitamin B_{12} (1), cystine (2) and H_2O_2 (3)) are indicated. (A) Whey was applied on Superdex 30. After gel filtration the tube was incubated at 25 °C for 0 h (open symbol) or 4 h (closed symbol). (B) YM-3 filtrate was pre-incubated at 25 °C for 30 min and then applied. After gel filtration the tube was incubated at 25 °C for 0 h (open symbol) or 4 h (closed symbol).

Gel filtration of whey and YM-3 filtrate

An H_2O_2-generating substance must be present in milk. All 14 samples were examined and the representative results are shown in Fig. 2. Whey was subjected to gel filtration (Fig. 2A). No peaks for H_2O_2 were detected immediately after gel filtration, but after 4 h of incubation, a single peak was seen in fraction 84. When a comparison was made with the molecular mass marker positions, the H_2O_2-generating substance had an apparent molecular mass of 600. To test the heat stability, fraction 84 was placed in

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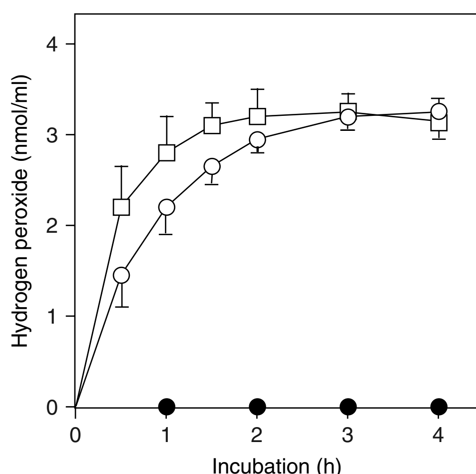

Fig. 3. Time-dependent production of H_2O_2. The YM-3 filtrate was incubated with (closed symbol) or without (open symbol) catalase and at the times indicated, the amount of H_2O_2 was determined using System 1; 25 °C (circle) and 38 °C and 45 °C (square). Values are means ± SD (n=4).

boiling water for 5 min. The production of H_2O_2 was unchanged after boiling.

The YM-3 filtrate was prepared from whey used above. To generate H_2O_2 before gel filtration, the YM-3 filtrate was incubated at 25 °C for 30min and then applied (Fig. 2B). In fraction 84, the concentration of H_2O_2 increased from 0 to 1·2 nmol/ml during the 4-h incubation. Another peak was detected at position 94, the same as that for pure H_2O_2, but the height was unchanged by the 4-h incubation after gel filtration. H_2O_2 was clearly separated from the H_2O_2-generating substance.

The total amount of H_2O_2 from fractions 80–97 (Fig. 2B) was correlated with that from fractions 80–90 (Fig. 2A) (r>0·93, P<0·01, n=14).

Except in a void volume (fraction 41), the OD profile of whey was essentially the same as that for YM-3 (results not shown).

Time-dependent production of H_2O_2

YM-3 filtrate was incubated at 25°, 38° and 45 °C. Time-dependent production of H_2O_2 is shown in Fig. 3. At 25 °C, the production of H_2O_2 increased at 30 min (P<0·01 compared with that at 0 h) and increased in a curvilinear manner with time. The production peaked after 3 h of incubation. Production at 38 °C increased faster than that at 25 °C and peaked at 2 h of incubation. The time-dependent production at 45 °C was comparable to that at 38 °C (results not shown). Maximun production was the same at either 25, 38 or 45 °C. No H_2O_2 was detected in the presence of catalase.

In our unpublished observations, the YM-3 filtrate was degassed in a vacuum and incubated in an atmosphere of water-saturated nitrogen. No production was observed at 25, 38 and 45 °C. Incubation was done under light-protected and unprotected conditions, and no difference was found.

Effects of whey incubation on production of H_2O_2

Whey was preincubated on ice and at 38 °C for 2 h and the YM-3 filtrate was then prepared. After 4 h of incubation of the YM-3 filtrate, production of H_2O_2 was determined. With preincubation at 38 °C for 0 and 2 h, production of H_2O_2 was 4·1 ± 1·5 nmol/ml and 1·6 ± 0·9 nmol/ml (mean ± SD, n=14), respectively. The difference was significant (P<0·01). In three milk samples, however, the degree of this decrease was relatively small (29, 33 and 39%). With preincubation on ice, no decrease of the ability to produce H_2O_2 was observed.

Discussion

Initially, we found that a TDPO-reactive substance was generated during incubation of the YM-3 filtrate. Several methods are available for the determination of H_2O_2 in the nanomolar range. System 1 had the highest sensitivity and no background interference in determining H_2O_2. Colorimetry was also successful in determining H_2O_2. However, high background levels were occasionally observed in Systems 3 and 4, thus suggesting that the amount of H_2O_2 should be calculated by the difference between the presence and absence of catalase.

Catalase decomposes H_2O_2 to water and oxygen, and we confirmed the complete decomposition of H_2O_2 in the standard H_2O_2 solution by catalase. The TDPO-reactive substance also disappeared on incubation with catalase. LPO decomposes H_2O_2 efficiently in the presence of oxidizable halides and thiocyanate (Furtmüller et al. 2002). Thiocyanate in milk (Zapico et al. 1991; Althaus et al. 2001; Fonteh et al. 2002) is converted to hypothiocyanite through the decomposition of H_2O_2 by LPO (Pruitt et al. 1982; Thomas et al. 1991; Furtmüller et al. 2002). Thiocynate in milk is able to pass through the YM-3 filter (unpublished data) as determined by ferric nitrate dye assay (Fonteh et al. 2002). Thus, LPO is capable of decomposing H_2O_2 in the YM-3 filtrate. The TDPO-reactive substance was decomposed by incubation with LPO.

TDPO, an aryl oxalate ester in the peroxyoxalate chemiluminescence reaction, was developed for the high-sensitivity detection of H_2O_2 (Imai et al. 1986). The TDPO-reactive substance oxidized ABTS with LPO in System 2 (Shindler & Bardsley, 1975) and oxidized phenol red with HRPO in System 3 (Pick, 1986). Ferrous ions (Fe^{2+}) are converted to Fe^{3+} by H_2O_2 and Fe^{3+} binds with xylenol orange (Jiang et al. 1990). A purple colour proportional to TDPO-reactive substance was produced in System 4. The catalase-dependent decreases were within the same range in all detection systems used in this study.

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Based on the high correlations, all detection systems appeared to detect the same compound generated during incubation. On gel filtration, the TDPO-reactive substance was eluted at the same position as H_2O_2. All these observations indicate the presence of H_2O_2 and we thus concluded that the TDPO-reactive substance is H_2O_2.

Gel filtration of whey indicated that a H_2O_2-generating substance had an apparent molecular mass of 600. A H_2O_2-generating substance could completely pass through the ultrafiltration membrane and the molecular mass remained unchanged. At present, it is uncertain whether the H_2O_2-generating substance is a non-proteinic compound.

Based on the decrease of H_2O_2 production after incubation of whey, it is clear that H_2O_2 is generated in milk. However, H_2O_2 in whey was not detected in this study, which confirms previous findings (Björck et al. 1975; Korhonen & Reiter, 1983). This is probably due to the decomposition of H_2O_2 by LPO in milk, as the oxidation of thiocynate by LPO in the presence of H_2O_2 occurs very rapidly (Furtmüller et al. 2002). As shown here, the generation of H_2O_2 terminated once a H_2O_2-generating substance had been consumed. It is reasonble to speculate that the production of H_2O_2 is started after the release of the substance into the alveolar lumen. As the interval is longer after milking, the volume of milk in the udder is larger. The concentration of H_2O_2-generating substance in milk present in the alveolar lumen is higher than that reported here.

In conclusion, a H_2O_2-generating substance is present in bovine milk and is degraded in whey. Thus, a novel H_2O_2-supplying system is identified in this study.

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