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COWPEA LIPOXYGENASE: PART 1 PHYSIOCHEMICAL CHARACTERIZATION

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ABSTRACT

Lipoxygenase (linoleate: oxygen oxidoreductase E.C.1.13.11.12) level was determined in ten varieties of cowpea, five varieties of soybean and six varieties of winged bean. Cowpea lipoxygenase specific activity was highest (205 -316 units/mg protein) as compared to that of winged bean (14 - 30 units/mg protein) and soybean (11 - 48 units/mg protein).

Crude cowpea lipoxygenase was observed to have optimum activity at about pH 6.2 and 30°C. The apparent Km and substrate concentration at Vmax of this enzyme which follows Michaelis-Menten kinetics were 0.195×10^{-3} and 1.6×10^{-3} M, respectively.

Lipoxygenase activity was located in 40 - 50 % and 50 - 60 % ammonium sulfate precipitates with 3 and 5 fold enrichment in said fractions.

Disc gel electrophoresis followed by specific enzyme staining revealed four isozymes of cowpea lipoxygenase with a major isozyme of Rf = 0.21 – 0.27. The average molecular weight of this enzyme is 68,000 daltons as shown by Sephadex G-50 gel chromatography.

The possible role of lipoxygenase in the formation of the strong beany flavor of cowpea is discussed.

INTRODUCTION

Among legumes, cowpea (*Vigna unguiculata* (L.) Walp.) has been reported to possess a lower concentration of antinutritional factors (1). It can be grown relatively easy, producing high yields in tropical regions that are not suitable for the growth of other legumes (2). High cowpea production, therefore, can be achieved in many areas of the world. Thus, cowpea with its high protein content of about 24%, lower antinutritional factors and high yielding ability has increasingly become an important source of high-protein food.

In recent years cowpea has received considerable attention in food processing and product utilization. However, like other legumes, a beany

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flavor was detected in cowpea products, which consequently limits its use (3). Okaka and Potter (4) were able to reduce the beany flavor in cowpea powder by using acid and heat treatment and they attributed this to the inactivation of lipoxygenase. However, lipoxygenase activity before and after treatments was not reported.

Lipoxygenase (linoleate: oxygen oxidoreductase E.C.1.13.11.12) catalyzes the formation of odorous carbonyl compounds which account for the off- and beany flavor in soybean, peanut and other legume products (5) through the oxidation of polyunsaturated fatty acids containing *cis, cis*—1—4—pentadiene system.

We have initiated the determination of lipoxygenase levels in different cowpea varieties and have compared these with this enzyme's activities in several soybean and winged bean varieties. We also report on the characterization of cowpea lipoxygenase, a step preparatory to its purification. Both steps, characterization and purification, are necessary to understand its mechanism of action and role in the formation of the beany flavor in cowpea.

MATERIALS AND METHODS

Materials

Seeds of several varieties and lines of cowpea, soybean and winged bean were obtained from the Institute of Plant Breeding, University of the Philippines at Los Baños. Linoleic acid, oleic acid, bovine serum albumin, and Tween 20 were purchased from Sigma Chemical Company. All other chemicals used were of analytical grade.

Sample preparation

Cowpea seeds were ground in a Wiley Mill to pass a 60-mesh screen. The meal was defatted with excess hexane at 4°C. The defatted meal was pulverized, air dried and stored at -20°C until used.

Preparation of Crude Extracts

Defatted cowpea meal was extracted with 0.05 M citrate-phosphate buffer, pH 5.8, 4°C in a chilled mortar and pestle for 5 minutes, at a meal: solvent ratio of 1:20 (w/v). The slurry was then passed through four layers of cheesecloth and then centrifuged at 12,000 x g for 20 minutes at 4°C in Sorvall RC5. The clear supernatant was used for the enzyme assay.

Ammonium Sulfate Fractionation

One hundred ml of crude extract was treated with ammonium sulfate to make 40% saturated solution. The precipitate formed was removed by

centrifugation and discarded. More ammonium sulfate was added to the supernatant to reach 50% and then 60% saturation. The corresponding 40-50% and 50-60% precipitates were spun down by centrifugation and dissolved with 0.05 M phosphate buffer, pH 7.0.

Gel Filtration Chromatography

The crude extract was prepared as described above using 40 g hexane-defatted cowpea meal and 0.05 M citrate-phosphate buffer, pH 5.8, at 1:10 meal-solvent ratio. The extract was treated with ammonium sulfate to make 40-60% saturated solution and the resulting protein precipitate was separated by centrifugation at 12,000 x g for 30 min. The precipitate was dissolved in a small amount of 0.05 M phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer. The dialyzate was applied on a Sephadex G-150 column (90 x 2.5 cm) and eluted with 0.05 M phosphate buffer, pH 7.0, with a flow rate of about 17 ml/hr. Five ml fractions were collected.

Disc Gel Polyacrylamide Electrophoresis

Electrophoresis was carried out according to the procedure of Davis (6) using 7.5% polyacrylamide gel, pH 9.3 with 1% soluble starch for enzyme activity staining (7). Electrophoresis proper was done at 3 mA per tube for two hours at 4^{o} C. Protein was stained by immersing the gels in 1% amido-black 10B. Destaining was performed in a Bio-Rad diffusion destainer.

To detect lipoxygenase activity, the gels were immersed in 0.1 M phosphate buffer, pH 6.2, containing 1.6×10^{-3} M linoleic acid and 0.05% Tween 20 for 30-45 min at room temperature. The gels were then rinsed with distilled water and the brown color bands were developed by starch-iodine staining (7).

Protein Determination

Protein was determined by the method of Lowry *et al.* (8) or by measuring absorbance at 280 nm. Bovine serum albumin was used as standard.

Assay for Lipoxygenase Activity

Lipoxygenase activity was determined spectrophotometrically by measuring the increase in absorbance due to the formation of conjugated diene hydroperoxide at 234 nm (9). An initial lag period of about two minutes was observed especially with newly prepared substrate. This lag period decreased upon storage of the substrate. The initial velocity of the reaction was taken in the linear part of the enzyme reaction progress curve.

The substrate solution was prepared by adding 0.1 NaOH dropwise to a mixture of 0.05 ml linoleic acid and 0.05 ml Tween 20 until the solution became clear after which deionized distilled water was added to make a total volume of 10 ml. This stock solution had a concentration of 16.1×10^{-3} M

linoleic acid and could be used for several days if stored at 4°C under nitrogen.

Each reaction mixture contained 0.3 ml of substrate solution, 0.02 ml of diluted enzyme preparation and 2.7 ml of 0.1 M phosphate buffer, pH6.2.

One unit of enzyme activity is defined as the amount of enzyme which produces a change of one unit of absorbance at 234 nm per minute.

Assays were done at 30°C with a Gilford Model 250 spectrophotometer attached to a recorder.

The procedures described above had been established as optimal for crude extract preparation and lipoxygenase assay of cowpea (10). For winged bean and soybean lipoxygenase, optimal conditions followed were those established by Truong (11) and Diel and Stan (12), respectively.

RESULTS AND DISCUSSION

Lipoxygenase Activity in Different Varieties and Lines of Cowpea, Soybean and Winged Bean

The lipoxygenase activity of all the 10 cowpea lines is higher than any of the soybean and winged bean lines assayed. CES 26-12 exhibited the highest specific activity of 316 units/mg protein while TVX 289-4G had the lowest of 205 units/mg protein (Table 1). The latter value is 4 and 7 times more than the highest activity obtained for soybean (UPSSY$_2$, 48 units/mg protein) and winged bean (Batangas medium, 30 units/mg protein), respectively. Soybean has been reported previously as the richest source of lipoxygenase (13). The present finding indicates that cowpea could be an even richer source of the enzyme and this could possibly account for the strong and persistent beany flavor in processed cowpea products. The wide variation in the lipoxygenase level of the ten accessions of cowpea indicates that suitable materials may be obtained for breeding low lipoxygenase-containing varieties.

The outstandingly high levels of lipoxygenase activity obtained in the cowpea extracts prompted a study to establish if the high linoleate-oxidizing activity was due to a lipoxygenase-type enzyme or to a heme-containing enzyme like catalase or peroxidase. The following observations show that the linoleate-oxidizing activity was due to a lipoxygenase-type enzyme (summarized in Table 2).

(a) the cowpea crude extract did not oxidize oleic acid;

(b) the activity was directly proportional to the extract concentration;

(c) the activity was inhibited by high concentrations of Tween 20;

and (d) boiling the crude extract for 5 minutes destroyed the enzyme activity.

Tappel (15) reported that the activity of hemoprotein-catalyzed oxidation is directly proportional to the square root of the enzyme concentra-

Table 1. Lipoxygenase activity in cowpea, soybean and winged bean extracts.

Legume Variety or Line	Lipoxygenase Specific Activity (Unit/mg protein)
Cowpea	
CES 26–12	316
VCS–6-1	296
BPI–Imp Gr # 1	279
CES 42–2	272
Mecan Pea	260
VCS–18	258
VCS–12	242
V59–41	211
All Season	209
TVX – 289-4 G	205
Soybean[a]	
UPSSY 2	48
Cobb	37
CES–12	33
Commercial	14
Commercial Defatted meal (14)	11
Winged Bean (11)	
Batangas Meduim	30
PI 7041	30
TPT–2	28
TPT–1	24
UPS 31	23
PI 7256	14

[a] Assayed at pH 6.2.

tion and that the reaction is not specific only to the substrates containing *cis, cis*-1,4-pentadiene system as linoleic acid. Oleic acid can thus be oxidized

Table 2. Some properties of the linoleate-oxidizing activity of crude cowpea extract.[a]

Treatment	Activity
Oleic acid as substrate	None
Activity vs extract concentration	Proportional increase
EDTA, 1mM	No effect
KCN, 1mM	Inhibit
Boiled enzyme	None

[a]Crude extract of cowpea variety VCS-18 was prepared according to Methods.

by a hemoprotein but not by lipoxygenase. Ben-Aziz *et al.* (9) showed that Tween 20 acted as a competitive inhibitor of lipoxygenase but it did not inhibit hemoproteins. Furthermore, Erikson *et al.* (16) reported that hemoprotein derived from catalase and peroxidase maintained their ability to oxidize linoleic acid even after heat treatment whereas the linoleate-oxidizing activity reported herein was inactivated after heat treatment. This study also shows that KCN reduced the linoleate-oxidizing activity of cowpea extract by 50% at 1 mM KCN. However, the effect of KCN on lipoxygenase activity is not well defined and contradictory reports on this have been published (17, 18, 19).

Some Physical and Chemical Properties of Cowpea Lipoxygenase

1) pH dependence. The optimum pH for cowpea lipoxygenase activity is about 6.2 (Figure 1) which is close to that reported for lipoxygenase from other legume sources (11, 12, 17, 20, 21).

2) Temperature effect. No appreciable differences in total activity were observed when the reaction mixture was incubated at a temperature range of 25° to 35°C. The optimum activity was obtained at 30°C.

3) Substrate dependence. Lipoxygenase in cowpea crude extract exhibited Michaelis-Menten type of saturation curve with an apparent Km of 0.195×19^{-3} M calculated from a Lineweaver-Burk plot (Figure 2). Maximum activity was attained at 1.6×10^{-3} M linoleic acid; higher substrate concentra-

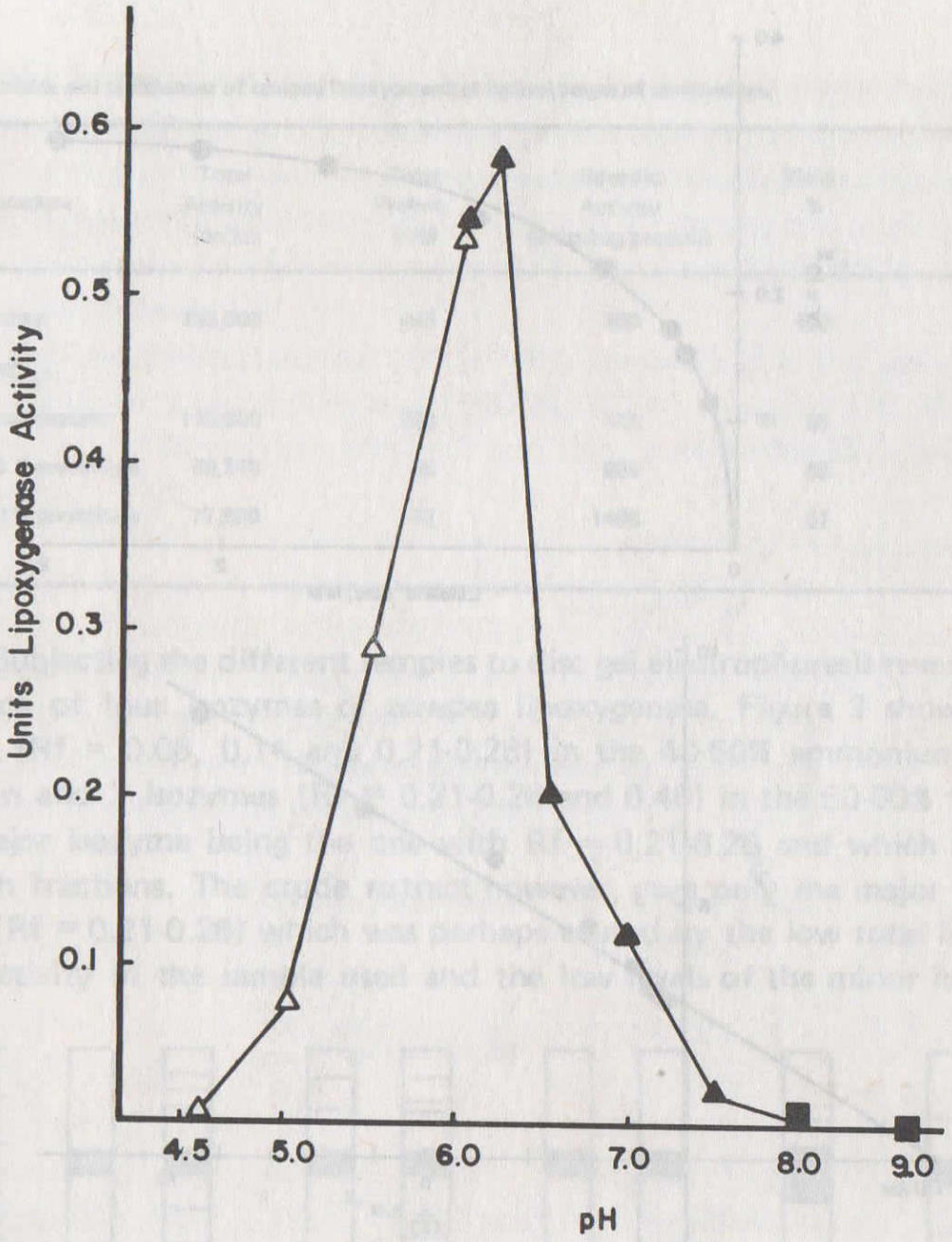

Figure 1. pH-activity profile of crude cowpea lipoxygenase. 0.1 M citrate-phosphate buffer (△——△), 0.1 M phosphate buffer (▲——▲), or 0.1 M Tris-HCl buffer (■——■) of desired pH was used instead of the regular buffer employed in the routine assay as described in Methods.

tion produced only a slight increase in enzyme activity. On the other hand, an optimal linoleic acid concentration of 5.47×10^{-5} M followed by a rapid decrease of enzyme activity beyond this value, has been reported for crude barley lipoxygenase (22). Km values of 0.025×10^{-3} and 0.61×10^{-3} M at pH 9.0 were observed with soybean lipoxygenase in the absence of Tween 20 and at 0.25% Tween 20, respectively (9). Shastry and Rao (23) reported a Km value of 0.35×10^{-3} M for rice bran lipoxygenase.

Bubbling oxygen into the buffer for 10 to 20 minutes at psi pressure before adding the substrate and the enzyme solution in the reaction mixture had no effect on the activity. The dissolved oxygen in the buffer mixture appeared to be sufficient to saturate the enzyme for its optimum activity.

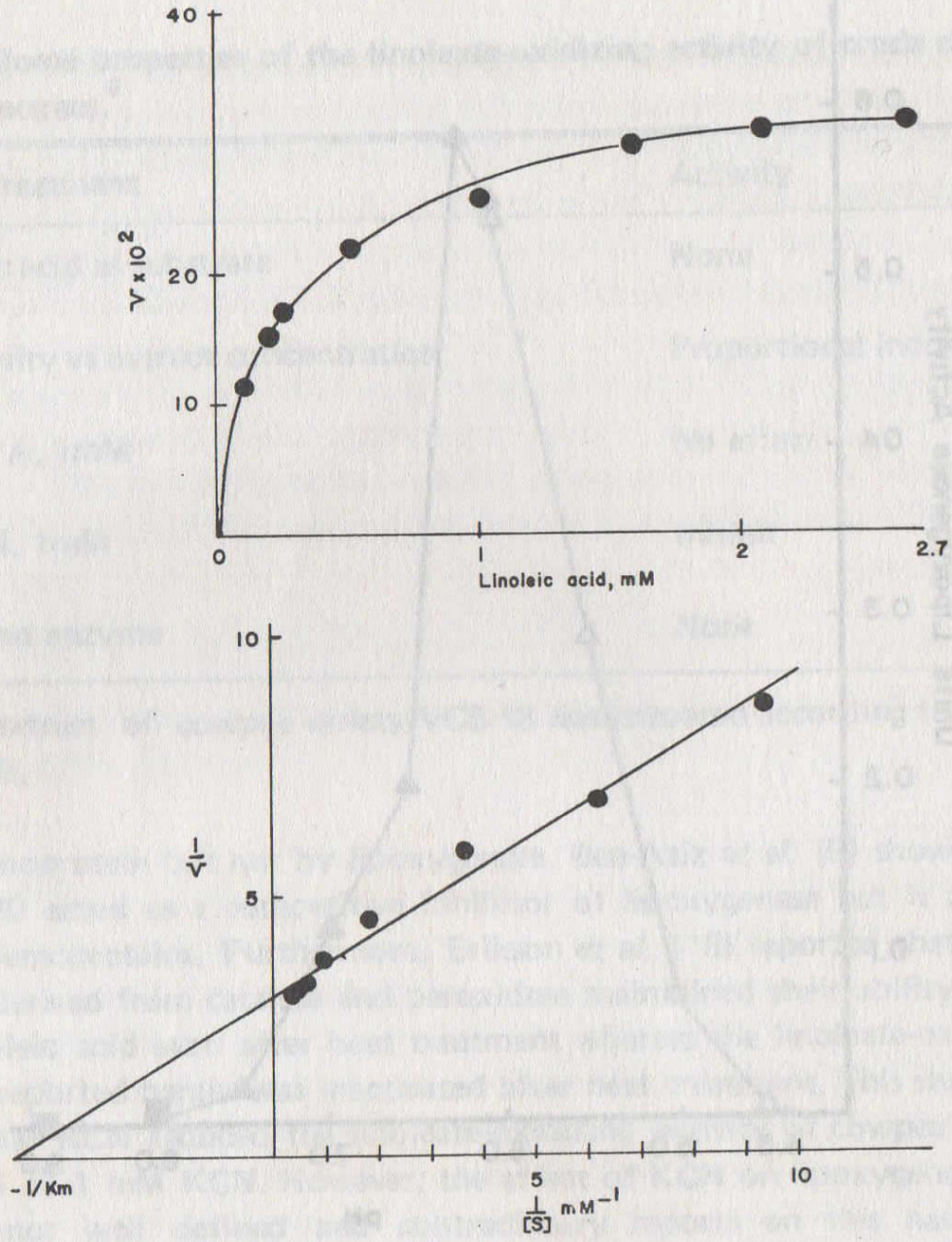

Figure 2. Activity of crude cowpea lipoxygenase versus substrate concentration.

The concentration of 0.05% Tween 20 in the reaction mixture was optimum for cowpea lipoxygenase while higher concentrations decreased enzyme activity (unpublished data). As mentioned earlier, Ben Aziz *et al.* (9) found that Tween 20 acted as a competitive inhibitor of soybean lipoxygenase. They reported a drastically decreased soybean lipoxygenase with 0.25% Tween 20.

4) Fractionation in ammonium sulfate; isozymes indicated by polyacrylamide gel electrophoresis. As a preliminary step to purifying the cowpea lipoxygenase, the crude extract was treated with ammonium sulfate at different saturating levels. Lipoxygenase activity, enriched 3.2 and 4.9 fold, respectively, was observed in the 40-50% and 50-60% ammonium sulfate fractions (Table 3).

Table 3. Yields and enrichment of cowpea lipoxygenase at various stages of purification.

Procedure	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg protein)	Yield %	Purification Factor
Crude Extract	135,000	445	303	100	1.0
$(NH_4)_2SO_4$:					
40 % supernatant	115,500	278	415	85	1.3
40 - 50 % precipitate	65,300	66	989	48	3.2
50 - 60 % precipitate	77,800	52	1496	57	4.9

Subjecting the different samples to disc gel electrophoresis revealed the presence of four isozymes of cowpea lipoxygenase. Figure 3 shows 3 isozymes (Rf = 0.06, 0.14 and 0.21-0.26) in the 40-50% ammonium sulfate fraction and 2 isozymes (Rf = 0.21-0.26 and 0.46) in the 50-60% fraction, the major isozyme being the one with Rf = 0.21-0.26 and which is found in both fractions. The crude extract however, gave only the major isozyme band (Rf = 0.21-0.26) which was perhaps caused by the low total lipoxygenase activity in the sample used and the low levels of the minor isozymes.

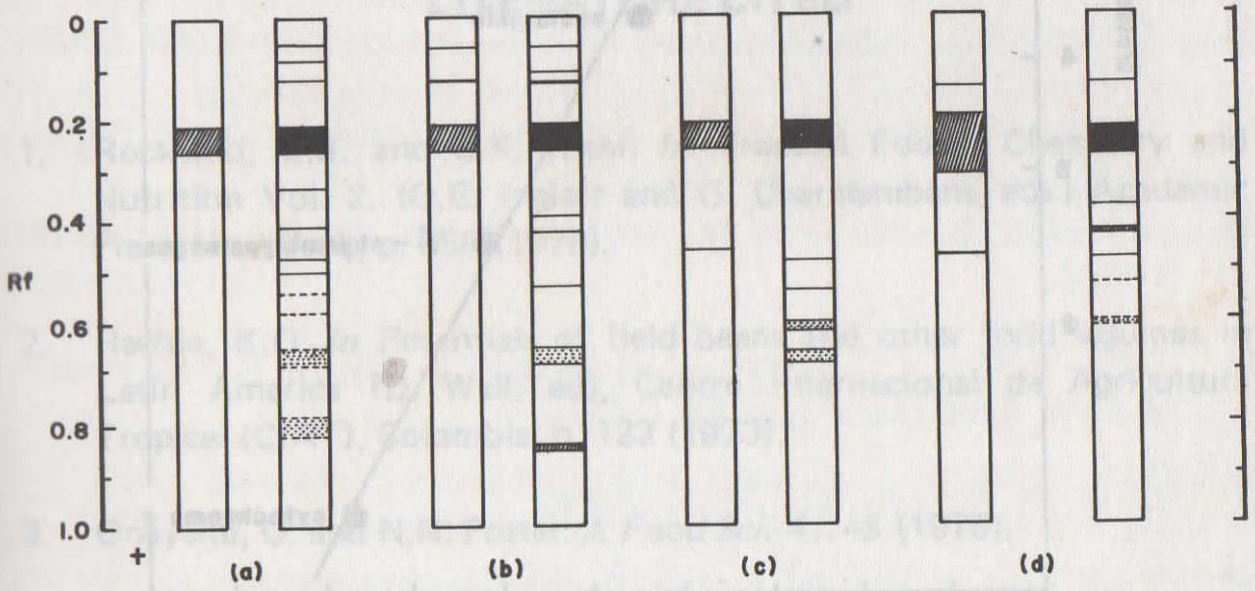

Figure 3. Disc gel electrophoretic patterns of cowpea lipoxygenase in fractions at various stages of purification. Patterns on the left and right side of each sample correspond to specific lipoxygenase stained gel and general protein stained gel, respectively. (a) crude extract; (b) dialyzed 40-50% ammonium sulfate fraction; (c) dialyzed 50-60% ammonium sulfate fraction; and (d) pooled active fractions from Sephadex G-150. Lipoxygenase activity was detected by a lipoxygenase-starch-iodine specific staining technique (7) while protein was stained with 1% amido black 10B.

Isozymes of lipoxygenase have also been reported for soybean (24) and small faba bean (25). In both cases, a major isozyme of Rf = 0.22-27 was present which was similar to that observed above.

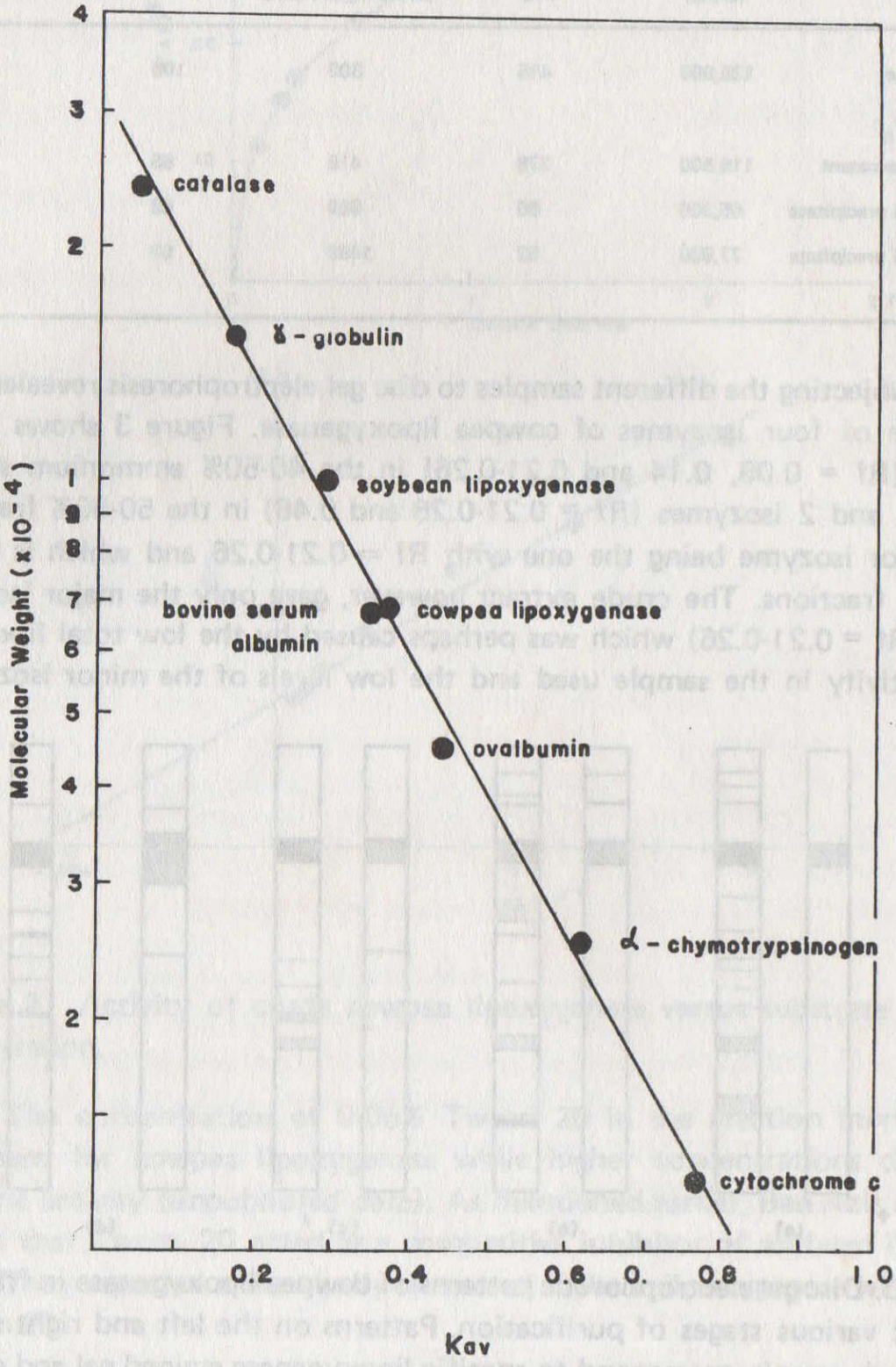

Figure 4. Standard curve for molecular weight determination of cowpea lipoxygenase by gel chromatography on Sephadex G-150 column (2.5 x 90 cm). Eluant used was 0.05 M phosphate buffer at pH 7.0 K_{av} is defined as V_e-V_o/V_t-V_o where V_e is elution volume, V_t is total volume and V_o is void volume. The standard curve was obtained using the least squares method.

5) Molecular weight. Cowpea lipoxygenase was observed to elute from Sephadex G-150 column at a volume corresponding to a molecular weight of 68,000 daltons. The molecular weight was obtained from a plot of Kav vs molecular weight of standard proteins (Figure 4). Peanut and soybean lipoxygenase have been reported to have molecular weights of 73,000 and 100,000 daltons, respectively (12, 17).

The electrophoretic pattern of the pooled active fractions revealed the presence of three isozymes (Rf = 0.14, 0.19-0.31 and 0.47). The lowest isozyme Rf = 0.06 was not observed and this could be due to its low level in the sample.

Purification and isolation of each of the isozymes would be necessary to establish their exact molecular sizes and other properties.

Work is presently being undertaken to further purify and characterize the different cowpea lipoxygenase isozymes. It will also be interesting to compare the properties of cowpea lipoxygenase isozymes with those of soybean. Like the soybean lipoxygenase, the cowpea lipoxygenase may cause the formation of compounds which give cowpea its strong beany flavor.

LITERATURE CITED

1. Rockland, L.B. and S.K. Nishi. *In* Tropical Foods: Chemistry and Nutrition Vol. 2. (G.E. Inglett and G. Charalambans, eds.) Academic Press, New York, p. 656 (1979).

2. Rachie, K.O. *In* Potentials of field beans and other food legumes in Latin America (D. Wall, ed), Centro Internacional de Agricultura Tropical (CIAT), Colombia, p. 123 (1973).

3. Onayemi, O. and N.N. Potter. *J. Food Sci. 41:*48 (1976).

4. Okaka, J.C. and N.N. Potter. *J. Food Sci. 44:*1235 (1979).

5. Rackis, J.J., D.J. Sessa and D.H. Honig. *J. Am. Oil Chem. Soc. 56:*262 (1979).

6. Davies, B. *Ann. N.Y. Acad. Sci. 121:*404 (1964).

7. Guss P.L., T. Richardson and A. Stahmann. *Cereal Chem. 44:*607 (1967).

8. Lowry, O.H., N.J. Rosenbrough, A.I. Farr and R.J. Randall. *J. Biol. Chem. 193:*265 (1951).

9. Ben-Aziz, A., S. Grossman, I. Ascarelli and P. Budowski. *Anal. Biochem 34:*88 (1970).

10. IPB Annual Report. Institute of Plant Breeeding, University of the Philippines at Los Baños, Philippines (1979).

11. Truong Van Den. Ph.D. Dissertation, University of the Philippines at Los Baños, Philippines (1980).

12. Diel, E. and H.J. Stan. *Planta 142:*321 (1978).

13. Scott, D. *In* "Enzymes in Food Processing" (G. Reed, ed.), Academic Press, New York, p. 232 (1975).

14. Flurkey, W.H., L.W. Young and J.J. Jen. *J. Agric. Food Chem. 26*:1474 (1978).

15. Tappel, A.L. *In* Autoxidation and Antioxidation Vol. 1 (W.O. Lundbert, ed.) Interscience, New York, p. 325 (1961).

16. Erikson, C.E., P.A. Olsson and S.G. Svensson, *Lipids 5:*365 (1970).

17. Sanders, T.H., H.E. Patter and J.A. Singleton, *Lipids 10:*681 (1975).

18. Grossman, S., M. Trop, R. Avtalion and A. Pinsky. *Lipids 7:*467 (1972).

19. Flick, Jr. G.J., F.S. Burnette, L.H. Aung, R.L. Ory and A.J. St. Angelo. *J. Agric. Food Chem. 26:*1000 (1978).

20. Surrey, K. *Plant Physiol. 39:*65 (1964).

21. Yoon, S. and B.P. Klein *J. Agric. Food Chem. 27:*955 (1979).

22. Lulai, E.C. and C.W. Baker. *Cereal Chem. 53:*777 (1976).

23. Shastry, B.S. and M.R.R. Rao. *Cereal Chem. 52:*597 (1975).

24. Kalac, J. *Chem. Abstr. 80:*(5):24128 a (1974).

25. Eskin, N.A.M. and H.M. Henderson. Proc. IV. Int. Congress Food Sci. and Technol. Vol. 1, Spain (1974).