Lactoperoxidase

II. ISOLATION*

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Early methods for the isolation and purification of lactoperoxidase were based on ammonium sulfate or acetone fractionation (1-3). Theorell et al. (4, 5), using an ammonium sulfate method, were the first to obtain a highly purified preparation. Their procedure was never fully detailed, however, and the reported yields were low, although they did obtain crystals of the enzyme.

Polis and Shmukler (6) developed a more reproducible procedure in which the final purification was achieved by chromatography on tricalcium phosphate columns. More recently, Morrison et al. (7) developed a method employing ion exchange resin for the isolation of the enzyme. It is the purpose of this paper to present the details of a modification of this latter procedure which not only simplified the method, but also resulted in greater yields of a more highly purified preparation.

EXPERIMENTAL PROCEDURE

In the final procedure adopted, 15 g of the sodium form of the resin, CG 50 type 2, were added to each liter of raw skim milk. The resin had been previously treated in order to remove impurities and resin fines. The resin was stirred with the milk for 2 or 3 minutes and then the pH was adjusted to 7.0 by adding 6 N acetic acid or 6 N ammonium hydroxide. The milk-resin suspension was stirred for 1 hour and then allowed to stand approximately 30 minutes until the resin settled. Throughout this procedure, the temperature of the milk was not allowed to rise above 8°.

The milk was then removed either by decantation or siphoning, and an additional 7.5 g of resin per liter added. The pH was readjusted to 7.0 and the milk-resin suspension stirred again for 1 hour and then allowed to settle. The combined resin was washed with distilled water in batches by alternately suspending the resin and allowing it to settle. This was repeated until the optical density of the wash water was less than 0.02.

The resin was then washed into a chromatographic tube with distilled water. The resin required for 20 liters of milk made a column approximately 45 cm in height and 6 cm in diameter. Distilled water was passed through the column until the eluates were clear and nearly free of 280 mp absorbing material.

The lactoperoxidase was eluted at room temperature with 0.5 M sodium acetate. The enzyme moved down the column as a dark green band, increasing in size as it descended. The colored material was collected in 500-ml flasks on a fraction collector. The crude lactoperoxidase thus obtained was precipitated by the addition of 53 g of ammonium sulfate for each 100 ml of solution at 4-10°. Additions were made slowly and with constant stirring to avoid denaturation of the protein. After the addition of ammonium sulfate, the precipitate was allowed to stand at least 2 hours in the cold and then collected by centrifugation for 20 minutes at 20,000 x g. The precipitate was suspended in a small volume of distilled water and dialyzed repeatedly against large volumes of distilled water. The resulting solution was adjusted to pH 7.0 with 0.2 M NaHPO₄ or Na₂HPO₄ and then centrifuged at 20,000 x g for 30 minutes. The crude lactoperoxidase obtained in this way usually had a 412/280 absorbancy ratio of 0.3 to 0.5.

Purification of Crude Lactoperoxidase—The sodium form of the resin was buffered at pH 7.0 before it was used in the final purification procedure. The resin was suspended in 0.1 M phosphate buffer at pH 7.0, stirred, and then allowed to settle. The pH of the supernatant fluid was adjusted to 7.0 with 6 N HCl, and this process repeated until the pH remained constant at 7. The resin was allowed to settle and the supernatant fluid decanted. The resin was then suspended in 0.01 M phosphate buffer and the pH adjusted to 7.0 after equilibrium was established. The process of adjustment of pH and stirring was repeated until the pH of the suspension remained constant at 7. The resin was finally washed with distilled water and was then ready to be used for chromatography. A column, 25 cm x 2.5 cm, was prepared with the resin.

Approximately 20 ml of the crude lactoperoxidase solution, containing approximately 250 mg of the crude lactoperoxidase, were allowed to enter the column at a very slow rate in order to obtain a concentrated band of the material at the top of the column. The column was then washed with 0.02 M sodium acetate. After 300 ml of this solution had passed through the column, the concentration of the sodium acetate was raised to 0.25 M. After the column had been eluted with the 0.25 M sodium acetate, the molarity of the salt solution was raised to 0.5 M. The eluent from this column was collected automatically and its absorbancy at 280 and 412 mp was determined. A typical purification experiment is shown in Fig. 1.

The lactoperoxidase could also be purified by chromatography on carboxymethyl cellulose. In this procedure, the CM-cellulose was buffered to pH 5.7 with acetate buffer and finally washed...
with 0.01 M acetate buffer. Approximately 6 mg of crude lactoperoxidase, which had been dialyzed against the same buffer, were then allowed to enter the 1 × 25-cm column. The walls of the tube were washed with two 2.5-ml aliquots of 0.01 M buffer. The column was then eluted with 100 ml of 0.05 M acetate buffer, which removed the extraneous protein, leaving lactoperoxidase on the column. The lactoperoxidase was eluted with 0.05 M acetate buffer containing 0.2 M sodium chloride. Lactoperoxidase moved off the column as a single material, leaving a small amount of denatured protein on the column. Lactoperoxidase purified in this manner has an \( A_{433}/A_{280} \) ratio of 0.86.

Final purification of the lactoperoxidase was achieved by passing the protein through a column of Sephadex G-100. Five or ten milliliters of a concentrated solution of purified lactoperoxidase with an \( A_{433}/A_{280} \) ratio of 0.7 to 0.85 were passed through a column 45 cm in height and 6 cm in diameter containing the Sephadex in equilibrium with 0.05 M phosphate buffer, pH 7.0. The central cut of the lactoperoxidase fraction now had an \( A_{433}/A_{280} \) ratio of 0.90.

This procedure removed traces of two extraneous proteins. One was a red protein fraction with a higher molecular weight than lactoperoxidase, which preceded it off the Sephadex column. The other was a slower moving, colorless fraction which followed lactoperoxidase on the Sephadex column.

As shown in Table I, the recovery of the enzyme is much higher than the 20% or lower recoveries reported by others (4, 6). The ratio of the absorbancy at 412 m\( \mu \) to 280 m\( \mu \) which is employed as an index of purity is also higher than the values reported by these workers. Folks and Shimukler (6) obtained values as high as 0.9, whereas a value of 0.77 was reported for the preparation of Theorell et al. (4, 5). The activity of the enzyme, when expressed on a hemoglobin basis, is, however, not improved over earlier reports (6, 7).

### DISCUSSION

The chief advantage of the modified procedure are that fewer steps are required to produce a crude preparation of lactoperoxidase and yet higher yields of the enzyme are obtained. In application, it was found that between 60 and 80% of the total lactoperoxidase present in milk could be recovered in the crude preparation. This represents a marked improvement over previous methods. In the ionic environment presented by milk, there appears to be a distribution of the enzyme between the soluble phase and the insoluble resin phase. Approximately 50 to 80% of the enzyme present in milk is recovered from the first batch absorption, and 15 to 25% is recovered from the second batch of resin. Thus, although nearly complete removal of the enzyme can be achieved, the effort required to obtain maximal recovery is not really fruitful.

The present method varies from the previously reported procedure (7) in that it minimizes possible proteolytic activity during the course of the isolation. The earlier method used rennin, a proteolytic enzyme, to remove casein. Since the present method does not require removal of casein, the use of the proteolytic enzyme as well as the need to raise the temperature of the milk are eliminated. The control of temperature helped to minimize bacterial growth, and thus proteolytic activity resulting from the growth of microorganisms in the milk. The importance of inhibiting bacterial growth is clear from the recent work of Kiermeier and Kayser (8), who have pointed out that lactoperoxidase is inactivated by microorganisms. The degree of this inactivation parallels the growth of the organisms.

It had previously been suggested (7) that the two forms of the enzyme which have been isolated might be the result of proteolytic activity. Application of the new isolation method resulted in at least four instances in producing a single form of lactoperoxidase. However, two forms of the enzyme were isolated at other times. In all cases, the major form of the enzyme was that least strongly absorbed to the resin, thus ruling out the possibility that a selective isolation had occurred.

Although the data suggest that the two forms of the enzyme may be the result of proteolytic activity, other possibilities must still be considered. A suggestion has been made (7) that the two forms represent two chemically different proteins produced by genetically different animals. Since a single form of the enzyme could be isolated from pooled milk samples from both Rochester, New York, and Duarte, California, this suggestion would appear less likely. A study of the lactoperoxidase obtained from individual animals however, would be most illuminating at this point.

It is interesting to note that after the crude lactoperoxidase has been eluted from the resin, a red colored protein can be obtained by washing the resin with dilute ammonium hydroxide.

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**Table I**

<table>
<thead>
<tr>
<th>Protein Type</th>
<th>( A_{433}/A_{280} ) Ratio</th>
<th>Enzyme Activity Recovered</th>
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<tbody>
<tr>
<td>Raw skim milk</td>
<td>0.30-0.50</td>
<td>70-75</td>
</tr>
<tr>
<td>Crude lactoperoxidase</td>
<td>0.80-0.85</td>
<td>50-60</td>
</tr>
<tr>
<td>Purified lactoperoxidase</td>
<td>0.91-0.95</td>
<td>48-63</td>
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This red protein, similar to that isolated in previous procedures, contains iron, but it is not a hemoprotein. In the present method, the greater part of this protein is separated from the lactoperoxidase at the crude stage. Immunochemical methods\(^1\) have, however, shown it to be present even after column chromatographic purification. The red protein is readily separated from lactoperoxidase on Sephadex G-100, however, since it appears to have a higher molecular weight than lactoperoxidase and is eluted before the enzyme.

**SUMMARY**

An improved method for the isolation of the enzyme lactoperoxidase is presented. This method depends upon the direct extraction of the enzyme from the milk with a weak cation exchange resin. The enzyme is then purified by column chromatography on the cation exchange resin and Sephadex G-100.

**REFERENCES**

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