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A CATALASE FROM TOMATO FRUIT

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Key Word Index—*Lycopersicon esculentum*; Solanaceae tomato; fruit; catalase; protein modification; metal ion inhibition; leaf.

Abstract—Catalase from the pericarp of green tomato fruit (*Lycopersicon esculentum*) has been purified to homogeneity as judged by silver staining of SDS-gels. The M_r of the holoenzyme was found to be 225 000 and that of each subunit.polypeptide, 55 000, indicating a tetrameric structure. Catalatic activity was found to be optimal in the pH range of 6.0–6.5, while half-maximal activity was observed at pH 4.5 and 8.0. Tomato pericarp catalase and bovine liver catalase activities were compared on the basis of their inhibition by heavy metals and protein-modifying reagents. The concentrations of silver nitrate which caused equivalent inhibition of activity differed by four orders of magnitude, with pericarp catalase being the more sensitive. Catalase from the pericarp was antigenically related to that in leaf and stem tissue. Catalase activity correlated with the amount of immunodetectable protein in pericarp tissue.

INTRODUCTION

Fruit development and ripening have been characterized as being in part a redirection of protein synthesis. Changes in protein composition of tomato fruit are the possible consequence of: changes in synthesis rates, changes in stability or the development of transport or processing mechanisms [1]. The activity of catalase (EC 1.11.1.6), the principal enzyme responsible for the breakdown of potentially harmful hydrogen peroxide, has been observed to reach a maximum level at the climacteric stage of tomato fruit development then decline to 10–20% of that amount as ripening proceeds [2, 3]. Concomitant accumulation of peroxides during fruit ripening has been reported and may be due to the decline in catalase level [4]. A role for peroxides in the process of fruit ripening has been suggested [5]. As a first step towards studying the molecular events involved in regulation of catalase synthesis and degradation, we have purified and characterized the enzyme from tomato, comparing some of its properties with commercially purified enzyme from bovine liver.

RESULTS

The enzyme was purified 32-fold to yield a specific activity of 36.7 μkat/mg protein (Table 1). The purified catalase appears as a single band after SDS-PAGE and silver staining (Fig. 1). The degree of catalase enrichment, as judged by gel profiles of progressively purified fractions, appears to be greater than the net increase in specific enzyme activity. We have observed a slow inactivation of highly purified tomato catalase which may explain this result, although it should be noted that the effects of enzyme inhibitors were similar whether fractions enriched 8- or 32-fold for catalase activity were tested.

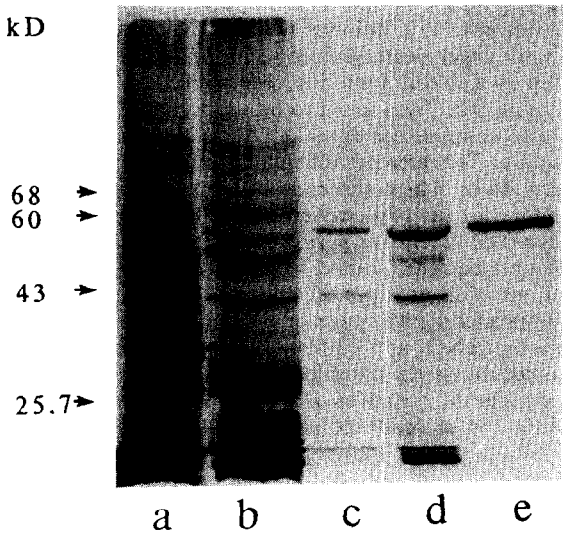

Fig. 1. Gel analysis of catalase-containing fractions by SDS-PAGE. The gel was silver-stained as described in the Experimental. Lane (a) crude filtered extract, 10 μg; (b) 25–40% $(NH_4)_2SO_4$ fraction, 15 μg; (c) Sephacryl S300 fraction, 7 μg; (d) DEAE-Sephacel fraction, 9 μg; (e) hydroxylapatite fraction, 2.5 μg. Arrows identify the positions of the indicated M_r standards.

The native M_r of the holoenzyme was calculated to be 225 000. Using SDS-polyacrylamide gel electrophoresis, the apparent subunit M_r was found to be 55 000, thus indicating that the holoenzyme consists of four subunits.

Catalatic activity of the tomato enzyme was found to be optimal in the range pH 6.0–6.5 in Pi buffer, while half-

Table 1. Purification of catalase from tomato pericarp

Fractions	Total units	Total protein (mg)	Specific activity* (μkat/mg)	Purification (fold)
Crude extract	334	290	1.15	1
25–40% $(NH_4)_2SO_4$	315	45.3	6.95	6.1
Sephacryl S-300	117	12.7	9.24	8.1
DEAE-Sephacel	38.0	2.2	17.3	15.1
Hydroxylapatite	37.8	1.03	36.7	32.2

Determined by measuring oxygen evolution as described in the Experimental.

maximal activity was found at pH 4.5 and 8.0 in K-Pi–citrate and Tris–HCl buffers, respectively.

The inhibition of tomato catalase by KCN, DTT, 2-mercaptoethanol and sodium azide was assayed in 50 mM K-Pi (pH 6.3) with 50% inhibition occurring at 6×10^{-5}, 2×10^{-2}, 1×10^{-1} and 1×10^{-6} M, respectively. These results are in general agreement with values reported for catalase purified from other plants [6, 7].

The effect of heavy metals on the activity of purified tomato pericarp catalase was investigated and compared with the results obtained when the bovine liver enzyme was used. The metal salts $AgNO_3$, $HgCl_2$, $CuCl_2$ and $Cd(Ac)_2$ were separately tested by addition to a reaction mixture prior to addition of enzyme. The reagent concentrations which decreased product formation by 50% are given in Table 2. Inhibitory effectiveness, given in decreasing order, was found to be: Ag > Hg > Cu > Cd for tomato catalase and Hg=Cu > Ag > Cd for bovine liver catalase. The greatest differential effect was observed using silver, where the concentrations which were inhibitory for the two enzymes differed by four orders of magnitude. Extensive purification of tomato catalase is not a prerequisite for inactivation by nanomolar concentrations of silver, since the enzyme-enriched fraction at the Sephacryl-S300 stage of purification was inhibited 50% by $AgNO_3$ at 5×10^{-8}M. The inhibition of tomato catalase by silver thiosulphate was also tested in this study. The thiosulfate form of silver must be present at *ca* 5×10^{-7} M in order to inhibit catalatic activity of the tomato enzyme by 50%. By preincubating silver thiosulphate in 1 mM ascorbic acid, 50% inhibition of tomato catalase was achieved at 1×10^{-7} M.

Protein-modifying reagents were assayed for their effect on the activities of tomato and bovine liver catalase and the results summarized in Table 3. The tomato enzyme was inhibited by lower concentrations than the bovine liver enzyme when NEM, pCMBA or DEPC was added. Iodoacetamide was slightly more effective at inhibiting the mammalian catalase. The hierarchy of inhibition was determined to be: (DEPC > *p*CMBA > NEM > iodoacetamide) for tomato catalase and in the case of bovine liver catalase: (DEPC=*p*CMBA > iodoacetamide > NEM).

Antiserum raised against purified catalase from tomato pericarp was used to probe SDS-PAGE electro-blots made from tomato extracts. Figure 2 illustrates a correlation between the previously reported [2, 3] levels of catalase activity in the pericarp, which is maximal at the breaker stage, and the presence of immuno-detectable enzyme protein. Subunit molecular masses appear to be identical when blots of purified tomato pericarp catalase (lane D) and fresh pericarp extracts (lanes E, F and G) are compared, indicating that degradation had not occurred during isolation. Leaf and stem samples (lanes A and B) that originally had catalase activity *ca* equal to that in the green pericarp sample (lane E) revealed polypeptides which react specifically with the antiserum after immunoblotting.

Table 2. Effects of metals on the catalatic activity of purified catalase from tomato pericarp and bovine liver

	Catalase (M)	
Metal	Tomato	Bovine liver
$AgNO_3$	2×10^{-8}	1×10^{-4}
$HgCl_2$	1×10^{-7}	3×10^{-5}
$CuCl_2$	5×10^{-6}	3×10^{-5}
$Cd(Ac)_2$	1×10^{-2}	$> 1 \times 10^{-2}$

Concentrations given are those required to reduce activity by 50% compared to a control.

Table 3. Effect of protein-modifying reagents on catalatic activity

	Catalase (M)	
Reagent	Tomato	Bovine liver
Iodoacetamide	5×10^{-2}	2×10^{-2}
NEM	5×10^{-3}	5×10^{-2}
p-CMBA	5×10^{-4}	1×10^{-2}
DEPC	1×10^{-4}	1×10^{-2}

Concentrations given are those which result in a 25% loss of activity compared to a control assay.

Reactions were run at pH's which are compatible with the solubilities and reactivities of the listed reagents.

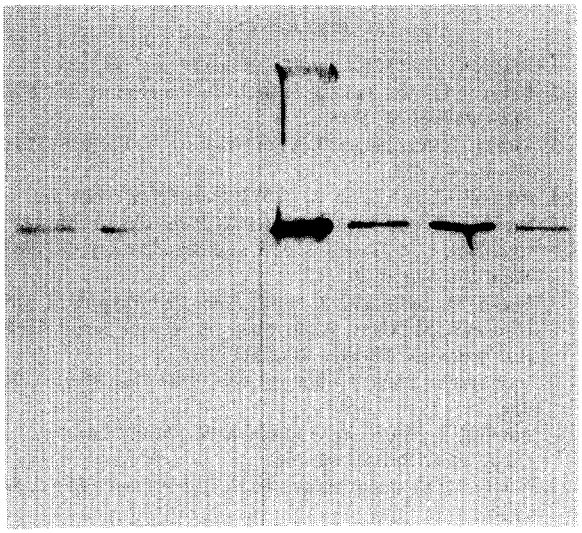

Fig. 2. Western blot analysis of tomato tissues using polyclonal antiserum raised against purified pericarp catalase. Lane (A) leaf, 0.2 μg; (B) stem, 0.3 μg; (C) prestained M_r standards; (D) purified pericarp catalase, 2 μg; (E) extract from ripe red pericarp, 4 μg; (F) extract from breaker stage pericarp, 4 μg; (G) extract from green pericarp, 4 μg.

DISCUSSION

Catalase subunits from the purified tomato pericarp enzyme have the same apparent M_r as do those from spinach [8], pumpkin cotyledon [9], cucumber [10] and cotton [11]. Larger subunits (59–65 000) have been reported for sweet potato root [7], maize isoenzymes CAT-1, CAT-2 and CAT-3 [6] and sunflower cotyledon [12]. In terms of holoenzyme and subunit M_r, inferred tetrameric structure, and inhibition by typical inhibitors of catalase, the enzyme from tomato pericarp is similar to catalase which has been characterized in other eukaryotes. A comparison of purified catalase from bovine liver and tomato pericarp, based upon enzyme susceptibility to inactivation by metal ions, demonstrated an extreme sensitivity of the tomato enzyme to silver. The overall hierarchy of metal toxicity towards purified tomato catalase which was found (Ag > Hg > Cu > Cd) is similar to that reported for cultured tomato cells (Hg > Ag- > Cu > Cd) [13]. In that study, the relative ranking of silver was questionable due to components in the culture medium which caused the metal to precipitate out of solution. Since enzyme inhibition by silver ion has been correlated with effects on protein sulphydryl groups [14], reagents which specifically react with these residues were used to compare the two catalases. Examination of the effects of these reagents upon catalatic activity again showed that the tomato enzyme is more readily inactivated. Similarly, modification by DEPC affects catalase activities differentially. But compared with silver ion, the differential effects of the sulphydryl-specific reagents on the two catalases are small. This indicates that sulphydryl groups readily available for alkylation or mercaptide formation are not likely to be the primary targets of the observed silver effects.

The detection of catalase in leaf and stem tissue extracts of tomato by means of Western blotting indicates that antigenically related forms of the enzyme are expressed in these tissues. In general, catalase enzyme activity appears to correlate with the level of immunoreactive protein. It is possible that more detailed study may reveal other forms of catalase in tomato tissue. Two forms of catalase have been detected in pumpkin cotyledon [15], consisting of subunits of M_r 55 000 and 59 000, respectively. Three different isoforms of catalase have been purified from maize [6], each form possessing subunits of the same (60 000) M_r. In each of the above studies, different specific activities were found for catalase enzymes from different tissues. The results obtained in the present study show the overall physical structure of catalase from tomato pericarp to be very similar to that of the enzyme isolated from other eukaryotes.

EXPERIMENTAL

Plant material. Green tomatoes (*Lycopersicon esculentum* Mill, cv FTE-674; Florida Tomato Exchange proprietary cultivar) were obtained from local wholesalers. The fruit was peeled and the pericarp sliced and stored at −70° until used.

Extraction. Frozen pericarp was pulverized in a Waring blendor, mixed with an equal amount (wt/vol) of 200 mM K-Pi (pH 7) containing 1 mM EDTA and 1 mM freshly prepared phenylmethylsulphonyl fluoride then stirred until the temp. of the slurry reached 4°. The crude extract was then filtered through 4 layers of cheesecloth. Catalase activity was pptd with $(NH_4)_2SO_4$ between 25 and 40% satn at 0°.

Purification scheme. The $(NH_4)_2SO_4$ pellet was resuspended in 50 mM K-Pi (pH 7) containing 150 mM NaCl, centrifuged, then chromatographed on a Sephacryl S300 column (110 × 2 cm) equilibrated with the same buffer. In some cases the $(NH_4)_2SO_4$ pellet was resuspended in column buffer made up to 50% (v/v) glycerol then stored at −20° prior to chromatography. The catalase peak eluting from the Sephacryl column at a vol. of 215 ml was pptd with $(NH_4)_2SO_4$ (90% satn) then resuspended in and dialysed against 20 mM Tris–HCl (pH 7.5). The dialysed sample was loaded onto a DEAE-Sephacel column (5 × 1 cm) equilibrated with the same buffer, and eluted with a gradient of 0–300 mM NaCl, in 20 mM Tris-HCl (pH 7.5). The collected fractions were assayed for catalase activity. Fractions containing catalase were pooled, dialysed against 10 mM K-Pi (pH 7), loaded onto a hydroxylapatite column (5 × 1 cm) then eluted with a gradient of 10 to 200 mM K-Pi (pH 7). All steps were performed at 0–5° unless otherwise noted, using a scheme modified from ref. [6].

Assays. Catalase activity was assayed by monitoring the evolution of O_2 with a Clark oxygen electrode and amplifier. Routine measurements were carried out in a stirred reaction vessel filled with 4 ml of 50 mM K–Pi (pH 6.3) containing 20 mM H_2O_2. Reactions were run at 25° and the activities routinely calculated from the initial linear rates except where noted. Studies on inhibition by metals and diethylpyrocarbonate (DEPC) were performed at pH 6.3 in a 50 mM MES buffer system while assays containing *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzoic acid (*p*-CMBA) were run at pH 7.0 and 7.45, respectively. Due to rapid enzyme inactivation caused by some inhibitors, the test substances were in all cases included in the assay mixture and the catalatic activity measured between 1 and 2 min after sample injection. The determination of catalase activity by measurement of O_2 evolution is in stoichiometric agreement with the breakdown of H_2O_2 as determined by spectrophotometric assay. There is no indication of other signifi-

cant H_2O_2-consuming activity in the initial 25–40% $(NH_4)_2SO_4$ fraction. Protein was determined using a modification [16] of the method of ref. [17], with bovine serum albumin as the standard. Each sample was precipitated with TCA at 0° then resuspended in 1 M NaOH prior to assay.

Gel electrophoresis. For denaturing gels, protein samples were solubilized by incubation in 2% SDS, 75 mM Tris–HCl (pH 6.8) and 30 mM dithiothreitol for 60 sec at 100°. The samples were electrophoretically separated in 11% polyacrylamide slab gels, using the discontinuous buffer system of ref. [18]. Bovine serum albumin (M_r 68 000), bovine liver catalase (M_r 60 000), ovalbumin (M_r 43 000) and alpha-chymotrypsinogen (M_r 25 700) were used as marker proteins. Gels were silver stained by the method of ref. [19].

Native molecular mass estimation. The M_r of tomato catalase was estimated using a Superose-12 (Pharmacia Inc.) gel filtration column equilibrated with 50 mM K-Pi (7), containing 150 mM NaCl and calibrated with Blue Dextran (average M_r 2 000 000), aldolase (M_r 158 000), bovine liver catalase (M_r 232 000), ferritin (M_r 440 000) and thyroglobulin (M_r 669 000).

Production of antiserum. Antibodies against purified tomato catalase were raised in female New Zealand white rabbits by an initial injection of 0.1 mg of antigen mixed with Freund's complete adjuvant followed by three biweekly injections of antigen mixed 1:1 with incomplete adjuvant.

Western blot protocol. Proteins were electrophoretically transferred from SDS-PAGE gels [20] onto an Immobilon-P membrane (Millipore Corp.) at 0° in buffer consisting of 25 mM Tris, 192 mM glycine and containing 0.01% SDS. Blots were blocked with 3% dry milk and polyclonal antiserum applied at a 1/1000 dilution. Detection was carried out using horseradish peroxidase-coupled secondary antibody [19].

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