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Purification of polyphenol oxidase from opium poppy latex

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Opium poppy (*Papaver somniferum* L.) remains an economically important medicinal plant, because it is the only commercial source of several pharmaceutical alkaloids. The biosynthesis of benzylisoquinoline alkaloids begins with the condensation of dopamine and 4-hydroxyphenylacetaldehyde to form the first alkaloid intermediate, (*S*)-norcoclaurine [1]. Polyphenoloxidase (PPO) is assumed to be involved in the formation of dopamine from tyrosine [2]. It was shown [3] that PPO in latex is located in organelles which sediment at $1000 \times g$. These organelles are supposed to accumulate and store the opium poppy alkaloids. The substrate specificity of the latex PPO was particularly studied by Roberts [4], but the latex PPO was not isolated to homogeneity up to now, and its molecular characteristics are not known so far. In this paper the affinity purification of the latex PPO to homogeneity and determination of its molecular weight are described.

The matrix prepared by coupling p-aminobenzoic acid (PABA) to Sepharose-4B by the divinylsulphone activation method was used for purification of PPO [5, 6]. The crude enzyme was prepared by sonication and freeze-thawing of $1000 \times g$ organelles in the presence of 1% Triton X-100. The enzyme preparation was loaded directly on the affinity column equilibrated in 50 mM sodium phosphate buffer (pH 6.0). The column was washed by a pH gradient (from pH 6.0 to pH 8.0) of a 50 mM Na^+ -phosphate buffer. The PPO active protein fractions were obtained by elution with 1 M NaCl (Fig. 1).

The specific activity of crude enzyme preparation was $2.78 \text{ U} \cdot \text{mg}^{-1}$ and that of enzyme solution after affinity chromatography was $260 \text{ U} \cdot \text{mg}^{-1}$. By this single step a

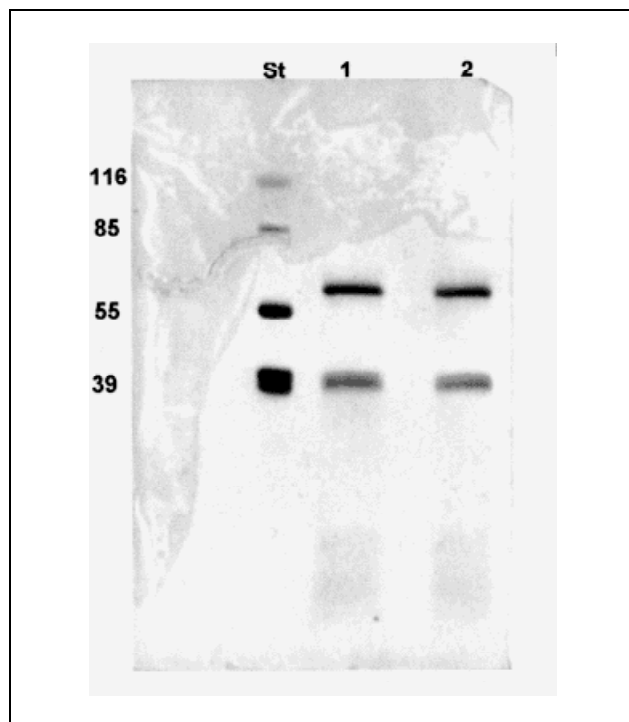


Fig. 2: SDS-PAGE of opium poppy latex PPO. St — molecular weight standards, 1 — PPO under reducing conditions, 2 — PPO under non-reducing conditions. Proteins were stained with silver [11]

93.5-fold purified enzyme solution was achieved with 95.5% recovery of enzyme activity.

The purified enzyme was analysed by SDS-PAGE [7]. Two bands were obtained by reducing and non-reducing condition (Fig. 2). The M_r were determined using standard proteins and were estimated to be 65–66 kDa and 40 kDa.

The M_r of the larger isoenzyme is similar to those of other plants. M_r of different plants reported were between 60–65 kDa [5, 8]. A 40-kDa isoenzyme was reported in

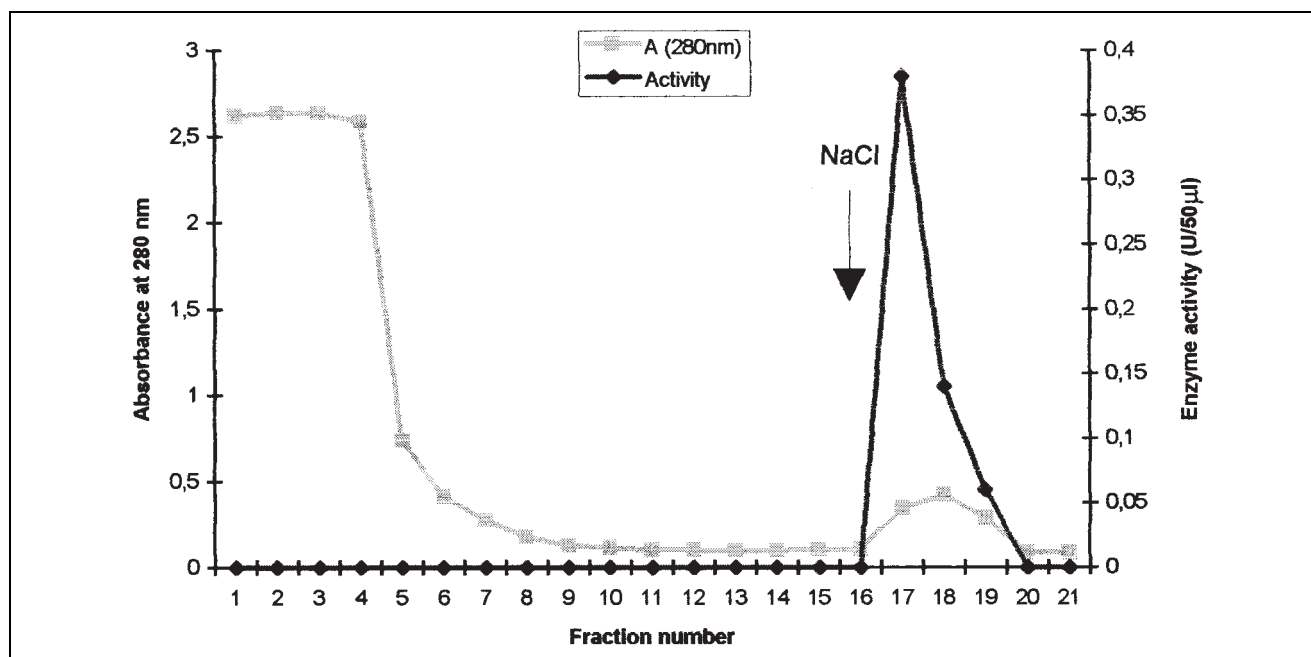


Fig. 1: Isolation of opium poppy latex PPO by affinity chromatography on PABA-Sepharose. The column was equilibrated with 50 mM Na^+ phosphate buffer pH 6.0. Enzyme solution was applied to it and the column was washed with phosphate buffer pH gradient 6.0–8.0 (fractions 1–16). Elution was performed with 1 M NaCl in phosphate buffer pH 8.0 (fractions 17–21)

mung bean and sweet potato [9, 10], but it was suggested, that the 40-kDa subunit is a result of proteolytic cleavage of the 65-kDa isoenzyme.

Experimental

The opium poppy latex was collected into test tubes containing 0.5 M mannitol/0.1 M phosphate buffer pH 6.5 in a way that the final ratio of latex/mannitol-phosphate buffer 1:1. Organelles were sedimented by centrifuging the collected latex at $1000 \times g$ for 30 min and suspended to the original volume in mannitol-phosphate buffer [3].

The organelles were desintegrated by three-fold freeze/thawing and sonication in the presence of 1% Triton X-100. After centrifugation ($12000 \times g$, 15 min, 4 °C) the obtained soluble fraction was used as a crude extract.

Sephacrose 4B (10 ml) was activated using divinylsulphone [5, 6]. The activated Sepharose was suspended in 1 M sodium carbonate (pH 11) containing PABA. Coupling was carried out at 4 °C for 24 h with continuous stirring.

PABA-Sepharose was filled in a glass column. Crude enzyme (3 ml; 7.5 mg of proteins) was applied to the column (12 cm \times \varnothing 14 mm). The column was washed with a gradient of 50 mM phosphate buffer, pH 6.0 to 8.0 (flow-rate 12 ml \cdot h⁻¹). The PPO activity was eluted with 1 M NaCl in 0.05 M phosphate buffer, pH 8.0.

The activity of PPO was measured colourimetrically using 2 mM DOPA as the substrate. One unit of enzyme activity was defined as a change of 1.0 absorbance per min at 475 nm.

SDS-PAGE was performed as described by Laemmli [7]. Under reducing conditions the sample was boiled with β -mercaptoethanol; under non-reducing conditions without boiling and β -mercaptoethanol. Proteins on the gel were stained with silver [11].

The protein content was determined using the Bradford method [12].

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Composition of the essential oil of *Micromeria thymifolia* (Scop.) Fritsch and its chemical variation

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Micromeria thymifolia (Scop.) Fritsch (Lamiaceae) is a perennial dwarf shrub. The habitat of this aromatic plant are gorges and crevices in limestone ranging from 30 m to 2000 m above sea level. Since it grows over the whole area of the Dinarides and in some small regions in northern Croatia and Hungary, the plant is a typical representative of the Illyrian flora [1, 2]. The previous research on *M. thymifolia* revealed the presence of flavonoids [3–5], saponins [6], triterpenic acids [7] and essential oil [8]. The first study of the essential oil composition was reported in 1988. The oil which was obtained in 1.2% yield contains mostly oxygenated monoterpenes. The main component of the oil is pulegone (64%) [9]. Antimicrobial and diuretic effects of the ethanolic extract and the essential oil isolated from this plant were investigated. High activity against a large number of bacteria and fungi, especially dermatophytes, was confirmed [10–12]. The essential oil and ethanolic extract also showed a strong diuretic effect [13]. These observations led us to investigate the essential oil composition in details. Results of a comparative study of the essential oils isolated from wild plants collected at three different localities are presented here.

The yields of essential oil obtained by hydrodistillation from aerial parts of *M. thymifolia* were 1.5% v/w (Učka), 0.6% v/w (Dariva) and 1.3% v/w (Ivančica). The oils (samples A, B and C) constituted a complex mixture of 36, 25 and 18 compounds respectively. Of these 24, 19 and 17 could be assigned by GC and combined GC/MS, representing 96.4%, 98.4% and 99.8% of the total oils. In the Table the compounds are listed in the order of elution on a WCOT fused silica column CP-Sil 8CB. All samples were characterised by a very high content of oxygenated monoterpenes: 77.4% in sample A, 94.0% in sample B, and 83.3% in sample C. Other components were mono- and sesquiterpene hydrocarbons and sesquiterpene alcohols. The content of monoterpene hydrocarbons (+1.8 cineole) was 11.7% and 15.7% in samples A and C, but much lower in sample B (3.2%). Sample A presented the highest value of sesquiterpenes (7.3%), while they constituted 1.2% and 0.8% of the other two oils. There were great differences in the relative amounts of cyclic oxygenated monoterpenes (ketones, oxides and alcohols) as the main constituents of the oils. Sample A was characterised by a high ketone fraction (69.3%) of which pulegone was the main component representing 67.5% of the total oil. Oxides and ketones in a ratio of 3:1 were the main constituents of sample B. Piperitenone oxide (69.9%) was identified as the major component followed by lower amounts of pulegone (12.9%) and piperitone (7.3%). Sample C was composed mainly of isomenthol (71.9%). Significant contents of myrcene (11.2%) and linalool (7.5%) were also detected. These results showed a major composition difference between the investigated oils and led to the conclusion that *M. thymifolia* forms chemotypes. Sample A was isolated from a pulegone chemotype which was rich in essential oil with a high level of pulegone and very small contents of other cyclic oxygenated monoterpenes. This chemotype was collected in the Submediterranean zone of

Table: Composition of the essential oil of *Micromeria thymifolia* (Scop.) Fritsch from different localities

Component	Locality		
	Učka (%)	Dariva (%)	Ivančica (%)
α -Thujene	0.3	0.1	0.6
α -Pinene	6.1	0.5	3.2
Sabinene	0.1	0.1	0.4
β -Pinene	0.5	—	0.1
Myrcene	0.8	2.2	11.2
α -Terpinene	0.2	—	—
<i>p</i> -Cymene	0.2	—	—
Limonene + 1,8 Cineole	3.5	0.3	0.2
Unidentified	0.3	0.1	0.2
Unidentified	0.7	0.1	—
Linalool	5.2	—	7.5
Camphor	0.4	0.1	0.5
Menthol	1.3	0.4	0.3
Isomenthol	1.0	0.7	71.9
Pulegone	67.5	12.9	0.3
Isopulegone	0.5	0.3	0.1
Piperitone	0.1	7.3	0.7
Carvone	0.6	0.3	—
Thymol	0.4	0.6	—
Carvacrol	0.1	0.3	—
Unidentified	0.1	0.2	—
Unidentified	0.1	0.3	—
Unidentified	0.5	—	—
Piperitenone	0.2	1.2	0.2
Piperitenone oxide	0.1	69.9	1.8
Caryophyllene	4.6	0.3	0.5
Cadinene	2.0	0.6	—
Unidentified	0.9	0.7	—
Unidentified	0.1	0.2	—
Ledol	0.3	0.3	0.3
Unidentified	0.1	—	—
Unidentified	0.2	—	—
Unidentified	0.2	—	—
Unidentified	0.1	—	—
Elemol	0.4	—	—
Unidentified	0.3	—	—

Croatia, at an altitude of 1200 m (Učka – the western part of the Dinarides). A smaller amount of essential oil (sample B) yielded from the plant material collected in the central continental region of Bosnia and Herzegovina (Dariva – the middle part of the Dinarides, 680 m above sea level). The oil which was constituted mainly of piperitenone oxide associated with lower contents of pulegone and piperitone represented the piperitenone oxide chemotype. The isomenthol chemotype was rich in essential oil with a clearly different composition from the other two samples. The oil (sample C) was characterised by a high alcohol fraction (mainly isomenthol) but by very low percentages of ketones and oxides. This chemotype was collected in the north-western continental part of Croatia (Ivančica, 500 m above sea level) where it grows in a small restricted region beyond the main distribution area of the Dinarides.

The results of our examination also confirmed the assumption that the biological effects of the essential oil of *M. thymifolia* can be different depending on the origin of the plant material.

Experimental

1. Materials

Aerial parts of wild *M. thymifolia* in the flowering stage were collected at three different regions in Croatia and Bosnia and Herzegovina: Učka

(above Lovran, altitude 1200 m, south-eastern exposure, July 1994), Ivančica (Belečgrad, altitude 500 m, southern exposure, June 1995), Dariva (near Sarajevo, altitude 680 m, south-eastern exposure, August 1994). Voucher specimens No. 812 (1–3) are deposited at the Herbarium Croaticum (ZA), Department of Botany, Faculty of Science, University of Zagreb, Croatia.

2. Isolation of the essential oil

Air dried plant materials were hydrodistilled for 3 h using a Clevenger-type apparatus. The obtained oils, after drying over anhydrous sodium sulfate, were submitted for capillary GC and GC/MS analysis.

3. GC and GC-MS analysis

The analysis performed with a Pye Unicam Pu 4550 GC, equipped with a FID coupled to a PU 4810 integrator; column: WCOT fused silica CP-Sil 8CB (25 m \times 0.32 mm i.d., coating thickness 0.13 μ m). Working conditions: injector temperature 220 °C, detector temperature 250 °C; oven temperature program: 60 °C (1 min), 60–240 °C at 4 °C \cdot min⁻¹; carrier gas: 0.3 ml H₂/min, split ratio 1:50. GC-MS analyses were performed on a Shimadzu QP 1000 GC/MS-EI 70 eV connected to a data station. The components were identified by comparing their retention times to those of authentic samples, as well as by comparing their MS spectra with literature data. The quantitative data were obtained by the peak normalisation technique using integrated FID responses.

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