Brattin, W. J., Smith, E. L., *J. Biol. Chem. 246*, 2400 (1971).
Chen, L., Richardson, T., Amundson, C. H., *J. Milk Food Technol.* **38**, 89 (1975).
Creamer, L. K., Roeper, J., Lohrey, E. H., *N.Z. J. Dairy Sci. Technol.* **6**, 107 (1971).
Endo, Y., *Biochim. Biophys. Acta* **523**, 207 (1978).
Erbersdobler, H., in "Protein Metabolism and Nutrition", Cole et al., Ed., Buttersworth, London, 1976, pp 139–158.
Fields, R., in "Methods in Enzymology", Hirs, C. H. W., Timasheff, S. N., Ed., Academic Press, New York, 1972, pp 464–468.
Finot, P., Bujard, E., Arnaud, M., *Adv. Exp. Med. Biol.* **86**, 51 (1977a).
Finot, P. A., Mottu, F., Bujard, E., Mauron, J., Paper No. 58, 174th National Meeting of the American Chemical Society, Division of Agricultural and Food Chemistry, Chicago, IL, Aug 1977b.
Grant-Green, M. C., Friedberg, F., *Int. J. Protein Res.* **2**, 235 (1970).
Gounaris, A. D., Perlmann, G., *J. Biol. Chem.* **242**, 2739 (1967).
Gounaris, A., Ottesen, M., *C. R. Trav. Lab. Carlsberg* **35**, 37 (1965).
Groninger, H. S., Jr., *J. Agric. Food Chem.* **21**, 978 (1973).
Groninger, H. S., Jr., Miller, R., *J. Food Sci.* **40**, 327 (1975).
Habeeb, A. F. S. A., Cassidy, H. G., Singer, J. S., *Biochim. Biophys. Acta* **29**, 587 (1958).
Hamilton, P. B., Lou, M. F., *Biochem. Med.* **6**, 193 (1972).
Hass, L. F., *Biochemistry 3*, 535 (1964).
Hugli, T. E., Moore, S., *J. Biol. Chem.* **247**, 2828 (1972).
Janolino, V. G., Swaisgood, H. E., *J. Biol. Chem.* **250**, 2532 (1975).
Karibian, D., Laurent, C. Labouvesse, J., Labouvesse, B., *Eur. J. Biochem.* **5**, 260 (1968).
Kruckenberg, W., U.S. Patent 2731480, Jan. 17, 1956.
Leclerc, J., Benoiton, L., *Can. J. Biochem.* **46**, 471 (1968).
Li, C. H., Bertsch, L., *J. Biol. Chem.* **235**, 2638 (1960).
Lou, M. F., *Biochemistry* **14**, 3504 (1975).
Lou, M. F., Hamilton, P. B., *Biochem. Med.* **8**, 485 (1973).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193**, 265 (1951).
Meighen, E. A., Schachman, H. K., *Biochemistry* **9**, 1163 (1970).
Miller, R., Groninger, H. S. Jr., *J. Food Sci.* **41**, 268 (1976).
Mühlrad, A., Corsi, A., Granata, A. L., *Biochim. Biophys Acta* **162**, 435 (1968).
Nakamura, K., Fujita, K. *Agric. Biol. Chem.* **36**, 903 (1972).
Neuberger, A., Sanger, F., *Biochem. J.* **37**, 315 (1943).
Paik, W. K., Benoiton, L, *Can. J. Biochem. Physiol.* **41**, 1643 (1963).
Riordan, J. F., Vallee, B. L., *Biochemistry* **2**, 1460 (1963).
Riordan, J. F., Vallee, B. L., *Biochemistry* **3**, 1768 (1964).
Tanaka, M., Lee, T., Chichester, C. O., *J. Nutr.* **105**, 989 (1975).
Varnish, S. A., Carpenter, K., *J., Br. J. Nutr.* **34**, 339 (1975).
White, H. C., Britton, E. C., U.S. Patent 2879164, Mar 24, 1959.

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Some Properties of Pea Lipoxygenase Isoenzymes

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Four pea lipoxygenase isoenzymes were isolated from pea seeds (Little Marvel variety) by ammonium sulfate fractionation, gel filtration, and ion-exchange chromatography. The two major isoenyzmes, pea lipoxygenase I and II (PL I and PL II), were further purified by isoelectric focusing in a granulated gel. PL I and II were homogeneous proteins and had isoelectric points of 6.25 and 5.82, respectively. The molecular weight of both PL I and II was 95 000 and they had optimum activity between pH's 5 and 7 and no activity above pH 8. The two minor isoenzymes were present at very low concentrations and were active at pH 9, but not at neutral to acid pH's. PL I and PL II differed in pH profiles, substrate specificity, carotene- and chlorophyll-bleaching activity, and in their ability to produce carbonyl compounds during the linoleate oxidation reaction.

Quality deterioration, such as off-flavors, off-odors, and off-colors, in unblanched frozen vegetables has been ascribed to the oxidative degradation of unsaturated lipids by certain naturally occurring enzymes (Joslyn, 1949; Lee and Wagenknecht, 1951, 1958). Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12), which catalyzes the peroxidation of unsaturated fatty acids, is recognized as one of the main oxidative catalysts in plants. Interest in the role of lipoxygenase in the formation of flavor and volatile substances from lipids and in the cooxidation of pigments ranges from practical use to basic questions about the assumed secondary reactions (Eskin et al., 1977).

The development of rancid off-flavor in unblanched vegetables during frozen storage was associated with increases in hydroperoxides and carbonyl compounds which were thought to be the result of lipoxygenase action on lipids in vegetables (Siddiqi and Tappel, 1956; Wagenknecht and Lee, 1958; Bengtsson and Bosund, 1966). The role of lipoxygenase in the development of beany and bitter flavors from lipids during the processing of soybeans has been demonstrated (Mustakas et al., 1969; Kon et al., 1970; Kalbrener et al., 1974). However, the routes leading to the formation of flavor and aromatic compounds and the involvement of lipoxygenase in these reactions remain unclear.

Lipoxygenase, which was named "carotene oxidase" initially, was also thought to cooxidize carotene and chlorophyll (Grossman et al., 1969; Kies et al., 1969; Holden, 1965; Arens et al., 1973). Much of the work concerning the participation of lipoxygenase in pigment bleaching was done with enzyme preparations of varying degrees of purity, but differences in bleaching potential among isoenzymes has been suggested. This indicates the importance of using purified enzymes for studying the bleaching activity of lipoxygenase (Kies et al., 1969; Grosch et al., 1976; Pistorius, 1974).

The existence of multiple lipoxygenases in soybeans is well-established (Guss et al., 1967; Yamamoto et al., 1970; Verhue and Francke, 1972; Christopher, 1972) and their occurrence in other vegetables has been indicated. The

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possible presence of several lipoxygenase isoenzymes in peas was intimated by Hale et al. (1969) and confirmed by Arens et al. (1973), Anstis and Friend (1974), Haydar et al. (1975), and Klein (1976). In other work (Eriksson and Svensson, 1970; Haydar and Hadziyev, 1973), isoenzymes of lipoxygenase were not clearly identified in peas.

In this investigation, lipoxygenase isoenzymes isolated from pea seeds were characterized by pH profile, substrate specificity, isoelectric point and carbonyl-forming ability. The bleaching of carotene and chlorophyll in pea lipoxygenase–linoleate systems were studied.

MATERIALS AND METHODS

Lipoxygenase Isolation and Purification. The isolation and purification procedure was a modification of that used by Klein (1976). All steps in the procedure were performed at 4 °C unless otherwise stated.

Acetone-defatted ground pea seeds were extracted with 10 volumes of 50 mM sodium phosphate buffer, pH 6.8, for 5 h. The slurry was forced through two layers of cheesecloth and centrifuged at 16000*g* for 15 min in a Sorvall RC-2B centrifuge. The precipitate was discarded and the supernatant (SI) was brought to 25% saturation with ammonium sulfate. The solution was allowed to stand for at least an hour and the precipitate was spun down at 16000*g* for 15 min and discarded. The supernatant (SII) was brought to 60% saturation with respect to ammonium sulfate and allowed to stand for at least an hour. The resulting precipitate (PIII) was centrifuged down at 16000*g* for 15 min, dissolved in 50 mM sodium phosphate buffer, pH 6.8, and then dialyzed overnight against two changes of 50 volumes each of 50 mM sodium phosphate buffer, pH 6.8.

The dialysate was applied to a Sephadex G-200 column (5 × 60 cm) equilibrated with 50 mM sodium phosphate buffer, pH 6.8. Fractions of 9 mL were eluted from the column with the same buffer, at a flow rate of 50–75 mL/h. Lipoxygenase activity and protein concentration were determined on alternate fractions. Those with lipoxygenase activity were pooled and concentrated by ultrafiltration (Amicon membrane PM-10) under nitrogen pressure of 60 psi. The concentrated sample solution was dialyzed overnight against three changes of 50 volumes of 50 mM NaCl in a 50 mM sodium phosphate buffer, pH 6.8.

The dialysate was applied to a DEAE-Sephadex A-50 column (2.6 × 35 cm) and eluted with a linear salt gradient increasing from 50 to 400 mM NaCl in 50 mM sodium phosphate buffer, pH 6.8, in a total volume of 800 mL. Fractions of 6 mL were eluted from the column at a flow rate of 20–25 mL/h and lipoxygenase active fractions were pooled.

Pooled lipoxygenase active fractions were dialyzed against deionized distilled water for about 6 h before using preparative gel electrofocusing. Preparative isoelectric focusing was run in a granulated gel using the LKB 2117 Multiphor system. The procedure followed was described in LKB Application Note 198 (Winter et al., 1975). The enzyme solution was mixed with 5 mL of Ampholine solution (40%, w/v), pH 5–7, and deionized water and diluted to a final volume of 100 mL. Four grams of Ultrodex was added slowly to the Ampholine–enzyme solution. The gel bed was dried until a water loss of 35% occurred. Electrofocusing was performed at 10 °C for 14 h, with a constant power of 8 W. The initial voltage was about 0.3 kV with a current of 20 mA. After 14 h, the voltage increased to 1.15 kV and the current dropped to 7 mA. The separated zones were collected and eluted with 50 mM phosphate buffer, pH 6.8. Each fraction was assayed for lipoxygenase and the optical density measured at 280 nm.

Assay Procedures. Protein concentrations were determined both by optical density at 280 nm and by the Lowry method (Lowry et al., 1951). The absorbance of protein at 280 nm was used as an index of protein concentration in the column eluants.

Lipoxygenase activity was determined spectrophotometrically at 234 nm by the diene conjugation method ("Worthington Enzyme Manual", 1972). One unit of lipoxygenase activity was defined as an increase in absorbance of 0.001/min.

A polarographic method, using a biological oxygen monitor Model 28, equipped with a Clark electrode (Yellow Springs Instruments, OH), was also employed for the lipoxygenase assay. One unit of lipoxygenase activity corresponded to the consumption of 1 μmol of oxygen/min.

Throughout the purification, an aqueous linoleate solution (Rackis et al., 1972) was used. A stock solution was prepared by adding 0.1 mL of linoleic acid to 0.1 mL of Tween 20; 0.3 mL of 1 N KOH was then slowly added to obtain a clear solution and brought to a final volume of 25 mL with deionized water. The stock linoleate solution was diluted with an appropriate buffer to give a final concentration of 2.57 mM linoleate at the desired pH.

Lipoxygenase activity was routinely assayed at pH 6.0 and 7.2. Both the polarographic and spectrophotometric methods were employed at both pH's and for the pH profiles.

Assay mixtures employed in the substrate specificity studies were similar to those of Koch et al. (1958). One-tenth milliliter of linoleic acid was dissolved in 15 mL of 95% ethanol and the volume was brought to 25 mL with water. Stock methyl linoleate solution was prepared by dissolving 0.1 mL of methyl linoleate in a mixture of 10 mL of acetone and 15 mL of 95% ethanol. These stock solutions were diluted one to five with buffer immediately before use. The final concentrations used were 2.57 mM linoleic acid or 2.41 mM methyl linoleate.

Carbonyl-producing activity of lipoxygenase isoenzymes was determined spectrophotometrically by measuring the increase of absorbance at 280 nm. The reaction conditions were the same as for the lipoxygenase assay. One unit of carbonyl production activity corresponded to an increase in absorbance of 0.001 at 280 nm per min.

Carotene- and chlorophyll-bleaching activities of lipoxygenase isoenzymes were determined spectrophotometrically using the methods of Ben-Aziz et al. (1971) and of Arens et al. (1973) with some modifications. Stock carotene solution was made by dissolving 2.5 mg of β-carotene and 0.01 mL of Tween 80 in 25 mL of chloroform. One milliliter of the carotene solution was evaporated to dryness under vacuum and the residue was dissolved in 10 mL of water. The aqueous carotene solution was then mixed with the aqueous linoleate solution to give a final concentration of 2.57 mM linoleate and 8.3 mg of β-carotene in 2 mL of 50 mM buffer. After addition of enzyme, the change of the absorbance at 460 nm was determined against the blank. The blank contained linoleic acid and β-carotene but no enzyme.

A stock chlorophyll solution was prepared by dissolving 1 mg of chlorophyll *a* and 0.036 mL of Tween 80 in 10 mL of acetone. Aqueous chlorophyll–linoleate solution was prepared in the same way as the carotene–linoleate solution. The reaction mixture contained 2.57 mM linoleic acid and 8.3 μg of chlorophyll *a* in 2 mL of 50 mM buffer. After addition of enzyme, chlorophyll bleaching was followed by measuring the change of absorbance at 664 nm

Table I. Specific Activity during Isolation of Pea Seed Lipoxygenase Isoenzymes

fraction	spectrophotometric assay (units × 10^{-4}/mg of protein) pH 7.2[a]	pH 6.0[b]	polarographic assay (units/mg of protein) pH 7.2[b]	pH 6.0[c]
crude extract (SI)	1.24	7.94	0.23	1.30
25% $(NH_4)_2SO_4$ supernatant (SII)	1.29	8.26	0.24	1.32
60% $(NH_4)_2SO_4$ precipitate (PIII)	1.95	12.61	0.36	2.01
Sephadex G-200 pooled fractions	6.65	42.01	1.46	5.95
DEAE-Sephadex fraction I	21.53	82.76	1.83	4.44
fraction II	3.33	63.77	0.54	10.02

[a] Values are means for eight replications. [b] Values are means for two replications. [c] Values are means for five replications.

against the blank. One unit of carotene- or chlorophyll-bleaching activity of the enzyme was defined as a change in absorbance of 0.001/min.

Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed at pH 7.5 by a modification of the method of Gabriel (1971) and at pH 9.0 by the method of Davis (1964). The stacking gel was omitted and the small pore gel contained 1% starch. A sample containing approximately 100 μg of protein per tube was subjected to electrophoresis at 4 °C with a current of 3 mA/tube.

The lipoxygenase-specific iodine staining technique applied was that of Guss et al. (1967). Protein staining was done by immersing the gels in 0.025% Coomassie Blue R-250 in 25% isopropyl alcohol and 10% acetic acid.

Estimates of molecular weights of lipoxygenase isoenzymes were done by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis (Weber and Osborn, 1969). Bovine serum albumin (mol wt 134000), amylase (50000), enolase (45000), aldolase (39000), and trypsin (24000) were used as molecular weight standards. The gel concentration employed was 5%. Electrophoresis was performed at room temperature at 8 mA/tube.

Analytical Isoelectric Focusing. Analytical polyacrylamide gel isoelectric focusing was performed by the procedure described by Catsimpoolas (1976). Gel electrofocusing was performed in the conventional apparatus for gel electrophoresis using cylindrical gels. The gel mixture contained 2% carrier ampholytes (pH 5–7, 40%) and 6% acrylamide monomer. The anode and cathode compartments were filled with the electrode solutions, 0.4% sulfuric acid and 0.4% ethanolamine, respectively. The starting voltage was usually about 200 V and as the current dropped, voltage was gradually increased up to a maximum of about 400 V. Electrofocusing was carried out for approximately 24 h at 4 °C. After the run, the pH gradient in the set of gels was determined. A gel, identical with those to be stained, was cut into 10–20 equal sections, and the pH of a 1-mL water extract of each piece was measured.

The activity of lipoxygenase isoenyzmes after isoelectric focusing was detected by incubating the gels in a medium containing 2.57 mM linoleic acid and 0.02% 3,3′-dimethoxybenzidine hydrochloride (Verhue and Francke, 1972; DeLumen and Kazeniac, 1976). During the incubation, oxygen supply was assured by frequent inversion of the tubes. Orange hydroperoxide bands developed slowly and remained upon storage without color deterioration. Protein staining was done by the method described by Catsimpoolas (1976).

Table II. Fold Purification during Isolation of Pea Seed Lipoxygenase Isoenzymes

fraction	spectrophotometric assay pH 7.2	pH 6.0	polarographic assay pH 7.2	pH 6.0
crude extract (SI)	1.00	1.00	1.00	1.00
25% $(NH_4)_2SO_4$ supernatant (SII)	1.04	1.04	1.04	1.02
60% $(NH_4)_2SO_4$ precipitate (PIII)	1.57	1.59	1.57	1.55
Sephadex G-200 pooled fractions	5.36	5.29	6.35	4.58
DEAE-Sephadex fraction I	17.36	10.42	7.96	3.42
fraction II	2.69	8.03	2.35	7.71

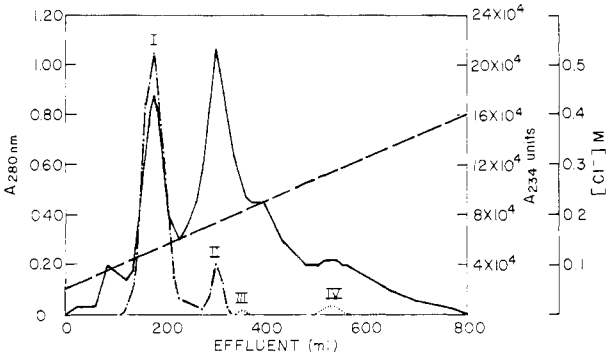

Figure 1. Isolation of pea seed lipoxygenase isoenzymes on DEAE-Sephadex: column dimensions, 2.6 × 40 cm; 250 mg of protein applied (- - -) NaCl gradient, 50–400 mM NaCl in 50 mM sodium phosphate buffer, pH 6.8; (—) A_{280nm}; (·—·) lipoxygenase activity at pH 7.2; (···) lipoxygenase activity at pH 9.0.

RESULTS AND DISCUSSION

Purification of Pea Seed Lipoxygenase Isoenzymes. The isolation of lipoxygenase isoenzymes from pea seeds was accomplished by employing conventional procedures for protein purification: ammonium sulfate fractionation, gel filtration on Sephadex G-200, and DEAE-Sephadex ion-exchange chromatography. The two main isoenzymes were further purified by means of preparative isoelectric focusing in a granulated gel. Specific activity and fold purification during isolation of lipoxygenase isoenzymes from pea seeds are summarized in Tables I and II, respectively.

Gel chromatography of PIII on Sephadex G-200 resulted in the separation of one protein peak with lipoxygenase activity. About fivefold purification of the enzyme was obtained regardless of pH or assay method.

When the pooled eluate from gel filtration was applied to a DEAE-Sephadex ion-exchange column, two major protein peaks were obtained. The activity profile from a representative elution is shown in Figure 1. Activity at pH 7.2 (as shown) and 6.0 (not shown) corresponded to the protein absorption peaks. The elution profile is similar to those of Arens et al. (1973) and Weber et al. (1973), who reported the presence of at least two pea lipoxygenases after ion-exchange chromatography. However, Eriksson and Svensson (1970) and Haydar and co-workers (1973, 1975) obtained only one pea lipoxygenase after ion-exchange chromatography, resulting in a considerable loss of the total enzyme activity.

Although previous work with pea lipoxygenase had indicated that there was no activity at pH 9.0, lipoxygenase activity was detected in crude pea extracts after disc gel

electrophoresis at pH 9.0. Lipoxygenase activity at this pH was not detectable in the two major peaks. Therefore, the DEAE-Sephadex eluant fractions were reassayed at several pH's, and fractions III and IV were identified, and their elution profiles are shown in Figure 1. These fractions were not active at pH 7.2 and were not present at as high levels as fractions I and II. Based on the elution profile, pH optimum and electrophoretic behavior, fraction IV appears to be the "Theorell enzyme" or soybean lipoxygenase 1 (Christopher, 1972). This isoenzyme, previously reported to be absent in peas, does appear to be present but at very low concentrations. Because such small quantities of fractions III and IV were obtained, no additional characterization could be done.

The pea lipoxygenase isoenzymes that were eluted from the ion exchanger in the first protein peak (fraction I) and the second peak (fraction II) resemble the soybean lipoxygenase isoenzymes 3 and 2, respectively, isolated by Christopher (1972) based on elution profile, substrate specificity, and formation of 280-nm absorbing compounds (see following sections). This suggests that similar lipoxygenase isoenzymes may be present in different plant materials, as well as in different varieties of the same seed as postulated by Postel et al. (1978).

Ion-exchange chromatography resulted in further purification of the major isoenzymes. In the case of fraction I, a lower fold purification was achieved when lipoxygenase activity was expressed as oxygen uptake than as conjugated diene formation (Table II). However, with fraction II, the fold purification was comparable with either assay method. Preliminary studies showed that the specific activity of fraction I as measured by oxygen uptake was lower at higher enzyme concentrations, while the specific activity of fraction II was not dependent on enzyme concentration. This difference between the two isoenzymes may be explained by their behavior in carbonyl formation (see the following section).

Values for the fold purification varied depending on the pH. Regardless of assay method, a higher purification fold was obtained with fraction I at pH 7.2 than at pH 6.0. However, fraction II demonstrated a higher purification fold at pH 6.0 than at pH 7.2. This result can be explained in part by the fact that the two isoenzymes differ in their pH optima.

Preparative isoelectric focusing of fractions I and II in a granulated gel with a pH gradient from 5 to 7 resulted in further separation of lipoxygenase active bands. Fraction I separated into one major and two minor bands, focused at pH's 6.25, 6.35, and 6.15, respectively. Isoelectric focusing of fraction II resulted in one major and one minor lipoxygenase band with isoelectric points of 5.82 and 5.75, respectively. The major lipoxygenase bands of each fraction were eluted from the gel and were the final enzyme preparations used in subsequent studies: pea lipoxygenase I (PL I) and II (PL II).

Arens et al. (1973) and Verhue and Francke (1972) reported the occurrence of multiple lipoxygenases with small differences in their isoelectric points. They suggested that these differences resulted from small variations on the surface of the protein molecule, without fundamental differences in the molecules. Therefore, the multiplicity of the enzymes would not necessarily point to multifunctional behavior. Catsimpoolas (1977) pointed out that isoelectric focusing techniques can separate molecules differing as little as 0.01 pH unit in their isoelectric points. Because of the extremely high resolution, isoelectric focusing may detect other charge differences not strictly related to the "gross homogeneity" of the protein. Catsimpoolas, therefore, suggested that isoelectric focusing patterns should be interpreted with caution.

Analytical Electrophoresis and Electrofocusing. During the course of the study, the isoenzyme patterns in crude pea extracts and the separated fractions were examined by analytical gel electrophoresis using the specific lipoxygenase staining procedure developed by Guss et al. (1967). When the crude pea extract was subjected to electrophoresis at pH of 9.0, two brown lipoxygenase active bands with R_f's of 0.21 and 0.54 were obtained. In addition, a white band that did not give a positive lipoxygenase reaction appeared at R_f 0.29. When fractions I and II were tested, this same white band appeared but no lipoxygenase activity could be detected on the gels. Similarly, the final preparations, PL I and PL II, produced only white bands with an R_f of 0.29. Arens et al. (1973) were also unable to obtain an enzymatically active band with a purified pea lipoxygenase after electrophoresis, and it was assumed that enzyme activity was lost during the procedure.

The pH profiles of PL I and II indicated that these isoenzymes exhibited activity between pH 5.5 and 7.5 but not above 8.0. Therefore, a gel system operating at pH 7.5 was used and both PL I and II reacted to give brown bands with an R_f of 0.23. Presumably these correspond to the white band on the pH 9.0 gels. Both PL I and II gave single bands when either the 7.5 or 9.0 gels were stained for protein, as did fractions I and II, indicating that they were homogeneous proteins.

After fractions III and IV were identified in the DEAE-Sephadex eluants by the spectrophotometric assay, they were applied to pH 9.0 gels. Fraction III gave a lipoxygenase-active brown band with an R_f of 0.21, corresponding to the upper brown band in the crude extract. Fraction IV produced a lipoxygenase-active band with greater mobility, R_f of 0.54, which appeared to be the lower band in the crude extract.

In previous investigations, an operating pH of 9.0 for polyacrylamide gel electrophoresis was used for the identification of lipoxygenase isoenzyme patterns (Guss et al., 1967; Hale et al., 1969; Haydar et al., 1975). This study, however, demonstrates that employing a gel system with an appropriate pH is necessary to obtain enzymatically active bands. The presence of at least three isoenzymes was demonstrated using the two sets of gels.

PL I and II were also applied to analytical isoelectric focusing polyacrylamide gels, using a pH gradient from 5 to 7. After the proteins were focused isoelectrically on polyacrylamide gels, those having lipoxygenase activity were localized by a specific staining technique based on the ability of enzymatically formed hydroperoxide to react with a dye, 3,3'-dimethoxybenzidine hydrochloride (DBH), producing an orange color (Verhue and Francke, 1972; DeLumen and Kazeniac, 1976). This system was successful in detecting the activities of PL I and II on polyacrylamide gels after isoelectric focusing and confirmed that PL I and II produced enzymatically active bands on the gel system in a neutral to acid pH range. Both PL I and II produced single protein bands on isoelectric focusing gels, indicating that they were homogeneous proteins. Although R_f values for PL I and II were too similar for the isoenzymes to be distinguished from each other after polyacrylamide gel electrophoresis, on the electrofocusing gels PL I and II could indeed be differentiated according to their isoelectric points. This provided further evidence that PL I and II were distinct isoenzymes.

Molecular Weight Determination. The average values for the molecular weights of PL I and II as determined by $NaDodSO_4$ gel electrophoresis were 95 000 for

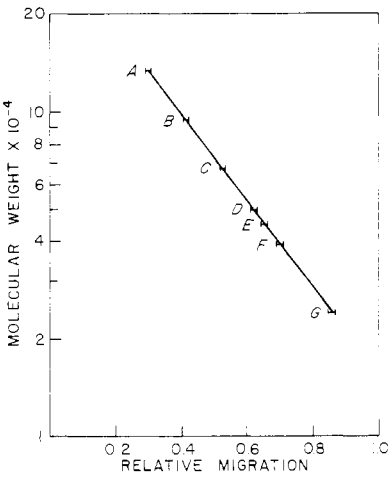

Figure 2. Plot of molecular weight vs. relative migration. (A) Bovine albumin dimer (134 000), (B) PL I and II, soybean lipoxygenase (95 000), (C) bovine albumin monomer (67 000), (D) amylase (50 000), (E) enolase (45 000), (F) aldolase (39 000), (G) trypsin (24 000).

both PL I and II (Figure 2). Commercial soybean lipoxygenase had a similar molecular weight to PL I and II under the same conditions. This result compares well with the molecular weight of about 100 000 reported for soybean lipoxygenases 1, 2, and 3 (Christopher, 1972). Haydar et al. (1975) also reported that the storage-resistant pea lipoxygenase had a molecular weight equal to that of Christopher's soybean lipoxygenase 3. Arens et al. (1973) obtained a molecular weight of 78 000 for pea lipoxygenase using a gel filtration method.

Available information about the subunit composition of lipoxygenase is contradictory. According to Stevens et al. (1970) treatment of soybean lipoxygenase with guanidine hydrochloride or sodium dodecyl sulfate after reduction produced dissociation of soybean lipoxygenase into multiple bands of molecular weights of 95 000, 64 200, 31 000, and 14 500. Haydar et al. (1975), however, observed no decrease in the protein content of pea lipoxygenase after the enzyme was treated with $NaDodSO_4$ and DTT, suggesting that lipoxygenase is a single polypeptide. Christopher (1972) also failed to achieve any dissociation of soybean lipoxygenase isoenzymes with sodium dodecyl sulfate and mercaptoethanol. Similarly, PL I and II appear to be comprised of single polypeptide chains.

Activity Profiles. The purified pea isoenzymes, PL I and II, were further characterized by pH profiles and substrate specificity. Since very small quantities of fractions III and IV were obtained, they were not used in the subsequent studies.

With linoleic acid dispersed with Tween 20 as a substrate, PL I had a rather broad pH optimum between 6.0 and 7.0. The dip at pH 6.5 was characteristic and occurred consistently (Figure 3). PL II had a bell-shaped pH profile with a distinct pH optimum of 6.0 (Figure 4). No activity was detected at pH's below 4.0 or above pH 8.0. The pH profiles were similar for oxygen uptake and conjugated diene formation with both isoenzymes.

The relationship between oxygen uptake and diene conjugation was different with each isoenzyme. When lipoxygenase activity was expressed as conjugated diene formation, the specific activities of the two isoenzymes were similar at pH 6.0 (optimum pH). However, with the oxygen uptake method which required 100 times more enzyme than the diene formation assay, PL I exhibited much lower oxygen uptake than PL II. Preliminary studies indicated that PL I had lower specific activity at higher

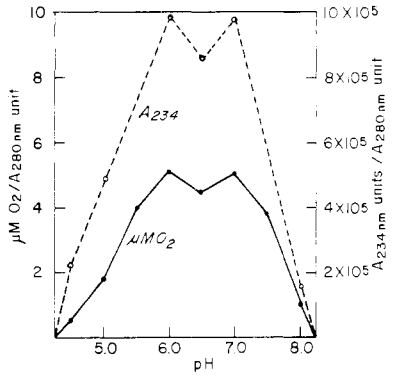

Figure 3. Activity of PL I as a function of pH, using linoleic acid dispersed in Tween 20 as substrate.

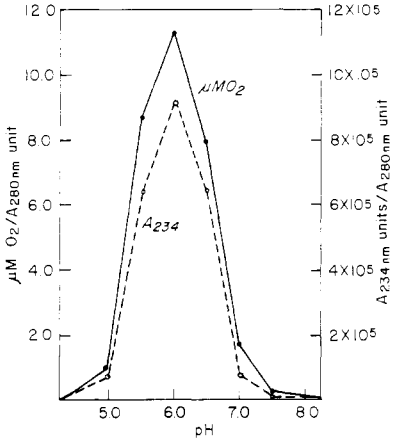

Figure 4. Activity of PL II as a function of pH, using linoleic acid dispersed in Tween 20 as substrate.

enzyme concentration, while the specific activity of PL II was not dependent on enzyme concentration. According to Christopher (1972), soybean lipoxygenase 3 exhibited lower specific activity at higher enzyme concentrations, but lipoxygenase 2 demonstrated constant specific activity over a 50-fold range of enzyme concentration. On the other hand, the lack of agreement between specific activity determined by oxygen uptake and by diene formation for PL I but not PL II may be due to differences in the reaction pathways. This may be demonstrated by the fact that PL I is capable of catalyzing 280-nm absorbing material production, but PL II is not (see following section). Using Christopher's soybean lipoxygenase isoenzymes, Pistorius (1974) also observed that lipoxygenase 3 catalyzed the production of 280-nm absorbing material as a secondary reaction, while lipoxygenase 2 catalyzed only conjugated diene formation. These findings further suggest that PL I is similar to soybean lipoxygenase 3, and PL II, to lipoxygenase 2.

The substrate specificity of PL I and PL II was studied using linoleic acid and methyl linoleate under conditions similar to those used by Koch et al. (1958). PL I demonstrated less activity with methyl linoleate as substrate than with linoleic acid in the pH range from 4.5 to 8. Arens et al. (1973) also obtained a pea lipoxygenase which had less activity toward methyl linoleate than toward linoleic acid at pH 6.3.

PL II used both linoleic acid and methyl linoleate as substrates, but activity with the ester was higher than with the acid. Using methyl linoleate as substrate PL II showed a broad pH optimum of 8.5 to 10. Based on the pH optimum and the higher activity with the ester than with the acid form of substrate, PL II appears to be similar to the soybean lipoxygenase 2 characterized by Christopher

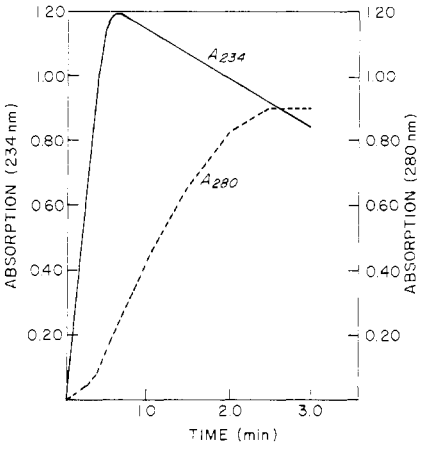

Figure 5. Formation of conjugated dienes (—) and carbonyl products (---) by PL I.

(1972) and the "ester" enzyme described by Verhue and Francke (1972).

Formation of Carbonyl Products and Hydroperoxide Destruction. It is known that under certain reaction conditions, lipoxygenase produces not only hydroperoxides, but also carbonyl compounds (Holman and Burr, 1945; Vioque and Holman, 1962; Garssen et al., 1971; Pistorius, 1974). Pistorius (1974), however, pointed out that the formation of carbonyl compounds was not a general property of all lipoxygenase isoenzymes. In the present study, the isoenzymes could be distinguished on the basis of their carbonyl-producing ability. The reaction products generated during peroxidation of linoleic acid by PL I and II were compared with respect to ultraviolet absorption spectra. PL I generated two peaks: a major peak around 234 nm and another smaller peak near 280 nm. However, with PL II there was only one peak with a maximum near 234 nm. A peak around 234 nm corresponds to that of linoleic acid hydroperoxide and a peak at 280 nm was attributed to carbonyl compounds (Holman and Burr, 1945; Vioque and Holman, 1962).

The spectra obtained with the reaction products of PL I and II were in accord with the following observations. When PL II was incubated with linoleic acid at pH 6.0 (the optimum pH), an increase in conjugated dienes absorbing at 234 nm occurred, but no increase in absorbance at 280 nm was noted. However, at the same enzyme (0.016 A_{280nm} unit) and substrate (2.57 mM linoleic acid) concentrations PL I produced conjugated dienes and 280-nm absorbing material (Figure 5). The production of conjugated dienes increased rapidly to a maximum within a minute. However, with increasing reaction time, a gradual decrease in absorption at 234 nm was observed. This decrease in conjugated dienes was accompanied by an increase in the formation of compounds absorbing at 280 nm. Some studies which reported the disappearance of hydroperoxides attributed this to the presence of a second enzyme which destroys hydroperoxides (Gini and Koch, 1961; Zimmerman and Vick, 1970). The production of carbonyl compounds by PL I could be due to the breakdown of hydroperoxides. However, the fact that PL I was an electrophoretically homogeneous protein with a single isoelectric point does not support the hypothesis that there are two distinct enzymes responsible for peroxidation and decomposition of peroxides. A more plausible explanation is the existence of lipoxygenase isoenzymes which differ slightly in their mechanism of action, yielding different end products. Previous findings that different isoenzymes of lipoxygenase produced either the 9- or 13-hydroperoxides or a mixture of these two may be important in understanding the observations made in this study (Christopher, 1972; Galliard and Matthew, 1975; Gardner, 1975). The hydroperoxides may differ in their resultant breakdown products with one giving rise to 280-nm absorbing carbonyls, or one of the intermediates may decompose to carbonyls without prior hydroperoxide formation.

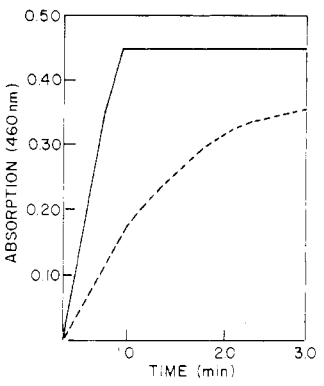

Figure 6. Carotene bleaching (ΔA_{460nm}) with PL I (—) and PL II (---).

Carotene and Chlorophyll Bleaching Activities. With PL I, carotene bleaching occurred in an almost linear fashion until all the carotene disappeared from the reaction mixture (containing 2.57 mM linoleate, 8.3 μg of carotene, 0.016 A_{280nm} unit of enzyme). However, in the presence of PL II carotene was bleached more slowly and the rate decreased with increasing reaction time (Figure 6). When the carotene bleaching potentials of PL I and II were compared, PL I exhibited a higher activity ratio of carotene bleaching to peroxidation than PL II. The ratios of the carotene bleaching to peroxidation activity at pH 6.0 were 0.43 with PL I and 0.23 with PL II. However, the ratio of carotene bleaching to peroxidation activity was found to be variable depending on the carotene, linoleic acid, and enzyme concentrations. The pH profile for carotene bleaching by PL I was similar to that for peroxidation, but did not match exactly. PL II exhibited the same pH optimum of 6.0 for carotene-bleaching and peroxidation activity.

The direct relationship between lipoxygenase action and carotene-bleaching ability has been questioned since Kies (1947) discovered that carotene-bleaching activity of lipoxygenase preparations was extremely susceptible to heat treatment as compared to the peroxidation activity of the same soybean enzyme preparation. Later several investigators (Kies et al., 1969; Pistorius, 1974; Grosch et al., 1976) reported that there are differences in carotene-bleaching potential among lipoxygenase isoenzymes. This appears to be true for pea as well as soybean lipoxygenases. Grosch et al. (1977) and Ramadoss et al. (1978) reported that the soy lipoxygenase isoenzymes which have pH optima of about 6.5 were responsible for the coupled oxidation of carotene. Ramadoss et al. (1978) pointed out that lipoxygenase 3, in combination with either lipoxygenase 1 or 2 promoted the cooxidation of carotene. PL I most closely resembles soy lipoxygenase 3 and is most effective in carotene bleaching, although both pea isoenzymes bleached carotene as Arens et al. (1973) reported.

Lipoxygenase has also been implicated in the degradation of chlorophyll (Wagenknecht et al., 1952; Holden, 1965). However, Zimmerman and Vick (1970) reported that in addition to lipoxygenase, hydroperoxide isomerase activity was necessary for chlorophyll destruction. In the present study, chlorophyll bleaching occurred with PL I, but not with PL II. This difference between the two isoenzymes indicates that chlorophyll bleaching may be

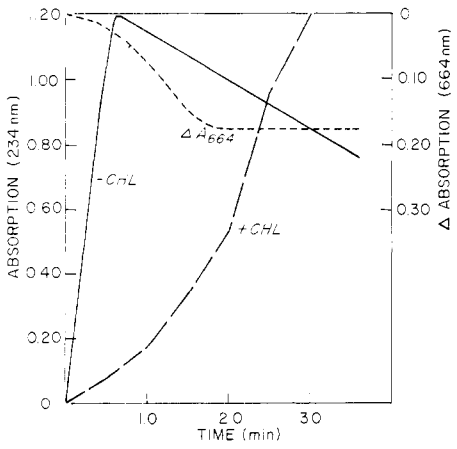

Figure 7. Formation of conjugated dienes in the absence of chlorophyll (—) and in the presence of chlorophyll (– –) and chlorophyll bleaching (ΔA_{664nm}, - - -) by PL I.

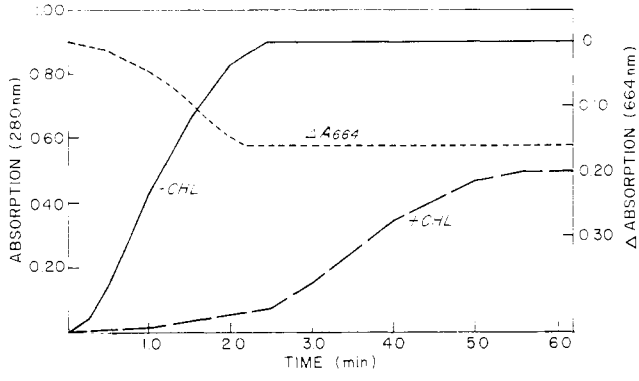

Figure 8. Formation of carbonyl products in the absence of chlorophyll (—) and in the presence of chlorophyll (– –) and chlorophyll bleaching (ΔA_{664nm}, - - -) by PL I.

involved with a reaction other than diene formation. Since PL I and II differed in carbonyl-producing ability, this property may be related to chlorophyll bleaching. The isoenzyme which was effective in carbonyl production (PL I) had chlorophyll-bleaching activity, while the other did not produce carbonyls nor bleach chlorophyll. The pH profile for the formation of 280-nm absorbing carbonyls by PL I was found to be in parallel with that of chlorophyll-bleaching activity.

In the presence of chlorophyll, the rate of production of conjugated dienes from linoleic acid by PL I was markedly decreased until the chlorophyll bleaching ceased (Figure 7). Similarly, carbonyl production was very low while chlorophyll bleaching was occurring (Figure 8). These findings suggest that the chlorophyll-bleaching reaction required intermediates formed during the peroxidation of linoleic acid by PL I, thus preventing formation of conjugated dienes and carbonyl compounds. It is also possible that the decomposition of hydroperoxides to nonabsorbing material occurred.

When Pistorius (1974) compared soybean lipoxygenase isoenzymes in their ability to catalyze the destruction of 2,6-dichlorophenol indophenol (DCPIP), she observed that only the isoenzyme which was capable of producing 280-nm absorbing material could reduce DCPIP. This finding with the reduction of DCPIP is similar to that for chlorophyll bleaching in terms of the involvement of carbonyl production. Therefore, it is possible that the same mechanism is responsible for the reduction of DCPIP and bleaching of chlorophyll.

Grosch (1968) proposed that the unstable intermediate peroxide radicals formed during the peroxidation of linoleic acid by lipoxygenase attack the double bonds of lipid-like unsaturated compounds such as carotene and chlorophyll, thus resulting in pigment bleaching. On the other hand, Pistorius (1974) suggested that singlet oxygen might be responsible for the destruction of the pigment and that the difference in the carotene-bleaching activity of the lipoxygenase isoenzymes might arise from competition between linoleic acid and carotene for the activated oxygen. The differences observed with pea lipoxygenase isoenzymes in their carbonyl-forming ability and in pigment-bleaching activity suggests that the mechanism of linoleate oxidation is not the same for the two isoenzymes. Further study is needed to clarify the reaction mechanism for carbonyl production and its possible relation to pigment bleaching.

LITERATURE CITED

Antis, P. J. P., Friend, J., *Phytochemistry* **13**, 567 (1974).

Arens, D., Seilmeier, W., Weber, F., Kloos, G., Grosch, W., *Biochim. Biophys. Acta* **327**, 295 (1973).

Ben-Aziz, A., Grossman, S., Ascarelli, I., Budowski, P., *Phytochemistry* **10**, 1445 (1971).

Bengtsson, B. L., Bosund, I., *J. Food Sci.* **31**, 474 (1966).

Catsimpoolas, N., "Isoelectric Focusing", Academic Press, New York, 1976, Chapter 4, p 99.

Catsimpoolas, N., "Food Proteins", Whitaker, J. R., Tannenbaum, S. R., Ed., Avi Publishing Co., Westport, CT, 1977, p 116, Chapter 5.

Christopher, J., Ph. D. Thesis, Purdue University, West Lafayette, IN, 1972.

Davis, B. J., *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).

DeLumen, B. O., Kazeniac, S. J., *Anal. Biochem.* **72**, 428 (1976).

Eriksson, C. E., Svensson, S. G., *Biochim. Biophys. Acta* **198**, 449 (1970).

Eskin, N. A., Grossman, S., Pinsky, A., *CRC Crit. Rev. Food Sci. Nutr.* **9**(11), 1 (1977).

Gabriel, O., "Methods in Enzymology", Vol. XXII, Jakoby, W. B., Ed., Academic Press, New York, 1971, p 577.

Galliard, T., Matthew, J. A., *Biochim. Biophys. Acta* **398**, 1 (1975).

Gardner, H. W., *J. Agric. Food Chem.* **23**, 129 (1975).

Garssen, G. J., Vliegenthart, J. F. G., Boldingh, J., *Biochem. J.* **122**, 327 (1971).

Gini, B., Koch, R. B., *J. Food Sci.* **26**, 359 (1961).

Grosch, W., *Z. Lebensm. Unters. Forsch.* **139**, 1 (1968).

Grosch, W., Laskawy, G., Weber, F., *J. Agric. Food Chem.* **24**, 456 (1976).

Grosch, W., Weber, F., Fischer, K. H., *Ann. Technol. Agric.* **26**, 133 (1977).

Grossman, S., Ben-Aziz, A., Budowski, P., Ascarelli, I., Gertler, A., Birk, Y., Bondi, A., *Phytochemistry* **8**, 2287 (1969).

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

Hale, S. A., Richardson, T., Von Elbe, J. H., Hagedorn, D. J., *Lipids* **4**, 209 (1969).

Haydar, M., Hadziyev, D., *J. Sci. Food Agric.* **24**, 1039 (1973).

Haydar, M., Steele, L., Hadziyev, D., *J. Food Sci.* **40**, 808 (1975).

Holden, M., *J. Sci. Food Agric.* **16**, 312 (1965).

Holman, R. T., Burr, G. O., *Arch. Biochem. Biophys.* **7**, 47 (1945).

Joslyn, M. A., *Adv. Enzymol.* **9**, 613 (1949).

Kalbrener, J. E., Warner, K., Eldridge, A. C., *Cereal Chem.* **51**, 406 (1974).

Kies, M. W., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **6**, 267 (1947).

Kies, M. W., Haining, J. L., Pistorius, E., Schroder, D. H., *Biochem. Biophys. Res. Commun.* **36**, 312 (1969).

Klein, B. P., *J. Agric. Food Chem.* **24**, 938 (1976).

Koch, R. B., Stern, B., Ferrari, C. G., *Arch. Biochem. Biophys.* **78**, 165 (1958).

Kon, S., Wagner, J. R., Guadagni, D. F., Horvat, R. J., *J. Food Sci.* **35**, 343 (1970).

Lee, F. A., Wagenknecht, A. C., *Food Res.* **16**, 239 (1951).

Lee, F. A., Wagenknecht, A. C., *Food Res.* **23**, 584 (1958).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193**, 265 (1951).

Mustakas, G. C., Albrecht, W. J., McGhee, J. E., Black, L. T., Bookwalter, G. N., Griffin, E. L., Jr., *J. Am. Oil Chem. Soc.* **46**, 263 (1969).

Pistorius, E., Ph.D. Thesis, Purdue University, West Lafayette, IN, 1974.
Postel, W., Görg, A., Westermeier, R., *Lebensm.-Wiss. Technol.* **11**, 202 (1978).
Rackis, J. J., Honig, D. H., Sessa, D. J., Moser, H. A., *Cereal Chem.* **49**, 586 (1972).
Ramadoss, C. S., Pistorius, E. K., Axelrod, B., *Arch. Biochem. Biophys.* **190**, 549 (1978).
Siddiqi, A. M., Tappel, A. L., *Arch. Biochem. Biophys.* **60**, 91 (1956).
Stevens, F. C., Brown, D. M., Smith, E. L., *Arch. Biochem. Biophys.* **136**, 413 (1970).
Verhue, W. M., Francke, A., *Biochim. Biophys. Acta* **284**, 43 (1972).
Vioque, E., Holman, R. T., *Arch. Biochem. Biophys.* **99**, 522 (1962).
Wagenknecht, A. C., Lee, F. A., Boule, F. P., *Food Res.* **17**, 343 (1952).
Wagenknecht, A. C., Lee, F. A., *Food Res.* **23**, 25 (1958).
Weber, F., Arens, D., Grosch, W., *Z. Lebensm.-Unters. Forsch.* **152**, 152 (1973).
Weber, K., Osborn, M., *J. Biol. Chem.* **244**, 4406 (1969).
Winter, A., Perlmutter, H., Davies, H., LKB Application Note 198, Application Laboratory, LKB-Productor AB, Stockholm, Sweden, 1975.
"Worthington Enzyme Manual", Worthington Biochemical Corporation, Freehold, NJ, 1972.
Yamamoto, A., Yasumoto, K., Mitsuda, H., *Agric. Biol. Chem.* **34**, 1169 (1970).
Zimmerman, D. C., Vick, B. A., *Plant Physiol.* **46**, 445 (1970).

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Comparative Nutritive Value and Amino Acid Content of Different Varieties of Sorghum and Effect of Lysine Fortification

Krishan C. Sikka* and Rajendra P. Johari

A study was conducted to evaluate the nutritive value, i.e., PER (protein efficiency ratio), NPR (net protein retention), and amino acid content of different varieties of sorghum (hybrid and old selected varieties). The protein quality index based on PER and NPR at a 8.5% protein level was found to be highest in the variety CSH-1, followed by CSH-2 and Swarna. A chemical score based on the essential amino acid content of egg protein and FAO/WHO provisional pattern of protein (1973) indicated the levels of amino acids which are limiting in three different varieties of sorghum. The EAAI (essential amino acid index) and BV (biological value) were also calculated. Fortification of sorghum grain with lysine at 9% level has increased the PER and NPR values significantly.

Sorghum grain is one of the important crops in the world. This cereal plant is cultivated on all six continents and widely used as food in Africa and Asia. In Western countries the grain is used as feed and the whole plant as forage (Wall and Ross, 1970).

Although sorghum is a staple food in India, the nutritional value of sorghum grain is inferior to that of maize and barley (Xavier Filho, 1974; Howe et al., 1965). The amino acid composition of its proteins shows deficiency of lysine and threonine, and their digestibility is less than that of other cereal grain proteins. The reason for this low digestiblity is not known (Wall and Ross, 1970).

Preliminary data of Miller et al. (1964) on sorghum indicated that wide variation in protein content was associated with location, hybrids, and fertilization. Little work has been reported on the amino acid composition of sorghum (Baumgarton et al., 1945, 1946; Lyman et al., 1956; Deyoe and Shellenberger, 1965; Singh and Axtell, 1973) and on the nutritive value of hybrid sorghum (Breuer and Dohm, 1972; Jambunathan and Mertz, 1973).

It was considered important to study systematically the nutritive value based on PER, NPR, and amino acid content in different varieties of hybrid sorghum and compare it with that of older selected variety, in order to explore the possibility of improving the nutritional quality by breeding and to learn what other amino acids are limiting besides lysine. The effect of lysine fortification at different levels on the nutritional quality was also studied in this investigation. The present paper describes results of such investigation on different varieties of sorghum developed at the Indian Agricultural Research Institute, New Delhi.

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MATERIALS AND METHODS

Sorghum grain samples were collected from a field experiment conducted at Division of Agronomy, Indian Agricultural Research Institute, New Delhi.

Methods. Samples were dried in a hot air oven at 105 °C to determine moisture content. The protein content of the samples was calculated by multiplying the Kjeldahl N by 6.25. The amino acid composition was studied using a Technicon sequential multisample amino acid analyzer (TSM). Defatted samples containing 5 mg of protein were hydrolyzed by refluxing with 5 mL of 6 N HCl acid for 22 h. After removal of acid by evaporation under reduced pressure, the residue was dissolved in 2 mL of citrate buffer (pH 2.875). An aliquot (0.4 mL) was used for determination of amino acids according to the method of (Spackman et al., 1958). Tryptophan was determined by the method of (Spies and Chambers, 1949).

Diets. The diets for all the biological experiments were prepared at a 8.5% protein level. The composition of 100 g of diet was as follows; test sample flour weight calculated to give 8.5% protein; ground nut oil 10 g (containing 1 mg or 100 IU vitamin E; 4% mineral mixture U.S.P. XVII 4) (Sikka et al., 1975); 1 g of a complete vitamin mixture

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