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Kinetics of thermal inactivation of pea seed lipoxygenases and the effect of additives on their thermostability

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Abstract

Mature pea seeds contain two major lipoxygenases (LOX) isoenzymes designated LOX-2 and LOX-3. The thermal inactivation of crude pea LOX and the recombinant LOX (rLOX) were studied. Heat-inactivation plots for crude extracts of pea LOX were linear from which thermodynamic activation parameters, $\Delta H^{\#}$, $\Delta S^{\#}$ sand $\Delta G^{\#}$ have been estimated. The enzymatic activity was relatively stable with a respective half-life ($t_{1/2}$) at 60 °C of 54.2 min for LOX from pea (*Pisum sativum* L. cv. Birte) or 18.4 min for a mutant line lacking LOX-2. At 50°C the thermostability of LOX-3 present in crude extracts of the mutant strain ($t_{1/2}=66.8$ min) was 90% greater than purified recombinant LOX-3 (rLOX-3; $t_{1/2}=34.6$ min). However, rLOX-3 was more heat-stable than rLOX-2. Both rLOX-3 and pea mutant line lacking LOX-2 possessed considerable thermostability at 60°C ($t_{1/2}=16.5$ min and 18.4 min, respectively). Even at the higher temperatures of 70°C the $t_{1/2}$ values were 84 and 51, respectively. It is suggested that LOX in crude enzyme extracts was stabilised at 50°C due to protection by other constituents, possibly including starch and proteins. Separate tests at 70°C in the presence of additives (polyols, detergents and small ions) showed that sucrose was the most effective stabiliser and increased the stability of pea LOX by 400–600%.

1. Introduction

The lipid oxidation pathway via sequential action of lipoxygenase (LOX) and hydroperoxide lyase (HPL) plays a significant role in raw materials quality through the generation of the characteristic tastes of a range of fresh and processed foods, including mushrooms, tomatoes, melons, cucumbers, bananas, frozen vegetables and legume seed products. The aromas responsible for the characteristic flavours include short chain aldehydes, such as hexanal and nonenal, that are formed by the action of LOX and HPL on polyunsaturated fatty acids such as linoleic acid (Robinson et al., 1995). LOX, found throughout the plant and animal kingdoms, also have key roles in some metabolic pathways, including the synthesis of prostaglandins and jasmonates (Siedow, 1991). As aids for food processing, the main uses of LOX to-date are the bleaching of white flour (via the co-oxidation of carotenoids) and improvement of the rheological properties of bread dough during baking, probably through co-oxidation of thiol groups in wheat proteins (Frazier et al., 1973; Casey, 1997). LOX is an ideal biocatalyst using just atmospheric oxygen for the conversion of polyunsaturated fatty acids into many potentially useful and valuable chemicals, which may include leukotrienes and lipoxins. It is possible that the plant enzymes will have other uses as biocatalysts for production of aroma compounds when available in larger quantities and at a lower price.

Recently we have characterised a number of products, including the hydroperoxides, hydroxy- and keto-acids, produced by LOX from a pea cultivar and mutant strains, using HPLC and mass spectrometry (Wu, Robinson, Domoney, & Casey, 1995). It is now possible to consider making LOX on a larger scale by expressing the plant cDNA in suitable microbial hosts and using the expressed enzymes as potent new biocatalysts. To this end, it is timely to assess the thermostability of the crude and any available recombinant LOX and also determine how thermostability is affected by simple additives such as salts, sugars, polyols and detergents.

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This paper examines the thermostability of LOX from two pea lines and two recombinant LOX (rLOX-2 and rLOX-3) preparations produced by expressing pea cDNA in *Escherichia coli* (Hughes et al., 1997).

2. Materials and methods

2.1. Reagents

Linoleic acid, sugars (glucose and sucrose), neutral salts (Na_2SO_4, NaCl, $NaNO_3$, NaSCN and NH_4Cl, KCl, $MgCl_2$), polyols (mannitol, sorbitol, glycerol and erythritol) and poly(ethylene glycol) (PEG200, 400, 600, 1000 and 8000) were obtained from Sigma and Tween 20 and 80 from Fluka. All other chemicals were of analytical grade.

2.2. Pea seed material

Dry mature seeds of two pea (*P. sativum*) lines were used for the preparation of LOX-crude extract. The standard line, cv. Birte, contains two major seed LOX polypeptides with predicted amino acid sequences similar to those of LOX-2 and LOX-3 from soybean (Ealing & Casey, 1988, 1989; Domoney et al., 1990). The LOX-2-null line lacks the LOX-2 type polypeptide and has been derived from John Innes germplasm accession JI1006 (North, 1990) by crossing and backcrossing to cv. Birte (unpublished results). rLOX-2 and rLOX-3 were produced as described by Hughes et al. (1997).

2.3. Extraction procedure

Pea seeds were ground to a fine powder in a coffee grinder, suspended in 50 mM sodium phosphate buffer (pH 6.25; 1/10 w/v) and stirred gently for 3 h at 4°C. The homogenate was centrifuged (17 000 rpm or ~25 000 ***g***, 45 min) at 4°C and the supernatant stored at approximately −204°C as a crude LOX extract.

2.4. Measurement of LOX activity

Two LOX assays were used. The first, involving the use of 1% w/w Tween-20 to disperse the enzyme substrate, was used for assaying crude LOX extracts and rLOX-3. Linoleic acid (0.3mM) was dispersed by homogenization (approximately 4 min, 8000 rpm, Ultra-turrax T25) in sodium phosphate buffer (50 mM, pH 6.25) containing Tween 20 (1.0 % w/v). Lipoxygenase activity was determined spectrophotometrically by adding 50 µl of a 25–75-fold diluted crude enzyme extract to 0.95 ml sodium phosphate buffer (50 mM, pH 6.25) and 2 ml substrate solution. The samples were incubated for 15 min at 25°C. Hydrochloric acid (0.2 N, 0.5 ml) was added to stop the enzyme reaction. The formation of a conjugated diene as a result of LOX oxidation of linoleic acid was determined from A_{234} readings recorded using a double beam UV spectrophotometer. The reference-blank contained all reaction components except that LOX was added after the addition of HCl to the reaction system.

rLOX-2 appeared to be sensitive to high concentrations of Tween 20. Consequently, rLOX-2 was assayed according to the procedure described by Chen and Whitaker (1986). The concentration of Tween 20 employed for this assay is 0.0785% (v/v). The assay medium was phosphate buffer (50 mM, pH 6.25) as described before for crude LOX or rLOX-3. The reaction was stopped by adding 0.5 ml Tris-HCl buffer (1.5 M, pH 8.0). This also had the effect of clarifying the substrate suspension before A_{234} readings were taken.

Samples of rLOX-3 were normally stored as ~10mg ml^{-1} suspensions in saturated ammonium sulphate. 50 µl of enzyme sample was centrifuged and the pellet resuspended in a final volume of 10 ml of phosphate buffer before assaying as above. Under the conditions of this study, A_{234} values increased linearly with time over the first 30 min of reaction. Samples of rLOX-2 were also prepared as described for rLOX-3. However, the pellet of rLOX-2 was resuspended in 1 ml of phosphate buffer.

The kinetics of LOX heat inactivation were measured at pH 6.25 in 50 mM sodium phosphate buffer by incubating the enzyme at a range of temperatures. To ensure rapid heating, concentrated enzyme samples (~0.2 ml) were added to 10–15 ml of preheated buffer. The residual enzymic activity was measured at 25°C.

The rate constant (k) for heat inactivation was estimated from a plot of log percent residual activity vs time. To determine the activation energy ($\Delta E^{\#}$) for thermal inactivation, an Arrhenius plot was constructed, and the line slope and intercept determined by linear regression analysis (Owusu & Berthalon, 1993). Transition state parameters such as the activation enthalpy ($\Delta H^{\#}$) were determined according to the equation

$$\Delta H^{\#} = \Delta E^{\#} - RT \quad (1)$$

where R is the universal gas constant and T is the absolute temperature. The activation free energy ($\Delta G^{\#}$) was determined using the relation

$$\Delta G^{\#} = -RT\ln(kh/KT) \quad (2)$$

where $h(= 6.6262 \times 10^{-34} Js)$ is the Planck constant, and $K(= 1.3806 \times 10^{-23} JK^{-1})$ is the Boltzmann constant. From Eqs. (1) and (2) the activation entropy ($\Delta S^{\#}$) for LOX heat inactivation was calculated from Eq. (3).

$$\Delta S^{\#} = (\Delta H^{\#} - \Delta G^{\#})/T \quad (3)$$

A procedure for obtaining activation parameters from a non-linear Arrhenius plot is described below. Where crude enzymes samples were studied, then activation parameters should be viewed as apparent values.

2.5. Effect of additives

To examine the effect of additives on the heat stability of LOX, crude enzyme extracts from pea cv. Birte and the LOX-2 null line were heated at 70°C, for 8 min and 2 min, respectively, in sodium phosphate buffer (50mM, pH 6.25) containing known quantities of either sugars, salts, polyols or Tweens. The heating times employed were 2.5 × greater than the half-life of each enzyme at 70°C. Heating was terminated by rapid cooling in ice. Residual enzyme activity was measured at 25°C as described above.

3. Results and discussion

The thermal stabilities of crude LOX from mature seeds of (wild-type) pea (*Pisum sativum* L. cv. Birte), LOX from a mutant pea variety lacking LOX-2 (LOX-2 null pea line) and purified recombinant LOX-2 (rLOX-2) and LOX-3 (rLOX-3) were investigated at 45, 50, 55, 60, 65 and 70°C.

The number of LOX isoenzymes found in *P. sativum* varies with the cultivar, plant organ and degree of maturation. Chen and Whitaker (1986) extracted three LOX isoenzymes from immature pea seeds, as used in the frozen food industry. The two main LOX isoenzymes from immature pea seeds were classed as type 2 enzymes (pI 5.8 and 5.6 by chromatofocusing) according to their neutral pH-activity optima and relatively low thermostability when compared with soybean LOX. Studies of mature Birte pea seeds showed two main LOX polypeptides with apparent molecular weights of 97 kDa and 90 kDa (). These are type-2 enzymes (LOX-2 and LOX-3) judging from their high activity at pH 4-7. In contrast, the LOX-2 null line contains mainly LOX-3 (Wu et al., 1995).

Figs. 1–3, show semi-logarithm graphs of enzyme residual activity vs heating time (t). In all cases the graphs were linear with a correlation coefficient (R^2) value of 0.91–0.99. The straight-lines graphs in Figs. 1–3 show that mature pea seed LOX is heat-inactivated in accordance with simple 1st order kinetics. Values for the enzyme half-life ($t_{1/2}$) in minutes were calculated from;

$$t_{1/2} = \ln(2)\, k\ (\mathrm{min}^{-1} v) \quad (4)$$

A summary of $t_{1/2}$ values for the four enzymes is given in Table 1.

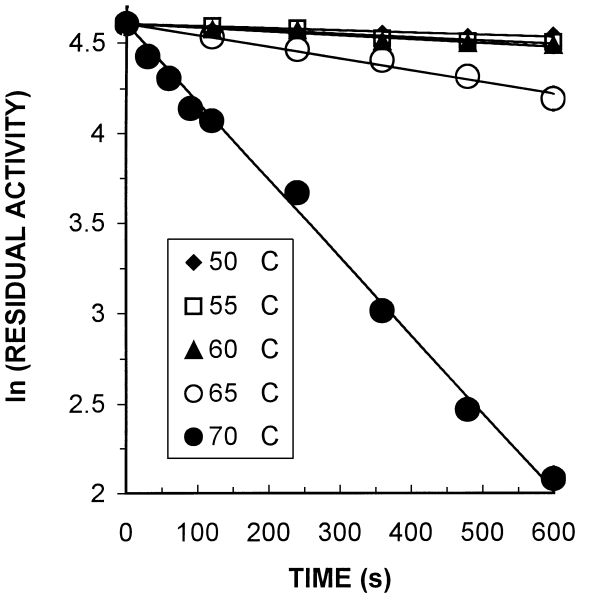

Fig. 1. Heat inactivation of lipoxygenase from pea (*P. sativum* L. cv. Birte). A semi-log. graph of ln (residual activity) vs time. Heating medium is phosphate buffer (50 mM, pH 6.25).

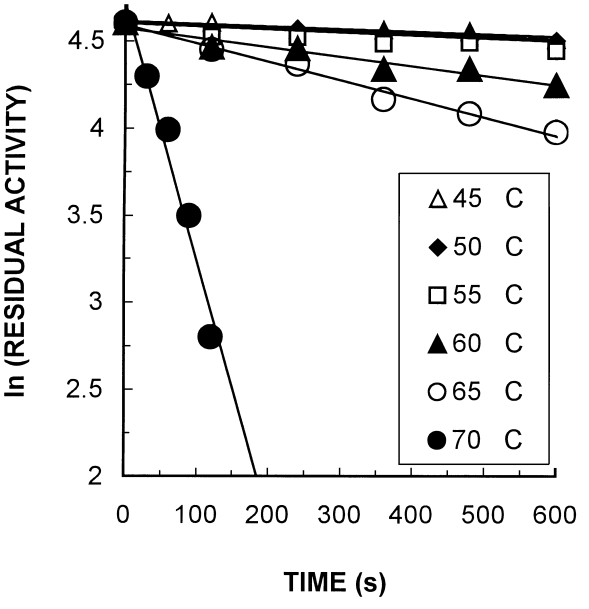

Fig. 2. Heat inactivation of lipoxygenase from a LOX-2 null mutant pea variety . A semi-log. graph of ln (residual activity) versus time. Heating medium is phosphate buffer (50 mM, pH 6.25).

From the linear graphs in Fig. 1 the crude LOX sample from the pea cv. Birte responded as if it contained only one LOX isoenzyme. However, a straight line 1st order plot (Fig. 1) is consistent with two conditions: (a) crude Birte Pea extract has a single LOX isoenzyme (known not to be true), or (b) Birte Pea extract has more than one LOX isoenzyme and these have broadly similar inactivation characteristics in the crude enzyme extract at 50–70°C. Any differences in LOX isoenzyme stability extract was apparently not sufficient to be

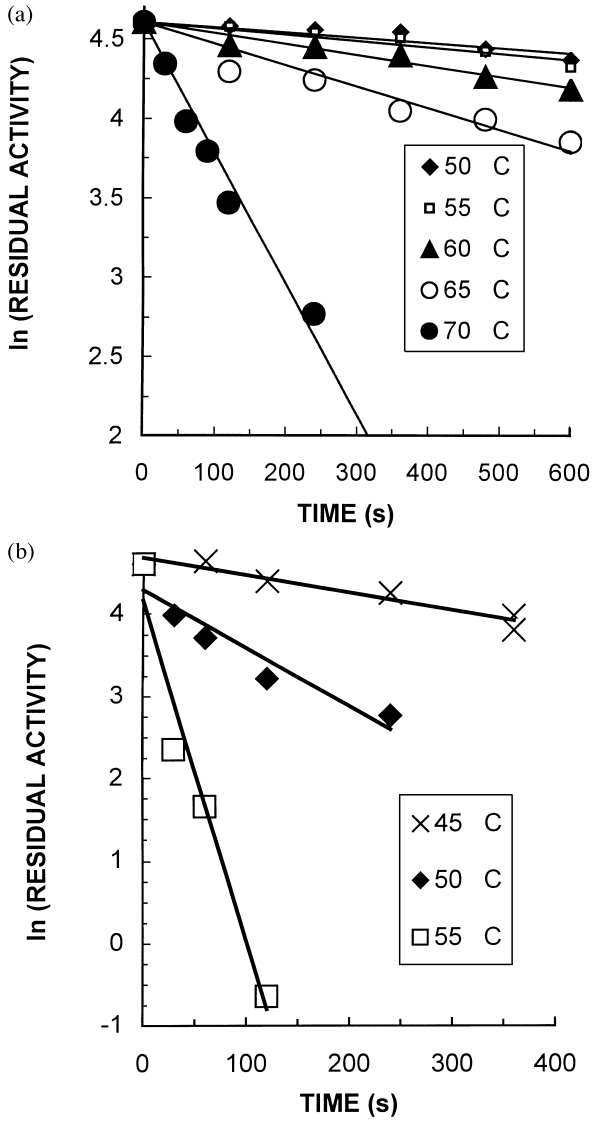

Fig. 3. Heat inactivation of purified pea recombinant lipoxygenase (rLOX) from *E. coli*. A semi-log. graph of ln (residual activity) vs time. A and B show results for rLOX-3 and rLOX-2, respectively. Heating medium is phosphate buffer (50 mM, pH 6.25).

resolved by the 1st order plot. Heat inactivation of crude wheatgerm LOX at 60°–68°C produced a biphasic 1st order plot due to the presence of two LOX isoenzymes with an order of magnitude difference in their heat stability (Bhirud & Sosuslki, 1993). Figs. 2 and 3A,B show linear graphs such as expected in the presence of a single LOX isoenzyme, i.e. LOX-3, rLOX-3 or rLOX-2, respectively.

Table 1 summarises $t_{1/2}$ values for pea LOX at 45–70°C. It is assumed that $t_{1/2}$ values are additive and therefore values from pea cv. Birte show combined $t_{1/2}$ values for LOX-2 and LOX-3 isoenzymes. By comparing $t_{1/2}$ values from cv. Birte with those from the LOX-2 null pea mutant, we may derive separate $t_{1/2}$ estimates for LOX-2 and LOX-3 in the crude Birte pea enzyme extract. At 50°C such calculations lead to respective $t_{1/2}$

Table 1
Thermal inactivation half lives for pea lipoxygenase and the recombinant enzymes (r-LOX)

Enzyme	Half life (min)					
	45°C	50°C	55°C	60°C	65°C	70°C
cv. Birte	–	98.0	63.4	54.2	18.1	2.7
cv. LOX-2 null	75.1	66.8	35.0	18.4	10.5	0.85
rLOX-3	–	34.6	28.7	16.6	8.5	1.4
rLOX-2	4.1	1.2	~0.3	–	–	–

estimates of 67 and 31 min for LOX-3 and LOX-2 in the crude enzyme extract from pea cv. Birte. Therefore, crude LOX-3 was about twice as heat-stable as LOX-2 at 50°C. On the other hand, $t_{1/2}$ calculations at 60–70°C suggest that crude LOX-2 was more heat-stable than crude LOX-3. At 70°C the relative heat stabilities of crude LOX-2 and LOX-3 were not that different. As crude enzyme samples are being considered, then differences in stability will only partially reflect intrinsic enzyme properties. Interaction with other constituents present within the crude samples may contribute to the observed statbilities.

At 50–55°C purified rLOX-3 was between 35 and 95 times more heat resistant than rLOX-2 (Table 1). This compares with a 2-fold difference in the heat stabilities of *crude* LOX-2 and LOX-3 (above). Such results suggest that, removal of extraneous material, from crude enzyme samples during purification, destabilises LOX-2 to a greater degree than LOX-3 (see below).

The thermostability of crude LOX-3 at 50°C (i.e. LOX activity in the LOX-2 null line) was about twice that observed with purified rLOX-3 (Table 1). Svensson and Eriksson (1974) also showed that the thermostability of crude pea LOX is greater than the stability of the purified enzyme at the same pH. The presence of starch and proteins may stabilise LOX activity in crude enzyme extracts. Such extraneous materials may become ''denatured'' at higher temperatures leading to a loss of the LOX protective action. At an elevated temperature (70°C), the stability of purified rLOX-3 was greater than the heat stability of crude LOX-3 (present in the LOX-2 null line; Table 1). At 65°C both the rLOX-3 and the (LOX-2 null line) pea LOX-3 extract possessed considerable thermostabilities with $t_{1/2}$ values of 510 and 630 sec, respectively.

3.1. The temperature dependence of pea LOX inactivation

There is surprisingly little information published concerning transition state parameters for pea LOX heat inactivation. Estimates ΔE, ΔH, ΔS and ΔG for pea LOX heat inactivation were reported by Svensson and Eriksson (1972, 1974).Fig. 4 shows Arrhenius plots for pea LOX heat-inactivated at 45–70°C. Clearly, all the graphs are curvilinear. At temperatures of >65°C, the

Table 2
Thermal inactivation parameters for pea lipoxygenase and the recombinant enzymes (r-LOX)

Enzyme	Temperature (°C)	$\Delta E^{\#}$ (kJ mol^{-1})	$\Delta H^{\#}$ (J mol^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})
cv. Birte[a]	50–60	53.0	50.2	104–106	−166.0
	65–75	456	457	104–93.5	1043
cv. LOX-2 null[a]	45–60	89.6	86.9	102–103	−48.0
	65–70	485	482	103–96.9	1122
rLOX-3[a]	50–60	65.5	62.8	101–102	−119
	65–70	348	345	102–99.8	717
rLOX-2	45–55	233	230	89.3–93.6	992

[a] Two-phase Arrhenius plots (see Fig. 4) assuming parameters are independent of temperature.

positive $\Delta H^{\#}$ and $\Delta S^{\#}$ values (Table 2) are in general agreement with values expected for enzyme heat-inactivation (Adams, 1991). However, both parameters were unusually low and sometimes negative at 50–60°C suggesting that the simple Arrhenius equation

$$\ln k = \Delta E^{\#}/RT + C \quad (5)$$

may be inapplicable for pea LOX. In Eq. (5) C is a constant. Eq. (5) is applicable for reactions where $\Delta E^{\#}$, $\Delta H^{\#}$, $\Delta S^{\#}$ and $\Delta G^{\#}$ are independent of temperature. The effect of temperature on the preceding thermodynamic parameters can be reconciled by referring to the activation heat capacity change, $\Delta Cp^{\#}$. Eq. (5) is applicable for systems where $\Delta Cp^{\#}$ is zero. Results (Fig. 4) were fitted to a polynomial Arrhenius equation

$$\ln k = Ax^2 + Bx + C \quad (6a)$$

and the constants A, B and C determined by nonlinear regression. In Eq. (6a) $x = 1/T$. The slope from Eq. (6a) yields a value for $\Delta E^{\#}/R$ at any specified temperature, for example

$$d(\ln k)/dx = \Delta E^{\#}/R = 2Ax + B \quad (6b)$$

(Apenten, 1995). Values for $\Delta E^{\#}$ were determined at 68°C to enable comparison with literature data by substituting $x = 1/(68 + 273)$ into Eq. (6b). $\Delta Cp^{\#}$ was obtained from the definition, $\Delta Cp^{\#} = d(\Delta H^{\#})/dT$. Combining (1) and (6b):

$$\Delta H^{\#} = 2ART^{-1} + RB + RT \quad (6c)$$

$$\Delta Cp^{\#} = 2ART^{-2} + R \quad (6d)$$

Values for $\Delta H^{\#}$, $\Delta S^{\#}$, $\Delta G^{\#}$ and $\Delta Cp^{\#}$ were calculated at 68°C using Eqs. (1)–(3) and (6). A summary of results is given in Table 3. From here it can be seen that

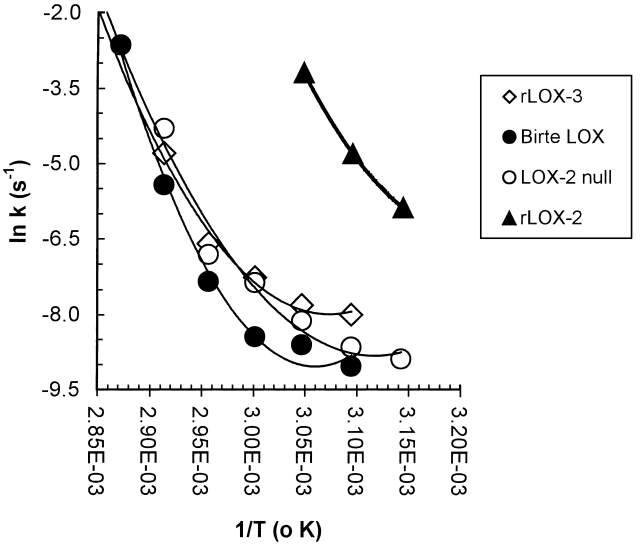

Fig. 4. Arrhenius plot for the heat inactivation of pea (cv. Birte and LOX-2 null) lipoxygenase and recombinant lipoxygenases (rLOX-3 and rLOX-2) . Continuous lines show results calculated according to Eq. (6). A, B and C are constants reported in Table 3.

Table 3
Thermal inactivation parameters for pea lipoxygenase and the recombinant enzymes (r-LOX) at 68°C

Parameter[a]	cv. Birte	cv. LOX-2 null	rLOX-3	rLOX-2
A	1.77×10^{8}	1.02×10^{8}	1.21×10^{8}	1.27×10^{8}
B	-1.08×10^{6}	-0.635×10^{6}	-0.744×10^{6}	-0.811×10^{6}
C	1645	980	1136	1294
$\Delta H^{\#}$ (kJ mol^{-1})	375	313	284	578
$\Delta S^{\#}$ (J mol^{-1} K^{-1})	801	629	541	1476
$\Delta G^{\#}$ (kJ mol^{-1})	102	99.2	99.7	75.0
$\Delta Cp^{\#}$ (kJ mol^{-1} K^{-1})	25.2	14.5	17.3	18.1

[a] A, B and C are constants in Eq. (6)—see text for details.

$\Delta Cp^{\#} \neq 0$ for LOX heat-inactivation, thereby justifying the use of Eq. (6a) in place of Eq. (5).

The $\Delta G^{\#}$ value for Birte pea crude LOX inactivation agrees with a figure of 100.68 kJ mol^{-1} reported by Svensson and Eriksson (1972, 1974) for the heat inactivation of purified pea LOX in phosphate buffer (0.1M, pH 6) at 68°C. The $\Delta G^{\#}$ value for the LOX-2 null system and rLOX-3 show these to be less stable than pea LOX studied by Svensson and Eriksson (1972, 1974). Except for rLOX-2, $\Delta H^{\#}$ and $\Delta S^{\#}$ estimates obtained in this study were noticeably lower than values of 581.6 and 1401.8 kJ mol^{-1} K^{-1} reported formerly for purified pea

LOX (Svensson & Eriksson, 1972, 1974). Values for $\Delta H^{\#}$ and $\Delta S^{\#}$ are extremely sensitive to solvent conditions, e.g. pH and ionic strength. Moreover, $\Delta H^{\#}$ and $\Delta S^{\#}$ changes may occur, due to entropy-enthalpy compensation, without a net change in enzyme heat stability and $\Delta G^{\#}$. Unlike the literature study cited, the current study involved crude samples as described above.

At moderate temperatures, the rate-limiting step for the irreversible heat-inactivation of enzymes is the formation of an unfolded enzyme (**U**) state. Describing irreversible inactivation as a two-stage reaction (Ahern & Klibanov, 1985, 1988; Owusu & Berthalon, 1993);

$$\mathbf{N} \Longleftrightarrow \mathbf{U} \rightarrow \mathbf{I} (\textit{and } \mathbf{N} \rightarrow \mathbf{I} \textit{ overall}) \quad (7)$$

N is the native conformation for LOX, **U** is the heat-unfolded enzyme and **I** is the irreversibly inactivated LOX. Results in Table 3 show thermodynamic parameters associated with the formation of a transition state (**Tn***) according to Eq. (8):

$$\mathbf{N} \leftrightarrow \mathbf{Tn}^{*} \leftrightarrow \mathbf{U} \quad (8)$$

Thus, $\Delta H^{\#}$ and $\Delta S^{\#}$ are, respectively, the heat and entropy change for the **N** → **Tn*** reaction. The two parameters provide a measure of the number of non-covalent bonds broken and the net enzyme/solvent disorder change associated with the **N** → **Tn*** transition, respectively. Large values for $\Delta H^{\#}$ are associated with increased enzyme stability unless coupled with large compensatory increase in the value of $\Delta S^{\#}$ which is destabilising. Finally, the magnitude of $\Delta Cp^{\#}$ is largely related to apparent changes in protein nonpolar surface area brought into solvent contact via the **N** → **Tn*** transition. Increasing values for $\Delta Cp^{\#}$ lead to curvature in the Arrhenius plots shown in Fig. 4.

3.2. The effect of additives on pea LOX

The effect of sugars and polyhydroxy compounds (polyols) on the stability of LOX preparations from pea cv. Birte pea and LOX-2 null is summarised in Table 4. The results are shown in terms of the stabilisation factor (*SF*), where *SF* is the enzyme residual activity (+ additive) divided by residual activity (with no additive). An additive with a stabilising action is one for which $SF > 1$. For an additive with a destabilising effect on LOX, $SF < 1$. When $SF = 1$, an additive is considered to have no effect on LOX thermostability.

For a substantial number of polyols examined, sucrose had the most stabilising effect. Thus 1–2 M sucrose increased pea LOX stability at 70°C by 400–600%. Other sugars (mannitol, sorbitol, erythritol and glycerol), were less efficient stabilisers or had no effect. The stabilisation effect produced by sugars has been related to the number of OH-groups contained per molecule of sugar. On the basis of such an hypothesis, the order of stabilisation produced by various sugars is expected to be sucrose > glucose, mannitol > sorbitol > erythritol > glycerol. Glucose had an unexpectedly destabilising effect on LOX considering the number of hydroxy groups. However, glucose was the only reducing sugar tested. The stabilising effect of sucrose can be explained in terms of exclusion of sucrose from the immediate surroundings of LOX and greater cohesive force of the sucrose-water solvent system (Lee & Timasheff, 1981; Timasheff & Arakawa, 1989).

Table 4
The effect of additives (polyols) on pea lipoxygenase heat stability

Additive (concentration)	Name	cv. Birte[a] *SF*[b]	cv. LOX-2 null[a] *SF*[b]
	Control	1	1
Sugars (1 M)	Sucrose (2 M)	5.4	6.2
	Sucrose	4.0	5.2
	Mannitol	2.0	2.3
	Sorbitol	1.8	1.7
	Glycerol	1.3	1.2
	Erythritol	1.2	1.2
	Glucose	0.7	0.9
	Glucose (2 M)	0.8	0.5
PEGS (5% w/v)	PEG 200	0.9	0.8
	PEG 400	0.8	0.7
	PEG 600	0.8	0.6
	PEG 1000	0.4	0.4
	PEG 8000	0.9	0.5
Detergents (1% w/v)	Tween 80	0.9	0.5
	Tween 20	0.4	0.2

[a] LOX from cv. Birte and LOX-2 null were heated at 70°C for 8 and 2 min respectively (see text for explanation). Heating medium was phosphate buffer (50 mM, pH 6.25).

[b] Stabilisation factor (*SF*) is residual activity (+ additive) divided by residual activity (+ no additive). $SF > 1$ or $SF < 1$ for stabilising and destabilising additives, respectively.

Polyethyleneglycols (PEGs) and the non-ionic surfactants (Tween 20 and Tween 80) were found to be destabilising at levels of 1–2%. It is important to distinguish the effect of destabilising additives from possible inhibitory action. To avoid inhibition effects, *SF* values were calculated from residual activity measurements. For each additive, activities of LOX before and after heating in the presence of an additive were compared so that any possible inhibitory effects were normalised.

The effect of salts on LOX was examined by heating the enzymes in the presence of a series of anions (Y^{-}) in the form of the sodium salts. A series of cations (X^{n+}) in the form of the chlorides were also examined. A summary of *SF* values achieved with various ions is presented in Table 5. In general, the effect of various ions on the stability of LOX varies in accordance with their position in the Hoffmeister series (Timasheff &

Table 5
The effect of additives (salts) on pea lipoxygenase heat stability[a]

Additive type	Name	cv. Birte *SF*[b]	cv. LOX-2 null *SF*[b]
Cations	NH_4^+	1.6	1.4
	K^+	1.3	1.5
	Na^+	0.9	0.8
	Mg^{2+}	0.6	0.4
Anions	SO_4^{2-}	1.8	1.7
	Cl^-	0.9	0.8
	NO_3^-	0.7	0.6
	SCN^-	0.7	0.6
	Acetate	1.7	1.4

[a] Cations and anions were used as chloride or sodium salts, respectively. All additives were tested at 1 M concentration.
[b] Stabilisation factor (*SF*) is residual activity (+ additive) divided by residual activity (+ no additive).

Arakawa, 1989). The order of stabilisation observed for cations was NH_4^+, > K^+ > Na^+ > Mg^{2+}. The order of stabilisation for cations was essentially the same for LOXs associated with pea cv. Birte and the LOX-2 null line.

In conclusion, the stabilities of pea lipoxygenases appear to differ, depending on the type of enzyme and the presence of natural "additives" (associated with crude enzyme extracts) or artificial additives. The apparently greater thermostability of LOXs from pea cv. Birte and the LOX-2 null line could be a consequence of studying these enzymes as crude extracts. Overall, our preliminary results suggest that rLOX-3 may be produced without serious loss of enzyme heat stability as compared with enzymes from wild-type pea. The heat stability of purified rLOX-2 was lower than expected.

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