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Heterogeneity of soybean lipoxygenase 2

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Soybean lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) have been analysed by chromatofocusing. Four major protein peaks with lipoxygenase activity at pH 6.6 eluting between pH 7.0 and pH 5.0 were found. The peak with the lowest p*I* is identical to the classical lipoxygenase 1. The other peaks belong to the type-2 lipoxygenases which have further been characterized by isoelectric focusing, amino acid analysis and metal analysis. The type-2 enzymes differ in their isoelectric points and in some respects of the amino acid composition. They all contain one atom of iron per enzyme molecule and show a preference for the introduction of oxygen at C-9 of linoleic acid. They do not precipitate with antibodies directed against lipoxygenase 1. It is concluded that in sobyeans four lipoxygenases exist, which can be divided into two classes.

250 g soybeans (*Glycine max* (L.) Merr. var. Williams) were ground and defatted with cold acetone (−10°C). After drying, the acetone powder was extracted with 2 litres of 0.2 M sodium acetate buffer (pH 4.5, 2 h). These and all further steps were carried out at 4°C. After centrifugation (15 000 × *g*, 40 min) the precipitate was brought to 60% saturation with finely ground $(NH_4)_2SO_4$. Finally, after standing for 2 h and centrifugation (20 000 × *g*, 40 min), the precipitate was suspended in 0.05 M sodium acetate buffer (pH 4.8) and then dialysed against this buffer.

Ion-exchange chromatography on CM-Sephadex C-50 was performed in 0.05 M sodium acetate buffer (pH 4.8) with a linear gradient from 0.1 to 0.4 M NaCl.

Ion-exchange chromatography on DEAE-Sephadex A-50 was performed as described earlier [1]. Before applying the protein to the column, it was dialysed against 0.02 M sodium phosphate buffer (pH 6.8).

After dialysing the protein against 0.025 M imidazole/HCl buffer (pH 7.4), chromatofocusing was carried out in the pH range 7.0–5.0. In a subsequent experiment the resolution could be enhanced by adjusting the pH of the starting buffer to 6.8. Proteins were liberated from Polybuffer by precipitation with $(NH_4)_2SO_4$ (60% saturation).

Lipoxygenases 1 and 2 were found to have absorbances at 280 nm of 1.6 and 1.4 (1 mg/ml), respectively. These values were routinely used to measure enzyme concentrations.

Lipoxygenase activity was assayed polarographically with a Clark electrode in air-saturated solutions at 25°C at pH 9.0 and pH 6.6. Substrate solutions contained 1.8 mM ammonium linoleate in 0.1 M sodium borate buffer (pH 9.0) or 0.1 M sodium phosphate buffer (pH 6.6). In the former case 2 mM $CaCl_2$ was added to counteract substrate inhibition. Enzyme units are defined as μmol O_2 uptake per min.

Analytical isoelectric focusing was carried out

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in 6% polyacrylamide slab gels containing Ampholytes (LKB, Zoetermeer, The Netherlands) for the pH range 8.0–5.0. Preparation of the gels and staining for protein and lipoxygenase activity were carried out according to Verhue and Francke [2]. A high-p*I* calibration kit from Pharmacia (Woerden, The Netherlands) was used.

The immunoreactivities of the pooled and Polybuffer-free proteins were tested by double gel immunodiffusion according to the method of Ouchterlony [3] as described earlier [4]. Antibodies against lipoxygenases 1 and 2 were a generous gift from Dr. M. Vernooy-Gerritsen.

M_r values were determined with SDS-polyacrylamide under dissociative conditions essentially according to Weber and Osborn [5]. Gels were calibrated with calibration sets for high and low molecular weights (Bio-Rad, Utrecht, The Netherlands).

The relative amounts of the amino acids in the proteins were determined after hydrolysis with 6 M HCl in vacuo for 24 h and 48 h. In separate experiments, lysine was determined as its 2,4-dinitrophenyl derivative and tryptophan was determined after hydrolysis in the presence of thioglycollic acid [6]. Sulphydryl groups were assessed in 1% SDS with dithionitrobenzoic acid [7]. Sulphydryl groups were modified by treating the mixture of the type-2 lipoxygenases with CH_3HgI for 40 h at 4°C. (cf. Ref. 8). The excess of modifying reagent was removed by gel filtration on Sephadex G-25 (Pharmacia, Woerden, The Netherlands).

Fe, Mn and Cu were determined with a Perkin Elmer graphite furnace atomic absorption spectrometer.

Incubations of linoleic acid (100 μM) with type-2 lipoxygenases were carried out as described by Van Os et al. [9]. Reactions were monitoried by repetitively taking spectra from 200–300 nm. The reaction products were treated with $NaBH_4$, converted into their methyl esters with diazomethane and then analysed by SP-HPLC on a Partisil-5 column with hexane/isopropanol (99.3 : 0.7, v/v) as the eluent. The eluent was monitored at 234 nm and the signal was analysed further with a Hewlett-Packard 3390A integrator.

In order to study oxodiene formation during the oxygenation, 10-μl portions of the purified enzyme solutions were incubated with 1 ml of an air-saturated solution of ammonium linoleate (40 mM, 0.1 M sodium phosphate buffer, pH 6.6). Diene and oxodiene contents were monitored with a Cary 118C spectrophotometer at 234 and 285 nm, respectively.

Formation of prostaglandin-like substances from arachidonic acid (5*Z*, 8*Z*, 11*Z*, 14*Z*-eicosatetraenoic acid (50 μM)) by type-2 lipoxygenases was investigated as described by Bild et al. [10]. After reduction with dithionite the incubation mixture was extracted with disposable C_{18}-coated silica columns (J.T. Baker, Deventer, The Netherlands) and analysed by RP-HPLC on a Nucleosil 5-C-18 column (Chrompack, Middelburg, The Netherlands) with methanol/water/acetic acid (70 : 30 : 0.1 v/v) as the eluent. The eluent was monitored at 205 nm. Prostaglandins B_2 and $F_{2\alpha}$ were used as reference compounds.

Fig. 1 contains the elution pattern after chromatofocusing of a whole soybean extract without a preceding ion-exchange chromatography step. In addition to the protein peak with a relatively high specific activity, which was identified as the classical lipoxygenase 1, two other peaks with lower specific activity are present. These peaks showed no activity at pH 9.0 unless substrate inhibition was counteracted with $CaCl_2$ and gave precipitation only with antibodies directed against lipoxygenase 2. Ion-exchange chromatography on Sephadex CM-C50 of a whole soybean extract gave a good resolution of type-1 and type-2 enzymes. Lipoxygenase type-2 fractions obtained with CM-C50 chromatography were pooled and then sub-

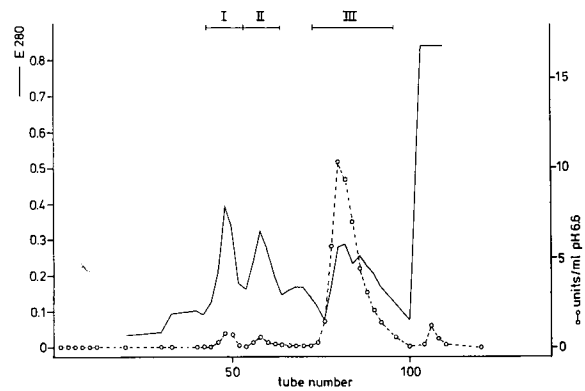

Fig. 1. Chromatofocusing of a whole soybean extract.

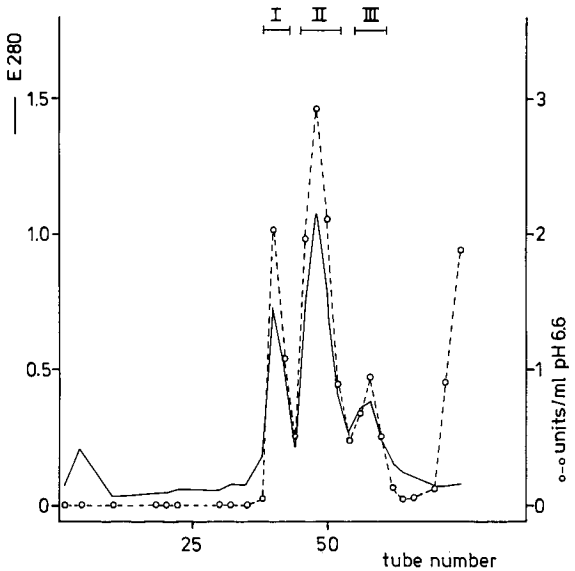

Fig. 2. Chromatofocusing of lipoxygenase 2 after Sephadex CM-C50 ion-exchange chromatography.

jected to chromatofocusing. The result is given in Fig. 2. Beside the two type-2 lipoxygenases already discernible in Fig. 1, a third peak with type-2 activity is present. The fractions were pooled, designated I, II and III (Fig. 2) and analysed by isoelectric focusing. Relevant p*I* values are tabulated in Table I. The fractions show slight mutual contaminations. The activity stain clearly demonstrated that the main bands of pools I and II have lipoxygenase activity, whereas the main band of pool III exhibited only a weak activity. Since none of the main bands of pools I and II is present in III to such an extent as to account for its activity in the assays, it is considered to be a true lipoxygenase species. At least four different lipoxygenases (including lipoxygenase 1) can thus be isolated from soybeans. The fractions isolated from pools I, II and III are designated lipoxygenase 2a, 2b and 2c, respectively.

M_r values as determined by SDS-polyacrylamide gel electrophoresis are listed in Table I. For all type-2 lipoxygenases, two closely migrating bands were observed. Under these circumstances, a similar feature was observed for lipoxygenase 1. An M_r value of 92000 was found for all lipoxygenases, including lipoxygenase 1. Each of the lipoxygenases appeared to have 1 atom of iron per enzyme molecule (Table I). Manganese and copper are present in subequivalent quantities, most probably as contaminants. The amino acid analyses (Table II) of the type-2 enzymes show many similarities, although lipoxygenase 2c has relatively high values for leucine and low values for cysteine, lysine and tryptophan. Upon comparison of lipoxygenase 1 with the type-2 lipoxygenases some significant differences are noteworthy: lipoxygenase 1 appears to contain more Glx and fewer arginine residues. Also, lipoxygenase 1 has more sulphur-containing residues (methionine, cysteine). The lack of an immunological relationship between lipoxygenases 1 and 2 may be caused by these differences. No indication could be found for the presence of disulphide bridges. Upon reacting the type-2 enzymes with CH_3HgI [8], two sulphydryl groups appeared to be modified, whereas with lipoxygenase 1 three such groups were found to react with the mercurial. In contrast to lipoxygenase 1, modification of type-2 lipoxygenase with CH_3HgI does not affect its activity. Therefore, the native type-2 lipoxygenases might, in comparison to the type-1 enzyme, lack one sulphydryl group, probably near the active site. Immunoreactivity tests have shown that lipoxygenases 2a, 2b and 2c give precipitation bands

TABLE I

PROPERTIES OF SOYBEAN LIPOXYGENASES
n.d., not determined.

	p*I*	M_r	Fe	Cu	Mn	$\Delta A_{285}/A_{234}$
Lipoxygenase 2a	6.12	92000	1.12	0.13	0.03	0.33
Lipoxygenase 2b	6.00	92000	0.97	0.02	0.04	0.86
Lipoxygenase 2c	5.87	92000	0.98	0.03	0.03	0.99
Lipoxygenase 1	5.71	92000	1.14	0.11	0.06	n.d.

TABLE II

AMINO ACID COMPOSITION OF SOYBEAN LIPOXYGENASE

Amino acid	Lipoxygenase 2a (M_r 92000)	Lipoxygenase 2b (M_r 92000)	Lipoxygenase 2c (M_r 92000)	Lipoxygenase 1 [11] (M_r 92000)
Asx	84	88	77	87
Thr	45 [a]	47 [a]	45 [a]	44
Ser	54 [a]	55 [a]	51	52
Glx	82	87	86	111
Gly	59	61	62	66
Ala	49	51	55	66
Cys	4	4	3	5
Val	47	49	56	52
Met	12	11	12	17
Ile	47	48	49	50
Leu	72	72	87	82
Tyr	33	33	37	43
Lys	42	36	29	52
His	32	32	30	28
Arg	51	47	53	36
Pro	49	45	43	45
Trp	12	10	7	11
	809	813	813	883

[a] Determined by extrapolation to zero hydrolysis time.

only with antibodies directed against lipoxygenase 2, and not with those against lipoxygenase 1.

Each of the type-2 lipoxygenases was incubated with linoleic acid to establish possible differences in regiospecificities. The results are compiled in Table 3. As the regiospecificity of each of the isoenzymes is close to that determined for the mixture of the isoenzymes, the relatively low regiospecificity of lipoxygenase 2 cannot be due to its heterogeneity. The results of a study on oxodiene formation accompanying the dioxygenation of linoleic acid at pH 6.6 are summarized in Table 1. Lipoxygenase 2a appears to be significantly different from the other two, its oxodiene formation being much less pronounced.

Lipoxygenase 2a, which is presumably the enzyme designated lipoxygenase 2 by other authors [1,10], was found not capable of forming prostanoic acid derivatives, despite the fact that the experimental conditions were virtually identical to those described by Bild et al. [10]. Soybeans thus contain two major types of lipoxygenases. The type-2 enzyme lacks one sulphydryl group which is present in lipoxygenase 1. This feature may be responsible for some of the characteristic differences between these enzymes.

TABLE III

REGIOSPECIFICITIES OF TYPE-2 LIPOXYGENASES

	%13-HPOD	%9-HPOD
Lipoxygenase 2a	40	60
Lipoxygenase 2b	40	60
Lipoxygenase 2c	45	55

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