The use of lactoperoxidase for the bleaching of fluid whey

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ABSTRACT

Lactoperoxidase (LP) is the second most abundant enzyme in bovine milk and has been used in conjunction with hydrogen peroxide (H_2O_2) and thiocyanate (SCN^-) to work as an antimicrobial in raw milk where pasteurization is not feasible. Thiocyanate is naturally present and the lactoperoxidase system purportedly can be used to bleach dairy products, such as whey, with the addition of very little H_2O_2 to the system. This study had 3 objectives: 1) to quantify the amount of H_2O_2 necessary for bleaching of fluid whey using the LP system, 2) to monitor LP activity from raw milk through manufacture of liquid whey, and 3) to compare the flavor of whey protein concentrate 80% (WPC80) bleached by the LP system to that bleached by traditional H_2O_2 bleaching. Cheddar cheese whey with annatto (15 mL of annatto/454 kg of milk, annatto with 3% wt/vol norbixin content) was manufactured using a standard Cheddar cheesemaking procedure. Various levels of H_2O_2 (5–100 mg/kg) were added to fluid whey to determine the optimum concentration of H_2O_2 for LP activity, which was measured using an established colorimetric method. In subsequent experiments, fat-separated whey was bleached for 1 h with 250 mg of H_2O_2 /kg (traditional) or 20 mg of H_2O_2 /kg (LP system). The WPC80 was manufactured from whey bleached with 250 mg of H_2O_2 /kg or 20 mg of H_2O_2 /kg. All samples were subjected to color analysis (Hunter color values and norbixin extraction) and proximate analysis (fat, protein, and moisture). Sensory and instrumental volatile analyses were conducted on WPC80. Optimal LP bleaching in fluid whey occurred with the addition of 20 mg of H_2O_2 /kg. Bleaching of fluid whey at either 35 or 50°C for 1 h with LP resulted in >99% norbixin destruction compared with 32 or 47% destruction from bleaching with 250 mg of H_2O_2 /kg, at 35 or 50°C for 1 h, respectively. Higher aroma intensity and increased lipid oxidation compounds were documented in WPC80 from bleached whey compared

with WPC80 from unbleached whey. Monitoring of LP activity throughout cheese and whey manufacture showed that LP activity sharply decreased after 30 min of bleaching (17.01 ± 1.4 to <1 U/mL), suggesting that sufficient bleaching takes place in a very short amount of time. Lactoperoxidase averaged 13.01 ± 0.7 U/mL in unpasteurized, fat-separated liquid whey and 138.6 ± 11.9 U/mL in concentrated retentate (11% solids). Lactoperoxidase may be a viable alternative for chemical whey bleaching.

Key words: whey, flavor, bleaching, lactoperoxidase

INTRODUCTION

Lactoperoxidase (LP) is an oxidoreductase enzyme belonging to the peroxidase family and is found in a wide range of mammalian milks, including humans (Seifu et al., 2005). This enzyme is heat stable and is inactivated after 15 s at 78°C (de Wit and van Hooijdonk, 1996). Historically, the LP system has been used to inhibit microbial growth in bovine milk. The LP system consists of 3 components: LP, thiocyanate (SCN^-), and hydrogen peroxide (H_2O_2). The system is not active unless all 3 components are present in sufficient amounts (Seifu et al., 2005). The major intermediate oxidation product of the LP-catalyzed oxidation of SCN^- is the hypothiocyanate ion (OSCN^-), which is bactericidal (Seifu et al., 2005). Hydrogen peroxide is sometimes added to activate the system if no H_2O_2 is naturally present. Catalase-negative organisms (such as lactic acid bacteria) can generate H_2O_2 under aerobic conditions and, thus, can also activate the LP system. Many lactobacilli, lactococci, and streptococci produce sufficient H_2O_2 under aerobic conditions to activate the LP system (Seifu et al., 2005). Exogenous H_2O_2 must be added to activate the system (Reiter and Harnulv, 1982) if it is not supplied by catalase-negative organisms. Gram-negative, catalase-positive organisms (such as *Pseudomonas* spp., coliforms, salmonellae, and shigellae) are not only inhibited by the LP system, but may be killed, provided that H_2O_2 is supplied exogenously (Seifu et al., 2005). Gram-positive, catalase-negative bacteria (such as streptococci and lactococci) are generally inhibited but not killed by the LP system

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(Seifu et al., 2005). If raw milk is stored at $\leq 15^{\circ}\text{C}$, the LP system can effectively preserve raw milk for 24 to 26 h (Reiter and Harnulv, 1982).

Measurement of LP can be done using a variety of methods and, as such, LP activity values vary widely in the literature. In 1994, a method was established to quantify LP activity (Pruitt and Kamau, 1994). This assay uses 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (**ABTS**) as a chromophore and the measurement is carried out at 412 nm. This method has been widely accepted (Pruitt and Kamau, 1994). Bovine milk contains 1.2 to 19.4 U of LP/mL (Seifu et al., 2005), although levels in liquid whey are reported to be 30 mg/L or about 0.5% (wt/wt) of whey proteins (de Wit and van Hooijdonk, 1996). Levels of LP can vary depending on the lactation cycle of the cow, season, feeding regimen, and breed (Kussendrager and van Hooijdonk, 2000). Like LP, thiocyanate can vary widely due to feeding regimen (Seifu et al., 2005). The third component of the LP system, H_2O_2 , is not normally detected in raw milk and is typically added exogenously. Hydrogen peroxide can be generated endogenously by bacteria, although amounts sufficient to activate the LP system may not be generated (Seifu et al., 2005). Depending on the milk, any 1 of the 3 components that make up the LP system could become the limiting factor.

In addition to inhibiting microbial growth, OSCN⁻ (produced when LP reacts with SCN⁻), also has bleaching capabilities. The strong oxidizing capacity allows OSCN⁻ to react with carotenoids, leading to destruction of conjugation and subsequent color loss of norbixin. Very few papers have investigated the use of the LP system for decolorization of whey, and no scholarly journal articles to our knowledge have been published. Bottomley et al. (1989) published a patent describing the decolorizing of whey and products derived from whey using the LP system. Though some process details were provided, quantitative norbixin destruction was not addressed, LP activity was not monitored throughout processing, and the flavor of the finished product was not addressed. Subsequently, all of these items were a focus of the current study. The United States is a major consumer of yellow Cheddar cheese, in which the natural colorant, annatto, comprising the carotenoids bixin and norbixin, is added. Some of the color remains in the liquid whey following curd separation and it is necessary to bleach the whey to achieve a desired lack of color in dried whey ingredients (Kang et al., 2010). The objectives of this study were to quantify the amount of H_2O_2 necessary for optimal bleaching using the LP system in fluid whey, to monitor LP activity throughout cheesemaking and whey processing, and to compare the flavor of whey protein concentrate 80%

(**WPC80**) bleached by the LP system to that bleached by high levels of H_2O_2 (traditional chemical bleaching). The overall goal of this study was to determine if whey bleaching via the LP system would be a viable alternative to chemical H_2O_2 bleaching for the dairy industry.

MATERIALS AND METHODS

Experimental Design Overview

Two experimental components were involved: liquid whey trials and the production of WPC80. Optimum H_2O_2 levels to activate the LP system were first determined. Liquid whey trials were then conducted as a 2 by 3 factorial design with temperature (35 or 50°C) and bleach treatment [control, 20 mg of H_2O_2 /kg (LP), or 250 mg of H_2O_2 /kg (**HP**)]. The samples with the highest bleaching efficacy were then selected for WPC80 manufacture. All samples within each trial were made from the same lot of milk. Lactoperoxidase and SCN⁻ were monitored throughout the entire process of cheese and whey manufacture. All experiments were conducted in triplicate.

Production of Liquid Whey

Cheddar whey was manufactured from vat pasteurized whole bovine milk (195 kg) as described by Campbell et al. (2011). Double strength annatto color (3% norbixin wt/vol; Danisco USA Inc., New Century, KS) was added at 15 mL/454 kg of milk and diluted 20 times in deionized water before addition to pasteurized milk. The whey was drained from the curds at pH 6.35 and a sieve was used to remove any remaining particles. The whey was immediately processed with a hot bowl cream separator (model SI600E; Agri-Lac Tecnologia Lactea, Miami, FL) to decrease the fat content.

Activation of the LP System

The optimum level of H_2O_2 to activate the LP system was determined by adding 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 mg of H_2O_2 /kg to unpasteurized, fat-separated liquid Cheddar whey. Bleaching was then carried out as described below. The concentration of H_2O_2 that resulted in the most bleaching [20 mg/kg, according to percent destruction via the degree of yellowness or blueness (**b***) reflectance values] was selected for further trials.

LP and HP Bleaching

Small aliquots (50 mL) of liquid whey were placed in amber glass jars to prevent light degradation. The

jars were placed in a water bath at either 35 or 50°C and allowed to equilibrate. To activate the LP system, 20 mg of H₂O₂/kg (35% wt/vol; Nelson Jameson Inc., Marshfield, WI) was added and allowed to bleach in the water bath at either 35 or 50°C for 1 h with gentle agitation. For HP chemical bleaching, 250 mg of H₂O₂/kg was added to liquid whey and allowed to bleach for 1 h with gentle agitation. The latter concentration of H₂O₂ was selected because it represents the mid range of the legally allowed amount of H₂O₂ for traditional chemical bleaching of whey and also represents a concentration that might be applied by industry (Kang et al., 2010; Listiyani et al., 2011, 2012). Peroxide test strips (EMD Chemicals, VWR International LLC, West Chester, PA) were used to determine if any H₂O₂ was leftover after the bleaching treatment. If so, catalase (20 mg/kg, FoodPro CAT; Danisco USA Inc.) was added at 20 mg/kg to deactivate H₂O₂. The samples were then pasteurized (63°C for 30 min) and then immediately cooled on ice. Measurements, including the amount of LP and SCN⁻; luminosity (**L***, the degree of lightness from black to white), the degree of redness or greenness (**a***), and **b*** values (collectively designated **L*a*b*** values); and volatile compound analyses were performed immediately. Samples were frozen at -80°C for norbixin, mineral, and proximate analysis at a later date (within 90 d).

Production of WPC80

Treatments were selected based on current industrial practices, previous bleaching studies, and from the liquid whey trials [LP (20 mg of H₂O₂/kg) at 35°C, HP (250 mg of H₂O₂/kg) at 50°C, and control at 50°C] for manufacture of WPC80. Hydrogen peroxide was a less effective bleaching agent at lower temperatures, so 50°C was selected for chemical bleaching (Listiyani et al., 2012). The optimum temperature for LP activity was 37°C and was selected for LP. The control was heated to 50°C to account for any effect of heat treatment (worst-case scenario). Colored, fat-separated cheese whey was freshly manufactured as described previously. The separated whey was transferred into a 102-L stainless vat (Fermenator; Blichmann Engineering LLC, Lafayette, IN) equipped with a coil heater (1.3 cm o.d.; PAC Stainless Ltd., Seattle, WA). One of 3 treatments: LP (35°C, 20 mg of H₂O₂/kg (35% wt/vol; Nelson Jameson Inc.), HP (50°C, 250 mg of H₂O₂/kg), or control (50°C, no bleach added) was administered. The whey was allowed to bleach or was held at 50°C (control) for 1 h while recirculating using a peristaltic pump (model 77410-10; Millipore Inc., Billerica, MA). If any H₂O₂ remained after treatment, it was deactivated using 20 mg of catalase/kg (FoodPro CAT, Danisco USA Inc.).

Wheys were then heated to 63°C for 30 min to pasteurize. Following pasteurization, the whey was cooled to the appropriate temperature (either 35 or 50°C) before UF commenced. The UF system (model Pellicon 2; Millipore Inc.) was equipped with 5 polyethersulfone cartridge membrane filters (model P2B010V05, 10-kDa nominal separation cutoffs, 0.5 m² surface area; Millipore Inc.). Each sample was run through a peristaltic pump (model 77410-10) and the UF assembly using silicone tubing (model 96440-73) that was connected to the vat. Pumps, pump heads, and tubing were all obtained from Cole-Palmer Instrument Co. (Vernon Hills, IL). This process continued until the retentate reached 80% protein (wt/vol) content confirmed by a Sprint rapid protein analyzer (CEM Corp., Matthews, NC). Retentates were then collected and spray dried (Model Lab 1; Anhydro Inc., Søborg, Denmark). The inlet temperature was 150°C and the outlet temperature was at 80°C. The total spray-drying time was about 1 h. The powder was collected and stored in Mylar bags at -80°C following production. All treatments were manufactured from the same lot of milk and the experiment was carried out in triplicate.

Composition Analysis

Total solids of liquid whey and WPC80 were determined by air oven drying (AOAC, 2000; method number 990.20; 33.2.44). In powdered WPC80, fat was quantified by Soxhlet ether extraction (AOAC, 2000; method number 947.05). In liquid wheys, fat was quantified using the Pennsylvania modified Babcock method (AOAC, 2000; method number 989.04). Protein content was determined using the Kjeldahl method in powdered wheys (AOAC, 2000; method number 991.20; 33.2.11) or using the Sprint rapid protein analyzer (CEM Corp.) if the whey was liquid. Mineral analysis (phosphorus, calcium, magnesium, potassium, sulfur, sodium, and iron) was done by the North Carolina State University Analytical Services Laboratory (Raleigh, NC) using a standard dry ash method with inductively coupled plasma optical emission spectroscopy (Lloyd et al., 2009). All samples were measured in duplicate.

Hunter L*a*b* Values

Whey protein concentrate 80% samples were measured in both powder form (10 g) and liquid form (10 mL of 10% wt/vol solution). Ten milliliters of the sample (rehydrated at 10% wt/vol solids, if necessary) was placed into the bottom of a 60 × 15-mm polystyrene Petri dish (Becton Dickinson and Co., Franklin Lakes, NJ). The color of the samples was measured using a Minolta Chroma meter (CR-410; Konica Minolta

Sensing Americas Inc., Ramsey, NJ). Each sample was measured in duplicate and duplicate measurements were taken. Before measurements being taken, a factory-supplied calibration plate was used to calibrate the instrument. The Hunter Commission on Illumination (CIE) $L^*a^*b^*$ color scale was used. Reflectance values were taken with a white calibration plate as the background.

Norbixin Extraction and Quantification

Norbixin is the primary carotenoid in water-soluble annatto extracts and was extracted and measured to determine percent annatto destruction and bleaching efficacy (Kang et al., 2010). Norbixin was extracted using the methods described by Campbell et al. (2011) and quantified by HPLC. Briefly, 0.6 g of sample was weighed into a 50-mL centrifuge tube (Nalge Nunc International Corp., Rochester, NY). To this, 6 mL of water was added and the sample was vortexed. Three milliliters of ethanol was added and the solution was then vortexed again. Three milliliters of chloroform (VWR International LLC) and 1 mL of glacial acetic acid (1% wt/vol; JT Baker Chemical Co., Phillipsburg, NJ) were added to the previous solution. The sample was vortexed and centrifuged at $16,500 \times g$ for 10 min at 4°C (model RC5B; Thermo Scientific Inc., Waltham, MA). The bottom chloroform layer containing the norbixin was collected and the volume was measured. The extraction procedure and measurements were performed with premium full-spectrum F885 flat sheet filters covering all lights (Ergomart, Dallas, TX) to minimize norbixin isomerization and degradation (Mercadante, 2008).

To further filter and purify the extracted norbixin, solid-phase microextraction (SPME) was used. The column selected was the Strata-NH₂ solid-phase extraction (SPE) column (500 mg/3 mL; Phenomenex Inc., Torrance, CA). To condition the column, 4 mL of *n*-hexane (VWR International LLC) was run through the column. An aliquot of the extract collected previously (1 mL) was transferred onto the conditioned SPE column. The column was rinsed with 2.5 mL of *n*-hexane:diethyl ether (1:1, vol/vol) and 1 mL of acetone (VWR International LLC). The norbixin was eluted with 2 mL of methanol:glacial acetic acid (7:3, vol/vol). The final volume was measured and samples placed in vials for quantification by HPLC (Waters 1525 binary pump; Waters Corp., Milford, MA). Isocratic mobile phase [70% acetonitrile /30% water with 0.1% (wt/vol) formic acid (all from EMD Chemicals, VWR International LLC)] was used at a flow rate of 1 mL/min pumped through a binary pump (Waters

1525, Waters Corp.). Fifteen microliters of the sample was injected (Waters 2707 autosampler) onto the column (Phenomenex Kinetex 2.6- μ m particle size, 10-cm length, 4.6-mm i.d., 100Å pore size; Phenomenex Inc.), which was heated to 40°C. The injector temperature was set to 4°C. The sample was sent through a photodiode array detector (Waters 2998). A standard curve was created by rehydrating norbixin powder (45% (wt/vol), Chr. Hansen, Milwaukee, WI) in 2.5% (wt/vol) potassium hydroxide (BDH; VWR International LLC) and then diluting in methanol:glacial acetic acid (7:3, vol/vol). The maximum used for calculation was 460 nm. Norbixin concentration was calculated by TS and correction for dilution during the extraction and SPE processes.

LP Measurement

Lactoperoxidase measurements and calculations were performed according to the International Dairy Federation method (Pruitt and Kamau, 1994). Two milliliters of ABTS stock solution [1 mM ABTS (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) in 0.1 mM phosphate buffer (pH 6.0; Electron Microscopy Sciences, Hatfield, PA)] was placed in a disposable plastic cuvette (VWR International LLC). To start the reaction, 0.10 mL of sample and 1.0 mL of H₂O₂ solution (0.3 mM; Nelson Jameson Inc.) were added simultaneously and mixed thoroughly. The absorbance was monitored at 412 nm every 5 s until the reaction reached a plateau (2–5 min). Calculations were determined using the least squares regression of the absorbance data and Beer's law (Pruitt and Kamau, 1994). Samples for LP and thiocyanate measurement were taken from raw milk, pasteurized milk, whey (at drain), pasteurized whey, and pasteurized bleached whey.

SCN⁻ Measurement

Thiocyanate measurements were performed according to the International Dairy Federation method (IDF, 1988). Four milliliters of sample was mixed with 2.0 mL (wt/vol) of 20% TCA solution (BDH; VWR International LLC). The mixture was blended well and allowed to sit for at least 30 min. The mixture was then centrifuged at $16,500 \times g$ for 10 min (model RC5B; Thermo Scientific Inc.). After centrifugation, 1.5 mL of the supernatant was mixed with 1.5 mL of ferric nitrate solution [16 g of Fe (NO₃)₃ (EMD Chemicals, VWR International LLC) dissolved in 50 mL of 2 M nitric acid (HNO₃; BDH; VWR International LLC) and then diluted with deionized water to 100 mL). The measurement was performed at 460 nm within 10 min

of ferric nitrate solution addition. The concentration of SCN^- (Sigma-Aldrich Co. LLC, Milwaukee, WI) was determined by the use of external standard curves.

Descriptive Sensory Analysis

Sensory analysis was conducted on rehydrated WPC80 (10% wt/vol) using a trained descriptive sensory panel and an established dairy flavor language (Drake et al., 2003, 2009). Panelists ($n = 8$) each had more than 150 h of previous experience with the sensory analysis of fluid and dried whey products using the Spectrum descriptive analysis method (Meilgaard et al., 2007). All sensory testing was conducted in accordance with the North Carolina State University Institutional Review Board for Human Subjects guidelines.

Reconstituted WPC80 (10% solids wt/vol) was evaluated by placing 30 mL in 3-digit-coded 60-mL lidded cups (Solo Cup Co., Urbana, IL). Preparations were conducted with overhead lights off to avoid exposure to light. The WPC80 were evaluated within 7 d of production. Samples were evaluated by each panelist in duplicate. Sensory data were collected on paper ballots or using Compusense 5 (release 4.8; Compusense Inc., Guelph, ON, Canada).

GC-MS

Volatile compounds in fluid whey and WPC80 powder were extracted by SPME using selective ion monitoring (SIM). Compounds were then separated and identified by GC-MS using a modified method of Liaw et al. (2010). Liquid samples were tested the day of manufacture and spray-dried powders were reconstituted at 10% solids (wt/vol) and evaluated within 7 d. All samples contained 10% (wt/vol) sodium chloride (Fisher Scientific, Pittsburgh, PA), and 10 μL of internal standard solution (2-methyl-3-heptanone in methanol at 81 mg/kg; Sigma-Aldrich Co. LLC) in 20-mL autosampler vials with steel screw tops containing silicone septa faced in Teflon (MicroLiter Analytical Supplies Inc., Suwanee, GA). Samples were injected using a Combi PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) attached to an Agilent 6890N gas chromatograph with 5973 inert mass selective detector (MSD; Agilent Technologies Inc., Santa Clara, CA). Samples were maintained at 5°C before fiber exposure. Samples were equilibrated at 40°C for 25 min before 30-min fiber exposure of a 1-cm divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco Inc., Bellefonte, PA) at 31 mm with 4 s of pulsed agitation at 250 rpm. Fibers were injected for 5 min at a depth of 50 mm.

The GC method used an initial temperature of 40°C for 3 min with a ramp rate of 10°C/min to 250°C held for 5 min. The SPME fibers were introduced into the split/splitless injector at 250°C. A Zb-5ms column (Zb-5ms, 30-m length $\times$ 0.25-mm i.d. $\times$ 0.25- μm film thickness; Phenomenex Inc.) was used for all analyses at a constant flow rate of 1 mL/min. The purge time was set at 1 min. The MS transfer line was maintained at 250°C with the quad at 150°C and source at 250°C. Compounds were identified using the National Institute of Standards and Technology (NIST) 2005 library of spectra and comparison of spectra of authentic standards injected under identical conditions. Relative abundance for each compound was calculated using the calculated recovery of the internal standard concentration to determine relative concentrations of each compound. Retention indices were calculated using an alkane series (Sigma-Aldrich Co. LLC; van den Dool and Kratz, 1963).

Statistical Analysis

Data were analyzed by one-way ANOVA using a general linear model (PROC GLM) in SAS (version 9.2; SAS Institute Inc., Cary, NC). Replication was designated as a random effect. Principal component analysis was conducted and biplots were generated using XLSTAT (version 2010.5.02; Addinsoft, New York, NY).

RESULTS

Liquid Whey

The most effective bleaching with LP in unpasteurized, fat-separated liquid whey occurred with addition of 20 mg of $\text{H}_2\text{O}_2/\text{kg}$ (Figure 1) and this concentration was chosen for subsequent sets of experiments. Liquid whey was treated at either 35 or 50°C and received 1 of 3 treatments: HP (250 mg of $\text{H}_2\text{O}_2/\text{kg}$), LP (20 mg of $\text{H}_2\text{O}_2/\text{kg}$), or nothing (control). Samples did not differ in composition (solids 6.49 ± 0.08 , protein 0.82 ± 0.05 , and fat 0.03 ± 0.01). Samples bleached by the LP system showed the greatest bleaching efficacy (>99% destruction of norbixin). Bleaching by LP did not vary with temperature for the bleaching time evaluated; however, bleaching by H_2O_2 was more effective at 50°C than at 35°C with 46.9 and 31.8% norbixin destruction, respectively ($P < 0.05$). Reflectance values were consistent with norbixin extraction values in that LP bleached better than H_2O_2 ($P < 0.05$).

Both LP and SCN^- were monitored from raw milk through whey pasteurization, which immediately fol-

Figure 1. Bleaching efficacy of lactoperoxidase across various levels of hydrogen peroxide in unpasteurized, fat-separated fluid Cheddar whey at 35°C for 1 h. b^* = degree of yellowness or blueness. Color version available in the online PDF.

lowed bleaching to ensure that neither of these changed over time or limited the system. The SCN^- did not vary with bleaching type (LP, HP, or control), nor did it decrease over time ($P > 0.05$). Using an external standard curve for quantification, the SCN^- level was 3.8 ± 2.2 mg/kg in fluid whey, which is consistent with previously reported values of 3.2 to 4.6 mg/kg (Seifu et al., 2005). Lactoperoxidase was monitored throughout cheesemaking and was still active in samples receiving no bleach treatment after whey pasteurization (17.01 ± 1.4 U/mL). Lactoperoxidase activity levels in unpasteurized fluid whey were not affected by fat separation ($P > 0.05$) but slightly increased following whey pasteurization, with values averaging 13.01 ± 0.7 U/mL before pasteurization and 17.01 ± 1.4 U/mL after pasteurization ($P < 0.05$). Previous studies have shown an LP activity decrease in milk after milk pasteurization (Seifu et al., 2005). We observed a consistent increase in LP activity in fluid whey after pasteurization. It is unknown why this increase occurred. A possible expla-

nation may be due to small decreases in whey pH that occur between whey drain and vat pasteurization. The pH optimum for LP activity is between 5 and 6. Whey is drained at pH 6.4 and may decrease to 6.3 due to starter activity while being pumped to the vat pasteurizer and the temperature is increased to 63°C. As the pH decreases closer to the optimal pH, the activity of LP increases (Kussendrager and van Hooijdonk, 2000). In samples that received bleach treatment [either LP (20 mg of H_2O_2 /kg) or HP (250 mg of H_2O_2 /kg)], no LP activity was detected after 30 min. This suggests that bleaching by the LP system was a fast process, occurring within 30 min.

Volatile compound differences indicated that treatment had more of an effect than did temperature, with very few interactions. Octanal and 2,6-nonadienal were affected by temperature, whereas 2-pentylfuran, 1-octen-3-one, octanal, 2,4-nonadienal, 2,5-nonadienal, hexanal, and diacetyl were affected by treatment ($P < 0.05$; Table 1). Only 2 interaction effects (temperature $\times$ treatment) were found: nonanal and heptanal ($P < 0.05$; Table 1). Methional, dimethyl trisulfide, and 1-hexen-3-one were not different between treatments or temperature ($P > 0.05$; Table 1). These volatile compounds have been previously documented to be associated with off flavors in both liquid and dried whey protein (Croissant et al., 2009; Whitson et al., 2010, 2011; Listiyani et al., 2011).

WPC80

Whey protein concentrate 80% did not differ in composition ($3.5 \pm 0.1\%$ ash, $77.1 \pm 0.7\%$ protein, and $4.7 \pm 0.1\%$ fat). Mineral composition was also not different (results not shown). Whey protein concentrate 80% from LP-bleached whey had lower norbixin content than did WPC80 from HP-bleached whey (99.4 vs.

Table 1. Relative abundance ($\mu\text{g/kg}$) of selected volatile compounds in unpasteurized, fat-separated fluid Cheddar whey after bleaching for 1 h¹

Compound	Control 35°C	Control 50°C	HP 35°C	HP 50°C	LP 35°C	LP 50°C
Hexanal	0.146 ^b	0.099 ^b	0.894 ^b	1.08 ^b	4.00 ^a	3.17 ^a
Heptanal	0.110 ^c	0.088 ^c	0.366 ^c	0.476 ^{bc}	2.20 ^a	1.21 ^b
Octanal	0.152 ^c	0.078 ^c	0.221 ^{bc}	0.211 ^{bc}	1.21 ^a	0.658 ^b
Nonanal	0.415 ^{bc}	0.259 ^c	0.384 ^{bc}	0.358 ^{bc}	1.29 ^a	0.759 ^b
2,4-Nonadienal	0.020 ^b	0.020 ^b	0.034 ^b	0.027 ^b	0.137 ^a	0.107 ^a
2,6-Nonadienal	0.043 ^{ab}	0.023 ^b	0.027 ^b	0.022 ^b	0.054 ^a	0.039 ^{ab}
Methional	0.022 ^a	0.018 ^a	0.016 ^a	0.019 ^a	0.016 ^a	0.015 ^a
Dimethyl trisulfide	0.979 ^a	0.960 ^a	0.966 ^a	0.845 ^a	0.973 ^a	0.929 ^a
2-pentylfuran	0.431 ^b	0.451 ^b	1.05 ^b	1.37 ^b	13.3 ^a	11.2 ^a
1-Octen-3-one	0.084 ^b	0.084 ^b	0.198 ^b	0.203 ^b	0.647 ^a	0.450 ^a
1-Hexen-3-one	0.015 ^a	0.164 ^a	0.034 ^a	0.033 ^a	0.095 ^a	0.085 ^a
Diacetyl	0.184 ^a	0.169 ^{ab}	0.116 ^{bc}	0.094 ^c	0.156 ^{ab}	0.161 ^{ab}

^{a-c}Means in a row not sharing a common superscript are different ($P < 0.05$).

¹Control samples received no bleach treatment, HP samples received 250 mg/kg addition of hydrogen peroxide, and LP samples received 20 mg/kg addition of hydrogen peroxide, thus activating the lactoperoxidase (LP) system. Samples were bleached for 1 h at either 35 or 50°C.

Table 2. Hunter color values¹ for dried and rehydrated whey protein concentrate 80% (10% wt/vol)

Treatment ²	Powder			Rehydrated		
	L*	a*	b*	L*	a*	b*
Control	87.4 ^b	−0.7 ^a	22.0 ^a	64.6 ^b	−0.3 ^a	33.2 ^a
HP	92.0 ^a	−0.8 ^a	16.8 ^b	69.7 ^a	−2.3 ^c	27.3 ^b
LP	91.5 ^a	−0.6 ^a	17.8 ^c	70.0 ^a	−1.7 ^b	18.7 ^c

^{a-c}Means in a column not sharing a common superscript are different ($P < 0.05$).

¹L* = luminosity (the degree of lightness from black to white); a* = the degree of redness or greenness; b* = the degree of yellowness or blueness.

²Control samples received no bleach treatment, HP samples received 250 mg/kg addition of hydrogen peroxide, and LP samples received 20 mg/kg addition of hydrogen peroxide, thus activating the lactoperoxidase (LP) system.

48.3% destruction, respectively ($P < 0.05$), consistent with fluid whey trials. Reflectance values in rehydrated WPC80 (Table 2) were consistent with norbixin extraction results. Powdered WPC80 reflectance values were not in agreement with norbixin extraction results (Table 2). This is not unusual, as b* measurements are an indirect measure of all yellow pigments, including naturally present carotenoids in milk and Maillard browning products, which can also contribute to color.

Whey protein concentrate 80% from bleached wheys (either HP or LP) were higher in aroma intensity and cardboard flavor and lower in sweet aromatic and cooked/milky flavors compared with the control WPC80 ($P < 0.05$; Table 3). Bleached whey proteins varied from each other in flavor in that LP-bleached WPC80 contained a very noticeable cabbage flavor, whereas WPC80 from HP-bleached whey displayed a fatty flavor ($P < 0.05$). Volatile results were consistent with sensory results. Whey protein concentrate 80% from either LP- or HP-bleached wheys were higher in hexanal, decanal, 1-hexen-3-one, 2,3-octadienone, benzacetaldehyde, heptanal, octanal, and 2-pentylfuran than the control unbleached WPC80 ($P < 0.05$; Table 4). Whey protein concentrate 80% did not differ in pentanal, DMS, dimethyl trisulfide, sotolon, or diacetyl ($P > 0.05$; Table 4). Whey protein concentrate 80%

from whey bleached with LP was significantly higher in heptanal, octanal, dimethyl disulfide, and 2-pentylfuran than those bleached with HP ($P < 0.05$; Table 4).

DISCUSSION

Liquid Whey

Lactoperoxidase is one of the most heat-stable enzymes, even retaining activity during normal pasteurization of milk (Seifu et al., 2005). Monitoring LP throughout cheesemaking (raw milk, pasteurized milk, whey, and pasteurized whey) showed minimal loss throughout processing, with LP still active in pasteurized whey. Lactoperoxidase constitutes about 1% of whey proteins in raw bovine milk, with reported activity ranging widely from 1.2 to 19.4 U/mL; however, most recent literature in bovine milk points to the average being between 1.5 and 2.7 U/mL (Seifu et al., 2005). Variations of enzyme level in bovine milk can be attributed to the sexual cycle of the cow, season, feeding regimen, and breed (Kussendrager and van Hooijdonk, 2000). Thiocyanate levels were not different between samples or during processing ($P > 0.05$). Thiocyanate levels can vary depending on the feeding regimen of the animal (Reiter and Harnulv, 1984).

Table 3. Descriptive sensory profiles of whey protein concentrate 80% (WPC80)¹

Treatment ²	Aroma intensity	Sweet aromatic	Cardboard	Cabbage	Cooked/milky	Fatty	Astringency
Control	2.1 ^b	1.9 ^a	1.1 ^b	ND ³	2.2 ^a	ND	2.0 ^a
HP	2.8 ^a	0.5 ^b	2.3 ^a	ND	1.5 ^b	1.2	1.8 ^a
LP	2.6 ^a	0.6 ^b	2.2 ^a	2.1 ^a	1.5 ^b	ND	1.8 ^a

^{a,b}Means in a column not sharing a common superscript are different ($P < 0.05$).

¹Attribute intensities were scored on a 0 to 15 point universal intensity scale (Meilgaard et al., 2007). Most dried ingredient flavors fall between 0 and 4 (Croissant et al., 2009; Listiyani et al., 2011).

²Control samples received no bleach treatment, HP samples received 250 mg/kg addition of hydrogen peroxide, and LP samples received 20 mg/kg addition of hydrogen peroxide, thus activating the lactoperoxidase (LP) system.

³ND = not detected.

Table 4. Relative abundance ($\mu\text{g/kg}$) of selected volatile compounds in whey protein concentrate 80% (WPC80)¹

Compound	Control	HP	LP
Pentanal	3.00 ^a	2.84 ^a	3.32 ^a
Hexanal	3.08 ^b	8.06 ^a	6.00 ^a
Heptanal	0.916 ^c	2.77 ^b	4.27 ^a
Octanal	0.481 ^c	1.89 ^b	3.63 ^a
Nonanal	2.87 ^b	4.00 ^{ab}	5.44 ^a
Decanal	0.070 ^b	0.124 ^a	0.131 ^a
Dimethyl sulfide	0.387 ^a	0.371 ^a	0.424 ^a
Dimethyl disulfide	0.131 ^b	0.313 ^b	3.12 ^a
Dimethyl trisulfide	0.483 ^a	0.519 ^a	0.515 ^a
1-Hexen-3-one	0.143 ^b	0.432 ^a	0.460 ^a
2,3-Octadien-one	2.00 ^b	5.09 ^a	5.49 ^a
Benzacetaldehyde	0.470 ^b	1.09 ^a	1.35 ^a
Sotolon	0.051 ^a	0.039 ^a	0.040 ^a
2-Pentyl furan	4.80 ^c	15.9 ^b	49.1 ^a
Diacetyl	0.048 ^a	0.027 ^a	0.040 ^a

^{a-c}Means in a row not sharing a common superscript are different ($P < 0.05$).

¹Control samples received no bleach treatment, HP samples received 250 mg/kg addition of hydrogen peroxide, and LP samples received 20 mg/kg addition of hydrogen peroxide, thus activating the lactoperoxidase (LP) system.

The bleaching of whey by LP has not been well documented. Bottomley et al. (1989) found decolorization of whey to be between 50 and 85%, depending on the amount of SCN⁻, HP, and LP. The level of bleaching achieved in this study was >99%, suggesting that the LP system was operating under optimal conditions. Previously, LP activity had been reported to be optimal between 11 and 15 mg/kg (Bottomley et al., 1989), which is in general agreement with the curve generated in this paper, as LP is known to vary slightly, which was previously discussed (Figure 1).

Previous research has not reported the volatile compounds produced specifically from the bleaching of whey using the LP system. Bleaching whey (by either H₂O₂ or benzoyl peroxide) increased lipid oxidation compounds (Croissant et al., 2009; Listiyani et al., 2011, 2012). Bleaching whey via the LP system yielded levels of hexanal, heptanal, octanal, nonanal, 2,4-nonadienal, and 2,6-nonadienal higher than unbleached wheys ($P < 0.05$) and concentrations similar to or higher than concentrations found in wheys with chemical bleaching (Table 1), suggesting that LP catalyzes lipid oxidation. The LP system generates OSCN⁻ and ferrous iron, not only allowing for bleaching efficacy from OSCN⁻, but also from Fenton-type reactions that may further participate in bleaching efficacy and possible side reactions such as lipid oxidation. Lipid oxidation compounds are the source of fatty and cardboard off-flavors in dried whey proteins (Wright et al., 2009; Whitson et al., 2010). Further, lipid oxidation products formed in fluid whey contribute to these off-flavors in spray-dried whey

proteins (Croissant et al., 2009; Listiyani et al., 2011; Whitson et al., 2011).

WPC80

In agreement with the first part of the study, WPC80 from whey bleached using the LP system contained <1% residual norbixin (more norbixin destruction than those samples from whey bleached by HP). Reflectance values were consistent with norbixin destruction values in that LP bleached better than H₂O₂ alone ($P < 0.05$). Whey products devoid of any color are highly desirable (Kang et al., 2010).

Bleaching and bleach type have been previously shown to negatively affect the flavor of whey and spray-dried whey proteins (Croissant et al., 2009; Listiyani et al., 2011, 2012). Lipid oxidation products are the primary off-flavors found in dried whey proteins (Whitson et al., 2010, 2011) and contribute to undesirable off-flavors. Consistent with previous studies, bleached whey and WPC80 from bleached whey had higher concentrations of lipid oxidation products (such as aldehydes), cardboard flavor, and higher aroma intensities compared with unbleached whey and WPC80 from unbleached whey ($P < 0.05$). Source of bleaching also influenced flavor. Whey protein concentrate 80% from HP-bleached whey displayed a fatty flavor, whereas WPC80 from LP-bleached whey contained cabbage flavor. In addition, WPC80 bleached with LP were higher in heptanal, octanal, dimethyl disulfide, and 2-pentylfuran than those bleached with HP ($P < 0.05$). Bleaching with LP resulted in a whiter product (more norbixin destruction) than chemical bleach (HP), but also resulted in increased off-flavors compared with unbleached WPC80. The high amount of norbixin destruction (>99%) using LP suggests that conditions might be optimized to decrease the amount of norbixin destruction while minimizing flavor contributions or to use LP in combination with another bleaching method.

CONCLUSIONS

The lactoperoxidase system has long been used in the dairy industry as an antimicrobial in raw milk but shows other potential applications, such as the bleaching of fluid whey. The LP system was activated by the addition of 10 to 40 mg of H₂O₂/kg, with an optimal concentration of 20 mg/kg. Compared with HP bleaching, enzymatic bleaching was more effective in norbixin destruction, bleaching >99%, in both fluid whey and WPC80. Lipid oxidation products were higher in concentration in bleached wheys and WPC80 (either HP or LP) than in unbleached wheys or WPC80. Concur-

rently, cardboard flavors were also increased in HP- or LP-bleached WPC80. These results support that sufficient LP bleaching may be obtained in less time, thereby optimizing bleaching efficacy while decreasing volatile loads and optimizing flavor. Future experiments should focus on the optimization of enzymatic bleaching in both fluid whey and liquid retentate.

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