

Thermal inactivation of lipoxygenase and hydroperoxytrienoic acid lyase in tomatoes

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Abstract

Lipoxygenase (LOX) and hydroperoxytrienoic acid lyase (HPL) activities were determined in tomato juice from four cultivars of processing tomatoes. Large differences in total activity levels were found between different cultivars. Thermal inactivation kinetics of these two enzymes were determined for two cultivars used in cold break processing. LOX inactivation did not follow simple first order kinetics. The data could be fitted by assuming that three isoforms of LOX are present. HPL inactivation also indicated the presence of labile and resistant forms with the resistant form showing simple first order inactivation kinetics. The inactivation kinetics for both enzymes indicate that they would be rapidly inactivated at the cold break target temperature of 60 °C. It is questionable whether the activity of these two enzymes contributes to the superior flavour associated with products produced by the cold break process.

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1. Introduction

Tomato juice, which may be used as such or concentrated into paste or sauces, is typically processed by one of two methods. In the “hot-break” process, the tomatoes are homogenized and rapidly heated to 95 °C to inactivate the endogenous enzymes. During the “cold-break” process the tomato homogenate is only heated to about 60 °C. At this temperature the enzymes, pectin methylesterase (PME) and polygalacturonase (PG), are known to be active. The action of these enzymes breaks down some of the pectins, which has the beneficial effect of reducing the viscosity of products, such as soup and juice. Another benefit of the cold-break process is better colour and flavour of the product as compared to hot break juice (Hayes, Smith, & Morris, 1998; Thakur, Singh, & Nelson, 1996). These quality improvements may be due to a reduced level of thermal abuse as compared to the hot-break process. It is also possible that, during the cold-break process, enzymes

responsible for the formation of flavour and aroma compounds are active and generate higher levels of desirable compounds.

Volatile aldehydes, which are derived from the oxidative breakdown of fatty acids, are significant aroma compounds in tomatoes and many other foods. Most notable of these in tomatoes is *cis*-3-hexenal, derived from the breakdown of linolenic acid (or other ω -3 fatty acids), which is often described as having a tomato-like aroma (Buttery, Teranishi, & Ling, 1987; Tandon, Baldwin, & Shewfelt, 2001). It has been shown that lipid derived volatile aldehydes such as *cis*-3-hexenal are present at low levels in intact tomatoes. Following homogenization of a tomato, however, the level of *cis*-3-hexenal rapidly increases (Buttery et al., 1987). The formation of volatile aldehydes in the homogenate is thought to involve the concerted action of two enzymes, lipoxygenase (LOX) and hydroperoxytrienoic acid lyase (HPL), acting on fatty acid substrates (Galliard, Matthew, Wright, & Fishwick, 1977; Hatanaka, 1993). Fatty acids are first oxidized to their hydroperoxy derivatives by the action of LOX. These peroxy fatty acids are then cleaved to form 6-carbon aldehydes by HPL. The role of LOX has been questioned because the LOX

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enzyme purified from tomatoes produces, exclusively, 9-hydroperoxy lipids, whereas the desirable lipid-derived aroma compounds are 6-carbon aldehydes derived from 13-hydroperoxy lipids (Suurmeijer, Perez-Gilbert, van der Hijden, Veldink, & Vliegthart, 1998). In addition, in transgenic tomatoes where the expression of two of the isozymes of LOX were suppressed, and little or no LOX activity could be detected, the level of volatile aldehydes formed was no different from that found in control tomatoes (Griffiths et al., 1999). It is possible that an additional and very minor form of LOX is the enzyme responsible for the formation of the 13-hydroperoxy lipids. It is also possible that the 13-hydroperoxy lipids are produced non-enzymatically. Whatever their origin, 13-hydroperoxy lipids are cleaved into a 12-oxoacid and a six carbon aldehyde by the action of HPL (Suurmeijer et al., 2000). HPL is thus the enzyme directly responsible for the formation of *cis*-hexenal and hexanal.

Since the volatile aroma aldehydes are generated enzymatically after homogenization, it is possible that one benefit of the cold break process is to allow the enzymes responsible to remain active longer and increase the level of these desirable compounds. Thermal inactivation data has not been previously reported for LOX or HPL in tomatoes. Our goal was to determine the inactivation kinetics of the two enzymes to determine the extent to which they are active during processing. We have measured these inactivation kinetics in juice rather than on a purified enzyme preparation in order to obtain information more relevant to actual tomato juice processing.

2. Materials and methods

2.1. Tomatoes

Field grown tomatoes of the cultivars CXD-199 and CXD-152, which are used primarily for cold-break processing, as well as BOS 3155 and H9494, which are primarily used for hot-break processing, were obtained from Campbell's Soup Research Center, Davis, CA. Tomatoes were washed and sliced in half; seeds and locular gel were removed; then they were cut into a half-inch dice. The diced tomatoes were frozen in a -80°C blast freezer then stored in polyethylene bags at -20°C or colder until use.

2.2. Heating of homogenates

Homogenates were prepared by grinding about 100 g of thawed diced tomatoes in a mortar and pestle, then passing this homogenate through a fine metal screen to remove pieces of skin and any seeds. Using a syringe with a blunt needle, the homogenate was transferred to

glass capillary tubes, sealed at one end, with a capacity of 200 μl (1.5 mm i.d. \times 2.3 mm o.d. \times 125 mm length). Two tubes (for a total of 0.4 ml of tomato juice) were heated in a circulating water bath (model 20B, Julabo, Allentown, PA) to the indicated temperatures (between 50 and 65 $^{\circ}\text{C}$) for the times specified. The temperature of the waterbath was verified with a calibrated mercury thermometer (model 1005-3FC, Ertco, West Patterson, NJ) and was controlled to $\pm 0.1^{\circ}\text{C}$. The come-up time for the capillary tube was determined by placing a fine thermocouple in the solution at the centre of the tube and recording the time necessary for the solution in the tube to reach that of the waterbath. This time was determined to be less than 10 s. Following heating, samples were cooled in ice water and stored on ice until assay.

2.3. LOX activity

Following heat treatment, samples were prepared for assay by mixing 0.4 ml of heated homogenate with 0.1 ml of a solution containing 0.5 M phosphate buffer, pH 6.5, and 0.5% Triton X-100 in a 1.5 ml microcentrifuge tube. Preliminary experiments indicated that this final concentration of Triton X-100 (0.1%) was required to prevent the LOX enzyme from sticking to the particulate fraction, which is consistent with other reports (Smith, Linfoth, & Tucker, 1997). Samples were centrifuged for 10 minutes at $10,000 \times g$ and the pellet was discarded. The supernatant was then desalted by centrifuging through a column (2.0 ml bed volume) of Sephadex G-25 equilibrated with 0.1 M phosphate buffer, pH 6.5 (Helmerhorst & Stokes, 1980). Lipoxygenase activity was measured by the formation of conjugated dienes from linoleic acid (Axelrod, Cheesbrough, & Laakso, 1981). The linoleic acid substrate was prepared by mixing 140 mg (155 μl) of linoleic acid and 280 mg (257 μl) of Tween-20 in 5 ml water. The solution was clarified by the addition of 0.6 ml of 1 N NaOH, then brought to a final volume of 25 ml and stored frozen under N_2 in 1.0 ml aliquots. Assays were performed at room temperature in 1.0 ml of a medium containing 100 mM phosphate buffer (pH 6) and 20 μl of linoleic acid substrate to give a final linoleic acid concentration of 0.5 mM. Reactions were started by the addition of 50 μl of homogenate and the rate was calculated from the change in absorbance at 234 nm over this time span.

2.4. HPL activity

Samples were prepared as described for LOX. As with LOX, the inclusion of Triton X-100 was required to solubilize the enzyme, which is consistent with previous reports showing that HPL is membrane-bound (Blee & Joyard, 1996; Suurmeijer et al., 2001). Activity was assayed using a coupled enzyme assay (Vick, 1991).

Incubations were performed at room temperature and contained 0.95 ml sodium phosphate buffer (pH 6.5), 10 μ l of 10 mM NADH, 10 μ l of 5 U/ μ l alcohol dehydrogenase, and 10 μ l of 10 mM 15-hydroperoxylinolenic acid in ethanol, prepared enzymatically from linolenic acid using soybean LOX according to Gibian and Vandenberg (1987). Reactions were started by the addition of 50 μ l of the extracted tomato enzyme and the decrease in absorbance at 340 nm was monitored.

2.5. Calculations

First order inactivation rate constants and activation energies for inactivation were calculated as described previously (Anthon & Barrett, 2002). Where multiple isoforms of the activity are proposed, the decrease in total activity versus time was assumed to be the sum of the inactivation of each of the individual isoforms or

$$A(t) = \sum A_n \exp(-k_n t) \quad (1)$$

where $A(t)$ is the total activity as a function of time, and A_n is the initial activity and k_n is the first order inactivation rate constant for isoform n . To model the data, values for k_n and A_n were allowed to vary and the expected residual activity versus time calculated from Eq. (1). For this it was assumed that each k_n varied independently with temperature while the initial proportion of the total activity due to each isoform, $A_n/A_{t=0}$, was the same at each temperature.

3. Results and discussion

3.1. LOX and HPL activity levels

The total levels of LOX and HPL activity were determined for four tomato cultivars, two of which are used primarily for hot break processing, BOS 3155 and H9492, and two which are used primarily for cold-break processing, CXD 152 and CXD199. The two cold-break cultivars had higher levels of HPL than either of the two hot-break cultivars (Table 1). The CXD 152 tomatoes also had the highest level of LOX activity. Curiously,

the CXD 199 tomatoes, which had by far the highest HPL activity, also had the lowest total LOX activity. A recent study of 12 fresh market cultivars (Yilmaz, Tandon, Scott, Baldwin, & Shewfelt, 2001) also showed significant differences in the levels of these two enzymes between different tomato cultivars. These authors did not, however, find any correlation between these differences in activity and the levels of volatile aldehydes.

3.2. LOX inactivation

For thermal inactivation studies, the two cold-break varieties with the highest total activity levels, CXD 152 for LOX and CXD 199 for HPL, were used. Inactivation kinetics for LOX from CXD 152 tomatoes are given in Fig. 1. The inactivation did not follow simple first order kinetics, nor could the inactivation be reasonably resolved into two linear phases. The simplest model which gave a good fit to the data was to assume three isoforms of the enzyme and the inactivation parameters in Table 2. The lines in Fig. 1 were drawn by assuming that the total observed activity is the sum of the activities of three isoforms, present in the relative amounts of 0.62, 0.30, 0.08 and inactivated according to

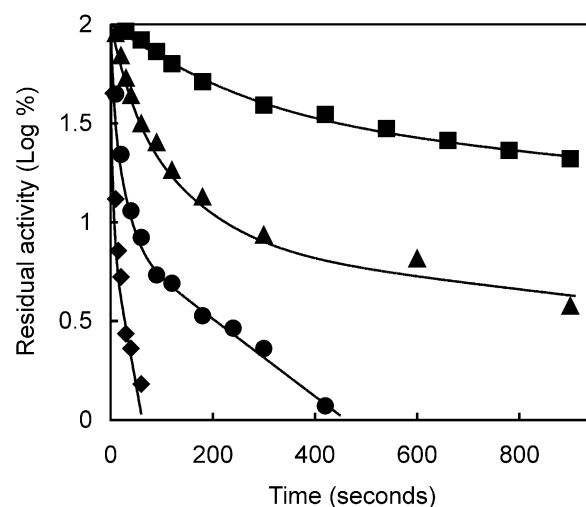


Fig. 1. Heat-inactivation of lipoygenase in tomato juice. Juice prepared from CXD 152 tomatoes was heated for the times indicated to 50 °C (■), 55 °C (▲), 60 °C (●), or 65 °C (◆). Lines were calculated according to the parameters in Table 2.

Table 1
Total activity levels of LOX and HPL in different tomato cultivars

Tomato cultivar	Enzyme activity (nmols min ⁻¹ g fresh weight ⁻¹)	
	LOX	HPL
BOS 3155	151	169
Heinz 9492	189	135
CXD 152	248 ± 92	195 ± 42
CXD 199	79 ± 13	457 ± 52

Table 2
Rate constants for inactivation of LOX

	% Of total activity	Inactivation rate constants × 10 ³ (s ⁻¹)				
			50 °C	55 °C	60 °C	65 °C
LOX 1	62	k_1	6.4	37.0	220.0	1000
LOX 2	30	k_2	0.9	10.0	50.0	250
LOX 3	8	k_3	0.01	0.7	4.5	33

the rate constants given in Table 2. Arrhenius plots (Fig. 2) of the constants in Table 2 were linear with slopes indicating activation energies for thermal inactivation of 308, 336, and 349 kJ/mol for the three isozymes. These activation energies are less than those reported for LOX from peas (Svensson & Eriksson, 1972) and beans (Indrawati, Van Loey, Ludikhuyze, & Hendrickx, 1999), but higher than that reported for potatoes (Park, Kim, & Lee, 1988).

Tomato fruits are known to contain three isozymes of lipoxygenase (Griffiths et al., 1999). It is possible that these three isozymes have different thermal stabilities and that this explains why three forms of LOX need to be assumed to get a good fit of the data. On the other hand, by assuming three isozymes we have a model with six adjustable parameters (three rate constants and three values for the relative amounts of each isozyme) at each temperature, so the fact that a good fit can be obtained is perhaps not surprising. Nonetheless, this fit is obtained without making unreasonable assumptions, such as allowing the proportion of the isozymes to be different at each of the inactivation temperatures used. Furthermore, the rate constants show a reasonable temperature dependence and form linear Arrhenius plots with reasonable slopes. The calculated activation energies are in the range of what has been reported for LOX inactivation from a number of other sources. In studies of LOX from other sources (Indrawati et al., 1999; Park et al., 1988) non-first order kinetics were also observed and has been taken to indicate the presence of isozymes. It is also possible that the non-first order behaviour in both tomatoes and other sources is due to other factors, such as a sequential inactivation pathway involving intermediate forms with reduced activity (Adams, 1991).

The temperature dependence of LOX inactivation indicates that LOX in tomatoes is not particularly thermally stable. At 60 °C, the target temperature used in

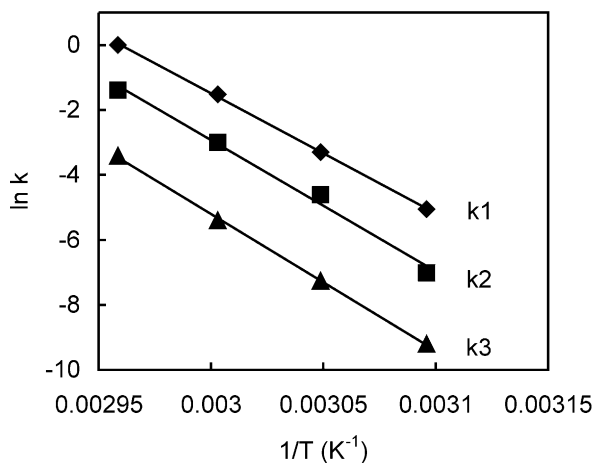


Fig. 2. Arrhenius plot of the inactivation rate constants for LOX, from Table 2.

the cold-break process, LOX is rapidly inactivated and reduced to less than 10% of its original level in less than 1 min. This would imply that, during cold-break processing LOX is rapidly inactivated. However, our inactivation kinetics were determined with very short come-up times. During actual processing the come up time is likely to be significantly longer, depending on the viscosity of the juice and the rate of heat penetration. LOX activity during this come-up time may be substantial and be significant in the formation of volatile aroma compounds. We also cannot rule out the possibility that, while the bulk of the LOX activity is rapidly inactivated, a minor form of the enzyme may be more thermally stable and is in fact the enzyme responsible for 13-hydroperoxy lipid formation.

3.3. HPL activity

The enzyme HPL splits 13-hydroperoxy linolenic acid into a 12-oxo acid and *cis*-3-hexenal. This latter compound is one of the volatile aldehydes responsible for the characteristic fresh tomato aroma. Thermal inactivation of HPL from CXD 199 tomatoes showed two phases. A rapid phase, which resulted in the loss of about 50% of the total activity, occurred at temperatures below 50 °C and was not characterized. The second phase, which was responsible for the loss of the remainder of the activity, showed first order inactivation kinetics at temperatures between 50 and 65 °C (Fig. 3). An Arrhenius plot of the rate constants for inactivation was linear (Fig. 4) and indicated an activation energy of 197 kJ/mole.

We know of no thermal inactivation data for this enzyme in tomatoes or other plant sources. The data that we present here indicate that this is a relatively

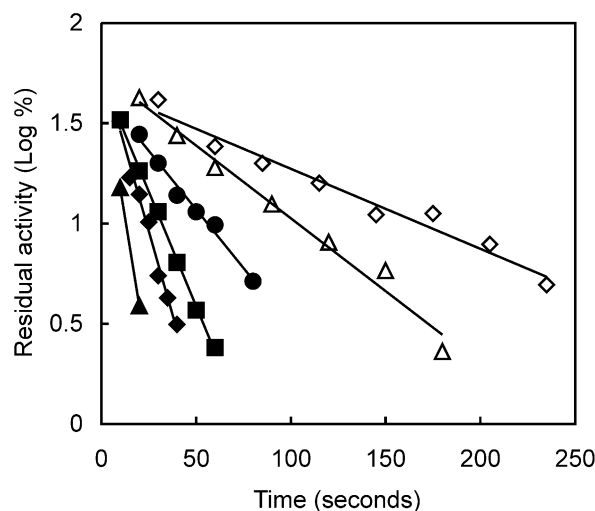


Fig. 3. Heat-inactivation of hydroperoxide lyase in tomato juice. Juice prepared from CXD 199 tomatoes was heated for the times indicated to 52.5 °C (◇), 55 °C (△), 57.5 °C (●), 60 °C (■), 62.5 °C (◆), or 65 °C (▲).

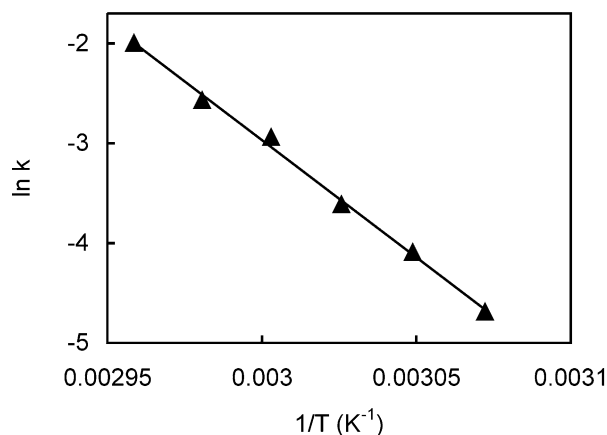


Fig. 4. Arrhenius plot of the inactivation rate constants for HPL.

heat-labile enzyme and it would be reduced to only a few percent of the initial activity level in just one minute at a cold break temperature of 60 °C. Rapid inactivation of this enzyme may reduce the amount of *cis*-3-hexenal formed and lead to a level of this aroma compound that is less than maximal. On the other hand, the limiting factor in the formation of volatile aldehydes may not be the level of HPL activity but the availability of its substrate. This is consistent with the fact that when 12 cultivars of fresh market tomatoes were compared, the level of aldehydes formed upon homogenization did not correlate with the level of HPL activity (Yilmaz, Tandon, Scott, Baldwin, & Shewfelt, 2001). It is possible that the burst of volatile aldehyde formation that occurs upon tomato homogenization results from the consumption of a preexisting pool of 13-hydroperoxy lipids (Griffiths, Leverentz, Silkowski, Gill, & Sanchez-Serrano, 2000) rather than the ongoing generation of hydroperoxy lipids via LOX. If this is the case, then sufficient HPL activity may be present in the period between homogenization and complete inactivation to convert this pool.

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