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BRIEF COMMUNICATION

Soapwort oxidoreductase is involved in trinitrotoluene detoxification

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Abstract

Plant enzymes participating in degradation of nitroaromatic compounds have not been biochemically characterized in details so far. From suspension culture of soapwort (*Saponaria officinalis* L.) we isolated a novel plant oxidoreductase involved in degradation of trinitrotoluene (TNT). The enzyme catalyses first steps of reduction of TNT nitro groups in the presence of NAD(P)H under anaerobic conditions. The enzyme is monomeric with molecular mass 29 kDa, its two isoforms have pI 4.8 and 5.1. According to the spectral and activation analysis the enzyme contains flavinmononucleotide as a prosthetic group. The structure properties suggest an evolutional relationship to oxophytodienoate reductase. The N-terminal amino acid sequence shows homology to family of Old Yellow Enzyme (E.C. 1.6.99.1).

Additional key words: flavoprotein, Old Yellow Enzyme, oxidoreductase, phytoremediation, *Saponaria officinalis*.

Nitroaromatic compounds represent an environmental problem, mainly due to their persistency for decades in the soil and due to their high toxicity to many organisms (Kaplan and Kaplan 1982). The biodegradation of nitroaromatic compounds (NACs) was performed by microbial cells (Nishino *et al.* 1993, Boopathy *et al.* 1994, Scheibner *et al.* 1997, Samson *et al.* 1998), by lower plants (Tadros *et al.* 2000), as well as by higher plants (Larson *et al.* 1999, Sens *et al.* 1998, 1999, Bhadra *et al.* 1999a,b, Vaněk *et al.* 2003, Snellix *et al.* 2001). Only a few papers were focused on the study of enzyme(s) participating in the degradation of nitroaromatics (Mishkiniene *et al.* 1998) and tests of nitroreductase activity (Shah and Spain 1996, Shah and Campbell 1997). The TNT degradation pathway in plants is based on reduction of TNT nitrogroups (Vanderford *et al.* 1997, Rivera *et al.* 1998, Scheidemann *et al.* 1998, Larson *et al.* 1999, Vaněk *et al.* 2003, Nepovím *et al.* 2004). The reaction leads to formation of aminodinitrotoluenes (Fig. 1). Hydroxylamino- and aminoderivates of TNT were found to be transient intermediates forwarding to formation of different compounds, mainly conjugates or polymers. As an alternative degradation pathway oxidation processes on a methyl group were observed (Bhadra *et al.* 1999b, Snellinx *et al.* 2002). TNT degradation was studied using radiolabeled [^{14}C]-TNT (Bhadra *et al.* 1999a, Sens *et al.* 1999, Nepovim *et al.* 2005). In the roots of wheat (*Triticum aestivum*) 43 % of ^{14}C was observed in the cytoplasm and 57 % was found in the cell wall. In the cell wall 27 % of ^{14}C taken up or adsorbed by wheat was bound in the lignin structures (Sens *et al.* 1999). Characterization and identification of TNT degradation products is difficult due to the insoluble/inextricable fractions bound in the plant cells.

Characterization of the enzymatic system participating in degradation of TNT or other nitro-aromatics in plants still has not been done sufficiently. We present here the first results, that TNT is not metabolised by special nitroreductase, but by the constitutive enzyme with nitroreductase activity.

Soapwort (*Saponaria officinalis*) grown in the area of former ammunition factory in the soil contaminated with

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Abbreviations: BAP - N^6-benzylaminopurine; 2,4-D - 2,4-dichlorphenoxyacetic acid; MS - Murashige and Skoog; TNT - trinitrotoluene;
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TNT was chosen as a model plant. Callus culture of soapwort was initiated from surface sterilized seeds on the basal MS medium (Murashige and Skoog 1962) supplemented with 10 mg dm^{-3} of 2,4-dichlorphenoxy-acetic acid (2,4-D), 1 mg dm^{-3} of N^6-benzylaminopurine (BAP) and 1 mg dm^{-3} of kinetin and solidified by agar (0.7 %). The callus culture was transferred into the liquid medium and cultivated in Ehrlenmayer flasks on horizontal shaker in the dark at 25 °C. The suspension culture was subcultured at 2-week intervals (Fig. 2).

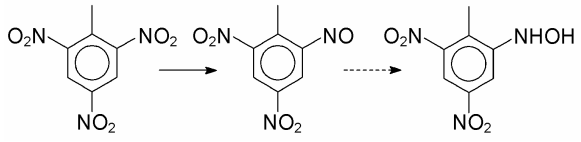

Fig. 1. First steps of reducing degradation pathway of nitrogroup of TNT.

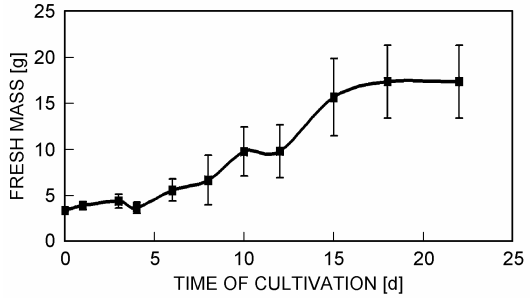

Fig. 2. Growth characteristic of suspension culture of soapwort (*Saponaria officinalis*) in liquid medium in dark.

The cell culture was harvested after 15 d and filtered through non-woven textile. 350 g fresh mass (f.m.) of cells was processed subsequently in 30 g batches. Cells were washed with 10 cm^{-3} of extraction buffer (100 mM Tris-HCl, 20 % glycerol, 50 mM $Na_2S_2O_5$, 50 mM ascorbic acid, 5 mM dithiotreitol (DTT), 5 μM FAD/FMN, 2.5 mM EDTA, pH 7.5) and frozen in liquid nitrogen and homogenized in a mortar with addition of polyvinylpyrrolidone (PVP) (0.5 g). 60 cm^3 of extraction buffer were subsequently added. The extract was filtered through cheesecloth and centrifuged at 11 000 *g* at 4 °C for 20 min. Supernatant was precipitated with ammonium sulphate to 40 % saturation and subsequently to 80 %. Resuspended pellet was dialysed against 25 mM Tris-HCl (pH 9.0) and 10 % glycerol.

The yield of soluble proteins extracted by homogenization of cells was 217.2 mg per 350 g(f.m.), which corresponds to 1.49 % of dry mass. The Table 1 summarizes yield of protein and the enzyme activity in the individual isolation steps. The enzyme activity was tested in the assay buffer [25 mM Tris-HCl (pH 9.0), 10 % glycerol, 5 μM FAD/FMN)], in the presence of cofactors NAD(P)H (3 mM), catalase (1 500 U cm^{-3}), and TNT (0.3 mM). The mixture was saturated by argon and incubated for 3 h at 30 °C under argon atmosphere. The enzyme activity was characterised as the decrease of TNT concentration in the reaction mixture in time. Quantification of TNT was determined by the spectrophotometric method based on colour reactions (Cumming reactions) of TNT and some related compounds under alkaline conditions (Channon *et al.* 1944). The rapid spectrophotometric method according to Oh *et al.* (2000) was used in combination with multi-well ELISA reader with a filter for 450 nm. The advantage of the multi-well arrangement is the possibility of almost simultaneous analysis of a large amount of samples excluding the effect of most degradation products like aminoderivates of TNT. 0.050 cm^3 of reaction mixture (0.05 $cm^{3)}$ was diluted to 0.2 cm^3 with distilled water and alkalised by 0.002 cm^3 of 1.5 M NaOH in a multi-well plate. The absorbance at 450 nm was determined after 8 min using microplate reader *SLT-Lab Instruments EAR 340AT (Labequip,* Ontario, Canada). The TNT concentration was calculated from the calibration curve. The enzymatic activity was detected in both the cytosolic and microsomal fractions, preferentially under anaerobic conditions. Comparison of the enzymatic activity in the crude extract of TNT pre-treated [TNT application in three doses (20 mg dm^{-3} each) 4 d before harvest] and untreated cell cultures revealed lower specific activity in the crude extract of treated culture. The results indicate that the detoxification reactions in plant are performed by a system of constitutive enzymes with nitroreductase activity. The level of nitroreductase activity detected in the individual fractions during the isolation procedure could be modified by participation of other enzymes exhibiting this activity.

In next step the 80 % ammonium sulphate fraction was separated by ion-exchange chromatography using *DEAE-cellulose* column (*Whatman Scientific LTD*, UK) equilibrated with 10 % glycerol in 25 mM Tris-HCl (pH 9.0). Elution was done with a linear gradient of KCl (0 - 0.5 M, 200 cm^3). Concentrated fractions with nitroreductase activity were applied on *Sephadex G100* column equilibrated with 25 mM Tris-HCl, 0.2 M KCl and 10 % glycerol (pH 9.0). Collected active fractions were separated by ion-exchange chromatography on *Mono Q 515* column (*Pharmacia LKB,* Uppsala, Sweden) using *FPLC* (*ÄKTAexplorer*, *Pharmacia Amersham Biotech*, Piscataway, NJ, USA). Linear gradient of KCl (0 - 0.5 M) in Tris-HCl buffer (25 mM, 10 % glycerol, pH 9.0) was applied in 100 min. The flow rate was 1 cm^3 min^{-1}.

The fraction isolated by ion-exchange chromatography on column *Mono Q 515* was separated by electrophoresis under native conditions. The SDS polyacrylamide gel electrophoresis was performed according to Laemmli (1970), for a native electrophoresis SDS was omitted and concentration of glycerol in resolving gel was increased to 15 %. The concentration of polyacrylamide was 4.5 % for the stacking gel and 10 % for the resolving gel. The SDS gels were run using 40 mA and native gels were run using 20 mA at 4 °C. The protein

Table 1. Mass balance and nitroreductase activity during isolation of the enzyme.

Step	Protein yield [mg]	Spec. activity [mmol g^{-1} min^{-1}]	Final activity [%]
Homogenisation - crude extract	217.2	0.58	11.1
$(NH_4)_2SO_4$ precipitation (40 - 80 %)	102.5	0.87	16.7
Ion-exchange chromatography (*DEAE-cellulose*)	37.5	2.18	41.8
Gel filtration (*G100*)	3.9	3.59	68.9
Ion-exchange chromatography (*MonoQ*)	0.8	5.21	100.0

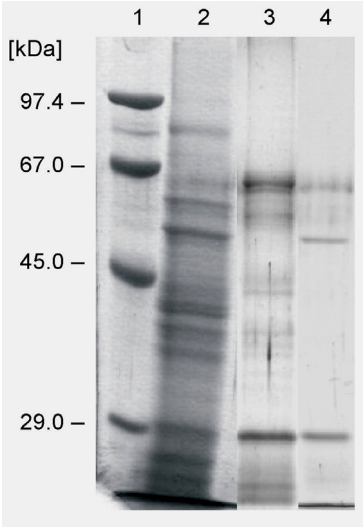

Fig. 3. SDS electrophoresis of fractions obtained during isolation. The stacking gel (6.6 % PAA, pH 6.8) and resolving gel (12 % PAA, pH 8.8) contained 0.1 % SDS. The gels were stained by Coomassie Brilliant Blue (0.1 %). 1 - molecular markers: phosphorylase B (97.4 kDa), serum albumin (67 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa); 2 - concentrated fractions (60 - 70) obtained by ion-exchange chromatography on DEAE Cellulose; 3 - fractions (43 - 53) from gel chromatography on *Sephadex G100*; 4 - fractions from ion-exchange chromatography on *MonoQ*.

gave two bands corresponding to two isoforms, with pI 4.8 and 5.1 as determined by isoelectric focusing. The enzyme activity of the protein was tested directly in the gel after in-gel visualization, which is a novel approach. Test of the enzymatic activity in the zymograms was based on the assay directly in the agarose gel overlaying the polyacrylamide gel. The agarose mixture (1 %, low-melting agarose, *Sigma*, St. Louis, USA) containing the same components as for the enzyme assay [NADH (3 mM), catalase (1500 U cm^{-3}), and TNT (0.3 mM)] was incubated at 35 °C and saturated by argon before overlaying the polyacrylamide gel. The gels placed in the polyethylene foil were incubated in the argon atmosphere at 30 °C for 2 h (Fig. 4). A strip of the poly-acrylamide gel with separated proteins after native electrophoresis was separated in second dimension in presence of SDS to verify the molecular mass of the active enzyme. The gel shows a single band formed by isoforms of the protein with molecular mass 29 kDa (Fig. 5).

The molecular mass of the isolated protein was determined by SDS electrophoresis using 15 % polyacrylamide gels under reducing and as well as non reducing conditions (Fig. 3). The precise mass spectrum was measured on a matrix-assisted laser desorption/ ionisation reflectron time-of-flight *MALDI-TOF* mass spectrometer *BIFLEX* (*Bruker-Franzen*, Bremen, Germany) equipped with a nitrogen laser (337 nm) and gridless delayed extraction ion source. Ion acceleration voltage was 19 kV. Spectrum was calibrated externally using $[M+H]^+$ and $[M+2H]^{2+}$ ions of protein standard carbonic anhydrase (*Sigma*). Saturated solution of 3,5-dimethoxy-4-hydroxy-cinnamic acid in 30 % aceto-nitrile/0.05 % trifluoroacetic acid was used as a *MALDI* matrix. 0.5 mm^{-3} of matrix solution was mixed with 0.5 mm^{-3} of the sample on the target and the droplet was allowed to dry at ambient temperature. The result (29 - 285 Da) confirm our previous measurement.

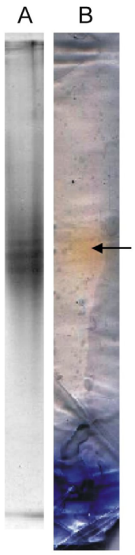

Fig. 4. Zymogram shows the enzymatic activity of the isolated enzyme in the agarose gel (B) after native electrophoresis (A). The *arrow* shows a spot characterizing the activity of the protein.

The enzyme classification was done according to the amino acid sequence. Seventeen terminal amino acids were sequenced from N-terminus by protein sequencer (*Protein Sequencer 491*, *PE Applied Biosystems*, Foster City, CA, USA) based on Edman degradation.

The determined sequence SSGVDVAEFSPPRRLLT

was checked using *Blast* protein data bank (NCBI 2001) and the highest sequence homology found was with 12-oxophytodienoate reductase (OPR1) isolated from *Arabidopsis thaliana* (Biesgen and Weiler 1999) and tomato (*Lycopersicon esculentum*) (Breithaupt *et al.* 2001). OPRs were classified as enzymes belonging to the family of Old Yellow Enzyme. An Old Yellow Enzyme (OYE, EC. 1.6.99.1) is a flavoprotein, which contains flavinmononucleotide as a prosthetic group (Theorell *et al.* 1935). The determined sequence SSGVDVAEFSPPRRLLT shows 66.7 % homology to the sequence SNGIDEALFTPPRRL (compared region: 139 - 153) analysed from *Arabidopsis thaliana* (Biesgen and Weiler 1999) and 60 % homology to sequence SNGIDIAHFTRPRRL (compared region: 143 - 158) analysed from tomato (*Lycopersicon esculentum*) (Breithaupt *et al.* 2001). The amino acid sequence SNGIDIAHFT corresponds to N-terminus of 12-oxo-phytodienoate reductase isolated from tomato (Strassner *et al.* 1999).

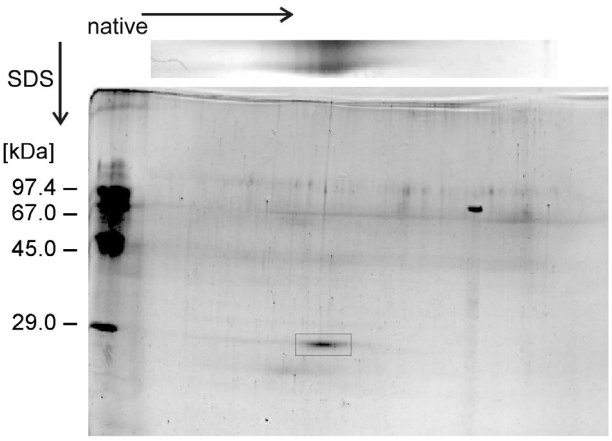

Fig. 5. Two-dimensional electrophoresis of the protein fraction obtained by ion-exchange chromatography on *MonoQ*. Native electrophoresis was performed on polyacrylamide gels (SDS- and EDTA-free, 10 %) in the first dimension. A strip with separated protein was cut off, saturated by SDS and polymerised into the resolving SDS polyacrylamide gel (6.6 %) and separated in the second dimension in the stacking gel (12 %). The band of determined protein is terminated.

The results will be further used for studies of enzymes participating in degradation of TNT and other explosives/ nitroaromatics. The isolated enzyme could be used for preparation of antibody, which will be utilized as a marker for detection of the enzyme in different plant species. The enzyme sequence determination is the starting point of search of the appropriate gene. The next step will be construction of transgenic plants via enhancement or multiplication of the gene in the genome or insertion of the specific or stronger promoter, which will have hyper-degradation capacity for elimination of nitroaromatics from the soil. The final step of our work will be focused on the production of transgenic plants potentially used as multipurpose technical crop.

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