STUDIA UBB CHEMIA, LXIV, 2,Tom I, 2019 (p. 49-60)
(RECOMMENDED CITATION)
DOI:10.24193/subbchem.2019.2.04

Dedicated to Professor Florin Dan Irimie on the Occasion of His 65th Anniversary

ISOLATION, PURIFICATION AND CHARACTERIZATION OF ASCORBATE OXIDASE AND PEROXIDASE FROM *CUCURBITA PEPO MEDULLOSA*

ALINA-MARIANA CRAINIC[a], AUGUSTIN C. MOȚ[a], RADU SILAGHI-DUMITRESCU[a*]

ABSTRACT. Ascorbate oxidase is a well-known copper-containing enzyme however, its biological role and mechanism are still unclear. Reported here is a new protocol for purification of ascorbate oxidase from *Cucurbita pepo medullosa* (field pumpkin), with improved yields. The enzyme is biochemically characterized, including Michaelis-Menten parameters. Also, purification and characterization of the peroxidase from the same source is described, featuring an unusually high affinity for hydrogen peroxide(low-micromolar).

Keywords: *ascorbate oxidase, peroxidase, field pumpkin, purification*

INTRODUCTION

Ascorbate oxidase (AO) is a homodimeric enzyme (2×70 kDa) that catalyzes the oxidation of ascorbate to dehydroascorbate, using O_2 as electron acceptor. The active site features a mononuclear type I (T1) blue copper center—where the color is due to a cysteine-to-sulfur charge transfer, and the rest of the coordination sphere consists of two histidines and a methionine—where ascorbate is oxidized by one-electron donation to Cu(II); the resulting ascorbyl radical is thought to immediately dismutate to ascorbate and dehydroascorbate. In order to provide the oxidizing equivalents, AO also features a three-copper active site (type III, T3, colorless and magnetically coupled, ligated only by histidines and with an oxo or hydroxo bridge in the oxidized form), where the ascorbate-derived electrons are stored as Cu(I) and

[a] *Department of Chemistry, Babes-Bolyai University, 1 Mihail Kogalniceanu street, Cluj-Napoca 400084, Romania*
** Corresponding author: rsilaghi@chem.ubbcluj.ro*

then used for reducing molecular oxygen to water [3, 8] [9] [10]. These features, as well as the multimeric nature of the protein (with three equivalent units, each containing a trinuclear and a mononuclear site, are shared by several other multicopper oxidases (also known as blue copper oxidases), such as laccase and ceruloplasmin [1-3]. AO is selective towards ascorbate, due to interactions at the surface of the protein, where histidine and tryptophan residues bind to the lactone ring of the substrate [11]. Nevertheless, other compounds can also serve as reducing substrates, such as catechol or dichlorohydroquinone [2]. Beyond this, the mechanistic details of AO remain unclear, especially at the T3 center.

AO was the first of the blue copper oxidases whose structure was solved by x-ray diffraction, with several examples now available [11, 14]. Biochemical features of the enzyme, including thermostability, pH dependence and Michaelis-Menten parameters for the ascorbate oxiase cycle were also reported [3].

AO is found predominantly in higher plants, especially in *Cucurbitacee*, although it has also been observed in microorganisms such as *Acremmonium sp* (fungi) [8]. By comparison, laccase is found in microbes and in some plants, while ceruloplasmin is exclusively found in blood [3]. The biological role of AO is still unclear. In plants, ascorbate is part of the defense mechanism against oxidative stress and is located predominantly in leaves, extracellularly. AO is also found extracellularly, in the apoplast, where its expression is upregulated by light [12]. A role for AO in regulating the redox potential of the cells has been proposed, via tuning the ratio of ascorbate vs. dehydroascorbate. Also noted was an involvement of AO in cellular elongation [15].

AO also has practical applications, such as in biochemical sensors [15], or as a useful reagent for generating anaerobic conditions in biochemical laboratory experiments. It also provides an opportunity for exploring the mechanistic features of multicopper oxidases, with implications for enzymes with even larger numbers of practical applications – laccases and ceruloplasmin.

AO purification was first reported in 1940, from the yellow pumpkin *Cucurbita pepo condensa* [1]; a number of other purification protocols have since been reported [1, 2, 8].

Peroxidases (PX) are found in a wide range of organisms and have physiological/biological functions involving defense against oxidative stress, biochemical synthesis, and possibly others. By far the most common peroxidases are the ones featuring a heme at the active site (as seen, e.g., in horseradich peroxidase HRP or in chloroperoxidase CPO) – to the extent that the term “peroxidase” is generally assumed to refer to heme-containg peroxidases; other types of peroxidases feature active sites based on cysteine, or selenocysteine, or vanadium, or a binuclear di-iron center. The practical applications of peroxidases are vast and have been described elsewhere [16-19].

Reported here is an improved protocol of purification for AO from green pumpkin *Cucurbita pepo medullosa*, as well as a biochemical characterization of this enzyme. Purification of a peroxidase is also reported from these preparations, as a side-product.

RESULTS AND DISCUSSION

AO isolation and purification

Figure 1 shows the hydrophobic interaction chromatogram of the resuspended total protein pellet obtained by ammonium sulfate and acetone treatment. The brown color of the latter, alongside the general knowledge of peroxidases (PX) as relatively abundant enzymes in plant extracts, is the reason why the fractions were monitored not only at 610 nm (T1 AO – specific) but also at 405 nm (heme – specific). It may be seen that the presumed peroxidase impurity is present in most AO-containing fractions. Considering that the extinction coefficient of the heme is one order of magnitude larger than that of the charge-transfer band in the T1 copper of AO, the ratio of the 610 nm to 405 nm absorbances in the fractions suggests that AO is the dominant component over PX in most fractions, except at ~50 min where the two are sensibly equal.

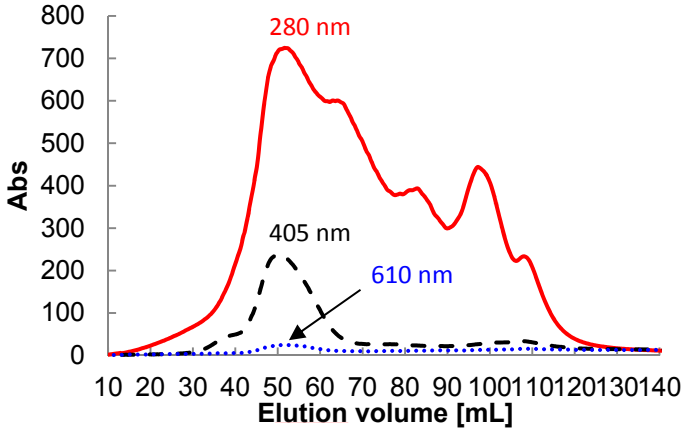

Figure 1. Hydrophobic interaction chromatogram of the resuspended total protein pellet by ammonium sulfate and acetone

Figure 2 shows that anion exchange chromatography does allow for separation of the peroxidase component to a good degree. However, the major AO fraction does still feature absorbance at 405 nm. The PX was mainly present in the flow-through fraction, which was collected for further analysis and purification in parallel with the AO-containing fraction. At this stage the two fractions contained predominantly the proteins of interest as shown by SDS-PAGE analysis.

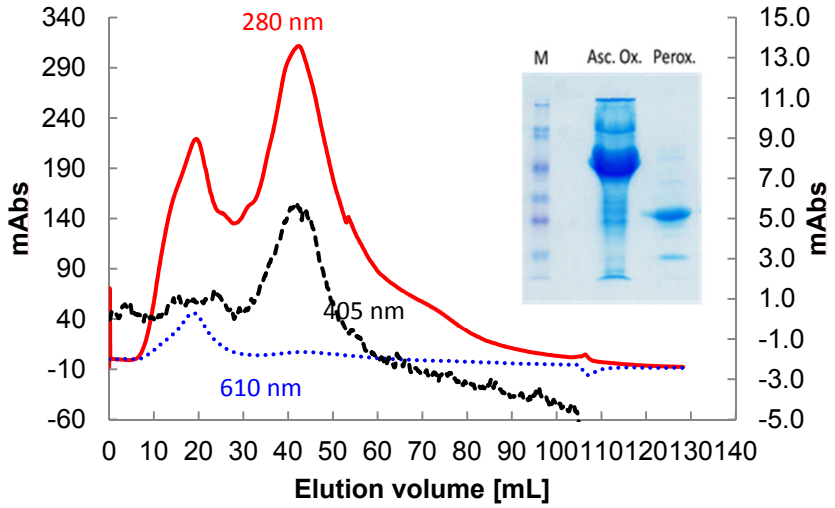

Figure 2. Anion-exchange chromatogram of the AO-containing fraction collected from hydrophobic interaction separation. Inset: SDS-PAGE analysis of the same fractions.

The AO and PX fractions were further subjected to preparative native gel electrophoresis, followed by a final purification step (hydrophobic interaction for AO, cation exchange for PX). As seen in Figure 3, this yields proteins of excellent purity.

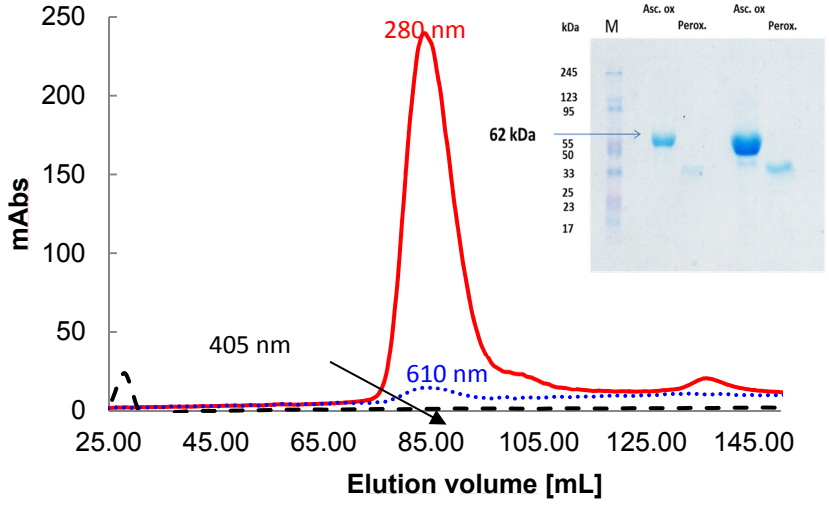

Figure 3. Hydrophobic exchange chromatogram for the final purification step of AO. Inset: SDS-PAGE of the purified AO and PX at two levels of concentration.

The UV-vis spectra of the purified AO and PX are shown in Figure 4. The ratio of absorbances due to the protein component (280 nm) vs. specific to the copper center (610 nm) can be used as purity indicator; a value of 25.3 was noted, in excellent agreement with an average of 25±0.5 seen in the literature.

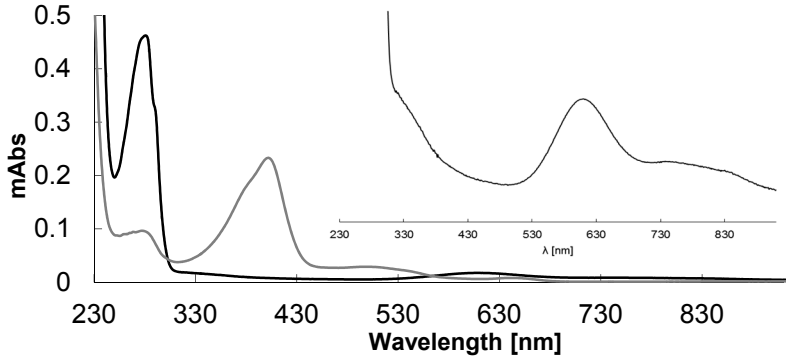

Figure 4. Molecular absorbtion UV-vis spectra of AO (black) and PX (grey). Inset – enlarged visible domain for AO spectrum.

Figure 5 summarizes the progress of the puritication steps; the maximum specific activity was 1056 μmoli/(min × mg), and the purification factor was 91.3. The latter value is ~ double to that of 43 reported for previous protocols [21], while the quantitative yields were 12.5% vs. 18%.

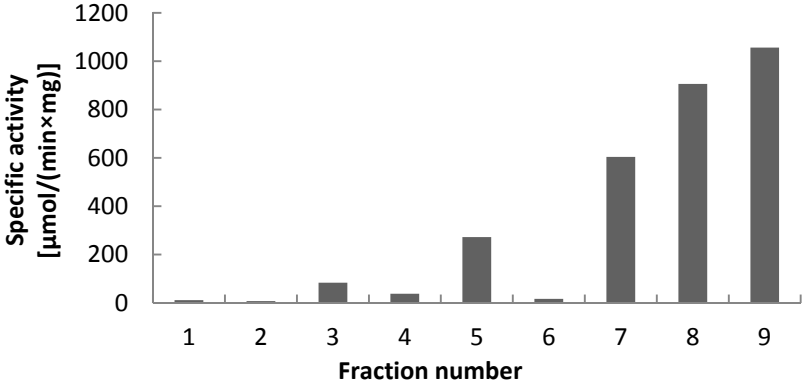

Figure 5. Specific activity of AO across the purification procedure. Fraction description:1-extract from blended peels (7200 mL), 2-After first ammonium sulfate procedure (1850 mL), 3-After precipitation with acetone (750 mL), 4-After first ammonium sulfate procedure (1240 mL), 5-AO fraction from first hydrophobic interaction purification, after dialysis (335 mL), 6-PX fraction after anion exchange (350 mL), 7-AO fraction after anion exchange (63 mL), 8-AO after native electrophoresis (42 mL), 9-AO at the end of purification protocol (26 mL). Fraction volumes are derived from a 50 kg starting point of raw material.

Figure 6 shows similarly obtained data as in Figure 5 – in this case for the purification of the PX. The purification factors at the final two fractions are 5.5 – 6.7, two orders of magnitude lower than for AO. This may be explained by the fact that in the initial extract several peroxidases are expected to be present – and several fractions were also present at various chromatographic steps, out of which only the dominant one, from the anion-exchange flow-through, was selected for purification. The highest specific activities were 162.5 and 118.9 μmol/(min×mg), respectively.

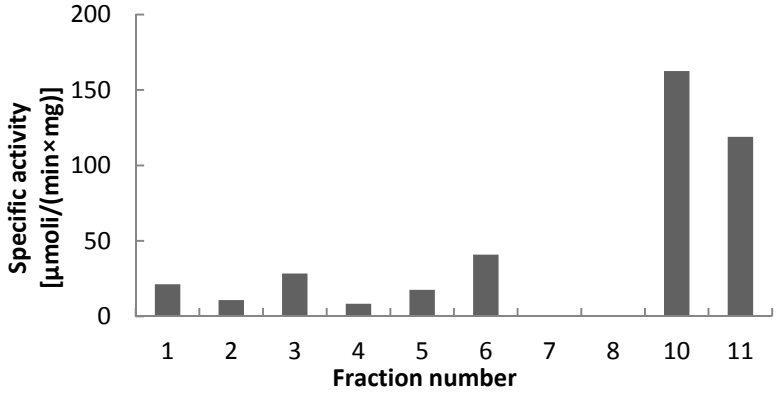

Figure 6. Specific activity of PX across the purification procedure.
Fraction description: as in Figure 6, with two additions –
10 PX after cation exchange, fraction 1 (11 mL),
11 PX after cation exchange, fraction 1 (6.5 mL).

Michaelis-Menten analysis

For ascorbate, a K_m value of 124 μM at V_{max} = 2.356 UA/min was measured vs. AO, using an Eadie Hofstee plot as shown in Figure 7.

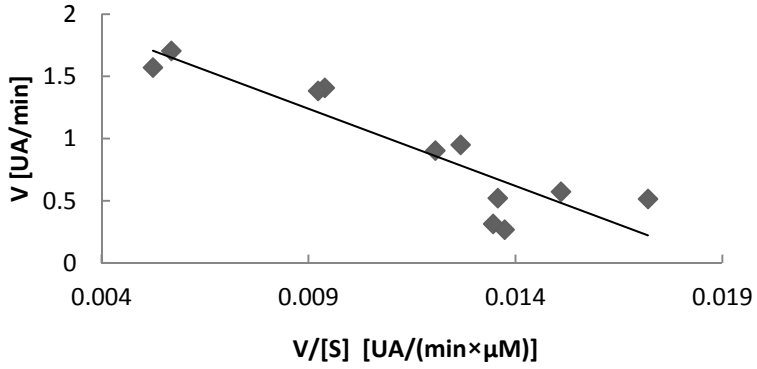

Figure 7. Eadie Hofstee plot for AO activity vs. ascorbate concentration.

In the case of PX, the Km values for the two substrates were 9 μM for H_2O_2 (at V_{max} = 0.017 UA/min) and 664 μM for ABTS (which was employed as model reducing substrate; V_{max} = 0.012 UA/min), according to Figure 8. Typical affinities of heme enzymes for peroxide are closer to the range of 1 mM. From this point of view, the PX purified here constitutes a particular exception, rivalling the non-heme peroxidases in this respect [16].

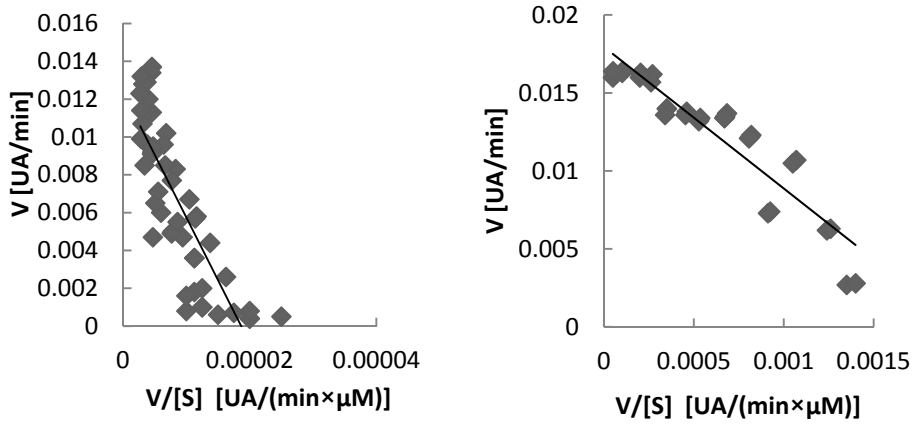

Figure 8. Eadie Hofstee plot for PX activity vs. ABTS (left) and peroxide (right) concentration.

Stability and dependence on reaction conditions

Figure 9 shows the pH dependence of the activity, for the two enzymes purified – AO and PX. The peroxidase shows a distinctly higher pH dependence, losing almost all of the activity above pH 5 – unlike AO for which the activity varies to a much smaller degree between pH 4 and 8.

Working at a pH close to optimum pH for both enzymes but not very close to the denaturation limit (pH 4, cf. Figure 9), the temperature dependence was examined cf. Figure 10. First, when measuring the activity immediately after exposing to the respective temperature, an optimal temperature of 50°C is noted for AO. Moreover, at 60°C essentially all AO activity is lost within 20 minutes – while at 50°C 80% of the activity is retained after 1 hr (data not shown), while for PX the maximum is at 70°C yet with only very small differences to 80°C.

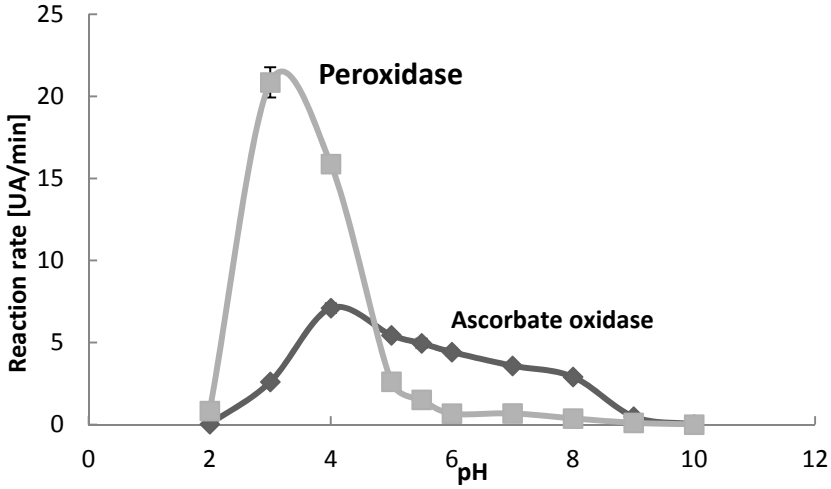

Figure 9. pH dependence of AO and PX activities.

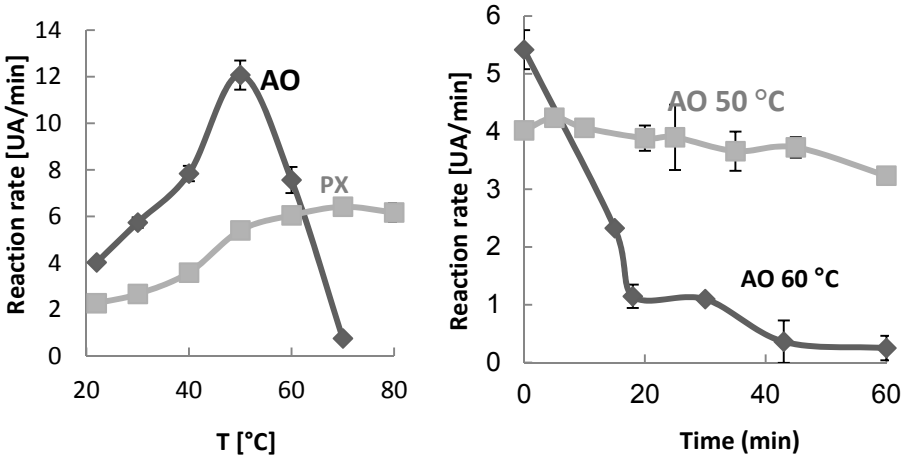

Figure 10. Influence of temperature on AO and PX.
Left: exposure immediately after mixing. Right: thermostability.

A number of potential inhibitors were also tested on AO and PX, cf. Figure 11, from a range typical of biotechnological applications. Expectedly, SDS is efficient, presumably via denaturation; notably, AO is completely inhibited even at 1 mM SDS, where PX retains most of its reactivity; this, alongside the temperature-dependent reactivity discussed above, reinforces PX as a more stable enzyme than AO. On the other hand, AO is much more

stable than PX towards ethanol, remarkably withstanding 20% without major loss of activity. Azide and fluoride (as typical exogenous metal ligands) inhibit peroxidase less than they do than AO.

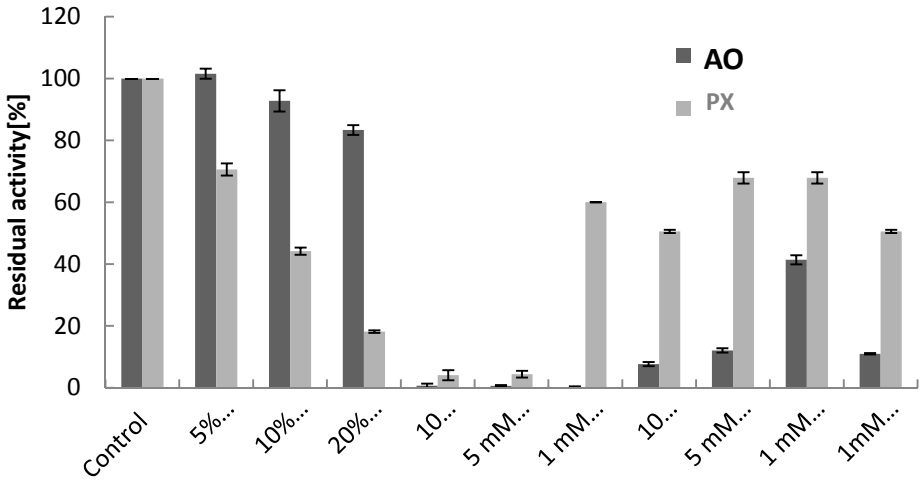

Figure 11. Influence of tested inhibitors on the activities of AO and PX.

MATERIALS AND METHODS

TrisHCl and ascorbic acid were purchased from Sigma-Aldrich (Germany), sodium tetraborate and sodium chloride from Reactivul Bucharest (Romania), ammonium sulfate from Lachner (Czech Republic), ABTS from TCI (Japan), sodium hydrogen phosphate from Chempur (Germany).

The AO purification protocol was based on previous reports [2], and involved 50 kg of green pumpkins per run. The majority of ascorbate oxidase is located in the peel of the fruit [6], which accounts for 20% of the total weight of the fruit. The pumpkins were washed, after which the peels were removed, with a thickness of 3 mm. The peels were subsequently homogenized in a blender (Kempo Juice Extractor, Green power) and squeezed thoroughly through a layer of cloth to maximize juice extraction. The pH of the ruslting suspension was adjusted to 6.7-6.9 by adding solid $Na_2B_4O_7$ (approximately one spatula per 500 mL). Solid ammonium sulfate (65%, (408 g/L) was subsequently added, and the mixture was stirred overnight in a cold room. The precipitate was collected decanted and centrifuged (5000 rpm, 10 min). At this stage the supernatant had negligible AO activity. The precipitate was then resuspended in ultrapure water (4℃, 1 L per 500 g solid), and then

centrifuged (10000 rpm, 10 min). At this stage, AO activity was by far confind to the supernatant. Solid sodium chloride was then dissploved in the supernatant to 13 g/L, after which acetone (pre-cooled to -30°C) was gradually added under stirring to a final volume ratio of 0.9 (acetone) : 1 (supernatant). The precipitate was decanted and centrifuged (5000 rpm, 10 min; AO activity was confined to the precipitate) and was then resuspended in ultrapure water (300 mL water to 150 mL precipitate). The resulting suspension was centrifuged (13000 rpm, 10 min), then the supernatant was subjected to chromatographic purification. The precipitate, when resuspended in water overnight with stirring, also contained residual AO activity but was not further employed.

The supernatant was adjusted to contain 1.6 M ammonium sulfate and was subjected to hydrophobic interaction chromatography on a *HiTrap Phenyl HP* 5 mL column (A: TRIS 10 mM, $(NH_4)_2SO_4$ 1.6 M; B: TRIS 10 mM) using a GE Healthcare FPLC chromatograph. Among others, this procedure allows for removal of nucleic acids, as they do not bind to the column. The fractions were monitored at 610 nm (absorbance specific to the AO T1 center), 280 nm (protein-specific) and 405 nm (heme-specific, chosen due to the brown color of the extract and on the assumption that this color was due to a heme protein). The major AO-containing fraction (40-70 mL elution volume cf. Figure 1) was collected and dialyzed against Tris 5 mM, pH=7.6, then centrifuged (15000 rpm, 15 min) loaded onto a *HiTrap QFF* 5 mL anion exchange column (A: TRIS 5 mM; B: TRIS 5 mM, NaCl 1 M), again monitored at 280, 405 and 610 nm. At this stage two separate fractions were collected: one with high 405 absorbtion (flow-though) and one with high 610 nm absorption (35-50 mL elution volume).

The resulting AO and PX fractions were then each concentrated using Amicon concentrators and were subjected to native electrophoresis using a preparative native PAGE gel. The migration time was kept to a minimum in order to avoid heating of the gel and autoreduction of AO as witnessed in loss of the blue color (nevertheless, this color is readily recovered upon elution from the gel).

The resulting AO fraction was adjusted to 1.6 M ammonium sulfate and subjected to one more round of hydrophobic interaction chromatography. The PX fraction obtained from native electrophoresis was dyalized and then loaded onto a cation-exchange column *HiTrap SP FF* 1 mL (A: Na_2HPO_4 5 mM; B: Na_2HPO_4 200 mM). A 12.5% yield was noted at the end of the procedure, for AO purification starting from the initial blended peels.

Protein contents were estimated using the Bradford method, with bovine serum albumin as standard and using a range of protein concentrations from 0 to 12 µg/mL. For ech measurement, 950 µL of Bradford reagent were mixed with up to 50 µL of sample as needed, after which ultrapure water was added to a total volume of 1 mL. Measurements were performed in duplicates.

AO activity was monitored at 240 nm in pH 5.5 acetate,10 mM, at room temperature. ABTS peroxidase reactivity was monitored at 420 nm [20].

Enzyme purity was verified by SDS-PAGE by standard protocols [16].

CONCLUSIONS

Reported here is a new protocol for purification of ascorbate oxidase from *Cucurbita pepo medullosa* (field pumpkin), with improved yields. The enzyme is biochemically characterized, including Michaelis-Menten parameters. Also, purification and characterization of the peroxidase from the same source is described, featuring an unusually high affinity for hydrogen peroxide (low-micromolar).

ACKNOWLEDGEMENTS

AMC thanks the World Federation of Scientists for a scholarship. Funding from the Romanian National Authority for Scientific Research and Innovation, CNCS – UEFISCDI, grants PCE 488/2012 (RSD) and PN-II RU-TE-2014-4-2555 (ACM), is gratefully acknowledged.

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