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QUINOPROTEIN AMINE OXIDASE FROM SAINFOIN SEEDLINGS

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Abstract—Amine oxidase (SAO) from sainfoin (*Onobrychis viciifolia*) was isolated by a new purification procedure involving three chromatographic steps. The homogeneous enzyme exists as a dimer with a M_r of 145 k, as demonstrated by gel permeation chromatography under non-denaturing conditions. SDS–PAGE revealed a M_r of 77 k for the single subunit. SAO is a slightly acidic protein (pI of 6.5), and it is pink with an absorption maximum (A_{max}) at 500 nm. Spectrophotometric titration of the enzyme by *p*-nitrophenylhydrazine demonstrated one reactive carbonyl group per subunit. The pH shift of A_{max} of SAO *p*-nitrophenylhydrazone and redox-cyclic quinone staining after SDS–PAGE were taken as evidence for the presence of topa quinone as the cofactor. The *N*-terminal amino acid sequence of SAO shows a high degree of identity to other plant and microbial Cu-amine oxidases. Substrate specificity of SAO and its interactions with inhibitors are similar to those of pea seedling amine oxidase.

INTRODUCTION

Copper-containing amine oxidases [amine: O_2 oxidoreductase, deaminating (EC 1.4.3.6)] catalyse the oxidative deamination of biogenic amines, producing the corresponding aldehydes, H_2O_2 and ammonia [1]. Copper and the carbonyl cofactor, recently identified as topa quinone [2,3], mediate the enzymatic reaction following a ping-pong mechanism [4]. Although these enzymes have been found in bacteria, fungi and various plants and animals, their actual physiological relevance has not been sufficiently understood to date [5]. The Cu-amine oxidases are widespread in the Fabaceae. The enzyme from sainfoin (*Onobrychis viciifolia*) was first purified by Peč *et al.* [6]. An improved purification method providing the homogeneous enzyme and its characterization are described in this article.

RESULTS AND DISCUSSION

For isolation of amine oxidase (SAO), 8-day-old etiolated seedlings of sainfoin with high enzymatic activity were used [6]. In the first homogenization step (in 0.01 M K-Pi buffer, pH 6.0) SAO activity was retained in the remaining plant material, but a great amount of ballast proteins was extracted. In the second homogenization step of the residual seedlings (in 0.2 M K-Pi buffer, pH 7.0), an enzyme solution of relatively high specific activity was obtained. Fractionated ammonium sulphate precipitation is often used for initial purification of plant amine oxidases in order to reduce the volume of crude enzyme preparations with a concomitant increase of specific activity [7]. This procedure, however, could not be employed in the present investigation since SAO exposed to NH_4^+ ions was rapidly deactivated. This observation corresponds to results obtained with amine oxidase from *Escherichia coli* [8]. As a consequence, carboxymethyl cellulose was used for the concentration and further purification of the second sainfoin homogenate. After stirring the homogenate with the sorbent for 3 hr at low ionic strength, SAO was completely adsorbed and then eluted by high ionic strength from this column. The enzyme was further purified by chromatography on hydroxyapatite and finally isolated in homogeneous form by size-exclusion chromatography on Sephacryl S-300HR. A summary of the purification procedure is given in Table 1. The total activity of the second homogenate was arbitrarily

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Table 1. Purification of amine oxidase from sainfoin seedlings

Purification Step	Volume (ml)	Total protein (mg)	Total activity (mkat)	Specific activity (nkat mg^{-1})	Purification (fold)	Yield (%)
Dialyzed 2nd homogenate	1090	886	20.7	23.4	1	100
CM-23 Cellulose	28	24	18.7	779	33.2	90.3
Hydroxyapatite	60	9.0	11.4	1270	54.1	55.1
Sephacryl S 300-HR	25.6	3.6	8.1	2250	95.5	39.1

taken as 100%. From 1 kg of sainfoin seedlings, 3.6 mg of homogeneous SAO at about 40% yield could be isolated. Chromatography on CM-cellulose, hydroxyapatite and Sephacryl resulted in an almost 100-fold enhancement of SAO specific activity. The final product yielded an enzyme with an absorption maximum at 500 nm (ε_{500} = 2.9 mM^{-1} cm^{-1}), which is in accordance with those of other plant amine oxidases [9].

SDS–PAGE revealed a single protein band corresponding to a M_r of 77 k (Fig. 1). M_r of SAO in the native state was determined by gel permeation chromatography on a Superdex 200 HR column using M_r marker proteins. Analysis of the mobility of SAO showed a M_r of 145 k, which, together with the M_r of the single subunit deduced from SDS–PAGE, confirms the dimeric structure of the native enzyme. In addition, a small percentage of enzymatically active SAO appeared as a high M_r aggregate of *ca* 290 k in the gel permeation profile, probably due to partial tetrameric arrangement of subunits in the native state (data not shown). Similar aggregation was reported for several other amine oxidases [5]. Our results are in agreement with general features of plant amine oxidases from pea and lentil seedlings [10, 11], but

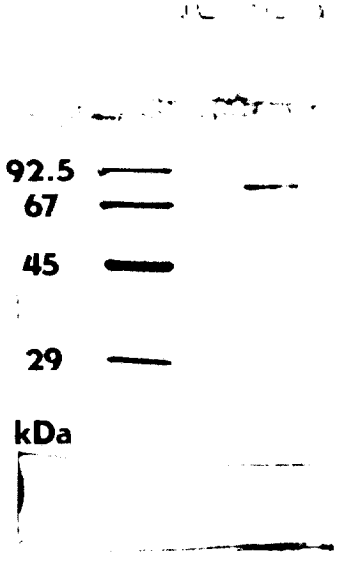

Fig. 1. SDS–PAGE of SAO (5 μg of purified enzyme); protein staining was done with Coomassie Brilliant Blue R-250. Standard protein M_r markers (see Experimental) were run in the left lane.

differ from previous observations made during the purification of SAO [6]. In that study, two isoforms of SAO were found by gel permeation chromatography. One major isoform was detected with an unusually low M_r of 25 k, whereas another minor isoform was eluted from a Sephadex G-50 column in fractions of higher M_r. Nevertheless, SAO purified with our new method did not show the occurrence of such isoforms. Apparently, it was the purification procedure used at that time, especially the application of the cationic precipitant methylalbumine, which led to a disintegration of the native enzyme. This assumption is further supported by the finding that the pI value of 8.5 for the low M_r fragment [6] is in clear contrast to the pI of 6.5 of the homogeneous enzyme determined in this work, which is similar to the pI of other plant amine oxidases [12]. In addition, the K_m values of the SAO substrates cadaverine (0.27 mM) and putrescine (0.72 mM) measured with the low M_r isoform [6] are different as well.

SAO showed a significant thermal stability. When incubated in 0.1 M K-Pi buffer, pH 7.0, the enzyme could be heated for 5 min at 60° without loss of activity. SAO was active towards substrates like 1,5-diaminopentane (cadaverine) with a K_m of 0.09 mM and 1,4-diaminobutane (putrescine) with a K_m of 0.24 mM. The maximal rate of oxidation for cadaverine at saturating concentrations is 2.7 fold higher than that of putrescine. In the case of pea amine oxidase, this ratio is significantly lower, reaching a value of only 1.3 [13]. A pH optimum of 7.5 for SAO was found with cadaverine as a substrate.

SAO, like other plant Cu-amine oxidases, is inhibited by substrate analogues (diaminoketones, *E*- and *Z*-1,4-diamino-2-butene), Cu^{2+} chelating agents (*o*-phenanthroline, 2,2′-bipyridyl), carbonyl reagents (aminoguanidine, phenylhydrazine) and some alkaloids (L-lobeline, cinchonine). Inhibition constants for *E*- and *Z*-1,4-diamino-2-butene, which are also oxidized by SAO, were determined as inhibition with an excess of substrate.

Irreversible inactivation of the enzyme with *p*-nitrophenylhydrazine gave rise to the coloured enzyme *p*-nitrophenylhydrazone with an absorption maximum at 460 nm at pH 7.0, and a different maximum at 585 nm in 0.5 M NaOH. This is taken as evidence for the presence of topa quinone as enzyme cofactor, as already observed for several amine oxidases that contain topa quinone [3]. The *p*-nitrophenylhydrazone of

the topa quinone analogue, 2-hydroxy-5-methyl-1,4-benzoquinone, had similar spectral characteristics showing an absorption maximum of 470 nm at pH 7.0 and of 610 nm in 0.5 M NaOH. SAO also gave positive quinone staining after SDS–PAGE. Titration of SAO with *p*-nitrophenylhydrazine showed consumption of *ca* 1.7 moles of reagent per mole of the enzyme ($\varepsilon_{460} = 25.8$ mM^{-1} cm^{-1} per subunit), which indicates the presence of one reacting carbonyl group in each subunit.

The *N*-terminal amino acid sequence of SAO blotted to PVDF membranes was determined by automatic Edman degradation. Fifteen residues were identified yielding a sequence of A-V-T-P-L-H-F-Q-H-P-L-D-P-L-T. This sequence is almost identical to sequences obtained from *c*DNA encoding the amine oxidases from lentil and pea seedlings [14, 15] and similar to those of a number of microbial amine oxidases [16].

EXPERIMENTAL

Plant material. Seeds of sainfoin were soaked for 24 hr in H_2O, transferred onto a layer of Perlite and germinated in the dark at 23° for 8 days [6].

Enzyme preparation. All procedures were performed at 0–5°. 8-day-old seedlings (1 kg after removal of peels) were homogenized by a Moulinex hand blender in 2 l 10 mM K-Pi buffer, pH 6, for 10 min and left standing for a further 30 min. Crude homogenate was passed through a nylon mesh cloth. The filtered soln was discarded, and the remains of the seedlings were subjected to a second homogenization in 1 l of 0.2 M K-Pi buffer, pH 7. The second homogenate was passed through a nylon cloth again and the filtrate was centrifuged for 60 min at 5000 *g*. The ppt was discarded and the supernatant dialysed against 10 mM K-Pi buffer, pH 6, overnight. The dialysate was centrifuged for 60 min at 20 000 *g* and then gently stirred for 3 hr with 30 cm^3 of CM-23 cellulose (Serva, Heidelberg, Germany), equilibrated in dialysis buffer. The material was packed in a glass column (1.5 × 20 cm) and washed with 10 mM K-Pi buffer, pH 6.). Then the enzyme was eluted with 0.2 M K-Pi buffer, pH 7, containing 2 M NaCl. Active frs were pooled and dialysed against 20 mM K-Pi buffer, pH 7. The dialysed enzyme soln was loaded onto a hydroxyapatite column [17] (2.5 × 20 cm), equilibrated with 20 mM K-Pi buffer, pH 7, and the column was extensively washed with the same buffer. After initial washing steps with 0.1 and 0.3 M K-Pi buffers, pH 7, SAO was eluted by 0.75 M K-Pi buffer, pH 7. Fractions of highest amine oxidase activity were pooled, dialysed against 20 mM K-Pi buffer, pH 7, overnight and concd in an ultrafiltration cell (Amicon, Danvers, MA, U.S.A.) equipped with a XM 50 filter. The concd enzyme soln was submitted to size-exclusion chromatography on a Sephacryl S-300 HR (Pharmacia Biotech, Uppsala, Sweden) column (2.5 × 50 cm) and eluted with the same buffer at a flow rate of 1.25 ml min^{-1}. Frs with the highest enzymatic activity were pooled and concd in the ultrafiltration cell as described above.

Activity and protein assay. Amine oxidase activity was measured using a coupled reaction of horseradish peroxidase and guaiacol [18] with cadaverine as a substrate. The protein concn was determined according to Bradford [19] with BSA as a standard.

M_r *determination.* SDS–PAGE was performed by a standard method [20] on a vertical slab gel (10% T, 2.5% C). Samples were treated for 10 min at 100° in the presence of 6% SDS before application to the gel. After electrophoresis, the protein bands were visualized by staining with Coomassie Brilliant Blue R-250. M_r marker proteins (Pharmacia) were used as references: phosphorylase *b* (92.5 k), BSA (67 k), ovalbumin (45 k) and carbonic anhydrase (29 k).

Size-exclusion chromatography was carried out on an LC 4A liquid chromatograph equipped with an SPD M6A photodiode array detector (Shimadzu, Kyoto, Japan) and a (1 × 30 cm) Superdex 200 HR column (Pharmacia). 0.1 M K-Pi buffer, pH 7, containing 0.2 M NaCl was used as a mobile phase. Proteins were eluted at a flow rate of 0.5 ml min^{-1} and detected at 214 nm and 280 nm. The following calibration proteins (Oriental Yeast, Tokyo, Japan) with the indicated M_r were used as references: glutamate dehydrogenase (290 k), lactate dehydrogenase (142 k), enolase (67 k), adenylate kinase (32 k) and cytochrome *c* (12.4 k).

Isoelectric focusing was performed on a polyacrylamide disc gel [21] with Ampholine 3–11 (Pharmacia) and acetylated cytochromes *c* (Oriental Yeast, Tokyo, Japan) as pI markers (pIs of 4.1, 4.9, 6.4, 8.3, 9.7 and 10.6). A sample gel (50 μg of SAO) was stained with Coomassie Brilliant Blue G-250.

Detection of the quinone cofactor. For quinone staining [22], proteins were electro-transferred onto a 0.45 μm Trans-Blot nitrocellulose membrane (Bio-Rad, Richmond, CA, U.S.A.) after SDS–PAGE in 25 mM Tris base, 0.192 M glycine buffer (pH 8.3), containing 20% MeOH, for 2 hr at 50 V. The membrane was stained for quinone for 30 min in the dark with 10 ml of 0.24 mM nitroblue tetrazolium in 2 M potassium glycinate buffer, pH 10.

Titration of SAO with p*-nitrophenylhydrazine.* The SAO sample (1.24 nmol) was titrated in 0.2 ml of 10 mM K-Pi buffer, pH 7, with a 0.5 mM EtOH soln of *p*-nitrophenylhydrazine by adding 1 μl aliquots at 2 min time intervals to the enzyme. After each addition the soln was briefly shaken and an absorption spectrum was recorded on a U3210 spectrophotometer (Hitachi, Tokyo, Japan).

Preparation of a benzoquinone analogue. The *p*-nitrophenylhydrazone of 2-hydroxy-5-methyl-1,4-benzoquinone was prepared immediately before the measurement by extracting the synthesized quinone [23] from 1 ml of a 0.47 mM soln in CH_2Cl_2 with 0.7 ml of 10 mM K-Pi buffer, pH 7. Then 0.3 ml of 1 mM *p*-nitrophenylhydrazine freshly dissolved in the same

buffer was added and the reaction mixture was left standing for 10 min at room temp. and centrifuged for 10 min at 15 000 *g* to remove traces of CH_2Cl_2.

N-terminal sequencing of SAO was performed by automatic Edman degradation on a Model 476A protein sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Samples were obtained by SDS–PAGE and subsequently the protein was transferred to PVDF membranes by semi-dry blotting. Phenylthiohydantoin derivatives of the respective amino acids were separated on Brownlee PTH C 18 microbore columns (2.1 × 220 mm, 5 μm particle size). Composition of the mobile phases, gradient programs, temp., flow rates and wavelength of detection are given in ref. [24].

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