[CONTRIBUTION FROM THE MULTIPLE FELLOWSHIP OF THE COTTON RESEARCH FOUNDATION, MELLON INSTITUTE]

Composition of Cottonseeds. IV. Lipase of Germinated Seed[1]

BY H. S. OLCOTT AND T. D. FONTAINE

Although cottonseed containing 12 to 25% moisture may develop large amounts of free fatty acids during storage, numerous experiments designed to demonstrate the presence of a lipase in such seed gave consistently negative results.[2] During the course of the work, it was found that, as in other plants, lipase activity appears during the germination process. The effects upon this enzymatic system of *p*H, gossypol and some inorganic salts have now been determined.

Experimental

Germinated cottonseed lipase preparations were obtained as follows. Cottonseeds were soaked overnight in water, then placed between sheets of wet paper. When the germination had proceeded to the allotted time, usually three to five days, the hulls were removed by hand and the seedlings were dried in vacuum desiccators over sulfuric acid. Dehydration was complete in two to three days, and the residues could be powdered easily. Preparations dried by grinding the seedlings in acetone were appreciably less effective, as were also direct aqueous extracts or emulsions of the wet seed.

The activity of the lipase was assayed by a modification of the method described by Longenecker and Haley.[3] To a weighed sample of the preparation in a homeopathic vial were added 1 cc. (0.92 g.) of refined cottonseed oil (Wesson oil), 3 cc. of water, and 1 cc. of buffer solution (0.1 *M*).[4] The vial was stoppered with a paraffined cork and shaken for sixteen hours at room temperature in a mechanical shaker. The contents were then centrifuged and the solid residue was washed four times (by centrifugation) with isopropanol–petroleum ether (2:1) mixture. The original supernatant layers were combined with the washings and titrated with 0.1 *N* sodium hydroxide in isopropanol with aniline blue indicator.[5] When less than 20 mg. of lipase preparation was used, the entire assay mixture was titrated. Appropriate blanks were run and the recorded data have been corrected for these determinations.

The results of analyses of several seed constituents during successive stages of germination are given in Table I. There is a progressive loss in dry weight and total lipids with increasing free fatty acid and lipase contents. The lipase reaches maximum activity on the third or fourth day and thereafter does not appear to change. Similarly the free fatty acid level increases only slowly after this stage, although when expressed as percentage of total lipids, the relative increase appears significant (from 8% on the third day to 20% on the sixth). Possibly the fatty acid stage is intermediate in the transformation of lipids to nonfatty materials. Ultimately the lipid content is reduced to very low levels (after two to three weeks of growth).

TABLE I

CHANGES OCCURRING DURING THE GERMINATION OF COTTONSEED

Time of germination, hrs.	Root length, cm.	Water content, %	Dry weight,[a] g.	Oil content,[b] g.	Free fatty acids,[c] g.	Lipase activity[d]
0	0	6	6.40	2.75	0.05	0
23	0	48	6.02	2.62	.04	0.8
42	1–2	63	5.68	2.57	.14	7.8
67	2–5	73	5.56	2.11	.18	17.7
91	5–8	78	5.51	1.83	.21	20.1
115	8–15	83	5.51	1.38	.21	21.9
139	10–18	84	5.40	1.23	.25	20.0

[a] Weight of 100 dried seedlings minus hulls, corrected for residual moisture. [b] By continuous hot chloroform extraction (Soxhlet) for 24 hours. [c] Expressed as oleic acid. [d] Expressed as percentage of Wesson oil hydrolyzed by 17 mg. of germinated cottonseed (calculated on oil and moisture-free basis) in 16 hours, at *p*H 7.2 (phosphate buffer). All figures are the average of duplicate determinations.

The roots, on a dry weight basis, were approximately 30% more active lipolytically than were the whole seedlings. A powdered root preparation was used to obtain the observations presented in Fig. 1.

The lipase was most effective in the *p*H range 6 to 9, and the particular buffers employed were apparently without specific effect. In the presence of calcium chloride (3 mg. per assay) maximal activity was observed at *p*H 8–9, but some difficulty was experienced in obtaining consistent results. The data graphically set forth in the figure are typical of most of the runs. The reactivity of cottonseed lipase at different *p*H levels thus resembles that of pancreatic lipase and con-

(1) Presented in part before the Division of Agricultural and Food Chemistry at the Detroit meeting of the American Chemical Society, September, 1940. Other papers in this series appeared in THIS JOURNAL, **61**, 2037, 2417 (1939); **62**, 1334 (1940).

(2) To be described in another publication.

(3) H. E. Longenecker and D. E. Haley, THIS JOURNAL, **57**, 2019 (1935).

(4) Sandberg and Brand (*J. Biol. Chem.*, **64**, 59 (1925)) recommended the addition of water first and olive oil last in their study of papain lipase. The order of addition in the germinated cottonseed lipase assays appeared to be more important in the alkaline than in the neutral or acid ranges. At *p*H 9.0 increased lipolysis was observed when the aqueous reagents were added first.

(5) "Report of Committee on Indicators, American Oil Chemists' Society," *Oil and Soap*, **16**, 132 (1939).

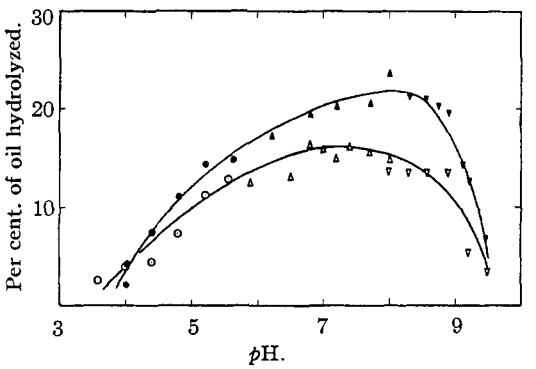

Fig. 1.—*p*H–activity relationships of germinated cottonseed lipase: ⊙, acetate buffer, 0.1 *M*; △, phosphate buffer, 0.067 *M*; ▽, ammonia buffer, 0.1 *M*; ● ▲ ▼, calcium chloride (3 mg. per assay) present.

trasts with that of castor bean preparations, in which case maximal activity is obtained at *p*H 4.5–5.0.[6]

Gallup and Reder[7] reported that gossypol inhibits the action of pancreatic lipase. As shown in Table II, the inhibition is more effective in an alkaline medium. The cottonseed lipase was not affected. The activity of castor bean lipase (at *p*H 4.5) also was not changed in the presence of gossypol.

TABLE II

EFFECT OF GOSSYPOL ON THE LIPASES OF GERMINATED COTTONSEED AND PANCREATIN

Additions	*p*H[b]	Per cent. of total added cottonseed oil hydrolyzed[a] Germinated cottonseed	Pancreatin[c]
None	5.6	13.7	19.6
10 mg. gossypol	5.6	14.4	15.1
None	7.0	22.5	30.5
10 mg. gossypol	7.0	21.6	23.2
None	8.8	21.0	27.6
10 mg. gossypol	8.8	21.0	5.8

[a] Twenty mg. of the lipase preparation and 3 mg. of $CaCl_2$ were used in each assay. [b] The following buffers were used: *p*H 5.6, acetate (0.1 *M*); *p*H 7.0, phosphate (0.67 *M*); *p*H 8.8, ammonia (0.1 *M*). [c] U. S. P. Pancreatin obtained from the Fisher Scientific Co.

The cottonseed preparation thus differs from the castor bean enzyme in the *p*H range of optimum activity, and from pancreatic lipase in its susceptibility to inhibition by gossypol. However, since the preparations were crude, it is not possible to conclude that the enzyme is a different entity. Willstätter's[8] observations indicate that the properties of lipases may change markedly during purification procedures.

(6) K. J. Falk, THIS JOURNAL, **35**, 601, 616 (1913), and others.
(7) W. D. Gallup and R. Reder, *J. Agr. Research*, **52**, 65 (1936).
(8) R. Willstätter, F. Haurowitz and F. Memmen, *Z. physiol. Chem.*, **110**, 203 (1924).

According to Glick and King,[9] hexylresorcinol and octyl alcohol are effective activators for the hydrolysis of tributyrin by pancreatic lipase preparations. These compounds, in the concentrations which they used, did not affect the activity of the cottonseed lipase when either cottonseed oil or tributyrin was used; nor was the activity of commercial pancreatin influenced under the conditions described. Bile salts and cholesterol were also without effect on the cottonseed enzyme. Possibly the presence of significant amounts of soluble protein and a solid phase in the lipase assay mixtures modified the influence of these surface active reagents.

Sodium cyanide (1% of the aqueous phase) and cysteine (1%) neither inhibited nor activated the reaction but sodium fluoride in the same concentration accomplished approximately 60% inhibition (*p*H 7.2). Inasmuch as sodium oxalate (1%) did not affect the assay results (in the absence of added calcium) while sodium hexametaphosphate (Calgon) activated the reaction, the fluoride inhibition is probably not due to a removal of calcium ions.

The best preparations from germinated cottonseed were approximately equal in lipolytic action to that of commercial pancreatin and roughly one-fourth to one-tenth as active as petroleum ether-extracted castor beans.[10] The powder is fairly stable; one preparation had lost approximately 30% of its activity after four months of storage at 0°.

In a limited series of experiments, it was found that the germinated cottonseed product was capable also of hydrolyzing triacetin, tributyrin and benzyl butyrate.

The preparation of extracts and concentrates was attempted without marked success. Water and dilute salt solutions extracted some of the lipase but a large part of the activity remained in the solid residue. Better extractions were accomplished with an alkaline buffer (*p*H 8.8.) or glycerol (87%). The activity of these extracts did not decrease markedly on standing, an observation in contrast to that of Longenecker and

(9) D. Glick and C. G. King, *J. Biol. Chem.*, **97**, 675 (1932).
(10) Studies of castor bean lipase, the most active plant lipase yet investigated, have been hampered by the toxic properties of fat-free castor bean meal.[11] In the present investigation, one of us (T. D. F.) suffered severe asthmatic attacks after working with such a preparation for only three weeks, although no unpleasant symptoms were encountered during 18 months of contact with the germinated cottonseed lipase. The Baker Castor Oil Co., Newark, N. J., kindly furnished us with castor beans.

Haley[11] who described an inactivation of castor bean lipase while in contact with water.

Summary

Dormant cottonseeds contain no lipase. Lipolytic activity develops during germination. Concurrently, total lipids decrease and free fatty acids increase. The germinated cottonseed lipase is effective in the *p*H range 6 to 9 (optimum 7 to 8). Calcium chloride activates the enzyme, particularly in alkaline solutions. The effects of several other reagents on its activity are described.

(11) H. E. Longenecker and D. E. Haley, THIS JOURNAL, **59**, 2156 (1937).

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[CONTRIBUTION FROM THE INSTITUTE OF MATERIA MEDICA, SHANGHAI]

Study of Gelsemine. II. The Bromination and Nitration of Gelsemine

BY T. Q. CHOU AND T. T. CHU

In a previous paper,[1] the reduction of gelsemine to dihydrogelsemine, isogelsemine, and a crystalline substance having the composition $C_{18}H_{22}O_4N$, was reported. The ease with which gelsemine forms new bases by taking up one molecule of water or hydrogen chloride,[2] indicates probably the presence in its molecule of a double bond which is more highly reactive than the double linkages of a benzene ring. The fact that gelsemine readily forms an addition product with bromine appears to support this view. Dibromogelsemine, $C_{20}H_{22}O_2N_2Br_2$, is obtained when gelsemine is allowed to react with two atoms of bromine at a low temperature. It is sufficiently stable to be isolated and purified, but loses easily one molecule of hydrogen bromide to form monobromogelsemine, $C_{20}H_{21}O_2N_2Br$, when treated with acids and alkalies. The nitration of gelsemine with a mixture of concentrated nitric and sulfuric acids at $-7°$ did not lead to satisfactory results as the products were amorphous, without definite melting points and difficult to be purified. On the contrary, dihydrogelsemine, when nitrated under similar conditions, gives rise to dinitrogelsemine, $C_{20}H_{22}O_6N_4$, with an almost quantitative yield. It forms well crystallized nitrate and methiodide.

Experimental

I. Dibromogelsemine.—To 1 g. of gelsemine (acetone-free) in 25 cc. of chloroform cooled in a freezing mixture 0.5 g. of bromine (2 atoms) in 5 cc. of chloroform is slowly added with stirring. The addition product, which separates out at first, redissolves on shaking. After about one hour in the freezing mixture, the slightly pink chloroform solution is distilled and the residue taken up with alcohol whereupon dibromogelsemine crystallizes in colorless rhomboidal crystals. Dibromogelsemine in a pure state is only sparingly soluble in most organic solvents such as ether, benzene, chloroform, methanol, ethanol, carbon tetrachloride, and ethyl acetate. When carbon tetrachloride is substituted for chloroform as the solvent for bromination, the same result is obtained.

II. Monobromogelsemine.—A solution of 0.5 g. of dibromogelsemine in dilute oxalic acid, is made alkaline with sodium carbonate, and the precipitate extracted with chloroform. The chloroform solution is dried with anhydrous sodium sulfate and distilled to a small volume. Ether is then cautiously added until a turbidity begins to appear. On standing overnight at room temperature, monobromogelsemine crystallizes out in colorless prisms. It is easily soluble in chloroform or ethanol and much less so in ether.

III. Dinitrogelsemine.—One gram of powdered dihydrogelsemine is slowly added with stirring to a mixture of 8 cc. of sulfuric acid (sp. gr. 1.84) and 1 cc. of nitric acid (sp. gr. 1.42) which is maintained at $-7°$ in a freezing mixture. At the end of the reaction, the temperature is allowed to rise gradually and remain at 5° for about ten minutes. A clear orange solution thus obtained is poured into a sufficient quantity of a mixture of ice and water, made alkaline with potassium carbonate, and the precipitate extracted with chloroform. The chloroform solution is dried with anhydrous sodium sulfate and concentrated until fine yellow needles separate out. Recrystallized from a mixture of chloroform and absolute alcohol, dinitrogelsemine forms bright yellow, long, soft needles. The yield is almost quantitative. It is dextrorotatory, $[\alpha]^{18}D$ +6.6°. Dinitrogelsemine is found to be easily soluble in chloroform and pyridine, less so in acetone, methanol, ethanol, benzene, and ether and insoluble in petroleum ether. It is slightly soluble in water and its aqueous solution has a neutral reaction toward litmus paper. It is soluble in concentrated sulfuric acid to form a light yellow solution which becomes green on the addition of a crystal of potassium dichromate. The acetone solution of dinitrogelsemine becomes cherry red and then blood red on the addition of a few drops of 10% potassium hydroxide solution—Janovsky's reaction for aromatic polynitro compounds. When hydrogenated in the presence of Adams platinum catalyst, it takes up six moles of hydrogen, but

(1) Chu and Chou, THIS JOURNAL, **62**, 1955 (1940).

(2) Moore, *J. Chem. Soc.*, **99**, 1231 (1911).