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Characteristics of crude lipoxygenase from commercially de-oiled lupin flakes for different types of lupins (*Lupinus albus*, *Lupinus angustifolius*)

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Abstract

To use effectively lupin protein products in food formulations, the two mainly cultivated and processed sweet lupins (*Lupinus albus*, *Lupinus angustifolius*) were investigated related to lipoxygenase activity. Optimum pH of purified lupin lipoxygenase was reported at 6.0, however, optimum pH of crude lipoxygenase from *L. angustifolius* and *L. albus* was found to be 7.5 and 8.0, respectively. This crude extract showed 10 times of total activity of purified lipoxygenase. Lipoxygenase from *L. angustifolius* showed higher thermal stability up to 80 °C than *L. albus*. Soybean crude lipoxygenase showed 9–12 times activity of lupin lipoxygenase both at pH 7.5 and 8.0. Our results indicate that research on crude extract is useful to know the changes of lupin lipoxygenase activity and thermostability of lipoxygenase in the food applications.
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Keywords: *Lupinus albus*; *Lupinus angustifolius*; Lipoxygenase; Protein; Industrial application

1. Introduction

Lupins are leguminous plants and in Mediterranean countries, lupin seeds have been used as a source of protein and oil since ancient times. Two types of lupins are mainly cultivated today (*Lupinus albus* and *Lupinus angustifolius*): white lupins (*L. albus*) are mainly cultivated in Europe and blue lupins (*L. angustifolius*) are often grown in Australia. As reported previously, there are potential applications for lupin protein products (concentrates, isolates) in foods. (Wäsche, Müller, & Knauf, 2001). The interest in lupins as an alternative protein source has increased recently even though the factors that can affect the quality of lupin protein during storage and processing have not been sufficiently studied. Some enzymes from lupin seeds, namely lipoxygenase (LOX), polyphenol oxidase and peroxidase, might be important factors for altering the quality of the lupin protein. Protein isolate from lupins showed interesting physico-chemical functionality compared to the original protein (Wäsche et al., 2001). However, not only protein functionality but also enzyme activities are important for the utilisation of lupin protein.

It is well known that the enzyme LOX affects the quality of food and catalyses the hydroperoxidation of fatty acids having the *cis*,*cis*-1,4-pentadiene structure (Axelrod, 1974). The reaction can be detected by the increase in absorbance at 234 nm, due to the formation of hydroperoxide (Surrey, 1964). LOX has been identified as the enzyme responsible for the development of off-flavours in green peas (Williams, Lim, Chen, Pangborn, & Whitaker, 1986), green beans (Velasco, Lim, Pangborn, & Whitaker, 1989), soybeans (Sheu & Chen, 1991) and corn (Barrett & Theerakulkait, 1995) during storage. LOX from legumes which were purified to apparent homogeneity and characterised are the enzymes from soybean seeds (Christpher, Pistorius, & Axelrod, 1972), tomatoes (Suurmeijer, Perez-Gilabert, van der

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Hijden, Veldink, & Vliegenthart, 1998), olives (Jaren-Galan, Carmona-Ramon, & Minguez-Mosquera, 1999; Ridolfi, Terenziani, Patumi, & Fontanazza, 2002), English peas (Chen & Whitaker, 1986) and broad beans (Clemente, Olias, & Olias, 2000). Until now, some of the food processing methods utilised LOX to brighten the colour, however, controlling the activity is difficult. Therefore, many processes have aimed to annul or reduce the LOX activity.

There are a few reports of lupin seed LOX (Beneytout, Najid, & Tixier, 1988; Najid, Beneytout, Leblanc, Tixier, & Rigaud, 1988), and those reports have focused on the purification of lupin LOX and characterising the pure LOX. They reported that the optimum pH of 377 times purified lupin LOX was pH 6.0, and that purified fraction expressed approximately 10% of total activity. However, from our preliminary experiment, we have reached that this optimum pH is available only for purified fraction, crude extract which is keeping 10 times of total LOX activity showed different optimum pH. From a food processing point of view, it is more important to know the changes of LOX activity in crude extract basis. Purification procedure of LOX does not give us the information of the differences of LOX activity in food processing. We have reported the extraction of useful protein fraction from lupin (Wäsche et al., 2001), however, we did not know the changes of LOX activity in that extraction procedure. Although there are many reports on the purification and characterisation of common legume LOX, no report is available that compares the activity using the same processing method and on the same scale. Since soybean is important plant protein source, many researches have been done to explain several types of LOX. For the basic research of food processing, information of legume LOX in the large-scale extraction is important. Therefore, the LOX activity from lupin and soybean was compared in the same manner in this study.

As a first step to providing information about lupin LOX activity *for food processing*, this study set out to determine the lipoxygenase activity of commercially available de-oiled flakes produced on a large scale.

2. Materials and methods

Lupinus albus seeds were obtained from Chile and *L. angustifolius* seeds were obtained from Australia. Soybeans were obtained from the United States. Lupin seeds and soybeans were commercially de-oiled using hexane, and the de-oiled flakes were milled using a Retsch ZM-100 mill into powder form (<0.1 mm). Linoleic acid and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the other reagents used for the experiments were analytical grade.

LOX extract was prepared following the method of Ridolfi et al. (2002) and Clemente et al. (2000) with some modification. About 2.5 g of the de-oiled lupin powder or soybean powder was homogenised with 50 mL of 50 mM sodium phosphate buffer solution (pH 6.8) containing 0.3 mM DTT, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium metabisulphite, 0.1% Triton X-100 and 5 g of insoluble polyvinylpolypyrrolidone (PVPP) for 30 min using a magnetic stirrer. The sample was centrifuged at 10,000*g* for 30 min. The supernatant was dialysed to remove small molecular weight fractions and the dialysate was used as a crude extract for enzymatic assay. The remaining wet solid was recovered as pellet in the bottom of the centrifugation tube.

Partial purification of the enzymatic extract was performed by ammonium sulphate precipitation. The supernatant was mixed with 75% saturated ammonium sulphate for 30 min and set aside for 1 h to facilitate protein precipitation. The sample was then centrifuged for 30 min at 10,000*g*. The pellet (P0–75) was re-suspended in a small volume of 50 mM sodium phosphate (pH 6.8) and dialysed with the same buffer. The fractionation of lupin LOX was initiated by the addition of 75% saturated ammonium sulphate solution to provide 25% saturation. The suspension was allowed to stand for 1 h and then centrifuged (10,000*g*, 30 min) to obtain the precipitate (P0–25). The resulting supernatant was brought up to 50% saturation by adding solid ammonium sulphate, and the precipitate was obtained after centrifugation (P25–50). The subsequent supernatant was further saturated with solid ammonium sulphate up to 75% saturation, and the precipitate (P50–75) was obtained by centrifugation. The precipitated fractions (P0–25, P25–50 and P50–75) were re-suspended in minimal amounts of sodium phosphate buffer (50 mM, pH 6.8) and dialysed against the same buffer. The obtained dialysates (P0–75, P0–25, P25–50 and P50–75) were subjected to enzymatic assay and molecular weight analysis. All of the extraction procedure was performed under refrigerated condition (3–5 °C).

For the linoleic acid substrate, a stock solution of 10 mM concentration containing Tween 20 (0.1%, v/v) in 10 mM sodium tetraborate buffer (pH 9) was prepared. The preparation was carried out under a continuous nitrogen flow to maintain anoxic conditions. The substrate was then stored in the dark at −20 °C until used.

The LOX activity was determined spectrophotometrically at room temperature by measuring the increase in absorbance at 234 nm over a 3 min period for the oxidation of linoleic acid (Axelroad, Cheesborough, & Laakso, 1981). The reaction mixture (total volume 1.5 mL) contained sodium phosphate buffer (200 mM, pH 4.5–8.5) with Tween 20 (0.1%, v/v) and an appropriate quantity of enzymatic extract to give an increase in absorbance of more than 0.001/min. The reaction was

started by adding 120 μL of substrate and 10 μL of enzymatic extract to the medium. The final concentration of linoleic acid was 800 μM. The specific activity was expressed as units of enzyme per milligram of protein, where a unit of enzyme is defined as the amount of enzyme capable of causing an absorption increment of 0.001/min.

The protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) based on the Bradford–dye binding procedure using bovine serum albumin as standard (Bradford, 1976).

The optimum pH for lupin LOX assay, using linoleic acid as substrate, was determined in a pH range from 4.5 to 9 at room temperature using the following buffers: sodium phosphate and sodium borate at a final concentration of 200 mM. Since the enzymatic activity is temperature-dependent, the LOX extracts (P0–75) were treated at different temperatures (0–99 °C) for 10 min. After cooling on the ice, the LOX activity was determined under standard assay conditions (pH 7.5 for *L. angustifolius*, pH. 8.0 for *L. albus*).

The kinetic constants of LOX were measured under standard assay conditions using linoleic acid concentrations ranging from 25 to 800 μM at pH 7.5 for *L. angustifolius,* and at pH 8.0 for *L. albus*. The respective kinetic parameters were evaluated by plotting the data on a Lineweaver–Burk double reciprocal graph (Lineweaver & Burk, 1934).

The apparent molecular weight of LOX was determined by HPLC using a Bio-Sil Sec 250-5 (Bio-Rad, 300 × 7.8 mm) separation column and a Bio-Sil Sec 250 Guard (80 × 7.8 mm) guard column heated to 35 °C by the column oven. The eluent contained 150 mM sodium chloride and 50 mM sodium phosphate buffer (pH 6.62–6.65), and the flow rate was 0.9 mL/min. The proteins were detected at 220 and 280 nm. The molecular weight was calibrated using Bio-Rad gel filtration standards including thyroglobin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B12 (1.35 kDa).

All the experiments were carried out more than three times.

3. Results and discussion

As shown in Fig. 1, for *L. albus* the optimum pH was 8.0, whilst *L. angustifolius* showed the highest activity at pH 7.5. Soybean has been reported to have two types of LOX (LOX-1 and LOX-2), with LOX-1 having an optimum at pH 9.0 and LOX-2 having an optimum at pH 6.5 (Sheu & Chen, 1991). The optimum pH of lupin LOX was also different for both soybean LOX-1 and 2. Najid et al. (1988) showed that the optimum pH of purified LOX from *L. albus* was pH 6.0 and that the purified fraction had 9–10% of the total activity. In our study, LOX precipitated by 75% saturated ammonium sulphate (P0–75) had an optimum at pH 8.0 and had 90% of total activity. When lupin protein isolates were prepared to express better characteristics in the food industry in our laboratory (Patent No. EP 1024 706 B1, 2002), we also needed to have information of the changes of LOX activity. This optimum pH difference was an important information to tell us how useful to continue the research of LOX activity and characteristics on crude extract basis for food processing. We therefore used pH 8.0 for our further studies on the activity of LOX from *L. albus*. For the same reasoning, we fixed the pH at 7.5 for the enzymatic assay of LOX from *L. angustifolius*.

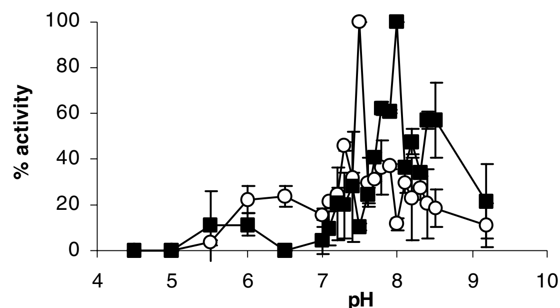

Fig. 1. Effect of pH on lupin lipoxygenase activity using linoleic acid as the substrate. *L. angustifolius* (○) and *L. albus* (■).

The results of the thermal stability tests are shown in Fig. 2. When the temperature increased above 60 °C, the activity of the LOX from *L. albus* decreased dramatically. However, the *L. angustifolius* LOX, was thermally stable up to 80 °C. This dramatic decrease in the activity was also mentioned by Najid et al. (1988). The activity of *L. angustifolius* LOX decreased gradually. The biggest difference between the activities of the LOX from *L. albus* and *L. angustifolius* was at 70 °C.

As shown in Table 1, more than 90% of the protein in the crude extract was collected by 75% saturated ammonium sulphate precipitation, i.e., 201 mg of protein was collected in the P0–75 fraction from 214 mg protein

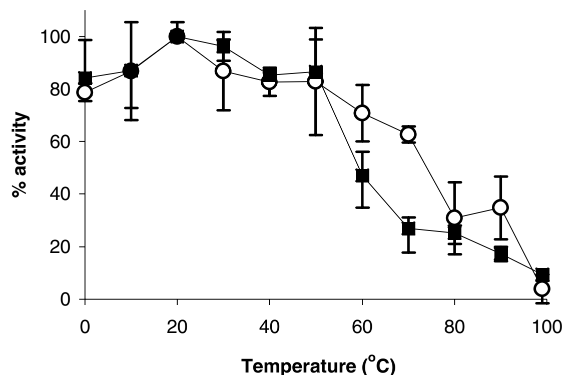

Fig. 2. Thermal stability of lupin lipoxygenase. *L. angustifolius* (○) and *L. albus* (■).

Table 1
Comparison of the lipoxygenase activity of lupin and soybean flakes de-oiled using hexane

Step	Protein (mg)		Total activity (units)		Specific activity (units/mg protein)		Yield (%)	
A: At pH 8.0	*L. albus*	Soybean	*L. albus*	Soybean	*L. albus*	Soybean	*L. albus*	Soybean
Crude extract	214	173	65,300	597,900	305	3460	100	100
$(NH_4)_2SO_4$ (P0–75)	201	152	62,100	531,000	309	3500	95	89
B: At pH 7.5	*L. angustifolius*	Soybean	*L. angustifolius*	Soybean	*L. angustifolius*	Soybean	*L. angustifolius*	Soybean
Crude extract	115	173	49,000	469,000	426	2605	100	100
$(NH_4)_2SO_4$ (P0–75)	92	152	48,000	368,000	523	2830	98	75

in the crude extract of *L. albus*. From 1 g of *L. albus,* 214 mg of protein was extracted, in contrast only 115 mg of protein was recovered in the case of *L. angustifolius.* The amount of extracted protein was related to the difference in the total activity of the LOX from *L. albus* and *L. angustifolius.* The total activity of the LOX from *L. albus* was approximately 1.3 times that from *L. angustifolius*. In contrast, *L. angustifolius* showed higher specific activity than *L. albus* (approximately 1.4–1.7 times). Soybean LOX showed 9–12 times greater total LOX activity at both tested pH values (7.5 and 8.0) than did lupin LOX. The specific activity of soybean LOX was also 5–8 times greater than that of lupin LOX. The results have been converted to hydroperoxide (μmol) by using the factor $\varepsilon = 25,000\ M^{-1}\,cm^{-1}$. *L. albus* LOX crude extract produced 2612 μmol of hydroperoxide per minute according to the calculation. *L. angustifolius* LOX and soybean LOX produced 1960 and 24,000 μmol, respectively. Compared to LOX from durum wheat semolina (1570 μmol) (Barone et al., 1999) and olive extract (6800 μmol) (Jaren-Galan et al., 1999), lupin LOX had a similar range of activity to durum wheat semolina but less than half of the activity of olive LOX.

The LOX activity of each fraction separated by ammonium sulphate is shown in Table 2. *L. albus* and *L. angustifolius* showed different tendency for this separation, especially their specific activities. *L. albus* had highest specific activity in the P25–50 fraction at 822 units/mg protein. In contrast, the P0–25 fraction of *L. angustifolius* showed the largest specific activity at 711 units/mg protein. The protein distribution showed similar tendency in both types of lupins. The P50–75 fraction contained the highest percentage of total protein. The molecular weight of each fraction is shown in Table 3. The column (Bio-Sil Sec 250-5) can fit to measure the proteins with molecular weight between 10 and 300 kDa, and it was selected because of the usual LOX molecular weight (70–100 kDa). The molecular weight larger than 300 kDa including thyrogloblin standard is not precise, however, the values from calculation still give us the information of molecular weight distribution. The molecular weights of the P0–25 fractions were mainly in the range more than 1000 kDa

Table 3
Molecular weight of main peaks of crude extract and ammonium sulphate precipitated fractions

Step	Molecular weight (kDa)	
	L. albus	*L. angustifolius*
Crude extract	1800, 170, 18	1700, 570, 670, 18
$(NH_4)_2SO_4$ precipitation		
P0–75	1710, 170, 18	17–1800, 555, 170, 18
P0–25	1400	1800
P25–50	170–200	60–150
P50–75	160	56, 25

Table 2
Lipoxygenase activity of lupin protein fractionated via ammonium sulphate precipitation

Step	Protein (mg)		Total activity (units)		Specific activity (units/mg protein)		Yield (%)	
	L. albus	*L. angustifolius*	*L. albus*	*L. angustifolius*	*L. albus*	*L. angustifolius*	*L. albus*	*L. angusti folius*
Crude extract	214	115	65,300	49,000	305	426	100	100
$(NH_4)_2SO_4$								
0–75%	201	92	62,100	48,000	309	523	95	98
0–25%	21	16	9340	11,400	445	711	14	23
25–50%	33	36	27,100	23700	822	657	42	48
50–75%	141	39	12,400	11200	88	288	19	23

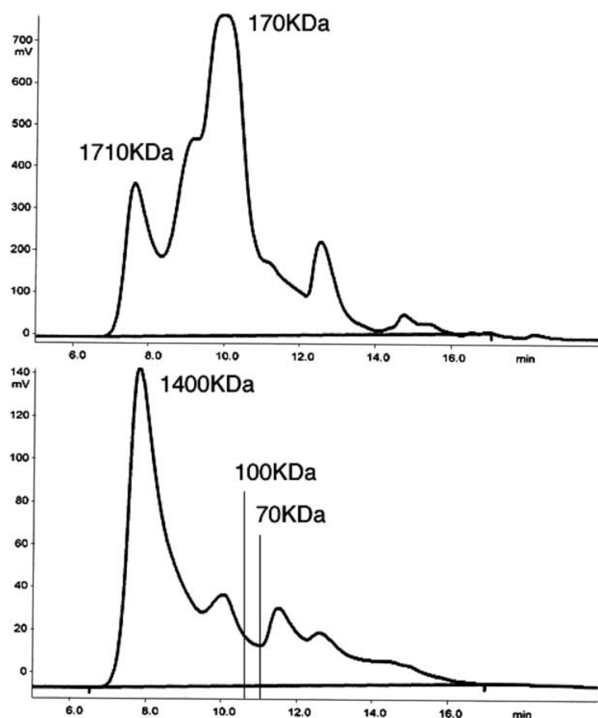

Fig. 3. Chromatograms showing molecular weight analysis of the fractions from *L. albus* precipitated using ammonium sulphate. Top: P0–75, Bottom: P0–25.

(1400–1800 kDa). There was no difference between the molecular weight distributions from *L. albus* and *L. angustifolius*. The P25–50 fraction contained proteins having a broader range of molecular weights (60–200 kDa). The P50–75 fraction contained proteins having a smaller range of molecular weight proteins (16–25 kDa). This table shows that the precipitation was effective in approximately separating LOX depending on the molecular weight. As shown in Fig. 3, the P0–75 fraction had many peaks in the gel permeation chromatography analysis for determining the molecular weight. After precipitation with 25% saturated ammonium sulphate (P0–25), the shape of the chromatogram became simpler. Since many of reports mentioned the molecular weight of highly purified LOX were 70–100 kDa, that area was also pointed out in Fig. 3. Najid et al. (1988) mentioned that purified LOX from *L. albus* had a molecular weight of 71 kDa, and their activity was approximately 10% of total activity of crude extract. From our chromatogram in Fig. 3, the rest of lipoxygenase activity (approximately 90%) still remain in the fraction with wide range of molecular weight. We have estimated there to be a similar molecular weight range for active LOX fractions in other plants (soybean LOX, 65–95 kDa (Sheu & Chen, 1991); tomato LOX, 85–95 kDa (Suurmeijer et al., 1998)), however, the LOX fraction (P0–25) of *L. angustifolius* showed the highest specific activity with higher molecular weight. From this result we conclude that well-purified LOX from *L. angustifolius* may have different characteristics to LOX from *L. albus*. In order to isolate protein from lupins in food processing plants, the differences in protein/LOX distribution between *L. albus* and *L. angustifolius* should be taken into account.

The Michaelis–Menten constant (K_m) and V_{max} values at pH 8.0 for *L. albus* and at pH 7.5 for *L. angustifolius* were calculated using Lineweaver–Burk plots. Respective K_m and V_{max} values for *L. albus* were 4.94×10^{-3} M and 1930 units/mg protein, and those for *L. angustifolius* were 1.22×10^{-3} M and 360 units/mg protein. Well purified LOX from *L. albus* has been reported to have a K_m value of 1.36×10^{-3} M with linoleic acid as the substrate (Najid et al., 1988), and partially purified LOX was reported to have K_m values of 2.3×10^{-3}, 2.2–3.6×10^{-3} and 2.0×10^{-4} M in, broad beans (Clemente et al., 2000), peas (Chen & Whitaker, 1986) and rapeseeds respectively (Khalyfa, Kermasha, & Ali, 1990). Partially purified LOX from olives (Jaren-Galan et al., 1999; Ridolfi et al., 2002) and tomatoes (Suurmeijer et al., 1998) showed smaller K_m values than for beans or other seeds. The lower K_m value was recorded in the LOX extract from *L. angustifolius*, which is about a quarter of the value of LOX from *L. albus*. However, it did not show a higher V_{max} than *L. albus*. This indicates that the LOX from *L. angustifolius* has higher affinity for the substrate (linoleic acid) but that LOX from *L. albus* has higher activity. As mentioned at the beginning of this report, we have a patent (EP 1024 706 B1, 2002) to extract useful protein isolate from lupin. In the next step of lupin LOX research, the changes of LOX activity from protein isolate processing should be analysed.

4. Conclusions

It is clear from our results that LOX from *L. albus* and *L. angustifolius* have different characteristics. The LOX activity in this large-scale study was also different from the activity in a purified LOX study. These data should be useful for lupin protein processing, bearing in mind that there has been an increasing demand for functional plant proteins over the last decade. We are now preparing lupin protein isolate using this information and are evaluating the characteristics and properties of the products for further application in the food industry.

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