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The thermostability of purified isoperoxidases from *Brassica oleracea* VAR. *gemmifera*

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Abstract

The thermostabilities of four previously purified isoperoxidases from Brussels sprouts (*Brassica oleracea* VAR. *gemmifera*.)have been determined. The heating time periods selected (10 s – 0.5 min intervals) are comparable to those used during commercial blanching. Non-linear regression (NLR) equation fitting, using common goodness of fit criteria (low chi-squared value, high regression coefficient and low residuals) points to a mechanism of peroxidase heat inactivation involving two consecutive reactions during the initial periods of heating. In the consecutive model, native peroxidase (E_0) is converted into a partially active from (E_1) and then into the inactivated enzyme (E_2) during short periods of heating. The order of calculated decimal reduction times for the two anionic (A1 and A2) and two cationic (C1 and C2) Brussels sprouts isoperoxidases was $A1 \geqslant C1 > A2 > C2$. Calculated concentration changes for E_0, E_1 and E_2 during heat inactivation were quite different for the four isoperoxidase preparations and indicated the generation of more stable forms of partially denatured peroxidases. The anionic isoenzymes showed greater regeneration of enzymatic activity after heat treatment and this could have been due to their greater ability to regain previously liberated haem.

1. Introduction

Peroxidase (EC 1.11.1.7) is a haem-containing enzyme whose primary function is to oxidise molecules at the expense of hydrogen peroxide. Peroxidases have a broad substrate specificity towards different H-donors, including phenols, aromatic amines (Burnette, 1977), anthocyanins, lignin and vitamin C. The enzyme's involvement in such a range of reactions has led to peroxidase being implicated in several development and postharvest changes in plant tissues (Haard, 1977); but this has also made its physiological role in living plants and its deleterious action on food quality difficult to assign precisely. A relationship with off-flavour in peas was claimed by Wagenknecht & Lee (1958) and Pinsent (1962). Wagenknecht & Lee (1958) also found that off-flavours developed when preparations of horseradish peroxidases were added to pea slurries. Peroxidases have also been implicated in loss of colour and changes in texture, e.g. toughening of asparagus tips due to lignification (Haard, 1977). Bruemmer et al. (1976) found a negative correlation between peroxidase activity and flavour scores for orange juices.

Peroxidase activity has been shown to arise from the presence of quite a large numbers of isoenzymes varying in substrate specificity, heat stability, molecular weight, isoelectric point and immunological properties (Lee et al., 1984; Vamos-Vigyazo, 1981; Robinson 1991a,b). However, unlike many other plant isoenzymes, the isoelectric points (pI values) for isoperoxidases traverse a wider range of pH values, generally from pH 4 to 10 as typified by Brassica species (McLellan & Robinson, 1987a; Moulding et al., 1987; 1988, 1989).

The use of peroxidase activity as an indicator of blanching for vegetables and fruits is sometimes questioned and reappraised in view of the high temperatures required to inactivate peroxidases. Nevertheless, Halpin & Lee (1987) and Halpin et al. (1989) have restated that other less stable enzymes, such as polyphenol oxidases, lipoxygenases and catalases, which are denatured at lower temperatures, are therefore not appropriate indicators of blanching and that peroxidases will continue to serve this requirement.

Blanching is the common term used to describe heat-inactivation of enzymes naturally present in the vegetables and always involves short time heating for periods

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of 2–3 min at temperatures between 70°C and 100°C. However, for such short time periods the relationship between blanching time at selected temperatures and residual peroxidase activity shows non-linear kinetics (Yamamoto et al., 1962; Adams, 1978a, b, 1997; Ling & Lund, 1978; McLellan & Robinson, 1981, 1987a; Moulding et al., 1987–1989; Robinson et al., 1989; Khan & Robinson, 1993). Typical, for many of the short time-heat treatments, are non-linear curves consisting of approximately three phases: first an initial steep nearly straight line for a relatively short period, an intermediate curved portion and finally a shallow almost straight line for the third phase. The rate constants for heat-inactivation of peroxidase activity decrease with heating time for many fruits and vegetables and apparent peroxidase activity, measured commonly with synthetic test substrates, can be detected after very long heating periods (the third phase). Several mechanisms have been proposed to explain the observed deviation from linear first order kinetics. Frequently the presence of a mixture of thermostable and heat-labile isoperoxidases has been suggested to account for the non-linear heat inactivation plots observed in crude samples. In many instances it is highly probable that mixtures of heat-resistant and heat-labile isoenzymes were present and responsible for the deviation from first order kinetics as described typically by Ling & Lund (1978). Alternatively, it has been suggested that the heat-inactivation process may involve the formation of thermostable aggregates (Winter, 1971; Lopez et al., 1994) or the process may follow series type inactivation kinetics with the formation of partially inactivated intermediates (Henley & Sadana, 1985). As an enzyme is a complex molecule with a three dimensional structure, heat-inactivation may well not follow first order kinetics. For peroxidases, which also contain a haem substituent, it seems even more likely that different types of intrinsic conformational and chemical changes will continually take place during the early stages of heat-inactivation. Tamura & Morita (1975) first suggested the loss of haem to form an apo-peroxidase. Adams has shown that haem fragments were formed during inactivation of a commercial preparation of horseradish peroxidase and that non-linear kinetics of heat-inactivation of horseradish peroxidase may be due to recovery and regeneration of peroxidase activity at acidic pH values (Adams, 1997).

In some instances the measurements of enzymatic activity have been made during long heat treatments up to 2,3, or 5 h (Saraiva et al., 1996; Adams et al., 1996) and not the first few seconds, nor indeed the first minute of heat-denaturation where the greatest loss of enzymatic activity has been observed (Yamamoto et al., 1962; Ling & Lund, 1978; Powers et al., 1984; McLellan & Robinson, 1987b; Robinson et al., 1989; Khan & Robinson, 1993).

Recently we have described procedures for purifying and assessing homogeneity using Western blotting of preparations of anionic and cationic types of Brussels sprout isoperoxidases. For each of the highly purified preparations referred to above that possess very different pI values and molecular weights (Forsyth & Robinson, 1998), the present investigation compares heat-inactivation by non-linear analysis (NLR). Equation fitting to consecutive reactions during the initial rates of heat inactivation, near to zero time, has been obtained by carrying out measurements of enzyme activity of 10 s and then 0.5 min intervals. Accepted goodness of fit criteria for NLR such as, a low chi-squared value, a high regression coefficient and an even distribution of residuals have been applied. The time periods selected are comparable to those used during commercial blanching. The ability to regenerate enzymatic activity for each type of peroxidase preparation has also been determined.

2. Materials and methods

Four preparations of isoperoxidases were purified as described and designated by Forsyth & Robinson (1998). Origin'™ (MicroCal Ltd) was supplied by Clecom Software Specialist, Edgbaston, Birmingham, UK.

2.1. Heat inactivation

Heat inactivation of the purified isoenzymes was carried out in triplicate at 60°C, 65°C, 70°C and 75°C following the method described by McLellan & Robinson (1981). The heat treatment was carried out in glass microtubes (100 mm long and 1 mm inside diameter), to permit rapid heat transfer, and filling was carefully exercised using a microsyringe before rapid heat-sealing at both ends. Each microtube contained about 70 μ1 of enzyme solution. Residual peroxidase activity was determined at 10 s intervals for the first 60 s followed by measurements at 30 s intervals. The *o*-dianisidine assay was used to measure the peroxidase activity. The activity of each isoperoxidase preparation was recorded and used to calculate the percentage peroxidase activity remaining after heat treatment.

2.2. Regeneration of peroxidase activity following heat-inactivation

Samples of the purified isoenzymes were heat treated for 2 min at 60°C. To monitor regeneration the heat-treated samples were placed at 30°C and aliquots were taken for assays of peroxidase activity using *o*-dianisidine during a subsequent 2.5 h period. Initial values of peroxidase activity prior to heating were used in order to

calculate regained enzymatic activity as a percentage of original activity.

3. Results and discussion

Non-linear 1st order inactivation kinetics have been observed previously for partially purified Brussels sprouts peroxidases and for a substantial number of other plant peroxidases. However, the magnitude of non-linearity observed has frequently been greater and more obvious during the very early stages (<1 min) of the heat-inactivation process as referred to above.

Fig. 1(a–d) show typical 1st order plots for the heat-inactivation of purified Brussels sprouts peroxidase isoenzymes (A1, A2, C1 and C2) at 60–75°C. The graphs were all non-linear as previously reported by McLellan & Robinson (1981) for crude peroxidase from Brussels sprouts. Examination of simple 1st order, *n*th order as well as a two-exponential function kinetics (Amiza et al., 1997) by non-linear regression (NLR) analysis using Origin™ showed that the non-linear 1st order Eq. (1) gave the best fit (Fig. 2(a–c) for describing the initial heat-inactivation process from almost 10 s to approximately 3 min heating of isoenzymes A1, A2, C1, and C2.

$$a = A_0 + B_1 \exp.(-k_1 t) - B_2 \exp.(-k_2 t), \quad (1)$$

where k_1 and k_2 are inactivation rate constants, a = residual enzyme activity after a heating time, t. The parameters B_1, B_2 and A_0 are constants. Commonly accepted goodness of fit criteria for NLR such as a low chi-squared value and a high regression coefficient were applied. The residuals (i.e., the difference between and calculated and experimental points) are shown in the three graphs. The best kinetic model function (Eq. (1)) led to the most even distribution of residuals (Eq. (2c)). Similar graphical results were obtained for all isoenzymes and heating temperatures examined. Values for A_0, B_1, B_2, k_1 and k_2 are given in Table 1.

Ling & Lund (1978) were first to suggest that peroxidase heat inactivation could be discussed using the two-exponential function, but in their case this was for an unpurified extract, where it can easily be assumed that the shape of the curve was due to at least two distinct isoperoxidases with different heat stabilities. A mixed extract of two isoperoxidases (E_0 and $E_0^{'}$) would inactivate by distinct (parallel) mechanisms:-

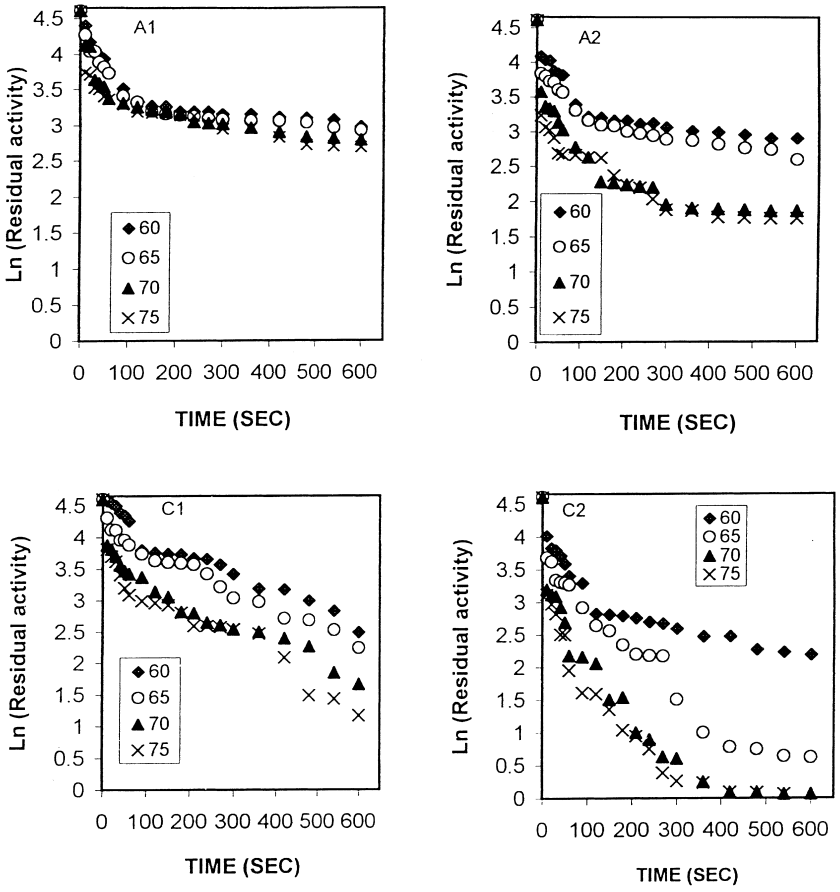

Fig. 1. Non-linear 1st order semi-log. graphs for Brussels sprouts isoperoxidase heat-inactivation at 60–75°C. Results for the purified isoperoxidase A1, A2, C1 and C2 are shown in (a)–(d) (see text for details).

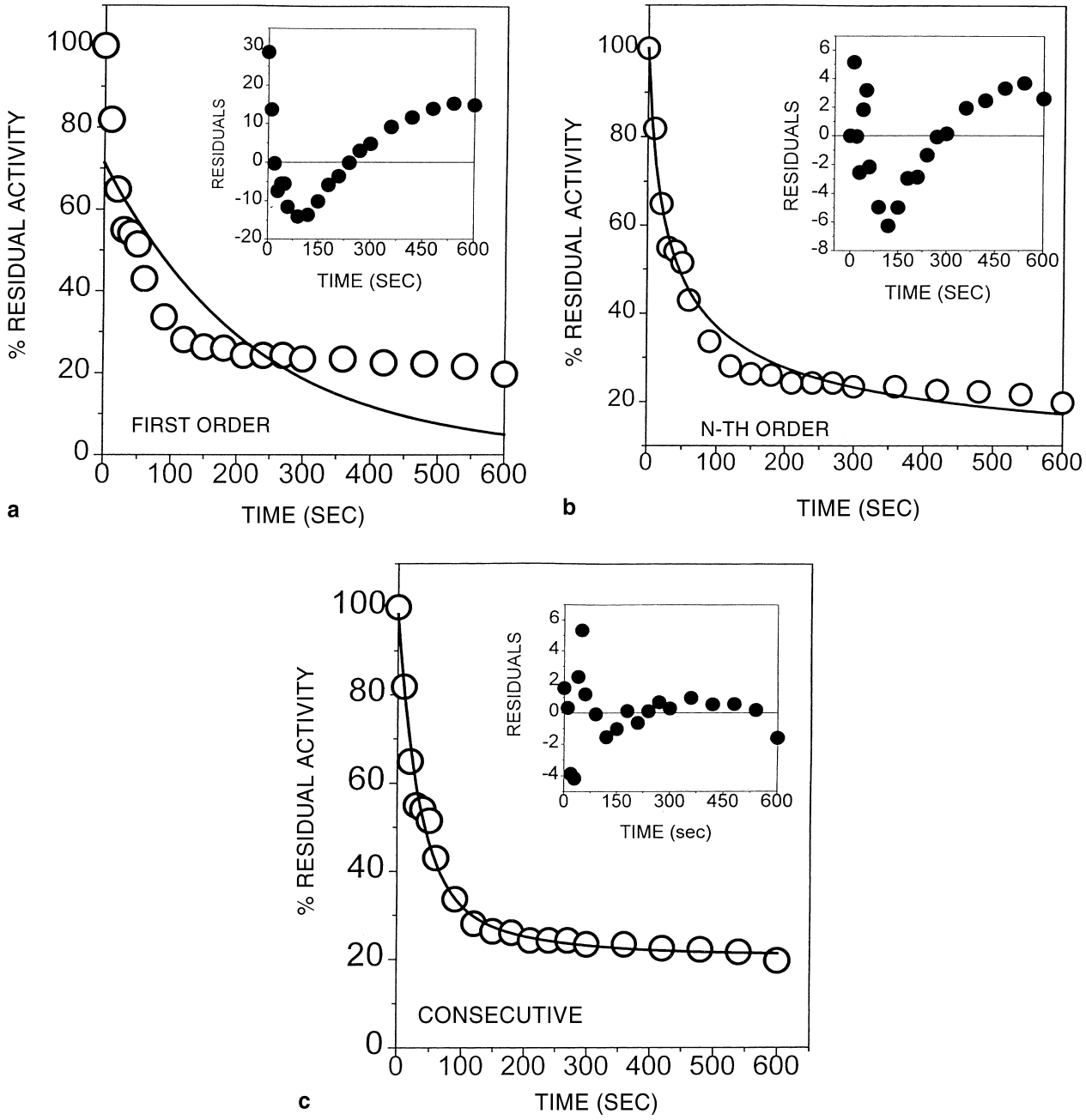

Fig. 2. Non-linear regression (NLR) analysis to establish a kinetic model for Brussels sprouts isoperoxidase heat-inactivation. Results for isoperoxidase A1 heat-inactivation at 60°C. Experimental points (○) were fitted to the 1st order (Fig. 2(a)), *n*th order (Fig. 2(b)) and non-linear 1st order or consecutive kinetic models (Fig. 2(c)). Inserts show residuals (see text for details).

$$E_0 \rightarrow I_1 \quad \text{and} \quad E_0' \rightarrow I_2 \tag{2a}$$

and Eq. (1) is applicable (Alberty & Silbey, 1992). The reactions leading to the inactivated enzymes (I_1 and I_2) are each associated with a rate constant, k_1 and k_2, respectively. In general, I_1 and I_2 are different, since distinct E_0 and E_0' isoenzymes would have different structures.

Alternatively, several forms of inactiated enzyme may be produced from a single highly purified isoenzyme (Eq. (2b)).

$$E_0 \rightarrow I_1 \quad \text{and} \quad E_0 \rightarrow I_2. \tag{2b}$$

Activity measurements are unlikely to differentiate between I_1 and I_2. The observed residual activity decrease due to two parallel inactivation processes (Eq. (2b)) follows a simple 1st order kinetic relation:-

Table 1
Heat-inactivation parameters for purified Brussel sprouts isoperoxidases from NLR equation fitting to a consecutive deactivation model

	Temp			
Isoperoxidase	60 (°C)	65 (°C)	70 (°C)	75 (°C)
A1				
k_1 (s^{-1}) × 10^3	29.4	29.71	57.7	209.7
k_2 (s^{-1}) × 10^3	6.6	2.75	3.44	5.56
A_0 (%)	21.3	20.9	14.0	14.6
B_1 (%)	62.4	66.4	66.3	62.5
B_2 (%)	15.0	7.2	18.3	14.6
α_1 (%)	32.9	27.4	31.2	28.8
α_2 (%)	21.3	20.9	14.0	14.6
R	0.9957	0.9960	0.9972	0.9914
A2				
k_1 (s^{-1}) × 10^3	397	992	992	281
k_2 (s^{-1}) × 10^3	16.07	11.1	13.13	6.46
A_0 (%)	20.0	16.0	6.8	5.0
B_1 (%)	22.8	48.0	61.6	78.0
B_2 (%)	57.3	36.1	31.7	17.0
α_1 (%)	75.0	51.7	38.1	21.6
α_2 (%)	20.0	16.0	6.8	5.0
R	0.9896	0.9981	0.9981	0.9990
C1				
k_1 (s^{-1}) × 10^3	14.3	80.8	228.1	147.0
k_2 (s^{-1}) × 10^3	2.1	2.96	7.28	5.2
A_0 (%)	2.6	0.00	7.4	3.8
B_1 (%)	54.8	41.6	53.4	64.0
B_2 (%)	48.5	58.2	39.1	31.0
α_1 (%)	44.0	56.1	45.3	33.7
α_2 (%)	2.6	0.0	7.4	3.8
R	0.9932	0.9960	0.9972	0.9914
C2				
k_1 (s^{-1}) × 10^3	2613.5*	3142.5*	3877.5*	4020.0*
k_2 (s^{-1}) × 10^3	9.53	14.8	20.3	17.4
A_0 (%)	3.0	1.6	1.5	12.2
B_1 (%)	58.7	70.6	72.2	37.1
B_2 (%)	38.3	27.8	26.3	47.7
α_1 (%)	41.2	29.3	27.7	59.7
α_2 (%)	3.0	1.6	1.5	12.2
R	0.9972	0.9981	0.9990	0.9868

$$a = A_0 + B_1 \exp.(-(k_1 + k_2)t) \quad (2c)$$

Non-linear 1st order kinetics for Brussels sprouts peroxidase isoenzymes can also arise from two consecutive 1st order inactivation reactions (Eq. (3))

$$E_0 \rightarrow E_1 \rightarrow E_2 \quad (3)$$

According to this model the starting enzyme (E_0) denatures via an intermediate species (E_1) before forming the fully inactivated enzyme (E_2). The process described by Eq. (3) (in common with the "mixed enzyme" model of Ling & Lund, 1978) leads to changes in residual activity with heating time in accordance with Eq. (1). This relation describes changes in the *combined residual activities* for E_0E_1 and E_2. The concentration of each enzyme form can be estimated as described later.

The "mixed enzyme" model (Eq. (2a)) of Ling & Lund (1978) may be rejected, in favour of a consecutive model (Eq. (3)), as a basis for the non-linear kinetics observed in this investigation (Fig. 1). Purified isoenzyme samples were used in this study. The four isoenzymes were homogeneous as determined by SDS-PAGE as well as native polyacrylamide gel electrophoresis, followed by activity staining. Analysis by isoelectric focusing, followed by activity staining, also showed that each isoenzyme preparation was homogeneous (Forsyth & Robinson, 1998). For some preparations, analysis by highly sensitive Western blotting showed the presence of a small number of antigenic polypeptides.

Owing to the purity of enzyme samples used it may be suggested that the consecutive model (Eq. (3)) is a more appropriate description of the heat-inactivation of enzymic activity over the time periods examined in this paper. Although a consecutive heat-inactivation model can be formulated such that it includes more than one irreversibly inactivated intermediate, a single intermediate leads to satisfactory agreement between calculated and observed data in most cases (Sadana, 1982; Henley & Sadana, 1985).

Calculated profiles showing changes in the concentrations of E_0 E_1 and E_2 during heat treatment at 60°C (Fig. 3(a–d)) are quite different for the four isoperoxidase preparations. The profiles were generated from data in Table 1 using Eq. (4) (Alberty & Silbey, 1992):

$$E = E_0 \exp(-k_1 t), \quad (4a)$$

$$E_1 = k_1 E_0[(\exp - k_1 t) - \exp.(-k_2 t)]/(k_1 - k_2), \quad (4b)$$

$$E_2 = E_0 + E_0[k_2 \exp(-k_1 t) - k_1 \exp(-k_2 t)]/(k_1 - k_2), \quad (4c)$$

where E is the total concentration of enzyme at $t = 0$ and in this analysis $E = 100\%$ enzyme activity. Fig. 3 also shows peroxidase residual activity *observed* as a function of heating time. The profiles, for the most thermostable isoenzymes C1 and A1, especially indicate a significant contribution, from the more stable E_0 and E_1 forms, to the measured enzymatic activity after 100 s of heating. Whereas, for the more heat-labile isoenzymes, A2 and C2, a major proportion of the residual activity, after 20 s of heating, was derived from partially inactivated species (E_1 and E_2); cf. Fig. 3(b) and Fig. 3(d).

The profiles also show that the residual activity, after the activities of E_0 and E_1 have decreased to nearly zero, is due to E_2. The order of decimal reduction times was $C1 \geqslant A1 > A2 \approx C2$ (Table 2). As expected from the D_v values, the concentration of native peroxidase (E_0) fell very rapidly during the heat treatment of A2 and C2. The time required for 100% inactivation of E_1 is $\approx 6/k_2$ or 2.6 D_v. For all enzyme forms considered in this study, complete inactivation occurred by about 48 min. For each isoperoxidase, E_1 and E_2 are unlikely to be distinct

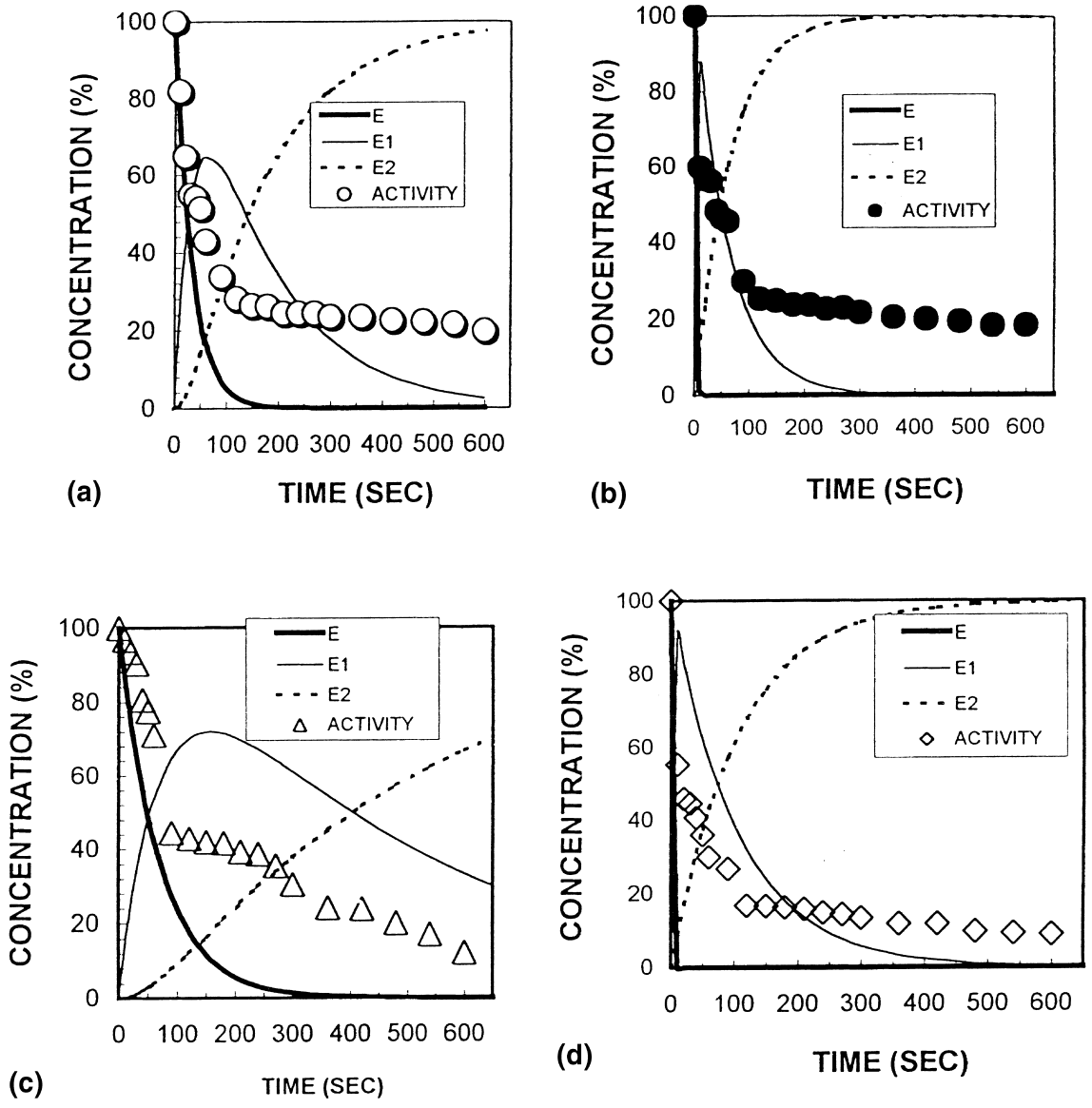

Fig. 3. Changes in the concentration profiles for E_0 (native enzyme), E_1 (partially inactivated form) and E_2 (fully inactivated enzyme). (a)–(d) show inactivaton profiles at 60°C for isoperoxidase A1, A2, C1 and C2, respectively.

Table 2
A summary of decimal reduction times (Dv) for purified Brussels sprouts isoperoxidases

Isoperoxidase *	(D_v) seconds	
	E_0	E_1
	Temp. 60°C	Temp. 60°C
C1	161.0	1096
A1	78.3	349.0
A2	5.8	143.3
C2	0.9	241.6
	Temp. 75°C	Temp. 75°C
C1	15.6	443.0
A1	11.0	414.2
A2	8.2	356.5
C2	0.6	132.3

* Dv values were calculated from rate constants k_1 and k_2 in Table 1. Enzymes are listed in the approximate order of heat stability.

homogeneous forms. It is feasible that E_1 and E_2 are probably mixtures of partially denatured enzymes with less specific activity relative to E_0.

Recent results with horseradish peroxidase published by Adams et al. (1996), which show that after longer heating times new forms of protein can be detected, is compatible with our equation fitting to consecutive reactions, albeit during the very early stages of heat-inactivation and the above proposal for the formation of heat-modified peroxidases, E_1 and E_2. In Fig. 1 it can be seen that the rate of the 1st order non-linear heat-inactivation decreases rapidly and becomes more linear with longer heating times where the concentration of E_2 approaches 100%. The components of E_2 are probably non-enzymic fragments of heat-denatured isoperoxidases able to slowly oxidise the test reagent, *o*-dianisidine. It

seems possible that such fragments may be haem-containing polypeptides and small amounts might even have been detected as antigenic polypeptides by the highly sensitive Western blotting technique used previously for assessment of purity of the Brussels sprout isoenzymes (Forsyth & Robinson, 1998). This finding prompts us to question whether other preparations, including commercial samples, also contain non-enzymic but antigenic polypeptide fragments.

We have calculated the *average* specific activities for the hypothetical $E_1(\alpha_1)$ and $E_2(\alpha_2)$ forms (Table 1) on a scale with unheated peroxidase (E_0) assigned a specific activity of 100%. Interestingly, α_2 was 12–18% for peroxidase isoenzymes A1 and A2 as compared to an α_2 value 3–4.6% for C1 and C2. It may be suggested that heat treatment leads to a more efficient elimination of the activity for the E_2 forms for isoperoxidase C1 and C2. Interestingly, E_2 may be considered a "final product" of peroxidase inactivation only within the timeframe used in this study. Specifically, E2 may be slightly heat-sensitive and capable of undergoing further degradation chemically at higher temperatures or with prolonged heating. As evidence, we note that average specific activities for the E_2 form for A1 and A2 decreased with rising temperature (Table 2).

3.1. Regeneration of Brussels sprouts peroxidase isoenzymes

Isoperoxidase A1 and A2 regenerated more efficiently than isoenzymes C1 and C2 (Fig. 4). Regeneration efficiencies, due to recombination of haem with apo-peroxidase, are higher when heat-inactivation proceeds with a "cleaner" dissociation of the haem group. On the other hand, inactivation processes, during which the haem moiety remains partially bound to the denatured apo-protein, appear to be followed by less efficient regeneration (Adams et al., 1996). From present results Brussels sprouts isoperoxidases are differentiated into the highly regenerable (A1 and A2) forms and the less regenerable cationic forms (C1 and C2).

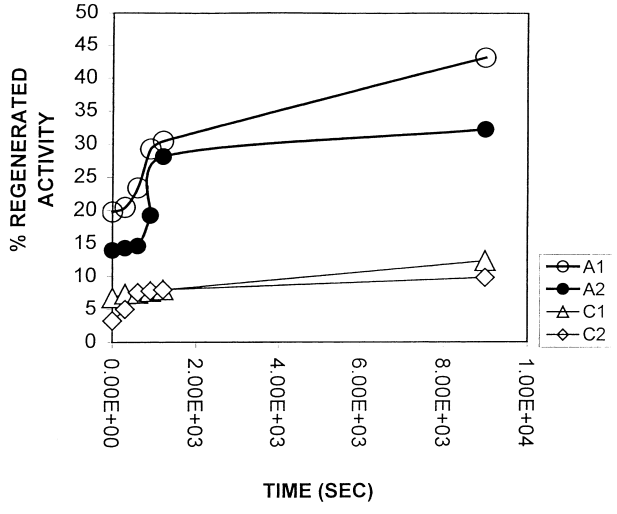

Fig. 4. Regeneration of purified Brussels sprout isoperoxidases after heat-treatment. (see text for experimental details).

3.2. Transition state parameters for the heat-inactivation of Brussels sprouts isoperoxidases

The activation energy ($\Delta E^{\#}$) for enzyme heat-inactivation was determined over successive temperature intervals using (Eq. (5a)).

$$\ln k_1 - \ln k_2 = \Delta E^{\#}/R(1/T_1 - 1/T_2) \quad (5a)$$

Transition state parameters were then calculated from the absolute reaction rate theory. Changes in the activation enthalpy ($\Delta H^{\#}$), free energy ($\Delta G^{\#}$) and entropy ($\Delta S^{\#}$) for enzyme heat-inactivation were determined for the $E_0 \rightarrow E_1$ or $E_1 \rightarrow E_2$ steps separately, using Eqs. (5b)–(5d)

$$\Delta H^{\#} = \Delta E^{\#} - RT \quad (5b)$$

$$\Delta G^{\#} = -RT \ln(kh/K_B T) \quad (5c)$$

$$\Delta S^{\#} = (\Delta H^{\#} - \Delta G^{\#})/T \quad (5d)$$

where h is Planck's constant, K_B, Boltzman constant. Transition state parameters for purified Brussels sprouts isoperoxidase heat-inactivation are given in Table 3.

The application of absolute rate theory to enzyme and protein denaturation reactions has been reviewed by Stearn (1949) and subsequently discussed by Rosenberg et al. (1971), Warren (1973) and Labuza (1980). For example, the $E_0 \rightarrow E_1$ reaction proceeds via the transition state E_{TS}.

$$E_0 \rightleftharpoons E_{TS} \rightarrow E_1 \quad (6)$$

The rate constant k_1 is determined by the equilibrium constant ($K^{\#}$) for E_{TS} formation and the rate (ϕ) for its breakdown;

$$k_1 = \phi K^{\#} \quad (\text{where} \quad \phi = \kappa K_B T/h) \quad (7)$$

κ is a probability (usually assumed to be one) that E_{TS}, once formed will proceed to E_1. The magnitude of $K^{\#}$ is related to standard thermodynamic parameters.

$$K^{\#} = \exp.(-\Delta G^{\#}/RT) = \exp.(-\Delta H^{\#}/R).\exp.(\Delta S^{\#}/RT). \quad (8a)$$

The magnitude of $\Delta H^{\#}$ and $\Delta S^{\#}$ is determined by the average number of non-covalent bonds broken and the net enzyme/solvent disorder change for the $E_0 \rightleftharpoons E_{TS}$ transition, respectively (Stearn, 1949).

Ranked in order of increasing stability, the value for $\Delta G^{\#}$ for the E_0 form of isoperoxidase A1, C1, A2 and C2 is 98.14, 96.4, 89.2 and 64.15 kJ mol^{-1}, respectively. The average $\Delta S^{\#}$ value for A1, C1, A2 and C2 is 78.4 J mol^{-1} °K^{-1}, 143.2 J mol^{-1} K^{-1}, −343.5 J mol^{-1} K^{-1},

Table 3
Activation enthalpy ($\Delta H^{\#}$), free energy ($\Delta G^{\#}$) and entropy ($\Delta S^{\#}$) change values for the heat-inactivation of purified Brussel sprouts isoperoxidases

Isoperoxidase [a]	Temp (°C)	$\Delta H^{\#}$ (J mol^{-1})	$\Delta G^{\#}$ (J mol^{-1})	$\Delta S^{\#}$ (J mol^{-1})
($E_0 \rightarrow E_1$)				
A1	60–65	−805.54	97328.40	−294.70
	65–70	125156.52	98802.97	77.97
	70–75	253284.56	98298.33	451.85
C1	60–65	321350.37	99448.01	666.37
	65–70	197265.30	95816.45	300.14
	70–75	−90059.04	94134.61	−537.01
A2	60–65	168665.86	89673.74	237.21
	65–70	−2821.96	88330.53	−269.68
	70–75	−252657.40	89681.86	−998.07
C2	60–65	31731.32	63816.36	−96.35
	65–70	37708.09	64268.82	−78.58
	70–75	4311.91	64627.38	−175.85
($E_1 \rightarrow E_2$)				
A1	60–65	−166626.95	101721.87	−805.85
	65–70	40349.01	105907.03	−193.96
	70–75	92448.77	106840.04	−41.96
C1	60–65	61476.45	105089.58	−130.97
	65–70	170686.63	105687.37	192.31
	70–75	−69638.46	104569.16	−507.89
A2	60–65	−72190.64	99104.82	−514.40
	65–70	29742.90	101744.55	−213.02
	70–75	−145487.58	102782.63	−723.82
C2	60–65	79618.58	100641.46	−63.13
	65–70	58109.14	100883.12	−126.55
	70–75	−33449.34	101462.75	−393.33

[*] Parameters for separate steps in the consecutive reaction (cf. Eq. (3)).

−116.6 J mol^{-1} °K^{-1}. Finally, the average $\Delta H^{\#}$ value for A1, C1, A2 and C2 is 126.0, 143.0, −29.0 and 24.5 kJ mol^{-1}, respectively.

$\Delta E^{\#}$ values are most frequently cited in the literature. Such values may be compared with present $\Delta H^{\#}$ estimates (since $\Delta H^{\#} = \Delta E^{\#} - RT$ and $RT \approx 2.8$ kJ mol^{-1} at 70°C). Some reported $\Delta E^{\#}$ values are, 194 kJ mol^{-1} cauliflower peroxidase (Lee et al., 1984), 143 kJ mol^{-1} cranberry peroxidase (Chan & Yang, 1971), 142 kJ mol^{-1} pea peroxidase (Svensson & Eriksson, 1974), 133.8 kJ mol^{-1} horse-radish peroxidase (Lu & Whitaker, 1974), 99.7 kJ mol^{-1} papaya peroxidase (Park et al., 1979), and 30 kJ mol^{-1} corn peroxidase (Lee & Hammes, 1979). It may be concluded that current $\Delta H^{\#}$ values are within the range reported for other peroxidases.

The activation Gibbs free energy change is the energy barrier for enzyme deactivation (Stearn, 1949). In contrast, isolated values for $\Delta H^{\#}$ or $\Delta S^{\#}$ are not good predictors of enzyme stability. The significance of $\Delta H^{\#}$ or $\Delta S^{\#}$ values may be illustrated by considering equilibrium thermodynamics principles as usually applied to enzyme unfolding (cf. Apenten, 1995 and refs. therein.). Analysis of simple enzyme unfolding reactions leads to the standard enthalpy (ΔH), entropy (ΔS) and Gibbs free energy (ΔG) for unfolding the native state. These thermodynamic parameters are usually very temperature-dependent due to a large heat capacity change (ΔC_p) for protein unfolding. At any temperature ΔH and ΔS can be described by:

$$\Delta H = \Delta H^0 + \Delta C_{\mathrm{p}}(T_{\mathrm{ref}} - T), \tag{9a}$$

$$\Delta S = \Delta S^0 + \Delta C_{\mathrm{p}} \ln (T/T_{\mathrm{ref}}), \tag{9b}$$

where T_{ref} is a reference temperature. The ΔC_{p} value for protein unfolding (also observed for the dissolution of low molecular weight nonpolar soluted in water) is due to changes in solvent structure, necessary to allow the insertion of nonpolar side-chain residues into the aqueous phase.

Eqs. (9a) and (9b) leads to expectations of "extra-thermodynamic" or linear free energy relationships between ΔH and ΔS since both parameters change in a similar fashion with changes of temperature or ΔC_{p}. Linear free energy relations are not usually derivable from accepted thermodynamic laws. For a range of reactions taking place in an aqueous solution, a graph of ΔH against ΔS often yields a straight-line. This is thought to be indicative of an enthalpy-entropy compensation phenomenon demonstrated for a very wide range of reactions whose only common link is that the reaction medium is water (Rosenberg et al., 1971; Lumry & Rajender, 1970; Warren, 1973; Labuza, 1980;

Connors, 1990). It has also been suggested that some enthalpy-entropy correlation may arise from statistical error because (i) $\Delta H^{\#}$ and $\Delta S^{\#}$ values are usually determined from the same rate-temperature data, and (ii) the determination of $\Delta S^{\#}$ involves a very substantial extrapolation of experimental data to the $1/T = 0$ intercept of an Arrhenius (and Eyring-type) graph (cf. Labuza, 1980; Connors, 1990 for an extensive discussion).

Fig. 5 shows a graph of $\Delta H^{\#}$ plotted against $\Delta S^{\#}$ values for the heat-inactivation of the four Brussels Sprouts isoperoxidases examined in this study. Clearly there is a correlation between values for $\Delta H^{\#}$ and $\Delta S^{\#}$. In Fig. 5, the equation of the straight line was $\Delta H^{\#} = m\Delta S^{\#} + c$. For the $E_0 \rightarrow E_1$ reaction, the slope (m), also called the isokinetic temperature, was 341.0(±3.5)°K whilst c was 95907(±1786) J mol^{-1}. A graph of $\Delta H^{\#}$ plotted against $\Delta S^{\#}$ for the $E_1 \rightarrow E_2$ reaction (results not shown) gave $m = 341.2(\pm 2.8)$°K and $c = 103911(\pm 1126)$ J mol^{-1}; in both cases $R^2 = 0.99$. A linear $\Delta H^{\#}$ vs. $\Delta S^{\#}$ graph suggests enthalpy-entropy compensation. Rosenberg et al. (1971) examined activation parameters for the heat-inactivation of a great number of proteins, viruses, yeasts and bacteria. For these systems the average isokinetic constants were $m = 329(\pm 2.6)$ K and $c = 89626(\pm 624)$ J mol^{-1}.

A plot of $\Delta G^{\#}$ vs. $\Delta S^{\#}$ is also shown in Fig. 5. The slope for this graph was zero. Apparently, there was no systematic relationship between $\Delta G^{\#}$ and $\Delta S^{\#}$ values. Therefore, similar values of $\Delta G^{\#}$ may arise from different absolute values for $\Delta H^{\#}$ and $\Delta S^{\#}$, owing to compensatory changes in these parameters. As with other linear free energy relations, the establishment of enthalpy-entropy compensation relations is taken as proof that one is dealing with a series of homologous reactions (here – inactivation of four isoperoxidases at four different temperatures) proceeding via the same mechanism.

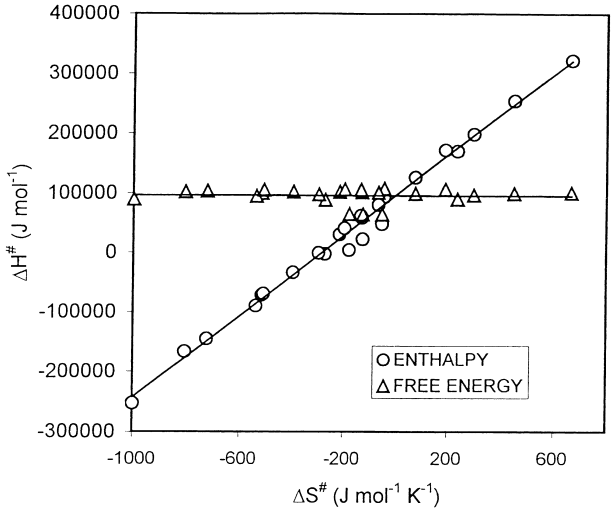

Fig. 5. A graph showing the relations between the activation entropy (ΔS), activation enthalpy (ΔH) and Gibbs free energy (ΔG) for the heat-inactivation of purified Brussels sprouts isoperoxidases. Graphs shows all values determined in this study.

At the isokinetic temperature, enthalpy-entropy compensatory changes, for a series of reactions will be the same, and the rate of (enzyme) inactivation will therefore be independent of values for $\Delta H^{\#}$ and $\Delta S^{\#}$ (Rosenberg et al., 1971). The relative stability of the native forms (E_0) was determined at the isokinetic temperature (66°C) from differences in $\Delta G^{\#}$ values (i.e., $\Delta\Delta G^{\#}$ values). In the order of decreasing stability for the four isoperoxidases, $\Delta\Delta G^{\#}$ values are 34.5 kJ mol^{-1} (A1) > 31.5 kJ mol^{-1} (C1) > 24.1 kJ mol^{-1} (A2) and 0.0 kJ mol^{-1} (C2). A similar calculation for the E_1 forms gives 5.2 kJ mol^{-1} (A1) > 4.8 kJ mol^{-1} (C1) > 0.86 kJ mol^{-1} (A2) and 0.0 kJ mol^{-1} (C2). These figures suggest that stability of native (E_0) forms of isoperoxidase differ by as much as $\leqslant$34.5 kJ mol^{-1}. In contrast, the stabilities of the E_1 forms differ by a modest $\leqslant$5.2 kJ mol^{-1}.

Further analysis of $\Delta H^{\#}$ and $\Delta S^{\#}$ values can provide information about the mechanism for enzyme inactivation. Assuming the linear-free energy type relation exists (cf. Fig. 5), it may be assumed that E_{TS} is the same for all the isoenzymes. That is, these enzymes are inactivated via the same pathway. Values of $\Delta H^{\#}$ will then increase with increasing numbers of H-bonds and electrostatic interactions within the distinctive E_0 state for each isoperoxidase. Severance of H-bonding interactions between water of hydration and E_0 during heating will also increase $\Delta H^{\#}$. These enzyme-stabilising (positive) contributions to $\Delta H^{\#}$ will be accompanied by compensating increases in the degree of disorder within E_0 and solvent disorder – leading to positive (destabilising) values of $\Delta S^{\#}$. Almost uniquely, increasing hydrophobic interactions within different E_0 states will lead to negative (enzyme stabilising) contributions to $\Delta S^{\#}$.

Enzyme heat-resistance is usually enthalpic and/or entropic in origin. Enthalpic stabilisation is indicated by a positive $\Delta H^{\#}$ value. Stabilisation is entropic when $-T\Delta S^{\#}$ is positive. Fig. 6(a,b) show bar-chart plots of $\Delta H^{\#}$ and $-T\Delta S^{\#}$ values for four isoperoxidases at 60 and 75°C. These are arranged along the X-axis (of Fig. 6) in order of decreasing enzyme heat-resistance. When the Y-axis is positive there is enthalpic and/or entropic stabilisation. Isoperoxidase C1 and A2 were stabilised by entropic factors ($-T\Delta S^{\#}$ was positive) at 60°C (Fig. 6(a)). However, stabilisation of these same enzymes was enthalpic at 75°C (Fig. 6(b)). Interestingly, heat resistance was equally likely to be associated with enthalpic or entropic stabilisation.

Much more work will be needed before the stability differences between purified isoperoxidases are fully understood. Forsyth & Robinson (1998) suggested, from molecular weight determinations, that Brussels sprouts isoperoxidases have variable degrees of glycosylation;

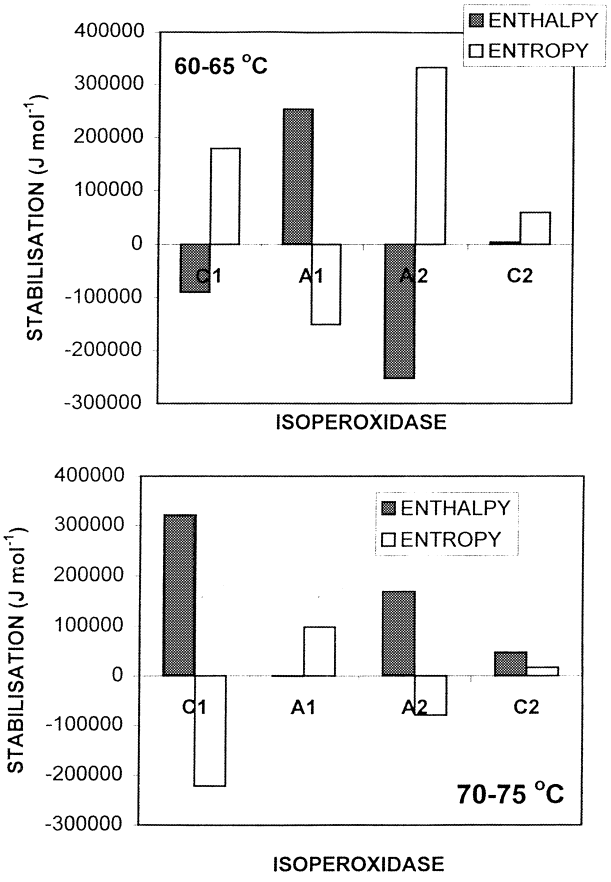

Fig. 6. Enthalpic versus Entropic stabilisation for Brussels sprouts isoperoxidases. Postive values for ΔH (enthalpy) or $-T\Delta S$ (normalised entropy) indicate enthalpic or entropic stabilisation, respectively. Negative values for ΔH and $-T\Delta S$ are destabilising.

A1 (MWt = 48 kDa) may be the most glycosylated isoform whereas C2 (MWt = 28 kDa) is probably the least glycosylated. Large differences in carbohydrate content might account for some of the stability differences observed. Deglycosylation of peroxidase, from horseradish or avocado, leads to a decrease in enzyme heat-resistance (Sanchez-Romero et al., 1994; Tams & Welinder, 1998).

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