Purification of hydroperoxide lyase from tomato fruits

Purvika Shah[1*] and Theodore Chase, Jr.[2]

Department of Biochemistry and Microbiology
Rutgers University, New Brunswick, NJ

[1*] pjshah@eden.rutgers.edu

[2] chase_c@aesop.rutgers.edu

[*]Rutgers Undergraduate Research Fellow

Abstract

Tomato fruit hydroperoxide lyase was initially purified using the procedure set forth in Fauconnier et al. (1997) for tomato leaves. The enzyme, hydroperoxide lyase (HPOOH lyase) was difficult to purify; for this reason, several changes to the procedure of Fauconnier et al. were explored. Tomatoes at different stages of ripening were extracted, purified, and assayed to determine the stage with the highest activity. Storage of the tomato fruit for future extractions and storage of the enzyme for future analysis and purification was tested. Two types of assays, one involving disappearance of absorbance and the other involving appearance of absorbance, were also tested.

It was shown that polyethylene glycol (PEG), used to precipitate leaf enzyme, could not precipitate fruit enzyme. Ammonium sulfate was later used to precipitate the fruit enzyme, but the activity significantly decreased. Centrifugation steps were eliminated to allow more time for further purification steps and to preserve enzymatic activity, which often decreased during each centrifugation step. From among the tomatoes in various stages of ripeness, green tomatoes showed the highest activity. These tomatoes could not be obtained after frost began and so they were lyophilized and also stored frozen. However, assays showed that the enzyme was not active when fruit were stored in the freezer or lyophilized. The assay involving appearance of NADH absorbance was tested, but activity was not observed. Instead, the assay involving disappearance of absorbance of unsaturated fatty acid hydroperoxides was used to monitor enzymatic activity. The behavior in purification of tomato fruit HPOOH lyase appeared, from our data, to be different from tomato leaf HPOOH lyase. We have not been able to sufficiently obtain a purified enzyme due to difficulties in precipitating the enzyme.

Introduction

The tomato enzyme, hydroperoxide lyase (HPOOH lyase), is important in the plant's defense against pathogens and in healing wounds (Schreier and Lorenz, 1982, Fauconnier et al., 1997). HPOOH lyase is a membrane-bound enzyme and is present in small amounts in plants. Its substrate 13-linolenic acid hydroperoxide, and five other hydroperoxides, which are not degraded by this enzyme, inhibit the enzyme. 13HPOOH lyase is important in aroma synthesis since the volatile aldehydes that it helps produce contribute to flavor and aroma. The first step in the pathway is catalyzed by lipoxygenase. Lipoxygenase is an enzyme that acts on pentadiene-containing fatty acids to form fatty acid hydroperoxides. This enzyme acts on linoleic and linolenic acids to form both 13 and 9 hydroperoxides, in varying amounts. The hydroperoxides are substrates for other reactions. For example, HPOOH lyase acts on hydroperoxides and breaks them into aldehydes and -oxo acids. There are two types of hydroperoxide lyases: 13-HPOOH lyase and 9-HPOOH lyase. Tomato fruits have 13-HPOOH lyase, which cleaves 13-linolenic acid to 3(Z)-hexenal and 12-oxo-9(Z)-dodecenoic acid; and 13-linoleic acid into hexanal and 12-oxo-9(Z)-dodecenoic acid. (Fauconnier et al., 1997).

The enzyme was isolated from tomato leaf by Fauconnier et al. (1997). Our original goal was to purify and isolate the enzyme from tomato fruit, starting by following the procedure set forth by Fauconnier et al. The original procedure was modified to increase the specific activity of the preparation of tomato fruit enzyme.

Schreier and Lorenz (1982) were able to isolate and purify fruit HPOOH lyase, but only 8.4 fold. The enzyme was purified to apparent homogeneity by Matsui et al. (1991) from tea leaves and by Shibata et al. (1995) from green bell peppers. Fauconnier et al. (1997) devised a relatively simple procedure by which HPOOH lyase was isolated from tomato leaves. We attempted to apply this procedure for isolation of HPOOH lyase from tomato leaves to tomato fruits, hoping to determine whether the fruit enzyme, important to flavor, is identical to the leaf enzyme or differs from it.

Materials

HPOOH lyase was purified from red tomatoes, bought from grocery stores. Dithiothreitol, EDTA, Triton X-100, PEG 4000, 2-mercaptoethanol, NADH, linolenic acid, soybean lipoxygenase, bovine serum albumin and Tris-HCl were products of Sigma Chemical Co. (St. Louis, MO). Sodium phosphate (monobasic and dibasic), NaCl, Na_2HCO_3, NaOH, glycine, diethyl ether (anhydrous), chloroform, methanol, ethanol, silicic acid, hexane, $MgSO_4$, Coomassie Blue R and $(NH_4)_2\ SO_4$ were obtained from Fisher Scientific Co.

Methods

Synthesis of substrate

The substrate for HPOOH lyase, linolenic acid 13-hydroperoxide, was synthesized by using oxygen and linolenic acid as substrates for soybean lipoxygenase at pH 9, where the 13-hydroperoxide is formed almost exclusively. The synthesis and purification of the substrate followed Gardner (1982).

In the synthesis, 150 mg linolenic acid was dissolved in 2 ml ethanol and diluted to 100 ml with 0.2M glycine buffer, pH 9.0. Soybean lipoxygenase was added to a concentration of 0.04 mg/ml. The solution was bubbled with a constant flow of oxygen for 45 minutes. In early preparations, Tween 20 (0.09% w/v) was added to solubilize the fatty acid. But later, it was decided that the Tween may have reacted in the solution and less of our hydroperoxides may have formed. In substrate solutions made later, Tween was not added; dilution of an ethanolic solution in alkaline buffer solubilized the fatty acid sufficiently.

The aqueous solution was acidified to pH 3 with HCl and extracted with an equal volume of either diethyl ether or a 50:50 mixture of methanol and chloroform (which proved more efficient). The nonaqueous layers were dried with $MgSO_4$ to remove water. The solvent was removed by rotary evaporation. The remaining residue was dissolved in 6 ml of hexane with 0.4 g silica. This slurry was applied onto a column of silica gel in hexane. The column was eluted with increasing concentrations of diethyl ether in hexane, following the procedure of Gardner (1982); the 13-hydroperoxide eluted in 25% (v/v) diethyl ether in hexane. The absorbances at 234 nm of samples of the fractions were checked; absorbance at 234 nm showed that a conjugated dienoic acid was present. All fractions with high aborbance at 234 nm were pooled together and evaporated down. The residue was dissolved in 2 ml methanol, and the concentration of the substrate was determined by checking the absorbance at 234 nm of a diluted aliquot and using the molar coextinction of the compound (25,000 $L \cdot mole^{-1}\ cm^{-1}$). This solution was then diluted to 12 mM with CH_3OH and stored at -20 ^{o}

The assay

The assay procedure was based on that of Schreier and Lorenz (1982). HPOOH lyase activity was measured by combining (3-x) ml 0.1M sodium phosphate buffer (pH 6.0), 0.01 ml of 12mM substrate (13-hydroperoxy linolenic acid), and x (0.1-0.3) ml of the enzyme preparation. The rate of disappearance of absorbance at 234 nm, due to cleavage of the conjugated double bond system, was observed for 1 minute. The initial slope was recorded and was assumed to be the activity of the enzyme. This method gave quick but not very specific measurement of disappearance of the hydroperoxide over time. In a typical assay, the observed reaction rate was about -.004 $A \cdot min^{-1}$ (0.48 $nmol \cdot min^{-1}$). The unit of activity is disappearance of 1 mole hydroperoxide$\cdot min^{-1}$.

The alternative assay

The assay observing the disappearance of absorbance at 234 nm is quick and sensitive, but it is not specific. There are other reactions of fatty acid hydroperoxides which also result in loss of absorbance at 234 nm. A more specific assay uses the aldehyde produced as a substrate of either alcohol dehydrogenase (oxidation of NADH) or aldehyde dehydrogenase (reduction of NAD^+). NAD^+ (0.3 ml 10mM), 0.03 ml 0.5 M DTT, 0.002 ml aldehyde dehydrogenase, and 3-x ml 0.1 M sodium phosphate buffer (pH 6.0) were combined. After x (0.1-0.3) ml enzyme was added, the rate of appearance of absorbance was observed. This assay, however, is less sensitive ($_{340}$ = 6220

$L \cdot mole^{l} cm^{-1}$). We attempted to use this assay with a tomato extract, but did not observe any change in absorbance at 340 nm. We set this assay aside pending preparation of more concentrated enzyme.

Protein determination

Protein determination was performed on four samples made in one day. The Coomassie Brilliant Blue procedure for protein determination (Bradford, 1976) was used to determine protein concentration, in the version of Zor and Selinger (1996), which increases sensitivity and linearity by measuring both A_{595} and A_{466} and plotting A_{595}/A_{466} vs. amount of sample. Bovine serum albumin (BSA), 0.0025 - 0.03 mg, was used as standard.

Purification: published procedure

The procedure of Fauconnier et al. (1997) was initially carried out on tomato leaves as a positive control. Leaves were homogenized in a blender with 200 ml 50mM NaP_i buffer (pH 6.7) containing 3 mM EDTA, 3 mM dithiothreitol, and 0.5% Triton X100. This extract was centrifuged for 10 min at 7250*g* (~6850 rpm). Our additional step: the supernatant was poured through a layer of Miracloth to remove some of the larger leaf fragments. The supernatant was stirred for 90 min and centrifuged for 20 min at 26,000*g* (~12,500 rpm).

Polyethylene glycol (PEG) 4000 (though published procedure says PEG 6000) was added to the supernatant to bring it to 7% (w/v) PEG. The solution was stirred for 30 minutes. The solution was centrifuged for 20 minutes at 26,000*g* (~12,500 rpm). PEG 4000 was added to the supernatant to a concentration of 23% (w/v) and the mixture was stirred for 30 minutes. The solution was centrifuged for 15 minutes at 12,000*g* (8500 rpm). The pellet was solubilized with 15 ml 20mM Tris-HCl buffer (pH 9, containing 0.5% Triton X-100). The supernatant solution was brought to 23% PEG and was stirred for 30 minutes, then centrifuged for 12 minutes at 12,000*g* (8500 rpm). Five ml TrisHCl buffer (pH 9, 0.5% Triton X-100) was used to dissolve the pellet, and 2mercaptoethanol was added to 0.1mM.

Modified procedure for tomato fruit

The procedure carried out on the tomato was modified to reflect the fact that the tomato fruit is quite different from the tomato leaf, containing large amount of acid liquid in vacuoles. Bicsak et al. (1982) used for extraction 0.09 ml 1.0 M NaHCO3 per g fruit in order to minimize dilution of tissue protein and achieve an extract pH ~ 7. 1,4Dithiothreitol (DTT) was added to the homogenate to obtain a final DTT concentration of 10mM. The pH was adjusted to 6.5-7.0 when necessary. The rest of the procedure previously described was carried out on the tomato fruit extract.

Results and discussion

Protein Determination

Table 1 compares the results from the Coomassie Brilliant Blue procedure for protein determination for BSA and samples 1, 2, 3, and 4. Sample 1 was from the supernatant after the first centrifugation of fruit extract (diluted 1:10), sample 2 was from the supernatant after the second centrifugation, sample 3 was from the 7% PEG solution (before centrifugation) and sample 4 was from supernatant with 7% PEG after centrifugation.

Table 1: Protein determination
(BSA standard curve, samples 1,2,3,4).

	1	2	3	4	5	6	7
Sample							
mg BSA	0	0.0025	0.005	0.01	0.015	0.02	0.03
A_{595}	0.389	0.448	0.538	0.649	0.719	0.784	0.909
A_{466}	0.606	0.587	0.600	0.565	0.512	0.470	0.412
A_{595}/A_{466}	0.642	0.762	0.896	1.148	1.404	1.67	2.21

		1			2	
Sample						
ml sample	0.05	0.15	0.30	0.05	0.15	0.30
A_{595}	0.415	0.445	0.441	0.464	0.581	0.661

A_{466}	0.632	0.651	0.612	0.599	0.574	0.471
A_{595}/A_{466}	0.658	0.682	0.720	0.774	1.01	0.0072 1.405
mg protein	0.0005	0.001	0.0017	0.0027		
mg/ml stock	0.1	0.06	0.056	0.054	0.048	0.0148
average		0.07			0.050	0.049
Sample		3			4	
ml sample	0.05			0.05	0.15	
A_{595}	0.483	0.15	0.561	0.444		
A_{466}	0.615			0.609	0.549	0.595 0.3
A_{595}/A_{466}	0.786	0.3	0.764	0.73	0.922	0.650 0.539
mg protein	0.0029	0.599		0.0019	0.0055	1.206
mg/ml stock	0.058	1.068	0.526	0.0374		
average		0.0083	1.454		0.0367	0.011
		0.055	0.0157		0.0369	0.0366
		0.055	0.0523			

The linear least squares line for A_{595}/A_{466} vs. mass BSA (mg) was used to determine amounts of protein in the samples.

Normally determination of protein concentration at each step of purification would be an integral part of enzyme purification. We initially decided to delay protein determination until we found conditions for the effective concentration of the enzyme. Satisfactory conditions were never found, hence we carried out determination only on samples from one purification.

Positive Control

As a positive control, to test that the procedure set forth by Fauconnier et. al. does indeed work in our hands, we carried out their procedure using garden-grown tomato leaves. Table 2 compares our experimental results with those of Fauconnier et al..

Table 2: Purification of HPOOH lyase from tomato leaves: comparison of these results to results of Fauconnier et al..

Fraction analyzed	(units/g wet weightTotal activity)	
	This work	Fauconnier et al.
Supernatant from first centrifugation	1.18	3.12
Supernatant from 7% PEG 6000 centrifugation	1.11	2.53
Pellet from 2nd 23% PEG 6000 centrifugation	1.26	1.47

*We used PEG 4000 since PEG 6000 was not available.

The difference in total activity may result from storage of the leaves at above 4 ^{o} prior to extraction.

Results from modified procedure

The purification of hydroperoxide lyase from tomato fruit met with two major problems: total activity was very low, barely detectable by the disappearance assay, and we were not successful in concentrating it by precipi-tation. We sought to purify the enzyme by making various changes on the original procedure.

We tested red, green, and breaker (changing from green to red) tomatoes to determine which had the most HPOOH lyase activity. We varied the amount of PEG added to the red and green tomato to determine at what % PEG the enzyme would be precipitated. We tried to purify the enzyme from lyophilized tomatoes (frozen under liquid nitrogen and vacuum dried) and frozen tomatoes (stored at -20 ^{o} without lyophilization). We also tested the storage of the enzyme at -20 ^{o} with or without addition of glycerol. As a final attempt at purifying the enzyme, we eliminated two centrifugation steps and attempted to precipitate the enzyme with $(NH_4)_2SO_4$.

Red tomato

Table 3 shows the results of purification from red tomatoes. The total activity was much lower than seen in tomato leaves and varied from sample to sample.

Table 3: Purification of HPOOH lyase from red tomatoes. Mass is given in grams of fresh tomato; activity is given in millU/g fresh mass. Each box shows results from one tomato sample.

Mass	Fraction analyzed	Activity
100	Supernatant after 1st centrifugation	7.90
	Supernatant after 7% PEG 4000 centrifugation	5.40
	Supernatant after 1st centrifugation at 23% PEG 4000	1.60
	Pellet after 2nd centrifugation at 23% PEG 4000	1.90
23.1	Supernatant after 1st centrifugation	6.06
	Supernatant after 7% PEG 4000 centrifugation	2.94
42.75	Supernatant after 1st centrifugation	17.50
	Supernatant after 2nd centrifugation	10.06
	Supernatant after 7% PEG 4000 centrifugation	4.91
	Supernatant after 1st centrifugation at 23% PEG 4000	7.95
	Pellet after 1st centrifugation at 23% PEG 4000	none
110.95	Supernatant after 1st centrifugation	62.64
	Supernatant after 2nd centrifugation	35.15
	Supernatant after 7% PEG 4000 centrifugation	25.40
	Supernatant after 1st centrifugation at 23% PEG 4000	15.86
	Pellet after 1st centrifugation at 23% PEG 4000	6.22
	Supernatant after centrifugation at 33% PEG 4000*	13.97

*33% PEG was used since we noted that the supernatant after 1st 23% PEG 4000 centrifugation still had some activity in it. We hoped that adding more PEG 4000 would precipitate the enzyme.

The pellets after each centrifugation were resuspended with Tris-HCl buffer (pH 9). Upon assaying the resuspended pellets, most absorbance vs. time plots did not show a clear, definable slope. Usually the slope varied so much that it was not easy to determine whether activity was present at all. In cases such as these, we normally assumed that the enzyme was not present and continued with purification. If the enzyme

was present in the supernatant (see data for fourth sample), then we tried to precipitate it with more PEG to concentrate it in the pellet.

From table 3, one can see that the fourth sample showed the highest total activity.

This is because more of the white locular tissue was removed and only the outer red (pericarp) tissue was used. This is one of the most recent changes made to our procedure.

Green tomatoes - fresh, lyophilized, and frozen. Since the HPOOH lyase concentration is higher in leaves, we thought that perhaps the concentration would be higher in green fruit than in red. We extracted and assayed 3 green tomatoes: 1 fresh, 1 lyophilized and extracted later, and 1 stored at -20 ^{o} and extracted later. Table 4 compares total activity of green tomato (extracted fresh) with that of a red tomato.

Table 4: Comparison of HPOOH lyase activity in green and red tomatoes. Masses are given for fresh samples.

Color	Mass(g)	Fraction analyzed	Total activity (milliU/g)
Green	47.35	Supernatant after 1st centrifugation	58.92
		Supernatant after centrifugation at 7% PEG 4000	
Red	42.75	Supernatant after 1st centrifugation	35.69
		Supernatant after 2nd centrifugation	17.54
		Supernatant after centrifugation at 7% PEG 4000	10.06
		Pellet after 1st centrifugation at 23% PEG 4000	4.91
			7.95

Green tomatoes are hard to find in late fall and winter, and for this reason purification from green tomatoes has not been repeated. It was expected that green tomatoes would be difficult to find in fall and winter. For this reason, a few tomatoes were lyophilized and a few were stored in the freezer. However, upon assaying after both types of storage, no activity could be definitely observed. The slope varied so much that it was difficult to tell if the slope was increasing or decreasing. We concluded that the enzyme is not stable to freezing of the fruit.

Breaker tomato. A breaker tomato is one that is just starting to turn red and thus appears yellow and pink. A breaker tomato was extracted, but upon assaying the extract a defined slope was not found. The slope varied a lot and did not give any information about the activity of the enzyme. Only one breaker tomato was used for purification because only one was available.

Various amounts of PEG added. Amounts of PEG-4000 were varied to determine at which concentration the enzyme would be most active in the supernatant and in the pellet. PEG (polyethylene glycol) is a solute that precipitates the enzyme by removing water from it. There were two ways in which we tested the amount of PEG needed to achieve maximum activity. In the first method, the extract was centrifuged twice, and equal amounts of supernatant were placed in small centrifuge tubes. PEG was added to reach concentrations from 7% to 32%, and the mixtures were then centrifuged. The supernatants and resuspended pellets were assayed. Red tomatoes showed the highest activity in the supernatant at 17% PEG and in the pellet at 32% PEG. In the green tomatoes, the highest activity seen in the supernatant was at 7% and in the pellet at 32% PEG.

The second procedure involved centrifuging the sample twice and adding PEG to the supernatant to bring it to 33% (w/v). Equal amounts of this solution were placed into test tubes and centrifuged. The pellets were resuspended with buffer containing various concentrations of PEG, and after 15 min incubation the solutions were centrifuged. The supernatant and the pellet (resuspended in Tris-HCl buffer) were assayed. The assay showed that in the red tomatoes, the highest activity seen in both the supernatant and in the pellet was at 10% PEG. In the green tomatoes, the highest activity seen in both the supernatant the pellet was at 25% PEG. It would be desirable to run this experiment again to verify the results, but after performing many trials, we concluded that PEG could not completely precipitate the enzyme into the pellet. A new precipitant, ammonium sulfate, was then used in an attempt to precipitate the enzyme.

Storage of HPOOH lyase. Because the enzyme is not stable, its activity decreases over time. This factor was the main reason why a new tomato preparation had to be made every two weeks (every week in some cases). Effective storage of the enzyme would allow more time to be spent on other aspects of the purification. Storage of the enzyme was tested by placing a sample of the enzyme into the freezer with or without glycerol. The samples were assayed 1 week later, and it was observed that the total activity remained approximately the same in both samples. The activity was a little higher in the sample with glycerol. Adding glycerol to our sample would dilute it, so we decided that since the activities with and without glycerol were approximately the same, it would be better to store the enzyme in the freezer without glycerol.

Omission of centrifugation steps and use of $(NH_4)_2SO_4$ to precipitate the enzyme. The activity of the red tomato (which is readily available) was very low in almost all cases.

We used less of the tomato, extracting only the outer pericarp. This helped to concentrate the enzyme. In January, it was decided that PEG was not effective in precipitating the enzyme. We switched to ammonium sulfate, since this chemical is commonly used to precipitate enzymes. To test at which concentration of ammonium sulfate the enzyme would be in highest amounts in the supernatant and the pellet, we centrifuged twice and added $(NH_4)_2SO_4$ to the supernatant to 95% saturation. This suspension was divided among several tubes, centrifuged, and the pellets were resuspended with various concentrations of ammonium sulfate (0-70% saturation). This solution was centrifuged and the supernatants were assayed. We discovered that at 50% ammonium sulfate, the highest level of the enzyme was seen in the supernatant. At 70% ammonium sulfate, the lowest activity of the enzyme was seen. Thus at 50% ammonium sulfate, most of the enzyme remained in the supernatant and at 70% ammonium sulfate, most of the enzyme remained in the pellet. See table 5 for exact values at various amounts of ammonium sulfate.

Table 5: Activity in samples resuspended at various % saturation in ammonium sulfate, after precipitation at 95% saturation. For these experiments a mass of 72.55 g (fresh) was taken.

Percent saturation	Activity $(NH_4)_2SO_4$ (units/ml)
0	0.037
30	0.024
40	0.035
50	0.044
60	0.026
70	0.007

Stirring (as in the original procedure) was eliminated because it was observed that the activity was the same whether the solution was stirred for 0, 30, 60, or 90 minutes before centrifugation and assay. See table 6 for exact values at different times of stirring.

Table 6: HPOOH lyase activity in supernatant after various times of stirring (extract of 165 g fruit).

Sample (after 1st centrifugation)	Activity (units/ml)	
Supernatant after 0 minutes of stirring, then centrifuging	0.136	
Supernatant after 30 minutes of stirring, then centrifuging	0.142	0.128
Supernatant after 60 minutes of stirring, then centrifuging	0.129	
Supernatant after 90 minutes of stirring, then centrifuging		

The second centrifugation step was therefore omitted since the activity merely decreased (some went into the pellet and the rest remained in the supernatant). After the first centrifugation, $(NH_4)_2SO_4$ was added, the suspension stirred for 20 minutes, and then centrifuged for 20 minutes at 12,500 rpm.

Table 7 shows the various results from using ammonium sulfate and the shortened procedure.

Table 7: Fractionation of HPOOH lyase with ammonium sulfate (extract of 158 g fruit).

Fraction assayed	Total activity (milliU/g)
Supernatant after 1st centrifugation	37.4
Supernatant after centrifugation at 55% saturation in $(NH_4)_2SO_4$	27.4
Pellet after centrifugation at 55% saturation	18.3
Pellet after centrifugation at 75% saturation	6.6

Activity in the 75% ammonium sulfate supernatant is not shown because no activity was observed. Often the activity would significantly decrease overnight when stored in the presence of ammonium sulfate. It seemed as though the enzyme was not stable in the presence of ammonium sulfate.

Our results have shown that the isolation and purification of the fruit enzyme is not the same as the isolation of the leaf enzyme, which was present at levels (units/g fresh weight) 100 times higher than in fruit, and could readily be precipitated at 23% PEG. More work needs to be done to explore how the enzyme can be precipitated or otherwise concentrated and to stabilize the enzyme for more than a few hours. PEG and ammonium sulfate do not seem to be able to precipitate the enzyme sufficiently to carry out further purification procedures.

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