

Biochemical comparison of *Pisum sativum* and *Brassica rapa* ascorbate peroxidase

by
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Abstract

All living systems use electron transport systems, which produce reactive oxygen species (ROS) even under optimal conditions. Although some of these reactive molecules are used in the regulation of cellular stress functions, an overabundance can cause photooxidative stress to the cell. A variety of ROS-scavenging enzymes are responsible for preventing a buildup of ROS, and are therefore responsible for protection from oxidative damage. Comparison of electronic spectra and steady-state kinetics of *Pisum sativum* cytosolic ascorbate peroxidase (PsAPX) and *Brassica rapa* cytosolic ascorbate peroxidase (BrAPX) were conducted, as well as a comparison of their sensitivity to inactivation by hydrogen peroxide. Electronic data suggested differences in coordination between the two enzymes. Steady-state analysis revealed that PsAPX and BrAPX had similar activities. PsAPX activity was not shown to significantly change following incubation in hydrogen peroxide. We were unable to attain usable data for inactivation of BrAPX by hydrogen peroxide because the protein had been compromised.

Introduction

Many metabolic processes produce active oxygen species (AOS), otherwise known as reactive oxygen species (ROS). ROS include free radicals such as superoxide (O_2^-) and the hydroxide radical, as well as hydrogen peroxide (H_2O_2) and singlet oxygen. These species are produced by electron transport systems in chloroplasts and mitochondria, as well as those bound to the cell membrane, even in an ideal environment. Both exogenous and endogenous processes can affect the level of intracellular ROS, which is controlled by several radical scavenging enzymes. Transient changes in expression or activity of these enzymes can have a significant influence on cellular redox potential (Yoshimura et al. 2000; Noctor & Foyer 1998; Adler et al. 1999).

Homeostatic control of ROS plays a significant role in the maintenance of cell growth pathways, which can include proliferation, apoptosis, and senescence (Adler et al. 1999). Like the hydroxyl radical, singlet oxygen is highly reactive due to the pairing of two electrons in a single pi molecular orbital that defies Hund's rule of maximum multiplicity (since each of the two electrons should occupy its own orbital). This results in an unoccupied molecular orbital and the species' excessive activity. However, singlet oxygen and the hydroxyl radical production must be minimized because of their extreme reactivity, biochemical adaptations have allowed the exploitation of the reactivity of ROS, and indeed O_2^- and H_2O_2 are synthesized at very high rates in the cell. For instance, plasmalemma-bound NADPH-dependent superoxide synthase catalyzes superoxide anion formation and

superoxide dismutase (SOD) catalyzes the reduction of the superoxide anion to hydrogen peroxide (Noctor & Foyer 1998).

When O_2^- and H_2O_2 are produced in excess, toxicity results from their ability to initiate signal transduction cascades that result in the production of the hydroxyl radical, as well as other destructive species such as lipid peroxidases (Noctor & Foyer 1998). It has been shown that altered redox or ROS levels affect almost every gene implicated in the response to stress. In addition, ROS can induce conformational changes in transcription factors that mediate instant cellular stress responses. These changes affect the activity of scavenging enzymes that are responsible for the control of redox potential (Adler et al. 1999).

To cope with environmental stressors, plant metabolism must be flexible and dynamic. Because ROS formation is accelerated under stress conditions, there must be a way to rapidly process these species to prevent oxidative damage. The antioxidative system determines the lifetime of ROS within the cell, providing vital protection against oxidative damage. This system is comprised of various enzymes and compounds of low molecular weight. Any compound capable of quenching ROS without itself undergoing conversion to a destructive radical is called an antioxidant. Antioxidant enzymes, by catalyzing such reactions or by directly processing ROS, function to interrupt the cascades of uncontrolled oxidation (Noctor & Foyer 1998). Table 1 lists the comparatively few classes of antioxidant enzymes that have been well characterized. Ascorbate (AsA) and glutathione (GSH) are of paramount importance among the antioxidants and carry out several roles in

defense reactions and are major assimilate sinks, present in many tissues at millimolar concentrations (Noctor & Foyer 1998).

Peroxidase is a ubiquitous enzyme, found in plants, mammals, fungi, and prokaryotes, which catalyzes the oxidation of cellular components by either hydrogen peroxide or organic hydroperoxides:

$$AH_2 + H_2O_2\ (ROOH) \rightarrow A + 2\ H_2O\ (ROH + H_2O)$$

Where (A) is the stable oxidation product. The selenoenzyme glutathione peroxidase scavenges hydrogen peroxide in mitochondria of mammalian tissues

Enzyme	Abbreviation in text	EC number
Superoxide dismutase	SOD	1.15.1.1
Ascorbate peroxidase	APX	1.11.1.11
Monodehydroascorbate reductase	MDHAR	1.6.5.4
Dehydroascorbate reductase	DHAR	1.8.5.1
Glutathione reductase	GR	1.6.4.2
Catalase	CAT	1.11.1.6
Glutathione peroxidase	GPX	1.11.1.9
Guaiacol-type peroxidases	—	1.11.1.7
Glutathione S-transferases	GST	2.5.1.18

Table 1 Major antioxidative enzymes (Noctor & Foyer 1998).

Whereas cytochrome *c* peroxidase is utilized by fungi in hydrogen peroxide detoxification. Prokaryotes also lack GSH peroxidase, and the flavoenzyme

NAD(P)H peroxidase seems to play the main roll in the scavenging of hydrogen peroxide and alkyl hydroperoxides. However, peroxidases using GSH, cytochrome *c* or pyridine nucleotides have not been found in plants (Asada 1992). AsA is the most important reducing substrate for hydrogen peroxide detoxification in plants; hydrogen peroxide scavenging in plants is achieved via heme peroxidase utilizing AsA, abbreviated APX (Noctor & Foyer 1998; Asada 1992). Class III peroxidases, like APX, can utilize ascorbate in the reduction of hydrogen peroxide, but have significantly greater enzymatic activity when using small phenolic substrates such as guaiacal or pyrrogallol (Kvaratskhelia, Winkel, & Thorneley 1997). Therefore, hydrogen peroxide-scavenging peroxidases are divided into heme, flavin, and seleno proteins with respect to prosthetic group; and into AsA, small phenolic substrates, GSH, NAD(P)H, and cytochrome *c* enzymes with respect to the electron donor (Asada 1992).

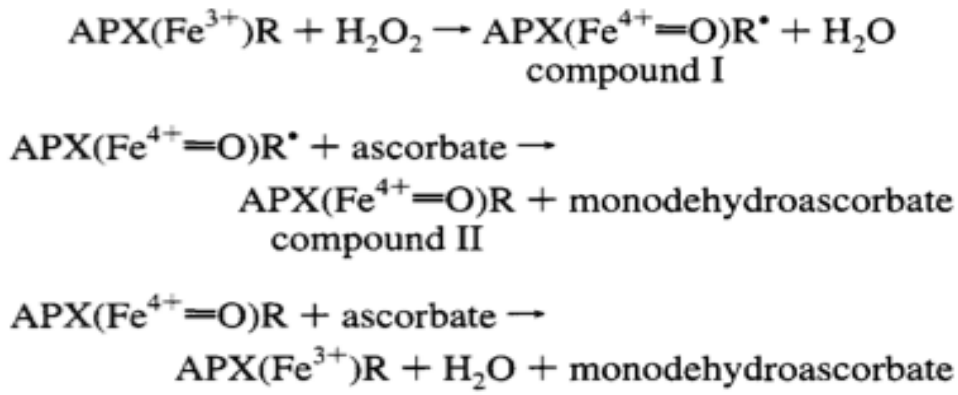


Figure 1 Three-step mechanism of ascorbate peroxidase. Two electrons are donated to an oxygen of hydrogen peroxide to form compound I in the first step. The following two steps use ascorbate as a reducing agent in order to restore the enzyme to its original state (Patterson & Poulos, 1995).

APX is a plant Class I heme peroxidase, along with cytochrome *c* peroxidase from yeast and catalase-like peroxidase from cyanobacteria, that uses two molecules of AsA to reduce hydrogen peroxide to water, with the concomitant generation of two molecules of monodehydroascorbate (MDHA; Figure 1) (Ishikawa & Shigeoka 2008; Noctor & Foyer 1998). Reduction of H_2O_2 happens in the first step of a three-step ping-pong mechanism (Figure 1). The heme first donates two electrons to one of the oxygen atoms of hydrogen peroxide (one from the iron itself and one from the porphyrin ring). The product of the first step is compound I, and water. In the second and third steps, APX uses two molecules of ascorbate to reduce the oxidated enzyme back to its resting state (where the iron is in a 3+ valence state). The radical porphyrin ring is first reduced by ascorbate, forming compound II, and monodehydroascorbate. The second reduction by ascorbate is the iron itself; this step produces water and monodehydroascorbate, as well as restores APX to its original state. Since APX utilizes multiple substrates, it does not follow traditional Michaelis-Menton kinetics (Patterson & Poulos, 1995).

The active site of ascorbate peroxidase is an iron-containing heme, in which the iron primarily displays high spin, five-coordinate characteristics. The iron is naturally ligated by four nitrogen residues of the porphyrin ring, in addition to the one nitrogen from the proximal histidine – His163 (Figure 2). But, there is also evidence of some six-coordinate high spin and low spin species due to the interaction of histidine with either water or hydrogen peroxide (or bis-histidine ligation). If there is bis-histidine ligation of the iron, the distal histidine – His42 –

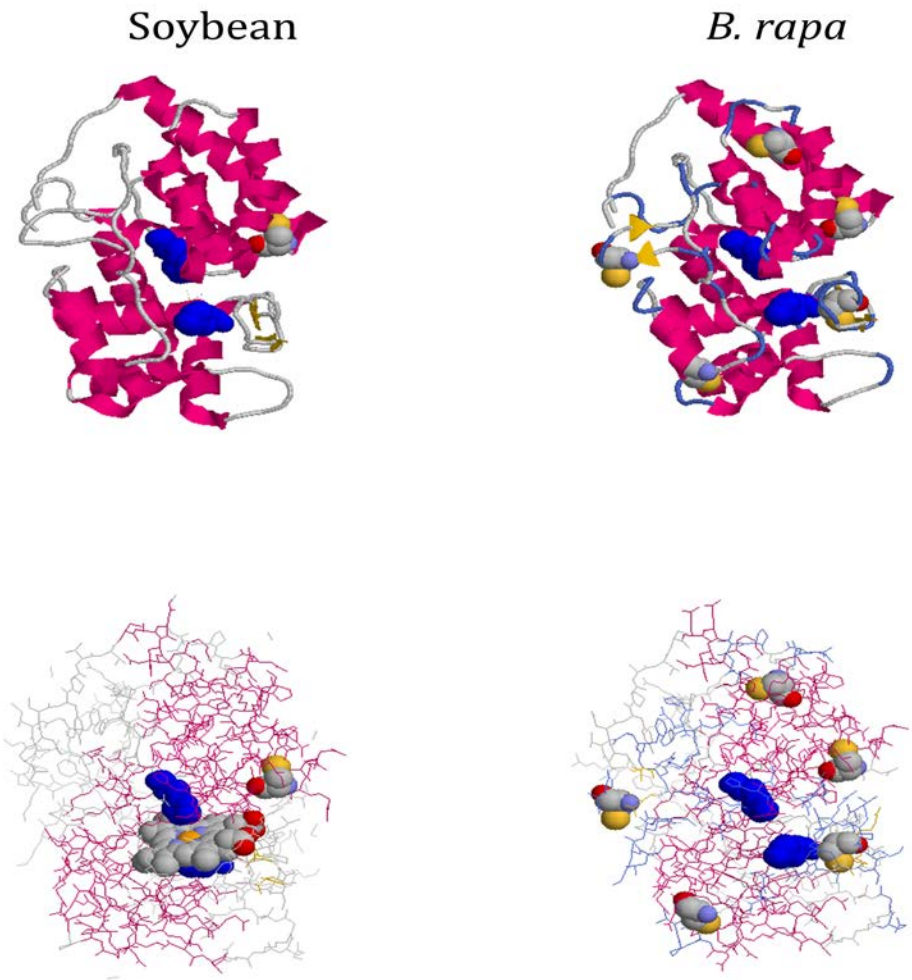


Figure 2 Soybean and *Brassica rapa* cAPX. Blue residues are the proximal (bottom) and distal (top) histidines. In addition to the heme, Cys32 is highlighted in the structure of Soybean APX. The corresponding residue is highlighted in the *B. rapa* structure, along with the other four cysteine residues encoded in its primary sequence.

serves as the sixth ligand (Cheek et al. 1999).

Isoenzymes of APX are distributed in at least four distinct cell compartments (Figure 3): the stroma (sAPX) and thylakoid membrane (tAPX) in chloroplasts, the

microbody (mAPX), and the cytosol (cAPX) (Yoshimura et al. 2000). Two or three isoenzymes are usually found in plant cytosol, indicating that cAPX is encoded by a multigene family (Ishikawa & Shigeoka 2008; Yoshimura et al. 2000).

Recently studies have been focused on changes in cAPX expression level under environmental stresses such as ozone, UV-B radiation, low temperature, high-light stress, salinity, water stress including drought, and pathogen infection. Yoshimura et al. (2000) studied the responses of all APX isoenzymes in spinach leaves to high-light stress, salinity, drought, and treatment with methyl viologen (MV) and abscisic acid (ABA) by northern-blot analysis using each cDNA as a probe. This study was needed because it seemed that APX isoenzymes are likely expressed by distinct regulatory mechanism and no studies (to date) had simultaneously analyzed the stress responses of all APX enzymes. Examination of steady state mRNA transcript levels revealed that only expression of the cAPX isoenzyme responds to high-light stress and MV treatment (which is known to cause oxidative stress), while the other isozymes are constitutively expressed under both normal and stress conditions. Furthermore, similar observations of cAPX induction by photo-oxidative stress have been reported in Arabidopsis, maize, rice, and pea. The induction of cAPX expression during an early stage of oxidative stress is expected to play an important role in removing hydrogen peroxide and minimizing photooxidative damage. Overexpression of cAPX in tobacco plants provides increased resistance against MV treatment (Yoshimura et al. 2000).

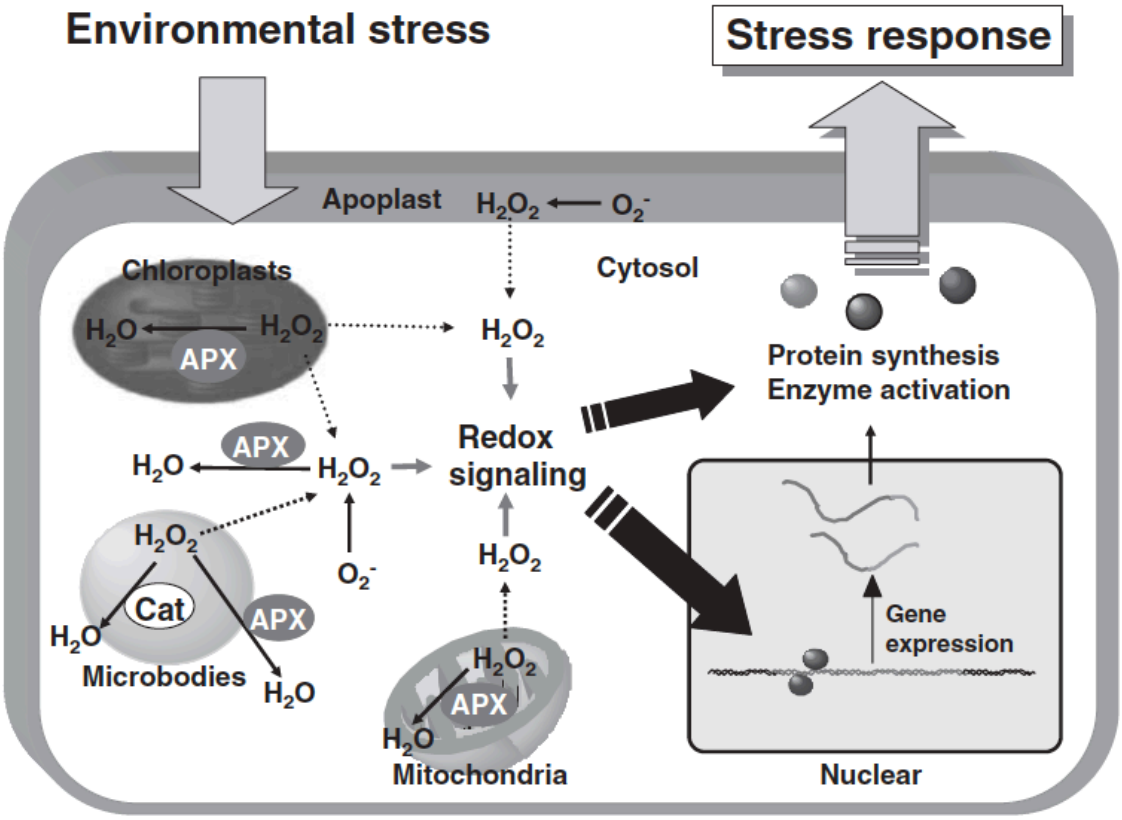


Figure 3 Outline of the physiological function of APX controlling cellular redox signaling (Ishikawa & Shigeoka 2008)

Even though cAPX expression increased, the level of cAPX protein was only slightly altered, and the cAPX activity was increased in response to the high light intensity. It is clear that the protein level of cAPX does not directly correlate with the increased transcript level and activity, and it is, therefore, likely that the activation state of cAPX pool increases under high-light stress. The hydrogen peroxide level peaked during the progression of high-light intensity, exhibited a rapid decrease, and finally returned to the level of control plants (Yoshimura et al. 2000). The response of spinach cAPX to photooxidative stress could potentially be mediated by a temporary buildup of hydrogen peroxide, which would corroborate

its suspected role as a second messenger in plants experiencing environmental stress (Yoshimura et al. 2000).

Pea (*Pisum sativum*) cAPX exists as a homodimer with a molecular weight of 57.5 kDa. Each homodimer consists of two identical monomers, each of which has 249 amino acids, as well as its own heme. The protein folding in Pea cAPX closely resembles that of cytochrome c peroxidase and their active sites are also similar. There is only one cysteine residue (Cys32) present in the amino acid sequence for the *Ps* cAPX monomer (Figures 2 & 4); the only cysteine known to be ubiquitous throughout APX isoenzymes (Patterson & Poulos, 1995). Analysis of sequences of cAPX from plants that are not legumes suggests that the number of cysteines in all other cAPXs is greater than one. The sequences of *Brassica rapa* and *Arabidopsis thaliana* each encode four cysteines in addition to the one found in legume cAPX (Figures 2 & 4). In addition to its hydrogen peroxide-scavenging role to reduce plant oxidative stress caused by ROS accumulation, there is strong support that cAPX holds an active role in the regulation of gene expression by modulating the concentration of hydrogen peroxide (cellular redox status) to adjust its activity or expression to a level sufficient for second messenger activity (Figure 3) (Ishikawa & Shigeoka 2008). Cysteine-rich molecules have been implicated in the response to altered redox potential and generation of ROS (Adler et al. 1999).

Hydrogen peroxide has been linked to the modulation of protein phosphorylation. It is unlikely, however, that it will exert its modulation through reversible binding with kinases or phosphatases because of its structural simplicity. Hydrogen peroxide's reactivity as a mild oxidant allows it to oxidize cysteine

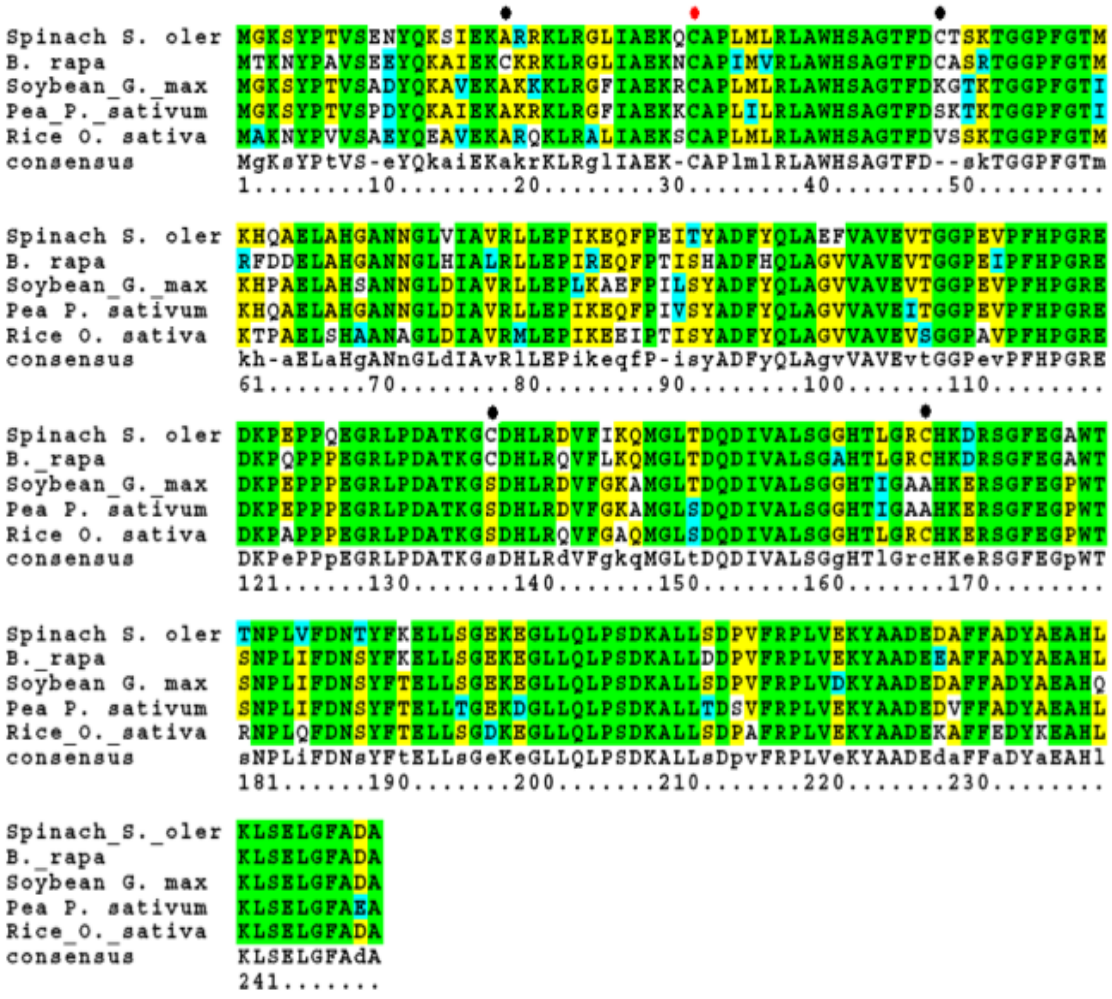


Figure 4 Amino acid sequence alignment of *Spinacia oleracea* (spinach), *Brassica rapa*, *Glycine max* (soybean), *Pisum sativum* (pea), and *Oryza sativa* (rice). Yellow shading signifies some conservation, whereas green shading indicates conservation throughout all five proteins. The red dot indicates the cysteine32 residue, which is conserved among all ascorbate peroxidases. The black dots point out the four additional cysteine residues found in *B. rapa* (Quinn-Elmore, 2010).

residues to cysteine sulfenic acid or disulfide. If cellular reactants are available, both of these species can be promptly reduced back to cysteine. It has been shown that the cysteine thiolate anion (Cys-S^-) is more readily oxidized by hydrogen peroxide than the sulfhydryl group of most cysteines (Cys-SH) due to its basic pK_a, of 8.5. Since cellular pH is neutral, there are few proteins with Cys-SH groups

vulnerable to oxidation by hydrogen peroxide. Thiolate anions exist in some proteins at neutral pH as a result of lowered pK_a values caused by charge interactions between the negatively charged thiolate anion and positively charged amino acids in close proximity (Rhee et al. 2000). Protein tyrosine phosphatases (PTPs) are one such family of proteins.

In all PTPs, an essential cysteine residue in the active site motif exists as a thiolate anion (at neutral pH). It has been observed that these residues are targets of specific oxidation by species such as hydrogen peroxide and are necessary for formation of a thiol-phosphate intermediate present in the catalytic mechanism of all PTPs. This modification can be reversed when reducing agents are present, suggesting the potential of cellular PTPs to undergo hydrogen peroxide-dependent inactivation (Rhee et al. 2000).

ROS can cause the formation of both intra- or inter-molecular disulfide bonds between respective cysteines. Intra-molecular disulfide bridges alter protein conformation, whereas inter-molecular disulfide bonds create dimers and multimers, potentially influencing association with other cellular proteins. Thioredoxin and glutathione S-transerase (GST) are redox-sensitive proteins that have been shown to undergo such changes. A linear relationship is apparent between the degree of oxidative stress, the formation of disulfide species, and the induction of stress responsive proteins (Adler et al 1999).

Thioredoxin has been extensively characterized and is a cysteine-rich redox-responsive protein implicated in the regulation of several stress responsive proteins, and has recently been identified as an inhibitor of stress kinase ASK1.

Thioredoxin association with ASK1 was found in non-stressed cells, required the amino-terminal domain of ASK1, and inhibited ASK1 kinase activity. Inhibition of ASK1 by thioredoxin can be attenuated by ROS, which induce dimerization of thioredoxin. Upon treatment with free radical scavengers, ROS-dependent dissociation of thioredoxin from ASK1 is abolished (Adler et al. 1999).

OxyR is a bacterial transcription factor that mediates the cellular response to both ROS and reactive nitrogen species (RNS) by controlling expression of the OxyR regulon, which encodes the proteins implicated in the metabolism of hydrogen peroxide, as well as the cytoplasmic thiol redox response. Oxidation converts OxyR from a repressor of OxyR expression to an inducer of the OxyR regulon, and it has been shown that the redox-reactive cysteine residues are required for the reversible induction of gene expression by ROS. This transition is highly sensitive to hydrogen peroxide concentration and is very rapid (approximately thirty seconds) (Barford 2004).

Cellular responses to ROS by OxyR have been shown to cease, when a mutation is present at Cys199. An additional conserved residue, Cys208, was also strongly implicated in the response to ROS because OxyR was less effective at inducing gene expression when this residue was mutated. Crystal structure, of the reduced state, of OxyR shows Cys199 and Cys208 separated by seventeen angstroms. When oxidized, the OxyR crystal structure reveals that Cys199 and Cys208 form an intramolecular disulfide bond. Disulfide bond formation is accompanied by a distorted alpha helix, as well as motion of a loop as the cysteines approach each other. This is due to the motion of Cys199, which disrupts the

domain interface, re-orienting the N- and C-terminal domains. These changes in domain confer a change in the tetrameric structure of OxyR, and might account for differences seen in selectivity and DNA binding affinity (Barford 2004).

A mechanism-based inactivation of horseradish peroxidase (HRP), a Class III peroxidase, by hydrogen peroxide and *m*-chloroperoxybenzoic acid (*m*-CPBA) has been observed. This can be thought of as an irreversible mechanism-based (suicide) inactivation (Silverman, 1995). Similar inactivation, in recombinant cytosolic Pea APX, has been examined by Hiner et al. (2000). In this kinetic study, the residual activity of APX, which had been incubated in varying concentrations of hydrogen peroxide, was tested at varying time points over a three-hour period. Findings suggest that APX inactivation by hydrogen peroxide is time dependent and that its inactivation proceeded an order of magnitude more slowly than that of horseradish peroxidase (HRP), whose affinity for, and sensitivity to, hydrogen peroxide is much lower than that of APX. Due to the important role of APX in cell defenses against oxidative stress, the study of enzyme inactivation by one of the major produces of stress (hydrogen peroxide) provides an indication of the limitations of resistance to attack by reactive oxygen species (Hiner et al. 2000).

The nature of structural changes that occur in formation of the inactive APX enzyme is unclear. Although preliminary UV/Visible spectroscopic analysis failed to identify similarities to the inactive compound P670 verdohaemochrome (seen in previous work on HRP), there was a noticeable fall in Soret peak intensity, suggesting that APX was experiencing some degradation of heme (Hiner et al., 2000). Knowing that cysteines are residues could be involved in

both the perception of hydrogen peroxide by scavenging enzymes, and their involvement in enzymatic inhibition, in the absence of a reducing agent, the goal of my study is to perform a comparative analysis of electronic spectra and steady state kinetics of *Pisum sativum* and *Brassica rapa* cAPX. In addition, the sensitivity of the two enzymes to inhibition by hydrogen peroxide will be compared.

Experimental

APX

IPTG-induced APX expression. Wild type *Pisum sativum* and *Brassica rapa* recombinant cells (*E. coli* pET15B), encoding His-tagged APX, were streaked onto LB agar (0.1 mg/mL carbenicillin) and incubated for 18-20 hrs (37° C). Overnight cultures, a single bacterial colony in 5 mL liquid LB (0.1 mg/mL carbenicillin), were incubated for 18-20 hrs (37° C, 250 rpm). Each overnight culture was added to separate 500 mL solutions of liquid LB (1 L DI H_20, 10 g NaCl, 5 g Difco Tryptone, 5 g Difco yeast extract, 0.05-0.10 mg/mL carbenicillin; autoclaved). Cultures were incubated for 2-4 hrs, or until OD_{600} reached 0.4-0.6 (37° C, 250 rpm). Expression was achieved through the addition of 750 μL 100 mM IPTG to each culture (final concentration IPTG: 0.15 mM) followed by incubation for 4-5 hrs (37° C, 250 rpm). Cultures were centrifuged for 15 min (4° C, 3000 x g); supernatant was decanted and pellets were stored in centrifuge bottles at -80° C.

Purification of APX protein. A His60 Ni Superflow™ Resin & Gravity Column kit was used in the purification process. Samples were prepared by first adding 10 mL His60 Ni xTractor buffer to each pellet, followed by mixing until pellets were

fully resuspended. To each resuspended pellet, 5 μL DNase1 was added and the solution was gently mixed. Samples were incubated for 15 min at room temperature, on a platform shaker, before undergoing centrifugation for 20 min (4° C, 10,000 x g). The resulting supernatant served as the starting sample for batch/gravity flow column purification.

All buffers and the His60 Ni Gravity Columns (1 mL each) were equilibrated to room temperature. Columns were washed with 5-10 column volumes (CVs) of His60 Ni Equilibration Buffer, and the bottom stoppers were secured to the bottom of the columns. The clarified samples were added to their respective columns and the top stoppers were attached. Protein was allowed to bind by inverting the columns for 1 hr (platform shaker at 4° C). After removing the stoppers, the columns were washed with 10 CVs His60 Ni Equilibration Buffer, then 10 CVs His60 Wash Buffer (710 μL His60 Ni Elution Buffer to 9.29 mL His60 Ni Equilibration Buffer). Target proteins (PsAPX and BrAPX) were eluted with approximately 10 500 μL aliquots His60 Ni Elution Buffer. Fractions with color were combined in a Centrifugal Filter Unit (from Millipore). Filter units were centrifuged six successive times (10 min each time; 4° C, 7,500 x g) following the addition of KPO4 to each tube the first 5 rounds. Concentrated proteins were stored in microcentrifuge tubes at 4° C.

Determination of protein concentration. Protein dilutions were made and absorbance was measured using a Beckman Coulter Du® 530 Life Science UV/Vis Spectrophotometer at 280 nm (A_{280}) and 403 nm (A_{403}). Enzyme concentrations

were calculated from the Beer-Lambert Law, using the *P. sativum* APX molar absorption coefficient (ε) at 403 nm of 91 $mM^{-1} \cdot cm^{-1}$ per heme.

APX Activity Assays

Preparation. First, a potassium phosphate (KPO_4) buffer was prepared (50 mM, pH 7.0, 1 mM EDTA). PsAPX and BrAPX enzymes samples were diluted to 0.857 and 1.132 μM, respectively, in buffer. From a solution of 50 mM sodium ascorbate (NaAsC; L-sodium ascorbate in DI water; ascorbate ε_{290} = 2.8 $mM^{-1} \cdot cm^{-1}$) a series of dilutions were prepared (0.4, 0.6, 0.8, 1.0 mM in buffer). A solution of 10 mM H_2O_2 (in buffer) was made from 1 M H_2O_2 (9.8 M 30 % H_2O_2 in DI water).

Data collection: ascorbate assay. Spectroscopic data was followed by the decrease in A_{290}, using a Beckman Coulter Du® 530 Life Science UV/Vis Spectrophotometer UV/Vis Spectrophotometer. The spectrophotometer was first blanked with buffer (100 μL). To a quartz cuvet (with a path length of 1 cm), 50 μL NaAsC dilution (final concentrations: 0.2, 0.3, 0.4, 0.5 mM) and 50 μL enzyme dilution (final concentration: 0.5 μM) were added; this was immediately mixed via micropipette and placed into the spectrophotometer. This solution was allowed to incubate, with the lid closed, for 2 min. At this point a 1 μL aliquot of 10 mM H_2O_2 was added to the cuvet, the lid was closed, and spectroscopic recording was initiated as quickly as possible. Initial rates were determined by dividing the changing in absorbance by the extinction coefficient for PsAPX, then dividing this value by the enzyme concentration – yielding K_{cat} (s^{-1}), or mM of ascorbate oxidation per mM enzyme per second.

Hydrogen Peroxide Sensitivity

Preparation. First, enzyme dilutions were made (1 μM in buffer). A 0.8 mM NaAsC dilution (in buffer) was made from a solution of 50 mM NaAsC and a series of dilutions of H_2O_2 (4, 8, 20, 30, 60 μM) was made from a solution of 1 M H_2O_2.

Data collection. To a microcentrifuge tube containing 100 μL enzyme dilution, 1 μL of H_2O_2 dilution was added. After 40 min incubation at room temperature, a 50 μL aliquot was removed and combined with 50 μL NaAsC dilution; spectroscopic data was obtained in the same manner as the ascorbate assay.

Chemicals

Sodium chloride (NaCl; M. W. 58.44) was from Fisher Scientific. Difco tryptone and Difco yeast extract were both from Difco Laboratories: Detroit, Michigan. Hydrogen peroxide (H_2O_2; 9.8 M 30 %) was from Spectrum Chemical MFG. Corp. and (+)-Sodium L-ascorbate (FW 198.11) was from Sigma Aldrich.

Results

Spectral Properties

To determine structural differences between the *Pisum sativum* and *Brassica rapa* cAPX, scans of UV/Visible wavelengths ranging from 380-420 nm and 475-700nm were used. Heme-containing proteins exhibit a characteristic Soret peak in the blue wavelength region of the visible spectrum (lower 400 nm range). This peak designates the wavelength of light that the iron, held in the heme complex, can absorb, and varies with the iron's ligation to the heme and surrounding protein. In

comparison to the Soret peak of Ps cAPX (407 nm), the Soret peak of Br cAPX is red shifted (411 nm) and is more defined (Figure 5 top).

A pronounced peak was seen at approximately 635 nm scans of Pea cAPX, but the B. rapa cAPX spectrum only showed a slightly visible shoulder at this location (Figure 5 bottom). A peak at 510 nm and a shoulder around 530 nm are visible in the PsAPX spectra, whereas these features are red-shifted in the BrAPX spectra.

Steady-State Kinetics

Steady-state kinetics was utilized to determine differences in the initial rate of the enzymes. Following the rate of ascorbate oxidation may reveal connections between how differences in structure (shown by electronic spectra) relate to enzymatic functioning. Analysis revealed that the rate of ascorbate oxidation did not differ significantly between PsAPX (9 to 20 s^{-1}) and BrAPX (12 to 22 s^{-1}; Figure 6). The recorded activities of PsAPX parallel findings by Quinn-Elmore 2010, in that the recorded values are within 2 s^{-1} of values from previous findings (at three separate ascorbate concentrations).

Hydrogen Peroxide Sensitivity

Neither enzyme showed an appreciable change in activity with incubation in solutions of increasing hydrogen peroxide concentration (Figure 7). Although PsAPX showed a similar activity to its steady state levels (12 to 16 s^{-1}), BrAPX activity was found to be extremely low, even following incubation in a hydrogen peroxide-free solution (all runs averaged < 2 s^{-1}). It was later shown, through a re-testing of steady state kinetics, that the BrAPX enzyme was compromised.

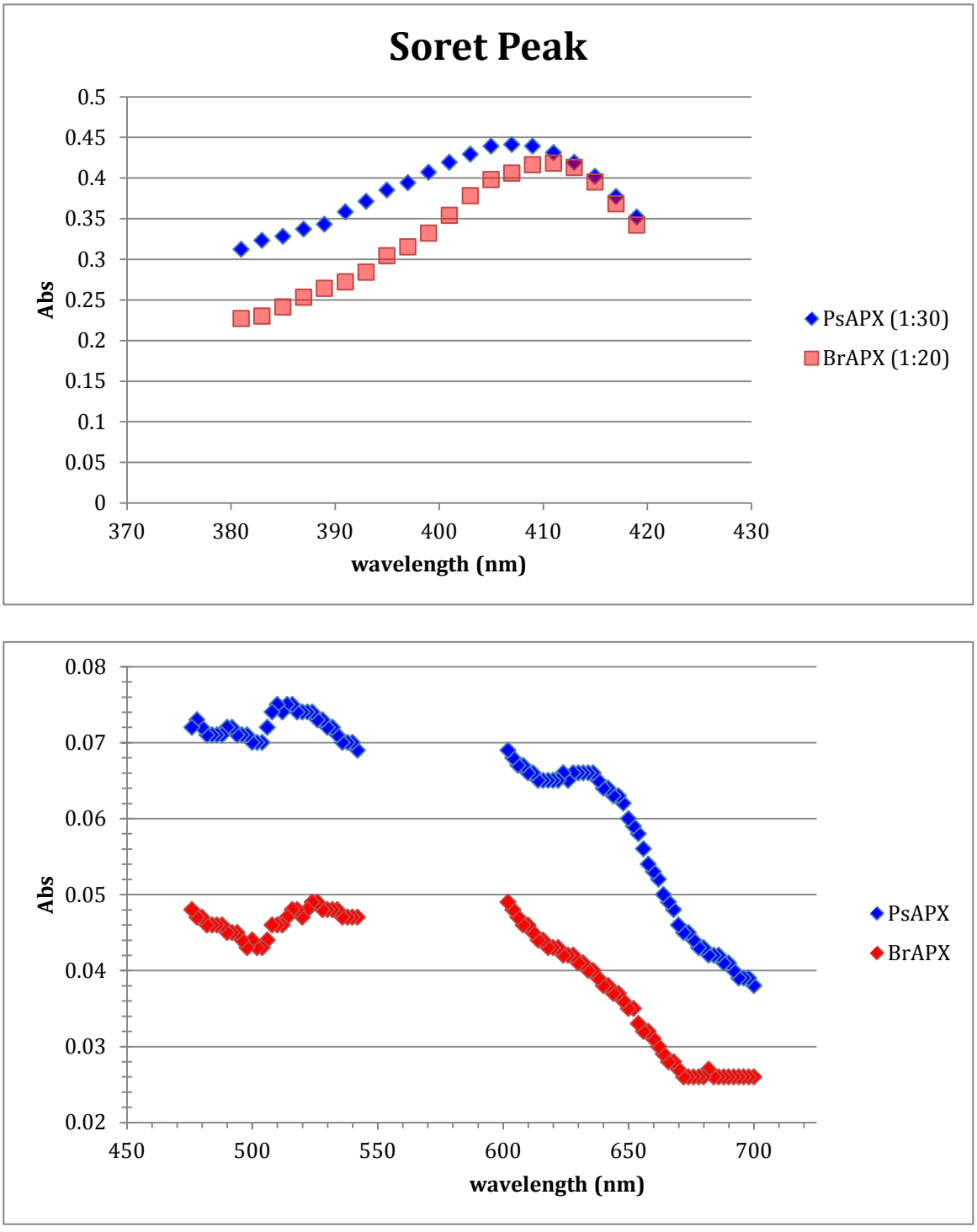


Figure 5 Electronic spectra of *Pisum sativum* and *Brassica rapa* cAPX (top: 380-420 nm; bottom: 475-700 nm). The gap in data between 540 and 600 nm (bottom) is a due to a mechanical event in the spectrophotometer necessary to capture data at shorter wavelengths.

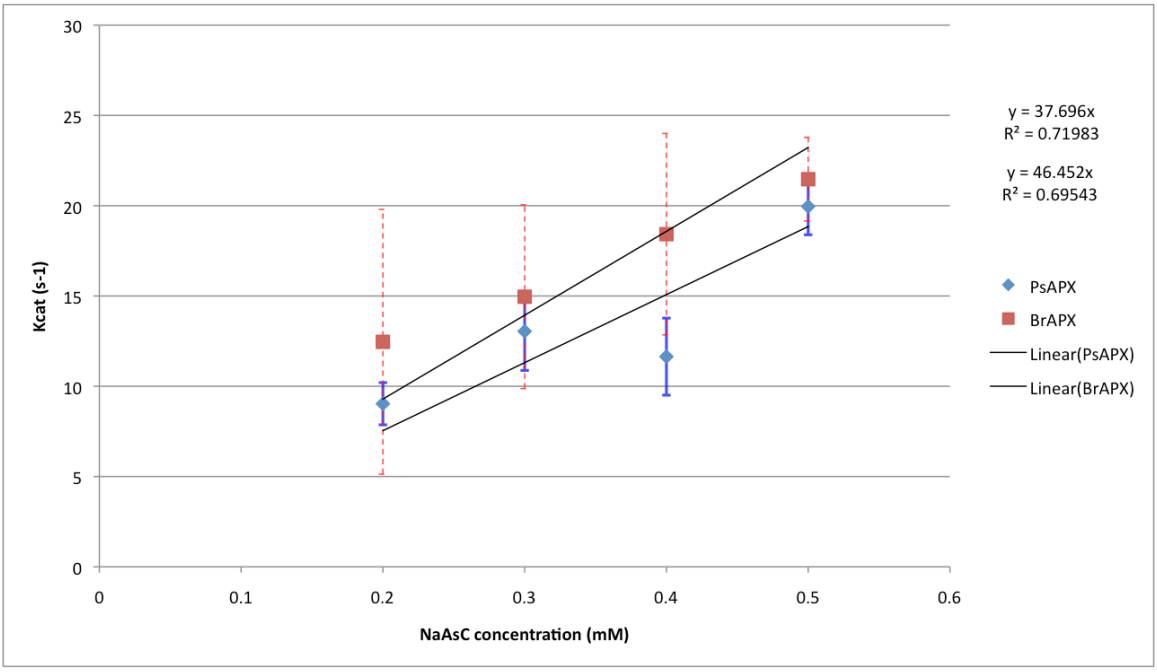


Figure 6 Ascorbate oxidation rates of PsAPX and BrAPX were assayed at final concentrations of 0.857 and 1.132 micromolar, respectively. Varying concentrations of ascorbate were used (0.2-0.5 mM) with a final hydrogen peroxide concentration of 0.1 mM. Data points are averages of three trials and error bars represent the standard deviation.

Discussion

The goal of this project was to investigate the difference in structure between *Pisum sativum* and *Brassica rapa* cAPX by comparing electronic spectra, and to see how these differences relate to enzymatic functioning by studying steady-state kinetics. In addition, the experiment served as a comparison hydrogen peroxide sensitivity between the two forms of cAPX.

The Soret peak in is found in electronic spectra of all heme peroxidases and the maximum absorbance depends on the state of the iron cation. A red shift in the Soret peak has been linked the oxidized form of APX known as Compound I. In this

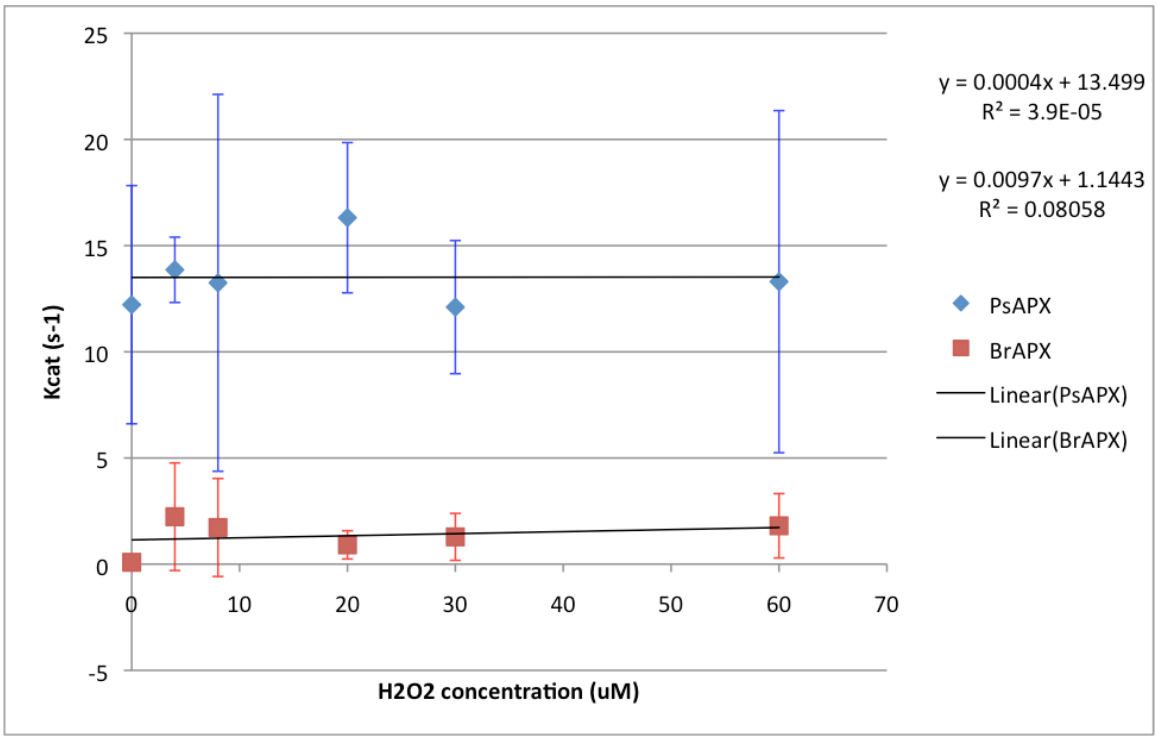


Figure 7 Ascorbate oxidation rates of PsAPX and BrAPX (1 uM) following a forty-minute incubation in varying hydrogen peroxide concentrations (0-60 uM). Following incubation, samples were added to a buffer solution with the final concentration of ascorbate 0.4 mM. The reaction was initiated by adding hydrogen peroxide (0.1 mM). Data points are averages of three trials and error bars represent the standard deviation.

oxidized state, the iron is coordinated by an additional sixth ligand – the oxygen atom left from the initial reduction of hydrogen peroxide (Mandelman et al. 1998). Since the Soret peak is red-shifted in B. rapa cAPX, this protein likely contains six-coordinate characteristics around the iron cation center.

The peaks at 510 nm and 530 nm in the PsAPX spectra indicate a pi to pi* electronic transition of d-orbital electrons from iron (whose orbitals are split due to its ligand coordination). Since these two peaks are red-shifted for BrAPX, it suggests that pi to pi* transitions require less energy for this species. This finding indicates the possibility of 6-coordinate heme existence.

Steady state findings indicate that PsAPX activity paralleled that of previous findings (Quinn-Elmore 2010) and that BrAPX had comparable activity levels. It was found that incubation in hydrogen peroxide does not cause a significant decrease in activity of PsAPX. While testing inactivation of BrAPX by hydrogen peroxide, it was obvious that the enzyme was compromised because it showed an increase in absorption, instead of a decrease, when we followed the absorbance of ascorbate. Upon re-testing the steady state kinetics for this protein, it was concluded that it had been compromised. This most likely is the result of the protein not being stable at room temperature.

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