Glutathione Accumulation in Lactococci

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ABSTRACT

Glutathione accumulation was quantified in cell-free extracts prepared from *Lactococcus lactis* ssp. *cremoris* C2 and Z8 cultures grown in milk and milk supplemented with the AA precursors to glutathione or to glutathione. Accumulation of glutathione was detected with Z8 in all three media (5.6, 12, and 470 nmol/mg of protein, respectively); however, no accumulation of glutathione was detected with C2. After growth of these strains in defined broth and defined broth supplemented with glutathione or the glutathione precursors, only the cell-free extract of Z8 grown with glutathione supplement contained detectable amounts of glutathione (372 nmol/mg protein). When [^{35}S]Cys was added to the defined broth containing the glutathione AA precursors, neither strain accumulated [^{35}S]glutathione. Supplementation of the defined broth with [^{35}S]glutathione resulted in accumulation of [^{35}S]glutathione in Z8, but not in C2. Although Z8 can transport glutathione, it is not capable of glutathione synthesis. However, C2 is incapable of both glutathione transport and glutathione synthesis. Additionally, Z8, C2, and a variety of other lactic acid bacteria were examined for γ-glutamyl-transferase activity; no activity was observed with any of the lactic acid bacteria examined.
(**Key words**: glutathione, lactococci, cheese flavor)

Abbreviation key: **GSH** = glutathione; **γ-GTP** = γ-glutamyl-transpeptidase.

INTRODUCTION

Glutathione (**GSH**) accumulation and hydrolysis by lactic acid bacteria is of interest because GSH is thought to play a role in cheese flavor development. Previous investigators demonstrated that the addition of GSH to cheese slurry systems increased bacterial growth, proteolysis, stability and activity of esterases, enhanced formation of methanethiol and hydrogen sulfide, and improved the development of characteristic Cheddar cheese flavor (7, 8, 10, 14). Additionally, incorporation of GSH during low fat cheese manufacture resulted in low fat cheese with higher flavor intensity and improved texture. The improvements in low fat cheese flavor and texture were correlated with faster production of soluble nitrogenous compounds and VFA (4). These results suggest that GSH may play an important role in the development of cheese flavor and that increased accumulation of GSH in low fat cheese may enhance the flavor and texture of these products.

The mechanism by which GSH influences cheese flavor development is unknown; however, possibilities include 1) contribution to the formation of a low redox potential; 2) stabilization of enzymes; 3) function as enzyme cofactors; or 4) function as a precursor for sulfur flavor compounds, such as methanethiol and hydrogen sulfide (14, 17). Results indicating that inhibition of γ-glutamyl transpeptidase (**γ-GTP**) reduced the accumulation of methanethiol and hydrogen sulfide (14) suggest that GSH hydrolysis products may serve as a precursors to these sulfur flavor compounds. Although γ-GTP is naturally present in milk, this enzyme is sensitive to pasteurization (12); therefore, we determined whether cheese-related lactic acid bacteria could serve as a source of this enzyme in the cheese matrix.

We previously demonstrated (6) that lactic acid bacteria differ significantly in their ability to accumulate GSH. Accumulation of GSH was detected in strains of *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*

Received November 3, 1993.
Accepted January 10, 1994.
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biovar. *diacetylactis, Lactobacillus helveticus, Streptococcus salivarius* ssp. *thermophilus,* and *Leuconostoc mesenteroides* ssp. *cremoris.* An examination of the effect of growth conditions on GSH accumulation by *L. lactis* ssp. *cremoris* Z8 indicated that growth medium composition was the most significant factor affecting GSH accumulation. Supplementation of milk with the GSH constituent AA (Glu, Cys, and Gly) increased GSH accumulation 5-fold; supplementation of milk with GSH increased GSH accumulation 95-fold. The enhancement of GSH accumulation as a result of supplementation with the GSH constituent AA suggested that Z8 can synthesize GSH; however, results were similar with *Saccharomyces cerevisiae,* an organism that does not synthesize GSH (1). The 95-fold increase in intracellular GSH concentration of Z8, when it is grown in milk supplemented with GSH, indicates that this organism is capable of transporting GSH.

An objective of this study was to determine whether the mechanism by which GSH is accumulated in lactococci is solely transport of the GSH that is present in the medium or whether lactococci can synthesize GSH.

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains employed in this study, growth conditions, and sources are listed in Table 1. To examine the ability of *L. lactis* ssp. *cremoris* Z8 and *L. lactis* ssp. *cremoris* C2 (previously designated *L. lactis* ssp. *lactis* C2) to synthesize GSH, a defined medium described by Jensen and Hammer (9) and containing 1% glucose was used. Working cultures were prepared from frozen stocks through two transfers in an appropriate medium.

Growth in Skim Milk

Pasteurized, homogenized skim milk (University of Wisconsin-Madison) was centrifuged at 15,300 × *g* for 15 min at 4°C to remove denatured proteins and other materials. For experiments examining the effect of supplements, the following 150-g samples were prepared: 1) unsupplemented milk, 2) milk supplemented with .1 m*M* GSH (final concentration; Sigma Chemical Co., St. Louis, MO), and 3) milk supplemented with .1 m*M* AA (final concentration of each, Glu, Cys, and Gly; Sigma Chemical Co.). Supplements were freshly prepared and filter-sterilized (.45 μm). The milk was inoculated with 2% of a 3-h Elliker broth (3) culture (4% inoculum from an overnight culture) for both strains Z8 and C2 and incubated for approximately 8 h. Before harvesting, the cultured milk was adjusted to pH 7.0 by the addition of 10*N* NaOH, followed by the addition of sodium citrate (10% wt/vol) to a final sodium citrate concentration of 2.5% (wt/wt). Cells were harvested by centrifugation at 15,300 × *g* for 10 min at 4°C, followed by two saline (.85% NaCl, wt/vol, 4°C) washes.

γ-GTP Assay

Cultures to be examined for γ-GTP activity were grown overnight using the conditions described in Table 1. One milliliter of these cultures was harvested by centrifugation at 4°C, washed once with 4°C .1 *M* K_3PO_4 (pH 7.0) buffer, and resuspended in .05 ml of the same buffer. The cell suspension (.02 ml) was then examined for γ-GTP activity using the γ-glutamyl-transferase kit from Sigma Chemical Co. As a blank, .02 ml of the cell suspension was added after addition of 2.0 ml of 1.7*N* acetic acid. Reaction mixtures were centrifuged at 1090 × *g* for 5 min at 20°C to remove the cells prior to determination of absorbance.

Growth in Defined Broth

Defined broth was prepared and filter-sterilized (.45 μm) <16 h prior to use. Sample size and supplements were as described for the milk samples. A 2% inoculum was prepared from a 14-h defined broth culture. The Z8 and C2 cultures were incubated at 30°C until 4 h after the beginning of the stationary phase, 16 and 12 h, respectively. Cells were harvested and washed as described for the milk samples.

For experiments with radioisotopes, approximately 100 μCi of either [^{35}S]GSH (Dupont, NEN Research Products, Boston, MA) or [^{35}S]Cys (Amersham Corp., Arlington Heights, IL) were included in the GSH or AA supplement, respectively. The experiments with isotopes were performed in duplicate.

Quantification of GSH and Cys

Bacterial cells were harvested by centrifugation (9630 × *g*) for 10 min at 4°C. Harvested cells (ca. .75 g of wet weight) were washed once with saline (.85% NaCl, wt/vol) at 4°C and once with deionized water at 4°C. The cell pellet was resuspended in 5 ml of lysis buffer [lysozyme (45,200 units/ml; Sigma Chemical Co.), mutanolysin (200 units/ml; Sigma Chemical Co.), 100 m*M* Tris·HCl, and 5 m*M* EDTA, pH 7.0]. The cell suspension was incubated for 30 min at 37°C. After the addition of SDS (.1% final concentration), incubation was continued at 37°C for 15 min. Bacterial cells were further disrupted by five cycles of freezing and thawing in an acetone-dry ice bath. Cellular debris were removed by centrifugation (27,000 × *g*, 30 min, 4°C), and supernatants were used as cell-free extracts. The protein content in the cell-free extract was determined by the method of Lowry et al. (11) with BSA (Sigma Chemical Co.) as the standard.

TABLE 1. γ-Glutamyl transpeptidase (γ-GTP) activity in *Escherichia coli* DH5α and a variety of lactic acid bacteria.

Strain	Medium[1]	Growth (°C)	γ-GTP (nmol/min)[3]	Source of bacteria[2]
Escherichia coli				
DH5α	LB	20	31	BRL
DH5α	LB	37	20	BRL
Lactococcus lactis ssp. *cremoris*				
Z8	ELL	20	BLD[4]	LLM
Z8	ELL	30	BLD	LLM
C2	ELL	20	BLD	LLM
C2	ELL	30	BLD	LLM
SK11	ELL	20	BLD	LLM
SK11	ELL	30	BLD	LLM
Lactobacillus helveticus				
CNRZ32	MRS	35	BLD	MEJ
CNRZ32	MRS	42	BLD	MEJ
Lactobacillus casei				
JLS20	MRS	20	BLD	SC
JLS20	MRS	37	BLD	SC
Leuconostoc mesenteroides ssp. *cremoris*				
CAFT9	MRS	20	BLD	SC
CAFT9	MRS	30	BLD	SC
ATCC 19254	MRS	20	BLD	SC
ATCC 19254	MRS	30	BLD	SC
Pediococcus pentosaceus				
NCDO 559	APT	20	BLD	SC
NCDO 559	APT	30	BLD	SC
ATCC 25745	APT	20	BLD	SC
ATCC 25745	APT	30	BLD	SC
Streptococcus salivarius ssp. *thermophilus*				
S6	ELL	35	BLD	LLM
S6	ELL	42	BLD	LLM
ST2	ELL	35	BLD	LLM
ST2	ELL	42	BLD	LLM

[1]LB, (13); ELL, Elliker's broth [(3), BBL Microbiology Systems, Cockeysville, MD]; MRS [(2), BBL Microbiology Systems]; and APT (Difco Laboratories, Detroit, MI).

[2]BRL, Bethesda Research Laboratories Inc., Gaithersburg, MD; LLM, L. L. McKay, Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN.; MEJ, M. E. Johnson, Center for Dairy Research, University of Wisconsin-Madison; and SC, stock culture collection.

[3]Expressed as nanomoles of *p*-nitroaniline released per minute.

[4]Below the limit of detection (5 units/ml).

Cell-free extracts were deproteinized by the addition of .25 volumes of a cold (4°C) 320 m*M* sulfosalicylic acid, 28 m*M* L-ascorbic acid, and 4 m*M* EDTA solution. Precipitated proteins were removed by centrifugation (27,000 × *g*, 15 min, 4°C), and the clear supernatants were stored at −70°C.

The GSH and Cys were quantified by reverse-phase HPLC. The deproteinized supernatants were returned to pH 7.0 by the addition of 17 μl of 10*N* NaOH/ml. Neutralized supernatants were added to 30 m*M* Na_3PO_4 buffer, pH 7.0, to a total volume of 1 ml. One hundred microliters of 10 m*M* 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma Chemical Co.) in 30 m*M* Na_3PO_4 buffer, pH 7.0, were added to begin the reaction. Samples were incubated at 25°C for 30 min prior to injection. The derivatized thiol compounds were separated isocratically using 30 m*M* sodium phosphate, pH 7.0, with 1.25% acetonitrile and 3 m*M* sodium azide on a 20-cm Hibar® LiChrosorb® RP-18 (5-μm) column (E. Merck, Darmstadt, Germany) with a flow rate of 2 ml/min at 20°C. The column effluent was monitored at 330 nm. Standards for GSH and Cys (Sigma Chemical Co.) were used to identify and to quantify the GSH and Cys peaks. All values were calculated from duplicate injections of duplicate samples.

Detection of Radioisotopes

The HPLC effluent of ^{35}S-labeled samples was collected for the Cys and the GSH peaks. Two milliliters of each sample were analyzed with a Beckman model LS5801 scintillation counter (Beckman Instruments, Palo Alto, CA). Counts per minute per milligram of protein were calculated for each sample.

RESULTS AND DISCUSSION

The ability of Z8 and C2 to accumulate GSH when they are grown in milk, milk supplemented with the GSH constituent AA, and milk supplemented with GSH was determined. Milk cultures of Z8 and C2 were harvested at pH 5.2 and 5.0, respectively. The amounts of GSH in Z8 cell-free extracts were 5.6, 12, and 470 nmol/mg of protein, respectively. These Z8 results are similar to those reported previously (6). The slightly elevated (2-fold) intracellular GSH accumulation in milk supplemented with the GSH precursors suggests that Z8 synthesized GSH from its constituent AA, that the supplements enhanced GSH transport, or that the supplements inhibited intracellular GSH degradation. The greatly elevated intracellular amounts of GSH (84-fold) in the Z8 culture that was grown in milk supplemented with GSH indicates that Z8 is capable of transporting the GSH that is present in milk. A GSH transport system has been described in *Streptococcus mutans* (16). The amounts of GSH in the C2 cell-free extracts, regardless of which milk-based medium was used, were below the limit of detection (.4 nmol of GSH/mg of protein). These results suggest that C2 is incapable of transport and synthesis of GSH or that GSH is rapidly degraded upon transport or synthesis.

The involvement of the γ-carboxyl group of Glu in the peptide bond with Cys requires a specialized enzyme, γ-GTP, for hydrolysis. As a positive control, a suspension of *Escherichia coli* DH5α was examined for γ-GTP activity; as expected, γ-GTP activity was detected at both incubation temperatures (Table 1). However, because reduction in the growth temperature from 37 to 20°C increased γ-GTP activity 7-fold (15), incubation temperature was expected to result in a greater difference in γ-GTP activity. To examine the ability of Z8 and C2 to degrade GSH, cultures were grown at 20 and 30°C, and the cell suspensions were assayed for γ-GTP activity. Regardless of the incubation temperature, no γ-GTP activity was detected with Z8 or C2 (Table 1), suggesting that neither strain is capable of hydrolysis of GSH. Additionally, because the hydrolysis of GSH may be important in the generation of flavor compounds from GSH in cheese, a variety of lactic acid bacteria were examined for γ-GTP activity. In all cases, γ-GTP activity was not detected (Table 1). The lack of γ-GTP activity in lactic acid bacteria may be particularly important in cheese made from pasteurized milk, because pasteurization inactivates the γ-GTP activity that is naturally present in milk (12).

To examine the ability of Z8 and C2 to transport or synthesize GSH, these organisms were grown in a chemically defined medium, supplemented with the GSH constituent AA or with GSH. Cell-free extracts from these cultures were then examined for GSH accumula-

tion. No accumulation of GSH was observed in C2 when it was grown in the defined medium supplemented with the GSH precursors or with GSH (limit of detection was .4 nmol of GSH/mg of protein). These results and the lack of γ-GTP activity suggest that C2 is incapable of transport and synthesis of GSH. The Z8 culture grown in the defined medium containing GSH accumulated 372 nmol of GSH/mg of protein, which is further evidence that Z8 is capable of transporting GSH. When Z8 was grown in the defined medium supplemented with the GSH precursors, no GSH accumulation was observed (limit of detection was .4 nmol of GSH/mg of protein), suggesting that this strain is not capable of GSH synthesis.

To confirm these results, these cultures were also grown in the defined medium containing [^{35}S]GSH or [^{35}S]Cys and the GSH constitutive AA. The counts per minute per milligram of protein for Z8 and C2 grown in the presence of [^{35}S]Cys were 8.0×10^3 and 9.0×10^3, respectively; these values decreased approximately 10-fold upon deproteinization. These results indicate that both strains were capable of transporting Cys and that the Cys was primarily incorporated into cellular proteins. The counts per minute per milligram of protein for Z8 and C2 grown in the presence of [^{35}S]GSH were 6.3×10^5 and 7.6×10^4, respectively. No significant decrease occurred with the Z8 sample upon deproteinization; however, the C2 sample decreased approximately 10-fold upon deproteinization. These results suggest that the majority of the [^{35}S]GSH present in C2 was bound to proteins, most likely as the result of disulfide bridges with cell-surface proteins; in Z8, the majority of [^{35}S]GSH was not bound to proteins.

The intracellular accumulation of [^{35}S]GSH and [^{35}S]Cys was then determined for these cultures (Table 2). The results lend further support to the hypotheses that C2 is incapable of both GSH transport and synthesis and that Z8 is capable of GSH transport, but not GSH synthesis. The ability of some strains of lactococci to transport GSH, but not to synthesize GSH, is consistent with the findings of Fahey and Sundquist (5) that Gram-positive bacteria do not synthesize GSH but are capable of transporting the GSH that is present in the growth medium. Additionally, the hydrolysis of [^{35}S]GSH to yield [^{35}S]Cys was not observed. These results suggest that no increase in GSH in the cheese matrix occurs via synthesis of GSH by the lactococcal starter. However, the lactococcal starter, if capable of transporting GSH, may influence the partitioning of the native milk GSH between the curds and the whey, thereby enhancing the amount of GSH in the cheese matrix. Once in the cheese matrix, GSH is thought to have a favorable influence on cheese flavor development (7, 8, 10, 14).

TABLE 2. Intracellular amounts of [^{35}S]Cys and [^{35}S]glutathione (GSH) in *Lactococcus lactis* ssp. *cremoris* Z8 and C2 grown in defined medium supplemented with either a mixture of the constituent AA of GSH (Glu, [^{35}S]Cys, and Gly) or [^{35}S]GSH.

	Z8		C2	
Supplement[1]	Cys	GSH	Cys	GSH
	(cpm/mg of protein)[2]		(cpm/mg of protein)[2]	
AA	BLD[3]	BLD	BLD	BLD
[^{35}S]GSH	BLD	2.5×10^5 $\pm 1.4 \times 10^5$	BLD	BLD

[1]Final concentration of each supplemented AA or GSH was .1 m*M*.

[2]Mean of duplicate determinations on two samples.

[3]Below the limit of detection (less than twice the counts in the blank).

CONCLUSIONS

Although lactococci are not capable of synthesis of GSH from its constituent AA, some strains are capable of transporting GSH. The lactococcal transport of GSH may influence the partitioning of the native milk GSH between the curds and the whey, thereby influencing the amount of GSH in the cheese matrix. The GSH in the cheese matrix would then be available to serve as a precursor to sulfur compounds that are thought to be important in the development of Cheddar cheese flavor. Lactic acid bacteria do not appear to express γ-GTP activity; therefore, the native milk γ-GTP, an enzyme which is sensitive to pasteurization, likely is the primary source of this enzyme in the cheese matrix. An explanation for the observed reduction in flavor of cheeses produced from pasteurized milk may be the reduction in γ-GTP activity.

ACKNOWLEDGMENTS

The authors thank James Harper and Leonides Fernández for their helpful suggestions and discussions. This project was supported in part by the College of Agricultural and Life Sciences at the University of Wisconsin-Madison and the Center for Dairy Research through funding from the National Dairy Promotion and Research Board.

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