JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Enrichment of Cheeses Manufactured from Cow's and Sheep's Milk Blends with Sheep-like Species-Related Alkylphenols

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Enhancement of concentrations of species-related sheep-like alkylphenols, *p*- and *m*-cresols and 3- and 4-ethylphenols, in experimental Manchego-type cheeses manufactured from cow's and sheep's milk blends (80:20) by using arylsulfatases was investigated. A food-grade arylsulfatase from *Aspergillus oryzae* (ATCC 20719) was produced using a stimulatory medium, and crude dried cells were used as the enzyme source. Exogenous arylsulfatases from *Helix pomatia* and *A. oryzae* were added to cheese curd, and the amounts of species-related alkylphenols were measured. Arylsulfatase from *H. pomatia* released limited amounts of alkylphenols in the cheese only when used at a high level. Arylsulfatase from *A. oryzae* released substantial amounts of alkylphenols during 2 months of ripening. The concentrations of alkylphenols in *A. oryzae* arylsulfatase-treated cheese were comparable to the previously reported levels present in aged Manchego-type cheeses manufactured from pure sheep's milk.

**KEYWORDS: Cheese; blended milk; enrichment; sheep-like; alkylphenols**

## INTRODUCTION

Sheep's milk has a unique flavor as compared to cow's and other ruminant species' milks, which is utilized mostly for production of specialty cheeses, such as feta, Roquefort, and Manchego, in various countries (*1*). Sheep's milk has higher dry matter content (16−20%) than cow's milk (11−13%; ref *2*). The average lactation period of sheep is 4−6 months, which is shorter than the average for cows that have a lactation period of 10 months. The milk yield per sheep is also less than that per cow. Because of its high dry matter content and scarcity throughout much of the year, sheep's milk is more expensive than cow's milk.

Freezing and holding at about −20 °C is a common practice to preserve sheep's milk and increase its availability throughout the year (*3*). Another practice to utilize sheep's milk is blending with cows' milk in processing, which also reduces the cost of manufacture and aids in meeting the continuing demand for sheep's milk-containing cheeses (*4*). However, blending leads to the dilution of characteristic flavor compounds of sheep's milk, and a reduction or loss of flavor identity in the resulting cheese commonly occurs (5).

Sheep's milk flavor has been attributed to volatile branched-chain fatty acids and alkylphenols, which differ in identity and distribution from cow's and other ruminant species' milks (*6*, *7*). Additionally, commercial specialty cheeses produced from sheep's milk have been characterized in part by these species-related flavor compounds (*8*, *9*). Alkylphenols contribute to the flavor at low concentrations as reflected by their low (ppb) threshold levels (*10*). 3-Ethylphenol and 4-ethylphenol were found to exhibit sheep- and sheep pen-like flavors, respectively (*10*). *p*-Cresol was reported to provide an animal-like flavor in pure form (*10*) and a sheepyard flavor note at a concentration of 100 ppb in a deodorized model butter (*11*). *m*-Cresol was reported to provide a sweetness and fullness at a concentration of 200 ppb along with *p*-cresol at a concentration of 2 ppb in a deodorized model butter (*11*). Alkylphenols were found to contribute to sheep-like flavors of the cheeses manufactured from sheep's milk (*5*, *8*, *9*).

Manchego-type cheeses manufactured from blended cow's and sheep's (80:20) milk have been found to contain substantially lower amounts of species-related alkylphenols as compared to cheeses from pure sheep's milk (*5*). Conjugated alkylphenols have been shown to be abundant in the skim milk (aqueous) fraction, whereas free alkylphenols partition into milk fat, and they have been associated mostly with the cream (fat) phase of milk (*6*, *12*−*15*). Therefore, it can be postulated that the flavor of cheeses manufactured from blended cow's and sheep's milk could be intensified by the utilization of the precursor reservoirs present as conjugated alkylphenols in milk.

Metabolically conjugated alkylphenols in milk have been readily released from conjugates for analysis as free phenols by suitable commercial hydrolytic enzymes, including acid phosphatase, arylsulfatase, and $\beta$-glucuronidase. Furthermore, species-related alkylphenols in sheep's skim milk have been found to be mostly conjugated with sulfate with minor amounts associated with glucuronide and phosphate conjugates (*16*). Milk also contains native conjugases that might be utilized for the hydrolysis of conjugated alkylphenols during processing and

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10.1021/jf0484834 CCC: $30.25 
Published on Web 02/05/2005

ripening of cheese (*17*, *18*). However, the levels of these enzymes were found insufficient to release alkylphenols from their conjugates (*10*).

Considering the current knowledge of the identity and distribution of conjugated species-related alkylphenols in sheep's milk, it was hypothesized that this information could be utilized for the development of technologies for the intensification of flavors in sheep's milk cheeses. Thus, the objective of this research was to investigate the utilization of arylsulfatase for intensification of the sheep-like flavors that would compensate for the flavor losses encountered in cheeses manufactured from cow's and sheep's milk blends.

## MATERIALS AND METHODS

**Materials.** Raw sheep's milk was obtained from Spooner Agricultural Research Station, University of Wisconsin−Madison (Spooner, WI). Milk was frozen and kept at −23 °C in the freezer until it was used for cheese making. Raw cow's milk was obtained from the Dairy Processing Plant of University of Wisconsin−Madison. Arylsulfatase from *Helix pomatia* (Type H-5, EC 3.1.6.1) and *p*-nitrophenyl sulfate were obtained from Sigma Chemical Co. (Milwaukee, WI). All solvents were HPLC grade.

**Preparation of a Food-Grade Arylsulfatase from *Aspergillus oryzae*.** A culture of *A. oryzae* (ATCC 20719, U.S. Patent 4 636 468; ref *19*) on a YM Agar slant was obtained from ATCC (Rockville, MD). The organism was grown on potato dextrose agar (PDA) slants with biweekly transfers of mycelia onto fresh media and incubation at 24 °C until sufficient sporulation was achieved. Cultures were stored on PDA slants covered by twice-autoclaved (121 °C for 15 min each) mineral oil at 24 °C. The morphology and sporulation of the organism were observed under a microscope by using lactophenol cotton blue stain (*20*).

The synthetic medium used by Burns and Wynn (*21*) was utilized as a base medium for broth cultures. The base medium contained 0.1 M NaCl, 0.05 M $NH_4Cl$, 1 mM $MgCl_2$, 1 $\mu$M $CaCl_2$, and 30 g of sucrose in 1 L of 0.1 M $KH_2PO_4$ in double-distilled water, which was adjusted to pH 7 with 0.1 M $K_2HPO_4$. Since this medium was found insufficient for growth, 0.5% $MgSO_4$ or 2, 5, or 10 mM taurine were supplied to stimulate growth and arylsulfatase production (*22*). The soybean medium used by Arbige and Neubeck (*17*) was also utilized, which included 6% soybean meal, 5% $(NH_4)H_2PO_4$, 0.5% $MgSO_4$, and 2% soybean oil.

Broth media were prepared in 200 mL quantities. Inoculations were made from PDA slants grown for two weeks at 24 °C. Spores were recovered in 8 mL of Tween-20 (0.025% w/v) by the surface of the slant being slightly scraped with a sterile wire loop. This suspension was transferred into broth in 1.5 mL quantities. Whole mycelia suspensions were used in the production of the enzyme. Inoculated broths were incubated at 30 °C on a reciprocal shaker at 100 rpm for 4 days. Mycelia were harvested by vacuum filtration through Whatman No. 1 filter paper. Mycelia were washed twice with 25 mL of double-distilled water and filtered through Whatman No. 1 filter paper. Mycelia were dried with the addition of 25 mL of dry ice chilled acetone in a cold mortar. Cells were disrupted in the cold mortar with a pestle, and the preparation was separated from the acetone solution by vacuum filtration. The acetone wash was repeated one additional time. Finally, mycelia were dried in aluminum dishes covered with filter paper in a desiccator under vacuum at room temperature for 3 h. Dried mycelia were used as the arylsulfatase source.

Arylsulfatase activity was measured using *p*-nitrophenyl sulfate as a substrate. Ten mg of dried cells in 0.5 mL of 0.1 M Tris-HCl buffer at pH 7.5 were mixed with 0.5 mL of *p*-nitrophenyl sulfate (10 mM in final mixture) solution in the same buffer. The reaction mixture was incubated at 37 °C for 20 min. The reaction was stopped by the addition of 1.5 mL of 0.5 N NaOH. The solutions were clarified by being centrifuged at 7000*g* for 5 min, and absorbances of clear supernatants were read with the spectrophotometer at 410 nm. Control samples were also prepared by the mycelia and substrate being combined after the incubation. Concentrations of *p*-nitrophenol released were calculated from a standard curve prepared by using authentic *p*-nitrophenol.

**Experimental Manchego-Type Cheese Manufacture.** Raw sheep's milk was thawed in a warm water (45 °C) bath and kept at 4 °C overnight for equilibration. Sheep's milk was filtered through a cheesecloth to remove particulate material. Experimental cheeses were manufactured in 7 kg batches from sheep's or blended cow's and sheep's (80:20) milk according to the procedures for Manchego cheese (*23*, *24*). Both sheep's and cow's milks were pasteurized at 63 °C for 30 min. Blended milk was warmed at 45 °C for 15 min by being stirred with a small propeller, and then it was cooled in an ice−water bath to 32 °C. A CH−N 11 specialty cheese culture, which consisted of multiple strains of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococus lactis* subsp. *lactis* biovar. *diacetylactis*, and *Leuconostoc mesenteroides* subsp. *cremoris* (0.15 g/kg milk) and Chy-maxII coagulant (0.1 mL/kg milk; Chr. Hansen's Laboratory, Inc., Milwaukee, WI) were used. Milk was incubated for 45 min at 32 °C after the addition of the starter. After the addition of rennet, milk was set for coagulation until a smooth curd formed (30−40 min). The curd was cut into 4−6 mm cubes with wire knives, and the temperature was increased to 38 °C in 15 min by being heated with manual stirring. After 15 min of holding at 38 °C, the curd was transferred in cheesecloth into plastic cheese molds with a 250 g capacity. Arylsulfatase preparations, including 30 and 300 U from *H. pomatia* and 30 U from *A. oryzae* (ATCC 20719; 0.3 g of dried whole cells), were mixed with 40 mL of distilled water in a sterile test tube and then stirred into 250 g of cheese curd. One U of activity was the amount of enzyme required to release 1 $\mu$mol of *p*-nitrophenyl sulfate per h at pH 7.5 for *A. oryzae* arylsulfatase and *p*-nitrocatechol sulfate at pH 5.0 at 37 °C for *H. pomatia* arylsulfatase. The curd was pressed with a hydraulic press (Carver Laboratory Press, Fred S. Carver, Inc., Summit, NJ) with a 50 kg load for 16−18 h at 20 °C. pH was measured during cheese making to maintain consistent manufacturing conditions for each batch of cheese. Cheeses had a pH of 5.2 after the manufacture.

After being pressed, cheeses were salted in brine (23% (w/v) NaCl in distilled water) for 40 min. Cheeses were then vacuum-packed in barrier pouches (0.5 mil polyester/2 mil polyethylene, Curwood Inc., New London, WI) and ripened for 2 months in a temperature-controlled room at 8−10 °C. Each batch contained two treated and two control cheeses. Samples of cheeses were kept at −80 °C in a freezer until analyses of alkylphenols and arylsulfatase activity.

**Analysis of Arylsulfatase Activity in Cheeses.** Arylsulfatase activity in enzyme-treated cheeses was determined at the beginning and end of ripening. Shredded cheese (5 g) was homogenized with a tissuemizer (Tekmar, Cincinnati, OH) in 10 mL of cold double-distilled water in an ice−water bath. The mixture was centrifuged at 15000*g* for 15 min at 4 °C. The aqueous phase without the fat layer was filtered through Whatman No. 4 filter paper. Filtrate (0.2 $\mu$L) was mixed with 0.3 mL *p*-nitrophenyl sulfate (10 mM in final mixture) in 0.2 M acetate buffer at pH 5. After the mixture was incubated at 50 °C for 1 h, the reaction was stopped with the addition of 1 mL of 1 N NaOH. Hydrolyzed *p*-nitrophenol was extracted by addition of 1 mL of chloroform and by being vortexed. The mixture was centrifuged at 4000*g* for 10 min to separate phases. Hydrolyzed *p*-nitrophenol in the chloroform phase was quantified by measurement of absorbance at 410 nm (DU-65 spectrophotometer, Beckman Instruments, Inc., Fullerton, CA). Concentrations were calculated based on a standard curve prepared by using *p*-nitrophenol in the enzyme assay.

**Analysis of Alkylphenols in Cheeses.** Volatile free alkylphenols were measured before and after ripening for 2 months according to the procedures of Han (*14*) and Zeng (*15*) with some modifications. Cheese shredded with a hand shredder (50 g) and 10 $\mu$L of *o*-cresol (10 mg/100 mL methanol; Aldrich Chemical Co., Milwaukee, WI) as an internal standard were combined. The cheese sample was then probe-homogenized after the addition of 100 mL of saturated NaCl solution in distilled water. Volatile free alkylphenols contained in fat were extracted by the addition of 50 mL of diethyl ether. After manual shaking, the mixture was centrifuged at 6000*g* at 10−15 °C for 30 min (Sorvall refrigerated centrifuge, DuPont Inst., Des Plaines, IL). The diethyl ether layer was removed, and the extraction was repeated for a second time with an additional 50 mL of diethyl ether. Each fatty

extract was concentrated by ether being removed using a rotary vacuum evaporator operated at room temperature. Then, each fatty extract was mixed with 30 mL of saturated NaCl solution and distilled for 1.5 h by using a microsimultaneous distillation and extraction apparatus (*25*). Volatile free alkylphenols were extracted into 5 mL of diethyl ether in the receiver flask. Each extract was dried with the addition of excess anhydrous $Na_2SO_4$ and then was concentrated under a slow stream of nitrogen to near dryness.

Each concentrate was then neutralized by addition of 100 $\mu$L of 0.2 M $NaHCO_3$, and the pH was adjusted to 11−12 by using 50 $\mu$L portions of 1 N NaOH monitored with pH indicator papers (Fisher Scientific, Itasca, IL). The concentrate was buffered with 100 $\mu$L of 0.2 M phosphate buffer at pH 12, and alkylphenols were dansylated by the addition of 600 $\mu$L of dansyl chloride (5 mg/mL acetone; 5-(dimethylamino)-naphthalene-1-sulfonyl chloride, Aldrich Chemical Co., Milwaukee, WI). The dansylation took place at 45 °C in the dark for 20 min. After excess dansyl chloride was hydrolyzed with 3 drops of 1 N NaOH, dansylated alkylphenols were extracted into 350 $\mu$L of hexane by being vortexed and centrifuged at 4000*g* for 1 min. Hexane extracts were filtered through 0.2 $\mu$m nylon filters (Acrodisc, Gelman Sciences, Fisher Scientific, Itasca, IL) before HPLC analysis. After concentration under a slow stream of nitrogen if necessary, 20 $\mu$L of each extract was used for HPLC analysis.

Normal phase HPLC was performed using a LichrosorbSi 60 column (5 $\mu$m, 250 × 4 mm i.d., Alltech Assoc., Inc., Deerfield, IL). The HPLC system included a Beckman 110B solvent delivery module (Beckman Instruments, Inc., Berkeley, CA) and a 20 $\mu$L sample loop. The mobile phase was 1.6% acetone in 2,2,4-trimethylpentane at a flow rate of 1.5 mL/min. Detection of dansyl derivatives was carried out with a fluorescence detector (Waters Model 420; Waters Associates, Inc., Milford, MA), which was set at an excitation wavelength of 360 nm and an emission wavelength of 425 nm and a gain setting of 8. Chromatograms were recorded using a Spectra-Physics Model 4200 computing integrator (Spectra-Physics, San Jose, CA) with a chart speed of 0.5 cm/min. Alkylphenols in chromatograms were identified by coincidence of retention times between unknown and authentic compounds. Concentrations were calculated based on the internal standard by using recoveries and response factors for individual compounds (*14*). Since *p*- and *m*-cresols and 3- and 4-ethylphenols were coeluted, these two compounds were reported together. All analyses were carried out in duplicate.

## RESULTS AND DISCUSSION

**Arylsulfatase Production by *A. oryzae*.** The strain of *A. oryzae* (ATCC 20719) used in these studies sporulated reluctantly even though different slant media, including PDA and YM Agar, were evaluated. Therefore, mycelial growth was limited upon transfer of spores into the fermentation broth. However, the amount of mycelia obtained was sufficient for laboratory research purposes. Further research is needed to identify conditions to obtain more mycelial mass for use in large-scale fermentations.

The organism was initially grown in a soybean medium that included soybean meal, soybean oil, and $MgSO_4$, which was the medium used by Arbige and Neubeck (*19*) for lipase production. Although the organism produced a large mycelial mass in this medium, low levels of arylsulfatase activity were obtained (**Table 1**). Use of inorganic sulfate in the growth media has been reported to repress the synthesis of arylsulfatase in bacterial as well as fungal species (*22*, *26*−*28*). Therefore, the synthetic media without a sulfur source used by Burns and Wynn (*21*) for producing arylsulfatase from *A. oryzae* was utilized in subsequent experiments. Even though this media was reported to be sufficient for growth of other strains of *A. oryzae*, this particular strain did not grow well in this media, and $MgSO_4$ was added back to the synthetic media to promote the growth. But, in this case, growth was obtained without an increase in the amount of arylsulfatase activity.

**Table 1.** Arylsulfatase Activity of *A. oryzae* (ATCC 20719) Grown in Different Media

| culture media | weight of dried cells (g) | arylsulfatase activity[a] ($\mu$mol/h g of dried cells) |
|---|---|---|
| soybean media[b] | 0.21 | 2.7 |
| control[c] + $MgSO_4$ (0.5%) | 0.25 | 1.3 |
| control + 2 mM taurine | 0.24 | 105.0 |
| control + 5 mM taurine | 0.25 | 107.1 |
| control + 10 mM taurine | 0.25 | 108.5 |

[a] Arylsulfatase assay was carried out using 10 mM *p*-nitrophenyl sulfate at pH 7.5 in 0.1 M Tris-HCl buffer at 37 °C for 20 min or 1 h depending on the activity present. [b] 6% soy bean meal, 5% $(NH_4)H_2PO_4$, 0.5% $MgSO_4$, and 2% soy bean oil by weight (*19*). [c] Control synthetic media (200 mL) included 0.1 M NaCl, 0.05 M $NH_4Cl$, 1 mM $MgCl_2$, 1 $\mu$M $CaCl_2$, and 30 g of sucrose in 1 L of 0.1 M $KH_2PO_4$ in double distilled water at pH 7 with 0.1 M $K_2HPO_4$ (*21*).

Earlier researchers had reported the use of sulfate-limited media and tyramine to induce arylsulfatase synthesis in some bacterial species (*21*, *27*, *29*, *30*). Therefore, tyramine was substituted for $MgSO_4$ in the synthetic medium in attempts to obtain higher levels of arylsulfatase, but the organism did not grow in this medium. Even though the synthesis of arylsulfatase has been reported to increase in the presence of tyramine in bacterial species, only in vitro activity was enhanced in the presence of tyramine for fungi (*31*). The strain of *A. oryzae* in the present study was insensitive to tyramine, and the results also indicated that this particular strain of *A. oryzae* required sulfur for growth.

Taurine was used as a sulfur source based on the reports for its induction of arylsulfatase synthesis in different fungi, including *A. oryzae* (*22*). Addition of taurine to the synthetic media allowed growth of the fungi and synthesis of arylsulfatase. Use of taurine in the synthetic media increased the arylsulfatase activity obtained 40- and 80-fold as compared to that obtained from the soybean media and the use of $MgSO_4$ along with the synthetic media, respectively. The yield of mycelia was equal for each media used in the study. Thus, taurine was effective in supplying the necessary sulfur for growth but also in induction of the synthesis of arylsulfatase. Since increases in the concentration of taurine beyond 5 mM did not produce additional increases in the synthesis of arylsulfatase, taurine was used at a concentration of 5 mM in subsequent experiments. The role of taurine has been claimed to be its ability to serve as an indirect intermediate in the pathway of sulfate assimilation to cysteine (*22*). On the other hand, direct intermediates, such as inorganic sulfate, cysteine, and thiosulfate, have caused repression of arylsulfatase synthesis.

**Use of Arylsulfatases in Cheeses.** To determine if arylsulfatases were able to resist destruction in cheese during ripening, arylsulfatase activity in aqueous extracts of the experimental cheeses was also measured at the beginning and after 2 months of ripening. The enzymes were retained in the cheeses to a significant extent as they were added to the cheese curd (**Table 2**). Arylsulfatases remained active during ripening even though both *H. pomatia* (300 U) and *A. oryzae* enzymes apparently lost some activity after 2 months.

***H. pomatia* Arylsulfatase.** The addition of 30 U of *H. pomatia* arylsulfatase in 250 g of cheese curd before being pressed did not consistently result in the release of additional amounts of alkylphenols as compared to the amounts present in control cheeses (**Table 3**). Of the small amounts observed, only the 3- and 4-ethylphenols levels were about 2-fold higher in treated cheese as compared to the levels in control cheeses. Arylsulfatase from *H. pomatia* fits the arylsulfatase type II

**Table 2.** Activities of Arylsulfatases in Experimental Manchego-Type Cheeses Manufactured from Blended Cow's and Sheep's Milk (80:20)

| arylsulfatase source[b] | activity ($\mu$mol/h 250 g of cheese)[a] | |
|---|---|---|
| | day 1 | day 60 |
| *H. pomatia* (30 U) | 9.0 | 9.6 |
| *H. pomatia* (300 U) | 34.0 | 27.0 |
| *A. oryzae* (30 U) | 14.8 | 9.6 |

[a] Assay conditions: *p*-nitrophenyl sulfate (10 mmol/kg of cheese), pH 5, 50 °C for 1 h. [b] Enzymes were added to 250 g of cheese curd. 1 U is the amount of enzyme required to release 1 $\mu$mol of *p*-nitrophenyl sulfate per h at pH 7.5 and 37 °C for *A. oryzae* arylsulfatase or *p*-nitrocatechol sulfate at pH 5.0 and 37 °C for *H. pomatia* arylsulfatase.

**Table 3.** Concentrations of Alkylphenols in Manchego-type Cheeses Manufactured from Blended Cow's and Sheep's Milk (80:20) by Using Arylsulfatase

| arylsulfatase source[b] | concentration[a] (ppb) | | | | | |
|---|---|---|---|---|---|---|
| | 3/4-ethylphenol[c] | | *p*/*m*-cresol[c] | | phenol | |
| | day 1 | day 60 | day 1 | day 60 | day 1 | day 60 |
| control | 1.2 c | 0.9 b | 4.2 b | 7.7 b | 3.5 a | 10.3 b |
| *H. pomatia* (30 U) | 2.0 c | 1.6 b | 4.3 b | 10.7 b | 4.4 a | 13.5 b |
| *H. pomatia* (300 U) | 8.9 b | 7.1 b | 12.9 b | 20.8 b | 3.3 a | 10.9 b |
| *A. oryzae* (30 U) | 24.1 a | 54.0 a | 146.1 a | 597.3 a | 8.2 a | 37.6 a |

[a] Means with different letters in the same column are significantly different ($P < 0.05$). [b] Enzymes were added to 250 g of cheese curd. 1 U is the amount of enzyme required to release 1 $\mu$mol of *p*-nitrophenyl sulfate per h at pH 7.5 and 37 °C for *A. oryzae* arylsulfatase or *p*-nitrocatechol sulfate at pH 5.0 and 37 °C for *H. pomatia* arylsulfatase. [c] Compounds coeluted.

category, which includes enzymes that have higher affinity toward *p*-nitrocatechol sulfate than for *p*-nitrophenyl sulfate, and which are also distinctly inhibited by phosphate ions (*32*). Since alkylphenol conjugates in milk are somewhat structurally similar to *p*-nitrophenyl sulfate, their hydrolysis by *H. pomatia* arylsulfatase might be predicted to be limited. However, as the enzyme activity measurements in cheeses indicated, *H. pomatia* arylsulfatase was capable of releasing free phenol from *p*-nitrophenol sulfate if the concentration of the substrate was at sufficiently high levels.

Arylsulfatase from *H. pomatia* has also been shown to be inhibited by major ions present in cheese, including phosphate, calcium, and sodium (*5*). Phosphate ions at a concentration of 50 mM at pH 5 inhibited the enzyme by 35%, and inhibition was 80% when the concentration was increased to 300 mM. Phosphate ions per kilogram of expressed aqueous cheese liquid from cheddar cheese aged for 1 month has been reported to be about 50 mmol (*33*). Therefore, sufficient amounts of phosphate ions were present in the aqueous phase of the experimental cheese to contribute a suppressive effect on the activity of *H. pomatia*. Calcium and sodium ions at concentrations commonly found in cheese (200 and 350 mM) also inhibited *H. pomatia* arylsulfatase by 33 and 15%, respectively (*5*). Dodgson and Powell (*32*) also earlier reported the inhibition of 80% of the activity of purified arylsulfatase from *H. pomatia* in the presence of 25 mM $Na_3PO_4$. Thus, it can also be concluded that the cations occurring in cheese also contribute to the suppression of *H. pomatia* arylsulfatase activity in the cheese matrix.

Although nearly complete inhibition of *H. pomatia* arylsulfatase was observed with the addition of 30 U of enzyme to cheese, the addition of 300 U of *H. pomatia* enzyme resulted in the release of notable amounts of alkylphenols as compared to concentrations found in control cheeses. Concentrations of *p*- and *m*-cresols were about 3-fold higher in 300 U of *H. pomatia* arylsulfatase-treated cheeses at the beginning and end of ripening as compared to those in control cheeses. In contrast, substantial amounts of 3- and 4-ethylphenols were released early in the ripening. The cause of this observation was not readily apparent, but the effects of inhibitory agents (e.g., calcium and phosphate ions) upon arylsulfatase in the compact structure of cheese after being pressed possibly may have been involved.

***A. oryzae* Arylsulfatase.** Arylsulfatase from *A. oryzae* performed better in releasing alkylphenols as compared to that from *H. pomatia*. Other studies had shown that the arylsulfatase from *A. oryzae* was more tolerant of the cheese environment (24% inhibition at 300 mM phosphate and no inhibition by sodium and calcium) than arylsulfatase from *H. pomatia* (*5*). The *A. oryzae* arylsulfatase-treated cheese contained 20- and 36-fold higher concentrations of 3- and 4-ethylphenols and *p*- and *m*-cresols, respectively, during the curd-pressing period (16−18 h) as compared to those in the control cheese. The concentrations of the alkylphenols increased in *A. oryzae* arylsulfatase-treated cheeses after 2 months of ripening. Concentration of phenol was also higher in the *A. oryzae* enzyme-treated cheese after ripening as compared to the control cheese.

The strain of *A. oryzae* (ATCC 20719) employed in this study was used by earlier researchers in the commercial production of an enzyme preparation called Flavor Age for use in accelerated ripening of cheese, especially Cheddar cheese (*19*). *A. oryzae* (ATCC 20719) provided both proteinases and peptidases that contributed to cheese ripening (*34*) but also uniquely produced a lipase that selectively enhanced the release of hexanoic and octanoic acids while suppressing the release of excessive amounts of butyric acid that cause undesirable rancid flavors in cheese (*19*). Fernandez-Garcia et al. (*24*, *35*) found that Flavor Age enzyme preparation from *A. oryzae* also accelerated ripening and enhanced the flavor of Manchego-type cheese manufactured from a cow's and sheep's milk blend. Flavor Age has also been utilized successfully in several other studies on accelerated ripening of cheese, including those by Guinee et al. (*36*), Shendy et al. (*37*), and Brandsma et al. (*38*). Rajesh and Kanawjia (*39*) reported that Gouda cheese manufactured from buffalo milk using Flavor Age exhibited the flavor of 3 month old cheese in only 1 month. *A. oryzae* (ATCC 20719) grown in the medium reported by Arbige and Neubeck (*19*) possessed only weak arylsulfatase activity, but by introducing taurine into the medium, a uniquely high arylsulfatase activity was produced in this study. Comparison of the amounts of enzymes between current and previous published studies was not possible, but it is theoretically possible that arylsulfatase might have contributed to some extent to the flavors of Flavor Age-treated cheeses in the earlier studies.

When the concentrations of alkylphenols in the experimental cheeses were compared, the cheese produced with *A. oryzae* arylsulfatase contained significantly higher levels of 3- and 4-ethylphenols and *p*- and *m*-cresols than the other cheeses ($P < 0.05$). Even though *H. pomatia* arylsulfatase at the 300 U level released these alkylphenols in the cheese, the difference as compared to the control cheese was not statistically significant. The method used to quantify alkylphenols at low levels led to a coefficient of variation in the range of 10−20%, and this possibly hindered the differences in the concentrations of alkylphenols between control and *H. pomatia* arylsulfatase-treated cheeses. Concentration of phenol was not different in the cheeses at the beginning of ripening, but *A. oryzae*

arylsulfatase-treated cheese contained higher levels of this compound as compared to the other cheeses after 2 months of ripening.

The taste threshold concentrations of *p*-cresol and 4-ethylphenol have been reported to be 2 ppb in water (300 ppb in Gouda cheese) and 100 ppb in water, respectively (*8*). The concentrations of the alkylphenols were lower than their respective threshold levels in both cheeses containing *H. pomatia* arylsulfatase. However, arylsulfatase from *A. oryzae* released flavor active amounts of *p*- and *m*-cresol in the cheese after 2 months of ripening. Even though substantial increases in the concentrations of 3- and 4-ethylphenols were obtained by *A. oryzae* arylsulfatase treatment of the cheese, the levels of these compounds were lower than the reported threshold concentration. However, concentrations of 3- and 4-ethylphenols and *p*- and *m*-cresols were 2.5- and 5.6-fold higher, respectively, in the blended milk experimental cheeses after 2 months of ripening as compared to the highest concentrations found in commercial aged Manchego-type cheeses manufactured from pure sheep's milk reported by Kılıç (*5*). Gallois and Langlois (*40*) also reported 20−30 ppb of 4-ethylphenol in Roquefort cheeses produced from sheep's milk aged for 210 days, and these concentrations were similar to those present in *A. oryzae* arylsulfatase-treated experimental Manchego-type cheese in this study.

Informal sensory assessments of the cheeses were carried out by the authors. The flavor of the cheese produced with *A. oryzae* arylsulfatase was sheepyard-like at the beginning of ripening because of high amounts of alkylphenols, but it lacked balanced cheese-like flavors. Concentrations of *p*- and *m*-cresols at 146 ppb would be expected to contribute sheepyard-like flavors in the cheese after being pressed, but other cheese-like flavors were developed later in the ripening that suppressed sheepyard-like flavors after 2 months of ripening. The levels of *p*- and *m*-cresols appeared to be important in producing desirable flavors in the cheeses. Dunn and Lindsay (*41*) using imprecise gas chromatographic analytical techniques estimated that *p*-cresol concentrations were generally lower than 100 ppb in clean-flavored Cheddar cheeses, whereas concentrations higher than 100 ppb exhibited unclean and utensil-type flavors. Additionally, Ramshaw et al. (*42*) have reported that even lower concentrations of *p*-cresol and 4-ethylphenol (>20 ppb) resulted in barn-like off-flavors in Cheddar cheese. In general, the flavor of *A. oryzae* arylsulfatase-treated cheese was fuller and more balanced with the contributions of alkylphenols, and perhaps other enzyme-generated flavor compounds (*19*), as compared to the bland flavors of the control cheese.

The results indicated that adjustments in the amounts of arylsulfatase activity and manufacturing procedures would be required to consistently provide desirable levels of alkylphenols in cheese for flavor enhancement. Furthermore, the release of alkylphenols within 2 months of ripening indicated that arylsulfatase from *A. oryzae* could be used in appropriate amounts to augment accelerated ripening of cheeses when used along with proteolytic and lipolytic enzymes.

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**Received for review September 10, 2004. Revised manuscript received December 5, 2004. Accepted December 16, 2004.**

JF0484834