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Crystalline Lipoxidase

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The purification of soy bean lipoxidase, an enzyme capable of promoting the oxidation of linoleic acid and related substances, has been attempted by several workers. Süllman [1] prepared an acetone powder of the enzyme containing full activity but only 27 % of the dry substance of the water extract. Cosby and Sumner [2] have reported a 60-fold purification of the enzyme, and Balls, Axelrod, and Kies [3] succeeded in effecting a purification 115 times that of a 2.5 % water extract of soy bean meal. Theorell, Bergström and Åkeson [4] obtained a preparation electrophoretically homogeneous having an activity of about 130 units per milligram. Later these authors [5] reported a preparation which was again homogeneous electrophoretically, but which this time showed an activity of 221 units per milligram.

The present investigation was begun in the attempt to improve the method of isolation of lipoxidase so as to obtain larger yields of pure enzyme intended to be used in the studies of the properties and activity of lipoxidase. A preliminary account of this work has appeared previously [6].

EXPERIMENTAL

The lipoxidase activity determinations were carried out using the spectrophotometric method described previously [5]. The only modification was the use of side-arm test tubes with rubber stoppers rather than the Thunberg tubes used previously. These were filled under a stream of oxygen, thus avoiding the danger of mixing by foaming encountered when evacuating the Thunberg tubes. The activity unit used is defined as the amount of enzyme that produces one micro-equivalent of peroxide (or conjugated diene) in one minute. It was often found convenient to express the purity of the enzyme preparations as units per density unit per cm at 280 mμ.

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RESULTS AND DISCUSSION

Preliminary experiments to determine the effect of pH of the extraction fluid upon the degree of extraction of lipoxidase were made using 6.6 ml fluid per gram of fat-free soy meal. The results shown in Table 1 indicated that an extraction with acetate buffer at pH 4.5 resulted in only a slight loss in yield, but a two-fold increase in purity over the water extraction was gained.

Table 1. The effect of pH of the extraction fluid upon the degree of extraction of lipoxidase.

	pH of the extraction fluid				
	4.0	4.5	5.0	5.5	Water
Units/ml	156	240	189	213	276
Units/mg N	95	104	60	52	48

Attempts to precipitate the enzyme from this extract with basic lead acetate, as had been done in previous preparations in this laboratory, indicated that excessive quantities of lead were necessary to cause any precipitation of the enzyme. In attempts to find another precipitant it was found that barium acetate precipitated non-active material, and that acetone reduced the amount of barium necessary. It was also found that small quantities of lead removed further inactive material. A comparison of the purity of the original extract itself with those after various treatments showed that the combined treatment with barium acetate, acetone, and basic lead acetate gave the largest increase in purity without loss in yield. However, attempts at ammonium sulfate fractionation of the extract thus obtained showed that the activity could not be recovered; but if the pH were raised to 6.7 with ammonia prior to the addition of barium acetate, acetone, and lead acetate, the activity could be recovered by precipitation with ammonium sulfate.

The use of buffer at pH 4.5 as extraction medium and the use of barium acetate as a precipitant of inactive material were prompted by the suspicion that the substance, which had been so difficult to separate from the enzyme in previous preparations, may have been a carbohydrate, perhaps phytic acid. The work of Fontaine, *et al.*[7], shows that the solubility of the phosphorus present in fat-extracted soy bean flour is greatly reduced at pH 4.0—4.5, and thus an extraction of soy flour in this pH range should probably reduce the amount of phytin extractable. Barium acetate was used in the belief that it would remove most of the remaining phytic acid. Preparations in which these procedures were not used always showed the presence of brown, sirupy material in the later stages of the purification, and this substance could not be separated

from the enzyme by any means tried. However, when the initial extraction was made at pH 4.5 followed by barium-acetone-lead precipitation at pH 6.7, this gummy material was no longer encountered.

Using the above preliminary experiments as a guide, a large preparation was made as follows: 6 kg of de-fatted soy meal were suspended in 40 liters $M/10$ acetate buffer at pH 4.5 and passed through a basket centrifuge to remove undissolved material. Yield — 4.2 million units. The supernatant fluid was adjusted to pH 6.7 with ammonia, and 5 volumes of 20 % barium acetate solution, 10 volumes of acetone and 2 volumes of 20 % basic lead acetate were added per 100 volumes of extract. The inactive precipitate was removed in a large separator. Yield 2.9 million units. The inactive slight precipitate formed upon the addition of 25 g of ammonium sulfate per 100 ml of extract was removed by sedimentation and decantation. The active precipitate formed upon the addition of ammonium sulfate to bring the concentration to 40 g per 100 ml of extract was removed in the same manner and redissolved in a minimum of water. Yield — 2.8 million units. After heating the solution at 63° for 5 minutes, a bulky, inactive precipitate was centrifuged off, and the remaining preparation containing 2.9 million units had an activity of 90 units per mg. Refractionation between 37 and 50 % saturation with ammonium sulfate reduced the yield to 2.4 million units but increased the activity to 230 units per milligram. This preparation was equal in activity to the best previously-reported preparation, but was still obviously not pure because of its intense, brown color.

The preparation was then dialysed against $M/50$ phosphate buffer at pH 5.5 and the precipitate which formed was discarded. Ninety per cent alcohol was added at 0° to give an alcohol concentration of 3.0 %. The active precipitate was centrifuged off and redissolved in $M/50$ phosphate buffer at pH 7.5. The preparation at this stage contained 0.85 million units at 502 units per milligram. Electrophoresis in the small Tiselius apparatus showed the presence of three components, of which lipoxidase was 63.5 %. Thus the pure enzyme should have an activity of about 790 units/mg. At the end of the electrophoresis a fraction having an activity of 748 units/mg was separated. The bulk of the preparation (502 U/mg) was subjected to electrophoresis in the large Tiselius apparatus, and a fraction containing 0.12 million units at 610 U/mg was obtained.

A larger-scale repetition of this procedure was made with 15 kg of soy flour and 100 l of $M/10$ acetate buffer at pH 4.5. At the stage after heating to 63° for 5 minutes 11.4 million units remained, but in the subsequent fractionation between 35 and 50 % saturation with ammonium sulfate, only 7.8 million units of enzyme at an activity of 45 U/D_{230} could be removed. Frac-

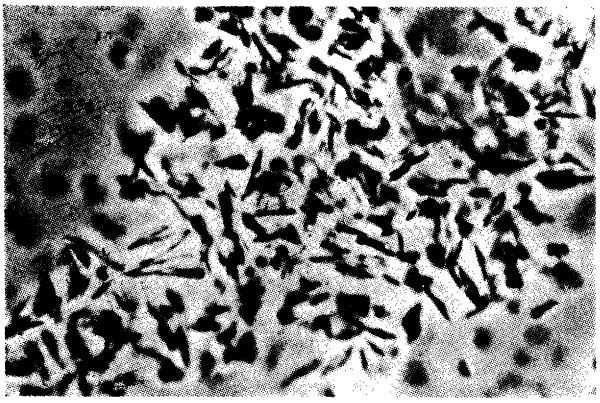

Figure 1. Crystalline lipoxidase (1200 ×).

tionation with alcohol this time required 12 % alcohol to precipitate the enzyme, of which 7.6 million units were recovered, having an activity of 257 U/D_{280}. In the fractionation of this preparation with ammonium sulfate, the fraction between 35 and 50 % saturation yielded 2.9 million units at 231 U/D_{280}, and the fraction between 50 and 60 % saturation yielded 1.8 million units at 358 U/D_{280}. Electrophoresis of this latter fraction showed it to contain two minor components and 58 % lipoxidase. The small fraction of supposedly pure enzyme obtained at the end of the electrophoresis had an activity of 725 U/mg and had a D_{280} of 1.75 per mg per ml per cm. The bulk of this fraction (50—60) was subjected to electrophoresis in the large Tiselius apparatus, and 0.34 million units of lipoxidase, having 471 U/D_{280}, or approximately 820 U/mg, were separated.

Crystallization of this latter preparation was accomplished by slowly dialysing a concentrated solution of the enzyme against ammonium sulfate of slowly increasing concentration. Microscopic, colorless plates or sheaves shown in Figure 1, crystallized out. The small amount of amorphous material remaining in the preparation was dissolved away by washing the preparation at a slightly lower ammonium sulfate concentration. The crystals, entirely free from amorphous material, assayed 479 U/D_{280} or 850 U/mg, and the washings assayed 457 U/D_{280}, or approximately 810 U/mg. The pure enzyme represents a purification of about 70 times that of the original extract, or about 150 times that of a water extract.

The fraction from which the enzyme was crystallized was shown to be homogeneous electrophoretically at pH 6.0 in the small Tiselius apparatus. The crystalline sample was found to be homogeneous according to sedimentation and diffusion patterns. The sedimentation constant, determined on the

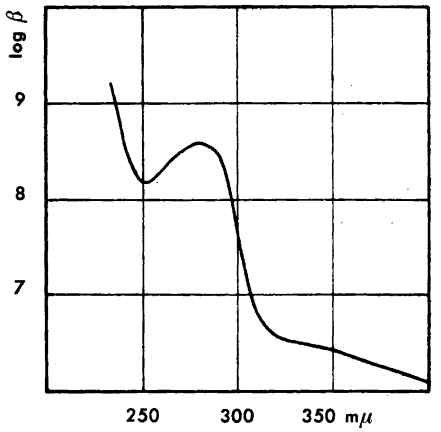

Figure 2. Absorption spectrum of crystalline lipoxidase.
($\beta = ln \frac{I_0}{I} \cdot \frac{l}{c} \cdot \frac{l}{d}$ when c = moles/ml).

crystalline material, was found to be 5.62×10^{-13}, the diffusion constant 5.59×10^{-7}, and the partial specific volume 0.750. The molecular weight was thus found to be 102,000. At pH 5.92 lipoxidase migrates anodically at a velocity equal to $u = 1.81 \times 10^{-5}$, and at pH 3.97 it migrates cathodically at a velocity of $u = 4.63 \times 10^{-5}$. Thus the isoelectric point is approximately pH 5.4. A mobility curve made with more impure material agreed very well with these values.

The absorption spectrum (Figure 2) is that of a common protein with a somewhat greater than average absorption at 280 mμ, indicating the probability that lipoxidase has a comparatively large amount of tyrosine or tryptophane in its structure. No evidence of a prosthetic group being present was indicated by the light absorption curve.

It was previously reported from this laboratory that lipoxidase contained nearly one atom of iron per molecule. In a series of determinations of iron on impure samples of lipoxidase obtained during the course of this investigation, the iron bore no relationship to the degree of purity. The iron content of a sample 94 % pure was found to be sufficient for one atom iron per molecule if its molecular weight were 370,000. It is thus clear that the iron must be present only as an impurity. This is in agreement with the observations that the crystalline lipoxidase is not inhibited by fluoride, azide, cyanide, pyrophosphate, or diethyl dithiocarbamate.

It should be pointed out that the procedure as outlined can not be rigidly applied without the use of pilot experiments. The two accounts of large preparations which have been given serve to illustrate the variation in method that can be expected from two batches of the same soy meal. Application of the same procedure to a sample of finely-ground soy meal from the Archer Daniels

Midland Company of Minneapolis gave a low yield of very impure enzyme. It is clear that the method of isolation has to be adapted to the soy meal available.

It is interesting to notice that this homogeneous enzyme preparation does not need any activitor under our assay conditions. The turnover number for lipoxidase acting upon linoleic acid at 20° is of the magnitude of 330 molecules per second per enzyme molecule. It is rather striking that this enzyme promoting the same reactions as heavy metal ions, does not use any heavy metal or detectable prosthetic group for its activity. This puts lipoxidase into a unique position among the oxidation enzymes known so far.

SUMMARY

The preparation of crystalline homogeneous soy bean lipoxidase is described. Lipoxidase is a colorless protein containing no iron or detectable prothetic group. The molecular weight is 102,000 and its isoelectric point is pH 5.4. The activity of the pure enzyme is of the magnitude of 330 moles of linoleic acid oxidized per mole enzyme per second under the conditions of the assay used.

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