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LIPOXYGENASE IN *VICIA FABA MINOR*

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Abstract—Lipoxygenase activity was demonstrated in partially purified preparations from small faba beans The enzyme was shown to possess a pH optimum of 6 5, and was inactivated by exposure to 70° for 15 min The K_m value for linoleic acid was calculated to be 0 57 mM Ammonium sulphate fractionation yielded two highly active preparations, which were both active towards linoleic and linolenic acids Neither fraction was inhibited by either cyanide or *p*-chloromercuribenzoate The two fractions showed markedly differing responses to calcium ions, suggesting the presence of two lipoxygenases in faba beans Activation of the enzyme by calcium ions was eliminated by the addition of EDTA

INTRODUCTION

THE SMALL faba bean, or horse bean, *Vicia faba* L. var. *minor* (Peterm.) Beck, is widely cultivated in Europe and Asia, and is currently being considered as a possible new crop for Western Canada.[1] There is considerable interest in this bean from the nutritional standpoint since its lysine content is comparable to that of casein. However, the limiting amino acids are methionine and cystine. Investigations are being carried out to determine the possibility of incorporating faba bean protein isolates into human foods for nutritional enrichment.

Analysis of the fatty acid content of faba beans has shown that linoleic acid accounts for over 50% of the total fatty acids present [1] This acid is particularly susceptible to oxidation which is responsible for the development of off-flavors. It has been found that processed faba beans develop rancidity very soon after grinding, representing a major storage problem

The purpose of this investigation was to study the development of rancidity in faba beans with respect to the enzyme lipoxygenase (linoleate. oxygen oxidoreductase, E.C. 1.13.1.13). This enzyme catalyses the oxidation, in the presence of molecular oxygen, of *cis,cis*-1,4-pentadiene systems in unsaturated fatty acids to conjugated *cis,trans* hydroperoxides It has been studied in a number of plants.[2–7] Activation of lipoxygenase by calcium

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ions was first demonstrated by Koch[6] in navy bean and soybean extracts. This work was extended by Koch *et al*.[7] who demonstrated calcium-activation effects which suggested that navy bean lipoxygenase may consist of two isoenzymes. Pinsky *et al*.[4] were unable to show any effect of calcium ions on either of their two potato lipoxygenases. Restrepo *et al*.[3] demonstrated that two soybean lipoxygenase isoenzymes differed markedly in their response to added calcium ions, one fraction being inhibited while the other fraction was activated.

The present study demonstrates the occurrence and isolation of two active lipoxygenase fractions in the faba bean, each differing markedly in their response to calcium ions.

Table 1. Yield and enrichment of faba bean lipoxygenase at various stages of purification

Procedure	Vol (ml)	Concn (units/ml)	Total units	Protein (mg/ml)	Specific activity (mM O_2/min/mg protein)	Yield (%)	Purification
Crude extract	425	9·5	4033·3	17·4	0·5455	100	1
Acid treatment	430	7·3	3156·2	10·0	0·7343	78·3	1·5
Heat treatment	415	6·7	2793·7	9·5	0·7064	68·1	1·5
$(NH_4)_2SO_4$ 40%	15	75·6	1134·0	48·0	1·5750	28·1	3·0
$(NH_4)_2SO_4$ 50%	5	60·4	302·0	37·2	1·4623	7·5	3·0
Dialysed $(NH_4)_2SO_4$ 40%	14	25·1	351·8	10·1	2·4951	8·7	5·0
Dialysed $(NH_4)_2SO_4$ 50%	5	20·8	104·0	11·0	0·7102	2·6	1·5

The standard reaction mixture composition was linoleic acid, 2·0 mM in Tris–maleate buffer, 0·2 M, pH 6·5, 3·0 ml; enzyme preparation, 10–30 µl. The substrate solution was oxygenated at 25° for 1·25 min, immediately prior to the addition of the enzyme. Oxygen uptake was monitored at 25° using an oxygen electrode.

RESULTS

Lipoxygenase activity was observed in two fractions obtained respectively from 30–40% and from 40–50% ammonium sulphate precipitation, and these were then purified by dialysis and reprecipitation (Table 1). There was a linear relationship between enzyme concentration and oxygen uptake in respect to all determinations carried out. The enzyme was completely inactivated by exposure to 70° for 15 min, it was found to conform to the Michaelis–Menten equation, and a Lineweaver–Burk plot gave a K_m value for linoleic acid of 0·57 mM. The enzyme was active over approx. 1·5 pH units, with the optimum at 6·5, it was completely inactivated at pH 8·2, and slightly active at pH 5·2.

Table 2. Substrate specificity of 40% and 50% ammonium sulphate fractions

Substrate	40% Fraction	50% Fraction	Substrate	40% Fraction	50% Fraction
Linoleic acid	100	70	Dilinolein	5	2
Linolenic acid	55	50	Monolinolein	5	3
Trilinolein	5	3	Methyl linoleate	5	3

Activity is expressed as percentage in terms of the activity of the 40% fraction upon linoleic

The two fractions from ammonium sulphate precipitation exhibited a high degree of specificity towards linoleic and linolenic acids. A slight activity was demonstrated when the free acids were replaced by methyl linoleate or the three glycerides (Table 2) Neither fraction was inhibited by 1 mM cyanide, or by 1 mM *p*-chloromercuribenzoate The addition of 1 mM EDTA interfered with the stimulation of lipoxygenase by calcium ions by eliminating any increase in activity

The two dialyzed fractions from ammonium sulphate precipitation were shown to differ markedly in their responses to calcium ions. Activity in the 40% fraction was significantly ($p < 0.01$) inhibited by calcium chloride at final concentrations in the reaction mixture of 0·75 mM and 1·00 mM, with the greatest amount (33 5%) of inhibition at 1 25 mM. However, activity in the 50% fraction was significantly ($p < 0.01$) stimulated by calcium chloride with maximum activation in the presence of 0·50 mM $CaCl_2$. The activity decreased until at 1 00 and 1·25 mM it was not significantly different from that observed in the presence of 0 25 mM $CaCl_2$ or the corresponding control

DISCUSSION

The differential response to calcium ions by the two dialyzed ammonium sulphate fractions suggests the presence of two separate lipoxygenases in faba beans This finding parallels work on soybeans by Restrepo *et al.*[3] who also reported two distinct lipoxygenase fractions differing in their response to calcium ions In the present study a 50% loss of activity was demonstrated following dialysis of the 40–50% ammonium sulphate fraction. This activity was increased by an increment of 64% on the addition of 0·50 mM $CaCl_2$ but was virtually eliminated by the addition of EDTA (1 mM) This indicates that EDTA has a higher binding affinity for the Ca^{2+} in comparison to the lipoxygenase fraction. Dialysis of the 30–40% ammonium sulphate fraction resulted in a 63% increase in activity. This activity was inhibited by 33·5% on the addition of 1·25 mM $CaCl_2$. Thus the two lipoxygenase fractions showed markedly differing responses to calcium ions.

EXPERIMENTAL

Source material Small faba beans (*Vicia faba* L var *minor* cv Ackerperle) were obtained from the Dept of Plant Science, University of Manitoba The beans were harvested in 1972 and subsequently stored at room temp

Enzyme preparation and assay Lipoxygenase was partially purified from buffered crude extracts of acetone powder by pH 5 0 treatment, heat treatment (55° for 10 min) and ammonium sulphate fractionation The activity was determined by following substrate oxidation at 25° using a biological O_2 monitor 3 0 ml of 2 0 mM substrate dispersed with 0 028% Tween 20 in Tris–maleate buffer, 0 2 M, pH 6 5 was placed in the reaction chamber, this solution including additions in the appropriate experiments The substrate solution was oxygenated at 25° for 1 25 min, after which 1–50 μl of enzyme preparation was added. and oxygen uptake recorded Protein was determined by the method of Lowry *et al* [8] using crystalline bovine serum albumin as the standard Control experiments with boiled enzyme preparations did not exhibit any activity Substrate solutions were made up fresh daily and were stored under N_2 when not in use

Enzyme activity units The specific activity was calculated as mM O_2 uptake/min/mg protein At zero time all substrate solns were 100% saturated with O_2 At 25°, the conc of O_2 in a 100% O_2-saturated aq soln is 1 26 mM [9]

Statistical analysis An analysis of variance was carried out to determine significant differences between the levels of $CaCl_2$ on the activity of lipoxygenase Duncan's multiple range test[10] was used to compare treatment means

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