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Department of Biochemistry

Study on Certain Enzymes of the Degradation Metabolism of Polyamines

Ph.D. Thesis

P1406 – Biochemistry

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Supervisor: prof. Mgr. Marek Šebela, Dr.

Olomouc

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I hereby declare, that this thesis has been written by myself using literature cited in reference part.

In Olomouc 15th April 2010 . . . . . .

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Abstrakt

Ornithin-δ-aminotransferasa (OAT, EC 2.6.1.13) katalyzuje reakci transaminace L-ornithinu na γ-semialdehyd L-glutamátu. Vyskytuje se v mitochondriích živočichů, člověka, rostlin i mikroorganismů. Fyziologická role OAT v rostlinách souvisí s katabolismem argininu vedoucího k syntéze glutaminu a s biosyntézou prolinu – mj. osmolytu známého svou důležitou úlohou při adaptaci rostlinných buněk na sucho a salinitní stres.

V prvních fázích doktorského studia byly optimalizovány metody detekce a stanovení aktivity OAT. Za pomoci vhodné homogenizační metody byla měřena aktivita enzymu v rostlinách (fazole, fazole mungo, hrách). Vhodným rostlinným materiálem pro izolaci OAT byl hrách. OAT byla částečně purifikována za použití technik precipitačních, nízkotlaké a střednětlaké kapalinové chromatografie. Výsledný enzym měl specifickou aktivitu 30 pkat.mg^{-1}, stupeň přečištění 43 a výtěžek 51 %. Vzorek však stále obsahoval nečistoty a OAT představovala pouze malý podíl zbylých proteinů. V tomto bodě bylo rozhodnuto o provedení rekombinantní exprese hrachové OAT.

Nejprve byla cDNA kódující PsOAT (OAT z hrachu – *Pisum sativum*, uloženo v databázi EMBL/GenBank pod přístupovým kódem EU414030) klonována a exprimována v *Escherichia coli*. Výsledkem byl rekombinantní protein s polyhistidinovou kotvou na C-konci. Rekombinantní PsOAT byla purifikována za nativních podmínek pomocí chelatační chromatografie a byly stanoveny její molekulové a kinetické vlastnosti. Identita proteinu byla potvrzena peptidovým mapováním po proteolytickém štěpení v gelu.

Přečištěná PsOAT (specifická aktivita 26,2 nkat.mg^{-1}, stupeň přečištění 7,7 a výtěžek 46,8 %) existuje jako monomer o molekulové hmotnosti 50 kDa a vykazuje typické spektrální vlastnosti enzymů obsahujících pyridoxal-5'-fosfát jako prostetickou skupinu. Obsah kofaktoru v PsOAT byl určen spektrofotometrickou analýzou s fenylhydrazinem jako 0,9 molu na 1 mol monomerního enzymu. PsOAT má bazické pH optimum (8,8) a vykazuje poměrně vysokou teplotní stabilitu (T_{50} = 58 °C). Optimální teplota pro transaminaci ornithinu je 43 °C. L-ornithin je jediný vhodný substrát PsOAT (K_m = 15 mM), avšak enzym pomalu katalyzuje i přeměnu N_α-acetyl-L-ornithinu. Při těchto reakcích slouží jako akceptor aminoskupiny výhradně 2-oxoglutarát (K_m = 2 mM). Diaminy a polyaminy nepůsobí jako substráty, naopak např. 1,3-diaminopropan, putrescin, spermin, spermidin a norspermidin představují nekompetitivní inhibitory s milimolárními inhibičními konstantami (K_i) na stejné úrovni, jako je Michaelisova konstanta (K_m) pro L-ornithin. Nejsilnějšími nekompetitivními inhibitory jsou diethylenetetramin a triethylenetetramin. Jediným nalezeným kompetitivním inhibitorem je L-norvalin.

Klíčová slova	ornithin-δ-aminotransferasa, L-ornithin, 2-oxoglutarát, pyridoxal-5'-fosfát, prolin
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Abstract

Ornithine δ-aminotransferase (OAT, EC 2.6.1.13) catalyzes the transamination of L-ornithine to L-glutamate-γ-semialdehyde. It has been found in mitochondria of human beings, animals, plants and microorganisms. The physiological role of OAT in plants is related to arginine catabolism resulting in glutamate and to biosynthesis of proline– osmolyte, which is known to play an important role in adaptation processes of plant cells to drought and salinity stress.

In initial phases of my Ph.D. study, methods of OAT detection and activity assays were optimized. OAT activity was measured in plants (bean, mung bean and pea) using an appropriate homogenization method. The most suitable material for OAT isolation was found to be pea. OAT was partially purified using precipitation techniques, low-pressure and fast protein liquid chromatography. The final enzyme had a specific activity of 30 pkat.mg^{-1}, an enrichment factor of 43 and a yield of 51 %. However, the sample still contained many impurities and OAT represented only a minor fraction of all proteins. At this point, it was decided to perform recombinant expression of pea OAT.

First, a cDNA coding for PsOAT (OAT from pea – *Pisum sativum*, deposited in the EMBL/GenBank database under the accession code EU414030) was cloned and expressed in *Escherichia coli* to obtain a recombinant protein with a C-terminal 6xHis tag. Recombinant PsOAT was purified under native conditions by immobilized metal affinity chromatography and its molecular and kinetic properties were characterized. Protein identity was confirmed by peptide mass fingerprinting after proteolytic digestion in gel.

Recombinant PsOAT (its purification resulted in a specific activity of 26,2 nkat.mg^{-1}, an enrichment factor of 7,7 and a yield of 46,8 %) existed as a monomer of 50 kDa and showed typical spectral properties of enzymes containing pyridoxal-5'-phosphate as a prosthetic group. The cofactor content of PsOAT was estimated to be 0.9 mol per mol of the monomer by a spectrophotometric analysis with phenylhydrazine. The enzyme had a basic optimal pH of 8.8 and displayed relatively high thermal stability (T_{50} = 58 °C). An optimal temperature for ornithine transamination was 43 °C. L-Ornithine was the best substrate (K_m = 15 mM) but PsOAT also slowly converted N_α-acetyl-L-ornithine. In these reactions, 2-oxoglutarate was the exclusive amino group acceptor (K_m = 2 mM). Diamines and polyamines were not accepted as substrates. Conversely, 1,3-diaminopropane, putrescine, spermine, spermidine and norspermidine represented non-competitive inhibitors with millimolar inhibition constants (K_i) being at the same concentration level like the Michaelis constant (K_m) for L-ornithine. The most potent non-competitive inhibitors were diethylenetetramine and triethylenetetramine. L-Norvaline was the only competitive inhibitor.

Keywords	ornithine δ-aminotransferase, L-ornithine, 2-oxoglutarate, pyridoxal 5'-phosphate, proline
Number of pages	74
Number of appendices	2
Language	English

Table of contents

Aims of the work

- ✓ to review a given issue
- ✓ to set up activity assay for ornithine δ-aminotransferase
- ✓ to isolate ornithine δ-aminotransferase from plants
- ✓ to clone a plant ornithine δ-aminotransferase
- ✓ to prepare a recombinant ornithine δ-aminotransferase
- ✓ to study biochemical properties of the enzyme – its molecular properties, substrate specificity and inhibitors

1 THEORETICAL PART

1.1 Current knowledge on ornithine δ-aminotransferase

Ornithine δ-aminotransferase (OAT, L-ornithine:2-oxoacid aminotransferase, EC 2.6.1.13) is a nuclear-encoded, pyridoxal-5'-phosphate (PLP) dependent enzyme. It is localized in mitochondrial matrix (Fig. 1) of most human, animal as well as plant tissues (Aral & Kamoun, 1997; Funck et al., 2008) and has been found also in insects (Yoshida et al., 1997) and microorganisms (Yasuda et al., 1979). Human OAT is expressed as a precursor, which is processed into its mature form following the cleavage of the mitochondrial transit peptide upon entry to the mitochondrion (Inana et al., 1986). Putative signal peptides occur also in sequences of plant OAT precursors (Delauney et al., 1993; Roosens et al., 1998). Interestingly, in the mushroom *Agaricus bisporus*, the enzyme is probably localized in the cytoplasm as its sequence lacks the mitochondrial targeting motif (Wagemaker et al., 2007). The N-terminal extension is also absent in the enzymes from the Saccharomycetaceae family (Jauniaux et al., 1978).

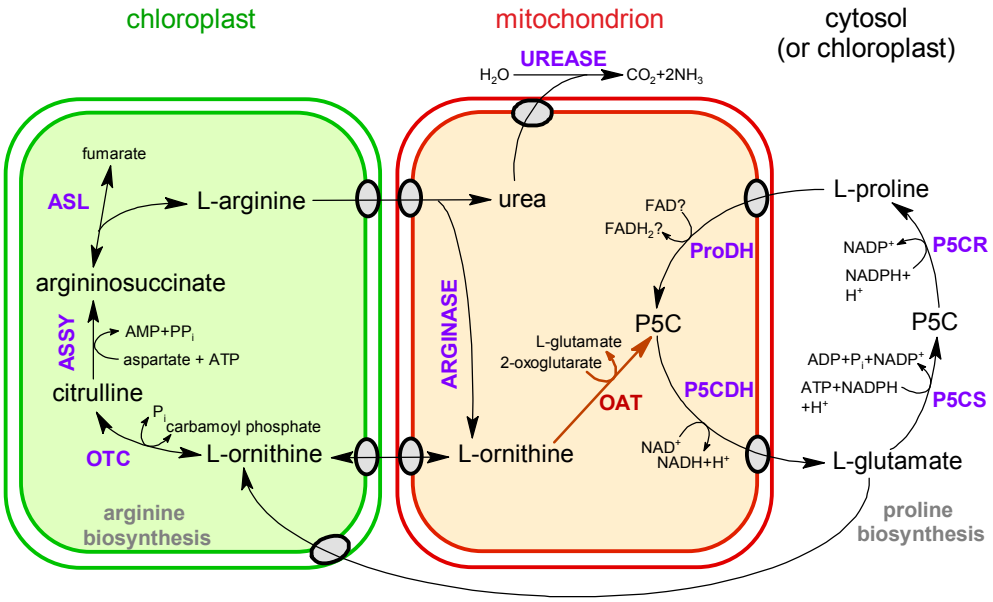

Fig. 1: Compartmentation of arginine and proline metabolic pathways (according to Funck et al., 2008). OAT links the degradation pathways for arginine and proline, which converge at the level of Δ^1-pyrroline-5-carboxylate (P5C) in mitochondria. Proline biosynthesis occurs in the cytosol or, during stress, in plastids, whereas arginine biosynthesis is constitutively localised in plastids. ASL, argininosuccinate lyase; ASSY, argininosuccinate synthetase; OTC, ornithine transcarbamylase; P5C, pyrroline-5-carboxylate; P5CDH, P5C dehydrogenase; P5CR, P5C reductase; P5CS, P5C synthetase, ProDH, Pro dehydrogenase.

OAT catalyzes the transfer of the δ-amino group of L-ornithine to 2-oxoglutarate, leading to the formation of L-glutamate-γ-semialdehyde and L-glutamate. The semialdehyde is in equilibrium with its chemically more stable cyclic

form Δ^1-pyrroline-5-carboxylate. Like in all PLP dependent aminotransferases, the reaction proceeds via a ping-pong mechanism that requires two half-reactions to complete one cycle of transamination (Storici et al., 1999). Some OATs can utilize pyruvate or glyoxylate as amino group acceptors instead of 2-oxoglutarate (Yasuda et al., 1979; Strecker, 1965), whereas *N*-acetylornithine represents an alternative substrate for OAT from *Plasmodium falciparum* (Gafan et al., 2001).

The human OAT has attracted a big scientific interest since the discovery of its association with gyrate atrophy, a recessive hereditary genetic disorder leading to progressive loss of vision and eventually blindness. Gyrate atrophy is characterized by elevated ornithine levels arising from OAT dysfunction (Seiler, 2000). In mammals, the role of OAT in providing glutamate for glutamine synthesis is well known, which allows for the detoxification of ammonia released by protein degradation (Boon et al., 1999). In plants, OAT has been associated frequently with the increased proline accumulation observed during salinity stress and water deficit (Roosens et al., 1998) or arginine metabolism (Funck et al., 2008) often with contradictory results (Roosens et al., 1998; Funck et al., 2008; Parida et al., 2008; Xue et al., 2009). In addition, arginine and ornithine are the precursors of polyamines (putrescine, spermidine and spermine), which are known as compounds with important roles in developmental processes and stress tolerance (Bouchereau et al., 1999). In chapter 1.2, there is our review from 2008 (Stránská et al., 2008), dealing comprehensively with the OAT topic.

As OAT is implicated in proline biosynthesis (Fig. 2), we may also find related sort of articles dealing with proline content, biosynthesis and degradation. Reviews are written on various topics – e. g. Lehmann et al. (2010) discussed proline metabolism and transport from the point of view of plant development, emphasizing regulatory aspects such as the influence of metabolites and hormones. Szabados & Savouré (2010) discussed the compartmentalization of proline biosynthesis, its accumulation and degradation in the cytosol, chloroplast and mitochondria and described the role of proline in cellular homeostasis, including redox balance and energy status. The possible roles of proline accumulation in stressed plants, the regulation of proline metabolism during development and stress, results of genetic manipulation of proline metabolism and current debate on proline toxicity in plants are presented in Verbruggen & Hermans (2008).

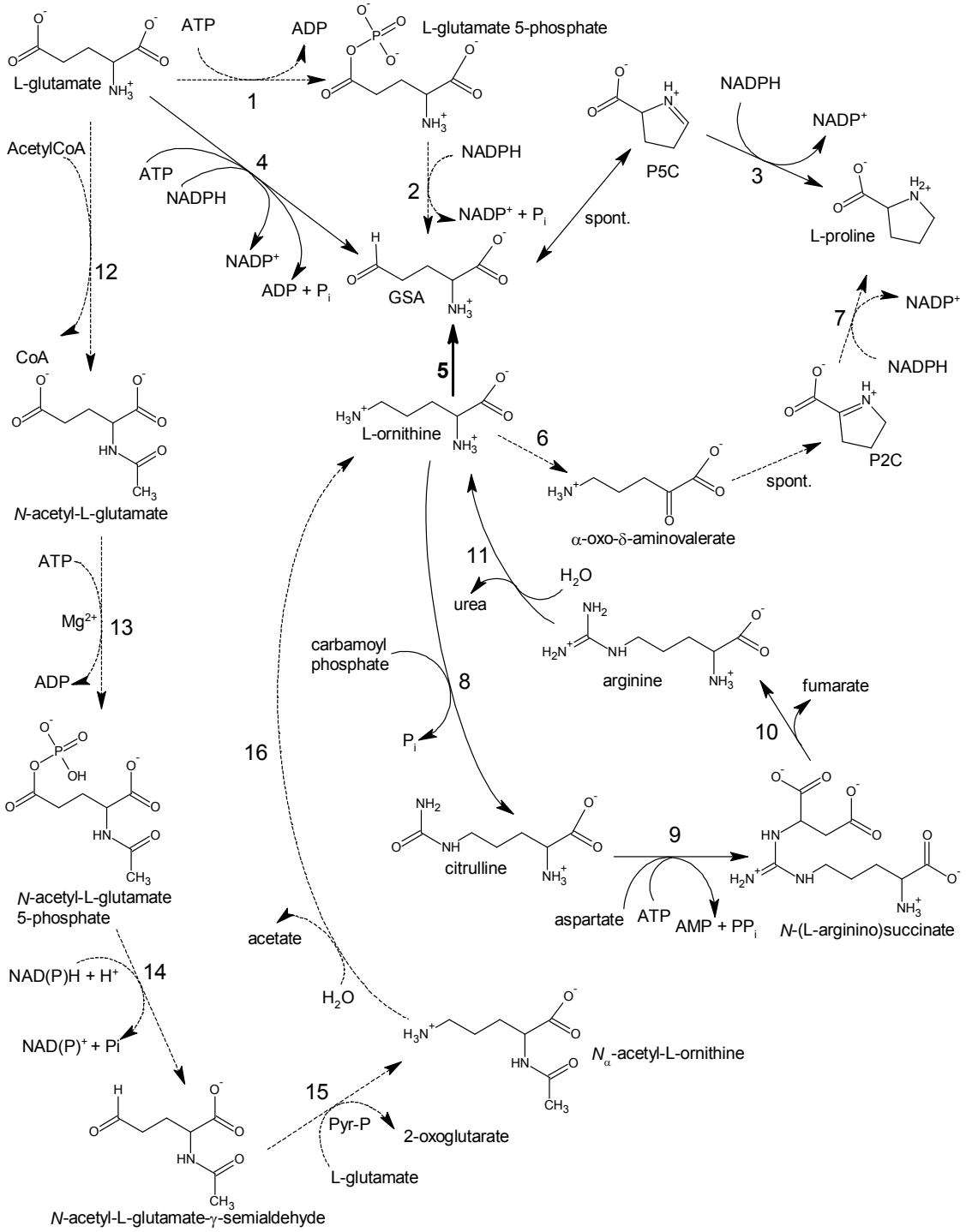

Fig. 2: Proline biosynthesis (according to Kavi Kishor et al., 2005; Thompson,1980; Stryer, 1975). GSA, L-glutamate-γ-semialdehyde; P2C, Δ^1-pyrroline-2-carboxylate; P5C, Δ^1-pyrroline-5-carboxylate; ⇢, typical of prokaryotes; →, characterized in plants. Enzymes involved in the network of proline biosynthesis:

No.	EC	enzyme	No.	EC	enzyme
1	2.7.2.11	glutamate 5-kinase	9	6.3.4.5	argininosuccinate synthetase
2	1.2.1.41	glutamate-γ-semialdehyde dehydrogenase	10	4.3.2.1	argininosuccinate lyase
3	1.5.1.2	pyrroline-5-carboxylate reductase	11	3.5.3.1	arginase
4	undefined	pyrroline-5-carboxylate synthetase	12	2.3.1.1	amino-acid *N*-acetyltransferase
5	2.6.1.13	ornithine δ-aminotransferase	13	2.7.2.8	acetylglutamate kinase
6	2.6.1.68	ornithine α-aminotransferase	14	1.2.1.38	*N*-acetyl-glutamate semialdehyde dehydrogenase
7	1.5.1.1	pyrroline-2-carboxylate reductase	15	2.6.1.11	*N*-acetylornithine aminotransferase
8	2.1.3.3	ornithine transcarbamylase	16	3.5.1.16	*N*-acetylornithine deacetylase

New insights into OAT field were introduced by *Arabidopsis thaliana* deletion mutants of *OAT*. Experiments clearly showed that the ornithine pathway is not crucial for proline biosynthesis under stress conditions, but the glutamate pathway plays a predominant or exclusive role. Ornithine pathway is essential for arginine catabolism and thus important for the recovery of nitrogen stored or transported in the form of arginine. In the same study, transgenic plants of Arabidopsis and *Nicotiana benthamiana* overexpressing a fusion protein of OAT and the green fluorescent protein were also prepared. Subcellular localization of the enzyme in mitochondria was confirmed in agreement with the sequence-based prediction (Funck et al., 2008). OAT transcription levels and activity were studied in *Pinus sylvestris* in embryos, germinating and developing seedlings in connection with the glutamate provision for glutamine biosynthesis. The expression of OAT was the highest at early stages of germination and seed development in all different organs, decreased in later stages and was undetectable in embryo. The activity of the enzyme corresponded with transcription levels (Cañas et al., 2008). A conclusion was made after studies on *Brassica napus*, that the ornithin route is activated in dependence on the severity of the stress. The upregulation of OAT expression was only detected during a prolonged severe osmotic stress. The results indicate that the stress-induced proline accumulation is caused by a reciprocal action of activated biosynthesis and inhibited proline degradation (Xue et al., 2009). For confusion, no changes were observed in OAT activity in drought tolerant and drought sensitive cotton genotypes after 14-days drought stress and following 7-days recovery (Parida et al., 2008).

Another directions in studying OAT have been oriented based on hypotheses of OAT involvement in arginine and nitric oxide accumulation and efflux (Tovar-Mendez et al., 2008) as well as suggested OAT participation in the formation of the major flavor compound in rice – 2-acetyl-1-pyrroline (Huang et al., 2008).

The amino acid ornithine is well known to be connected with polyamine metabolism. Ornithine decarboxylase (EC 4.1.1.17) represents the key enzyme of polyamine biosynthesis as its reaction produces putrescine (1,4-butandiamine) as a precursor of the most important (from the point of view of physiology) polyamines, spermidine and spermine. In the beginning of my Ph.D. study, an article was published (see List of Publications: Stránská et al., 2007) with experimental data on the inhibition of plant diamine and polyamine oxidase by *N*, *N´*-bis(2-pyridinylmethyl)-diamines. The topic is not directly related to ornithine δ-aminotransferase, hence the article is not provided *in extenso*.

1.2 Supplement 1: Ornithine δ-aminotransferase: An enzyme implicated in salt tolerance in higher plants

Stránská J., Kopečný D., Tylichová M., Snégaroff J., Šebela M.

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Review

Ornithine δ-aminotransferase

An enzyme implicated in salt tolerance in higher plants

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Abbreviations: DAT, diamine aminotransferase; GABA-AT, 4-aminobutyric acid aminotransferase; GSA, L-glutamate γ-semialdehyde; OAT, ornithine δ-aminotransferase; P5C, Δ^1-pyrroline-5-carboxylate; PLP, pyridoxal-5'-phosphate; PMP, pyridoxamine-5'-phosphate; P5CDH, Δ^1-pyrroline-5-carboxylate dehydrogenase; P5CR, Δ^1-pyrroline-5-carboxylate reductase; P5CS, Δ^1-pyrroline-5-carboxylate synthetase; ProDH, proline dehydrogenase

Key words: ornithine δ-aminotransferase, osmotic stress, proline, Δ^1-pyrroline-5-carboxylate, pyridoxal-5'-phosphate, semialdehyde, transamination

This review deals with biochemical and physiological aspects of plant ornithine δ-aminotransferase (OAT, EC 2.6.1.13). OAT is a mitochondrial enzyme containing pyridoxal-5'-phosphate as a cofactor, which catalyzes the conversion of L-ornithine to L-glutamate γ-semialdehyde using 2-oxoglutarate as a terminal amino group acceptor. It has been described in humans, animals, insects, plants and microorganisms. Based on the crystal structure of human OAT, both substrate binding and reaction mechanism of the enzyme are well understood. OAT shows a large structural and mechanistic similarity to other enzymes from the subgroup III of aminotransferases, which transfer an amino group from a carbon atom that does not carry a carboxyl function. In plants, the enzyme has been implicated in proline biosynthesis and accumulation (via pyrroline-5-carboxylate), which represents a way to regulate cellular osmolarity in response to osmotic stress. However, the exact metabolic pathway involving OAT remains a subject of controversy.

Introduction

Ornithine δ-aminotransferase or 5-aminotransferase (OAT; L-ornithine:2-oxoacid aminotransferase; EC 2.6.1.13) is a nuclear-encoded, pyridoxal-5'-phosphate (PLP)-dependent enzyme found in the mitochondrial matrix of most human and animal tissues.[1] The enzyme also exists in insects,[2] microorganisms[3] and plants.[4] It catalyzes the transfer of the δ-amino group of L-ornithine to 2-oxoglutarate, which produces L-glutamate γ-semialdehyde (GSA) and L-glutamate. Some enzymes can utilize pyruvate or glyoxylate as amino group acceptors instead of 2-oxoglutarate.[3,5] *N*-acetylornithine represents an alternative substrate for OAT from *Plasmodium falciparum*.[6] Human OAT is expressed as a precursor, which is processed into its mature form following the cleavage of the mitochondrial transit peptide upon entry to the mitochondrion.[7] Putative signal peptides occur also in sequences of plant OAT precursors.[4,8] The mitochondrial localization of OAT in Arabidopsis was demonstrated using a green fluorescent protein-based reporter fusion.[9]

Similar to all PLP-dependent aminotransferases, the transamination reaction proceeds via a ping-pong mechanism that requires two half reactions to complete one catalytic cycle of transamination[10] (Fig. 1). In the first half reaction, the PLP cofactor forms an aldimine with the δ-amino group of L-ornithine. Then a stereospecific proton transfer occurs from the δ-carbon of L-ornithine to the C4' carbon of PLP. The aldimine ("enzyme-substrate Schiff base") is converted to another aldimine ("enzyme-product Schiff base") between pyridoxamine-5'-phosphate (PMP) and GSA. Subsequent hydrolysis releases GSA, which spontaneously cyclizes to form Δ^1-pyrroline-5-carboxylate (P5C), and PMP. The first half reaction is virtually irreversible.[10] In the second half-reaction, 2-oxoglutarate undergoes transamination to L-glutamate, while the cofactor is restored to its PLP form.[10]

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Plant Ornithine δ-Aminotransferases

Transamination of L-ornithine in higher plants was demonstrated fifty years ago, namely in spinach (*Spinacia oleracea*)[11] and mungbean (*Phaseolus aureus*).[12] The mungbean enzyme was found to be associated with the mitochondria.[12] In the following two decades, OAT was partially purified for example from peanut (*Arachis hypogea*),[13] pumpkin (*Cucurbita maxima*)[14] and squash (*Cucurbita pepo*).[15] By activity measurements in cotyledon extracts, OAT was demonstrated in pea (*Pisum sativum*).[16] For the squash enzyme, the pH optimum of the transamination between L-ornithine and 2-oxoglutarate was 8.0 and the Michaelis constants were 4.7 and 6.3 mM, respectively. Squash OAT showed a molecular mass of 48 kDa as determined by gel chromatography.[15]

Proline auxotroph mutants of *E. coli* were transformed by mothbean (*Vigna aconitifolia*) cDNA expression library, and

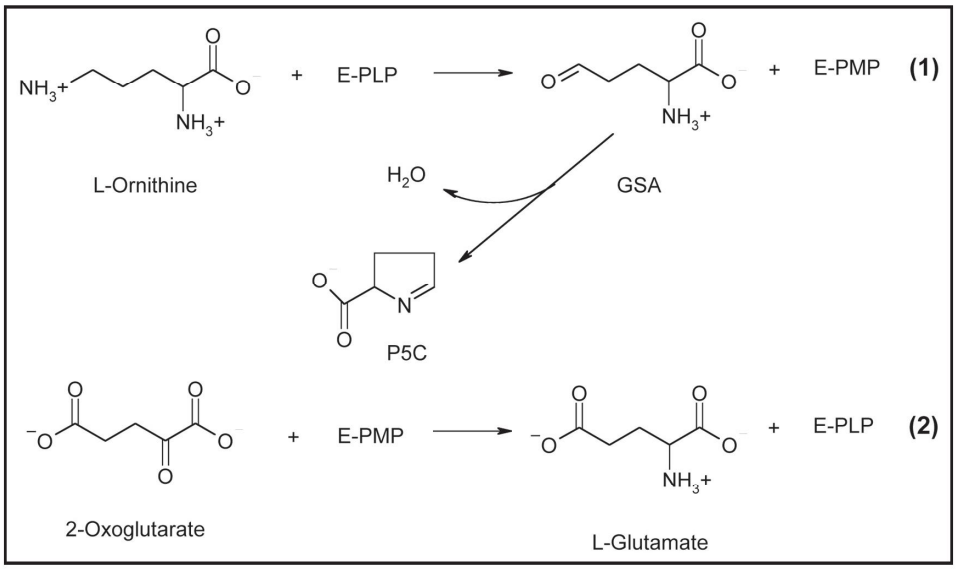

Figure 1. The catalytic mechanism of OAT. The presented reaction scheme[10] points out the role of the enzyme cofactor PLP. Numbers indicate the two separate half reactions.

ornithine and proline prototrophy was restored. This strategy called "trans-complementation" facilitated the isolation of cDNA clones encoding OAT.[4] The mothbean enzyme expressed in *E. coli* was partially purified (a monomer of 50 kDa) and its K_m values for ornithine (2 mM) and 2-oxoglutarate (0.75 mM) were determined at an optimal pH of 8.0.[4] Recombinant mothbean OAT was found to be inhibited significantly by isoleucine, valine and serine, whereas proline showed no apparent effect (at a concentration of 1 mM of each).[17]

Ten years ago, a cDNA encoding OAT from *Arabidopsis thaliana* was obtained and sequenced.[8] At present, a number of other plant OAT sequences (e.g., from aquilegia, barrel medic, grape, maize, pine, potato, rice, sorghum and soybean) are available in public DNA and protein databases. The amino acid sequence of pea OAT, UniProtKB accession no. B1A0U3, has been determined in our laboratory (Stránská J. et al., unpublished results) via cloning and sequencing the respective cDNA (EMBL/GenBank accession no. EU414030).

Physiological Role in Plants

L-Arginine is an important storage and transport form of organic nitrogen in many higher plants. For example, developing pea seeds accumulate arginine-rich storage proteins and arginine is the predominant free amino acid in pea cotyledons, accounting for 66.4% of all nitrogen in the amino acid pool of young cotyledons.[16] During seed germination, the first steps of arginine degradation are catalyzed by arginase (EC 3.5.3.1), OAT, and urease (EC 3.5.1.5). Arginase hydrolyzes L-arginine to yield urea, which is further degraded by urease to carbon dioxide and ammonia, and L-ornithine providing GSA by OAT reaction. This pathway appears to be related to the transfer of nitrogen from arginine to other amino acids.[16] In addition, arginine and ornithine are the precursors of polyamines (putrescine, spermidine and spermine), which are known as compounds with important roles in developmental processes and stress tolerance.[18]

The accumulation of compatible solutes such as polyols/sugars (e.g., mannitol, trehalose), quarternary ammonium compounds (e.g., glycine betaine) and neutral amino acids (e.g., proline) represents one of the most common responses for regulating cellular osmolarity.[19] These low-molecular-mass compounds are accumulated to high intracellular concentrations, in order to balance the osmotic pressure of the growth medium and thereby maintain both turgor and the driving gradient for water uptake.[19] In plants, proline is mainly synthesized in the cytosol from glutamate via P5C by the sequential action of P5C synthetase (P5CS; a bifunctional enzyme, EC 1.2.1.41/2.7.2.11) and P5C reductase (P5CR; EC 1.5.1.2). For degradation, proline is imported into mitochondria where it is converted back to glutamate by proline dehydrogenase (ProDH; EC 1.5.99.8) and P5C dehydrogenase (P5CDH; EC 1.5.1.12);[20-22] Figure 2. There is also evidence for a pathway of proline biosynthesis from ornithine, in which OAT has been implicated.[22]

OAT reaction results in GSA and glutamate. The semialdehyde is in spontaneous equilibrium with its cyclic form P5C, a common intermediate in proline metabolism. Formation of GSA/P5C from ornithine was postulated to constitute an alternative pathway of proline synthesis and accumulation.[4] The glutamate pathway is thought to be the primary route for proline synthesis in plants during conditions of osmotic stress and nitrogen limitation whereas the ornithine pathway might function under high nitrogen input.[4] A study performed on NaCl-treated cotyledons of radish (*Raphanus sativus*) seedlings using the specific OAT inhibitor gabaculine demonstrated the contribution of the ornithine pathway to proline synthesis.[23] In young *Arabidopsis thaliana* plantlets, free proline content, P5CS mRNA, OAT activity, and OAT mRNA were all increased by salt-stress treatment.[8] Moreover, transgenic *Nicotiana plumbaginifolia* plants overexpressing OAT from Arabidopsis synthesized more proline than the control plants and showed a higher biomass and a higher germination rate under

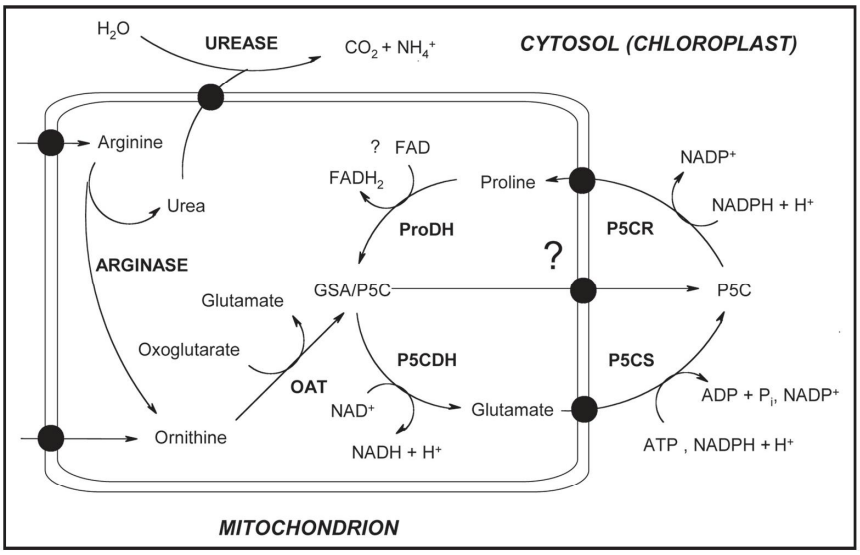

Figure 2. Involvement of plant OAT in proline synthesis. The scheme is based on that previously published by Funck D. et al., 2008.[9] OAT links the catabolic pathways for arginine and proline, which converge at the intermediate P5C in mitochondria. Proline biosynthesis occurs in the cytosol or, during stress, in plastids. Due to the chemical instability of GSA/P5C, an export from mitochondria seems unlikely but cannot be excluded. Abbreviations are those used throughout the text.

osmotic stress conditions.[24] All these data suggest that the ornithine pathway, together with the glutamate pathway, plays an important role in proline accumulation during osmotic stress in plants.

However, targeting OAT to mitochondria would strongly suggest that P5C enters the degradation pathway of proline rather than its biosynthesis. Proline production via ProDH is energetically unfavorable and due to the chemical instability of GSA/P5C, an export of this compound to the cytosol and thus a contribution to proline synthesis appears unlikely.[9] The ornithine pathway in rice leaves seems to contribute little, if any, to proline accumulation under water stress condition. When OAT activity decreased by 75% in consequence of a gabaculine inhibitory treatment under water stress conditions, the proline content was reduced only by 20%. Moreover, cycloheximide, a protein synthesis inhibitor, had no effect on OAT activity induced by water stress but significantly reduced proline accumulation.[25]

A new insight into this field has been introduced by demonstrating that OAT is essential for nitrogen recycling from arginine but not for the stress-induced proline accumulation.[9] Wild-type Arabidopsis plants could utilize either arginine or ornithine as the sole nitrogen source. In *oat*-knockout mutants, an accumulation of urea cycle intermediates occurred under such nutritional conditions. On the contrary, utilization of urea and stress-induced proline accumulation were not affected by the mutation. This provided strong evidence against a shortcut from arginine to proline that bypasses glutamate and cytosolic P5CS activity. OAT probably links the degradation pathways for arginine and proline (Fig. 2) converging in the formation of GSA/P5C in mitochondria, which is further metabolized to glutamate by P5CDH. It seems that under normal physiological conditions, ornithine can be converted to proline only via glutamate, while this conversion is not contributing substantially to stress-induced proline accumulation.[9]

Three-dimensional Structure

No crystal structure of a plant OAT has been solved so far. Neverthless, a homology modeling was recently performed for the enzyme from *Vigna aconitifolia*[17] based on the crystal structure of human OAT[10] (PDB accession code 1OAT). With this model, a flexible docking study with ornithine and competitive inhibitors was accomplished; see further.

Human liver OAT was first successfully crystallized as a recombinant protein obtained by expression of the entire gene in *E. coli*.[26] Later on, the crystal structure of the enzyme was determined.[10,27,28] The functional unit of the protein consists of a dimer built from two identical subunits. Each monomer contains 12 α-helices and 14 β-strands and can be structurally divided into three domains: a large 249 residue domain (PLP-binding domain), a small C-terminal domain of 95 residues and an N-terminal segment of 42 residues. Inter-subunit contacts occur mainly through the large domain.[10,27,28] The dimeric enzyme possess two equal active sites (in each subunit), where the cofactor PLP is covalently bound to Lys292 through a Schiff base forming the so-called internal aldimine. The cofactor is located in the large domain at the subunit interface and interacts non-covalently with residues from both subunits. Such a folding is common among several PLP-dependent enzymes.[27,28] The packing of the OAT dimers in the crystal yields a hexameric quaternary structure in which three dimers are arranged to form about one turn of a right-handed superhelix.[10]

Human OAT was also crystallized in the presence of L-canaline and gabaculine, which represent structural analogues of ornithine (the crystal structures are deposited under the PDB accession codes 2CAN and 1GBN, respectively).[27] The compounds mimic the natural substrate well enough to bind at the active site and enter the first steps of the transamination reaction. Unlike L-ornithine, however, they cause irreversible inhibition of the enzyme by producing covalent intermediates with the PLP cofactor.[27] OAT does not undergo a significant conformational changes upon binding of the inhibitors except for local sidechain movements. Because L-canaline (α-amino-γ-amino-oxybutyric acid) is virtually identical to ornithine, the structure of the enzyme-inhibitor complex allowed discovery of specific residues which participate in substrate recognition via the α-carboxylate and α-amino group (Arg180 and Tyr55, respectively). In this way, OAT specifically discriminates between α- and δ-amino groups of ornithine—only the latter is able to form the reactive Schiff base with PLP cofactor. The same residues were found to be mutated in inactivated OAT of patients suffering from gyrate atrophy.[29] Gabaculine (5-amino-1,3-cyclohexadienyl carboxylic acid) binding is facilitated by aromatic-aromatic interactions with two amino acid residues, Tyr85 and Phe177. It has been

proposed that a rearrangement ("switch") of Glu235 at the active site is important for the second OAT half-reaction, where the amino group abstracted by PLP from ornithine is transferred to 2-oxoglutarate as a terminal acceptor.[10,28]

Another ligand, which was used for co-crystallization of OAT, is (2S, 5S)-5-fluoromethylornithine (5FMOrn). The crystal structure of human OAT complexed with 5FMOrn (PDB accession code 2OAT) provided further information on the binding of ornithine to OAT.[28] The fluoro derivative is known as the only inhibitor exclusively specific for ornithine aminotransferase. It blocks the enzyme by a suicide reaction ("mechanism-based inhibition") leading to a covalent adduct with the cofactor. In the crystal structure, the cofactor adduct is detached from Lys292, but is nonetheless stabilized in its position by several non-covalent interactions with active site residues. The α-carboxylate and the α-amino group of the bound 5FMOrn are anchored to the enzyme by hydrogen bonding to Arg180 and Tyr55, respectively. Although not covalently bound, the adduct is firmly kept at the active site and cannot be removed by dialysis. The other diastereoisomers of 5-fluoromethylornithine cannot bind properly at the recognition site.[28]

As has been mentioned above, docking studies with the mothbean OAT model revealed interactions that are involved in binding of competitive inhibitors.[17] Based on the interaction energies, isoleucine and valine were the best enzyme ligands from this group. Valine showed even more favorable binding energy than ornithine itself. In the case of proline, which displayed no inhibitory properties in experiments performed in vitro, docking calculations revealed the lack of proper binding. Figure 3 shows a three-dimensional structure of pea seedling OAT obtained by homology modeling using human OAT (PDB accession code 1OAT) as a template. There are no significant differences in comparison with the structure of human OAT and the mothbean OAT model.

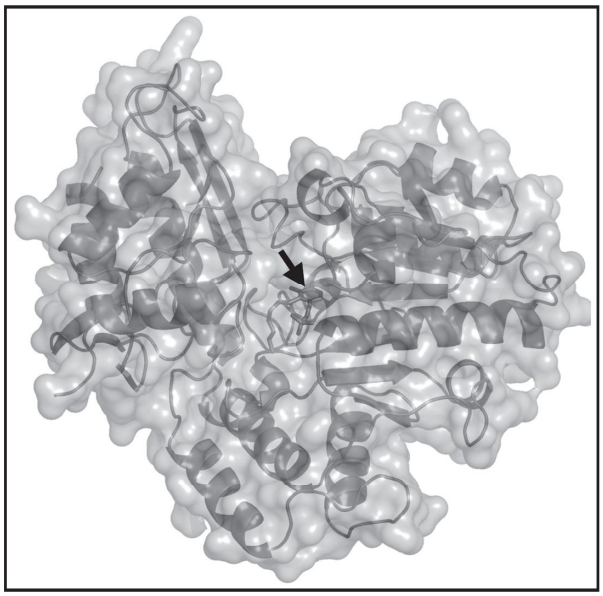

Figure 3. A homology-based molecular model of pea seedling OAT. The three-dimensional monomeric structure was modeled with human OAT as a template (PDB accession no. 1OAT) and pea OAT sequence (UniProtKB accession no. B1A0U3) using the program SWISS-MODEL (SIB-Biozentrum Basel, http://swissmodel.expasy.org/). The software used for picture drawing was PyMol (DeLano Scientific LLC, http://pymol.sourceforge.net/). The black arrow aims at the cofactor PLP.

Other Similar ω-Aminotransferases

OAT differs from other aminotransferases for which detailed biochemical information is available (e.g., alanine or aspartate aminotransferases) in that it is specific for an amino group other than the α-amino group adjoining a carboxyl function.[10] It belongs to the subgroup III of aminotransferases.[30] In the first half reaction, OAT functions as an ω-aminotransferase and attacks the distal δ-amino group of L-ornithine (interestingly the more reactive α-amino group is ignored), while it catalyses an α-transamination in the second half reaction.[31] The mechanism by which OAT is able to catalyze these two distinct reactions is not well understood and represents one of the most intriguing aspects of this enzyme. Based on the described crystallographic studies with human OAT[10,28] and a mutation study,[32] it seems that the reason resides in a rearrangement of the active site. Conversion of the enzyme to its PMP form disrupts the internal Glu235-Arg413 interaction, thus enabling 2-oxoglutarate to be a good substrate in the second half reaction.[32]

OAT shows structural and mechanistic similarity to other enzymes from the same subgroup like 4-aminobutyric acid aminotransferase (GABA-AT; EC 2.6.1.19) and glutamate-1-semialdehyde aminotransferase (glutamate-1-semialdehyde 2,1-aminomutase; EC 5.4.3.8).[27] Interestingly, mutation of Tyr85 in human OAT to Ile, as found in GABA-AT,[33] decreased the rate of the reaction of the enzyme with ornithine 1000-fold and increased that with 4-aminobutyrate 16-fold, indicating that Tyr85 is a major determinant of specificity toward ornithine.[32] *N*-acetylornithine aminotransferase from *Salmonella typhimurium* accepts both *N*-acetylornithine and ornithine as substrates but has higher affinity to the former one. Structural comparison of the *Salmonella* enzyme with human OAT suggests that its higher preference for N-acetylornithine may not be due to specific changes in the active site residues but could result from minor conformational changes in some of them.[34]

There is also a significant similarity of OAT to diamine (or polyamine) aminotransferases, which have been described to act in polyamine metabolism in some bacteria.[35-39] It should be noted here that the diamine aminotransferase (DAT; EC 2.6.1.29) from *E. coli* was described in the middle 60s and that time it was the first known aminotransferase utilizing a substrate with no carboxyl group.[35] Figure 4 shows the multiple sequence alignment of several OATs from Leguminosae plants, GABA-AT from *A. thaliana* and DAT from *E. coli*. The latter enzyme is able to accept several polyamine substrates including putrescine, cadaverine and spermidine utilizing 2-oxoglutarate, 2-oxobutyrate or pyruvate as terminal amino acceptors.[35] The enzyme also converts L-ornithine,[36] but it seems to be rather a weak substrate.[37] Polyamine aminotransferases were reported in the Gram-positive bacteria *Arthrobacter* sp. TMP-1,[38] and *Nocardioides (Arthrobacter) simplex*.[39] As amino donors, the enzymes accept particularly 1,3-diaminopropane, putrescine, agmatine, spermidine and cadaverine. Pyruvate and 2-oxoglutarate were found to be amino acceptors.

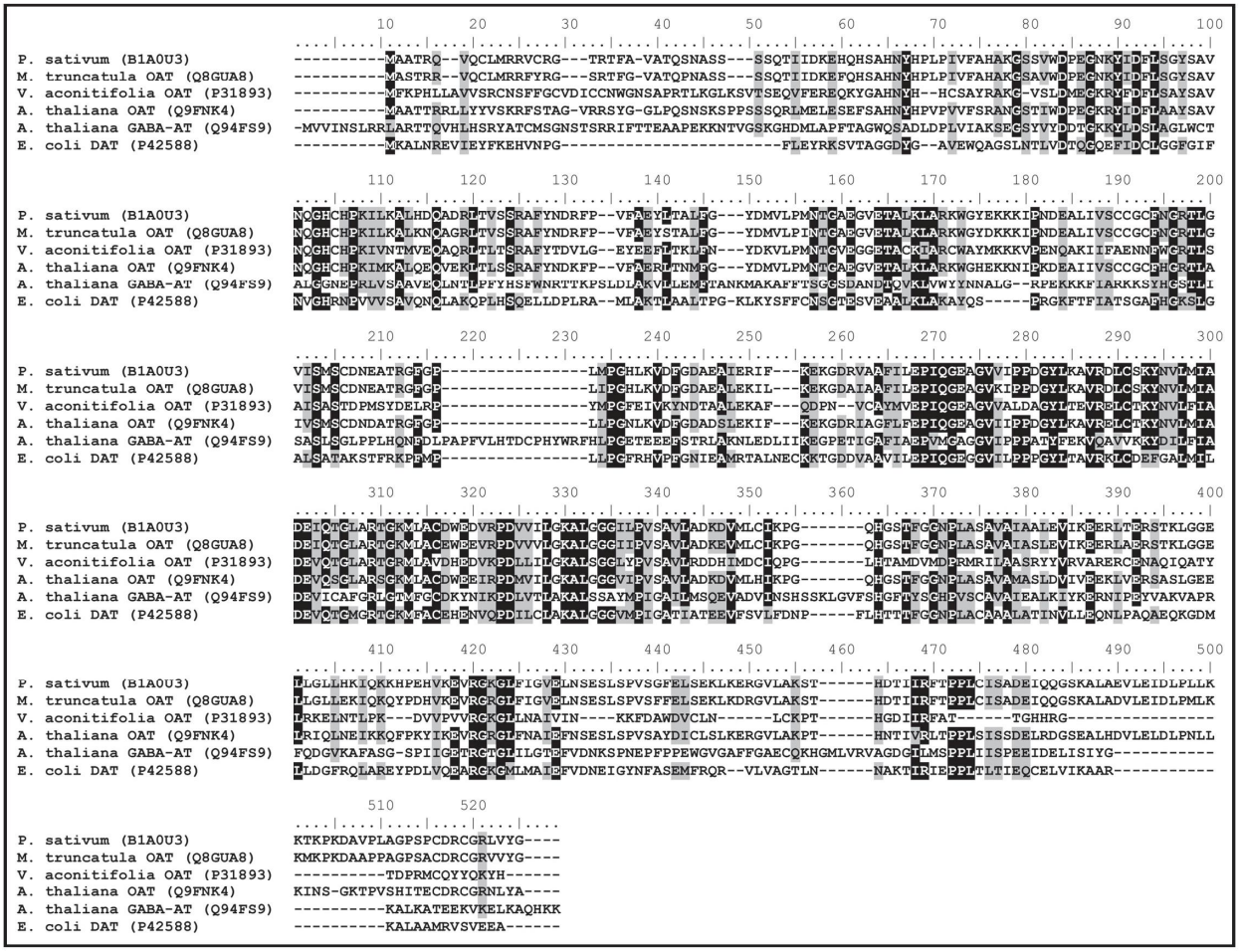

Figure 4. Amino acid sequence homology of plant OATs. A CLUSTAL W[40] multiple sequence alignment is shown comparing plant OATs from pea (*Pisum sativum*), barrel medic (*Medicago truncatula*), mothbean (*Vigna aconitifolia*) and Arabidopsis (*Arabidopsis thaliana*) with GABA-AT from *A. thaliana* and DAT from *E. coli* (annotated as putrescine aminotransferase). The sequences are deposited in the UniProtKB database, the respective accession numbers are given in parentheses. The graphic view was obtained using BioEdit v. 7.0.9.0.[41]

In microorganisms, the transamination of 1,3-diaminopropane results in 3-aminopropionaldehyde, which is then subjected to aminoaldehyde dehydrogenase (AMADH; EC 1.2.1.19) reaction producing β-alanine.[42] 3-Aminopropionaldehyde represents the best substrate of plant AMADHs.[43] However, still it is unclear, whether the compound is formed predominantly by an oxidative or transamination pathway in plants. The copper-containing diamine oxidase from pea seedlings for example converts 1,3-diaminopropane only negligibly.[43] There are some reports on the occurrence of DAT activity in plants,[44,45] but to our knowledge, no such an enzyme has been purified and characterized so far. Thus we reasoned that pea OAT, as a presumably similar enzyme, might accept some diamines as substrates. A recombinant pea seedling OAT was obtained by cDNA expression in *E. coli* and its substrate specificity was measured (Stránská J. et al., unpublished results). The enzyme was found to be strictly specific for L-ornithine showing practically no activity with putrescine, 1,3-diamimopropane and 4-aminobutyrate.

Conclusions

There are two research topics, which elucidate the significance of studying OAT: (i) genetic engineering of plants for increased production of the osmoprotectant proline; (ii) considering human OAT as a potential target for development of new therapeutic drugs. During the last years, interesting data appeared in this context. Future trends will probably involve site-directed mutagenesis of OAT enzymes to better understand the catalytic mechanism and relationships to other members of the same subgroup of aminotransferases.[32]

Despite the advanced agrotechnologies, which are available these days, drought and salinity are still the major factors affecting crop yield by reducing growth and plant productivity. Genetic engineering allows new ways to develop plants with enhanced osmotolerance and represents an important challenge to improve plant resistance. The accumulation of osmoprotectants has been a target for plant genetic engineering for many years.[19] A common strategy that is currently

being used in biochemical and experiments is based on overexpressing genes responsible for the biosynthesis of osmoprotectants i.e., coding for enzymes involved in the respective metabolic pathways (choline monooxygenase, betaine aldehyde dehydrogenase, P5CS, etc.,).[19,46] It has been shown that transgenic plants overexpressing OAT display enhanced tolerance to salt and drought due to increased proline content.[24,47]

Human OAT holds a significant scientific interest because of its association with gyrate atrophy, a recessive hereditary genetic dissorder leading to progressive loss of vision and eventually blindness in humans.[48] Gyrate atrophy is characterized by elevated ornithine levels arising from OAT dysfunction. Patients suffering from gyrate atrophy usually carry a point mutation in both copies of their OAT gene.[29] The OAT mRNA level appears to be normal. There is probably more than one mechanism of OAT inactivation which either involves an incorrect processing of the enzyme or of the correctly processed enzyme.[29]

Interestingly, human OAT has also been recognized as a potential target for chemotherapeutic drug development. In a study performed to evaluate the effect of selective blocking of mitosis in human cancer cells, OAT was identified as a protein, which binds the antimitotic drug diazonamide A and it has a role in regulating mitotic cell division.[49] Diazonamide A induces a G_2/M phase growth arrest in a variety of human cancer cell lines, which results from aberrant mitotic spindle formations. It seems that OAT facilitates spindle assembly but the mechanism is unknown. Although the activity may not be essential for normal development, it is critical for cell division in human cancer cells sensitive to diazonamide A.[49] The OAT-mediated mechanism of action of diazonamide A in inhibiting cell division implies this compound or their derivatives might show therapeutic activity comparable to clinically used tubulin-binding antimitotics (taxanes and vinca alkaloids) in the absence of toxicity.[50]

Acknowledgements

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2 EXPERIMENTAL PART

2.1 Material and methods

Common chemicals used in this work were kindly provided by the Department of Biochemistry (Palacký University in Olomouc, Czech Republic) and Department of Biological Chemistry (INRA/INAPG, Paris-Grignon, France) and were purchased in an ultra pure quality from Sigma-Aldrich Chemie (Steinheim, Germany) or local distributors. Exceptions are mentioned in the text.

2.1.1 Methods used to study OAT from *Pisum sativum* (PsOAT) in both native and recombinant forms

2.1.1.1 Activity and protein assay

PsOAT activity was assayed using a modified spectrophotometric method (Peraino & Pitot, 1963). This approach is based on the production of a yellow colored adduct by incubation of the P5C rising from PsOAT reaction with 2-aminobenzaldehyde (ABA). The reaction mixture in a test tube contained 50 mM Tris-HCl buffer, pH 8.0 (Tris stands for tris(hydroxymethyl)aminomethane), 35 mM ornithine, 5 mM 2-oxoglutarate and 0.05 mM pyridoxal-5'-phosphate. The assay was started by the addition of PsOAT in a total reaction volume of 1 ml. After incubation at 37 °C for 20 min, the reaction was stopped by adding 0.25 ml of 3 M perchloric acid. Later on, 0.25 ml of 0.5% (w/v) ABA in 96% ethanol was added into the reaction mixture for color development by incubating at 37 °C for 10 min. After centrifugation, the supernatant was measured at 440 nm.

Protein content was determined routinely by Bradford's spectrophotometric method with bovine serum albumin as a standard (Bradford, 1976) or for the pure enzyme from UV absorption at 280 nm using a theoretical ε_{280} value of 32,150 $M^{-1}.cm^{-1}$ deduced from the amino acid sequence (Pace et al., 1995).

2.1.1.2 SDS-PAGE and MALDI-TOF peptide mass fingerprinting

SDS-PAGE was performed using 4% stacking and 10% resolving polyacrylamide gel according to a standard procedure (Laemmli, 1970). Proteins were visualized by the Bio-Safe Coomassie Stain (Bio-Rad, Hercules, CA, USA). Gels were scanned by the computer program LabScan 5.00 (GE Healthcare, Uppsala, Sweden) and processed for images by Paint Shop Pro 8.00 (Jasc Software, Minnetonka, MN, USA).

Protein bands were excised from Bio-Safe Coomassie-stained SDS-PAGE gels. MALDI-TOF peptide mass fingerprinting after in-gel digestion (with an additional reduction and alkylation) by modified trypsin was conducted as described in Šebela et al. (2006). The instrument used was a Microflex LRF20 MALDI-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with a microScout ion source and a 337-nm nitrogen laser and operating in the reflectron mode for positive ions. The acquired spectra were processed by flexAnalysis 2.4 and Biotools 3.2 software (Bruker Daltonik). Database searches were performed against the Swiss-Prot and NCBInr databases using the program Mascot Server 2.2 (Matrix Science, London, UK). As variables, oxidation of methionine and carbamidomethylation of cysteine plus one missed cleavage were chosen for all searches performed without taxonomic restriction; a mass tolerance of 150 ppm was allowed.

2.1.2 Native PsOAT

2.1.2.1 Preparation and processing of plant material

Pea seeds (local distributor, *Pisum sativum* L., cv. Lantra) were soaked in distilled water for 24 h. After that, the seeds were sowed on a perlite layer (Perlit s.r.o., Šenov u Nového Jičína, Czech Republic) and were planted for 5 days in the dark. The etiolated seedlings were harvested and homogenized in 4 volumes of acetone (-15 °C) according to Lu & Mazelis (1975). After filtration, the retentate on filter (acetone powder) was left to air-dry for 24 h. Proteins from the acetone powder were extracted in 2 volumes of 50 mM MOPS [3-(*N*-morpholino)propanesulfonic acid] buffer, pH 7.4, containing 5 mM β-mercaptoethanol (β-ME; Ruitier & Kollöffel, 1983) and centrifuged (9,600 *g*, 4 °C, 25 min). The supernatant was saturated to 70 % (472 g/l) by ammonium sulfate. Precipitated proteins were separated by centrifugation (9,600 *g*, 4 °C, 25 min) and dissolved in 20 mM MOPS buffer, pH 6.5, containing 5 mM β-mercaptoethanol. The extract was centrifuged (9,600 *g*, 4 °C, 30 min).

2.1.2.2 Purification methods

All purification steps were performed in a cold environment (below 5 °C). Crude enzyme solution from the previous step was desalted by overnight dialysis (against 20 mM MOPS buffer, pH 6.5, 5 mM β-ME). After centrifugation (9,600 g, 4 °C, 25 min), the sample was purified by ion exchange low pressure chromatography on a SP-Sepharose FF column (25 mm i. d. x 250 mm, GE Healthcare, Uppsala, Sweden) previously equilibrated with 20 mM MOPS buffer, pH 6.5, containing 5 mM β-ME. The

column was operated at a flow rate of 2 ml.min^{-1} and the eluate was monitored by absorbance at 280 nm. The retained proteins were eluted by 0-100 % gradient of 2 M NaCl in the equilibration buffer.

This was followed by fast protein liquid chromatography (FPLC) including desalting on a Superdex 200 HR 10/30 column (10 mm i. d. × 300 mm; GE Healthcare) equilibrated with 20 mM MOPS buffer, pH 6.5, containing 5 mM β-ME and connected to a BioLogic Duo Flow medium-pressure chromatograph (Bio-Rad, Hercules, CA, USA). The mobile phase was pumped at a flow rate of 0.7 ml.min^{-1}, both absorbance at 280 nm and conductivity were monitored.

The enzyme solution was further loaded onto an ion-exchange HEMA Bio 1000 SB column for FPLC (8 mm i. d. x 250 mm; Tessek, Prague, Czech Republic) equilibrated with 20 mM MOPS buffer, pH 6.5 containing 5 mM β-ME. Flow rate of the mobile phase was 1 ml.min^{-1}; absorbance at 280 nm and conductivity were monitored. Elution buffer (buffer B; 20 mM MOPS, pH 6.5 containing 5 mM β-ME and 2 M NaCl) gradient was programmed as follows: an isocratic flow at 0 % B for 3 min, a linear gradient of 0-30 % B for 15 min, a linear gradient of 30-100 %B for 4 min, an isocratic flow at 100 % B for 3 min, a linear gradient of 100-0 % B for 5 min, and an isocratic flow at 0 % B for 10 min.

The final purification step was completed on an ion-exchange Mono S HR 5/5 column for FPLC (GE Healthcare) and proceeded under identical conditions as given above for the HEMA Bio 1000 SB column. Fractions showing OAT activity were processed by lyophilization.

During chromatography, all procedures were performed in cooling boxes. Eluates were collected into tubes placed on ice. Part of the fractions was used for activity measurements and determination of protein concentration; remaining parts were concentrated by ultrafiltration in an Amicon stirred cell (Millipore, Bedford, MA, USA) equipped with a 10-kDa cuttof membrane filters. In between the particular purification steps, samples were stored at -50 °C.

2.1.2.3 Post-source decay analysis

Lyophilized samples were dissolved to a concentration of 5 mg.ml^{-1} in 20 mM MOPS buffer, pH 6.5 containing 5 mM β-ME and separated by SDS-PAGE (see chapter 2.1.1.2). Peptides from excised and trypsin-digested protein bands were analyzed by MALDI-TOF peptide mass fingerprinting, acquired mass spectra were processed as given above (2.1.1.2).

MALDI post-source decay (PSD) spectra of selected peptides were recorded in 12–17 segments, with each succeeding segment representing a proportional reduction

in reflector voltage. About 300-500 laser shots were averaged per segment. Segments were pasted, calibrated, and smoothed under computer control by flexAnalysis 2.4, Biotools 3.2 and RapiDeNovo (Bruker Daltonik).

2.1.3 Recombinant PsOAT

A recombinant PsOAT for biochemical characterization was obtained by heterologous expression in *Escherichia coli*. To do that, a standard molecular biological strategy was employed involving experimental steps that proceeded in the following order: primer design, cDNA amplification and cloning, DNA sequencing, transformation of *E. coli* by an expression vector containing ligated *PsOAT* cDNA, cultivation of transformed cells, cell disruption with obtaining a lyzate, protein purification. Related results as well as the corresponding methods are described in our paper (chapter 3.3 of this thesis). Selected methods are mentioned in this chapter to point out some interesting experimental details.

2.1.3.1 Cloning of *PsOAT* gene and its expression in *E. coli*

A multiple sequence alignment of the database deposited nucleotide sequences and expressed sequence tags (ESTs) belonging to OATs of plant origin was obtained using the ClustalW function of BioEdit software version 7.0.5.3 (Hall, 1999). Based on the alignment, degenerated and nondegenerated oligonucleotide primers were designed in highly conserved areas. Total RNA was isolated from 10-day-old etiolated pea (*Pisum sativum* L., cv. Lantra) seedlings using the NucleoSpin® RNA Plant kit from Macherey-Nagel (Düren, Germany). The complete cDNA was obtained using rapid amplification of cDNA ends (RACE) – 3' RACE and 5' RACE Systems and AccuPrime™ *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Finally, it was cloned into a pCR®-Blunt plasmid (Fig. 3, Invitrogen) and sequenced. A transformation into NEB 5-alpha Competent *E. coli* (New England Biolabs, Hitchin, UK) was achieved by heat shock.

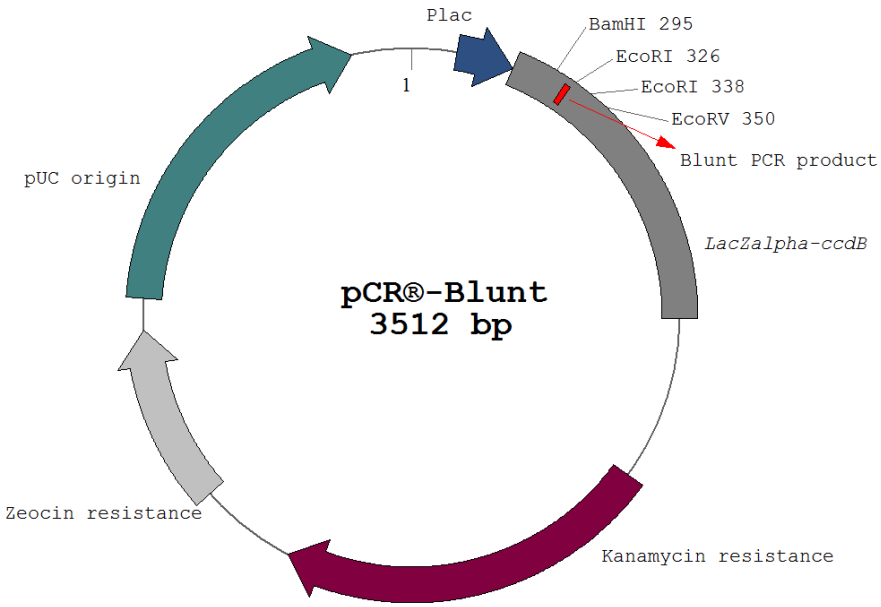

Fig. 3: The main features of the pCR®-Blunt vector. (According to Invitrogen, for more details see producer's Web site www.invitrogen.com) The pCR®-Blunt vector is designed to clone blunt PCR or DNA fragments with a low background of non-recombinants. It contains the lethal *E. coli ccd*B gene (control of cell death) fused to the C-terminus of LacZα. The *lac*Zα-*ccd*B gene fusion provides positive selection against nonrecombinant vector. The ccdB function is disrupted (inactivated) by an insert. P*lac* (*lac* promoter) allows expression of *lac*Zα-*ccd*B gene fusion; T7 promoter allows *in vivo* or *in vitro* transcription and translation; kanamycin and zeocin resistance genes allow selection and maintenance in *E. coli*; pUC origin allows high copy replication and maintenance of the plasmid in *E. coli*; multiple cloning site allows insertion of blunt PCR/DNA product to disrupt expression of the *ccd*B gene and allows convenient screening, restriction mapping, and excision of cloned insert; abbreviations accompanied with numbers show restriction enzymes used for a plasmid analysis and their cleavage position in the vector.

For expression in *E. coli*, a pair of primers including NcoI and XhoI restriction sites was designed based on *PsOAT* sequence and after PCR amplification of the cDNA (annealing at 61 °C, 35 cycles), the obtained product was inserted into a pET28b(+) expression vector (Fig. 4, Novagen, La Jolla, CA, USA).

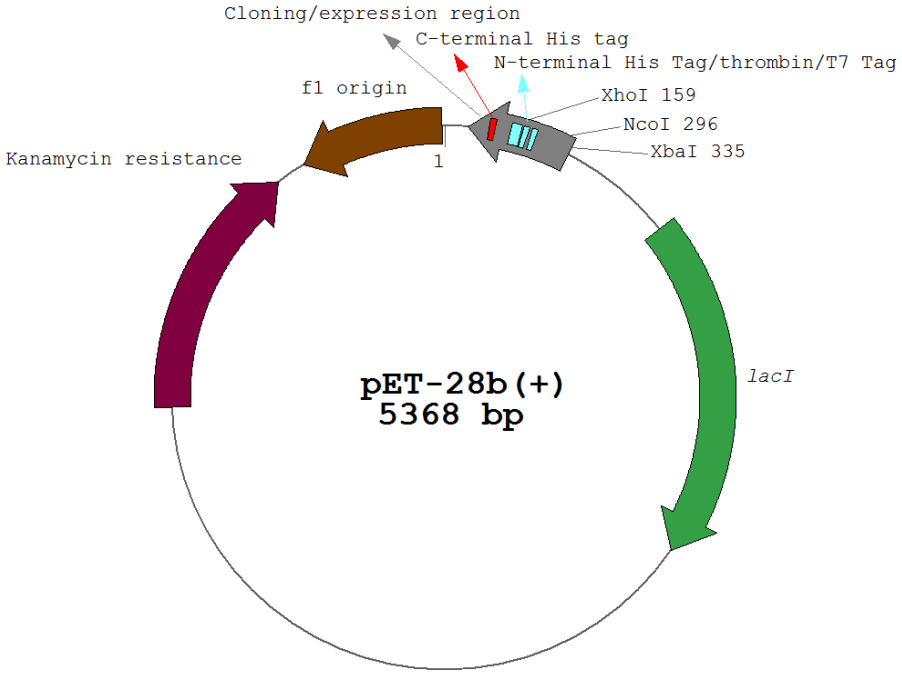

Fig. 4: The main features of the pET-28b(+) vector. (According to Novagen, for more details see producer's Web site www.novagen.com). The pET-28b(+) vector was developed for the cloning and expression of recombinant proteins in *E. coli*. Target gene was cloned in pET plasmid under control of strong bacteriophage T7 transcription and translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell; kanamycin resistance gene allows selection and maintenance in *E. coli*; f1 origin of replication allows the production of single stranded plasmid DNA for mutagenesis and sequencing applications; the vector carries an N terminal His•Tag®/thrombin/T7•Tag® configuration plus an optional C-terminal His•Tag sequence; abbreviations accompanied with numbers show restriction enzymes used for a plasmid analysis and their cleavage position in the vector.

The construct was then introduced by heat shock into T7 Express Competent *E. coli* cells (New England Biolabs). Transformed competent cells were precultured in Luria-Bertani (LB) medium containing 1% glucose and kanamycin (50 $\mu g.ml^{-1}$) at 37 °C overnight. Then PsOAT expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) when OD_{600} reached a value of 0.6. After overnight incubation, cells were harvested by centrifugation at 5,000 *g* for 15 min and stored frozen at -50 °C. A set of screening experiments was performed to find the most suitable IPTG concentration (0 mM as a non-induced control, 0.1, 0.5, 1 or 4 mM) and expression temperature (18, 22, 25 or 30 °C) was performed. Results were evaluated by SDS-PAGE and activity measurements according to standard protocols.

2.1.3.2 Extraction and purification of PsOAT

Harvested *E. coli* cells (~1 g) were resuspended in 1 ml of Bacterial Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA) and then in 10 ml of a lysis buffer containing 50 mM buffer, pH 7.0 or 8.0, 10 mM $MgCl_2$, 1 mM phenylmethylsufonyl fluoride, 1 µM pepstatine A and 10 mM imidazole. After addition of

lysozyme (0.3 mg.ml^{-1}), the mixture was incubated at 37 °C for 30 min and then supplemented to contain 1% (w/v) Triton X-100, 100 mM NaCl, 5% (w/v) glycerol, RNase A (0.01 µg.ml^{-1}) and DNase 1 (0.004 U.ml^{-1}). Finally, after a 30-min incubation at 37 °C, the lyzate was centrifuged at 9,000 *g* for 20 min and the supernatant containing recombinant PsOAT was recovered. A Tris-HCl buffer, pH 8.0, Bis-Tris [bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane] buffer, pH 7.0, or MOPS buffer, pH 7.0, were screened for extraction.

The enzyme solution was loaded onto a glass column (2.5 cm i. d. x 25 cm, Sigma-Aldrich Chemie) filled with an affinity matrix (suited for immobilized metal ion affinity chromatography) previously equilibrated with 20 mM MOPS buffer, pH 7.0, containing 100 mM NaCl, 10 mM imidazole and 5% (v/v) glycerol (EqB). At a flow rate of 2 ml.min^{-1}, the column was washed with the equilibration buffer. Weakly bound proteins were eluted with the same buffer containing higher concentration of imidazole. Elution of PsOAT was achieved using an additionally increased concentration of imidazole. The active fraction was desalted by a diafiltration using 10-kDa cutoff Centricon filters (Millipore) and stored frozen at -50 °C in 20 mM MOPS buffer, pH 7.0, containing 5% (v/v) glycerol. Two affinity matrices, i.e. Ni(II)-charged iminodiacetate-Sepharose 6B (IDA Sepharose, GE Healthcare) or HIS-Select® Cobalt Affinity Gel (Sigma-Aldrich Chemie), were compared. To determine conditions for an efficient elution of PsOAT, buffers containing varying concentrations of imidazole were tested.

To try further purification, the enzyme solution was loaded onto an ion-exchange Resource Q column for FPLC (16 mm i. d. x 30 mm, GE Healthcare) equilibrated with 20 mM MOPS buffer, pH 7.0 containing 5% (v/v) glycerol. Flow rate of the mobile phase was 1 ml/min; absorbance at 280 nm and conductivity were monitored. Elution buffer (buffer B; 20 mM MOPS buffer, pH 7.0, containing 5% (v/v) glycerol, 1 M NaCl) gradient was programmed as follows: an isocratic flow at 0 % B for 10 min, a gradient of 0-25 % B for 15 min, a gradient of 25-100 % B for 3 min, an isocratic flow at 100 % B for 3 min, a gradient of 100-0 % B for 3 min and an isocratic flow at 0 % B for 10 min.

The results were simultaneously studied by SDS-PAGE, activity measurements and peptide mass fingerprinting according to standard protocols (see chapter 2.1.1).

2.1.3.3 Molecular mass determination

Gel permeation chromatography was performed on a Bio-Silect SEC 125-5 column for FPLC (Bio-Rad). The column was equilibrated and run with 50 mM potassium phosphate buffer, pH 7.0, containing 150 mM NaCl at a flow rate of 1 ml.min^{-1}. The molecular mass of recombinant PsOAT was evaluated after calibration

of the column with protein standards (1.35, 17, 44, 158 and 670 kDa) purchased from Bio-Rad.

MALDI-TOF analysis for intact mass determination was performed on the Microflex LRF20 instrument (Bruker Daltonik). Prior to analysis, enzyme samples were dialyzed against 25 mM ammonium bicarbonate and concentrated by ultrafiltration in centrifugation cartridges with a cut off of 10-kDa to a concentration of 10 $mg.ml^{-1}$. Then, 1 µl of sample was mixed with 3 µl of matrix (10 $mg.ml^{-1}$ sinapinic acid in 0.1 % trifluoroacetic acid/acetonitrile, 1:1, v/v). Aliquots (1 µL) were placed onto the target plate previously modified by a thin layer of sinapinic acid (Vorm et al., 1994) and allowed to dry. Spectra were acquired in the linear mode for positive ions and calibrated externally using molecular ions of BSA.

2.1.3.4 Kinetic properties

Kinetic properties of recombinant PsOAT (for L-ornithine conversion) such as thermostability, pH optimum, optimum temperature as well as inhibition constants of the interaction with various inhibitors were measured using the protocols provided in Supplement 2 (Stránská et al., 2010). The inhibition constants were determined from double reciprocal Lineweaver-Burk graphs; 2-oxoglutarate concentration was fixed at 5 mM, L-ornithine concentration varied in the range of 3.33-25 mM. Two various additions of the inhibitor were used.

2.1.3.5 LC-MS/MS analysis

Protein samples from a lyzate of the transformed *E. coli* cells and flow-through plus eluate fractions from HIS-Select® Cobalt Affinity Gel (originating from a PsOAT purification run) were separated by SDS-PAGE with Bio-Safe Coomassie staining. Selected bands were excised from the gel slab and cut into pieces in PCR 0.5-ml microtubes. Then in-gel digestion with reduction and alkylation was performed by modified trypsin (Šebela et al., 2006). The obtained digests were purified using C_{18} StageTips following Rappsilber's et al. (2007) protocol.

All samples were analyzed on a Q-ToF Micro mass spectrometer (Waters, Milford, USA) coupled to a capillary liquid chromatography system (capLC, Waters) equipped with an autosampler. Prior to measurements, the MS system was tuned and calibrated using a Glu^1-Fibrinopeptide standard solution (1 pmol/µl, Sigma-Aldrich Chemie). Individual desalted and dried samples were freshly dissolved in 10 µl of 0.5% formic acid (FA). The reconstituted samples (4 µl) were injected into a nanoLC system, loaded directly onto a 75 µm × 130 mm nanocapillary column filled with 3 µm C18 AQ particles (Dr. Maisch GmbH, Ammerbuch, Germany) with buffer A [2% acetonitrile

(ACN)+0.5% FA] as a mobile phase at a flow rate of 350 nl/min for 20 min. Separation was achieved by a long binary gradient of buffer B (80% ACN+0.5% FA) programmed as follows: an isocratic flow at 0 % B for 5 min, a linear gradient of 0-10 % B for 5 min, a linear gradient of 10-60 % B for 80 min, an isocratic flow at 60 % for 5 min, a linear gradient of 60-0 % B for 5 min, and an isocratic flow at 0 % for 20 min.

All separated peptides were eluted into a coupled MS system. MS analysis was performed in a data-dependent analysis mode, where ions in the MS mode were screened in the *m/z* range of 350-1500 for a 1.0-s acquisition cycle, and MS/MS data were acquired in the *m/z* range of 80-1500 for the two most intensive ions with a charge state between 2 and 4 using a cycle time of 2 s for each selected ion. The MS and MS/MS switch threshold was set to 20 counts for a selected ion. All precursors scanned in the MS/MS mode were dynamically excluded from the consecutive fragmentation for the next 2 min with a window of 1.5 *m/z* unit.

Collected raw data were processed with Mascot Distiller 2.2 software (Matrix Science). MGF-formatted files were generated containing a list of precursors and their fragments. Protein identification was performed with Mascot server 2.2 that contains the Mascot searching algorithm installed on a Mascot in-house server (Matrix Science). Searches were done against the database NCBInr (release 20080616). The data analysis parameters were as follows: enzyme – trypsin; missed cleavage – one; fixed modification: carbamidomethylation of cysteine; variable modification – oxidation of methionine and deamination of asparagine and guanidine; mass tolerance in the MS and MS/MSmode: 1.3 Da and 0.3 Da, respectively; instrument type – ESI-QTOF.

3 RESULTS

3.1 Native PsOAT

Etiolated 5-day-old pea seedlings were homogenized in acetone. Then a protein extraction was performed by homogenization of the obtained acetone powder in 50 mM MOPS buffer, pH 7.4, containing 5 mM β-ME. The extract was saturated to 70 % by ammonium sulfate. Precipitated proteins were dissolved in 50 mM MOPS, pH 6.5, containing 5 mM β-ME. After dialysis, native PsOAT was partially purified using ion exchange chromatography on SP-Sepharose FF (Fig. 5), gel chromatography on Superdex 200 HR 10/30 (Fig. 6), and further by means of ion exchange chromatography on HEMA Bio 100 SB (Fig. 7) and Mono S HR 5/5 columns (Fig. 8). The process of purification is summarized in Table 1.

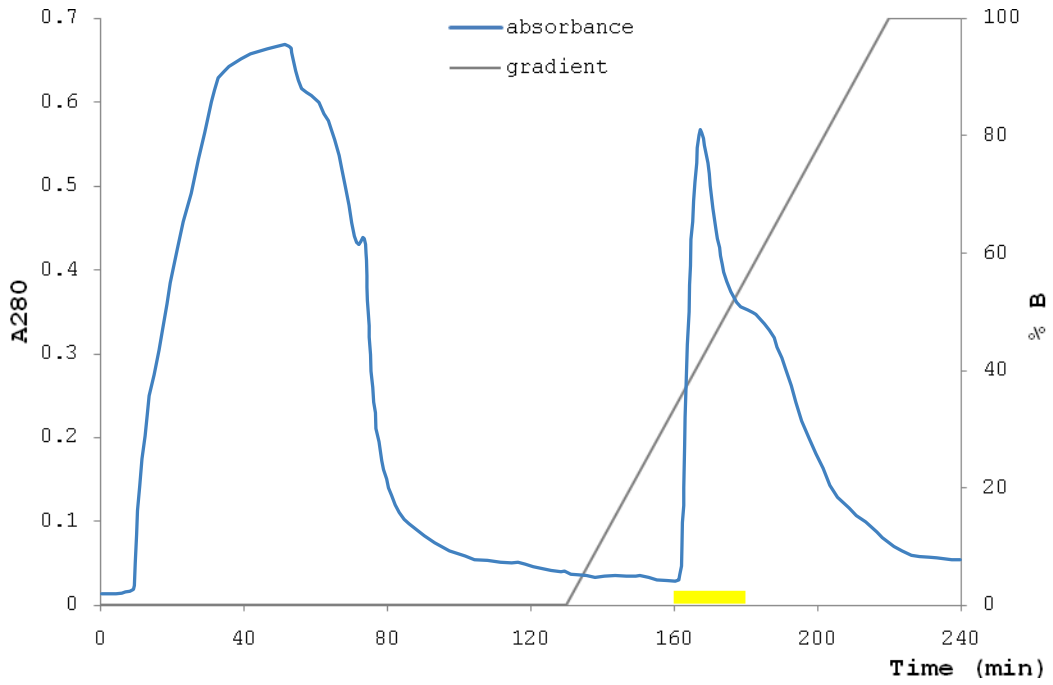

Fig. 5: Purification of PsOAT on SP-Sepharose FF column. The yellow box indicates PsOAT activity in fractions.

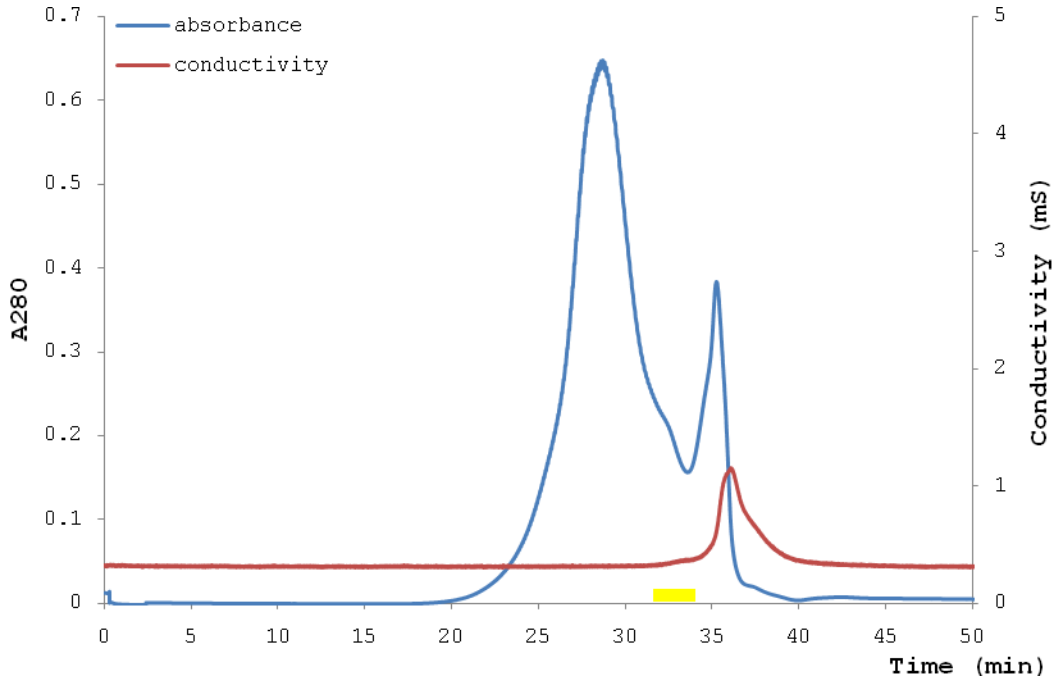

Fig. 6: Purification of PsOAT on Superdex 200 HR 10/30 column. The yellow box indicates PsOAT activity in fractions.

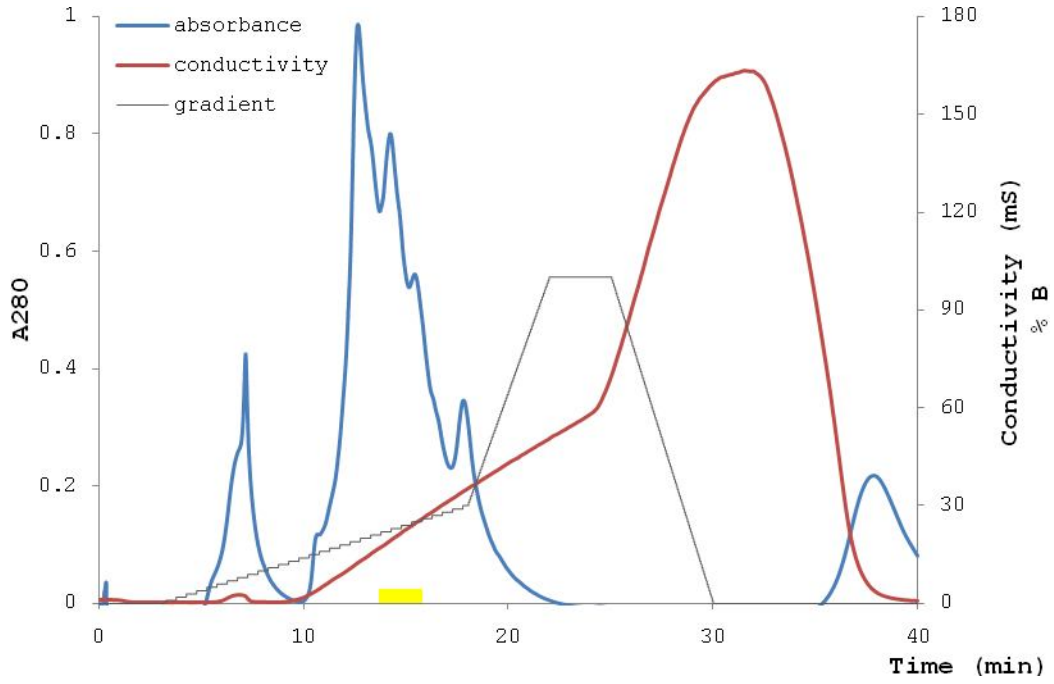

Fig. 7: Purification of PsOAT on HEMA Bio 100 SB column. The yellow box indicates PsOAT activity in fractions.

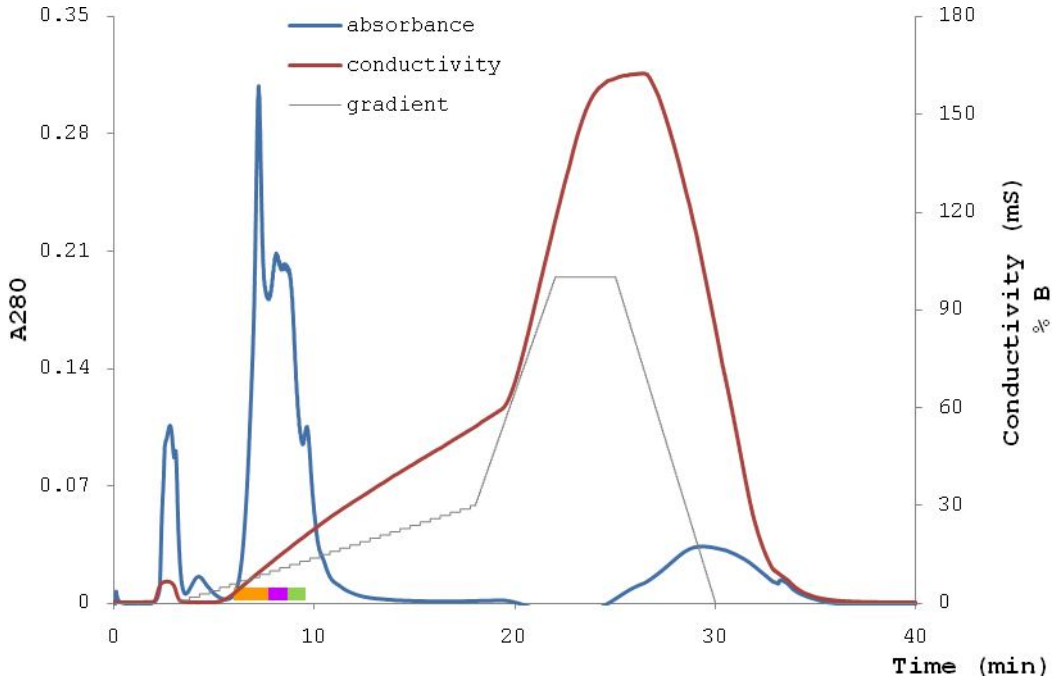

Fig. 8: Purification of PsOAT on Mono S HR 5/5 column. The orange, violet or green boxes indicate fractions 3, 4 or 5, respectively, used for SDS-PAGE and MALDI-TOF analysis.

Table 1: Purification of native PsOAT.

Purification Step	Total Volume (ml)	Total activity (pkat)	Protein content (mg)	Specific activity (pkat.mg^{-1})	Yield (%)	Enrichment Factor
Dialyzed extract	12	15.0	25.0	0.6	100	1
SP-Sepharose FF	30	12.0	2.8	4.3	80	7
Superdex 200 HR 10/30	10	10.2	1.8	6.5	68	11
HEMA Bio 1000 SB	12	9.6	1.2	8.0	64	13
Mono S HR 5/5, fraction 5	6	7.7	0.3	25.7	51	43

Fractions no. 3, 4 and 5 collected from FPLC on the Mono S HR 5/5 column were lyophilized, dissolved in 20 mM MOPS buffer, pH 6.5, containing 5 mM β-ME and separated by SDS-PAGE (Fig. 9). Protein bands no. 3-1, 3-2, 3-3, 3-4, 3-5, 3-6, 3-7, 3-8, 4-1, 4-2, 5-1 and 5-2 were analyzed by MALDI-TOF mass spectrometry.

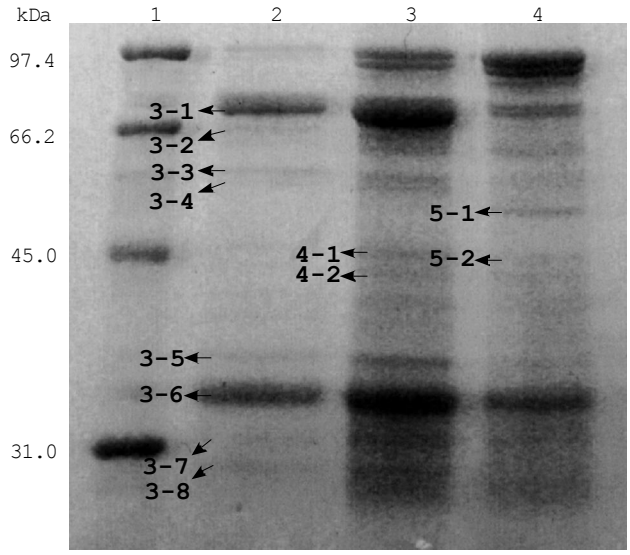

Fig. 9: Separation of fractions no. 3, 4 and 5 from gel chromatography by SDS-PAGE. Lane 1, protein molecular mass markers (low range, Bio-Rad); lane 2, fraction 3; lane 3, fraction 4; lane 4, fraction 5. Annotated protein bands were excised and after an in-gel digestion procedure analyzed by MALDI-TOF mass spectrometry.

MALDI-TOF peptide mass fingerprinting revealed interesting information. Database searches using Mascot allowed to assign the sample 3-1 to amine oxidase (MOWSE score value of 173), sample 3-6 to glutamine-rich protein (score 57) and sample 5-2 to lipoxygenase (score 95) (Table 2). Unfortunately, no high-quality spectra of the samples 3-2, 4-1 and 4-2 could be acquired. For the remaining proteins, identification was unsuccessful. A peptide with *m/z* value of 1503.77 that appeared in fingerprints of the sample 5-1 (Fig. 10) was further analyzed by MALDI-TOF post-source decay. Based on the observed series of b- and y-type fragment ions, a sequence tag SEI(L)SS was recognized (Fig. 11). A similar sequence segment (SESLS) occurs in the complete sequences of *M. truncatula* and *A. thaliana* OAT. Thus it was concluded that the analyzed protein might represent a PsOAT.

Table 2: MALDI-TOF peptide mass fingerprinting – matched proteins.

Band	Matched protein	Accession number	Molecular mass (Da)	Score value	Total peptides/matched peptides	Sequence coverage (%)
3-1	amine oxidase	Q9SXW5	76257	173	43/19	35
3-6	glutamine-rich protein	T51477	42528	57	43/7	35
5-2	lipoxygenase	S01142	97568	95	12/29	19

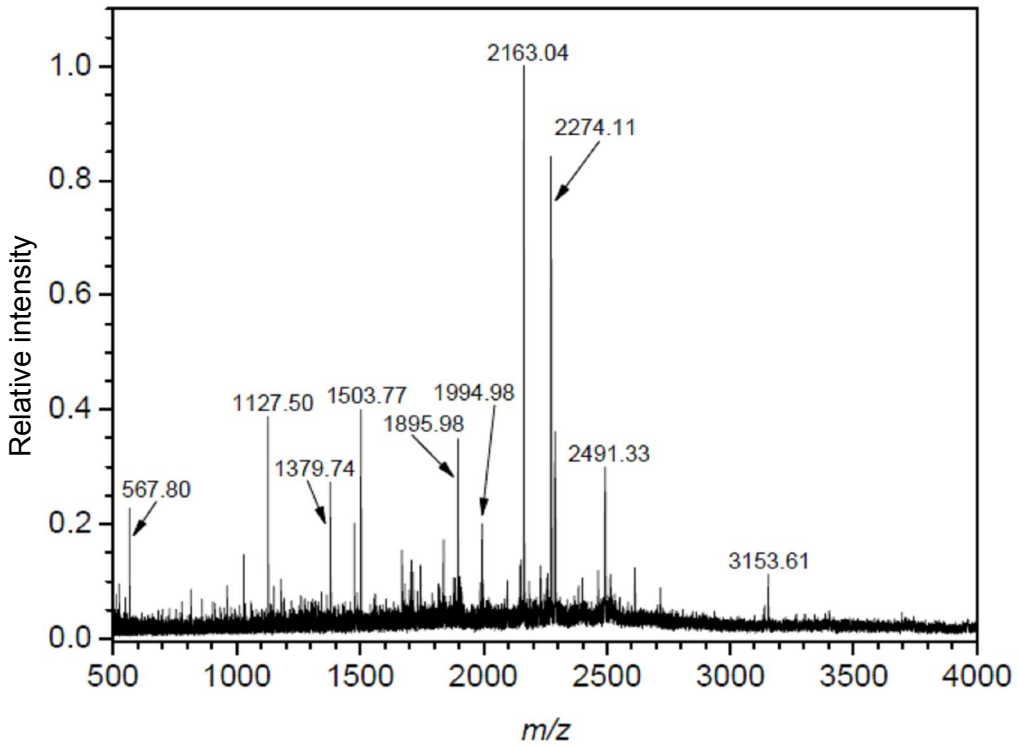

Fig. 10: MALDI-TOF peptide mass fingerprint of the sample 5-1.

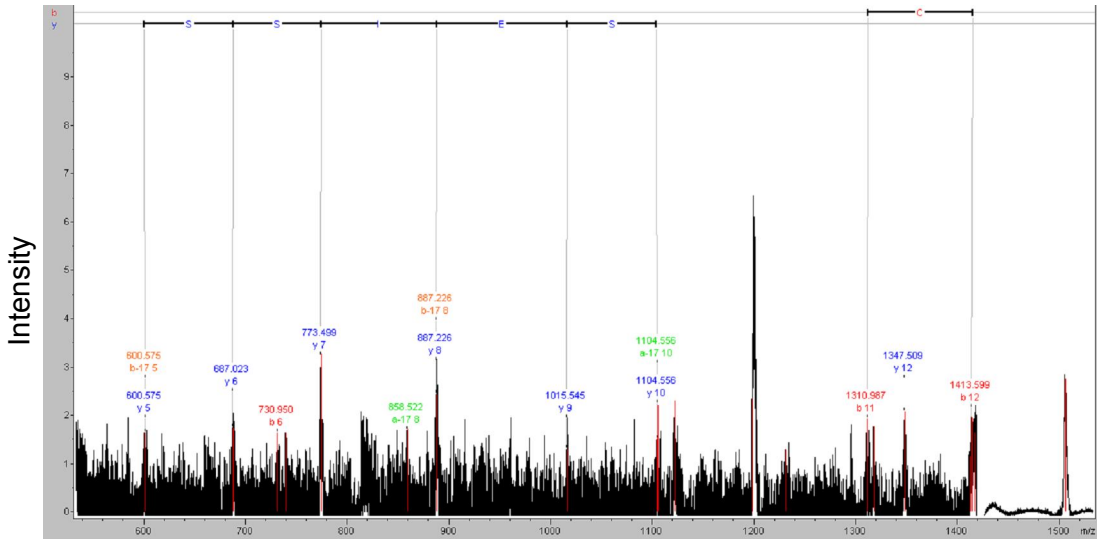

Fig. 11: Post-source decay spectrum of the peptide with *m/z* value of 1503.77 coming from the sample 5-1.

3.2 Recombinant PsOAT

A set of gene specific primers was designed based on the multiple sequence alignment of nucleotide sequences belonging to OATs of plant origin (Fig. 12). Total RNA was isolated and then a cDNA coding for PsOAT was obtained using 3' RACE and 5' RACE techniques. The final PCR product of was cloned into a pCR®-Blunt plasmid and sequenced. According to the obtained sequence, another set of primers including NcoI and XhoI restriction sites was designed. After amplification of the *PsOAT* cDNA (Fig. 13), the product of ~1,500 base pair (bp) was inserted into a pET28b(+) expression vector.

Fig. 12: Multiple sequence alignment of plant OATs with primers designed in this work and their schematic position and orientation in *PsOAT* cDNA strand. *OAT* sequences were from *Arabidopsis thaliana* (accession number AB006698), *Medicago truncatula* (AJ278819), *Gossypium* (EST CO101999 and TC41720), *Phaseolus vulgaris* (EST CB540884) and

Glycine max (DQ224372). Forward primers: peaOATd1, 5'-AGT GCC CAC AAT TAC CAY CC-3'; peaOATd2, 5'-TCW GCW GTT AAT CAG GGA CA-3'; peaOATd3, 5'-TTA AAG TTG ATT TTG GTG ATG C-3'; reverse primers: peaOATrv1, 5'-CAA TTA GTC CCT GTR ACR GT-3'; peaOATrv2, 5'-TTT CAA CTA AAA CCA CTA CG-3'; peaOATrv3, 5'-TGG ACC TGT CGT ACC TTC RTG-3'.

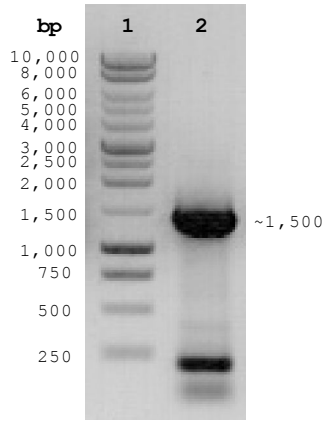

Fig. 13: Submarine electrophoresis of a *PsOAT* cDNA amplification product in 0.8% agarose gel. Lane 1, 1kb DNA Ladder (Promega, Madison, WI, USA); lane 2, *PsOAT* product.

Production of a recombinant PsOAT expressed in NEB T7 Express Competent *E. coli* cells was induced by IPTG. Based on screening experiments, the best expression was found in a culture induced by 0.1 mM IPTG and incubated at 25 °C (Fig. 14-16).

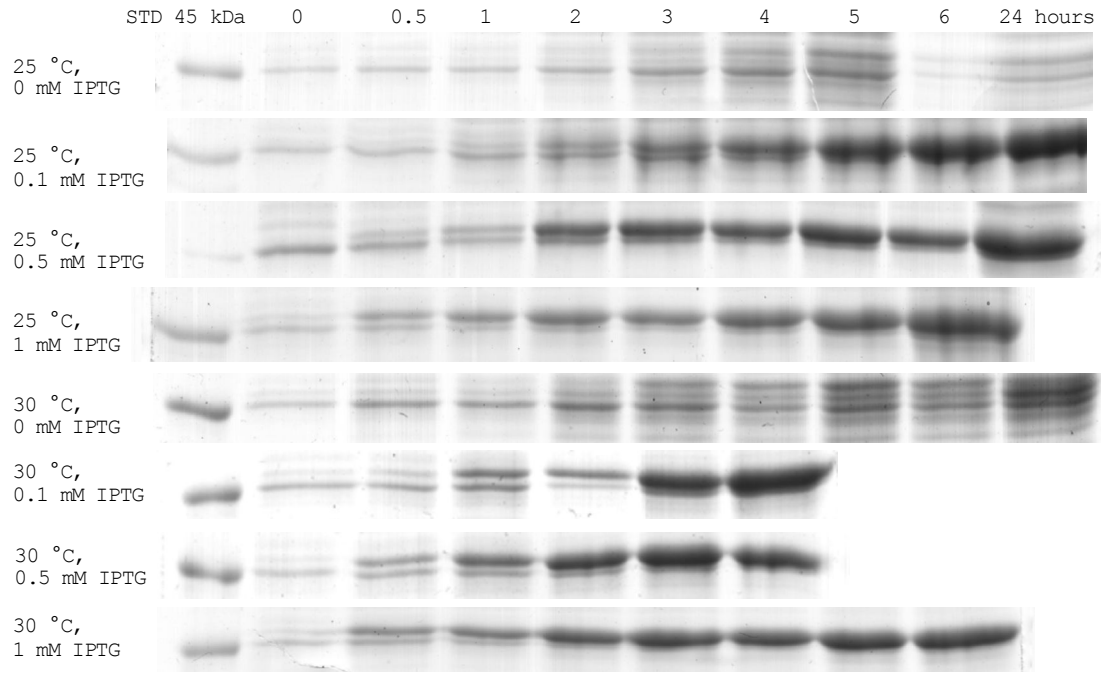

Fig. 14: SDS-PAGE separation of extracts from *E. coli* screening cultures grown under different conditions. Horizontal lanes illustrate the production of induced protein as a function of the incubation time; vertical lanes demonstrate the influence of the cultivation temperature and IPTG inductor concentration. Depicted parts of polyacrylamide gels were excised from areas corresponding to a molecular mass value of 45 kDa. Proteins were extracted from harvested cells (200 µl)

by 10% sodium dodecyl sulfate and mixed with the Laemmli sample buffer. After a 5-min incubation at 100 °C, the suspension was homogenized by repeated aspiration and discharge in a narrow syringe, span down and loaded on the gel.

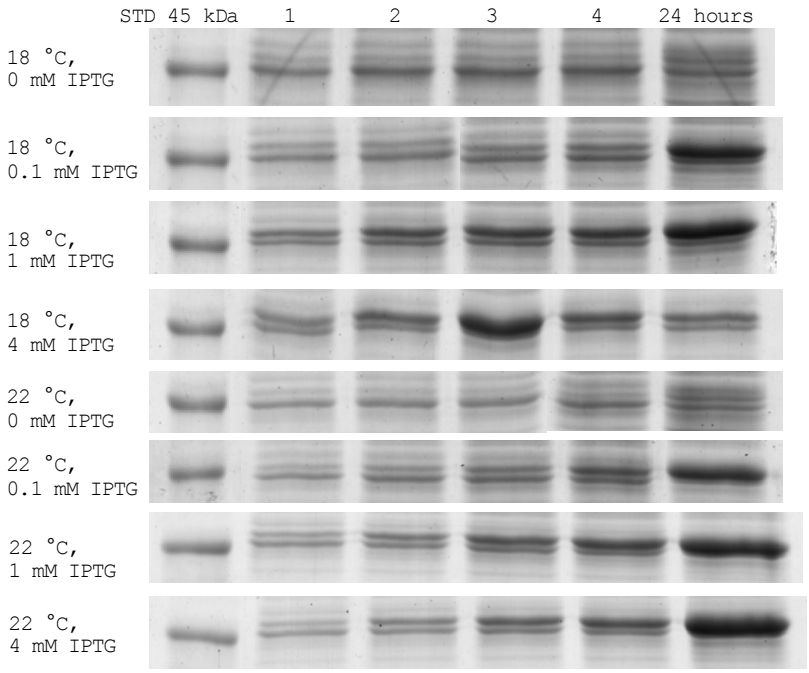

Fig. 15: SDS-PAGE separation of extracts from *E. coli* screening cultures grown under different conditions. Horizontal lanes illustrate the production of induced protein as a function of the incubation time; vertical lanes demonstrate the influence of the cultivation temperature and IPTG inductor concentration. Depicted parts of polyacrylamide gels were excised from areas corresponding to a molecular mass value of 45 kDa. Proteins were extracted from harvested cells (200 µl) by 10% sodium dodecyl sulfate and mixed with the Laemmli sample buffer. After a 5-min incubation at 100 °C, the suspension was homogenized by repeated aspiration and discharge in a narrow syringe, span down and loaded on the gel.

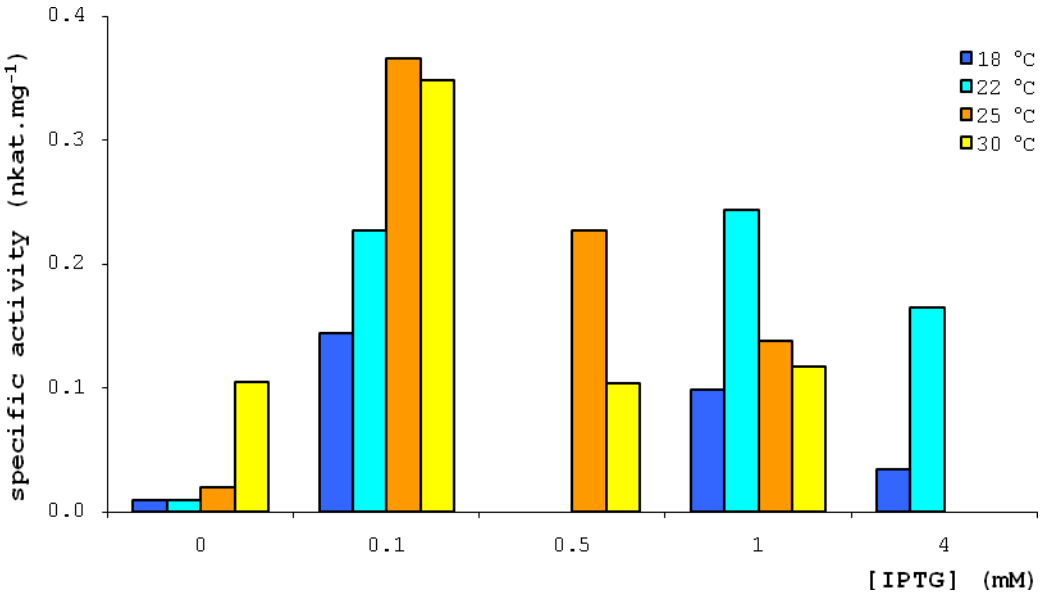

Fig. 16: Specific activity of extracts of *E. coli* screening cultures grown under different conditions. Harvested *E. coli* cells were resuspended in a lysis buffer containing 50 mM

Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2$ and 1 mM phenylmethylsufonyl fluoride, followed by standard extraction protocol (see chapter 2.1.3.2).

Screening experiments for the most efficient extraction procedure were performed with 50 mM Tris-HCl buffer, pH 8.0, 50 mM Bis-Tris buffer, pH 7.0 and 50 mM MOPS buffer, pH 7.0. Extracts were purified on HIS-Select® Cobalt Affinity Gel or IDA Sepharose sorbents (Fig. 17-18). A combination of MOPS buffer, pH 7.0, and IDA Sepharose was found to be the most suitable. The identity of PsOAT was confirmed by peptide mass fingerprinting (a sample eluted from IDA Sepharose using 250 mM imidazole; 27 matched peptides; MOWSE score: 194; sequence coverage: 69 %).

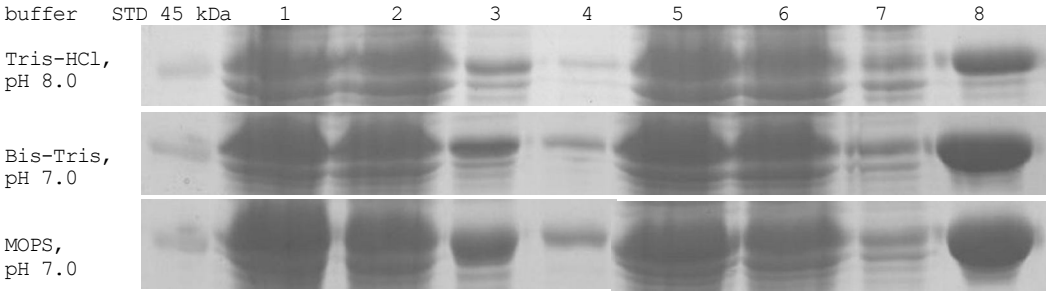

Fig. 17: SDS-PAGE separation of protein fractions from affinity chromatography performed under different conditions. Purifications on HIS-Select® Cobalt Affinity Gel (lanes 2-4) and IDA Sepharose (lanes 6-8) are compared. Lane 1 and 5, lyzate; lane 2 and 6; flow-through fraction; lane 3 and 7, wash fraction; lane 4 and 8, eluate. The legend on the left refers to the buffer system used for protein extraction (50 mM buffer) and chromatography.

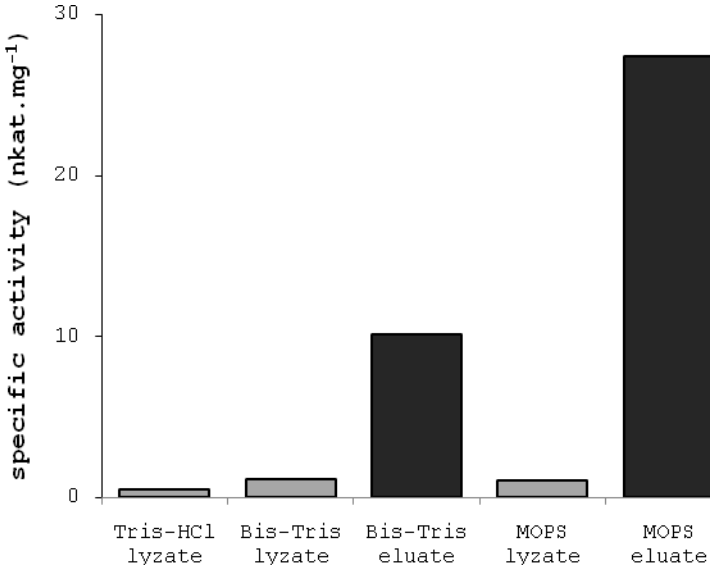

Fig. 18: A comparison of specific activity values for *E. coli* lyzates and the corresponding eluates from IDA Sepharose. The buffers used for extraction and chromatography were as follows: extraction buffer: 50 mM buffers Tris-HCl, pH 8.0, Bis-Tris, pH 7.0, or MOPS buffer, pH 7.0; equilibration buffer (EqB): 20 mM buffer containing 5% (v/v) glycerol, 100 mM NaCl and 10 mM imidazole; washing buffer: EqB with 50 mM imidazole; elution buffer: EqB buffer with 250 mM imidazole].

Elution conditions for the bound PsOAT were further optimized using washing buffers containing 50-250 mM imidazole (Fig. 19-20). The identity of PsOAT (a sample eluted using 250 mM imidazole) was confirmed by peptide mass fingerprinting (17 matched peptides; MOWSE score: 152; sequence coverage: 40 %). Finally, the optimized purification protocol used loading of the lyzate on the IDA Sepharose column equilibrated with 20 mM MOPS buffer, pH 7.0, containing 100 mM NaCl, 5% (v/v) glycerol and 10 mM imidazole (flow-through fraction), washing of the weakly bound proteins by EqB containing 100 mM imidazole (wash fraction) and PsOAT elution by EqB containing 250 mM imidazole (eluate fraction) (Fig. 21). The identity of PsOAT was confirmed by peptide mass fingerprinting (44 matched peptides; MOWSE score: 320; sequence coverage: 77 %).

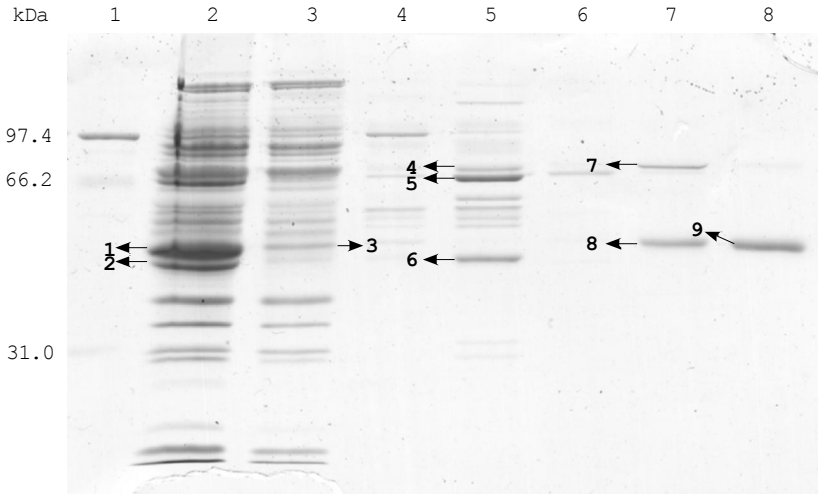

Fig. 19: SDS-PAGE separation of protein samples obtained during purification of PsOAT. The effect of imidazole concentration in the elution buffer from affinity chromatography on IDA Sepharose (equilibrated with 20 mM MOPS buffer, pH 7.0, containing 5% (v/v) glycerol, 100 mM NaCl and 10 mM imidazole) is demonstrated: lane 1, protein molecular mass markers (Bio-Rad); lane 2, lyzate extracted in 50 mM MOPS, pH 7.0; lane 3, flow-through fraction; lane 4, wash fraction with 50 mM imidazole; lane 5, wash fraction with 100 mM imidazole; lane 6, wash fraction with 150 mM imidazole; lane 7, wash fraction with 200 mM imidazole; lane 8, wash fraction with 250 mM imidazole. Protein bands indicated by arrows were analyzed by MALDI-TOF peptide mass fingerprinting after in-gel digestion. The presence of PsOAT was confirmed in the bands 1, 3, 8 and 9. Proteins from the other bands came from *E. coli*.

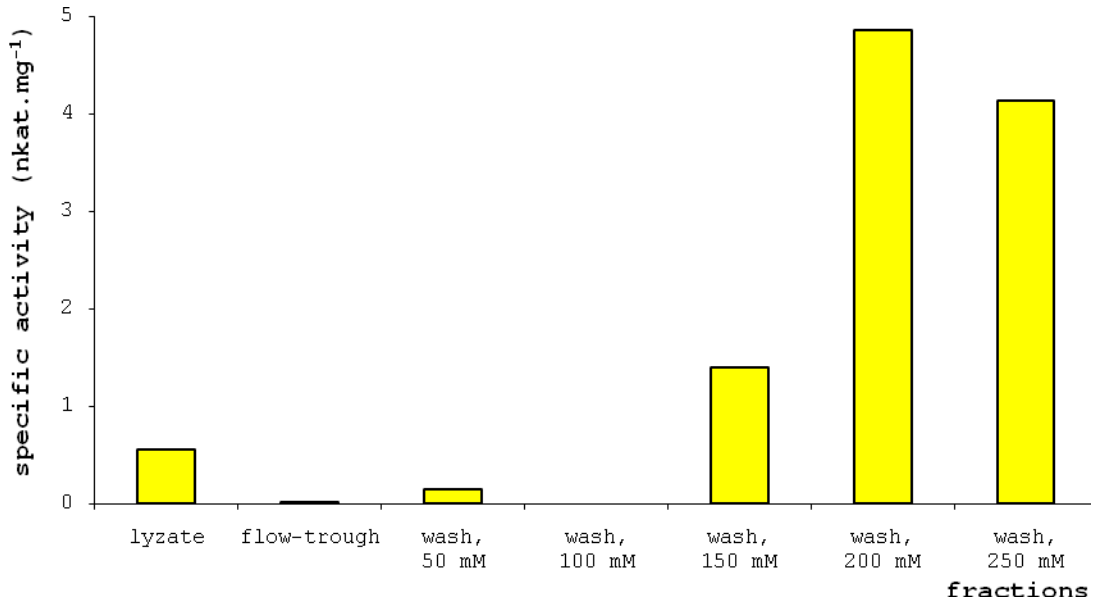

Fig. 20: A comparison of specific activity values for *E. coli* lyzate and fractions from IDA Sepharose. Imidazole concentration in the elution buffers (20 mM MOPS buffer, pH 7.0, containing 5% (v/v) glycerol, 100 mM NaCl and 50-250 mM imidazole) is indicated.

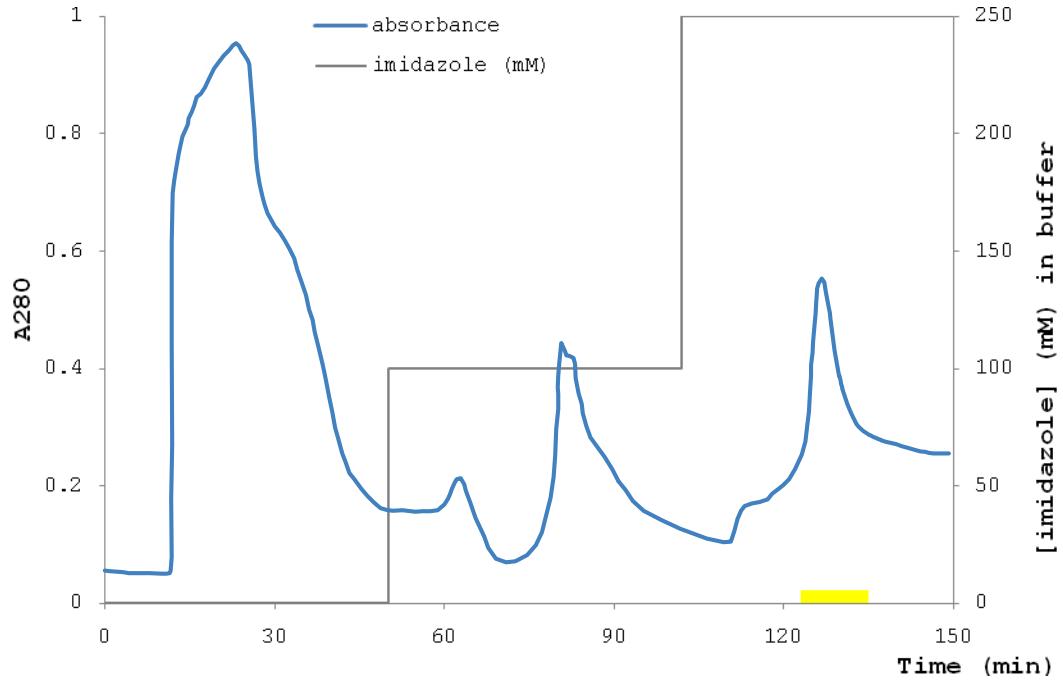

Fig. 21: The optimized PsOAT purification on IDA Sepharose column. PsOAT activity in fractions is indicated by the yelow box.

Initial unsuccesful attempts to obtain a homogeneous enzyme by immobilized metal ion affinity chromatography resulted in testing other possibilities of a conventional chromatographic separation (Fig. 22-25). These were abandoned upon introduction of the optimized protocol of affinity separation on IDA Sepharose. The final enzyme preparation was checked by SDS-PAGE giving a molecular mass estimate of 50 kDa. Gel permeation chromatography on a Bio-Silect SEC 125-5 column provided the same value and thus confirmed a monomeric character of the enzyme (Fig. 24). The identity of PsOAT was confirmed by peptide mass fingerprinting (21 matched peptides; MOWSE score: 156; sequence coverage: 54). Finally, an accurate molecular mass value of 51,988 Da was measured on the MALDI-TOF mass spectrometer (Fig. 26).

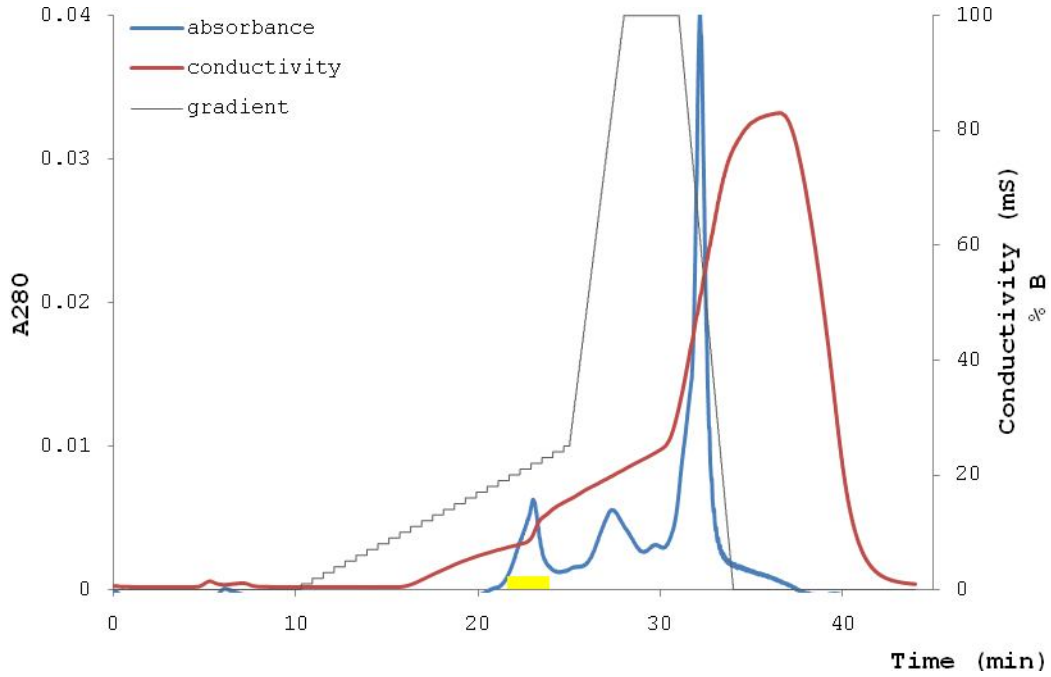

Fig. 22: PsOAT purification on Resource Q column. The yellow box indicates a major PsOAT elution fraction 1 (21-24 min). Other collected fractions were as follows: fraction 2, 25-28.5 min; fraction 3, 28.5-30.5 min, fraction 4, 30.5-34 min; fraction 5, 34-38 min.

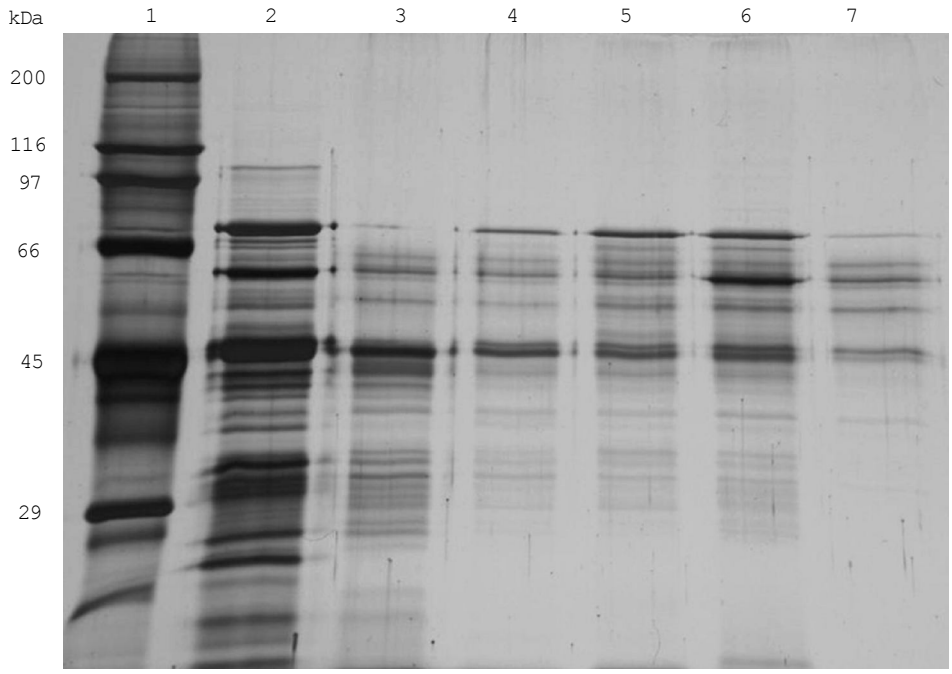

Fig. 23: Separation of fractions from the Resource Q column by SDS-PAGE. Lane 1, protein molecular mass markers (Sigma-Aldrich Chemie); lane 2, eluate from IDA-Sepharose; lane 3, fraction 1; lane 4, fraction 2; lane 5, fraction 3; lane 6, fraction 4; lane 7, fraction 5 (see Fig. 22 for fraction numbering).

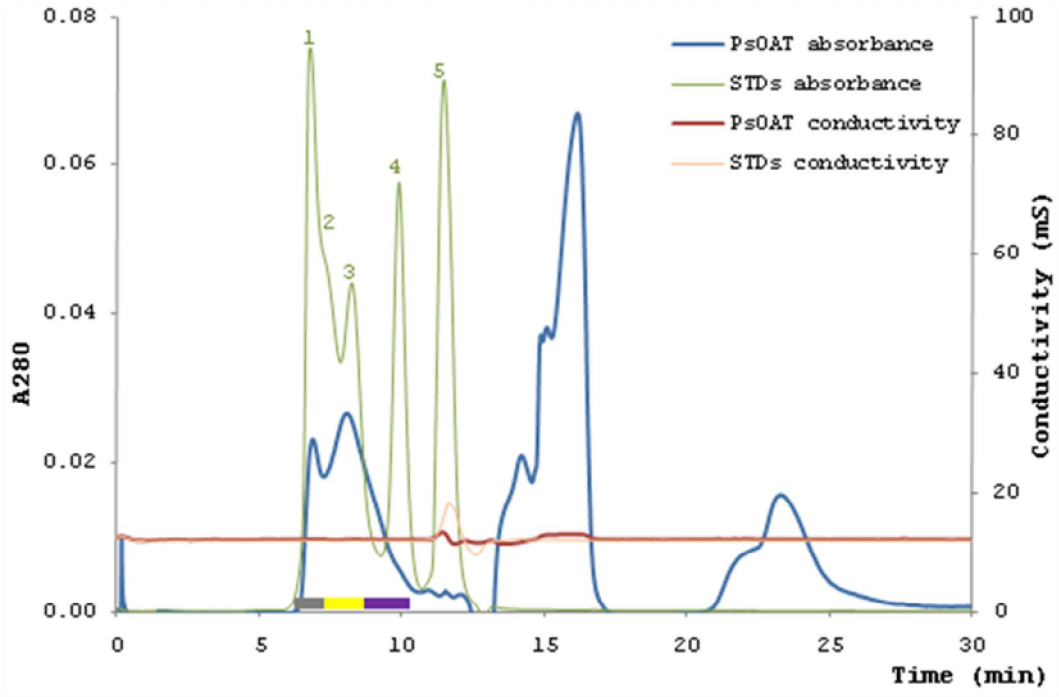

Fig. 24: Determination of PsOAT molecular mass on Bio-Silect SEC 125-5 column. The grey, yellow or violet boxes indicate protein fractions 1, 2 or 3, respectively, used for SDS-PAGE, in-gel digestion and subsequent MALDI-TOF mass spectrometric analysis. Peak numbering refers to gel molecular weight markers (Bio-Rad): 1, thyroglobulin (bovine), 670,000 Da; 2, γ-globulin (bovine) 158,000 Da; 3, ovalbumin (chicken), 44,000 Da; 4, myoglobin (horse), 17,000 Da; 5, vitamin B_{12}, 1,350 Da.

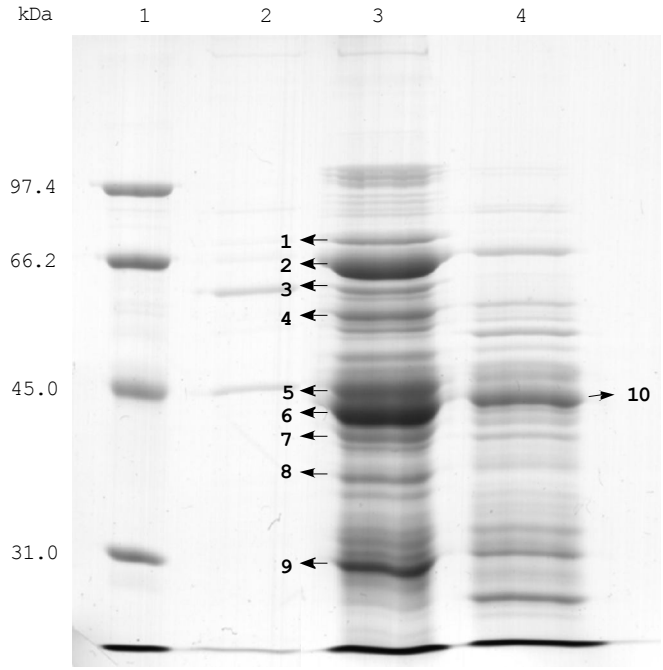

Fig. 25: SDS-PAGE separation of fractions from gel chromatography on Bio-Silect SEC 125-5 column. Lane 1, protein molecular mass markers (Bio-Rad); lane 2, fraction 1 (grey); lane 3, fraction 2 (yellow); lane 4, fraction 3 (violet); see fig. 24 for fraction numbering. Protein bands indicated by arrows were analyzed by MALDI-TOF peptide mass fingerprinting after in-gel digestion. The presence of PsOAT was confirmed in the bands No. 5 and 10. Proteins from the other bands came from *E. coli*.

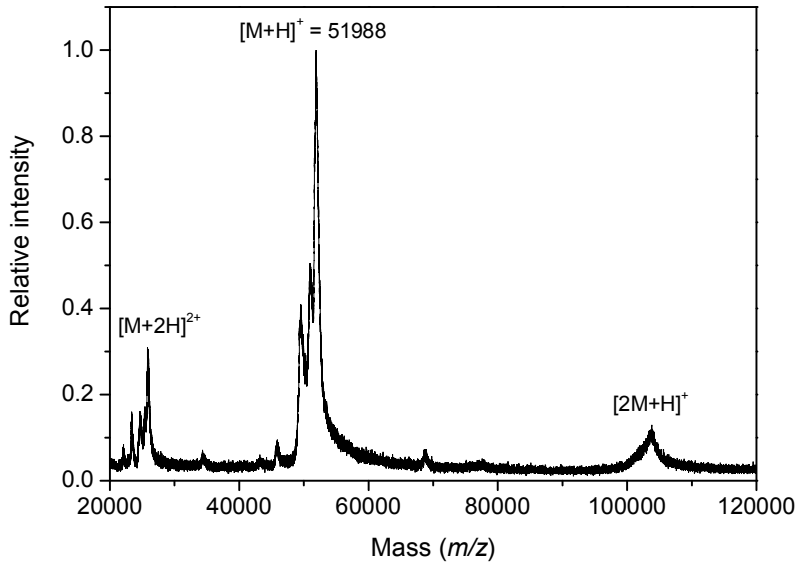

Fig. 26: Determination of PsOAT molecular mass by mass spectrometry.

Kinetic properties of recombinant PsOAT such as thermostability, pH optimum, optimum temperature for L-ornithine conversion and inhibition constants for the interaction with selected inhibitors were determined. The inhibition constants were determined by plotting experimental data on Lineweaver-Burk graphs. Some of the graphs are shown in Fig. 27-30.

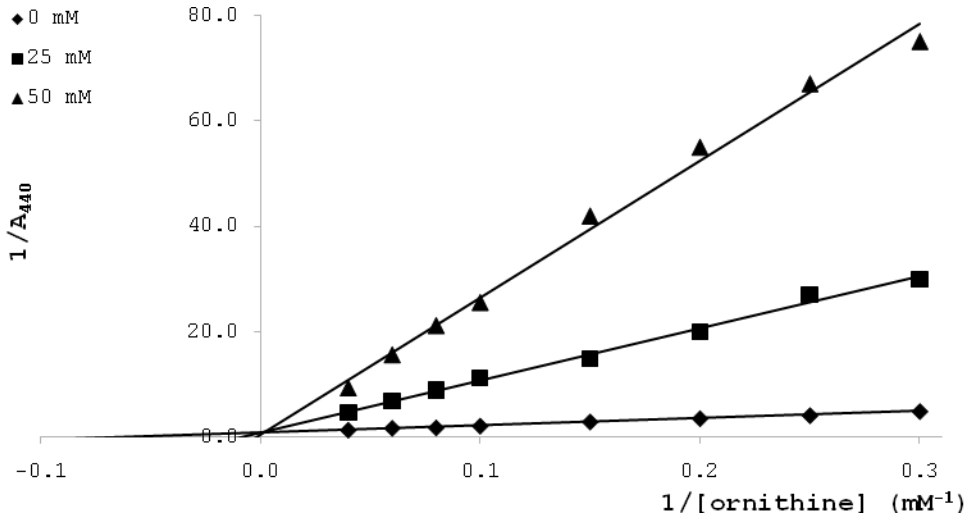

Fig. 27: A double reciprocal plot of competitive inhibition of PsOAT by norvaline. Ihibitor concentrations are shown in the legend. The corresponding secondary plot of slopes against the inhibitor concentrations (not shown) provided a K_i value of 13 mM.

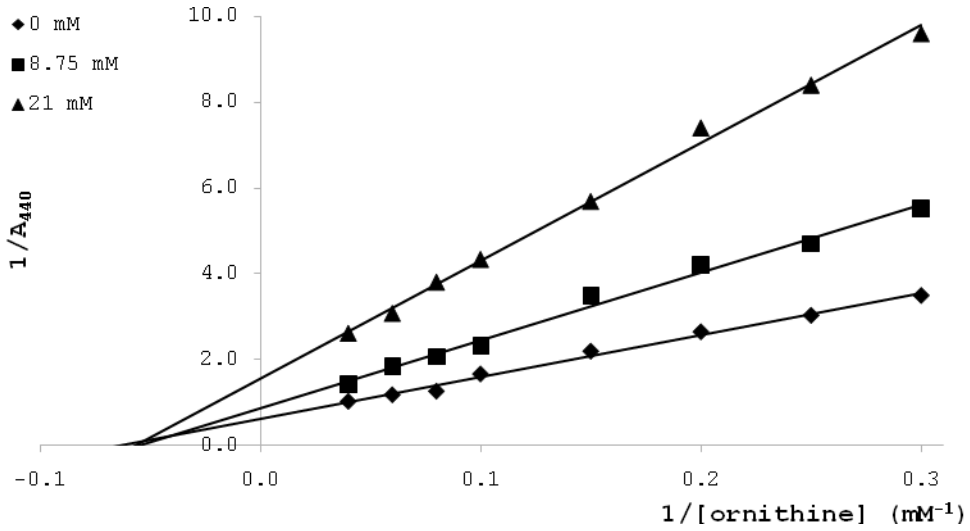

Fig. 28: A double reciprocal plot of non-competitive inhibition of PsOAT by spermine. The inhibitor concentrations are shown in the legend. The corresponding secondary plot of slopes against the inhibitor concentrations (not shown) provided a K_i value of 17 mM.

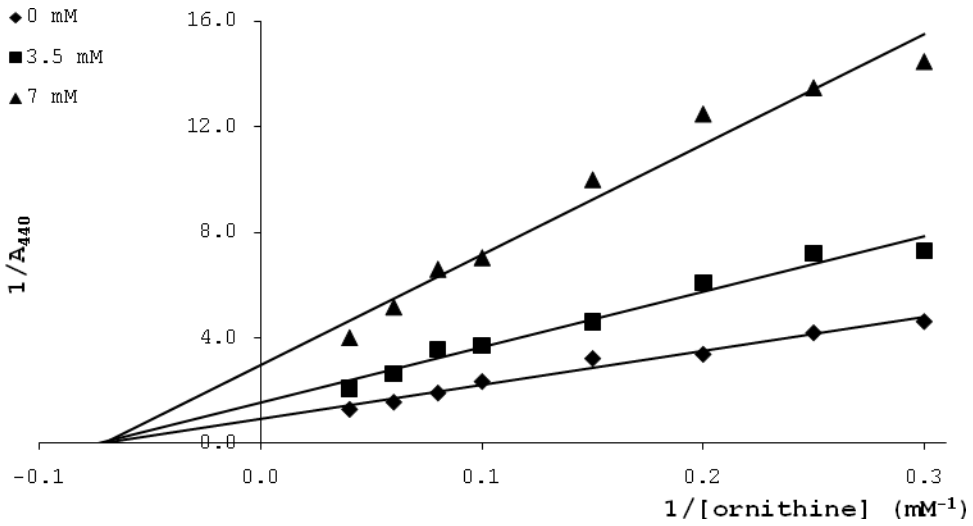

Fig. 29: A double reciprocal plot of non-competitive inhibition of PsOAT by diethylenetriamine. The inhibitor concentrations are shown in the legend. The corresponding secondary plot of slopes against the inhibitor concentrations (not shown) provided a K_i value of 3 mM.

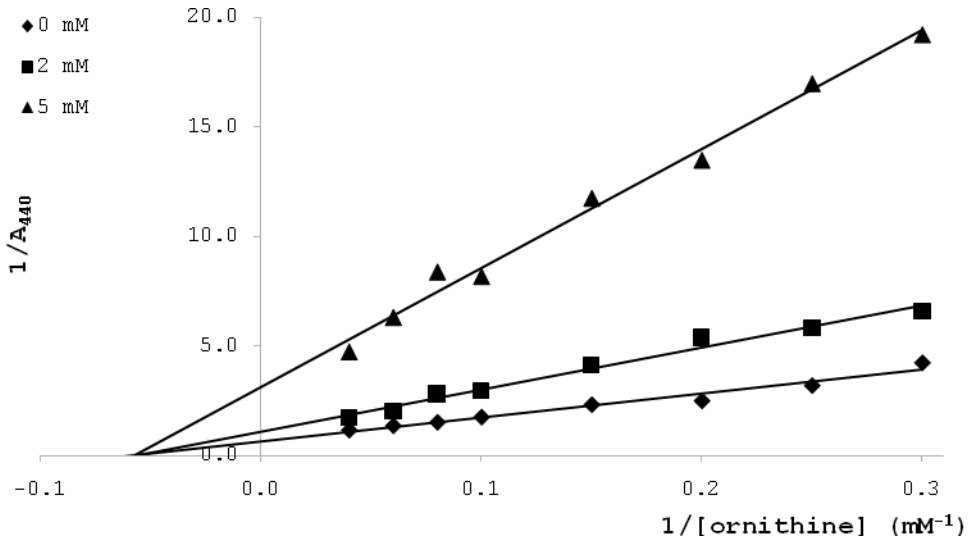

Fig. 30: A double reciprocal plot of non-competitive inhibition of PsOAT by triethylenetetramine. The inhibitor concentrations are shown in the legend. The corresponding secondary plot of slopes against the inhibitor concentrations (not shown) provided a K_i value of 0.8 mM.

The lyzate, flow-through and eluate fraction from HIS-Select® Cobalt Affinity Gel were separated by SDS-PAGE (Fig. 31). Selected bands were excised from the gel slab and in-gel digested by modified trypsin. Digest samples were further purified using C_{18} StageTips. All samples were analyzed on a Q-ToF Micro mass spectrometer coupled to a capillary liquid chromatography system. Collected raw data were processed with Mascot Distiller 2.2 software (Matrix Science). MGF-formatted files

were generated containing a list of precursors and their fragments. Protein identification was performed with Mascot server 2.2 (Matrix Science) (Fig. 32-33).

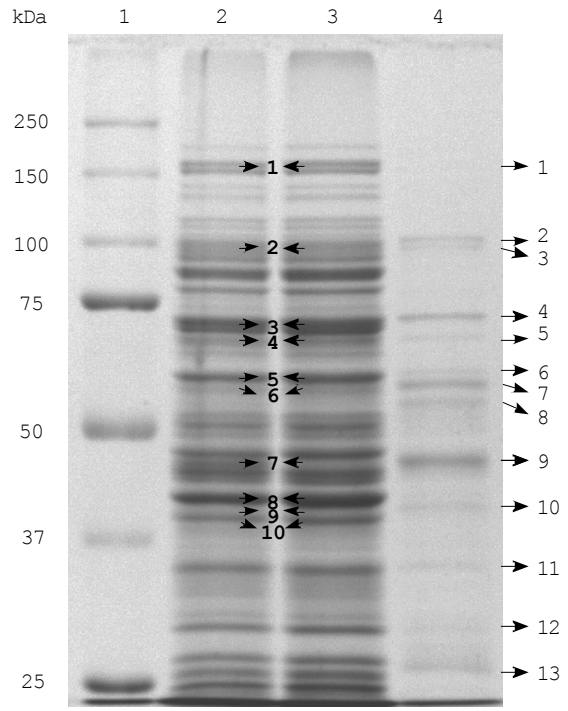

Fig. 31: SDS-PAGE separation of fractions from HIS Select® Cobalt Affinity Gel for LC-MS/MS analysis. Lane 1, protein molecular mass markers (Bio-Rad); lane 2, lyzate; lane 3, flow-through fraction; lane 4, eluate fraction containing PsOAT. Protein bands indicated by arrows were excised from the gel and digested by modified trypsin. Digest samples were analyzed by LC-MS/MS. All proteins were assigned in the NCBInr database (version June 16, 2008) to *E. coli* proteins except for the bands 4-9 (eluate fraction) and 2-7 (lyzate fraction) at a molecular mass position of around 50 kDa that contained PsOAT (annotated as gi|167047943).

Match to: **gi|167047943** Score: **554**
ornithine aminotransferase [Pisum sativum]
Found in search of D:\MascotDistiller_mgf\QT_000422.mgf

Nominal mass (M_r): **51664**; Calculated pI value: **7.90**
NCBI BLAST search of gi|167047943 against nr
Unformatted sequence string for pasting into other applications

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Deamidated (NQ), Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **39%**

Matched peptides shown in **Bold Red**

```
     1 MAATRQVQCL MRRVCRGTRT FAVATQSNAS SSSQTIIDKE HQHSAHNYHP
    51 LPIVFAHAKG SSVWDPEGNK YIDFLSGYSA VNQGHCHPKI LKALHDQADR
   101 LTVSSRAFYN DRFPVFAEYL TALFGYDMVL PMNTGAEGVE TALKLARKWG
   151 YEKKKIPNDE ALIVSCCGCF NGRTLGVISM SCDNEATRGF GPLMPGHLKV
   201 DFGDAEAIER IFKEKGDRVA AFILEPIQGE AGVVIPPDGY LKAVRDLCSK
   251 YNVLMIADEI QTGLARTGKM LACDWEDVRP DVVILGKALG GGILPVSAVL
   301 ADKDVMLCIK PGQHGSTFGG NPLASAVAIA ALEVIKEERL TERSTKLGGE
   351 LLGLLHKIQK KHPEHVKEVR GKGLFIGVEL NSESLSPVSG FELSEKLKER
   401 GVLAKSTHDT IIRFTPPLCI SADEIQQGSK ALAEVLEIDL PLLKKTKPKD
   451 AVPLAGPSPC DRCGRLVYG
```

Start-End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
20-39	1028.66	2055.31	2055.01	0.30	0	R.TFAVATQSNASSSSQTIIDK.E (Ions score 42)
60-70	588.38	1174.75	1174.53	0.22	0	K.GSSVWDPEGNK.Y (Ions score 48)
71-89	731.84	2192.50	2192.01	0.49	0	K.YIDFLSGYSAVNQGHCHPK.I (Ions score 19)
107-112	393.26	784.50	784.35	0.15	0	R.AFYNDR.F (Ions score 21)
174-188	835.55	1669.09	1668.74	0.35	0	R.TLGVISMSCDNEATR.G Oxidation (M) (Ions score 41)
189-199	577.35	1152.68	1152.61	0.07	0	R.GFGPLMPGHLK.V (Ions score 43)
189-199	585.43	1168.84	1168.61	0.23	0	R.GFGPLMPGHLK.V Oxidation (M) (Ions score 45)
200-210	611.40	1220.78	1220.57	0.21	0	K.VDFGDAEAIER.I (Ions score 79)
219-242	832.96	2495.85	2496.35	-0.50	0	R.VAAFILEPIQGEAGVVIPPDGYLK.A Deamidated (NQ) (Ions score 35)
251-266	912.16	1822.30	1821.93	0.37	0	K.YNVLMIADEIQTGLAR.T Oxidation (M) (Ions score 44)
270-287	1066.75	2131.48	2131.04	0.44	0	K.MLACDWEDVRPDVVILGK.A Oxidation (M) (Ions score 29)
288-303	741.07	1480.12	1479.87	0.25	0	K.ALGGGILPVSAVLADK.D (Ions score 41)
347-357	575.47	1148.92	1148.69	0.23	0	K.LGGELLGLLHK.I (Ions score 81)
406-413	471.84	941.67	941.49	0.18	0	K.STHDTIIR.F (Ions score 40)

Fig. 32: Identification of peptides from the band 4-9. The corresponding Mascot search result is shown.

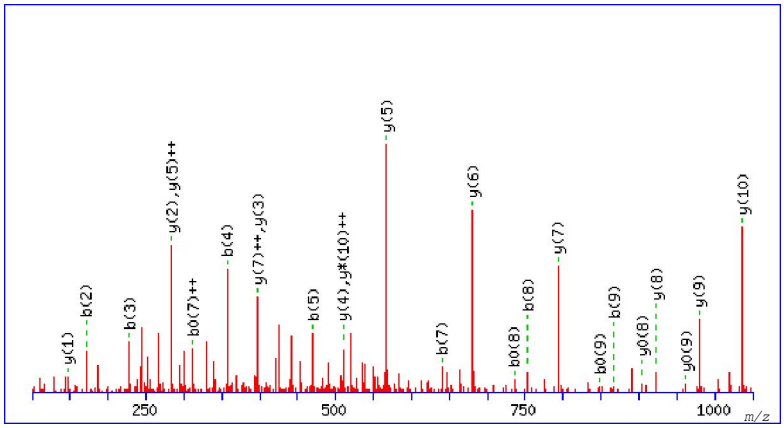

Fig. 33: Fragmentation of the peptide VDFGDAEAIER by MS/MS.

3.3 Supplement 2: Biochemical characterization of pea ornithine δ-aminotransferase: substrate specificity and inhibition by di- and polyamines.

Stránská J., Tylichová M., Kopečný D., Snégaroff J., Šebela M.

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Research paper

Biochemical characterization of pea ornithine-δ-aminotransferase: Substrate specificity and inhibition by di- and polyamines

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ABSTRACT

Ornithine-δ-aminotransferase (OAT, EC 2.6.1.13) catalyzes the transamination of L-ornithine to L-glutamate-γ-semialdehyde. The physiological role of OAT in plants is not yet well understood. It is probably related to arginine catabolism resulting in glutamate but the enzyme has also been associated with stress-induced proline biosynthesis. We investigated the enzyme from pea (PsOAT) to assess whether diamines and polyamines may serve as substrates or they show inhibitory properties. First, a cDNA coding for PsOAT was cloned and expressed in *Escherichia coli* to obtain a recombinant protein with a C-terminal 6xHis tag. Recombinant PsOAT was purified under native conditions by immobilized metal affinity chromatography and its molecular and kinetic properties were characterized. Protein identity was confirmed by peptide mass fingerprinting after proteolytic digestion. The purified PsOAT existed as a monomer of 50 kDa and showed typical spectral properties of enzymes containing pyridoxal-5′-phosphate as a prosthetic group. The cofactor content of PsOAT was estimated to be 0.9 mol per mol of the monomer by a spectrophotometric analysis with phenylhydrazine. L-Ornithine was the best substrate ($K_m = 15$ mM) but PsOAT also slowly converted N_α-acetyl-L-ornithine. In these reactions, 2-oxoglutarate was the exclusive amino group acceptor ($K_m = 2$ mM). The enzyme had a basic optimal pH of 8.8 and displayed relatively high thermal optimum. Diamines and polyamines were not accepted as substrates. On the other hand, putrescine, spermidine and others represented weak non-competitive inhibitors. A model of the molecular structure of PsOAT was obtained using the crystal structure of human OAT as a template.

1. Introduction

Ornithine-δ-aminotransferase or transaminase (L-ornithine:2-oxoacid aminotransferase; EC 2.6.1.13; OAT) is a nuclear-encoded, pyridoxal-5′-phosphate (PLP)-dependent enzyme found in the mitochondrial matrix of most human and animal tissues (with small amounts of activity in the soluble fraction). It also exists in microorganisms, insects and plants [1]. Transamination of L-ornithine in higher plants was reported 50 years ago [2]. Later on, plant OATs were partially purified for example from peanut [3] or squash [4]. Recombinant plant OATs from mothbean [5] and Scots pine [6] were subjected to a biochemical characterization only recently. Interestingly, in the mushroom *Agaricus bisporus*, the enzyme is probably localized in the cytoplasm as its sequence lacks the mitochondrial targeting motif [7]. The N-terminal extension is also absent in the enzymes from the Saccharomycetaceae family [1,8] (Fig. 1).

OAT catalyzes the reversible transfer of the δ-amino group of L-ornithine to 2-oxoglutarate producing L-glutamate-γ-semialdehyde and L-glutamate [12]. The semialdehyde is in equilibrium with its chemically more stable cyclic form Δ^1-pyrroline-5-carboxylate (P5C), which seems to be converted primarily to glutamate by P5C dehydrogenase in plants [13]. In some cases, the amino acceptor 2-oxoglutarate may be replaced with pyruvate or glyoxylate resulting in a decreased catalytic efficiency [4,14]. Besides L-ornithine, certain OATs accept N_α-acetyl-L-ornithine as a substrate [15]. Ornithine or 4-aminobutyrate (GABA) analogs like 5-fluoromethylornithine, canaline, gabaculine and 4-aminohex-5-ynoic acid are known as potent OAT inhibitors [12]. Carbonyl reagents and several amino acids also provide a significant inhibition, which can be reversed by the addition of PLP [16]. Similar to

Abbreviations: EST, expressed sequence tag; IDA, iminodiacetic acid; IPTG, isopropyl β-D-1-thiogalactopyranoside; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; OAT, ornithine-δ-aminotransferase; P5C, Δ^1-pyrroline-5-carboxylate; PLP, pyridoxal-5′-phosphate; PsOAT, *Pisum sativum* (pea) ornithine-δ-aminotransferase.

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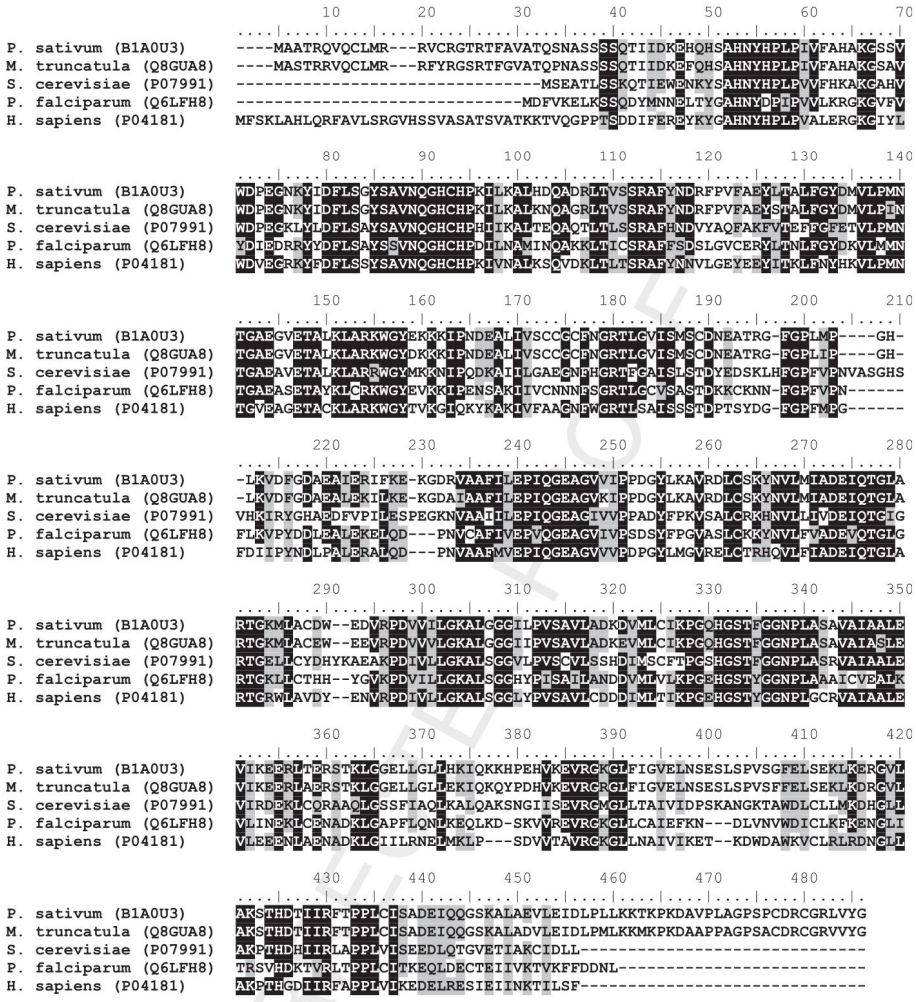

Fig. 1. Amino acid sequence homology among OATs from various organisms. The multiple sequence alignment of the enzymes from pea (*Pisum sativum*), this work, barrel medic (*Medicago truncatula*), baker's yeast (*Saccharomyces cerevisiae*), *Plasmodium falciparum* and human being (*Homo sapiens*) was performed by means of CLUSTAL W [9]; graphic view was obtained using BioEdit 7.0.9.0 [10] based on the similarity matrix BLOSUM 62 [11]. The accession codes in parentheses refer to the database UniProtKB (http://www.uniprot.org). In black, identical amino acids; in gray, similar amino acids.

other PLP-dependent aminotransferases, the reaction proceeds via a ping-pong mechanism comprising two half-reactions per one cycle of transamination [12]. There are several crystal structures of human OAT available [12,17–19] together with that of *Plasmodium yoelii* OAT [20].

Mammalian OAT is expressed mainly in the liver, kidney and intestine [21]. The human enzyme has attracted a big scientific interest since the discovery of its association with gyrate atrophy, a recessive hereditary genetic disorder leading to progressive loss of vision and eventually blindness in humans. Gyrate atrophy is characterized by elevated ornithine levels arising from OAT dysfunction [22]. In mammals, the role of OAT in providing glutamate for glutamine synthesis is well known, which allows for the detoxification of ammonia released by protein degradation [23]. In plants, contradictory results have been published as regards to the physiological function, which thus remains not well understood. The enzyme has been associated frequently with the increased proline accumulation observed during salinity stress and water deficit [24]. Conversely, Funck et al. demonstrated that proline accumulation under salinity stress was not affected in *Arabidopsis* mutants lacking OAT expression. Instead, it seems that plant OAT is essential for arginine catabolism, which yields glutamate as an end product. In this way, arginine-stored or transported nitrogen is recovered for further metabolic utilization [13]. Recent results with Scots pine support a role of OAT in the provision of glutamate to glutamine biosynthesis during the process of germination [6].

In this work, the enzyme from pea (*Pisum sativum*; PsOAT) was studied to evaluate possible reactions with di- and polyamines, which might be expected to occur based on a sequence homology with bacterial diamine and polyamine aminotransferases [1]. Moreover, *Escherichia coli* polyamine aminotransferase has previously been shown to accept L-ornithine as a weak substrate [25]. *PsOAT* cDNA of 1409 bp (EMBL/GenBank accession number EU414030) was cloned and expressed in *E. coli* to achieve

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a recombinant protein His-tagged at C-terminus. After affinity purification, biochemical properties of the purified enzyme including molecular properties, substrate specificity, pH optimum, thermostability and inhibitors were characterized. The enzyme was found to be strictly specific for L-ornithine. Although a series of di- and polyamines has been tested, none of the compounds served as a substrate but they inhibited the enzyme in a non-competitive manner.

2. Materials and methods

2.1. Chemicals

2-Aminobenzaldehyde; N_{α}-acetyl-L-ornithine; 1,3-diaminopropane dihydrochloride; hydrochlorides of L- and D-ornithine; iminodiacetic acid Sepharose® (IDA-Sepharose 6B), lysozyme; 2-(*N*-morpholino)ethanesulfonic acid (MES); 3-(*N*-morpholino)propanesulfonic acid (MOPS); L-norvaline; norspermidine; putrescine dihydrochloride; pyridoxal-5′-phosphate hydrate, spermidine trihydrochloride and spermine tetrahydrochloride were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Diethylenetriamine and triethylenetetramine tetrahydrochloride were from the same supplier; the former amine was converted to a trihydrochloride salt using a solution of hydrogen chloride in 2-propanol. Chemicals for electrophoresis were from Bio-Rad (Hercules, CA, USA); chemicals for MALDI mass spectrometry were from Bruker Daltonik (Bremen, Germany). Restriction enzymes were from New England BioLabs (Ipswich, MN, USA). AccuPrime™ *Pfx* DNA polymerase with a proofreading 3′ to 5′ exonuclease activity was from Invitrogen (Carlsbad, CA, USA). All other substances were of analytical purity grade or molecular biology grade.

2.2. Cloning and expression

Degenerated and nondegenerated oligonucleotide primers were designed in highly conserved areas deduced from database deposited nucleotide sequences and expressed sequence tags (ESTs) belonging to OATs of plant origin. Total RNA was isolated from 10-day-old etiolated pea (*Pisum sativum* L., cv. Lantra) seedlings using the NucleoSpin® RNA Plant kit from Macherey-Nagel (Düren, Germany). The complete cDNA was obtained using 3′ RACE and 5′ RACE Systems and AccuPrime™ *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Finally, it was cloned into a pCR-Blunt plasmid (Invitrogen) and sequenced.

PsOAT ORF (1410 bp) was then cloned into a pET28b+ expression vector (Novagen, La Jolla, CA, USA) digested by NcoI and XhoI restriction endonucleases. Thus, primers used for amplification of *PsOAT* cDNA contained NcoI (upstream primer: 5′-TCA T**CC ATG G**CT GCC ACA CGA CA-3′) and XhoI (downstream primer: 5′-GTG **CTC GAG** ACC ATA CAC GAG TCG ACC AC-3′) restriction sites (in bold). The construct was then introduced by heat shock into NEB T7 Express Competent *E. coli* cells (New England Biolabs, Hitchin, UK). Transformed competent cells were precultured in Luria-Bertani (LB) medium containing 1% glucose and kanamycin (50 μg ml^{-1}) at 37 °C overnight. Then PsOAT expression was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when OD_{600} reached a value of 0.6. After overnight incubation at 25 °C, cells were harvested by centrifugation at 5000 × *g* for 15 min and stored frozen.

2.3. Enzyme purification

The harvested *E. coli* cells (1 g) were resuspended in 1 ml of Bacterial Protein Extraction Reagent (B-PER, Thermo Scientific, Rockford, IL, USA) and then in 10 ml of a lysis buffer containing 50 mM MOPS, pH 7.0, 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatine A and 10 mM imidazole. After addition of lysozyme (0.3 mg ml^{-1}), the mixture was incubated at 37 °C for 30 min and then supplemented to contain 1% (w/v) Triton X-100, 100 mM NaCl, 5% (w/v) glycerol, RNase A (0.01 μg ml^{-1}) and DNase 1 (0.004 U ml^{-1}). Finally, after a 30-min incubation at 37 °C, the lyzate was centrifuged at 9000 × *g* for 20 min and the supernatant containing recombinant PsOAT was recovered. The enzyme solution was loaded onto Ni(II)-charged IDA-Sepharose 6B in a glass column (2.5 cm i.d. × 25 cm) previously equilibrated with 20 mM MOPS buffer, pH 7.0, containing 100 mM NaCl, 10 mM imidazole and 5% (v/v) glycerol. At a flow rate of 2 ml min^{-1}, the column was washed with the equilibration buffer. Weakly bound proteins were eluted with the same buffer containing 100 mM imidazole. Elution of PsOAT was achieved using an additionally increased concentration of imidazole to 250 mM. The active fraction was desalted by a diafiltration using 10-kDa cutoff Centricon filters (Millipore, Bedford, MA, USA) and stored frozen at −50 °C in 20 mM MOPS buffer, pH 7.0, containing 5% (v/v) glycerol.

2.4. Activity and protein assay

PsOAT activity was assayed using a modified spectrophotometric method [26]. This approach is based on the production of a yellow colored adduct by incubation of the Δ^1-pyrroline-5-carboxylate rising from PsOAT reaction with 2-aminobenzaldehyde (ABA). The reaction mixture in a test tube contained 50 mM Tris–HCl buffer, pH 8.0, 35 mM ornithine, 5 mM 2-oxoglutarate and 0.05 mM PLP. The assay was started by the addition of PsOAT in a total reaction volume of 1 ml. After incubation at 37 °C for 20 min, the reaction was stopped by adding 0.25 ml of 3 M perchloric acid. Later on, 0.25 ml of 0.5% (w/v) ABA in 96% ethanol was added into the reaction mixture for color development by incubating at 37 °C for 10 min. On centrifugation, the supernatant was measured at 440 nm. Protein content was determined routinely by Bradford's spectrophotometric method with BSA as a standard [27] or for the pure enzyme from UV absorption at 280 nm using a theoretical ε_{280} value of 32,150 M^{-1} cm^{-1} deduced from the amino acid sequence [28].

2.5. Characterization of kinetic properties

Thermostability of recombinant PsOAT was evaluated by activity measurements after incubation of enzyme samples at temperatures from 20 °C up to 75 °C (in 5 °C increments) for 30 min. The residual activity was measured with 35 mM L-ornithine as a substrate. To determine the pH optimum, 50 mM MES buffers (pH 6.0–7.0), 50 mM MOPS buffers (pH 6.5–8.0) and 50 mM sodium carbonate/bicarbonate buffers (pH 8.8–10.6) were used. The optimum temperature for L-ornithine conversion was obtained by activity assays at temperatures from 20 up to 52 °C. Kinetic parameters of substrates and inhibitors were characterized by measuring Lineweaver–Burk plots.

2.6. Cofactor analyses and SDS-PAGE

Absorption spectra of recombinant PsOAT and PLP were recorded on a DU7500 spectrophotometer (Beckman, Fullerton, CA, USA). Fluorescence spectra were acquired on an Aminco Bowman Series 2 spectrofluorimeter (Thermo Electron Corporation, Madison, WI, USA) at 23 °C. Spectrophotometric determination of the active-site cofactor was performed according to a published procedure [29]. Enzyme samples were brought to 0.2% (w/v) phenylhydrazine and 0.5 M H_2SO_4 [30], incubated for 30 min at 37 °C, and centrifuged to remove precipitated protein. The concentration of the released PLP was determined by absorbance at 410 nm; PLP

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solutions in the range of 0.2–5 μM were used for calibration [29]. SDS-PAGE was performed using 4% stacking and 10% resolving polyacrylamide gel according to a standard procedure [31]. Proteins were visualized by the Bio-Safe Coomassie Stain (Bio-Rad), gels were scanned by the computer program LabScan 5.00 (GE Healthcare, Uppsala, Sweden) and processed for images by Paint Shop Pro 8.00 (Jasc Software, Minnetonka, MN, USA).

2.7. MALDI-TOF peptide mass fingerprinting

Bands containing recombinant PsOAT (5 pmol) were excised from Bio-Safe Coomassie-stained SDS-PAGE gels. MALDI-TOF peptide mass fingerprinting after in-gel digestion by modified trypsin was conducted as described previously [32]. The instrument used was a Microflex LRF20 MALDI-TOF mass spectrometer (Bruker Daltonik) equipped with a microScout ion source and a 337-nm nitrogen laser and operating in the reflectron mode for positive ions. The acquired spectra were processed by flexAnalysis 2.4 and Biotools 3.2 software (Bruker Daltonik). Database searches were performed against Swiss-Prot (release 57.12) and NCBInr (release December 15, 2009) databases using the program Mascot Server 2.2 (Matrix Science, London, UK). As variables, oxidation of methionine and carbamidomethylation of cysteine plus one missed cleavage were chosen for all searches performed without taxonomic restriction; a mass tolerance of 150 ppm was allowed.

2.8. Molecular mass determination

Gel permeation chromatography was performed on a Bio-Silect SEC 125-5 column (Bio-Rad) connected to a BioLogic Duo Flow liquid chromatograph (Bio-Rad). The column was equilibrated and run with 50 mM potassium phosphate buffer, pH 7.0, containing 150 mM NaCl at a flow rate of 1 ml min^{-1}. The molecular mass of recombinant PsOAT was evaluated after calibration of the column with protein standards (1.35, 17, 44, 158 and 670 kDa) purchased from Bio-Rad. MALDI-TOF analysis for intact mass determination was performed on the Microflex LRF20 instrument (Bruker). Prior to analysis, enzyme samples were dialyzed against 25 mM ammonium bicarbonate and concentrated by ultrafiltration in centrifugation cartridges with a cut off of 10-kDa to a concentration of 10 mg ml^{-1}. Then 1 μl of sample was mixed with 3 μl of matrix (10 mg ml^{-1} sinapinic acid in 0.1% TFA/acetonitrile, 1:1, v/v). Aliquots (1 μl) were placed onto the target plate previously modified by a thin layer of sinapinic acid [33] and allowed to dry. Spectra were acquired in the linear mode for positive ions and calibrated externally using molecular ions of BSA.

2.9. Molecular modeling

A homology-based molecular modeling of pea seedling OAT was performed with human OAT as a template (PDB accession no. 1OAT) [18] and PsOAT sequence obtained in this work (UniProtKB/Swiss-Prot accession no. B1A0U3) using the program SWISS-MODEL (SIB-Biozentrum Basel, http://swissmodel.expasy.org/) [34]. The software used for picture drawing was PyMol 1.2 (DeLano Scientific LLC, http://www.pymol.org/).

3. Results and discussion

Aminotransferases (EC 2.6.1) represent a large group of PLP-dependent enzymes. They are ubiquitous in nature and play an important role in cellular metabolism of nitrogen. Because of properties that are considered advantageous from the point of view of industry like enantioselectivity and regioselectivity, substrate specificity and high reaction rate and stability, the enzymes have become useful in the production of various amino compounds, which are valuable intermediates or precursors for chiral drugs and agricultural products [35,36].

The present study deals with PsOAT as a representative of plant enzymes catalyzing the conversion of L-ornithine to L-glutamate-γ-semialdehyde. In general, OATs (EC 2.6.1.13) show a large structural and mechanistic similarity to other enzymes from the subgroup III of aminotransferases, which transfer an amino group from a carbon atom that does not carry a carboxyl function: ω-amino acid aminotransferase (2.6.1.18), 4-aminobutyrate aminotransferase (2.6.1.19), diamine aminotransferase (EC 2.6.1.29) etc. [1,19,37]. These aminotransferases usually show a broad substrate specificity. *E. coli* diamine aminotransferase was found to convert L-ornithine slowly in addition to its preferential action on putrescine, cadaverine and spermidine [25]. There are reports on the occurrence of a diamine aminotransferase activity in plants [38,39]. However, no such an enzyme has been purified and studied so far. Thus, we reasoned that PsOAT, as a homologous and presumably similar enzyme, might accept some diamines or polyamines as substrates in a non-oxidative reaction that would proceed in parallel with their oxidative conversion by amine oxidases. It is worthmentioning that diamine and polyamine levels in etiolated pea seedlings reach high levels of hundreds to several thousands nmoles per gram of fresh weight [40]. Interestingly, a weak inhibition of OAT by diamines was reported previously for the enzyme from rat liver [14].

Our first attempts to obtain PsOAT relied on its isolation from 500 g of 5-day-old etiolated pea seedlings (Moskalíková H. and Stránská J., unpublished results). The seedlings were homogenized in 50 mM MOPS buffer, pH 7.4, containing 5 mM 2-mercaptoethanol [41]. After ammonium sulfate precipitation, the enzyme solution was subjected to several steps of protein liquid chromatography involving ion exchange and gel permeation columns. The final enzyme had a specific activity of 30 pkat mg^{-1}, an enrichment factor of 43 and a yield of 51%. However, this procedure was time consuming and ineffective in providing enough material for further biochemical characterization. Moreover, the enzyme preparation still contained many impurities and thus PsOAT represented only a minor fraction. At this point, a decision was made to perform recombinant expression.

Cloning experiments were based on PCR amplifications with primers designed according to an alignment of nucleotide sequences of plant *OAT* genes deposited in public databases. Combinations of RACE and nested PCR runs followed by DNA sequencing allowed completing the whole cDNA sequence coding for PsOAT. This sequence has been deposited in the EMBL/GenBank database under the accession no. EU414030. On the DNA level, the *PsOAT* sequence shows the largest homology with sequences annotated as *Medicago truncatula OAT* (GenBank AJ278819; 80% identity) and *Glycine max OAT* (GenBank DQ224372; 74% identity), but it is also homologous with *OAT* sequences of microbial or animal origin. A multiple amino acid sequence alignment of the enzyme from pea and several other OATs is shown in Fig. 1. Recombinant expression of PsOAT with a His-tag at the C-terminus was achieved using pET28b+ vector. The cultivation conditions for growing of the transformed *E. coli* cells were optimized from a previous protocol [42]. Only the cultivation temperature after inducing expression by IPTG was increased to 25 °C. Table 1 shows a typical purification scheme from a culture volume of 400 ml. For protein extraction from the harvested cells, 50 mM MOPS buffer, pH 7.0, was found to be empirically better than 50 mM Bis-Tris–HCl buffer, pH 7.0, or 50 mM Tris–HCl, pH 8.0, containing the same additives. The enzyme was purified from lyzate by a single-step affinity chromatography on Ni(II)-charged IDA-Sepharose 6B with a specific elution by increased imidazole concentration. The final enzyme

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preparation had a specific activity of 26.2 nkat mg^{-1}, an enrichment factor of 7.7 and a yield of 46.8% (Table 1). The total amount of pure protein per one purification round was usually about 10 mg, much more than a yield of several hundred micrograms for the partially purified native enzyme.

Purity of the final enzyme preparation was checked by SDS-PAGE giving a molecular mass estimate of 50 kDa (Fig. 2) in agreement with other plant OATs [4–6]. Parallel experiments using gel permeation chromatography on a Bio-Silect SEC 125-5 column provided the same value and thus confirmed a monomeric character of the enzyme. Finally, an accurate molecular mass value of 51,988 Da was measured on a MALDI-TOF mass spectrometer (data not shown; the relative standard deviation from ten measurements was 0.03%). MALDI-TOF peptide mass fingerprinting after in-gel digestion of PsOAT using trypsin (Fig. 3) allowed unambiguous identification by searching the obtained list of peptide masses against NCBInr protein database. The enzyme was assigned to the accession no. gi|167047943 when 44 peptides matched the sequence providing a high coverage value of 77%. The corresponding probability-based score was 320.

Besides the determinations of molecular mass, recombinant PsOAT was also analyzed for other molecular properties including spectroscopic features and protein-to-cofactor stoichiometry. The final enzyme preparation was yellow colored due to the presence of PLP as a cofactor. Spectroscopic features of recombinant PsOAT were evaluated by measuring absorption and fluorescence spectra. An absorption spectrum of the purified enzyme is shown in Fig. 4A. In addition to a typical protein maximum of PsOAT at 280 nm ($\varepsilon_{280} = 31{,}500\ M^{-1}\ cm^{-1}$), there was another absorption band observed with a maximum centered at 420 nm ($\varepsilon_{420} = 4500\ M^{-1}\ cm^{-1}$), which was accompanied by a shoulder at 330 nm ($\varepsilon_{330} = 6150\ M^{-1}\ cm^{-1}$). The experimental value of the extinction coefficient ε_{280} is in agreement with a theoretical value of 32,150 $M^{-1}\ cm^{-1}$ calculated from the protein sequence [28]. Moreover, the observed ratios A_{280}/A_{420} and A_{330}/A_{420} (7.0 and 1.4, respectively) correspond with other PLP-containing enzymes [43,44]. The 420-nm absorption species is generally attributed to the ketoenamine form of the internal aldimine of PLP [45]. The assignment of the 330-nm absorption species is rather ambiguous, as several potential structures might have the same absorption, including an aldimine form in which the pyridinium nitrogen is deprotonated [45,46]. Free cofactor dissolved in water provided a major maximum at 390 nm ($\varepsilon_{290} = 2900\ M^{-1}\ cm^{-1}$) with a shoulder at 327 nm and a minor maximum at 298 nm (not shown). PLP in solution represents an equilibrium mixture of several different ionic and tautomeric structures with unique absorption properties [47]. The major maximum is attributed to the dipolar ionic aldehyde where N1 is protonated and O3′ is negative [48].

Fluorescence properties of PsOAT can be considered common when compared with those of other PLP-containing enzymes [49,50]. Due to the presence of bound PLP, resting oxidized PsOAT provided an emission spectrum with a maximum at 500 nm when excited at 420 nm (Fig. 4B), and had an emission peak at 392 nm when excited at 330 nm. When the enzyme was analyzed for emission at 390 nm and 500 nm, the excitation spectra exhibited maxima at 330 and 420 nm, respectively, corresponding to the absorption maxima of the holoenzyme. Spectrophotometric analysis of the cofactor with a phenylhydrazine reagent after its release from the active site [29,30] demonstrated by reading the absorbance at 410 nm that there was 0.9 mol PLP per 1 mol of the monomeric PsOAT. Direct spectrophotometric titrations of the bound cofactor via stepwise additions of aliquots of a phenylhydrazine solution were unsuccessful.

Substrates and inhibitors of recombinant PsOAT, which have been studied in this work, are summarized in Table 2. The enzyme

Table 1
Purification of recombinant PsOAT expressed in *Escherichia coli*.

Purification step	Total volume (ml)	Total activity (nkat)	Protein content (mg)	Specific activity (nkat mg^{-1})	Yield (%)	Enrichment factor
Lyzate	28	560	167	3.4	100	1
Affinity chromatography	55	262	10	26.2	46.8	7.7

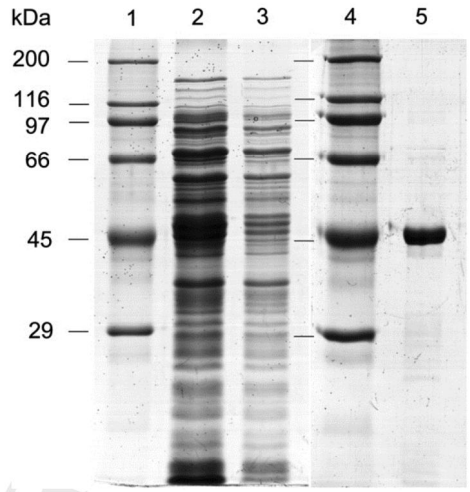

Fig. 2. SDS-PAGE of recombinant PsOAT. SDS-PAGE was performed using Laemmli's discontinuous buffer system [31] and then the separated proteins were visualized by Bio-Safe Coomassie Stain (Bio-Rad). Lane 1, protein molecular mass markers (from the top 200, 116, 97, 66, 45 and 29 kDa; Sigma-Aldrich Chemie); lane 2, *E. coli* lyzate (26 μg of protein); lane 3, the flow-through fraction from the affinity chromatography lacking OAT activity (9 μg of protein); lane 4, protein molecular mass markers (as in lane 1; 29–200 kDa); lane 5, pure recombinant OAT eluted from Ni(II)-charged IDA-Sepharose 6B (2–μg of protein) referring to a molecular mass estimate of 50 kDa.

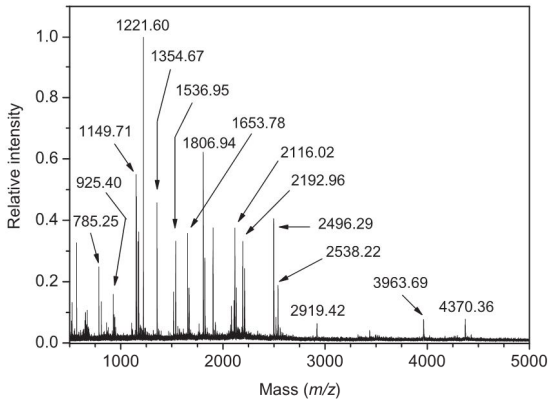

Fig. 3. MALDI-TOF peptide mass fingerprinting of recombinant PsOAT. The enzyme was in-gel digested with modified trypsin after prior reduction/alkylation. The presented mass spectrum of peptides from the digest was acquired on the Microflex LRF-20 instrument operating in the reflectron mode for positive ions. α-Cyano-4-hydroxycinnamic acid was used as a matrix. The fingerprint spectrum was assigned to the accession no. gi|167047943 in the NCBInr protein database.

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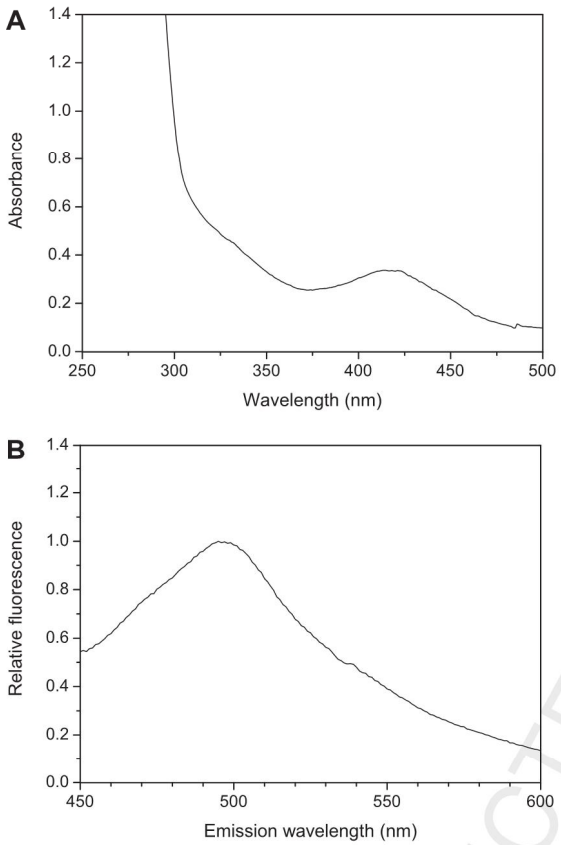

Fig. 4. Absorption and fluorescence emission spectra of recombinant PsOAT. (A) An absorption spectrum of PsOAT. The spectrum was obtained on a Beckman DU7500 spectrophotometer using 75 μM enzyme in 20 mM MOPS, pH 7.0. (B) A fluorescence emission spectrum recorded upon excitation at 420 nm of a sample of PsOAT (20 μM) in 20 mM MOPS, pH 7.0. The instrument used was an Aminco Bowman Series 2 spectrofluorimeter (23 °C).

showed a narrow substrate specificity as it readily accepted only L-ornithine as an amino group donor. PsOAT reaction with this compound was characterized by a high K_m value of 15 mM (under saturation with 2-oxoglutarate). The ornithine transamination by

Table 2
Substrates and inhibitors of recombinant PsOAT. For substrates (S) and inhibitors (I), the measured K_m or K_i values, respectively, are given; inhibition types: C, competitive; NC, non-competitive.

Compound	Substrate or inhibitor	K_m or K_i (mM)	Inhibition type
L-Ornithine	S	15[a]	–
N_α-Acetyl-L-ornithine	S	–[b]	–
L-Norvaline	I	7	C
Diethylenetriamine	I	3	NC
Triethylenetetramine	I	0.8	NC
Norspermidine	I	13	NC
Spermine	I	17	NC
Spermidine	I	25	NC
1,3-Diaminopropane	I	35	NC
Putrescine	I	90	NC

[a] $V_{max} = 60.4$ nmol s^{-1} mg^{-1}; $k_{cat} = 4.3$ s^{-1}; $k_{cat}/K_m = 286.7$ M^{-1} s^{-1}.
[b] The relative reaction rate towards L-ornithine is only 3%.

PsOAT was further characterized by the following kinetic parameters: $V_{max} = 60.4$ nmol s^{-1} mg^{-1}, $k_{cat} = 4.3$ s^{-1} and $k_{cat}/K_m = 286.7$ M^{-1} s^{-1}. As regards the observed k_{cat} value, it is in good order-of-magnitude agreement with other aminotransferases from the subgroup III [51,52]. Under saturation with L-ornithine, the amino group acceptor 2-oxoglutare provided a K_m value of 2 mM. Pyruvate and oxaloacetate do not seem to be alternative amino group acceptors. Their relative efficiency towards 2-oxoglutarate in the reaction with L-ornithine was negligible reaching scarcely 1–2%. The results are in accordance with a majority of previous observations made on other OATs [1]. For example, an overexpressed human OAT showed K_m values for L-ornithine and 2-oxoglutarate of 15 and 3 mM, respectively [53]. In the case of the squash cotyledon OAT, the K_m for L-ornithine was 4.7 mM and for 2-oxoglutarate 6.3 mM [4]. N_α-Acetyl-L-ornithine was a very weak substrate of PsOAT. Its relative conversion rate at a concentration of 30 mM was only 3% of that for L-ornithine. As expected, no activity was detected with D-ornithine, L-lysine, γ-aminobutyrate and ε-aminocaproate. Also the following diamine and polyamine compounds were ineffective as amino group donors: 1,3-diaminopropane; putrescine; 2-hydroxyputrescine; 2,3-dihydroxyputrescine; cadaverine; norspermidine, spermidine and spermine. This finding clearly disproved our previous speculations that PsOAT could act on diamines and polyamines.

Fig. 5A shows a graph used for the determination of pH optimum of the enzyme. PsOAT displayed the highest rate with L-ornithine at a basic pH of 8.8. This corresponds with basic pH optima of other enzymes from this group [5,54] and is probably related to the presence of a lysyl residue at the active site (see further). The enzyme had a temperature optimum for L-ornithine transamination at 43 °C (Fig. 5B). In addition, its thermal stability was relatively high as the T_{50} value, a temperature at which 50% of the activity was retained upon 30-min incubation, was found to be 58 °C (Fig. 5C). Various compounds were tested as inhibitors of the enzyme. Carbonyl reagents like phenylhydrazine or hydroxylamine, which are typical inactivators of PLP enzymes, showed considerable inhibition effects on PsOAT only at millimolar concentrations. We determined inhibition parameters (from Lineweaver–Burk plots) for several amino compounds at a fixed 2-oxoglutarate concentration of 5 mM (Table 2). From this group, only the amino acid L-norvaline inhibited competitively with a millimolar-range inhibition constant. The polyamines norspermidine, spermidine ((Fig. 5D) and spermine and the diamines 1,3-diaminopropane and putrescine were found to be non-competitive inhibitors with K_i values of the same concentration level like the Michaelis constant for L-ornithine. Diethylenetriamine and triethylenetetramine inhibited PsOAT non-competitively with significantly lower K_i values of 10^{-3} M. Putrescine and cadaverine were previously mentioned as weak inhibitors of the enzymes from rat and *Bacillus sphaericus* with no comment on the inhibition type and inhibition constant [14,16]. Polyamines appear up to millimolar concentrations in mitochondria and they are known to be involved as regulatory compounds in important mitochondrial processes like transport of calcium ions, mitochondrial permeability transition, free radical scavenging, apoptosis-related mitochondrial pathways (cytochrome *c* release) etc. [55,56]. They modulate activity of certain mitochondrial enzymes, e.g. ATPase, pyruvate dehydrogenase complex, glutaminase, endonuclease G [57–60]. It seems that increased polyamines could significantly reduce the activity of plant OAT *in vivo*, which would result in slowing down arginine catabolism.

The amino acid sequences of PsOAT (UniProtKB/Swiss-Prot accession no. B1A0U3, this work) and human OAT (UniProtKB/Swiss-Prot accession no. P04181) [61] show a large homology with 47.5% identity and 61.6% similarity. Therefore, a molecular model of

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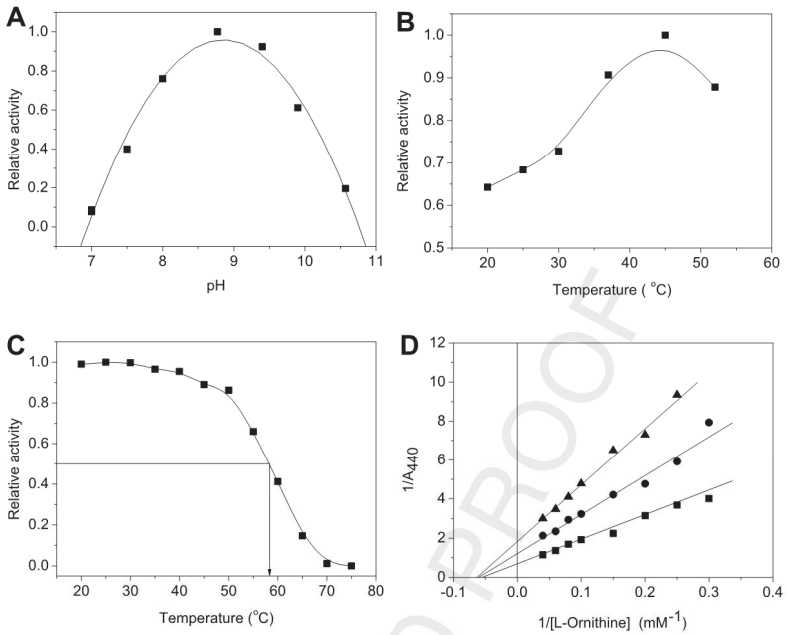

Fig. 5. Kinetic properties of recombinant PsOAT. In all cases, enzyme activity was assayed using the aminobenzaldehyde method with L-ornithine and 2-oxoglutarate. (A) Determination of pH optimum using MES, MOPS and carbonate/bicarbonate buffers (see Section 2). (B) Determination of temperature optimum; PsOAT activity was assayed at temperatures from 20 up to 52 °C in 50 mM Tris–HCl buffer, pH 8.0. (C) Thermostability of recombinant PsOAT; the enzyme was incubated at temperatures from 20 °C up to 75 °C for 30 min. After fast cooling of the samples in ice-cold water, they were assayed for residual activity at 37 °C. An arrow indicates the corresponding T_{50} value of 58 °C. (D) A double reciprocal plot of non-competitive inhibition of PsOAT by spermidine (fixed 2-oxoglutarate concentration); the inhibitor concentrations used were as follows: (■) 0 mM, (●) 20 mM, and (▲) 40 mM. A secondary plot of slopes against the inhibitor concentrations (not shown) provided a K_i value of 25 mM.

PsOAT was constructed using SWISS-MODEL program [34] operating in an automated mode. Root mean square deviation (RMSD) between the final model and human OAT (PDB accession code 1OAT) is 0.47 Å for 398 Cα atoms as calculated using SUPERPOSE from CCP4 6:1.2 program suite (http://www.ccp4.ac.uk). The modeled structure is thus almost identical to the template. Fig. 6 shows the overall topology of this model (residues 29–436), which contains 12 α-helices and 14 β-strands. The monomer structure consists of a large cofactor domain, a C-terminal small domain and an N-terminal region that contains an α-helix, a loop and a three-stranded antiparallel β-meander. The large domain contains a typical α/β fold and bears the covalent linkage of the cofactor PLP to Lys287. The small domain comprises an antiparallel β-sheet with accompanying α-helices. The three-stranded β-meander of the N-terminal region lies between the large and small domains and forms a roof of the active-site pocket like it appears in the template human OAT structure [18].

The active site arrangement of the PsOAT model corresponds with that of human OAT (Fig. 7). In the human enzyme [18], the cofactor PLP is covalently bound through a Schiff base linkage to the ε-amino group of Lys292 (Fig. 7A). The N1 atom and hydroxyl group of the pyridine ring are hydrogen bonded to Asp263 and Gln266. The non-ester phosphate oxygens of the cofactor are hydrogen bonded to the main-chain nitrogens of Gly142 and Val143. The side chain of Ile265 supports the cofactor pyridine ring from behind. In the dimer of human OAT, additional hydrogen bonds are donated by the side chain hydroxyls of Ser321* and Thr322* from the second subunit (not shown). The substrate binding site is located above Phe177 in front of Lys292 (Fig. 7A) and it is further flanked by aromatic residues Tyr85 and Tyr55. The latter residue and Arg180 donate the specific contacts for L-ornithine binding at the active site [17] via the α-carboxylate and α-amino group. In the PsOAT model

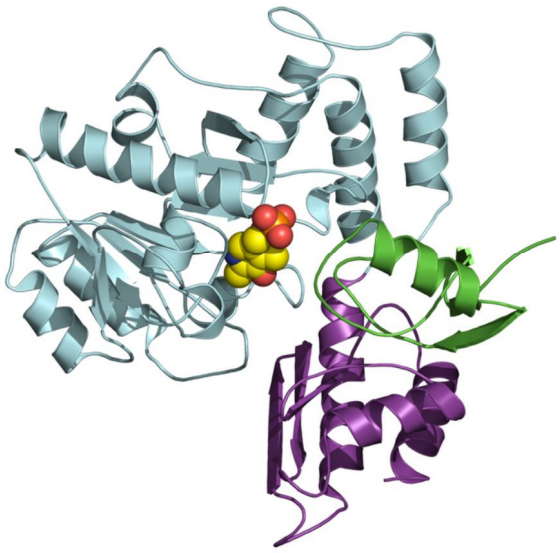

Fig. 6. PsOAT structure modeling. To demonstrate the global fold of PsOAT, a homology-based molecular model of the monomer is shown. The modeling was performed with PsOAT amino acid sequence (UniProtKB/Swiss-Prot accession no. B1A0U3) and the crystal structure of human OAT (PDB accession no. 1OAT) [18] as a template using the program SWISS-MODEL [34] operating in an automated mode (SIB-Biozentrum Basel, http://swissmodel.expasy.org). The N-terminal region is depicted in green, the large domain in pale blue and the small domain in violet. The cofactor PLP is shown in sphere representation with atom-coded colors. The picture was drawn using PyMol 1.2 (DeLano Scientific LLC, http://www.pymol.org).

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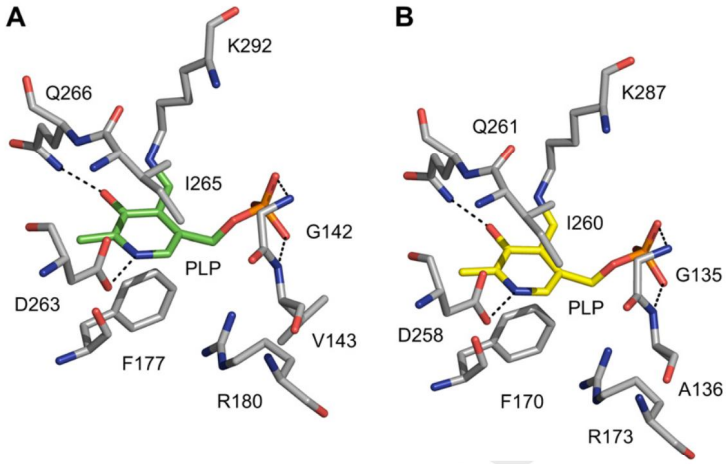

Fig. 7. A comparison of the active site arrangement of human OAT and PsOAT. The PLP cofactor carbon atoms are shown in (A) green (human OAT) or (B) yellow (PsOAT). Carbon atoms in adjacent amino acid residues are in gray. Other atoms are depicted using the following colors: nitrogen, blue; oxygen, red; phosphorus, orange. The picture was drawn using PyMol 1.2 (DeLano Scientific LLC, http://www.pymol.org).

(Fig. 7B), a similar configuration of the active site is observed. The cofactor PLP is covalently attached to Lys287. It interacts through hydrogen bonding with Asp258, Gln261, Gly135 and Ala136 and it is further supported by Ile260. The substrate binding pocket comprises conserved residues Phe170, Tyr48 and Tyr78 and thus it is analogous to that of the human OAT. In addition, Arg173 represents a counterpart of Arg180 from the human enzyme. Based on the model structure, it is clear that cationic polyamines cannot interact with Arg173 in such a way to become oriented properly with respect to the formation of the reactive Schiff base with PLP cofactor.

4. Conclusions

In this work, a cDNA coding for pea ornithine-δ-aminotransferase was obtained by PCR amplification and sequenced for the first time. The enzyme was prepared as a recombinant protein by heterologous expression in *E. coli*, purified to homogeneity and subjected to a biochemical characterization including molecular properties and enzyme kinetics. The latter measurements were intended especially for evaluation of possible reactions with di- and polyamines. PsOAT reaction with the compounds might be expected to occur based on a sequence homology of the enzyme with bacterial diamine and polyamine aminotransferases. It has been shown that the enzyme is strictly specific for L-ornithine and it does not accept di- and polyamines as substrates. Interestingly, the compounds were found non-competitive inhibitors. It seems that increased polyamines could significantly inhibit plant OAT *in vivo*, which would result in slowing down the process of arginine catabolism.

Acknowledgments

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4 DISCUSSION

The presence of OAT was discovered in plant species more than 50 years ago, namely in spinach (*Spinacia oleracea*; Scher & Vogel, 1957) and mungbean (*Phaseolus aureus*; Bone, 1959). In the following two decades, OAT was partially purified for example from peanut (*Arachis hypogea*; Mazelis & Fowden, 1969), pumpkin (*Cucurbita maxima*; Splittstoesser & Fowden, 1973), squash (*Cucurbita pepo*; Lu & Mazelis, 1975) and pea (*Pisum sativum*; Ruitier & Kollöffel, 1983). The highest activity level was detected during germination of seeds.

According to our previous results (Moskalíková H., Stránská J., unpublished), we preferred pea as a research subject to bean or mung bean, which showed lower OAT activity in initial experiments. For the analysis, 5-days-old etiolated pea seedlings were used. Proteins were extracted using acetone powder (Lu & Mazelis, 1975) and precipitated by 70 %-saturated ammonium sulfate. The crude enzyme was desalted by dialysis and partially purified by protein liquid chromatography on SP-Sepharose FF, Superdex 200 HR 10/30, HEMA Bio 1000 SB and Mono S HR 5/5 columns i.e. by a combination of gel permeation and ion exchange chromatography. The final enzyme preparation had a specific activity of 30 pkat.mg^{-1}, an enrichment factor of 43 and a yield of 51 %. The sample was separated by SDS-PAGE. Visualized protein bands were in-gel digested by trypsin. Peptides from the digests were characterized by MALDI-TOF mass spectrometry involving post-source decay analysis. Unfortunately, the amino acid sequence of pea OAT was not available that time and the complete pea genome has not been sequenced so far. Not surprisingly, any of the analyzed proteins was identified as PsOAT but there were some other pea proteins found. Tryptic digestion of a protein band with a molecular mass of 50 kDa (in agreement with other plant OATs; Delauney et al., 1993; Lu & Mazelis, 1975) provided a mass spectrum with sufficient intensity of several peptides. A peptide with *m/z* value of 1503.77 was analyzed by MALDI-TOF post-source decay and the formation of b- and y-type-fragment ions was observed. Reading distances in the individual fragment ion series yielded a sequence tag SEI(L)SS. A similar motif occurs in the complete sequences of *M. truncatula* and *A. thaliana* OATs (Fig. 34). This allowed a conclusion that the analyzed protein might be pea OAT.

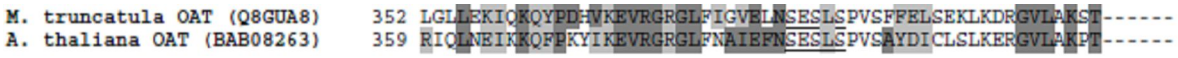

Fig. 34: A segment of the primary structures of OATs from *M. truncatula* and *A. thaliana* showing the presence of a sequence homologous to the tryptic peptide from *Pisum sativum* with *m/z* 1503.

However, the whole purification procedure was time consuming and ineffective in providing enough material for further biochemical characterization. Moreover, the

enzyme preparation still contained many impurities and thus OAT represented only a minor fraction. At this point, a decision was made to perform recombinant expression.

Cloning experiments were based on PCR amplifications with primers designed according to an alignment of nucleotide sequences of plant OAT genes deposited in public databases. Combinations of RACE and nested PCR runs followed by DNA sequencing allowed completing the whole cDNA sequence coding for OAT from *Pisum sativum* (PsOAT). This sequence has been deposited in the EMBL/GenBank database under the accession no. EU414030. On the DNA level, the *PsOAT* sequence shows the largest homology with sequences annotated as *Medicago truncatula OAT* (GenBank AJ278819; 80% identity) and *Glycine max OAT* (GenBank DQ224372; 74% identity), but it is also homologous with *OAT* sequences of microbial or animal origin. Recombinant expression of PsOAT with a His-tag at the C-terminus was achieved using pET28b(+) vector. The enzyme was purified from lyzate by a single-step affinity chromatography on Ni(II)-charged IDA-Sepharose 6B with a specific elution by increased imidazole concentration. The final enzyme preparation had a specific activity of 26.2 nkat.mg^{-1}, an enrichment factor of 7.7 and a yield of 46.8 %. The total amount of pure protein per one purification round (culture volume of 400 ml) was usually about 10 mg, much more than a yield of several hundred micrograms for the partially purified native enzyme (seedlings weight of 500 g). In comparison with the native enzyme (6 days of growth, 1 day of preparation of the acetone powder, 1 day of extraction and precipitation, several days of purification), the work with the recombinant OAT is much shorter and cheaper (3 days of *E. coli* cultivation, 1 day of extraction and purification); *E. coli* cells stored at -80°C are independent of the season and one preparation gives an amount of the enzyme, which is sufficient for several biochemical analyses.

Purity of the final enzyme preparation was checked by SDS-PAGE giving a molecular mass estimate of 50 kDa in agreement with other plant OATs (Lu & Mazelis, 1975; Sekhar et al., 2007; Cañas et al., 2008). Parallel experiments using gel permeation chromatography on a Bio-Silect SEC 125-5 column provided the same value and thus confirmed a monomeric character of the enzyme. Finally, an accurate molecular mass value of 51,988 Da was measured on a MALDI-TOF mass spectrometer. MALDI-TOF peptide mass fingerprinting after in-gel digestion of PsOAT using trypsin allowed unambiguous identification by searching the obtained list of peptide masses against NCBInr protein database. The enzyme was assigned to the accession no. gi|167047943 when 44 peptides matched the sequence providing a high coverage value of 77 %. The corresponding probability-based score was 320.

Besides the determinations of molecular mass, recombinant PsOAT was also analyzed for other molecular properties including spectroscopic features and protein-to-cofactor stoichiometry. The final enzyme preparation was yellow colored due to the presence of PLP as a cofactor. Spectroscopic features of recombinant PsOAT were evaluated by measuring absorption and fluorescence spectra. The experimental value of the extinction coefficient ε_{280} is in agreement with a theoretical value of 32,150 $M^{-1}.cm^{-1}$ calculated from the protein sequence (Pace et al., 1995). Fluorescence properties of PsOAT can be considered common when compared with those of other PLP-containing enzymes (Davies & Neuberger, 1979; Misono & Soda, 1977).

The present study deals with PsOAT as a representative of plant enzymes catalyzing the conversion of L-ornithine to L-glutamate-γ-semialdehyde. In general, OATs (EC 2.6.1.13) show a large structural and mechanistic similarity to other enzymes from the subgroup III of aminotransferases, which transfer an amino group from a carbon atom that does not carry a carboxyl function: ω-amino acid aminotransferase (2.6.1.18), 4-aminobutyrate aminotransferase (2.6.1.19), diamine aminotransferase (EC 2.6.1.29) etc. (Stránská et al., 2008; Markova et al., 2005; Hwang et al., 2005). These aminotransferases usually show broad substrate specificity. *E. coli* diamine aminotransferase was found to convert L-ornithine slowly in addition to its preferential action on putrescine, cadaverine and spermidine (Samsonova et al., 2003). There are reports on the occurrence of a diamine aminotransferase activity in plants (Hasse & Schmid, 1963; Wink & Hartmann, 1979). However, no such an enzyme has been purified and studied so far. Thus, we reasoned that PsOAT, as a homologous and presumably similar enzyme, might accept some diamines or polyamines as substrates in a non-oxidative reaction that would proceed in parallel with their oxidative conversion by amine oxidases. The enzyme showed a narrow substrate specificity as it readily accepted only L-ornithine as an amino group donor. PsOAT reaction with this compound was characterized by a high K_m value of 15 mM (under saturation with 2-oxoglutarate). The ornithine transamination by PsOAT was further characterized by the following kinetic parameters: V_{max} = 60.4 $nmol.s^{-1}.mg^{-1}$, k_{cat} = 4.3 s^{-1} and k_{cat}/K_m = 286.7 $M^{-1}.s^{-1}$. As regards the observed k_{cat} value, it is in good order-of-magnitude agreement with other aminotransferases from the subgroup III (Sandmark et al., 2004; Jeffery et al., 1988). Under saturation with L-ornithine, the amino group acceptor 2-oxoglutare provided a K_m value of 2 mM. Pyruvate and oxaloacetate do not seem to be alternative amino group acceptors. Their relative efficiency towards 2-oxoglutarate in the reaction with L-ornithine was negligible reaching scarcely 1-2 %. The results are in accordance with a majority of previous observations made on other OATs (Stránská et al., 2008). N_α-Acetyl-L-ornithine was a

very weak substrate of PsOAT. Its relative conversion rate at a concentration of 30 mM was only 3 % of that for L-ornithine. As expected, no activity was detected with D-ornithine, L-lysine, γ-aminobutyrate and ε-aminocaproate. Also the following diamine and polyamine compounds were ineffective as amino group donors: 1,3-diaminopropane; putrescine; 2-hydroxyputrescine; 2,3-dihydroxyputrescine; cadaverine; norspermidine, spermidine and spermine. Moreover, putrescine, spermidine and others represented weak non-competitive inhibitors. This finding disproved our speculations that PsOAT could act on diamines and polyamines.

Among other studied features, the pH optimum of PsOAT was determined. The enzyme displayed the highest rate with L-ornithine at a basic pH of 8.8. This corresponds with basic pH optima of other enzymes from this group (Ventura et al., 2009; Sekhar et al., 2007) and is probably related to the presence of a lysyl residue at the active site. The enzyme had a temperature optimum for L-ornithine transamination at 43 °C. In addition, its thermal stability was relatively high as the T_{50} value, a temperature at which 50 % of the activity was retained upon 30-min incubation, was found to be 58 °C.

Some of the analyzed kinetic properties of PsOAT are very interesting from the point of view of biochemistry (for example the relatively high thermal stability, very narrow substrate specificity and basic pH optima). We hereby propose to continue in studying PsOAT structure and function The aim is to obtain the crystal structure of the enzyme. Related experiments have already started and we hope in their progress. Another interesting goal involves site-directed mutagenesis of selected active site residues (based on homology modeling) to determine their contribution to the substrate specificity.

Aminotransferases (EC 2.6.1) represent a large group of PLP-dependent enzymes. They are ubiquitous in nature and play an important role in cellular metabolism of nitrogen. Because of properties that are considered advantageous from the point of view of industry like enantioselectivity and regioselectivity, substrate specificity and high reaction rate and stability, the enzymes have become useful in the production of various amino compounds, which are valuable intermediates or precursors for chiral drugs and agricultural products (Patel, 2000; Stewart, 2001). Human OAT has attracted a big scientific interest because of its association with gyrate atrophy (Seiler, 2000) and as a potential target for chemotherapeutic drug operating on mitotic cell division (Wang et al., 2007). In plants, OAT has been associated frequently with the increased proline accumulation observed during salinity stress and water deficit (Roosens et al., 1998) or arginine catabolism (Funck et al., 2008). Thus it could represent a potential target for genetic manipulations.

5 CONCLUSION

In this work, a native PsOAT was partially purified from extracts of etiolated pea seedlings. A cDNA coding for the enzyme was obtained by PCR amplification and sequenced for the first time. The enzyme was prepared as a recombinant protein by heterologous expression in *E. coli*, purified to homogeneity and subjected to a biochemical characterization including molecular properties and enzyme kinetics. The latter measurements were intended especially for evaluation of possible reactions with di- and polyamines. PsOAT reaction with the compounds might be expected to occur based on a sequence homology of the enzyme with bacterial diamine and polyamine aminotransferases. It has been shown that the enzyme is strictly specific for L-ornithine and it does not accept di- and polyamines as substrates. Interestingly, the compounds were found non-competitive inhibitors. It seems that increased polyamines could significantly inhibit plant OAT *in vivo*, which would result in slowing down the process of arginine catabolism.

List of abbreviations

ABA	2-aminobenzaldehyde
ACN	acetonitrile
Bis-Tris	bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane
bp	base pair
capLC	capillary liquid chromatography
DNase	deoxyribonuclease
EqB	equilibration buffer
ESI	electrospray ionization
EST	expressed sequence tag
FA	formic acid
FPLC	fast protein liquid chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
LC	liquid chromatography
MALDI-TOF	matrix assisted laser desorption/ionization-time of flight
β-ME	β-mercaptoethanol
MOPS	3-(*N*-morpholino)propanesulfonic acid
MOWSE	molecular weight search
MS	mass spectrometry
OAT	ornithine δ-aminotransferase
P5C	Δ^1-pyrroline-5-carboxylate
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PLP	pyridoxal-5‘-phosphate
PSD	post-source decay
PsOAT	ornithine δ-aminotransferase from *Pisum sativum*
QTOF	quadrupole time-of-flight
RACE	rapid amplification of cDNA ends
RNase	ribonuclease
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris	tris(hydroxymethyl)aminomethane

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