Spectrophotometric Method for Determination of Lipoxidase Activity[1, 2]

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Lipoxidase specifically catalyzes the oxidation of methylene-interrupted unsaturated fatty acids such as linoleic, linolenic, and arachidonic acids and their esters, to their respective peroxides (3). Various techniques have been devised for the determination of lipoxidase activity, including a colorimetric method based upon coupled oxidation, a manometric method, and spectrophotometric methods (6).

The spectrophotometric method was developed after Holman and Burr (4) and Bergström (1) independently observed an increase in ultraviolet light absorption, at 234 mμ, when lipoxidase acted upon essential fatty acids. The increase in UV-peak absorption was then related to the amount of peroxide formation which was found to be proportional to time and to enzyme concentration (7, 8). According to Holman and Bergström (3), both the manometric and the coupled oxidation methods give aberrant indices of lipoxidase activity because of variations in the degree of dispersion of fatty substrates. The spectrophotometric method, on the other hand, does not have these disadvantages because it employs more homogeneous substrates. Although satisfactory results are obtained when the method is operated at pH 9.0 or above, where the unsaturated fatty acids are present in a soluble form, nevertheless, this nonphysiological pH presents a major disadvantage of the spectrophotometric method. It was therefore concluded by Holman and Bergström (3) that the question of the effect of pH on the inherent activity of the enzyme could not be solved until a water-soluble substrate could be found.

The method described here is essentially a modification of the methods of Theorell et al. (8) and Tappel (7). The polyunsaturated fatty acid is solubilized by the addition of a detergent, and with this soluble substrate the activities of purified and crude lipoxidase are demonstrated over a wide range of pH. Also, lipoxidase activities in germinating mung beans, over a period of 80 hours, are presented.

Materials and Methods

Plant Material. The experimental material consisted principally of powdered soy beans (*Glycine max* L.), and gram seeds (*Cicer sp.*) which had been previously washed with petroleum ether and stored in a glass-stoppered bottle in a refrigerator.

For the determination of lipoxidase activities during the germination of mung beans (*Phaseolus aureus* L.), seeds were allowed to imbibe in the dark in a stainless steel screen container immersed in a water bath through which tap water was continuously circulated. At the desired stage of hydration a few seeds were taken from the water bath and their seed coats were removed. Cotyledons and embryos were weighed, ground with sand and extracted with water or 0.05M potassium phosphate buffer of the appropriate pH. The slurry was readjusted to the required pH and then centrifuged at 2000 × *g* for 15 minutes. The supernatant liquid was used as the enzyme source. Linoleic acid and purified soybean lipoxidase were obtained from Nutritional Biochemicals Corporation.

Lipoxidase Assay. For routine assay, substrate was prepared as follows: Tween 20, 0.5 ml, was dissolved in 10 ml of borate buffer of pH 9.0, and 0.5 ml of linoleic acid was added drop by drop. The contents were thoroughly mixed so as to disperse the acid into a fine emulsion. Then 1.3 ml of 1 N NaOH was added and the mixture once again agitated until a clear transparent solution was obtained. To this solution 90 ml of the borate buffer was added and the final volume made up to 200 ml with water. The resulting solution was approximately 7.5×10^{-3} M in linoleic acid and 0.25% in linoleate and Tween 20. Finally the solution was adjusted to the desired pH with concentrated HCl.

The reaction was carried out at room temperature in a mixture of measured volumes of enzyme and substrate, with O_2 passed continuously through the reaction mixture. Periodically 1 ml samples were transferred into each of several 12-ml centrifuge tubes containing 2 ml of absolute alcohol. To each tube 7 ml of 60% ethanol was later added to make a total volume of 10 ml. Tubes were centrifuged, if necessary. Optical densities of the clear alcoholic solutions were read at 234 mμ, against a suitable control, on a Cary recording spectrophotometer model 11. To ensure the maintenance of a constant operational pH during the reaction, pH of each reaction mixture was checked immediately after the termination of the sampling period. No variations from the starting pH's were noted.

The control solution was prepared using the same volumes of substrate and enzyme as those used in the test samples. Enzyme was added to 2 ml of absolute alcohol. After mixing, the tube was allowed to stand for a few minutes. Sixty percent alcohol was then added, followed by substrate. Further dilutions of both the test and the blank samples were made with 60% alcohol if necessary.

A unit of lipoxidase is defined as that activity

[1] Revised manuscript received June 24, 1963.

[2] This work was performed under the auspices of the U. S. Atomic Energy Commission.

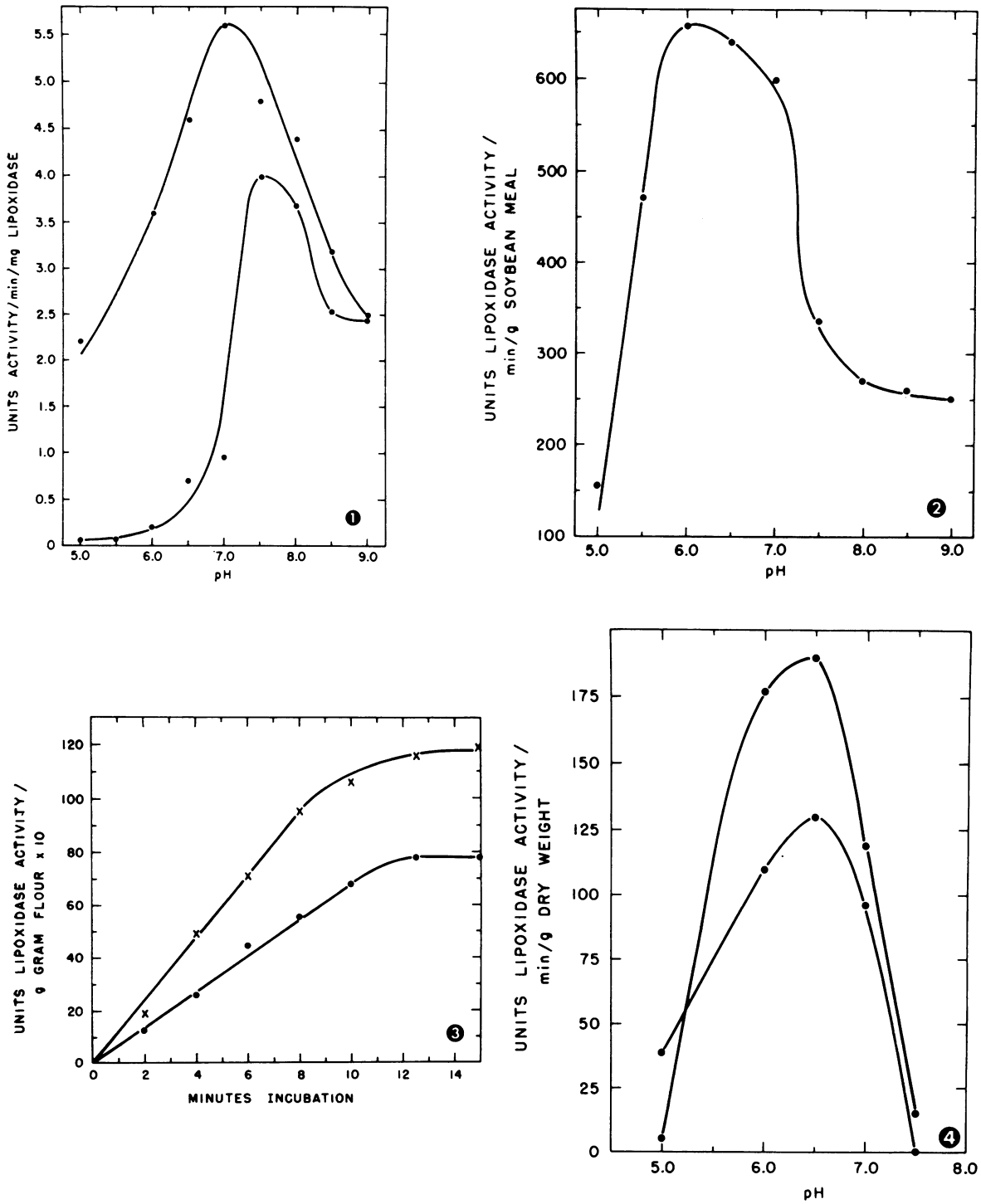

FIG. 1. Effect of pH on the activity of purified lipoxidase, with linoleic acid as the substrate. Top curve, with Tween 20; lower curve, without Tween 20. Enzyme was dissolved, in the ratio of 1 mg/ml, in each of the graded series of phosphate buffers; 1 ml of each enzyme solution was mixed with 19 ml of the substrate of corresponding pH value.

FIG. 2. Effect of pH on the activity of crude lipoxidase of soybean meal. Substrate was linoleate and Tween 20. Enzyme was extracted in the ratio of 1 g of meal per 200 ml of phosphate buffer for each pH value; 1 ml of this extract was incubated with 9 ml of the substrate of corresponding pH. The activities were determined over a period of 6 minutes.

which will produce an optical density of 1, at 234 mμ in 1 minute, in a total volume of 10 ml of 60% ethanol solution (cf. ref 6). To represent the rates of linoleate oxidation per minute, slopes of the least square lines were calculated from the linear portions of each activity curve.

Results

Effect of pH on Purified Soybean Lipoxidase. Periodic sampling indicated that the reaction of purified lipoxidase proceeded linearly with time for 10 minutes. The rates in relation to each corresponding pH value are shown in figure 1. Top curve shows the optimum pH of the enzyme activity to be 7.0, with the activity falling off symmetrically on either side. However, when Tween 20 was omitted from the reaction mixture (lower curve), 3 changes were noted: the pH optimum was shifted to 7.5; activities were lower over the entire pH range, except at pH 9.0 where the difference between the 2 points tends to disappear; and the activities declined abruptly below pH 7.5, presumably due to the unavailability of the substrate to the enzyme.

Effect of Tween 20 Concentration on Purified Soybean Lipoxidase. The effect of increasing amounts of detergent in a fixed concentration of linoleate is shown in table I. (For comparison of the first 2 concentrations, i.e., the 1: 0 and 1: 1 ratios, see also upper and lower curves, respectively, in fig 1.) At a ratio of 1: 1.5 of linoleate to Tween 20, the activities at pH 7.0 and below were slightly higher, and the activity at pH 7.5 was slightly lower, than those of the 1: 0 and 1: 1 ratios. The pH optimum, however, remained at 7.0. Further increase in detergent (ratio 1: 2) shifted the pH optimum to 6.5. Concomitantly, a decrease in oxidative activity was observed in the higher pH range. It is thus apparent that for minimal variation in activity over the pH range, the most effective ratio of linoleate to Tween 20 concentration lies close to 1: 1 (i.e., 0.25: 0.25%). No UV light absorption was recorded in an analogous experiment in which Tween 20 alone, 0.25% concentration at pH 7.0, was employed as substrate; thus although detergent itself does not interfere in the present assay procedure, its incorporation beyond 0.375% (ratio 1: 1.5) exerted an inhibitory influence on the enzymatic activity. A lower than 0.25% concentration of Tween 20, in a 0.25% concentration of linoleate, could not be employed in these experiments because of the precipitation of fatty acid in the acid pH.

Effect of pH on Soybean Meal Lipoxidase. Figure 2 shows that the optimal pH of crude lipoxidase of soybean meal was about 6.0. The activity declined sharply toward the acid side; on the alkaline side, the activity declined gradually up to pH 7, fell sharply between 7.0 and 7.5, and leveled off beyond pH 8.0.

Gram Flour Lipoxidase. Figure 3 shows the activity of crude gram flour lipoxidase with (top curve) and without (lower curve) Tween 20. In each case the reaction continued linearly for about 8 minutes. The presence of Tween 20 once again (see fig 1) resulted in enhanced oxidation of the linoleate substrate.

The effect of pH on the crude gram-flour lipoxidase, in the presence and in the absence of Tween 20, is shown in figure 4. In both substrates (with and without Tween) the enzyme showed its pH optimum to be 6.5. Virtually no activity was observed on either side of 5 to 7.5 pH range.

Effect of pH on Mung Bean Lipoxidase. In

Table I

Effect of Tween 20 on the Optimal pH for Oxidation of Linoleic Acid by Purified Soybean Lipoxidase

Ratio linoleate to Tween 20	Units of activity per mg lipoxidase/min			
	pH range			
	6.0	6.5	7.0	7.5
1: 0 (0.25: 0%)	0.20 ± 0.03	0.70 ± 0.04	0.96 ± 0.10	4.00 ± 0.24
1: 1 (0.25: 0.25%)	3.60 ± 0.31	4.60 ± 0.32	5.60 ± 0.36	4.80 ± 0.08
1: 1.5 (0.25: 0.375%)	4.04 ± 0.34	5.18 ± 0.40	5.76 ± 0.30	3.42 ± 0.12
1: 2 (0.25: 0.50%)	3.96 ± 0.08	4.46 ± 0.12	3.60 ± 0.02	3.50 ± 0.10

FIG. 3. Effect of Tween 20 on the activity of crude lipoxidase of gram flour. Substrate was linoleate. X, with Tween 20; ●, without Tween 20. Lipoxidase from 1 gram of petroleum ether-washed gram flour was extracted in 50 ml of phosphate buffer of pH 7.2; 1 ml of the extract was mixed with 9 ml of the substrate of the same pH. Oxidative activities of the enzyme were determined as usual.

FIG. 4. Effect of pH on the oxidation of linoleate by crude lipoxidase from gram flour. Top curve, linoleate and Tween 20. Lower, linoleate only.

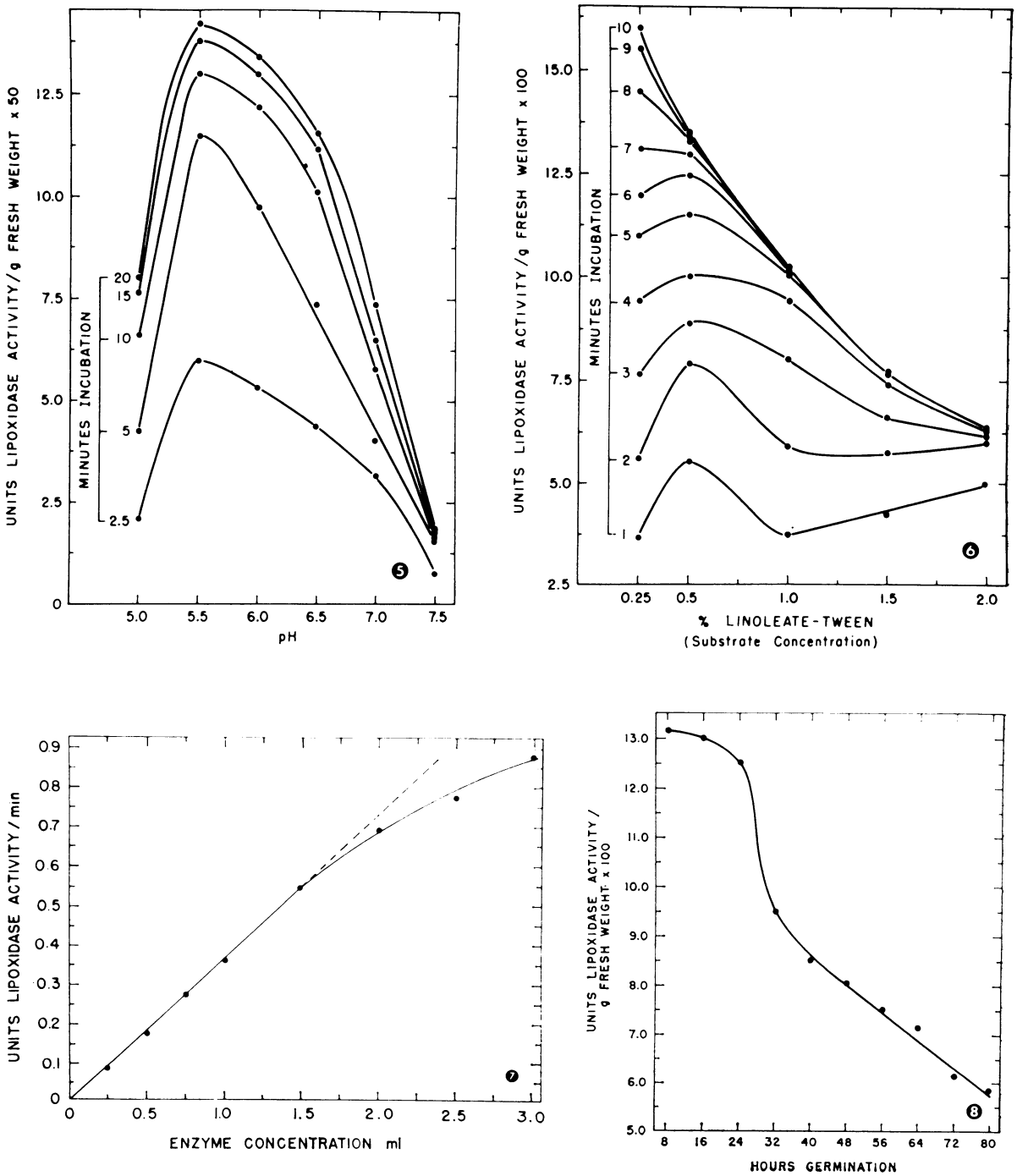

FIG. 5. Effect of pH on the oxidation of linoleate containing Tween 20 by crude lipoxidase of hydrated mung beans. Times of incubation at which the determinations were made are shown on the left side.

Sixteen grams of mung bean seeds hydrated for about 16 hours were ground in sand and in 200 ml of water. The water extract was divided into several portions, for which pH was adjusted with minimum quantities of HCl or NaOH before centrifugation. One ml of enzyme was mixed with 19 ml of the substrate of corresponding pH and the oxidation of linoleate was determined as before.

FIG. 6. Effect of substrate concentration on linoleate oxidation by crude lipoxidase of hydrated mung beans. Minutes of incubation at which the determinations were made are shown on the left side. The concentrations of linoleate as well as of Tween 20 were increased in equal proportions and the pH of each constituted substrate was adjusted to 5.5. Enzyme was extracted in water from seeds hydrated for about 18 hours in the ratio of 1 g/25 ml H_2O and the pH

figure 5 are represented the units of lipoxidase activity in relation to pH range over a period of 20 minutes of incubation. It can be seen that the enzyme had maximal activity at pH 5.5 with a considerable drop toward the acid side. On the other side, a gradual decline is manifested and after pH 7.5 no more oxidation was observed.

Effect of Substrate Concentration on Lipoxidase. Increasing the concentration of Tween 20 in a constant concentration of linoleate, partially inhibits the oxidation by purified lipoxidase, as was shown in table I. The effect of substrate concentration on the oxidative capacity of mung bean lipoxidase over a period of 10 minutes is shown in figure 6. In general, the highest activity was encountered at 0.5% substrate concentration but after 6 minutes of reaction, the activity began to level off. Although at the lower concentration (0.25%) the activity for each incubation time was lower than that for next higher concentration, linearity of activity was maintained over about 9 minutes of incubation; after 7 minutes the activity exceeded that for 0.5%. At the 1% substrate level, the activity was initially lower than at the 0.5% level; and for the first 4 minutes of incubation matched closely that at 0.25%. A slight upward trend, in relation to the 1% concentration, is also seen at the 1.5 and 2.0% levels during the first 2 minutes of oxidation; the activities level off much earlier than those encountered at the lower substrate concentrations. It is thus apparent that the optimal substrate concentration lies within the range of 0.25 to 0.5% and that the progressive increase in substrate concentration from 0.25 to 2.0% results in decreased enzyme activity.

It may be pointed out that this experiment (fig 6) does not discriminate between the differential inhibitions caused either by linoleic acid or by Tween 20. It rather illustrates the combined effects of these 2 components.

Effect of Enzyme Concentration on Crude Mung Bean Lipoxidase. In order to demonstrate that the present method is also applicable with crude enzyme extracts, where the presence of interfering substances could cause aberrant results, experiments were conducted to show that the amount of peroxide formation is proportional to the enzyme concentration. Figure 7 shows that strict linearity was maintained up to 1.5 ml of mung bean crude lipoxidase per 20 ml of reaction mixture. Beyond this level the activities start declining presumably because of the limitations in the substrate contents. Furthermore, concentrations above 1.5 ml also resulted in nonlinearity of activity with respect to the time of reaction, a phenomenon analogous to the one shown in figure 6 (for 1.0–2.0% substrate concentrations).

Lipoxidase and Germination of Mung Beans. Since the optimal pH of lipoxidase from hydrated mung bean lies close to 5.5, experiments were then conducted at this pH to determine lipoxidase activity at various stages of seed germination. The results are shown in figure 8 in which are represented 3 periods of enzyme activity: slow decline in the initial phase (8–24 hr), accelerated decline in the second phase (24–40 hr), and a linear decline of approximately 70 units/hour in the third phase (40–80 hr) of germination.

Discussion

The results of this investigation show that the optimal pH's of purified soybean lipoxidase and crude enzymes prepared from gram flour, soybean meal, and hydrated mung beans were 7.0, 6.5, 6.0, and 5.5 respectively. These differences in pH responses could be due to the interfering substances present in the crude extracts.

The presence of Tween 20 facilitates uniform distribution of fatty substrates, so that their pH can be varied over the entire pH range without the formation of an emulsion. Tween 20 did not absorb light at any wavelength of the ultraviolet spectrum and as such it did not interfere in the present spectrophotometric determinations. Although the incorporation of Tween 20 in the fatty substrate consistently contributed to higher lipoxidase activity (fig 1, 3, 4), its higher concentration exerted an inhibitory influence on the enzyme activity (table I, fig 6). Further evidence that Tween is not a substrate of lipoxidase comes from Holman (cited in ref 3) who observed that Tween prepared from linoleic acid and polyoxyethylenesorbitan was not attacked by lipoxidase.

The present procedure, without the incorporation of detergent (fig 1, 4, lower curves), did not result in an accelerated enzyme activity at pH 9.0 or above as was found by the original authors (7, 8, also described in ref 3).

Although the experiments on lipoxidase activity during mung bean germination (fig 8) are consistent with those reported for soybean (5) and maize seeds (2), the physiological significance of lipoxidase in the germination process remains to be determined.

adjusted to 5.5 before centrifugation. One ml of the enzyme was mixed with 19 ml of each substrate and the activities were determined.

FIG. 7. Effects of varied concentrations of crude mung bean lipoxidase on the oxidation of a fixed concentration of linoleic acid containing Tween 20.

Enzyme was prepared in the same way as in figure 6. To 17 ml of substrate (pH 5.5) water and enzyme were added so as to make a total volume of 20 ml of the reaction mixture. Enzyme concentration, ml, means ml of enzyme preparation per 20 ml of final reaction mixture. Activities were determined over a period of 4 minutes for each indicated enzyme concentration value.

FIG. 8. Lipoxidase activity of dark-imbibed mung beans, determined periodically at each indicated time interval. Enzyme was prepared in the same way as in figure 6, and the activities were determined after 30 minutes of incubation.

Summary

An improved spectrophotometric method for the determination of lipoxidase activity was developed and applied in studies of the purified enzyme and crude enzyme preparations from leguminous seeds, with linoleic acid solubilized in Tween 20 as the substrate. The optimum pH was found to be 7.0, 6.5, 6.0, and 5.5, for purified soybean lipoxidase and for the crude lipoxidases extracted from gram flour (*Cicer sp.*) soybean meal (*Glycine max* L.), and hydrated mung beans (*Phaseolus aureus* L.), respectively. The application of this test illustrates A) that the present method was free from the inherent limitations on pH present in the original methods, B) that increasing the amount of detergent, in a fixed concentration of fatty substrate, caused inhibition in the enzymatic activity; more pronounced inhibitions occurred when the concentrations of detergent as well as of fatty substrate were increased in equal proportions, and C) peroxide formation is proportional to time of reaction and to enzyme concentration.

Determination of lipoxidase activity in germinating mung beans indicated 3 periods of enzyme activity: a slow decline from 8 to 24 hours, a rapid decline from 24 to 40 hours, and a linear decline from 40 to 80 hours of germination.

Literature Cited

1. Bergström, S. 1946. On the oxidation of linoleic acid with lipoxidase. Arkiv Kemi Mineral Geol. Band 21A. No. 15.
2. Fritz, G. J. 1962. Studies on O_2-fixation by maize seedlings. Plant Physiol. 37: xxxii–xxxiii.
3. Holman, R. T. and S. Bergström. 1951. Lipoxidase or unsaturated-fat oxidase. In: The Enzyme Chemistry and Mechanism of Action, J. B. Sumner and K. Myrbäck, eds. Academic Press Inc., New York. vol. 2, Part 1, 559–74.
4. Holman, R. T. and G. O. Burr. 1945. Spectrophotometric studies of the oxidation of fats. IV. Ultraviolet absorption spectra of lipoxidase-oxidized fats. Arch. Biochem. 7: 47–54.
5. Holman, R. T. 1948. Lipoxidase activity and fat composition of germinating soy beans. Arch. Biochem. 17: 459–66.
6. Holman, R. T. 1955. Measurement of Lipoxidase Activity. In: Methods of Biochemical Analysis, D. Glick. ed. Interscience Publishers, New York. vol II, 113–19.
7. Tappel, A. L. 1962. Lipoxidase. In: Methods in Enzymology, S. P. Colowick and N. O. Kaplan, eds. Academic Press., New York and London. vol 5, 539–42.
8. Theorell, H., S. Bergström, and Å. Åkeson. 1946. Activity determination and further purification of lipoxidase. Pharm. Acta Helv. 21: 318–24.

Chemotropic Response of the Pollen of Antirrhinum majus to Calcium [1, 2]

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We reported earlier (A) our failure to identify among a large number of organic compounds active in biological systems any that exerted a chemotropic effect on the pollen tubes of snapdragon and (B) the results of extraction and fractionation procedures that suggested the chemotropic factor from gynoecia to be a quite small molecule, heat stable, water soluble, and associated with larger molecules from which it could be separated by various means (14). This led to a search among inorganic ions, particularly those reported at one time or another to enhance germination and tube growth of pollen. When we tested the chlorides of Ca, Mn, and Zn at several different concentrations, Mn and Zn proved to be inactive but Ca elicited a pronounced chemotropic response. The subsequent investigation of the chemotropic effect of Ca is reported in this paper. A brief summary of a part of these studies was previously published (15).

[1] Received May 6, 1963.

[2] Most of the research reported in this paper is part of a dissertation submitted by the first author to the Graduate Division of the University of California at Berkeley in partial satisfaction of the requirements for the degree of Doctor of Philosophy in the field of Plant Physiology. The work was supported by research grant G-7031 from the National Science Foundation.

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Materials and Methods

The basic procedures and materials are described in detail in the earlier report (14). Flowers of *Antirrhinum majus* (tetraploid) grown in the University Botanical Garden provided both gynoecia and pollen. For a brief period, when these plants were not available, cut flowers were purchased from a local florist.