

Effect of Freezing on the Activity of Catalase in Apple Flesh Tissue

Yiping Gong,[†] Peter M. A. Toivonen,* Paul A. Wiersma, and Changwen Lu

Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, 4200 Hwy 97, Summerland, British Columbia, V0H 1Z0, Canada

O. L. Lau

Okanagan Federated Shippers Association, in care of Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, 4200 Hwy 97, Summerland, British Columbia, V0H 1Z0, Canada

Catalase (CAT, EC 1.11.1.6) activity was measured in flesh tissue of six apple cultivars (*Malus domestica* Borkh. cvs. Braeburn, Gala, Jonagold, McIntosh, Red Delicious, and Spartan). Activity of CAT was determined for fresh and frozen tissue of the same fruit. Freezing resulted in reductions of 50 to 90% in CAT activity compared with the activity measured in crude extracts from fresh tissues. The rate of freezing had an impact on the level of reduction of CAT activity, with slower freezing procedures leading to greater losses in activity. Six additives to the extraction buffer were tested to evaluate their potential to reduce the inactivation of CAT from frozen tissue, but only EDTA and Tween 20 showed any benefit. However, EDTA and Tween 20 provided only partial recovery in CAT activity. In contrast, crude extracts prepared from fresh tissue showed no appreciable loss in CAT activity after frozen storage for two weeks at -80°C . Gel electrophoresis and immunological analysis indicated that the loss in CAT activity from tissue freezing could be attributed to loss of both the tetrameric CAT enzyme structure and total CAT protein. The implications of using freezing to preserve apple tissue samples prior to catalase activity analysis is discussed.

Keywords: *Apple; catalase; enzyme activity; freezing; inactivation*

INTRODUCTION

The enzyme catalase (CAT, EC 1.11.1.6) is widely found in plant and animal cells. CAT is known for its ability to protect cells from the oxidizing action of hydrogen peroxide (H_2O_2) produced by superoxide dismutase (EC 1.15.1.1) and flavoprotein enzymes (Thompson et al., 1987). It decomposes two molecules of H_2O_2 into molecular oxygen and water. CAT has received attention as one of the antioxidant enzymes that responds to stresses such as low temperature (Gianinetti et al., 1993; Wang, 1995), salt stress (Shalata and Tal, 1998), and high oxygen (Pritchard and Hudson, 1967). Changes in CAT activity are also associated with senescence (Kar and Mishra, 1976; Brennan and Frenkel, 1977) and dormancy of plant buds (Nir et al., 1986).

CAT enzyme from animal tissue has been reported to be inactivated or denatured during lyophilization, resulting in a much lower activity for lyophilized CAT than for the crystalline or native enzyme (Tanford and Lovrien, 1962; Deisseroth and Dounce, 1969). Shikama and Yamazaki (1961) reported an 80% reduction in CAT activity from ox liver following freezing and thawing. However, there have been no reports on the effect of freezing on CAT activity in plant tissues. Plant tissues are often frozen in liquid nitrogen (N_2) and stored for varying periods of time before enzyme assays are performed in plant biochemical experiments. Alter-

nately, grinding of fresh tissue to a fine powder to improve enzyme extraction is accomplished by freezing with liquid N_2 prior to and during the grinding procedure. Therefore, it is very important to document whether freezing procedures can affect CAT activity in plant tissue extracts. This study was conducted to assess the effects of freezing apple flesh tissues on subsequent CAT activity in crude enzyme extracts.

MATERIALS AND METHODS

Plant Material and Sampling Procedures. Apples (*Malus domestica* Borkh., cvs. Braeburn, Gala, Jonagold, McIntosh, Red Delicious, and Spartan) were harvested at commercial maturity from the experimental orchard at the Pacific Agri-Food Research Centre, or from commercial orchards in Summerland, British Columbia. The fruits were stored in air at 1°C prior to enzyme assays.

Flesh tissues were taken from the periphery of apple fruits with a 10 mm diameter cork borer and cut into <10 -mm-thick disks which were free of peel and core tissue. The tissue disks from a particular cultivar were collected into a single bulk sample. The bulk sample was mixed, using a spatula to ensure a uniformity within the sample. Subsamples of tissue disks were either frozen using different freezing procedures or immediately homogenized in extraction buffer.

Effect of Freezing Procedure. Braeburn apples (10 fruits) were removed from cold storage and held at room temperature for 3 days. The flesh tissue disks from each fruit were mixed and then divided into four parts (20 g each) for treatment: (1) not frozen, assayed for CAT activity immediately (control); (2) frozen in liquid N_2 and stored at -80°C ; (3) frozen and stored at -80°C ; (4) frozen and stored at -25°C . Treatments (2), (3), and (4) were stored at the designated temperatures overnight and the activity of CAT was assayed the next day. Each treatment was replicated three times.

* Corresponding author. Fax: (250)494-0755. E-mail: toivonenp@em.agr.ca (Centre Contribution No. 2035).

[†] Current address: USDA-ARS, Tree Fruit Research Laboratory, 1104 N. Western Ave., Wenatchee, Washington 98891.

Table 1. Catalase Activity in Crude Extracts of Apple Flesh Tissue (cv. Braeburn) after Freezing at Different Temperatures as Compared with a Non-frozen Control

| treatment | catalase activity (units/mg protein) ^a | relative activity | protein ^b (μg mL ⁻¹) |
|--|---|-------------------|---|
| nonfrozen (control) | 1.64 (0.02) ^c | 1.00 | 207 (1) ^c |
| frozen in liquid N ₂ and stored at -80 °C | 0.47 (0.02) | 0.29 | 196 (6) |
| frozen and stored at -80 °C | 0.35 (0.01) | 0.21 | 173 (6) |
| frozen and stored at -25 °C | 0.29 (0.01) | 0.18 | 170 (2) |

^a One unit = 1 mM H₂O₂ consumed per min. ^b Protein content of crude enzyme extract. ^c Means of three replicates; values in parentheses are standard errors.

Cultivar Differences. Six or more apples per cultivar (Braeburn, Gala, Jonagold, McIntosh, Red Delicious, and Spartan) were removed from 1 °C storage. Half of the fruit were sampled immediately and CAT activity assayed. The remaining fruit were held at room temperature (20 °C) for seven days. The disks of flesh tissue taken from the six fruit were pooled and thoroughly mixed before dividing into six uniform samples. Three of the six samples were assayed for CAT activity immediately (without freezing), and the other three were assayed after freezing in liquid N₂.

Effect of Extraction Buffer Additives. Braeburn apple flesh tissue was frozen in liquid N₂ and stored at -80 °C until assay. Frozen tissue (5 g, equivalent to approximately 10–12 disks) was homogenized in 15 mL of 0.1 M Tris-HCl buffer (pH 8.5) containing 10% polyvinylpyrrolidone (PVPP), and 10% glycerol plus one of six additives: (1) 1 and 2 mM dithiothreitol (DTT), (2) 1 and 2 mM phenylmethylsulfonyl fluoride (PMSF), (3) 0.25 and 0.50% Triton X-100; (4) 0.25 and 0.50% Tween 20, (5) 0.1 and 0.5% sodium dodecyl sulfate (SDS), and (6) 0.5, 2, and 5 mM ethylenediamine tetraacetate, disodium salt (Na₂-EDTA). CAT activity was calculated relative to a buffer-flesh tissue extract containing none of the six additives (control).

Frozen Storage of Crude Extract. Red Delicious and Gala flesh tissue (5 g) were homogenized with 15 mL of 0.1 M Tris-HCl buffer (pH 8.5) containing 2 mM Na₂-EDTA, 10% (w/v) PVPP, and 10% glycerol. The homogenate was centrifuged at 15 000*g* for 15 min at 4 °C. The supernatant was divided into aliquots and placed into microcentrifuge tubes. The CAT activity of the fresh, nonfrozen, extract was assayed immediately. The remainder of the tubes were frozen in liquid N₂ and then stored at -80 °C for up to four weeks. At 1, 2, and 4 weeks one tube was removed, thawed, and assayed for CAT activity.

Assay of CAT Activity. CAT activity was determined by the disappearance of H₂O₂ in the enzyme reaction mixture (Brennan and Frenkel, 1977; Du and Bramlage, 1995). Enzyme extract (0.25 mL) was added to 2 mL of assay mixture containing 50 mM Tris-HCl buffer (pH 6.8) and 5 mM H₂O₂. The reaction was allowed to proceed for 10 min at a 20 °C incubation temperature. The reaction was terminated by adding 0.25 mL of titanium reagent (20% titanate tetrachloride in concentrated HCl, v/v). A time zero sample was obtained by adding 0.25 mL of titanium reagent immediately after adding the enzyme extract to the assay mixture. Absorbance was read at 415 nm and the difference in absorbance at 10 min and time zero was calculated. The consumption rate of H₂O₂ was calculated using a calibration curve of H₂O₂ at a concentration range from 0.25 to 2.5 mM. Protein concentration of the extract was determined by the method of Bradford (1976) using bovine serum albumin as a standard. CAT activity is expressed as units per mg protein, with 1 unit defined as 1 mM H₂O₂ consumed per min.

Purification of CAT Protein. Fresh or frozen Braeburn apple tissue (30 g) was homogenized in 90 mL of 0.1 M Tris-HCl buffer (pH 8.5) containing 2 mM Na₂-EDTA, 10% (w/v) PVPP, and 10% glycerol. After centrifuging at 15 000*g* for 15 min at 4 °C, the supernatant was decanted into a clean tube. The protein in the supernatant was precipitated with addition of 85% saturated ammonium sulfate solution at 0 °C. This was centrifuged at 15 000*g* for 15 min at 4 °C, the supernatant was decanted, and the protein pellet was then re-suspended in 0.1 M Tris-HCl buffer (pH 7.0). The resultant purified

protein extract was dialyzed overnight using 3500 MW exclusion dialysis tubing (Spectra/Por, Spectrum, Houston, TX). Poly(ethylene glycol) (MW 8 000) was then employed as a solute-absorbing matrix to concentrate the protein solution (Pohl, 1990).

Nondenaturing PAGE. Nondenaturing PAGE was carried out as described by Bollag et al. (1996) using a Mini Gel System (Bio-Rad, Hercules, CA). Protein samples (12 mg protein for fresh tissue and 12 mg or 24 mg protein for frozen tissue) were loaded on a 5% polyacrylamide gel. The weight of protein loaded per lane was determined using the protein concentration of the extract and adjusting the volume of extract loaded, such that the target weight of protein was loaded. Electrophoresis of the gel was run at 100 V at a temperature of 4 °C. After the electrophoresis, the gel was cut in half vertically between lanes. One-half of the gel was stained for CAT activity with the method described by Clare et al. (1984). The proteins on the other half were transferred to a nitrocellulose membrane as described below.

SDS-PAGE and Western Blotting. SDS-PAGE and sample preparation were carried out as described by Bollag et al. (1996) using a Mini Gel System (Bio-Rad, Hercules, CA) with a 7.5% gel at 100 V. Following electrophoresis, proteins were electroblotted (30 V, overnight) onto nitrocellulose membrane (0.45 mm, Bio-Rad) using a Bio-Rad Trans-Blot cell containing transfer buffer (25 mM Tris, 0.192 M glycine, and 20% methanol). The nitrocellulose membrane was soaked for 2 h with 1% nonfat milk in PBST (i.e., phosphate buffer solution containing 10 mM phosphate buffer (pH 7.4) and 0.8% NaCl, 0.02% KCl, and 0.1% Tween 20). The membrane was then incubated in a medium of anti-catalase serum in PBST with 1% nonfat milk at a ratio of 1:1000 (Kunce and Trelease, 1986). After the membrane was washed with PBST three times, it was incubated for 2 h with alkaline-phosphatase conjugated goat anti-rabbit IgG diluted in PBST at a ratio of 1:3000. The membrane was then washed three more times with PBST. The membrane was then washed with an alkaline buffer (100 mM NaCl and 5 mM MgCl₂ in 100 mM Tris-HCl, pH 9.5) and then developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (NBT and BCIP) reagent (GIBCO/BRL, Life Technologies, Rockville, MD) in alkaline buffer until the bands were visible.

RESULTS

Effect of Freezing Procedure. CAT activity in Braeburn apple tissue was reduced by 70 to 80% as a result of freezing (Table 1). CAT activity for tissue from the "rapid freezing in liquid N₂ and storage at -80 °C" protocol was higher than the activities for tissues from the other two freezing protocols. This result is similar to results reported with catalase extracted from liver tissues (Shikama and Yamazaki, 1961). The samples frozen and stored at -80 °C had greater activity than those frozen and stored at -25 °C. The loss of activity in the liquid N₂ freezing protocol could not be attributed to loss of protein in the extract because protein content of the extract was similar to that from the nonfrozen, control extract. However, the -80 and -25 °C freezing and storage protocols resulted in lower protein contents in the crude tissue extracts. Therefore, loss of CAT

Table 2. Effect of Freezing on Catalase Activity for Six Apple Cultivars Which Were Sampled Immediately After Removal from 1 °C Storage and After an Additional 7 days Holding at 20 °C

| cultivar | from 1 °C storage | | | after 7 days at 20 °C | | |
|---------------|--|------------------------------|--------------------------------------|-----------------------------|------------------------------|-------------------------|
| | fresh (units/mg protein) ^b | frozen (units/mg protein) | loss in activity ^a (%) | fresh (units/mg protein) | frozen (units/mg protein) | loss in activity (%) |
| Braeburn | 2.87 (0.08) ^c | 0.71 (0.02) | 75.1 | 3.08 (0.02) | 0.55 (0.01) | 82.0 |
| Gala | 2.57 (0.01) | 0.53 (0.01) | 79.4 | 3.24 (0.01) | 1.57 (0.02) | 51.5 |
| Jonagold | 2.31 (0.02) | 0.54 (0.01) | 76.5 | 3.08 (0.03) | 0.91 (0.02) | 70.6 |
| McIntosh | 1.79 (0.02) | 0.17 (0.01) | 90.4 | 2.29 (0.03) | 0.20 (0.01) | 91.5 |
| Red Delicious | 2.98 (0.01) | 0.80 (0.01) | 73.3 | 3.88 (0.01) | 1.02 (0.03) | 73.8 |
| Spartan | 1.48 (0.02) | 0.26 (0.01) | 82.7 | 3.03 (0.28) | 0.68 (0.03) | 77.6 |

^a ((fresh activity – frozen activity)/fresh activity) x 100. ^b One unit = 1 mM H₂O₂ consumed per min. ^c Means of triplicates; values in parentheses are standard errors.

Table 3. Effect of Six Different Extraction Buffer Additives on the Activity of Catalase in Fresh and Frozen Braeburn Apple Tissues

| additive | additive concentration | relative activity in frozen tissue | relative activity in fresh tissue |
|--------------|---------------------------|---------------------------------------|--------------------------------------|
| control | | 1.00 ^a | 1.00 ^a |
| DTT | 1 mM | 0.30 | |
| | 2 mM | 0.31 | |
| PMSF | 1 mM | 1.01 | |
| | 2 mM | 0.66 | |
| Triton X-100 | 0.25% | 0.98 | |
| | 0.50% | 1.09 | |
| Tween 20 | 0.25% | 1.42 | 0.95 |
| | 0.50% | 1.22 | 1.00 |
| SDS | 0.10% | 0.36 | |
| | 0.50% | 0.18 | |
| EDTA | 0.5 mM | 2.56 | 0.98 |
| | 2.0 mM | 3.08 | 0.94 |
| | 5.0 mM | 1.25 | 0.87 |

^a Means of duplicates.

activity in these two freezing protocols could be attributed to protein loss in the extraction procedure.

Cultivar Differences. In this experiment, the inactivation of CAT by freezing was tested for six apple cultivars: Braeburn, Gala, Jonagold, McIntosh, Red Delicious and Spartan (Table 2). Freezing resulted in a loss of 70 to 90% of activity for the fruit upon removal from 1 °C. After one week of holding at 20 °C, the activities in fresh, nonfrozen, apple flesh tissue were higher than those for apples sampled immediately after removal from 1 °C. However, when these apple tissues were frozen in liquid N₂, the activity of CAT in all cultivars, except Gala, was reduced similarly as those samples frozen immediately after removal from 1 °C storage. The reduction of CAT activity in Gala from 20 °C was 51.5% after freezing. The greatest reduction in CAT activity due to freezing was seen for McIntosh apple tissue, whether sampled immediately after removal from 1 °C storage or after an additional week of holding at 20 °C. The differences in loss of CAT activity between cultivars and between the first and second sampling (i.e., after 1 °C versus after one week of holding at 20 °C) could not be attributed to differences in protein contents of the crude extracts (data not shown).

Effect of Extraction Buffer Additives. The results for preservation of CAT activity in extracts from frozen tissues using different additives are shown in Table 3. The activity of CAT was reduced to the greatest extent with the addition of DTT (1 and 2 mM) or SDS (0.10 and 0.50%) to the extraction buffer. Both concentrations of DTT and the low concentration SDS treatments resulted in similar reductions (~70%), whereas the higher SDS concentration reduced the CAT activity of

Table 4. Changes of Catalase Activity in Crude Extract Stored at –80 °C after Pre-freezing with Liquid N₂

| cultivar | weeks at –80 °C | catalase activity (units/mg protein) ^a | relative activity |
|---------------|--------------------|--|----------------------|
| Red Delicious | 0 | 2.98 (0.005) ^b | 1.00 |
| | 1 | 2.85 (0.016) | 0.95 |
| | 2 | 2.73 (0.012) | 0.92 |
| | 4 | 2.37 (0.013) | 0.79 |
| Gala | 0 | 2.57 (0.007) | 1.00 |
| | 1 | 2.52 (0.002) | 0.98 |
| | 2 | 2.57 (0.001) | 1.00 |
| | 4 | 2.14 (0.022) | 0.83 |

^a One unit = 1 mM H₂O₂ consumed per min. ^b Means of two replicates; values in parentheses are standard errors.

the extract by 82%, compared with that of the nonadditive control. Triton X-100 did not have any appreciable effect on the activity of CAT in the crude extract. PMSF had no effect at a 1 mM concentration, but at 2 mM PMSF CAT activity was reduced by 34%. Addition of Tween 20 (0.25 and 0.50%) and EDTA (0.5, 2.0, and 5.0 mM) resulted in an increase in CAT activity in the extracts from frozen tissue. The addition of 0.5 and 2.0 mM EDTA resulted in the greatest increase of CAT activity for the extract (256% and 308%, respectively). The highest concentration of EDTA had a much smaller effect on CAT activity. Tween 20 at a 0.25% concentration increased CAT activity by 40%, however, the 0.50% concentration of Tween 20 resulted in a much smaller increase. The addition of Tween 20 or EDTA to the extraction buffer did not improve CAT activity in fresh apple tissue extracts. The effect of EDTA could not be attributed to improved protein extraction because none of the EDTA treatments resulted in any change for total protein concentration in the extract in comparison with the control containing no additives (data not shown). Tween 20 did increase protein content of the extract by 47%, however, the protein content was independent of the concentration of this additive.

Frozen Storage of Crude Extract. The extraction solution from apple flesh tissue was assayed after storage at –80 °C for 1, 2, and 4 weeks. There was no appreciable change in the CAT activities of Gala and Red Delicious extracts after 1 and 2 weeks storage (Table 4). However, approximately 20% of activity was lost after 4 weeks in both cultivars. There was no appreciable decline in protein content of the extracts over the four week experiment (data not shown). The data therefore suggest that CAT activity in crude extract solutions made from fresh tissue was stable for at least 2 weeks at –80 °C.

Assay of CAT Protein. The freezing of apple tissue had a pronounced effect on the resulting pattern of CAT staining in nondenaturing activity gels (Figure 1A). Fresh tissue showed reproducibly higher intensity of

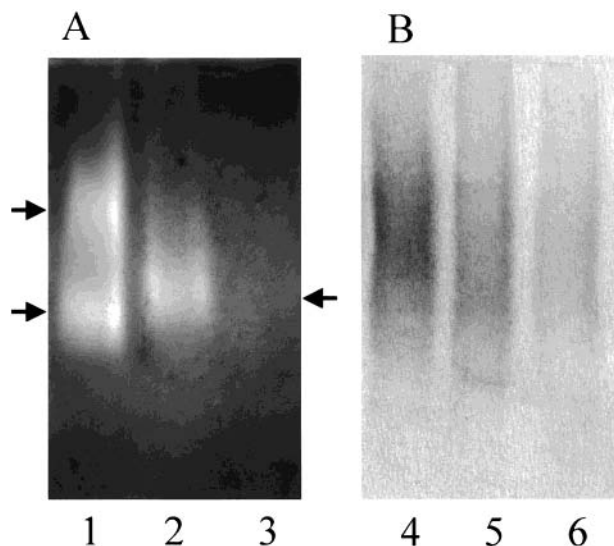


Figure 1. Comparison of CAT activity staining on a non-denaturing PAGE gel (A) and on anti-CAT (Kunce and Trelease, 1986) immunoblots of that gel (B) for protein extracted from fresh (lanes 1 and 4) and frozen tissue (lanes 2, 3, 5, and 6) from Braeburn apples. Lanes 1, 3, 4, and 6 were loaded with 12 mg of total protein; lanes 2 and 5 were loaded with 24 mg of protein. Arrows indicate bands of CAT activity from fresh (left of panel A) and frozen (right of panel A) tissues. Note that the PAGE gel was negatively stained for CAT activity.

signal, as well as a considerable quantity of slower migrating, presumably higher molecular weight, active CAT enzyme (lane 1). After freezing (lanes 2 and 3), the extracts demonstrated much less CAT activity. (For this analysis lanes 1 and 3 should be compared as each were loaded with an equivalent 12 mg of protein extract, but lane 2 contained double the amount of extract to better compare the positions of the bands of activity.) As indicated by the two arrows on the left of Figure 1A there were two broad but distinct bands of CAT activity in lane 1. When apple tissue was frozen before extraction only one band was seen (lanes 2 and 3) which is indicated by the arrow on the right side of Figure 1A. Whether this change was due to a different pattern of protein migration or to the loss of CAT protein from the extract was analyzed by blotting equivalent lanes in a Western blot and detecting with anti-CAT antibodies (Figure 1B). Although the non-denaturing conditions did not provide a good resolution separation of the CAT proteins, several general observations could be made. The first was a general increase in migration distance for the CAT protein from frozen tissue. This would be consistent with a breakdown of higher order protein structures. The second observation was that the total amount of protein labeled for fresh tissue (lane 4) appeared to be higher than that for either lane of frozen tissue (lanes 5 and 6) even though lane 5 was loaded with twice the quantity of protein. These results showed that inactivation of CAT was at least partially due to the reduction of recoverable CAT enzyme protein in the extract.

The Western blot of the SDS-PAGE gel indicates that there were two major CAT protein bands corresponding to molecular weights of between 50 and 55 kD in both fresh tissue and frozen tissue (Figure 2). The reaction bands intensities were smaller in frozen tissue than in fresh tissue, indicating a decrease in the amount of protein in these two bands. A third, minor band was found in only frozen tissue, suggesting that the freezing

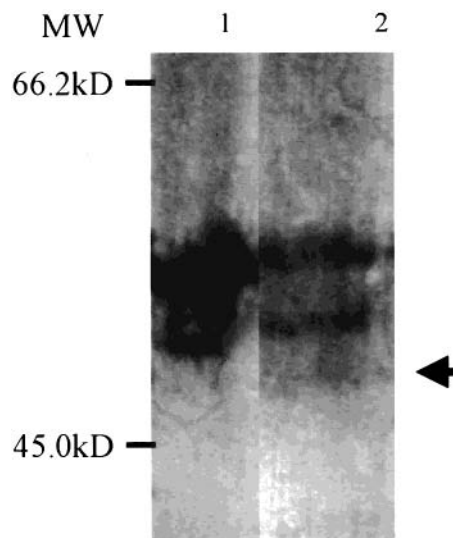


Figure 2. Western blot of an SDS-PAGE gel of soluble proteins from fresh (1) and frozen (2) Braeburn apple tissue. The bands shown are those which cross-reacted with anti-CAT (Kunce and Trelease, 1986). Twelve milligrams of protein was loaded to each lane of the SDS-PAGE. The arrow indicates a weak band of lower molecular weight CAT protein which developed only in the frozen tissue.

process resulted in the accumulation of a smaller subunit of CAT enzyme. These results suggest that the total amount of CAT protein was reduced in frozen tissue, and that freezing also resulted in at least some degradation of the protein into smaller molecular weight subunits.

DISCUSSION

Loss of CAT activity in apple tissue extracts was greatest when the sample was frozen at -25°C , slightly less when frozen at -80°C , and even less again if frozen in liquid N_2 . These results suggest that the inactivation which occurred during freezing of the tissue sample was greater if the freezing process was slower (i.e., liquid N_2 versus -80°C) or if it was performed at a higher temperature (i.e. -25°C). However, the loss of activity could not be attributed to loss of total protein. Therefore, the freezing process appears to be specifically reducing CAT enzyme activity in apples. This conclusion is supported by the fact that activities of other enzymes such as superoxidase dismutase, peroxidase, and polyphenol oxidase are not affected by freezing in apples (unpublished data). In addition, because freezing is a common practice for processing and preserving tissue samples in plant biochemical and molecular studies, it is fair to assume that freezing does not result in a loss of activity of many other enzymes.

Six apple cultivars were studied and it was determined that all six showed a major reduction in CAT activity due to freezing of tissue prior to extraction, and the range of activity loss was generally between 70 and 90%. The fact that there is a difference between cultivars in the level of activity loss is of concern in comparative studies. Currently, there is great interest in understanding postharvest disorders in relation to oxidative injury, and CAT is an important component of the endogenous antioxidant protectant system in plant tissues (Shewfelt and Purvis, 1995). The susceptibility of apples to certain physiological disorders is highly dependent on cultivar (Du and Bramlage, 1995).

In the case of apples, if samples are frozen prior to analysis, measurement errors for CAT associated with freezing may lead to misinterpretation of the importance of this enzyme in the development of the particular postharvest disorder in question.

Enzyme extraction and purification buffers often contain DTT, a thiol compound, to protect enzyme protein thiols from oxidation (Dignam, 1990), and PMSF as a serine protease inhibitor (Deutscher, 1990). In our experiments, when frozen apple tissue was extracted in buffer with different additives, addition of DTT and PMSF did not improve the recovery of CAT activity. The inactivation associated with freezing was, therefore, not likely to have been caused by protein thiol oxidation or serine protease activity.

EDTA, a chelator of divalent cations, increased the activity by 2.5 to 3-fold. The fact that EDTA addition did not affect protein content of the extracts rules out the possibility that its action was through enhancement of protein content of the extract. This rules out the possibility of EDTA acting as an anti-coagulant, an activity which is often associated with EDTA (West and Todd, 1963). Divalent ions such as Cu^{2+} may be released from the tissue during thawing, and Cu^{2+} is an inhibitor of CAT (Bergmeyer, et al., 1963). EDTA is known to sequester divalent cations and, therefore, may be acting to remove Cu^{2+} (or other inhibitory cations released during thawing) from the extract. However, EDTA is also an inhibitor of metallo-proteases (Deutscher, 1990). Therefore, the addition of EDTA may have improved the activity of CAT in extracts from frozen tissues either directly through sequestering divalent metal ions or through inhibition of metallo-proteases. The lower level of activity at higher EDTA concentration is likely to be related to its capability to sequester metal anions. CAT in its active form contains iron (Lardy, 1949), and it is therefore likely that at higher EDTA concentrations iron was being sequestered from CAT, thereby reducing its activity in the extract.

Addition of detergents affects solubilization of enzyme protein. SDS is a very strong surface-active agent and is often used for solubilizing membrane proteins. However, SDS is an anionic detergent and, as such, can dissociate protein into polypeptide subunits (Pit-Rivers and Impiombato, 1968) causing a loss of enzyme activity, particularly if enzyme activity is dependent on multimeric structure as is the case with CAT. Lardy (1949) indicates that CAT is highly susceptible to inactivation by anionic moieties, thereby supporting this line of argument. Therefore, it is most likely that dissociation of the active multimeric CAT protein to inactive subunits is the mechanism by which SDS reduced the CAT activity of the extract. Triton X-100 is also used to solubilize membrane-bound enzymes (Neugebauer, 1990). In this experiment Triton X-100 had no effect on CAT activity in frozen apple tissue. Tween 20 showed a large increase in recoverable CAT activity for extracts of frozen tissue. Tween 20 is also a detergent used to increase the solubilization enzymes from membranes such as phosphatidate phosphatase from microsomes of microspore-derived embryos (Kocsis, et al., 1996). This is the likely explanation for the effect of Tween 20 in the extraction buffer, because total protein concentration in the crude extract was higher. However, Kuwabara et al. (1997) reported that Tween 20 was essential for stabilization of a tetrameric protease. It may, therefore, also be possible that Tween 20 is also

acting to stabilize tetrameric protein structure of CAT. There is no clear explanation as to why Triton X-100 did not also improve the recovery of CAT activity in the crude extracts from frozen tissue.

Shikama and Yamazaki (1961) assayed CAT from fresh beef liver enzyme extract solution which had been frozen under different temperatures from -6 to -192 °C. After freezing for 10 min between -12 and -75 °C the loss of CAT activity was 20%. Between -120 and -192 °C, the solution retained its activity against freezing. In our experiment, CAT activity was retained in the enzyme extract solution for at least two weeks at -80 °C. Apparently, dissociation of CAT did not occur in frozen extract solution. Shikama and Yamazaki (1961) reported that concentrated enzyme extract solution and addition of protective reagents such as gelatin or glycerol may potentially prevent freezing-induced inactivation of CAT from ox liver. Further tests should be done for apple fruit to see if it may be possible to store the extract solution containing gelatin or glycerol below -80 °C for longer durations.

CAT activity in beef erythrocyte and beef liver was reduced 80–90% by lyophilization, as compared with native enzyme (Tanford and Lovrien, 1962; Deisseroth and Dounce, 1969). It was suggested that some of the CAT enzyme was partially dissociated to smaller molecular subunits by freezing, according to the results of sedimentation analysis, and that this resulted in inactivation of CAT. Similarly, in the present experiment, immunological analysis of proteins from fresh and frozen apple tissues indicated that the reduction of CAT activity in apple tissue by freezing was due to, at least in part, a reduction in the amount of active CAT protein. It is probable that the inactivation of apple CAT was, at least partially, due to dissociation of the multimeric form of the enzyme in a fashion similar to that of the animal protein. This was supported by the nondenaturing gel electrophoresis patterns for apple CAT suggesting a reduction in size after freezing. The dissociated enzyme subunits may be less stable and, therefore, more susceptible to protease cleavage or aggregation. In both of these cases the enzymatic activity would be reduced. Additives were tested to reduce the effect of freezing on CAT activity loss. While EDTA and Tween 20 showed some benefit, the level of CAT activity recovery was not great enough to consider them as alternate approaches to using extraction procedures which avoid freezing.

The results reported here have several implications on the interpretation of catalase activity analyses in plant tissues, specifically in apple tissues. As apples show this loss of activity in freezing of tissues, CAT extraction should only be done with fresh tissues. Green peppers do not appear to be subject to this problem (unpublished data) and this raises the question as to which types of plant tissues are susceptible to freeze-induced CAT inactivation. Considering that there is not a clear understanding of the basis for this loss in activity, it is prudent to suggest that each plant tissue be tested prior to deciding whether it can be frozen prior to extraction of crude extract for CAT assay. There have been some studies published regarding analysis of CAT activity in different apple cultivars using methods involving freezing procedures (Du and Bramlage, 1995; Masia, 1998), but the results here show that some cultivars show greater loss in activity than others. Therefore, it may be advisable to reconsider the interpretation of results for cultivar comparisons from these

previously published works. In respect to previous studies on CAT changes during ripening of apples in which frozen samples were used (Du and Bramlage, 1995; Masia, 1998), it is difficult to make comments regarding the impact of these results other than saying that the levels of CAT measured in these studies may have been significantly lower than the actual levels in the tissue. It may be that freezing could have a differential effect on fruits at different stages of maturity, however, this issue was not examined in this work. Nonetheless, it may be prudent to use fresh tissue in future studies involving apples of different stages of development and maturity. In conclusion, this work indicates that the use of freezing for processing or storage of samples prior to analysis of CAT activity is not recommended in apples. Use of freezing prior to CAT extraction for other plant tissues should be considered on a case by case basis until there is a better understanding of the scope of this problem.

ABBREVIATIONS USED

BCIP, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt; CAT, catalase; DTT, dithiothreitol; Na₂-EDTA, ethylenediamine tetraacetate, disodium salt; NBT, nitroblue tetrazolium; PBST, phosphate buffered saline with Tween 20; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

ACKNOWLEDGMENT

The authors thank Dr. Richard N. Trelease, Arizona State University, for the gift of catalase antibody.

LITERATURE CITED

- Bergmeyer, H. U.; Klotzsch, H.; Mollering, H.; Nelbock-Hochestetter, M.; Beaucamp, K. Biochemical reagents. In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Academic Press: New York, 1963; pp 971–972.
- Bollag, D. M.; Rozycki, M. D.; Edelstein, S. J. *Protein Methods*, 2nd ed.; Wiley-Liss, Inc.: New York, 1996.
- Bradford, M. M. A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
- Brennan, T.; Frenkel, C. Involvement of hydrogen peroxide in the regulation of senescence in pear. *Plant Physiol.* **1977**, *59*, 411–416.
- Clare, D. A.; Duong, M. N.; Darr, D.; Archibald, F.; Fridovich, I. Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. *Anal. Biochem.* **1984**, *140*, 532–537.
- Deisseroth, A. B.; Dounce, A. L. Comparison of the catalytic and physical properties of the components of lyophilized beef erythrocyte catalase with those of lyophilized beef liver catalase components. *Arch. Biochem. Biophys.* **1969**, *131*, 30–48.
- Deutscher, M. P. Maintaining protein stability. In *Methods in Enzymology*, Vol. 182, *Guide to Protein Purification*; Deutscher, M. P., Ed.; Academic Press: San Diego, CA, 1990; pp 83–85.
- Dignam, J. D. Extract preparation from higher eukaryotes. In *Methods in Enzymology*, Vol. 182, *Guide to Protein Purification*; Deutscher, M. P., Ed.; Academic Press: San Diego, CA, 1990; pp 194–198.
- Du, Z.; Bramlage, W. J. Peroxidative activity of apple peel in

- relation to development of poststorage disorders. *HortScience* **1995**, *30*, 205–209.
- Gianinetti, A.; Lorenzoni, C.; Morocco, A. Change in superoxide dismutase and catalase activities in response to low temperature in tomato mutants. *J. Genet. Breed.* **1993**, *47*, 353–356.
- Kar, M.; Mishra, D. Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. *Plant Physiol.* **1976**, *57*, 315–319.
- Kocsis, M. G.; Weselake, R. J.; Eng, J. A.; Furukawa-Stoffer, T. L.; Pomeroy, M. K. Phosphatidate phosphatase from developing seeds and microspore-derived cultures of *Brassica napus*. *Phytochemistry* **1996**, *41*, 353–363.
- Kunce, C. M.; Trelease, R. N. Heterogeneity of catalase in maturing and germinated cotton seeds. *Plant Physiol.* **1986**, *81*, 1134–1139.
- Kuwabara, T.; Masuda, T.; Aizawa, S. A dithiothreitol-sensitive tetrameric protease from spinach thylakoids has polyphenol oxidase activity. *Plant Cell Physiol.* **1997**, *38*, 179–187.
- Lardy, H. A. *Respiratory Enzymes*; Burgess Publishing Co.: Minneapolis, MN, 1949; pp 171–176.
- Masia, A. Superoxide dismutase and catalase activities in apple fruit during ripening and post-harvest and with special reference to ethylene. *Physiol. Plant.* **1998**, *104*, 668–672.
- Neugebauer, J. M. Detergents. An overview. In *Methods in Enzymology*, Vol. 182, *Guide to Protein Purification*; Deutscher, M. P., Ed.; Academic Press: San Diego, CA, 1990; pp 239–242.
- Nir, G.; Shulman, Y.; Fanberstein, L.; Shimon, L. Changes in the activity of catalase (EC 1.11.1.6) in relation to the dormancy of grapevine (*Vitis vinifera* L.) buds. *Plant Physiol.* **1986**, *81*, 1140–1142.
- Pit-Rivers, R.; Impiombato, F. S. A. The binding of sodium dodecyl sulphate to various proteins. *Biochem. J.* **1968**, *109*, 825–830.
- Pohl, T. Concentration of Proteins and Removal of solutes. In *Methods in Enzymology*, Vol. 182, *Guide to Protein Purification*; Deutscher, M. P., Ed.; Academic Press: San Diego, CA, 1990; pp 68–83.
- Pritchard, G. G.; Hudson, M. A. Changes in catalase activity in higher plants and fungi treated with oxygen at high pressure. *Nature* **1967**, *214*, 945–946.
- Shalata, A.; Tal, M. The effect of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii*. *Physiol. Plant.* **1998**, *104*, 169–174.
- Shewfelt, R. L.; A. C. Purvis. Toward a comprehensive model for lipid peroxidation on plant tissue disorders. *HortScience* **1995**, *30*, 213–218.
- Shikama, K.; Yamazaki, I. Denaturation of catalase by freezing and thawing. *Nature* **1961**, *190*, 83–84.
- Tanford, C.; Lovrien, R. Dissociation of catalase into subunits. *J. Am. Chem. Soc.* **1962**, *84*, 1892–1896.
- Thompson, J. E.; Legge, R. L.; Barber, R. F. The role of free radicals in senescence and wounding. *New Phytol.* **1987**, *105*, 317–344.
- Wang, C. Y. Effect of temperature preconditioning on catalase, peroxidase, and superoxide dismutase in chilled zucchini squash. *Postharvest Biol. Technol.* **1995**, *5*, 67–76.
- West, E. S.; Todd, W. R. *Textbook of Biochemistry*; The Macmillan Co.: New York, 1963.

Received for review May 18, 1999. Revised manuscript received July 25, 2000. Accepted August 11, 2000.

JF990525E