# Biochemical Aspects of Flavor Development in Cheddar Cheese Slurries

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A cheese slurry system, which permits cheese flavor development in a few days time, was evaluated in respect to its efficacy in studying cheese ripening and as a means of studying biochemical changes during the ripening process. Results revealed that the slurry systems replicated the ripening processes in natural cheeses. Flavor development in cheese appears to be under specific biochemical control in which glutathione plays a multiple role in respect to the slurry system, including the disassociation of peptides, making them more available for proteolytic attack, protection of enzyme groups, and feedback control relationships. Feedback control was found also in respect to diacetyl and acetaldehyde formation, suggesting a number of operative feedback systems. Lipid, protein, and carbohydrate fermentations are interrelated, and the direction of the fermentation can be influenced by the compositional balance of the milk constituents. End product formation is also interrelated, with carbohydrate fermentation being capable of producing fatty acids and amino acids for subsequent alteration.

As indicated by Sandine (1969), cheese flavor is recognized as a balanced blend of fatty acids, organic acids, amino acids, carbonyl compounds, esters, alcohols, and sulfur compounds. The development of cheese flavor is strongly dependent upon the starter microorganisms utilized (Schormüller, 1968) and appears to be under the influence of biological control of interrelated pathways. An understanding of the biochemical control systems significant to cheese flavor development is essential to the systematic control of flavor development by the manufacturer.

Cheese ripening investigations have long been hampered by the long term nature of the experimentation, with a single trial frequently taking 8 months to a year. The utilization of a cheese curd–brine slurry fermentation system that permits the controlled development of characteristic cheese flavor in a few days holds promise of being a powerful tool for facilitating cheese flavor research. This cheese–slurry system, developed at The Ohio State University by Kristoffersen *et al.* (1967), is applicable to a wide variety of cheese types that include Cheddar, Swiss, Romano, and Brick cheese. This paper explores some of the biochemical aspects of the rapid ripening process as related to the development of characteristic flavor.

## PROCEDURE

The slurry process as developed by Kristoffersen *et al.* (1967) was utilized in these investigations. Curd, made by conventional cheese manufacturing procedures, was the starting material. The pH of the curd was permitted to develop to about 5.1. Five hundred grams of curd was mixed with 250 ml of a brine solution, varying in concentration to give a 2 to 5% final salt concentration in the slurry, depending upon the type of cheese flavor being developed. The curd-brine mixture was blended in a high speed mixer to smooth paste, transferred aseptically to a sterile plastic jar, and then incubated in the dark at 30° C. During the fermentation, intermittent control was maintained over the pH and dissolved oxygen. The pH was measured with a glass electrode pH meter and dissolved oxygen was determined with a YSI-oxygen meter. Sodium hydroxide and lactic acid were used to adjust the pH, and desired oxygen content was controlled by changing the extent of agitation of the fermentation mixture. The fermentation rate and reproducibility of flavor development was controlled through the use of chemical additives. Glutathione has been of particular value as an additive and this cannot be substituted by cysteine (Kristoffersen *et al.*, 1967). In these studies slurries were made without and with 100 ppm added glutathione. Flavor evaluation was made of coded samples by a panel of three expert judges; characteristic flavor was scored from 0 (none) to 8 (maximum intensity).

As shown in Figure 1, the addition of glutathione results in more rapid characteristic flavor development. In addition to creating more rapid flavor development, the nature of the fermentation has been found to be much more reproducible in the presence of the glutathione additive at a concentration of 100 ppm. The control cheese slurries may or may not develop desired characteristic flavor, whereas glutathione treated cheese slurries developed characteristic flavor in about 22 of the 25 trials conducted for these investigations. The flavor development generally occurs in four characteristic stages. Between 1 and 2 days there is the appearance of a distinct diacetyl flavor which coincides with the maximum microbial cell population. This is followed by a slight fermented flavor and then a flat flavor. The first recognition of initial development of characteristic flavor generally occurs between the third and fifth day of the fermentation.

**Analytical Methods.** Chemical analyses of the cheese slurries included gas liquid chromatography (glc) of head space volatiles and fatty acid esters, peptide analysis by gel filtration, protein analysis by polyacrylamide electrophoresis, acetaldehyde and diacetyl by chromatography, and oxidized glutathione by enzymatic analysis.

Head space analyses of cheese volatiles were conducted using the head-space analysis method of Bailey *et al.* (1961). Analyses were made by glc under isothermal conditions at 90° C with direct inject of 5 ml gas samples onto a 6 ft column of 15% Carbowax 1500 on Chromosorb W.

Fatty acids were converted to methyl esters by the method by Van Wyngaarden (1967), following separation of the fatty acids from the cheese by silica gel extraction (Harper, 1965). Glc was used to separate the esters, using temperature programming from 100° to 200° C at 25° C per min. The column was a 6 ft 6% diethylene glycol succinate on Diatoport S. Peptides were separated by the method of Lindqvist and Storgards (1962), or by two-dimensional paper chromatography of phosphotungstic acid soluble material. Protein and enzyme separations were made by polyacrylamide electrophoresis (Ornstein and Davis, 1958), using acetone powders of the water extracts of cheese slurries as described by Carmona (1968). Acetaldehyde was measured by the method of Bills

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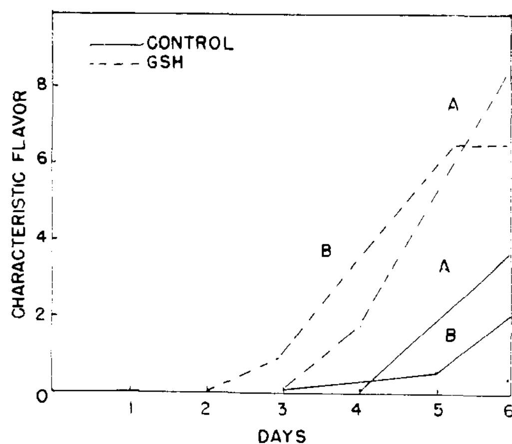


**Figure 1. Effect of glutathione on flavor development in Cheddar cheese slurries in two trials—A and B**

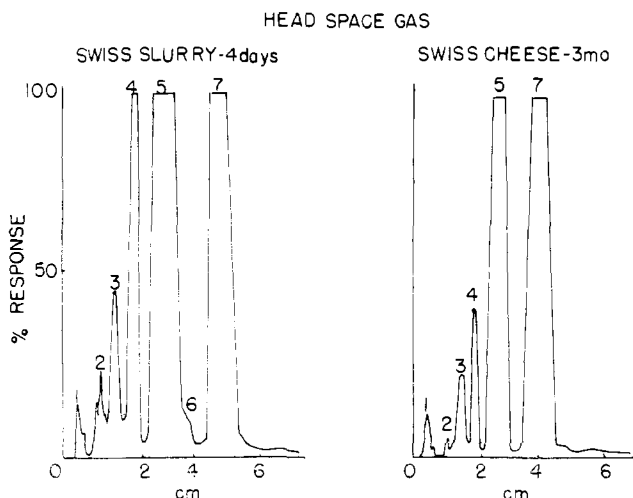


**Figure 2. A comparison of head space gas analyses of Swiss cheese and a Swiss cheese slurry**

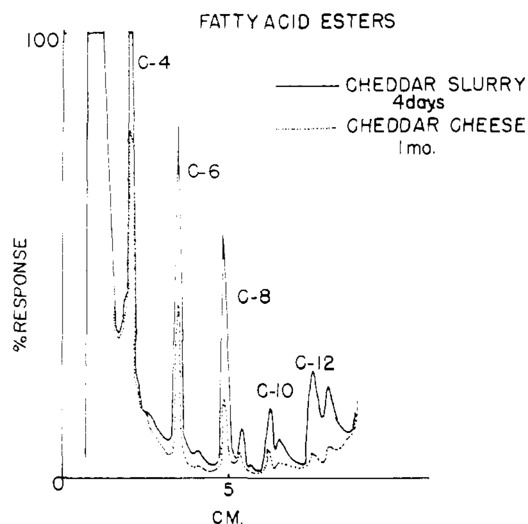


**Figure 3. A comparison of free fatty acid methyl esters of Cheddar cheese and of a slurry made from the same curd**

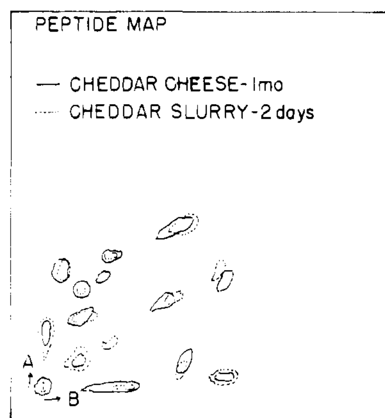


**Figure 4. A comparison of free peptides of Cheddar cheese and of a slurry made from the same curd [A solvent = pyridine: water (1:4); B solvent = butanol: water (1:2)]**

and Day (1966), diacetyl was measured by the method of Pack *et al.* (1965). The enzymatic method of Racker (1963) was used for oxidized glutathione.

## RESULTS

**Slurry Process as an Index of Normal Cheese Ripening.** The utility of the slurry process as a research tool depends on whether or not the fermentation in the slurry process is similar to that which occurs in the natural cheese system. One indication that they are similar has been based upon the fact that development of flavor in the slurry is typically characteristic of the cheese variety being slurried.

Various chemical analyses were made of cheese and slurries to determine the degree of similarity in biochemical changes in the two systems. Typical glc head space gas analysis of Swiss cheese and a Swiss cheese slurry is shown in Figure 2. The chromatographic patterns shown are qualitatively identical and are within the range found in different Swiss cheeses. Fatty acid methyl ester patterns from Cheddar cheese, and from a slurry made from the same curd used in the manufacture of the cheese itself, are shown in Figure 3. The patterns are visually the same, and calculations of the ratios of the individual fatty acid esters reveal that the ratios of the different fatty acid components are essentially identical in the two systems. Peptide maps obtained by two-dimensional paper chromatography of deproteinized water soluble extracts of Cheddar cheese and slurry made from the same curd are presented in Figure 4. Although different in slight details, the chromatograms are quite similar. These data, illustrative of our results, permit us to make the conclusion that the slurry fermentation and normal cheese fermentation are very similar, and thus we are justified in using the slurry process in studying flavor and biochemical processes in cheese ripening.

**The Role of Glutathione in the Ripening Process.** Glutathione is recognized as an ubiquitous material which serves multiple functions in cell biochemistry. These include the reduction of sulfide groups in proteins, protection of sulfhydryl enzymes, cofactor relationships, and implication in nucleic acid control systems of cells.

In the cheese slurry system the reduced glutathione is oxidized immediately upon its addition to the system (Kristoffersen, 1967). Figure 5 shows Sephadex G-25 elution patterns obtained by Carmona *et al.* (1968) of water extracts of the cheese slurry made with various cultures. These elution patterns, obtained immediately after the preparation of the slurry, and the disaggregation of proteins can explain the increased proteolysis previously noted in GSH treated slurries (Kristoffersen *et al.*, 1967). Glutathione also influences the rate of degradation of the caseins in the cheese slurry. Figure 6 compares the soluble protein as measured by the Sephadex analyses and the $\beta$-casein in control and glutathione treated

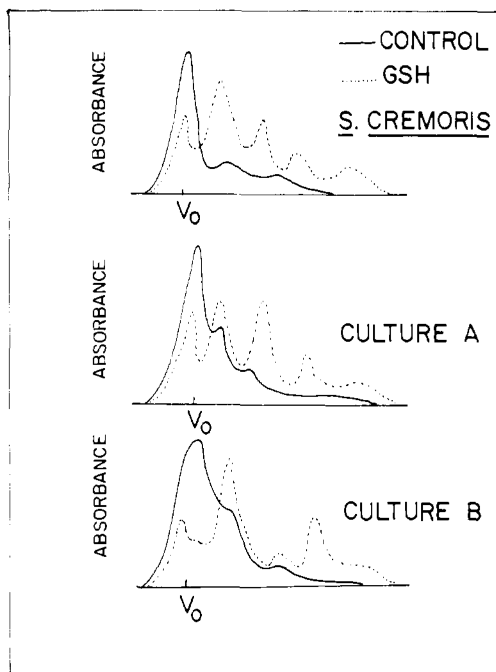


**Figure 5. The effect of glutathione on Sephadex G-25 elution patterns of the water soluble fraction of Cheddar cheese slurries made with different starter cultures (Control = —; added glutathione = .....)**

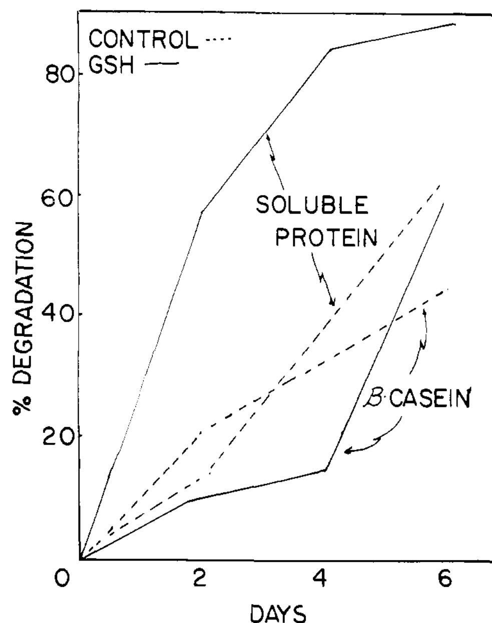


**Figure 6. The effect of glutathione on soluble protein and β-casein degradation in a Cheddar cheese slurry**

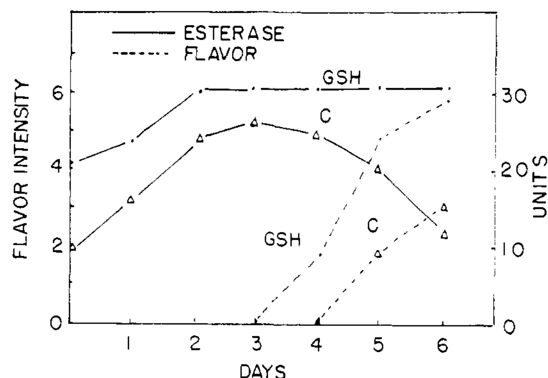


**Figure 7. The effect of glutathione (GSH) on characteristic flavor and esterase activity in Cheddar cheese slurries (C = control)**

systems. The soluble protein decay in the glutathione system is very rapid, and only when this degradation is more than 85% complete is the β-casein attacked. Thus, we consider that one of the roles of the glutathione is the dissociation of peptides which are preferentially utilized in building up a high protease level in the cheese in that the secondary attack then is directed to the β-casein. There is an apparent relationship between flavor development and casein degradation. Characteristic flavor intensity has no relationship to the α-casein content of the slurry but directly proportional to β-casein degradation. Since we know that amino acids are precursors of flavor components in the cheese system, we presume that the products derived from β-casein are more desirable to the development of flavor than those obtained from $\alpha$-$_s$casein.

The importance of proteins and protein balance in flavor development and in the biochemistry of the slurry system is illustrated in Table I, where blood serum albumin was added to the slurry system. The addition of the blood serum albumin caused slight decrease in characteristic flavor, a market increase in sulfhydryl production, and a distinct inhibition of fatty acid formation. A similar disruption of the normal fermentation in Cheddar cheese slurries was obtained by the addition of either 5% β-casein or 5% κ-casein. Thus, it would appear that there is a definite interrelation between protein lipid and also carbohydrate metabolism and the flavor development in the ripening system. A disruption of the composition of the milk system may be partially responsible for variability in the ripening of natural cheeses.

**Table I. Effect of Blood Serum Albumin Added to Slurry System**

| Sample | Flavor | | —SH | Acid Degree |
|---|---|---|---|---|
| | CF | Defect | | |
| Control | 6 | ... | 7.1 | 1.7 |
| +0.1 BSA | 5 | Fermented | 9.7 | 0.5 |
| +0.2 BSA | 4 | Sulfide | 17.3 | 0.2 |

Figure 7 illustrates another function of glutathione. The representative data concerns the α esterase activity of the cheese slurries and also flavor development reported by Harper *et al.* (1969). Addition of 100 parts of glutathione slightly accelerates total esterase activity, but, perhaps more significantly, it inhibits the degradation of the esterase activity during the later stages of the ripening process. Thus the ability of the added glutathione to protect enzymes in the slurry system is another significant function of this material.

The fact that the slurry, particularly with glutathione, appears to ripen similar to the normal cheese has suggested that glutathione may be a normal component of the cheese system. Figure 8 shows representative data for oxidized glutathione as measured by glutathione reductases, in a Cheddar cheese slurry with and without added glutathione. Oxidized glutathione was found in both the control slurry and the slurry with added glutathione. Essentially all of the glutathione in the slurry was found to be in the oxidized form. Initially the concentration of oxidized glutathione in five control slurries was found to vary from 25 to 75 ppm. For the

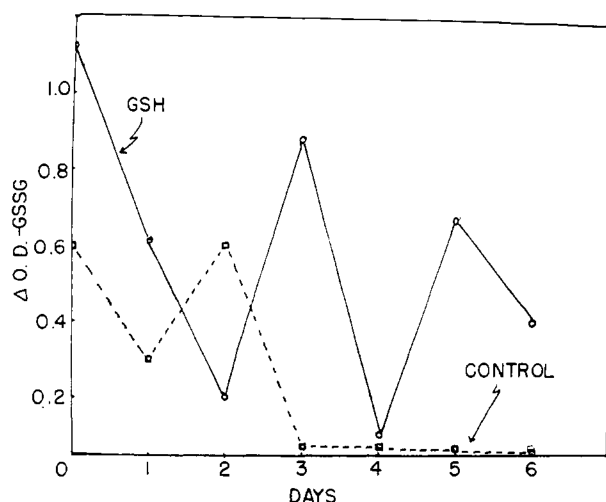


**Figure 8. The effect of ripening on oxidized glutathione in control (C) and glutathione (GSH) treated slurries**

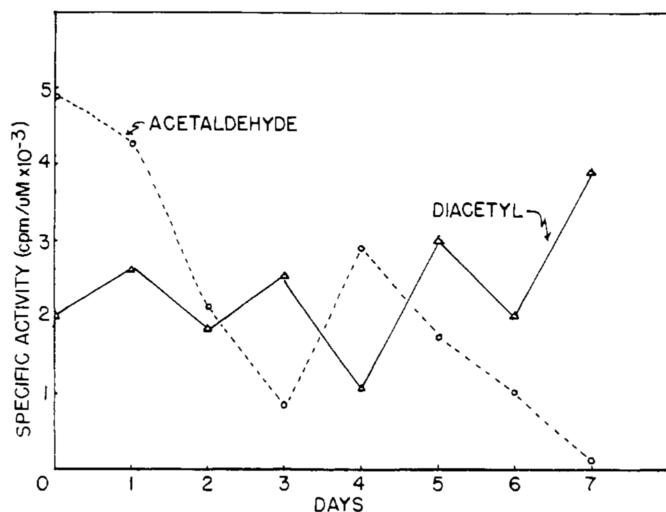


**Figure 9. The effect of ripening on the specific activity of acetaldehyde and diacetyl in glutathione treated slurry with added C-14 glucose**

slurry illustrated in Figure 11 there was a cyclic change in the oxidized glutathione in the control up to 3 days of age and then oxidized glutathione disappeared from the system. For the slurry with the added glutathione, there was a continued cycle change in the oxidized glutathione during the entire fermentation period. This suggests that there is feedback control regulation of the oxidized glutathione system. Limited data for reduced glutathione, as measured by the enzyme glyoxylase, suggest an alternate reduction–oxidation of the glutathione. In a number of similar trials, the same cyclic change in concentration of oxidized glutathione was noted where glutathione was added to the slurry; however, the results in the control slurry were totally unpredictable. Where the control slurries behaved normally and gave good flavor development, glutathione was detectable and there was generally the same cyclic relationship as observed in slurries with added glutathione. Slurries that failed to develop normal flavor also failed to show detectable glutathione after the first day of fermentation.

Potentially, glutathione has a multiplicity of functions in the cheese slurry system and appears to exert positive control on the fermentation process in a multi-faceted manner.

**Feedback Control.** The evidence for feedback control in respect to the glutathione system in cheese slurries suggests such control mechanisms may be generally operative in cheese fermentations. To gain a further insight into other feedback systems and to gain a better understanding of the interrelationships between carbohydrate, lipid, and protein degradations as they relate to cheese flavor, studies have been initiated to determine the fate of various key substrates. In this respect, radioactive glucose has been incorporated into the slurry system. In addition to lactic acid, radioactivity has been found in acetaldehyde, diacetyl, carbonyl compounds, fatty acids, and amino acids. This is in agreement with earlier studies (Harper, 1967) that suggested the expected interconnection in the various pathways in cheese ripening.

A plot of the specific activity of diacetyl and its precursor, acetaldehyde, is presented in Figure 9. If glucose carbon alone were involved in the formation of these compounds, then the specific activity would remain constant during the entire fermentation. The fact that specific activity is cyclic for both compounds is evidence for multiple precursors, as well as being indicative of feedback control. The fact that the specific activities of the two compounds are out of phase with one another is evidence for a coupled feedback control, involving two or more precursors and two or more enzyme systems. The data cannot be interpreted to answer the question as to whether one or more microbial population is operative.

The data in this study, while exploratory in nature, provide evidence for suggesting that the cheese ripening system is under the control of a member of interrelated feedback mechanisms. Thus unbalance in one system would tend to unbalance the entire fermentation, such as that observed with the addition of blood serum albumin. The data further infer that feedback suppression and induction can be expected in the cheese ripening system. The observed failure of added lactobacilli to enhance flavor may relate to a carefully balanced suppression and induction in the natural system. When added in large numbers at the beginning of the fermentation, the organisms grow "out of phase," and may lack enzyme systems essential to flavor development.

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