Thioctic Acid in Milk

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Abstract

Thioctic acid (a-lipoic acid) was isolated from milk and found to be associated with the fat globule membrane. The isolation procedure was as follows: Sweet cream buttermilk was hydrolyzed by refluxing for 3 hr with 6.0 N HCl. A benzene extract of the hydrolysate was extracted with 5% NaHCO₃, which, in turn, was acidified to pH less than 1 with 6.0 N HCl. The acidified solution was extracted with ethyl ether, and the extract evaporated to dryness and stored under nitrogen —10 C. Identification of thioctic acid was accomplished by UV absorption spectra, thin-layer and gas-liquid chromatography techniques.

Considerable research has been done on the relationship of sulfhydryl and disulfide groups to heat activated or cooked flavor in milk (3, 4, 6-10). Hutton and Patton (3) established that heat-labile sulfhydryl groups of β-lactoglobulin were responsible for this flavor defect.

Kiermeier and Hamed (8) showed that when heated milk was stored in the daylight, the sulfhydryl groups decreased more rapidly than the sulfhydryl groups of similar milk stored in the dark. Lyster (10) has shown that the sulfhydryl groups in ultrahigh temperature (UHT) processed milk decrease upon storage, both at —4 C and at room temperature, but the decrease is more rapid at room temperature. The possibility of a reversion of β-lactoglobulin to its native state does not fully account for this phenomenon. Lyster (10) has shown that only 1% of the protein reverted to its native state after UHT processing. These observations elicit the lack of information on the reactions of heat activated sulfhydryl groups in milk.

Kidder and Dewey (5) first reported the existence of an unknown growth factor essential for Tetrahymena geleii. It was found in many materials of plant and animal origin, including crude casein. In 1949 Stokstad et al. (14) further investigated the properties of the unknown growth factor. A variety of natural products were surveyed by microbiological assay for occurrence of the factor. Dried buttermilk was reported to contain about 2% of the activity exhibited by liver-presscake digest (the reference standard). Later, this factor was identified as thioctic acid. No further data have appeared in the literature concerning thioctic acid in relation to milk.

Thioctic acid is best known for its function as a co-enzyme in the redox system of oxidative decarboxylation reactions (1, 12, 13). However, Tollin and Green (16) were able to show that light could catalyze reactions involving free radicals between thioctic acid and chlorophyll in the following manner:

\[
\begin{align*}
\text{(a)} & \quad \text{S-S} \quad \text{COOH} \quad \downarrow \text{hv} \quad \rightarrow \quad \text{S-S} \quad \text{COOH} \\
\text{(b)} & \quad C + \text{hv} \quad \rightarrow \quad C^* \\
\text{(c)} & \quad \text{C}^* + \text{S-S} \quad \text{COOH} \quad \rightarrow \quad \text{C}^* + \text{S-S} \quad \text{COOH} \\
& \quad \downarrow \text{hv} \quad \rightarrow \quad \text{C} + \text{S-S} \quad \text{COOH}
\end{align*}
\]

Calvin (1) postulated that upon photo-oxidation, thioctic acid produced a compound, thought to be a thio-sulfenic acid, possessing both oxidizing and reducing powers, thus participating in the primary quantum conversion process of photosynthesis. Dahle and Sullivan (2) postulated that thioctic acid in the sulfoxide form could act as a sulfhydryl oxidizing agent in the dough maturation process, and demonstrated its presence in wheat flour. Morrison and Cousin (11) confirmed these results and later Sul-
livan et al. (15) successfully crystallized thioctic acid extracted from wheat flour. The fact that thioctic acid has been implicated in sulfhydryl-disulfide interchange reactions as a sulfhydryl oxidizing agent, and has been shown to produce free radicals upon photo-oxidation, led the authors to investigate the possible presence of thioctic acid in milk.

**Experimental Procedures**

Investigators studying the occurrence of thioctic acid in wheat flour (2, 11, 15) utilized UV absorption spectra of hydrolysate extracts as evidence of the presence of thioctic acid. Synthetic thioctic acid (Sigma Chemical Company, St. Louis, Missouri 63118) has a maximum absorption peak at 334 m\(\mu\) (1). Early investigators reported that crude casein and dried buttermilk exhibited thioctic acid activity (5, 14). Therefore, buttermilk, acid precipitated protein (pH 4.5), and whey proteins were prepared from the same lot of mixed-herd milk and each fraction hydrolyzed, extracted, and analyzed for thioctic acid by UV absorption methods. Results showed that thioctic acid of milk was concentrated in the buttermilk fraction and thereby most probably associated with the fat globule membrane. Both the acid precipitated protein fraction and the whey protein fraction failed to yield a maximum absorption peak in the 300 to 350 m\(\mu\) range. As a result of these findings spray dried, sweet cream buttermilk was used as the starting source for thioctic acid.

**Hydrolysis and extraction.** Several hydrolysis procedures have been reported, including refluxing in 2.0 N HCl (2, 11, 15) and autoclaving in 6.0 N H\(_2\)SO\(_4\) (12). The procedure used in this work was as follows: Four and one-half kilograms of spray dried sweet cream buttermilk were hydrolyzed by refluxing 3 hr in approximately 6.0 N HCl. Benzene was bubbled through the hydrolysate for 24 hr at a rate of 500 ml per hour. The benzene layer was collected and shaken with a 5% sodium bicarbonate solution and allowed to separate. The aqueous layer was removed, acidified with 6.0 N HCl, then extracted with ethyl ether. The ether extracts from 60 kg of buttermilk were combined, washed two times with distilled water, and dried over sodium sulfate. The ether was evaporated with a rotary flash evaporator and the residue stored under nitrogen at \(-10\) C until further analysis.

**Spectrophotometric analysis.** UV absorption studies were performed, using a Beckman Model DB Spectrophotometer equipped with a wave length drive and a chart recorder. The samples were analyzed in a 1-cm light path with 95% ethanol as the solvent, scanning from 360 to 230 m\(\mu\).

**Thin-layer chromatography (TLC).** Glass plates (20 by 20 cm) were coated with Silica Gel G, prepared according to Stahl (Brinkman Instruments, Inc., Cantiague Road, Westbury, N. Y. 11590), with a thickness of 250 \(\mu\). The solvent systems used were: water-saturated butanol and 5% butanol in benzene (v/v). The fractions were eluted from the silica gel with either methanol or ethanol. The position of the compounds was revealed by spraying the plates with 6.0% sulfuric acid, then heating the plates for 10 or 15 min at 120 C.

**Methylation.** Methyl esters of the free acids were prepared by refluxing 1 hr in 30 ml of absolute methanol to which two drops of conc HCl had been added. Sixty milliliters of distilled water were added and the methyl esters extracted with chloroform. The chloroform was evaporated and the esters dissolved in benzene. The benzene solution was passed through a Florisil column previously activated at 450 C for 2 hr, slurried with hexane, and packed in a 5- by 35-cm glass column. Thioctic acid was eluted with 20% benzene in hexane (v/v). The benzene-hexane was evaporated and the residue redissolved in ethyl-acetate for GLC analysis.

**Gas-liquid chromatography (GLC).** A Barber-Colman Gas Chromatograph equipped with a hydrogen flame ionization detector was used. The methyl esters were chromatographed on both polar and nonpolar columns. The polar column consisted of 10% ethylene glycol adi-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Column</th>
<th>Apiezon L</th>
<th>E.G. Adipate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection port temperature (C)</td>
<td>280</td>
<td>280</td>
</tr>
<tr>
<td>Detector block temperature (C)</td>
<td>280</td>
<td>280</td>
</tr>
<tr>
<td>Carrier gas-N(_2)- (cc/min)</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Sensitivity (amp)</td>
<td>(9 \times 10^{-9})</td>
<td>(9 \times 10^{-10})</td>
</tr>
<tr>
<td>Sample size ((\mu))</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Column temperature (C)</td>
<td>182</td>
<td>182</td>
</tr>
<tr>
<td>Column length (glass) (cm)</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

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Results and Discussion

The ether extract obtained from the hydrolyzed buttermilk contained impurities as well as thioctic acid. This was evidenced in the UV spectrum of the ether extract, since the peak maximum occurred at 330 μm rather than at 334 μm for pure thioctic acid. Figure 1 compares the spectra of known thioctic acid and the ether extract of hydrolyzed buttermilk. Morrison and Coussin (11) reported similar results when analyzing the hydrolysate of wheat flour. Figure 2 shows the effect of the addition of fatty acids from milk fat on the UV spectrum of known thioctic acid. The peak is not as sharp, and exhibits a maximum at 330 μm. This experiment served to illustrate the effect that added compounds, with no absorption maxima between 320 and 330 μm, can have on the UV spectra of known thioctic acid. Thus, it is not surprising that the UV absorption maximum of the buttermilk hydrolysate occurred at 330 μm.

Thin-layer chromatography was used for further purification and more conclusive evidence of the presence of thioctic acid in milk. Figure 3, A is a drawing of a typical thin-layer chromatogram of known synthetic thioctic acid and the ether extract of hydrolyzed buttermilk. At least four contaminating substances were found to be present, as well as a component with an Rf value corresponding to that of known thioctic acid. These contaminants may be responsible for the UV absorption peak shift discussed previously.

Further purification of the above fraction was accomplished in the following manner:
Fig. 3. Drawing of thin-layer chromatograms obtained from known synthetic thioctic acid and fractions isolated from spray-dried buttermilk. Chromatogram A is known thioctic acid and the ether extract obtained from buttermilk hydrolysate. Chromatogram B is known thioctic acid and a methanol eluate from the thin-layer fraction with an $R_f$ corresponding to known thioctic acid.

The thin-layer plate was prepared in the usual manner and developed with water-saturated butanol. The plate was not sprayed with $\text{H}_2\text{SO}_4$ to reveal components. Instead, the known thioctic acid was spotted on the left edge of the plate as a reference and the ether extract applied on the remainder of the plate. Following development with water-saturated butanol, a UV light source was used to locate the known thioctic acid spot. The silica gel corresponding to this $R_f$ value was removed from the section of the plate containing the ether extract of the buttermilk and any compounds present eluted from the silica gel with methanol. The eluate was evaporated to dryness and rechromatographed, using 5% butanol in benzene (v/v) as the solvent. Figure 3, B is a chromatogram of this fraction. The close proximity of two of the contaminating spots shown in Figure 3, A may account for the contaminants noted in Figure 3, B. The spectra of the ether extract of hydrolyzed buttermilk and those of the fraction purified by thin-layer chromatography, as described above, are compared in Figure 4. The purified fraction exhibits a peak maximum at 334 m$\mu$, confirming that the contaminants were responsible for the peak shift.

Gas-chromatographic results are shown in Figure 5. The methyl esters of synthetic thioctic acid and of the ether extract of hydrolyzed buttermilk were chromatographed on both polar and nonpolar columns. Chromatograms A and B are those of the ether extract and known thioctic acid, respectively, on the Apiezon L column. Chromatograms C and D are those of the ether extract of buttermilk hydrolysate and known thioctic acid, respectively, on a polar column, ethylene glycol adipate. In both instances retention times of a component in the ether extract of buttermilk hydrolysate corresponded with those of known methyl thioctate. Efforts to obtain a mass spectra of the component from the buttermilk fraction were not successful, because of insufficient concentration.
Impurities that remained during the isolation procedure are again clearly illustrated in the gas chromatographs of the ether extract of the buttermilk hydrolysate. It is of interest to note that some impurities are present in known synthetic thioctic acid. Good resolution was achieved, however, between contaminating compounds and the peak corresponding to the retention time of known thioctic acid.

In summary, the isolation and identification of thioctic acid in milk was accomplished. Thioctic acid was found to be associated with the fraction containing the fat globule membrane and apparently is tightly bound to protein components of this fraction. Efforts to detect the presence of thioctic acid without the hydrolysis step were unsuccessful.

Presence of impurities in the ether extract of buttermilk hydrolysate demonstrated the need for further purification, which was accomplished by thin-layer and gas-chromatographic techniques.

Thioctic acid is a naturally occurring substance in milk and is in sufficient concentration to play a significant role in the oxidation of activated sulfhydryl groups. Its participation in sulfhydryl-disulfide interchange reactions that occur in heated milks may explain some of the flavor changes observed upon storage of heated milk products. An understanding of these changes and the mechanisms involved could aid in achieving improved stability and flavor of heat-processed dairy products. Further investigations in this area are under way.

References


