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BRIEF COMMUNICATION

Characterization of polyphenol oxidase from the latex of opium poppy

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Abstract

Polyphenol oxidase from the latex of opium poppy was purified to the electrophoretic homogeneity by affinity chromatography using *p*-aminobenzoic acid as a ligand coupled to *Sepharose CL-4B* by divinyl sulphone activation method. The purified enzyme was used to prepare the polyclonal antibodies. The purified latex PPO exhibited high diphenolase activity in comparison with almost unmeassurable monophenolase activity. Both of these activities were sensitive to the activation with sodium dodecyl sulphate. Two isoforms (65 and 40 kDa) of latex PPO were separated by the gel filtration. There were no differences in substrate specifity (weak monophenolase and high diphenolase activity) and sensitivity to inhibitors between these isoforms, but they showed differences in electrophoretic mobility.

Additional key words: enzyme isoforms, *Papaver somniferum*.

The opium poppy (*Papaver somniferum* L.) is one of the oldest cultivated plants. The plant still remains important as the only source for the analgetic and antitussive drugs morphine and codeine, in addition to a number of other benzylisoquinoline alkaloids of pharmaceutical significance, such as the muscle relaxants papaverine and noscapine. The biosynthesis of benzylisoquinoline alkaloids starts with the condensation of dopamine and 4-hydroxyphenylacetaldehyde to form the first intermediate with benzylisoquinoline structure, (S)-norcoclaurine (Stadler *et al.* 1989). Dopamine may arise from L-tyrosine (Tyr) by decarboxylation first (by the action of decarboxylase) and by hydroxylation of tyramine by polyphenol oxidase, or L-tyrosine is first hydroxylated to 3,4-dihydroxyphenylalanine (DOPA) by PPO and then decarboxylated (Rueffer and Zenk 1987). Synthesis of 4-hydroxyphenylacetaldehyde from another molecule of L-tyrosine involves its decarboxylation and deamination. Deamination may be catalysed by transaminase (Rueffer and Zenk 1987) or by amine oxidase (Bilková *et al.* 2000a,b).

Tyr/DOPA decarboxylase is a candidate for control the accessibility of dopamine, tyramine and 4-hydroxyphenylacetaldehyde for biosynthesis of norcoclaurine. This enzyme is intensively studied at molecular level (Facchini and de Luca 1994). Contemporary research is focused on the regulatory sequences of the Tyr/DOPA decarboxylase gene family (Park *et al.* 1999).

The presence of the PPO in latex is known from the seventies (Roberts 1971), however the knowledge of this enzyme is still inadequate in opium poppy. Its molecular characteristics are missing completely and the role of enzyme in latex is unclear. The localization of the biosynthesis of the benzylisoquinoles in latex is doubted (Facchini and Bird 1998), but not definitively refused. It seems, that the main role of the PPO in latex is in defense response upon plant wounding.

In our previous work we described a method for isolation of opium poppy latex polyphenol oxidase in a single step to the electrophoretic homogeneity (Bilka *et al.* 2000). In this work we present further characterization of the purified polyphenol oxidase from latex of opium poppy.

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Abbreviations: *PABA-Sepharose* - *p*-aminobenzoic acid coupled to *Sepharose CL-4B* (*Sigma*, St. Louis, USA); PPO - polyphenol oxidase; SDS - sodium dodecyl sulphate; TMB - 3,3′,5,5′-tetramethylbenzidine.
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The opium poppy latex was harvested 5 - 7 d after flowering by incising the capsule with a scalpel. The latex was collected into the test tubes containing ice cold 0.5 M mannitol + 0.1 M sodium phosphate buffer, pH 6.5, to reach the final ratio of latex/mannitol-phosphate buffer 1:1. The organelles of latex were sedimented by centrifugation (1 000 *g*, 30 min, 4 °C) and suspended to the original volume in mannitol-phosphate buffer. The organelles were desintegrated by three-fold freezing/thawing and sonication in the presence of 1 % Triton X-100. After centrifugation (12 000 *g*, 15 min, 4 °C) the obtained soluble fraction was used as a crude extract.

The purification of the latex PPO using affinity chromatography on *PABA-Sepharose* was described previously (Bilka *et al.* 2000). An 93.5-fold increase of the specific activity was achieved and the purified PPO was homogeneous on SDS-PAGE.

To prepare polyclonal anti-PPO serum affinity chromatography purified PPO from latex was subjected to SDS-PAGE and PPO bands were excised from the gels. The gel slides were crushed with an equal volume of phosphate buffered saline (PBS). Two rabbits were injected subcutaneously with 200 μg of PPO (*ca.* 2 cm^3 of PBS). Two booster injections (200 μg of PPO) were given at intervals of 1 month. The rabbits were bled on the tenth day after last injection and the antisera were collected.

The separation of native isoforms of PPO (purified by affinity chromatography) was carried out by gel filtration on a *Sephadex G-100* (*Sigma,* St. Louis, USA) column (49 cm × ∅ 3 cm). As equilibrating and running buffer 0.1 mmol dm^{-3} sodium phosphate buffer, pH 6.5, was used. The flow rate was 9 cm^3 h^{-1} and collected fraction volume was 2.5 cm^3. The relative molecular masses of separated isoforms were determited using standard proteins (68, 45, 25 and 12.5 kDa).

The both activities of PPO (monophenolase and diphenolase) were measured colourimetrically using substrates in 2 mmol dm^{-3} concentration according to Escribano *et al.* (1997). One unit of enzyme activity was defined as a change of absorbance at 475 nm by 0.1 per min.

Native electrophoresis (PAGE) was performed according to Rodriquez and Flurkey (1992). Denaturating SDS-PAGE was performed according to Laemmli (1970). Samples in reducing conditions were prepared by boiling (100 °C, 3 min) in 0.5 volume of sample buffer [0.0625 mol dm^{-3} Tris/HCl, pH 6.8, 69 mmol dm^{-3} (2 %) SDS, 10 % glycerol, 5 % mercaptoethanol]. After electrophoresis, proteins were transferred to nitrocellulose using *Trans-Blot SD Semi-Dry Transfer Cell* (*Bio Rad,* Richmond USA) according to manufacturer instructions. The PPO was detected using imunoblot method with anti-PPO serum. The second antibody reaction was carried out using swine immunoglobulines against rabbit immunoglobulines, conjugated with peroxidase (*SwaR-Px, Sevac,* Praha, Czech Republic). Reaction was visualised with TMB stabilized substrate for horseradish peroxidase (*Promega,* Madison, USA). The protein content was determined using the method of Bradford (1976).

Monophenolase activity of latex PPO with L-tyramine as substrate was very low. With L-tyrosine as substrate, detectable activity was obtained only after extension of reaction time. In both cases, the monophenolase activity significantly increased (more than four times) in the presence of 3.45 mmol dm^{-3} SDS (Fig. 1).

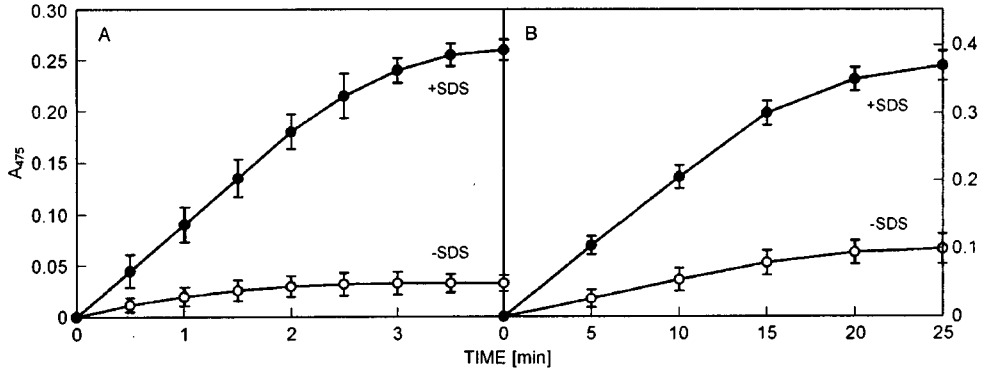

Fig. 1. Monophenolase activity of latex PPO. As substrates were used tyramine (*A*) and L-tyrosine (*B*). The reaction medium at 20 °C contained 2 mM of substrate in 1.8 cm^3 of sodium phosphate buffer (50 mM, pH 6.5) and 0.015 mg of purified PPO. The activity was measured without activation (-SDS) and in presence of 3.45 mM SDS. Means ± standard deviation from 5 separate experiments.

Diphenolase activity of latex PPO was studied with dopamine and DOPA as substrates. Latex PPO showed almost equal affinity to these substrates - K_m for dopamine was found to be 16.9 mmol dm^{-3} and for DOPA 17.9 mmol dm^{-3}. Enzyme exhibited a broad peak of pH optimum (6.5 to 10.0). The strong inhibitory effects were observed with potassium cyanide (IC_{50} ~ 0.42 mmol dm^{-3}) and cystein (IC_{50} ~ 0.3 mmol dm^{-3}). As possible activators $CuSO_4$ and SDS were tested. In case of $CuSO_4$ the maximal effect was observed at concentration 2 mmol dm^{-3}, when the activity of PPO increased about 33 % compared to the control. SDS

(up to 3.45 mmol dm^{-3}) appeared as a powerful activator of diphenolase activity of latex PPO (Fig. 2). SDS influenced the electrophoretic mobility of PPO, too. Incubation of sonicated latex (10 min, 20 °C) with different concentrations of SDS before native PAGE caused that the heavier PPO isoform became gradually undetectable (Fig. 3).

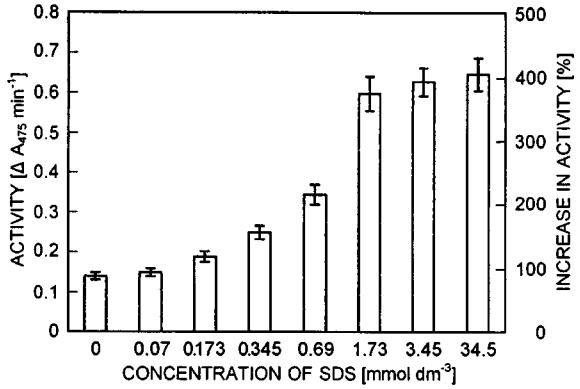

Fig. 2. Influence of various concentrations of SDS on PPO activity. Substrate 2 mM DOPA, 0.01 mg of purified PPO, 50 mM sodium phosphate buffer, pH 6.5. Means ± SD from 5 separate experiments.

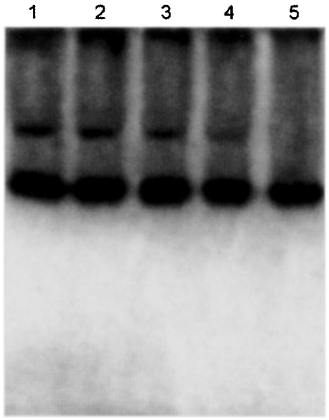

Fig. 3. Influence of SDS on the electrophoretic mobility of non-purified PPO. *Lane 1* without SDS, *lane 2* with 0.345 mM SDS, *lane 3* with 0.69 mM SDS, *lane 4* with 1.725 mM SDS and *lane 5* with 3.45 mM SDS (10 min incubation of sonicated latex with SDS at room temperature before loading to the 3 % native stacking gel; 7 % native separating gel).

Two isoforms of PPO were separated using gel filtration. The relative molecular masses of separated isoforms determined using standard proteins were *ca.* 65 and 40 kDa (Fig. 4). No differences were observed between these two isoforms - the monophenolase activity of both isoforms was almost unmeasurable without activation, their substrate specifity and sensitivity to activators and inhibitors of diphenolase activity were found to be identical (IC_{50} for KCN approximately 0.4 mmol dm^{-3}, IC_{50} for Cys *ca.* 0.3 mmol dm^{-3} and *ca.* 4.5-fold increase of activity with 3.45 mmol dm^{-3} SDS in both cases). Separated isoforms were submitted to denaturating electrophoresis and immunobloting. From the first peak (heavier isoform) one band (65 kDa) was detected. In the line with isoform from the second peak also one band (40 kDa) was identified (Fig. 5).

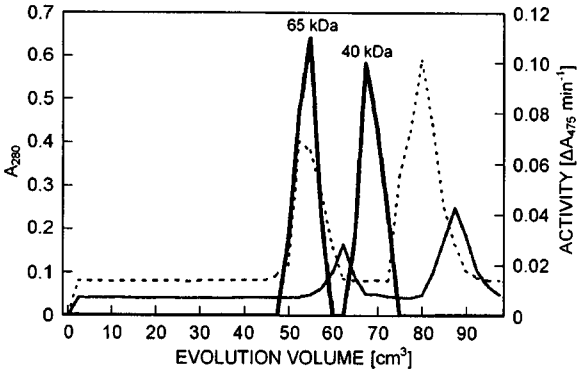

Fig. 4. Gel filtration of latex PPO on the column of *Sephadex G-100*. Standards of relative molecular mass: bovine serum albumine 68 kDa (elution volume 52.5 cm^3), egg albumine 45 kDa (elution volume 62.5 cm^3), chymotrypsinogen 25 kDa (elution volume 80 cm^3), cytochrome *c* 12.5 kDa (elution volume 87.5 cm^3). Standards of Mr were identified at 280 nm; PPO in reaction with DOPA at 475 nm. (*full line* - standards of 12.5 and 45 kDa, *dashed line* - standards of 25 and 68 kDa, *thick full line* - PPO).

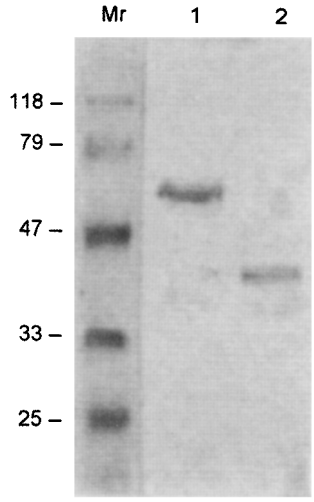

Fig. 5. Imunoblot of separated isoforms of latex PPO after denaturating PAGE in reducing conditions (12.5 % gel). *Lane 1* sample from the first peak from the separation by gel filtration of latex PPO, *lane 2* sample from the second peak. Mr - prestained protein molecular mass marker (*MBI Fermentas*, Hanover, USA) 118; 79; 47; 33 and 25 kDa.

The benzylisoquinoline biosynthetic pathway involves a number of hydroxylations which are catalysed with high specific cytochrome P-450-dependent hydroxylases. An exception is the hydroxylation of tyramine to

dopamine (L-Tyr to DOPA, respectively) where the specific enzyme was not identified yet (Chou and Kutchan 1998). So the PPO with its broad substrate specifity is the enzyme which participation in the conversion of tyramine to dopamine in benzylisoquinoline biosynthesis is presupposed. The concentration of dopamine in the opium poppy is significantly higher in comparison with those of tyrosine, tyramine and 4-hydroxyphenylacetaldehyde (Zichová *et al.* 1996a,b). Because the accessibility of these precursors could be a limiting factor of the endogeneous level of morphinanes, the PPO can become one of the potential targets for the biotechnological exploitation of alkaloid production in *Papaver somniferum*.

The latex PPO, like the PPO of other plants, catalyses two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity). The monophenolase activity of the latex PPO in comparison with diphenolase activity is very low. The occurrence of plant PPO with low monophenolase activity was described by Jiménez and García-Carmona (1996) and Escribano *et al.* (1997). Like in some other enzymes, the PPO from opium poppy latex is active in SDS concentrations that cause the loss of catalytic activity of many other enzymes (Moore and Flurkey 1990, Escribano *et al.* 1997). The mechanism of SDS activation of PPO is unknown. SDS might cause the conformational change of enzyme molecule, solubilization of enzyme or the removal of inhibitor. An *in vivo* regulatory mechanism which would mimic the effect of SDS activation is unknown yet (van Gelder *et al.* 1997).

Similar activating effect of $CuSO_4$ like in the latex PPO was observed on PPO from artichoke (Leoni and Palmieri 1990), but Fujita *et al.* (1995) described $CuSO_4$ as an inhibitor of the PPO from cabbage. Range of activity from pH 6.5 to 10 as in latex was described in artichoke with pyrocatechol as a substrate (Leoni and Palmieri 1990). The pH optimum of plant PPOs is usualy in a range of pH 6.5 - 7.5 (Escribano *et al.* 1997, Nozue *et al.* 1998).

Two isoforms of PPO were identified in opium poppy latex. There were no differences in substrate specifity and in sensitivity to effectors between these two isoforms. Because of similarity in enzymological properties we assumed that the heavier isoform is a dimer containining the 40-kDa isoform. This assumption might be supported by observation, that when the sonicated latex was before the native PAGE incubated with increased concentration of SDS, by 0.1 % (3.45 mmol dm^{-3}) SDS the band of heavier isoform became undetectable. In contrary with assumption of dimer-monomer relationship between isoforms is the result of immunobloting when after denaturating PAGE in reducing conditions, separated isoforms bands with different Mr were detected. This fact supports evidence about existence of premature PPO form (65 kDa) and mature form (40 kDa) like in polyphenol oxidases from chloroplasts of several plants (van Gelder *et al.* 1997). The possibility that adding of SDS to non-purified PPO can assist the maturation of PPO was not discussed yet. Only the knowledge of amino acid sequences of opium poppy latex PPO isoforms can give an answer about relationship between PPO isoforms.

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