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Charakterizace a regulace šikimátdehydrogenasy z kořene petržele

Enzyme kinetics and regulation of shikimate dehydrogenase from parsley roots

Diplomová práce

Master’s thesis

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Prohlášení (declaration)

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně, pod vedením školitelky RNDr. Veroniky Hýskové, PhD., a že jsem všechny použité prameny řádně citovala.

V Praze dne Podpis:

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Abstrakt

Šikimátová dráha spolu s navazující fenylpropanoidní drahou v rostlinách poskytuje vedle aromatických aminokyselin také celou řadu sekundárních metabolitů. Významným enzymem je šikimátdehydrogenasa, která je v rostlinách součástí bifunkčního proteinu 3-dehydrochinátdehydratasy/šikimátdehydrogenasy (DHD/SDH; EC 4.2.1.10 a EC 1.1.1.25). Ačkoliv regulace šikimátové dráhy je poměrně komplexní záležitostí, o regulaci rostlinné SDH je k dispozici informací velice málo. Cílem tohoto projektu bylo proto najít vhodný rostlinný zdroj o vysoké aktivitě SDH a zjistit, zda a jakým způsobem je SDH regulována látkami fenylpropanoidního metabolismu, především jednoduchými polyfenoly.

Enzymový preparát SDH o výsledné specifické aktivitě 470 μmol.min^{-1}.mg^{-1} byl připraven 3-krokovou purifikací z kořene petržele (*Petroselinum crispum*). Detekcí aktivity tohoto enzymu v gelu po nativní elektroforéze a po isoelektrické fokusaci byla zjištěna jedna isoforma. Relativní molekulová hmotnost SDH kořene petržele byla pomocí gelové chromatografie stanovena 60 000 a pomocí červené nativní elektroforézy 63 000. Isoelektrický bod odpovídal hodnotě 4,5. Optimální pH reakce katalyzované SDH bylo zjištěno v rozmezí pH 9,5 – 10,0.

Pomocí studie počátečních rychlostí dvousubstrátové reakce byly stanoveny kinetické parametry (K_m, V_{max}) pro substrát i koenzym šikimátdehydrogenasy. K_m SDH pro šikimát byla 161 ± 9 µM a pro NADP$^+$ 54 ± 11 µM. Mechanismus této dvousubstrátové reakce je pravděpodobně sekvenční.

Celá řada látek fenylpropanoidního metabolismu ovlivňovala aktivitu SDH z kořene petržele, z nichž kyselina *p*-kumarová a kyselina *t*-ferulová byly studovány podrobnou inhibiční studií. Pro obě látky byl určen smíšený typ inhibice a příslušné inhibiční konstanty. $ZnSO_4$ a $CuSO_4$ snižovaly aktivitu SDH.

Klíčová slova

šikimátdehydrogenasa, fenylpropanoidy, kyselina kumarová, kyselina ferulová, inhibice, šikimátová dráha

Abstract

In plants the shikimate pathway followed by the phenylpropanoid pathway leads to the formation of not only aromatic amino acids but also a plenty of secondary metabolites. The crucial enzyme shikimate dehydrogenase is there a part of bi-functional protein 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHD/SDH; EC 4.2.1.10 and EC 1.1.1.25). Whereas the regulation of the shikimic pathway is complex, little is known about the feedback regulation of plant SDH. The aim of this project was to find plant source with high SDH activity and to find out, if and in which way this SDH is regulated by compounds of phenylpropanoid metabolism, specifically by simple polyphenols.

SDH from parsley root (*Petroselinum crispum*) has been prepared by 3-step purification to final specific activity 470 μmol.min^{-1}.mg^{-1}. The enzyme exhibited one band after both isoelectric focusing and red native electrophoresis using detection of the activity. Relative molecular mass of native enzyme was determined by gel chromatography (M_r 60 000) and red native electrophoresis (M_r 63 000). Isoelectric point pI 4.5 was established by isoelectric focusing. Optimal pH for the reaction catalyzed by SDH was determined in rage pH 9.5 – 10.0.

The kinetic parameters (K_m, V_{max}) of SDH for substrate shikimate, and coenzyme NADP$^+$ were determined by the initial velocity studies. K_m SDH for shikimate was 161 ± 9 µM and for NADP$^+$ 54 ± 11 µM. The mechanism of two-substrate reaction catalyzed by SDH was determined as sequential. A number of compounds of secondary metabolism affected parsley SDH, from which *p*-coumaric acid and *t*-ferulic acid were subjected to detailed inhibition study. A mixed-type of inhibition and respective inhibition constants were determined for both inhibitors. A significant decrease of SDH activity was observed in the presence of $ZnSO_4$ a $CuSO_4$.

Key words

shikimate dehydrogenase, phenylpropanoids, coumaric acid, ferulic acid, inhibition, shikimate pathway

Contents

1. List of abbreviations

2,4 - D	2,4 - dichlorophenoxyacetic acid
4-HPP	4-hydroxyphenylpyruvate
AAs	amino acids
AD(H)T	arogenate (dehydrogenase) dehydratase
BPB	bromphenol blue („tracking dye“)
C(M)S	chorismate (mutase) synthase
DAHPS	3-deoxy-7-phosphoheptulonate synthase
DHD	3-dehydroquinate dehydratase
DHQS	3-dehydroquinate synthase
E4P	erythose-4-phosphate
EPSP(S)	5-enolpyruvylshikimate 3-phosphate (synthase)
FA	ferulic acid
GA	gallic acid
HPP-AT	4-hydroxyphenylpyruvate aminotransferase
INT	iodonitroterazolium blue
pCMB	p-chloromercuribenzoate
PD(H)T	prephenate (dehydrogenase) dehydratase
PEP	phosphoenolpyruvate
$pH_{opt.}$	optimum pH
PMS	phenazine metosulfate
PPA(PPY)-AT	prephenate (phenylpyruvate) aminotransferase
PPY	phenylpyruvate
PVPP	polyvinylpolypyrrolidone
S3P	shikimate 3-phosphate
SDH	shikimate dehydrogenase
SK	shikimate kinase
SKA	shikimic acid/shikimate
STs	protein standards

2. Introduction

2.1. The shikimate pathway

The shikimate pathway was discovered as the biosynthetic route of three aromatic amino acids tyrosine (Tyr), tryptophan (Trp) and phenylalanine (Phe) through the studies of American biologists Bernhard Davis and David Sprinson and their collaborators in the early 1950´s. The precuror shikimic acid had originally been isolated from plants of *Illicium* species, the aniseed (Japanese “shikimi-no-ki”) in 1885, long before its role in metabolism had been discovered [1]. David Sprinson of Columbia University contributed to elucidation of the shikimic acid atoms origin by using radioactive isotopes labeled in specific atoms. Three of the atoms came from phosphoenolpyruvate (PEP) and the other four from erythose-4-phosphate (E4P) [2]**.** The seven enzymes of the shikimate pathway were originally discovered in bacteria, mainly *Escherichia coli* and *Salmonella typhimurium* [3], where the pathway serves for production of the three aminoacids mainly. The shikimate pathway is responsible for the synthesis of chorismate, the metabolic intermediate for the synthesis of other aromatic compounds. In plants, the chorismate is a major building block for the synthesis of not only aromatic amino acids, but a variety of aromatic (so-called) secondary metabolites, including coumarins, anthocyanins, flavonols, flavones, condensed tannins, isoflavones, and no less important lignin. The synthesis of these compounds is ensured by phenylpropanoid pathway followed up the shikimate pathway (figure 1, page 11). The secondary metabolites with aromatic ring structures often make up a substantial amount of the total dry weight of a plant [4, 5]. The shikimate pathway intermediates can be considered as the branch point compounds that can serve as substrates for other metabolic pathways [6]. Bacteria also use chorismate for the biosynthesis of siderophores [7].

Shikimate pathway is absent from humans and animals [2, 3, 6]. The evidence of the shikimic pathway was intensively studied in some alveolates [8, 9], including the medically important parasitic protists from group Apicomplexa [10, 11]. For example, a full shikimate pathway has been identified in *Toxoplasma gondii* [8] and an incomplete pathway has been detected in the genomes of several plasmodia species [12, 13].

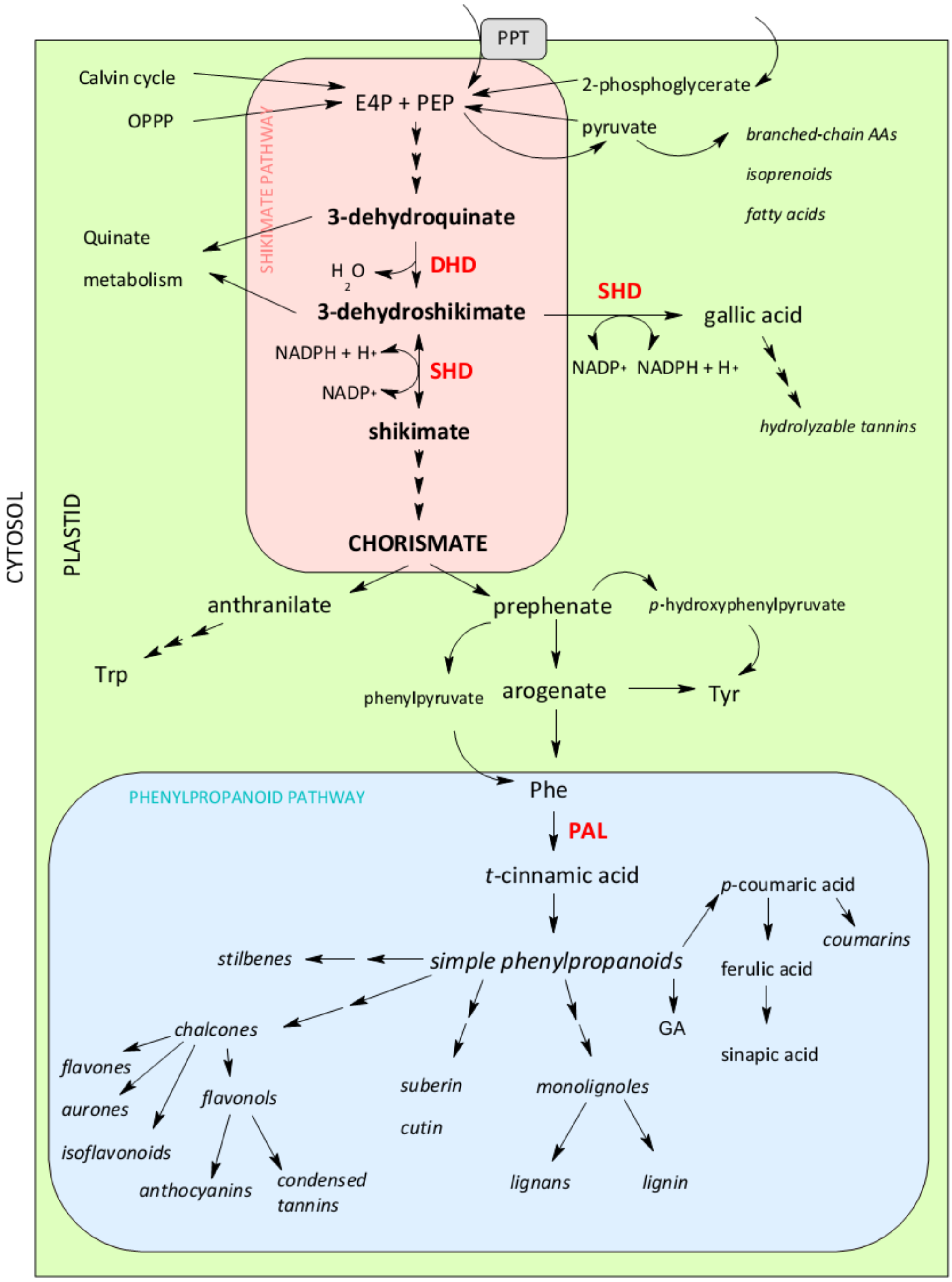


Figure 1 The shikimate pathway and the phenylpropanoid pathway in the plant plastids. Adapted from: [14, 15, 16]. DHD = 3-dehydroquinate dehydratase; E4P = D-erythrose 4-phosphate; GA = gallic acid; OPPP = oxidative pentose phosphate pathway; PAL = phenylalanin ammonium lyase; PEP = phosphoenolpyruvate; PPT = PEP/phosphate translocator; SDH = shikimate dehydrogenase.

It has been estimated that 20 % of the carbon fixed by plants can be directed towards the shikimate pathway [17]. In woody plants, up to one third of photosynthetically fixed carbon can be incorporated into lignin *via* Phe and hydroxycinnamyl alcohols [18]. However, how aromatic compounds serve as signals in the regulation of the shikimate pathway is only little investigated in higher plants [3].

2.1.1. Origin and reactions of the shikimate pathway

The substrates and products, and also intermediates of the shikimate pathway are identical for prokaryotic and eukaryotic organisms, indicating an ancient origin of the pathways [3]. Several examples in the literature suggested that genes of plant secondary metabolism have been recruited from ancestors of primary metabolism [19, 20]. Genes encoding enzymes of the entire shikimate pathway have been identified in *Arabidopsis* and other plant species mostly due to their homology genes from microbial organisms [15]. Genes of the shikimate pathway in plants apparently do not originate from a single prokaryotic ancestor of cyanobacterial origin, but it has been proposed that are likely derived from at least three different sources [21]. A complex, multistep shuffling of loss and gain of function occurred during phylogeny of this pathway, which might explain the multiple factors contributing to the genomic organization and expression of the pathway genes in plants. The seven genes of the shikimate pathway in plants have been serially acquired by endosymbiotic gene transfer and horizontal gene transfer [22].

The shikimate pathway can be divided into three parts. The first part, a pathway from PEP and E4P to chorismate is common for the synthesis of all three aromatic amino acids, and hence is often called the prechorismate pathway [23]. The plant prechorismate pathway comprises seven sequential reactions, localized in plastids, catalyzed by six enzymes summarized in table 1, page 13 [14].

Synthesis starts with condensation reaction catalyzed by 3-deoxy-7-phosphoheptulonate synthase (DAHPS, EC 2.5.1.54) between PEP and E4P yielding 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) and inorganic phosphate (figure 2, p. 14). PEP and E4P are derived from the glycolysis and the branch of the pentose phosphate pathway, respectively, thus connecting the shikimate pathway to central carbon metabolism. To be active, this enzyme requires Mn^{2+} (or Co^{2+}) and reduced thioredoxin, thereby linking carbon flow into the shikimate pathway with electron flow from photosystem I [23, 24].

Table 1

Enzymes of the shikimate pathway in plants, with corresponding genes in *E. coli*. Adapted from [14].

Enzyme	EC number	*E. coli* gene (Aro -)
DAHPS	EC 2.5.1.54	*AroF; AroG; AroH*
DHQS	EC 4.2.3.4	*AroB*
DHD**	EC 4.2.1.10	*AroD*
SDH**	EC 1.1.1.25	*AroE*
SK	EC 2.7.1.71	*AroL; AroK*
EPSPS	EC 2.5.1.19	*AroA*
CS	EC 4.6.1.4	*AroC*

** part of bi-functional enzyme in plants

Interestingly, three DAHPS isoenzymes found in *Escherichia coli* (type I isogenes: *AroF, AroG, AroH*) are influenced by feedback regulation by one of the aromatic amino acids L-phenylalanine (*aroF*), L-tyrosine (*aroG*), and L-tryptophan (*aroH*), whereas the corresponding type II enzyme found in plants is not inhibited by aromatic amino acids but it is instead activated by L-tryptophan [8, 25].

Step two, catalyzed by 3-dehydroquinate synthase (DHQS; EC 4.2.3.4), represents the formation of 3-dehydroquinnic acid from DAHP by elimination of phosphoric acid, followed by an intramolecular aldol reaction [26, 27]. Step three and four are catalyzed by bifunctional enzyme 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHD/SDH;

EC 4.2.1.10 and EC 1.1.1.25). Systematic name for SDH is shikimate: NADP$^+$- oxidoreductase, therefore other name used in literature is SORase. In higher plants, this bi-functional enzyme catalyzes reactions leading to formation of shikimate. Shikimic acid itself is formed from 3-dehydroquinic acid *via* 3-dehydroshikimic acid by dehydration and reduction steps [3].

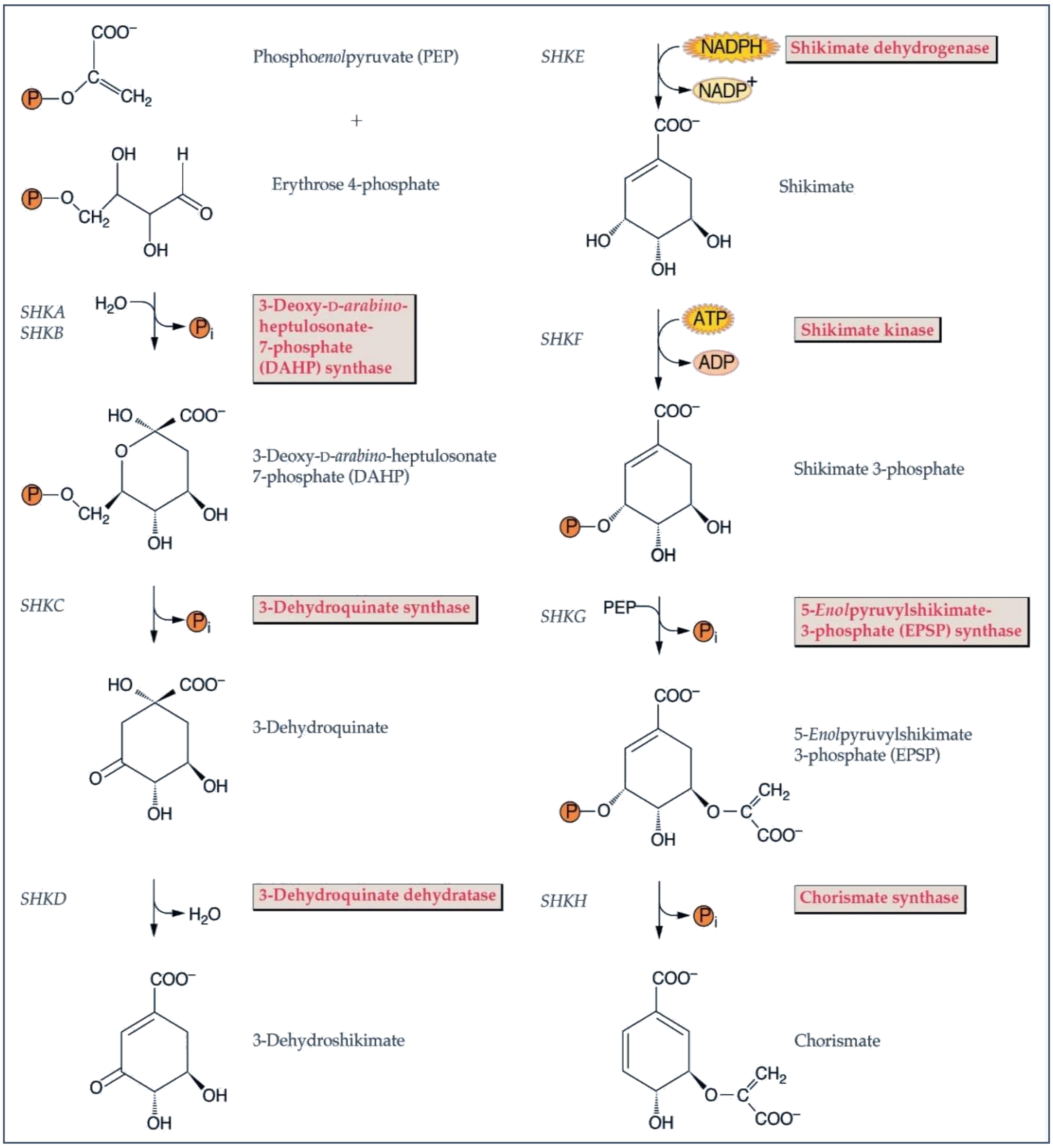


Figure 2 The shikimate pathway reaction steps from condensation of PEP with E4P to chorismate. Taken from [28].

Since the turnover number of 3-dehydroquinate dehydratase is one - ninth that of shikimate dehydrogenase [3], dehydroquinate is readily converted to shikimate without accumulation of dehydroshikimate, and the enzyme is generally known simply as shikimate dehydrogenase [29]. The enzyme is specific for cofactor NADPH predominantly [30, 31], but in some cases NAD^+ is preffered over the $NADP^+$, as described in some bacteria, *Corynebacterium glutamicum* [32]. Intermediates dehydroquinate and dehydroshikimate can be partitioned toward quinate metabolism. In plants, quinate is a precursor for chlorogenic acids which serve as antiherbivory compounds as well as antioxidants in animal diets [33].

The fifth reaction of the shikimate pathway catalyzed by shikimate kinase (SK; EC 2.7.1.71) converts shikimate to shikimate 3-phosphate (S3P) in simple ATP-dependent phosphorylation reaction [34, 35]. The second PEP enters the pathway in the sixth step and is condensed with S3P to yield 5-enolpyruvylshikimate 3-phosphate (EPSP) in the reaction catalyzed by 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS; EC 2.5.1.19) [36].

The last step 7 of the shikimate pathway is catalyzed by chorismate synthase (CS; EC 4.2.3.5). In this step the EPSP is transformed to chorismic acid, by means of 1, 4 - elimination of phosphoric acid. The reaction requires catalytic amounts of reduced flavin (FMN) for activity even though the overall reaction is redox neutral [17].

2.1.2. Biosynthesis of aromatic amino acids

Pathway to the production of Phe or Tyr (figure 3, page 15) includes chorismate mutase (CM, EC 5.4.99.5) that catalyzes the transformation of chorismate to prephenate *via* Claisen rearrangement. Prephenate aminotransferase (PPA-AT, EC 2.6.1.79) then catalyzes a reversible transamination between prephenate and arogenate using pyridoxal phosphate (PLP) as a cofactor, and L-glutamate or L-aspartate as amino donors. Other enzyme arogenate/prephenate dehydratase (ADT/PDT, EC 4.2.1.91/EC 4.2.1.51) catalyze the final steps for production of phenylalanine or tyrosine, respectively. And arogenate/prephenate dehydrogenase (ADH/PDH, EC 1.3.1.78/EC 1.3.1.12) catalyze the oxidative decarboxylation of arogenate and prephenate to Tyr and 4-hydroxyphenylpyruvate, respectively, using an NAD^+ or $NADP^+$ cofactor [2, 14, 17].

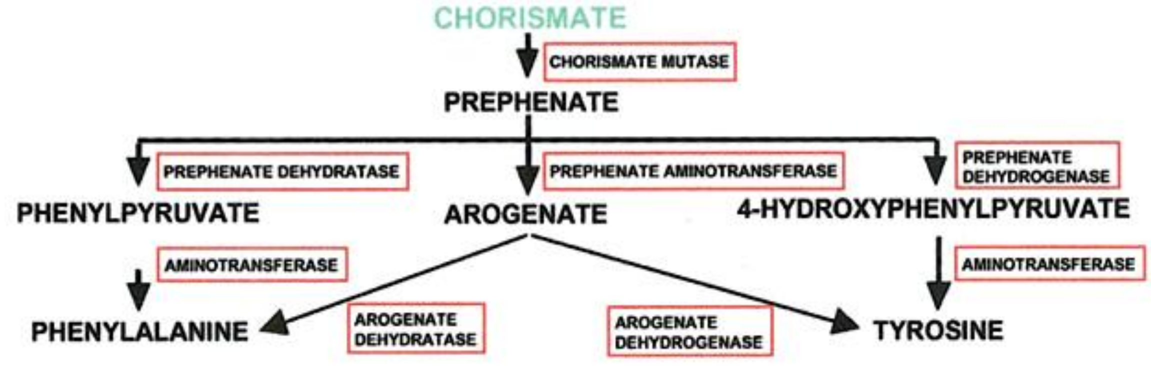


Figure 3 The overview of the phenylpropanoid pathway leading to Phe and Tyr. Taken fro m: [37].

2.1.3. Cell localization and genetic organization of the shikimate pathway enzymes through domains

The shikimate pathway is believed to be localized in plastids [38, 39]. However, it has been noted that the chloroplast-localized biosynthetic activity does not account for all of the aromatic amino acid biosynthesis observed. A spatially duplicated pathway in the cytosol has therefore been proposed, supported by more recent evidence that, at least in tobacco, the enzymes of the pathway can also be localized in the cytosol [40, 41]. In general, the shikimate pathway enzymes of higher plants are nucleus encoded, but many posses N-terminal plastid targeting peptides that direct them to the plastid [38, 39, 42]. It is still a matter of debate whether or not the shikimate pathway is operating in two different subcellular compartments in plants, and if so, how two separate pools of aromatic acids are maintained by the cell. The fractionation methods often produce results with a degree of inaccuracy, thus, this technique does not univocally solve the problem of the existence of a cytosolic shikimate pathway [24].

The pronounced difference between plant, fungal or bacterial pathway could be seen in the branch beyond chorismate leading to Phe and Tyr, where in plants, the biosynthetic sequence proceeds from chorismate *via* prephenate to arogenate and then to either Phe or Tyr (figure 4, p. 17). Also recent genetic evidence indicates that the arogenate pathway is the predominant route for Phe biosynthesis in plants (a major pathway for Tyr biosynthesis remains to be solved) [43].

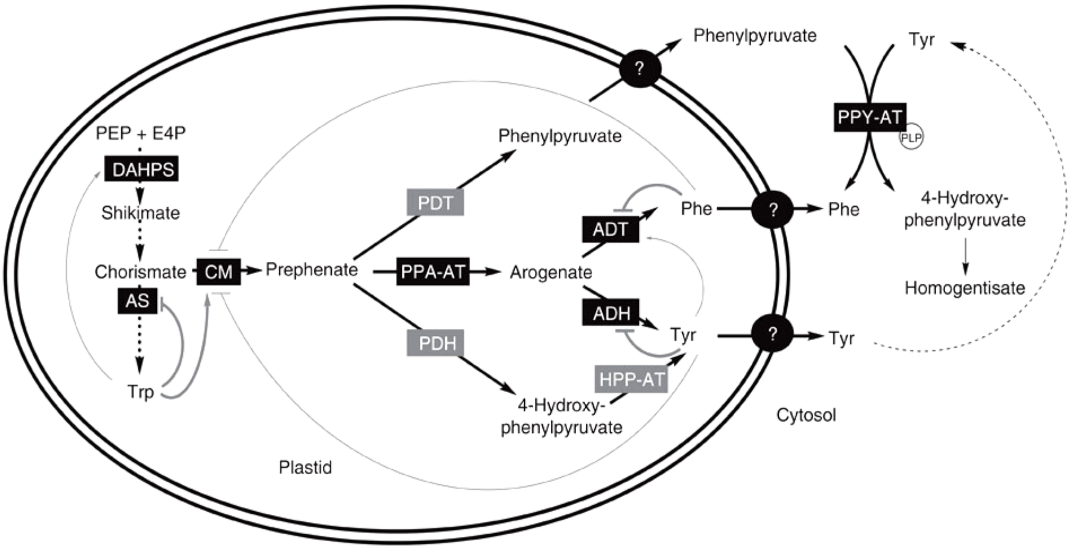


Figure 4 Revised model for phenylalanine biosynthesis in plants, with microbial-like phenylpyruvate pathway for Phe production. Taken from: [43]. The new model for Phe biosynthesis in plants now includes a cytosolic step linked to the catabolism of Tyr, which is mediated by PPY-AT. Circles with question marks correspond to undefined transport steps. Rectangles represent enzymes: ADH = arogenate dehydrogenase, ADT = arogenate dehydratase, CM = chorismate mutase, HPP-AT = 4-hydroxyphenylpyruvate aminotransferase, PDH = prephenate dehydrogenase, PDT = prephenate dehydratase, PPA-AT = prephenate aminotransferase, PPY-AT = phenylpyruvate aminotransferase.

In fungi *Neurospora crassa* and *Saccharomyces cerevisiae*, the pathway diverges already after prephenate, i. e. *via* phenylpyruvate to Phe, and *via* 4-hydroxyphenylpyruvate to Tyr. In many bacteria either of the pathways or even both are present [44, 45]. However, this embedded difference in the pathways has been questioned by recent research results, suggesting that plants also utilize a microbial-like phenylpyruvate pathway to produce Phe (figure 4). This alternative pathway contributes to phenylalanine biosynthesis in plants *via* a cytosolic tyrosine:phenylpyruvate aminotransferase (PPY-AT) that links the coordinated catabolism of Tyr to serve as the amino donor, thus interconnecting the extra-plastidial metabolism of these amino acids [43].

The great differences are found in the primary sturcture and properties of the prokaryotic and eukaryotic enzymes (figure 5). In bacteria, each enzyme is encoded by monofunctional genes, spread over the genome [46]. In fungi and certain apicomplexan species the genes of DHQS, EPSPS, SK, SDH and DHD are fused to a pentafunctional complex. As studied in *Neurospora crassa* [47], a cluster of five structural genes (arom) codes for an aggregate of five enzymes called AROM [48]. Plants have a similar organization like bacteria except for DHD and SDH which are fused to a bifunctional enzyme complex [49] (Figure 5).

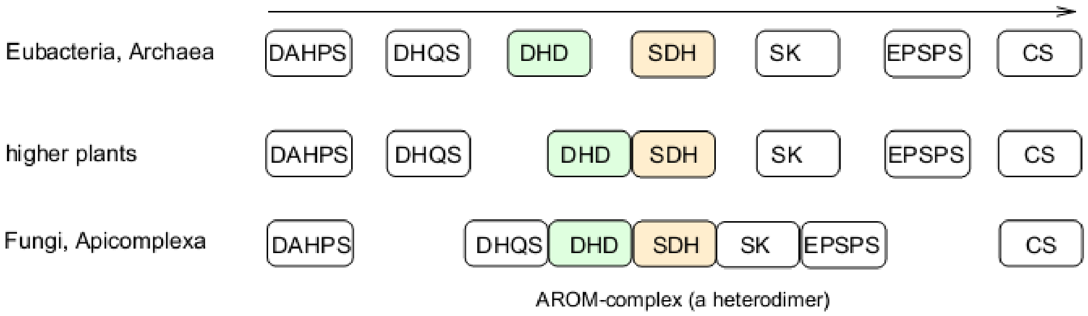


Figure 5 Genetic organization of the shikimate pathway in bacteria (first row), higher plants (second row), with a monomeric bi-functional DHD-SDH and in fungi (last row). Adapted from: [50]. The abbreviations of enzymes are clarified in table 1, page 13.

2.1.4. Regulation of the shikimate pathway

Regulation of the shikimate pathway is complex and varies greatly between organisms. In bacteria, the shikimate pathway is almost exclusively used to synthetize proteins, since bacteria use a significant amount of their metabolic energy for such biosynthesis. For most prokaryotes the three aromatic amino acids represent great output of aromatic biosynthesis, and regulatory mechanisms are triggered by the intracellular concentration of Phe, Tyr, Trp [51]. The regulation in bacteria therefore occurs at post-translational level by enzyme feedback-inhibition and at transcriptional level by repression, specifically modulation of expression of DAHP synthase genes [52 - 56]. In yeast, the pathway is under the so-called ‘‘the mechanism of general control’’, which enables the organism to respond to limited amino acid supply [57].

In plants, the situation is different; the three essential aminoacids are just starting points for production of plenty of other secondary metabolites, with multiple biological functions and biotechnological values [58]. The regulation of shikimate pathway in plants occurs mostly at gene expression level. Reduced levels of aromatic amino acids or their downstream products may act as a signal to induce the expression of the shikimate pathway genes and restore the carbon flux through the pathway in plants. The expression of many plant genes encoding enzymes involved in the shikimate pathway and aromatic amino acid metabolism is regulated developmentally [44, 59] and in response to various environmental stimuli, wounding [60, 61], ozone [62], pathogen infection, elicitors [62, 63] or heavy metals (Ni, Cd, …) that cause changes in free phenylalanine content [64].

In addition, regulation of shikimate pathway in plants is complicated by subcellular localization of the enzymes. The flux through the cytosolic phenylpyruvate route of Phe synthesis in plants is increased when stress factors limit a plant's ability to use the main pathway. (figure 4, page 17). This discovery uncovers another level of complexity in the plant aromatic amino acid regulatory network, unveiling new targets for metabolic engineering, e.g. to improve the plant response to pests, its nutritional capability or production of the components of cancer-treating drugs [43]. Similarly enzymes of shikimate pathway in plants are included in development of herbicides. EPSP synthase is the only cellular target for the herbicide N-phosphonomethylglycine (glyphosate), an analog of PEP (figure 6). Glyphosate compete with PEP in binding to the enzyme. EPSPS enzymes from different organisms show great variations in their sensitivities to the glyphosate [65, 66].

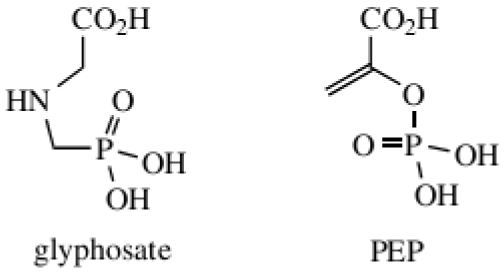


Figure 6 The glyphosate, an analog of PEP, inhibitor of EPSPS in the shikimate pathway.

Furthermore, the functional and structural characterization of enzymes belonging to microbial shikimate pathway is important for structure-based drug design, allowing development of new antimicrobial (especially anti-tuberculous) drugs lacking negative impact to humans [67]. Thus, there exists a high motivation to elucidate the overall regulation of the shikimic pathway and of the related phenylpropanoid biosynthetic pathway by their products and intermediates.

2.2. The shikimate dehydrogenase

2.2.1. Reactions catalyzed by the shikimate dehydrogenase

As was mention previously (figure 1, page 1111) the third and the fourth enzymatic steps of shikimate pathway in higher plants are catalyzed by the bifunctional enzyme 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHD/SDH; EC 4.2.1.10 and EC 1.1.1.25), Arabidopsis gene *At3g06350* (603 AAs) is located on chromosome 3 [15] (Figure 7). While Arabidopsis plants possess only a single AtDHQ/SDH gene, two genes were found in the experiment with the tobacco plants [41].

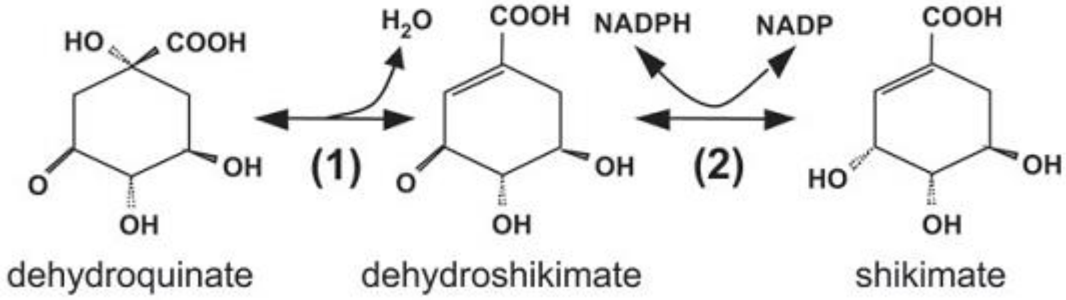


Figure 7 The reactions leading to formation of SKA catalyzed by the DHD/SDH. Adapted from: [4]. (1) reaction catalyzed by DHD; (2) reaction catalyzed by SDH.

This bifunctional enzyme has been well characterized in tomato (*Solanum lycopersicum*) [68], tobacco (*Nicotiana tabacum*) [69] or in *Arabidopsis thaliana* [70, 71]. In recent times, an early intermediate of the shikimate pathway, 3-DHS, was identified as a potential precursor for the gallic acid (GA) synthesis in plants, suggesting the third important catalytic activity of the SDH enzyme in plants and bacteria (figure 8, page 21). The proposed SDH reaction mechanism would result in continuous generation of both shikimate and gallic acid. This cycle could potentially continue as long as the supply of 3-DHS exists, with the result that both SKA and GA are produced, albeit not necessarily in equal amounts [16]. Plants and fungi produce high concentration of GA [72]. The GA is an active component responsible for reducing coronary arterial disease and arterial thrombosis in people who consume red wine, green tea, and fruit [73].

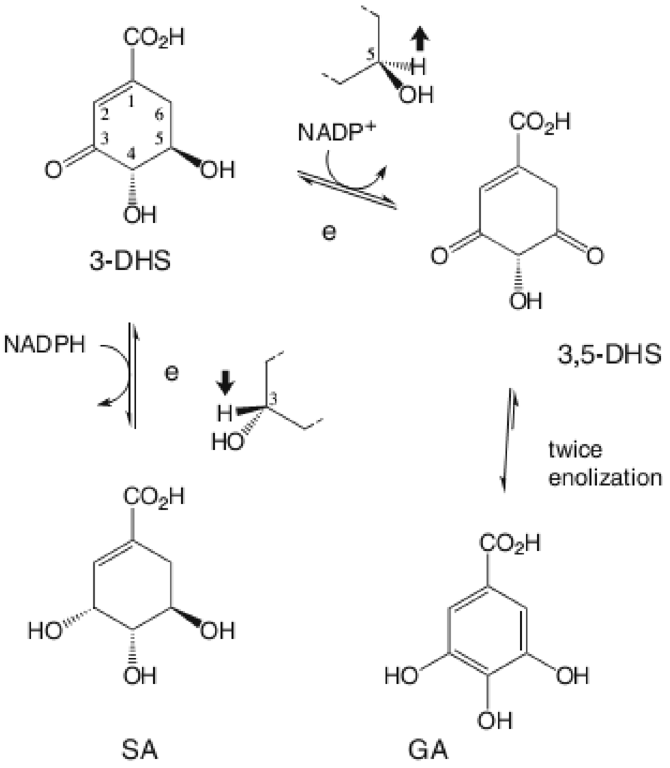


Figure 8 Mechanism of shikimic acid and gallic acid biosynthesis in plants and bacteria. Taken from: [16]. NADPH must be oriented properly in the enzyme-cofactor-substrate complex, with the orientation of 3- dehydroshikimate (3-DHS) in the way where the OH at C-5 is down and H is up, analogous to OH and H at C-3 of SA. The intermediate 3,5-DHS immediately and spontaneously converts to GA.

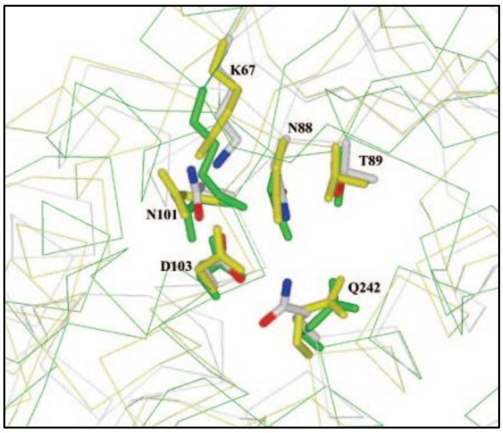
In recent years, increasing genomic data have revealed that some bacteria harbor additional homologs of SDH (marked AroE in E. coli), including YdiB, SDL (or SdhL), and RifI that show different enzymatic properties [74] (table 2, page 22). Characterization of AroE-type shikimate dehydrogenase has been done in many microorganisms such as in *Escherichia coli*, *Mycobacterium tuberculosis* [75], *Haemophilus influenza* and *Helicobacter pylori*

[76]. Some of them have been crystallized and their substrate binding structure determined [70, 74, 77, 78].

Table 2 The types of shikimate dehydrogenases in different organisms.

Enzmye	Organism	Properties	Ref.
AroE	all prokaryotes	prototypical SDH, accepts SA as substrate	[75]
YdiB	e. g. *E. coli, Pseudomonas putida*	accepts quinate and SA as substrate and uses NAD^+ and $NADP^+$ as cofactor	[77] [79]
YdiB2	e.g. *Pseudomonas putida*	accepts quinate and SA as substrate, but it is phylogenetically distinct from YdiB	[80]
RifI	e.g. *Amycolatopsis mediterranei, P. putida*	part of the aminoshikimate biosynthetic pathway that leads to the biosynthesis of the antibiotic rifamycin B	[81-83]
SDL (SdhL)	e. g. *E. coli,* *H. influenzae, P. putida*	catalyzes the oxidation of SA but not quinate and has a 1000-fold lower activty for shikimate than E. coli AroE	[70]
Ael1	*P. putida*	AroE-like with enzymatic properties distinct from any other SDH familiy	[80]

The active site is conserved among these classes of SDHs (Figure 9, page 22); variation from this conserved architecture confers substrate specificities [71].



AroE (green)

YdiB (yellow)

SDL (gray)

Figure 9 Superimposition of active site residues from E. coli AroE, YdiB, SDL [71].

2.2.2. Structural aspects of DHD/SDH enzyme

One of the first physical association of 3-dehydroquinate dehydratase and shikimate dehydrogenase in higher plants was suggested by investigation carried out on the moss (*Physcomitrella patens*), demonstrating that both enzyme activities are carried out by a two domains connected with a one polypeptide [49], what was confirmed later [70, 71].

The crystal structure of Arabidopsis DHD/SHD with shikimate bound at the SDH site and tartrate at the DHQ site has recently been elucidated [70]. The role of the active site residues for binding and catalysis in the Arabidopsis thaliana DHD/SHD domains has been assessed by site-directed mutagenesis. In the Arabidopsis DHD active site Arg279 functions as a key binding group and Lys241 with His214 are catalytic groups. In contrast, the SDH active site Lys385 and Asp423 (numbered with respect to the Arabidopsis protein) are conserved in SHDs and have been proposed to be involved in proton transfer during catalysis. They function probably as a catalytic dyad where the mutation of one residue results in the loss of functionality of the other (**Figure 10**figure 10).

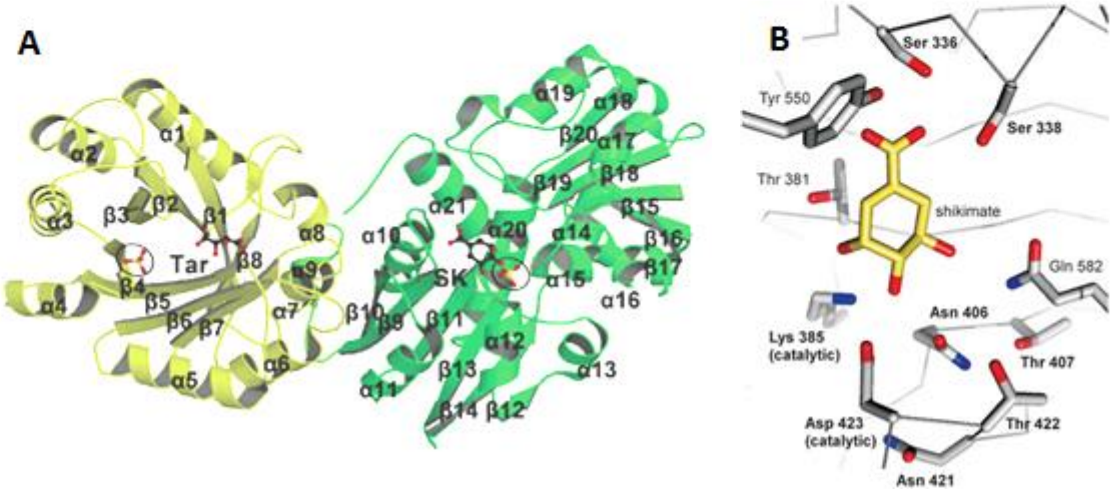


Figure 10 Arabidopsis DHQ-SDH structure (A); and the SDH (AroE type) - SKA complex that is present in both bacteria and plants (B). Taken from: [70, 80]. Ribbon diagram of the DHD (yellow) and SDH (green) domains bound with tartrate (Tar) and shikimate (here as SK) in the active sites. This topology is similar in all classes of the SDH enzyme with a few exceptions.

The crystal structure of the Arabidopsis SDH domain indicated that Ser336, Ser338, and Tyr550 are important for binding shikimate (interactions with C1 carboxylate of SKA) [70]. Mutagenesis of Ser338 to Ala (Ser338Ala) in the Arabidopsis enzyme resulted in a 10-fold reduction in the turnover rate, and this residue has been implicated to be involved in binding and also in positioning of the substrate for catalysis. The replacement of this critical residue by Gly (Ser338Gly) in tobacco NtSHD/DHD-2 most likely retains the ability of this cytosolic enzyme to synthesize SKA, although with a lower turnover rate [41].

2.2.3. Mode of substrate binding by SDH (DHD/SDH)

Catalytic efficiency is generally improved in bifunctional enzymes by different mechanisms. The fusion protein might be a kinetic solution to ensure a stoichiometric supply of products. Substrate channeling from the DHD to SDH site ensures a constant flux through the shikimate pathway towards chorismate biosynthesis. The bifunctional enzyme DHD/SDH plays an important role in controlling the partitioning of metabolites also to the quinate biosynthetic pathway [84]. The SDH adopts a concave architecture. The face-to-face organization of the DHD and SDH active sites provides a direct route for DHS to the SDH site (increasing the local DHS concentration at SDH active site), thus prefering the proximity effect (figure 11, page 25) over the two other proposed models (physical channeling of substrate; channeling *via* dimerization) [70]. Kinetic advantages caused by proximity effects are evident in several cases of plant enzymes (besides DHD/SDH also e. g. mitochondrial hydroxymethyl-dihydropterin-pyrophosphokinase-dihydropteroate-synthase, catalysing tetrahydrofolate synthesis in plants), but might not be the main functional advantage [85]. Because SDH is about nine times more active than DHD [3, 70, 86], the fusion protein would have a kinetic improvement. However, as mentioned before, DHS is also an intermediate in a pathway for quinate metabolism. Thus, this functional arrangement might be more useful for preferential routing of carbon to aromatic amino acid synthesis [84].

Figure 11 A bifunctional enzyme with two catalytic domains (A) [84], and the model of action of DHD/SDH domains, (B) [70].

2.2.4. Physical properties of SDHs

The physical properties of the plant SDHs have been described in a few plant species as summarized in table 3, page 25. Various characteristics are common in plant SDHs, e. g., an alkaline pH optimum, sensitivity to heat inactivation (with stabilization if substrates are bound) and higher affinity for $NADP^+$ than for shikimic acid. A lot of data about physicochemical properties have been collecetd in studies of medically important bacteria, with the molecular weight of SDH starting on value 27 000 [75]. The highest described molecular weight of plant SDHs was illustrated in the study of tomato (*Solanum lycopersicum*) – 73000 [31]. The pH optimum is generally found around pH 7.0 – 11.0 and the optimum temperature range is found between 22°C to 50 °C in plants. The enzyme usually became unstable above 50°C, with a great loss of activity. The isoelectric point (pI) values are limited. The pI value of SDH has been described in spinach (pI 6.4) or, differently, in *Bacillus anthracis*, with theoretical pI 6.55 [86], both suggesting the anionic character of the SDH surface AAs.

Table 3 Physical properties of SDHs from various plants.

Plant (segment)	Mr [×10^3]	$pH_{opt.}$	$T_{opt.}$ [°C]	pI	Ref.
Pisum sativum L. (seedlings)	-	10.0	-	-	[84]
Vigna mungo L. (seedlings)	57	8.0	-	-	[88, 89]
Camellia sinensis L. (shoot tips)	-	10.1; 7.7	-	-	[30]
Spinacia oleracea L. (chloroplasts)	59 – 67 (4 isoenzymes)	9.5	-	6.4	[90]
Cucumis sativus L. (pulp)	45 (2 isoenzymes)	8.7	40 - 55	-	[91]
Capsicum annuum L. (seedlings)	67 (4 isoenzymes)	10.5	50	-	[92]
Solanum lycopersicum L. (fruit)	73	9.1	-	-	[31]
Pinus taeda L. (needles)	35; 53	10.3	-	-	[93]
Nicotiana tabacum (plant)	-	6.8 – 7.2	30 - 45	-	[41]
Arabidopsis thaliana L. (leaves)	-	7.5	22	-	[70]

2.2.5. Kinetic properties of SDHs

A few initial velocity studies (table 4, page 27) indicated a sequential mode of addition of substrates to the SDH, i. e. both substrates must attach to the enzyme before any product is released. Some data suggested ordered mechanism of action [94]. The inhibition kinetic study with protocatechuic acid revealed a competitive-type inhibition [91]. Ions Zn^{2+} an Cu^{2+} have proven to be inhibitors of SDH regardless of species, related to inactivation of functional sulfhydryl groups of SDH by these metal ions [92]. Experimental papers available present slight protective effect of the Cl^- ions on SDH activity [91].

Table 4 Kinetic properties and regulation of SDHs.

Plant	Km (SA) [µM]	Km ($NADP^+$) [µM]	Regulator (degree of regulation)	K_i [mM]	Ref.
Pisum sativum L.	190 - 280 (pH 9)	7.0 (pH 9)	Iodoacetate (slight inhibition)	-	[87] [94]
Vigna mungo L.	87 (pH 8)	117 (pH 8)	-	-	[88] [89]
Camellia sinensis L.	430 µM (pH 9)	32 (pH 9)	p-CMB (reversible-inhibition)	-	[30]
Spinacia oleracea L.	200 (pH 9.5)	8 – 26 (pH 9.5)	Phe,Tyr, Trp, 4-HPP, PPY, chorismate, cinnamic acid, coumaric acid, prephenate, caffeic acid (no inhibition)	-	[90]
			3,4-dihydroxybenzoic acid quinic acid anthranilic acid	0.63 0.32 0.08	
			Glyphosate (no significant effect)	-	
Cucumis sativus L.	60 (pH 7.6)	10 (pH 7.6)	quinic, ferulic, cinnamic (no effect)	-	[91]
			Protocatechuic acid (strong inhibitory effect); Zn^{2+}, Cd^{2+} (inhibition) Na^+ or Cl^- (increasing activity)	0.02	
Capsicum annuum L.	87 (pH 9.0)	17 (pH 9.0)	Phe, chlorogenic acid, caffeic acid, coniferyl alcohol, vanillic acid, coumaric acid (no effect) coniferyl alcohol, gallic acid (slight inhibition)	-	[92]
			guaiacol, protocatechuic acid, 2.4 - D	0.38 0.27 0.16	
Solanum lycopersicum L.	38 (pH -)	10 (pH -)	Ca^{2+}, Mg^{2+}, Mn^{2+} (activation) Zn^{2+}, Cu^{2+} (strong inhibition)	-	[31]
Nicotiana tabacum	130 ±15 (pH 9.0)	31±7 (pH 9.0)	-	-	[41]
Arabidopsis thaliana L.	685 (pH 7.5)	131 (pH 7.5)	-	-	[95]

2.3. Phenylpropanoids

The plant shikimate pathway is the entry to the biosynthesis of many important phenylpropanoids. Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. They are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants, with more than 8 000 phenolic structures currently known, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins [96] (figure 12). These groups are readily oxidized, forming complexes with proteins and thus inhibit enzyme activity [97].

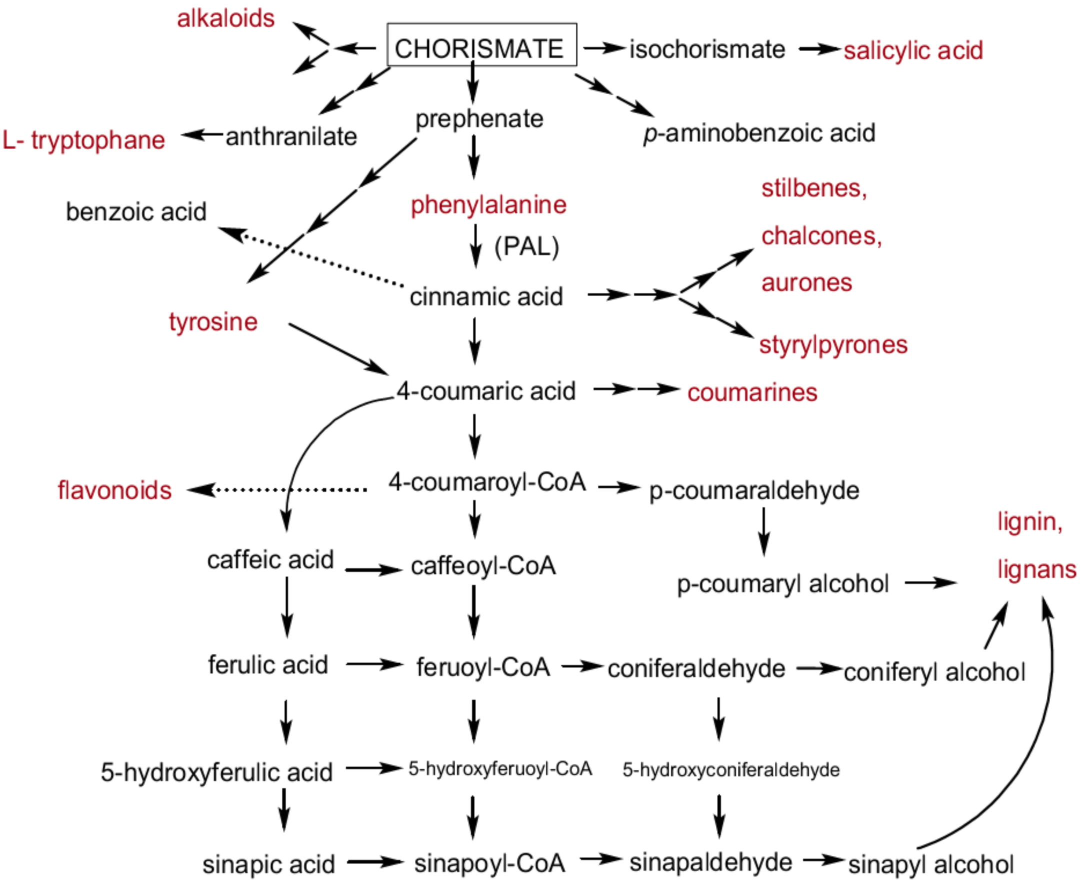


Figure 12 The branches for production of secondary metabolites – overview [14, 28, 44].

Polyphenols are known for their antioxidant activity, with their ability to scavenge free radicals and up-regulate certain metal chelation reactions by donation of hydrogen, which interrupts chain reactions of free radicals. The phenolics fulfill many functions in plants, such as determining the colour and taste of plants or having impact on reproduction [6]. The carbon flow is directed from the shikimate pathway to the various branches of the general phenypropanoid metabolism by phenylalanine ammonia lysae (PAL) and tyrosine ammonia lyase (TAL) that converts phenylalanine to cinnamic acid and tyrosine to *p*-coumaric acid, respectively [58]. The phenylpropanoid pathway comprises four types of reactions leading to different products): aromatic hydroxylations (from *p*- coumaric acid to 5-hydroxyferulic acid); O-methylations - methylating the meta-hydroxyl group (ferulic acid, sinapic acid); CoA ligations (ATP - dependent); two sequential NADPH-dependent reductions (monolignols → lignans, lignins) [28].

The main shikimic acid derivates are aromatic amino acids, benzoic acids (such as 4-hydroxybenzoic acid, salicylic acid, protocatechuic acid or gallic acid) and cinnamic acids (e.g. 4-coumaric acid, caffeic acid, ferulic acid, sinapic acid). The modifications of there precursors give rise to lignins and lignans, phenylpropenes (e.g. cinnamaldehyde, anethole, eugenol, or myristicin) and coumarins (e.g. coumarine; umbelliferone, scopoletine, scopolin) [98]. Cinnamic acids, and their coenzyme A esters, may also function as starter units for chain extension with malonyl-CoA units, thus combining elements of the shikimate and acetate pathways - giving rise to flavonoids and stilbenes [99, 100]. In some algae, fern, fern allies, seed plants and trees, lignins**,** complex aromatic heteropolymers**,** reinforce cell walls, allow water transport and provide various defence mechanisms [101]. Lignins have a variability, in both composition and structure, and an irregularity that defies exact description [102]. The related lignans**,** the major classes of phytoestrogens, can act as anti-phatogen protectors or antioxidants. Dietary lignan intake, for example consuming flaxseed or sunflower seeds, may be associated with a modest reduction in breast cancer risk [103, 104].

More than 4500 compounds form a group of the flavonoids, such as anthocynanins, flavanols, flavanones; flavones, isoflavones; flavonols. Flavonoids include a series of subclasses with a common general structure characterized by the presence of two aromatic rings linked by a three-carbon bridge (C_6 - C_3 - C_6 system). Nowadays it is accepted that natural flavonoids present in fruits and plant-derived-foods are relevant, not only for technological reasons and organoleptic properties, but also because of their potential healthpromoting effects, as suggested by the available experimental and epidemiological evidence. The beneficial biological effects of these food bioactives may be driven by two of their characteristic properties: their affinity for proteins and their antioxidant activity [105]. The hydrolyzable tannins are composed of a central sugar core and the gallic acid (gallotannins) or ellagic acid (elagitannins), mainly originated from the shikimate pathway. In recent research [106] hydrolyzable tannins chebulagic acid and punicalagin, isolated from the dried fruits of *Terminalia chebula Retz.* (Combretaceae) targeted and inactivated *Herpes simplex* virus 1 particles and could prevent binding, penetration, and cell-to-cell spread, as well as secondary infection. The coumarins, furanocoumarins, and stilbenes have protective functions. Many coumarins and their derivatives exert anti-coagulant, anti-tumor, antiviral, anti-inflammatory and antioxidant effects, as well as anti-microbial and enzyme inhibition properties, including acetylcholinesterase (AChE) inhibition, also important for Alzheimer's disease management [107]. Some coumarins, e. g. imperatorin, could be utilized to generate a new generation of bioherbicides and other pesticides, more ecologically friendly, using their allelopathic potential [108]. Recently, some stilbenes, such as resveratrol, pterostilbene and piceid (all found in V. vinifera) have been reported to show cytoprotective and growth inhibitory effects on mammalian cells [109].

3. The aims of the thesis

The aim of this study comprises several parts:

1. Searching for plant source of SDH with high enzyme activity.
2. Purification of SDH from this source and its characterization (K_m, pH optimum, pI, M_r).
3. Study of the regulation of plant SDH by various compounds of phenylpropanoid metabolism.
4. Detailed inhibition study with respective phenolics compounds.

4. Materials and methods

4.1. Laboratory equipment

Amicon Ultra - 0.5 mL Centrifugal Filters	(Merck Milipore, USA)
Centrifugal machine Universal 32R	(Hettich Zentrifugen, Germany)
Digital laboratory balances	(Kern, Germany)
Electronic analytical balances 100A	(Denver Instrument Company, USA)
Gel electrophoresis apparatus – Multigel	(Biometra, Germany)
Isoelectric focusing system with ECPS source	(Pharmacia Fine Chemicals, Sweden)
Magnetic stirrer	(IKA, Germany)
Peristaltic pump P-1	(Pharmacia Fine Chemicals, Sweden)
pH meter Ultra Basic UB-10	(Denver Instrument Company, USA)
Spectrophotometer Ultrospec 2100 Pro	(Biochrom, UK)
Spectrophotometer UV-Vis Helios α	(Thermo Spectronic, USA)
Thermostat EL-01	(Major Science, USA)
Vortex V-1 Plus	(Biosan, Latvia)

4.2. Chemicals and other material

Ampelopsin (APIChem); acrylamide/bis-acrylamide; 2-amino-2-methyl-1-propanol, iodonitrotetrazolium chloride, NAD^+, $NADP^+$, Nitro Blue Tetrazolium, phenazin methosulphate, poly(vinylpolypyrrolidon), protease inhibitor cocktail for plant tissues, secondary metabolites used in this study (with exceptions), shikimic acid, sodium L-lactate (Sigma – Aldrich, USA); 4-methylubelliferone (Fluka); quercetin (Riedel); Serva IEF Markers 3-10, myoglibine (equine), chymotrypsinogen A (Serva Electrophoresis GmbH, USA); Sephacryl S-300, Servalyt Precotes gels (Pharmacia Fine Chemicals, Sweden); ProSieve®Color Protein Markers (Cambrex Bio Science, USA); 2′,5′-ADP-Sepharose 4B (GE Health Care, Germany); DEAE cellulose (Whatman, UK); Ponceau Red RR (Loba Chemie, India).

4.3. Plant material

Type of vegetable	Latin name	Part of plant used in experiments
parsley	*Petroselinum crispum* L.	root
radish	*Raphanus sativus* L.	root
broccoli	*Brassica oleracea var. botrytis italica*	flowering head
kohlrabi	*Brassica oleracea var. gongylodes*	stem
carrot	*Daucus carota* L.	root
ginger	*Zingiber officinale*	rhizome
red cabbage	*Brassica oleracea var. capitata f. rubra*	leaved head
white radish	*Raphanus sativus var. Longipinnatus*	root
celery	*Apium graveolens var. dulce*	tuber
garlic	*Allium sativum* L.	bulb
pumpkin	*Cucurbita moschata*	fruit
beetroot	*Beta vulgaris* L.	root
string bean	*Phaseolus vulgaris* L.	fruit
zucchini	*Cucurbita pepo* L.	fruit
onion	*Allium cepa* L.	bulb

Fresh parsley roots of Czech origin and other vegetables were bought in a grocery store and stored at - 4°C overnight before processing the next day.

4.4. Samples preparation

4.4.1. Preparation of crude extracts from vegetable

One gram of specific fresh vegetable (see chapter 4.3.) was homogenized in a triple amount of 100mM Tris-HCl (pH 7.8) containing 1mM dithiothreitol (DTT), 1mM EDTA, 5mM $MgCl_2$ and 5 % glycerol (buffer A), with 0.02 g/ml of poly(vinylpyrrolidone) added to mixture, using a pestle and mortar. The homogenate was centrifuged at 16600*g* for 15 min. and the activity of the SDH measured immediately (chapter 4.6.1. page 35).

4.4.2. Concentration of the enzyme preparate

The purified protein samples (see next chapters) were concentrated using Amicon Ultra-15 or Amicon Ultra-0.5 centrifugal filter units, with centrifugation at 9400*g* for 20 min. (4 °C) and 16600*g* for 30 min. (4 °C), respecitvely. Enzyme was concentrated 30-fold.

4.4.3. Preparation of samples for electrophoretic separation

The suitable volumes of the samples from the individual SDH purification steps (see next chapters), corresponding to the protein content of 60 µg, were treated with 1 ml of 96 % ethanol overnight (at - 20 °C), in order to precipitate proteins. After precipitation, the samples were centrifuged at 16600*g*, 30 min. and stored (- 20°C).

4.5. Purification of shikimate dehydrogenase

4.5.1. Precipitation with ammonium sulphate

Raw parsley roots (100 g) were rinsed with cold water and homogenized in a triple amount of buffer A, with 1 g of poly(vinylpyrrolidone) added to mixture, using a kitchen blender. The homogenate was filtered through folded cheesecloth and the mixture was centrifuged at 9400g for 30 min. The supernatant (crude extract) was brought to 0 - 33% saturation, protein was collected by centrifugation at 9400*g* for 30 min. (4 °C). In the next step proteins in supernatant were precipitated with solid $(NH_4)_2SO_4$ to 33% - 60% saturation and centrifuged at 9400*g* for 30 min. (4 °C). Sediment was redissolved in buffer B containing 25mM Tris–HCl (pH 7.8) with 0.5mM DTT, 1mM EDTA, 5mM $MgCl_2$ and 5% glycerol, and dialyzed overnight against one litre of buffer B, with continual stirring at 4 °C.

The purification steps were monitored for SHD activity at 340 nm (chapter 4.6.1, page 35) and aliquots (1 ml) of each fraction were collected for protein determination and for SDS electrophoresis.

4.5.2. Ion exchange chromatography and gel filtration chromatography

The SDH purification was performed using a combination of elution from a column of DEAE-cellulose and a gel filtration on Sephacryl S-300. The dialysed protein was applied to a DEAE-cellulose column (1.5 × 12 cm) that had been pre-equilibrated with buffer B. The column was washed with buffer B until the A_{280} was below 0.1. The elution was performed with a 250 ml linear gradient of NaCl (0 – 300 mM NaCl) in buffer B (flow rate 1 ml/min). The eluted fractions were collected and monitored for protein content (at 280 nm) and for SDH activity (at 340 nm). Fractions containing the highest SDH activity were pooled, pre-cipitated with $(NH_4)_2SO_4$ to 80% saturation and centrifuged at 9400*g* for 30 min.

The pellet was dissolved in a minimal volume of buffer B and applied onto a column of Sephacryl S-300 (1.2 × 57 cm) previously equilibrated with buffer B. The column was washed with buffer B until the A_{280} was below 0.05. Elution with buffer B was performed (0.2 ml/min) and fractions with the highest SDH activity (chapter 4.6.1, page 35) were pooled.

4.5.3. Affinity chromatography

Further purification was done by 2′,5′-ADP-Sepharose 4B (1 × 10 cm), pre-equilibrated with buffer B. The SDH solution was applied to a column and absorbed. The column was washed with buffer B, until the A_{280} was below 0.05. Then the SDH was eluted with 5mM $NADP^+$ in buffer B, and fractions of 1 ml collected. The fractions with the highest SDH activity (chapter 4.6.1, page 35) were pooled and measured for the protein content.

4.6. Activity assay and determination of reaction rate of SDH

4.6.1. SDH activity assay

The shikimate dehydrogenase activity was monitored spectrophotometrically, following the increase in absorbance at 340 nm of forming NADPH during the oxidation of shikimic acid into 3-dehydroshikimic acid. The SDH assay mixture (total volume of 1 ml) contained 100mM AMP-NaOH buffer (pH 9.0), 0.2mM $NADP^+$ and 3mM shikimic acid. The reaction was started by addition of the enzyme (50 µl). Measurements (3 repeats minimally) were performed at room temperature.

The enzymatic activity “a” (µmol.min^{-1}.ml^{-1}) was calculated using equation (1) and related to the protein content as specific activity “$a_{sp.}$” (U.mg^{-1}) using eqation (2). The activity “a” was related to the fresh weight “F. W.” (µmol.min^{-1}.g^{-1} F.W.) of the plant material according to the equation (3).

$$a = \frac{{}_{\Delta}A \cdot V_t \cdot 1000}{\varepsilon \cdot t \cdot l \cdot V_e} \tag{1}$$

$$a_{sp.} = \frac{total\ \ activity}{total\ \ protein} \tag{2}$$

$$a\ (F.W.) = a\frac{V_p}{m} \tag{3}$$

$_{\Delta}$A - change in absorbance at 340 nm for 1 min.; V_t - total volume of the reaction mixture in a cuvette (1 ml); ε - the molar absorption coefficient for NADP(H) i. e. 6200 $mol^{-1}.l.cm^{-1}$; t - time of reaction (1 min.); l – cuvette length (1 cm); V_e - volume of SDH solution added to the reaction mixture (50 µl); V_p - volume of the extraction buffer used (ml); m - weight of fresh plant material used for extraction (g)

4.6.2. pH optimum of SDH

The pH optimum was measured as dependence of the SDH activity to pH of the reaction mixture. The 1 ml of SDH assay mixture contained 400 µl buffer of corresponding pH (80 mM), 3mM shikimate, 0.2mM $NADP^+$. The reaction was started by adding 50 µl of the enzyme preparate. All measurement were performed at the room temperature in a short time period. The optimum pH of the enzyme was determined over the pH ranges: 200 mM MES-NaOH buffer (pH 5.2 – 7.2); 200 mM Tris-HCl buffer (pH 6.8 – 8.6); 200 mM glycine-NaOH buffer (pH 8.6 – 10.6); 200 mM AMP buffer (pH 9.0 – 10.5); 200 mM Na_2CO_3-$NaHCO_3$ buffer (pH 9.5 – 11.0); 200 mM Na_2HPO_4-NaOH buffer (pH 11.0 – 12.0).

4.6.3 Determination of K_m and V_{max}

The initial study of an affinity of SDH for each of its substrates, i. e. shikimate and $NADP^+$, was performed by measuring the activity of the partially purified enzyme by varying the concentration of free $NADP^+$ (0.025 – 1mM) and varying the concentration of free shikimate (0.025 – 3mM), respectively, while the concentration of the second substrate was maintained at saturation level, i. e. 3 mM for shikimate (SKA) and 0.2 mM for $NADP^+$ respectively. The measurements were performed as described in the chapter 4.6.1. Kinetic parameters were obtained by fitting the data from a substrate saturation curve to the equation (4). The data were processed by non-linear regression with MS Excel program.

$$v = \frac{V_{\max} \cdot [S]}{K_m + [S]} \tag{4}$$

[S] - substrate concentration ($mmol.l^{-1}$); V_{max} - maximum velocity of enzyme reaction ($\mu mol.min^{-1}.ml^{-1}$); K_m - the Michaelis constant ($mol.l^{-1}$)

4.6.4. Determination of the kinetic mechanism of the two-substrate reaction catalyzed by SDH

Using the imaginative nomenclature of Cleland, two-substrate reactions can be classified as ping-pong or sequential. Sequential kinetics can be distinguished from ping-pong kinetics by initial rate studies by measuring the initial rates as a function of the concentration of one substrate while holding the concentration of the second substrate constant. The study of the kinetic mechanism of SDH was performed by varying the concentration of free NADP$^+$ (0.05 – 0.5 mM) and varying the concentration of free shikimate (0.2 – 3 mM) and measuring the activity of the SDH in these systems. Thus the SDH assay mixture (total volume of 1 ml) contained 100mM AMP-NaOH buffer (pH 9.0), 0.05 – 0.5mM NADP$^+$ and 0.2 – 3mM shikimic acid. The reaction was started by addition of the enzyme (50 µl). Measurements were performed at room temperature.

The experimental data obtained from the dependence of the reaction rate on the concentration of substrates (SKA, NADP$^+$) were fit into general reaction rate equations for the two-substrate reactions, classified as ping – pong (Eq. 5) and sequential (Eq. 6), and linearisation methods, as the double reciprocal plot, applied to resulting data.

$$v = \frac{V_{\lim} \cdot [A] \cdot [B]}{[B] \cdot K_{mA} + [A] \cdot K_{mB} + [A] \cdot [B]} \tag{5}$$

[A] - concentration of NADP$^+$ (mmol.l^{-1}); [B] - concentration of SKA (mmol.l^{-1}); $V_{\lim}$ - maximal velocity of enzyme reaction (µmol.min^{-1}. mg^{-1}); $K_{mA\,(B)}$ - the Michaelis constants (mol.l^{-1}) for the substrate A, B.

$$v = \frac{V_{\lim} \cdot [A] \cdot [B]}{[B] \cdot K_{mA} + [A] \cdot K_{mB} + [A] \cdot [B] + K_{A} \cdot K_{mB}} \tag{6}$$

[A] - concentration of NADP$^+$ (mmol.l^{-1}); [B] - concentration of SKA (mmol.l^{-1}); $V_{\lim}$ - maximal velocity of enzyme reaction (µmol.min^{-1}. mg^{-1}); $K_{mA\,(B)}$ - the Michaelis constants (mol.l^{-1}) for the substrate A, B; K_A - the dissociation constant for the complex enzyme – substrate A, B.

4.6.5. Effect of various secondary metabolites on the SDH reaction rate

25 different secondary metabolites (3-hydroxyflavone, 4-methylubeliferron, ampelopsin, caffeic acid, cinnamic acid, curcumin, flavanone, flavone, gallic acid, hesperetin, chrysin, morin, naringin, p-anisic acid, p-coumaric acid, quinnic acid, resveratrol, scopoletin, scopoline, sinapic acid, syringic acid, tannic acid, t-ferulic acid, vanillin, β-naftoflavone) were screened for their potential regulatory effect to the SDH activity and promising modulators were chosen for further inhibition studies (i. e. coumaric acid and ferulic acid). The SDH assay mixture for the inhibition study was composed of 100mM AMP-NaOH (pH 9.0), 0.2mM NADP$^+$, 3mM shikimate and 1mM inhibitor (diluted in 96% ethanol). In case of control (blank) the 100 µl of 96% ethanol were added to assay mixture instead of modulator. The reaction was started by addition of 50 µl of the enzyme, following the assay procedure described in the chapter 4.6.1, page 35.

4.6.6. Effect of various ions on the SDH activity

Potential regulatory effect of ions Zn^{2+}, Cu^{2+} (0.1mM); Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, Mn^{2+}, NH^{4+} (5mM) on SDH activity was tested. Substances were diluted in water and added to final enzyme activity assay mixture (100 µl) instead of water. The assay mixture was composed of 100mM AMP-NaOH (pH 9.0), 0.2mM NADP$^+$, 3mM shikimate. . The reaction was started by addition of 50 µl of the enzyme, following the assay procedure described in the chapter 4.6.1, page 35.

4.6.7. Inhibition studies

The inhibition constants and type of inhibition for chosen secondary metabolites (*t*-ferulic acid and *p*-coumaric acid) were established with 3 concentration of inhibitor, i. e. 0.1, 0.2, 0.3 mM, towards 5 various concentration of shikimate, i. e. 0.1, 0.3, 0.5, 1.0 and 3.0 mM, at room temperature and pH 9 (100 mM AMP-NaOH buffer). As control, same assay mixture was used, replacing the 100 µl of inhibitor with 96% ethanol.

The inhibition constants were obtained by fitting the experimental data to the three equations for three types of inhibition – non-competitive (Eq. 7), competitive (Eq. 8) and mixed inhibition (Eq. 9). The data were processed by non-linear regression with the MS Excel program.

$$v = \frac{V_{\mathrm{lim}}[A]}{(K_m + [A])\left(1 + \frac{[I]}{K_i}\right)} \tag{7}$$

$$v = \frac{V_{\mathrm{lim}}[A]}{K_m\left(1 + \frac{[I]}{K_{ic}}\right) + [A]} \tag{8}$$

$$v = \frac{V_{\mathrm{lim}}[A]}{K_m\left(1 + \frac{[I]}{K_{ic}}\right) + \left(1 + \frac{[I]}{K_{iu}}\right)[A]} \tag{9}$$

V_{lim} - the maximal reaction rate; [A] - the substrate concentration; [I] - the inhibitor concentration; K_m - the Michaelis constant for the substrate; K_{ic} and K_{iu} - the inhibition constants (competitive and uncompetitive, respectively) for the inhibitor derived from the slope and intercept, respectively in a Lineweaver - Burk plot, K_i - dissociation constant for the inhibitor.

4.7. Electrokinetic methods

4.7.1. SDS-PAGE

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out using a Biometra apparatus. The separations were performed on 12 % separating gel and 5% stacking gel of the common tabled composition [110].

The prepared samples of the precipitated proteins (chapter 4.4.3., page 34) were mixed with the SDS-PAGE sample buffer in ratio 1:1, then incubated in thermostat for 10 min. (at 100 °C) and immediately loaded into the wells of prepared stacking gel using Hamilton syringe. Protein standard was applied in one position (5 µl/well). The sample buffer consisted of 0.13M Tris-HCl buffer (pH 6.8), 0.005% BPB, 100mM DTT, 70mM SDS and 20% glycerol. Electrophoresis buffer was composed of 18.8 g glycine, 3 g Tris and 1 g SDS in 1 l of distilled water. The electrophoresis was performed at initial voltage of 70 V, changed to 140 V after the zone of tracking dye reached the top of the running gel.

After the separation, the gels (12 %) were stained for protein with Coomassie Brilliant Blue solution, consisting of Coomassie Brilliant Blue R250 (2 $g.l^{-1}$), Coomassie Brilliant Blue G (0.5 $g.l^{-1}$), ethanol (42.5 %), methanol (5 %) and acetic acid (10 %) in distilled water, overnight. Afterwards, the gel was placed in the destain solution, consisting of the acetic acid, ethanol and distilled water in ratio 1 : 2.5 : 6.5.

4.7.2. RN-PAGE

Method developed by Drab, et al. [111] was followed. In this modification of the native blue electrophoresis "red native electrophoresis" (RN-PAGE) the dye Ponceau Red is used instead of Coomassie Brilliant Blue (CBB) to impose uniform negative charge on proteins to enable their electrophoretic separation according to their relative molecular masses. As Ponceau Red binds less tightly to proteins, in comparison with Coomassie Blue, it can be easily removed after the electrophoretic separation and a further investigation of protein properties is made possible - e.g. an enzyme activity detection (thus determination of Mr) [111].

As the standard proteins the myoglobine from equine skeletal muscle (17 800), chymotrypsinogen A (25 000), ovalubumine (45 000) and BSA (mono-, di- and trimer, 67 – 201 000) were used.

Electrophoresis was performed using discontinuous system consisting of 4% stacking polyacrylamide gel and 13% separation polyacrylamide gel. Cathode buffer contained 50 mM Tricine, 15 mM BisTris-HCl, 0.012% Ponceau Red RR, pH 7.0. Anode buffer contained 50 mM BisTris-HCl, pH 7.0. And sample buffer contained 20% glycerol 50mM BisTris-HCl and 0.02% Ponceau Red RR, pH 7.0. The 13% sepataring polyacrylamide gel contained 13% acrylamide/bis-acrylamide, 0.012% Ponceau Red RR, 0.4% ammonium persulfate, 0.045% TEMED and 12.5mM anode buffer. The 4% stacking polyacrylamide gel contained 4% acrylamide/bis-acrylamide, 0.012% Ponceau Red RR, 0.23% ammonium persulfate, 0.27% TEMED, 12.5mM anode buffer and distilled water. The protein samples, mixed with sample buffer in ratio 1:1, were applied into the gell wells (60 µg/well). Electrophoresis was performed at 60 V for 30 min. and at 130 V for next 60 min. using water cooling system incorporated in electrophoresis apparatus.

After the separation, proteins were detected in gel using either standard CBB stain, and the SDH activity was detected in gel. The SDH assay mixture contained 100mM AMP-NaOH buffer (pH 9.0), 0.2mM NADP$^+$ and 3mM shikimic acid, with incubation at 37°C for 30 min. After the enzymatic reaction, 10 mg/ml of iodonitroterazolium chloride (INT) with 5 µg/ml phenazine methosulfate (PMS) were added. The dark red color, which is formed by reduction of INT and PMS corresponds to activity of the SDH.

4.7.3. Isoelectric focusing

The isoelectric point was determined by isoelectric focusing (IEF) performed with Pharmacia system and ECPS source. The 5 µl of the concentrated enzyme sample (chapter 4.4.2., page 33) was applied on the Servalyt Precotes gel with the application of the Serva IEF Marker 3-10 (5 µl) in the starting position parallel to that of sample. The IEF was run at initial voltage 300 V for 30 min. Then the voltage limit was set to 1200 V (4 mA) and the IEF was stopped after reaching 5000 V. h. (approximately after 4 hours). After the IEF procedure, the protein marker was stained separately with the standard CBB stain (chapter 3.7.1., page 41) and the band with the enzyme was detected for the SDH activity using method described in chapter 4.7.2, page 41, with INT and PMS reagens.

4.8. Protein content determination

Protein concentration in samples was determined by the method of Lowry (1951), using reagent “C” consisting of 2% Na_2CO_3 in 0.1M NaOH (A) and 0.5% $CuSO_4$ in 1% sodium tartrate (B), mixed in ratio A : B = 50 : 1, and Folin–Ciocalteu reagent (100 µl of the water diluted solution in ratio 1 : 1), added after 10 min. of incubation (at room temperature) of reagent C with added sample (200 µl). After 30 min. reaction protein content was measured at 500 nm, against control containing 200 µl of distilled water instead of the sample.

The bovine serum albumin (BSA) solution (concentration 0 – 0.5 mg.ml^{-1}) was used as standard protein for a protein calibration line determination.

4.9. Molecular weight determination by gel chromatography

Molecular weight of SDH was determined on Sephacryl S-300 (1.2 × 57 cm) column with ovalubumin (45 000), BSA (67 000) and lactate dehydrogenase (LDH; 140 000) as molecular weight standards, in an individual analytical gel chromatography separations. Each of the protein preparates was applied onto a column and washed with buffer B (pH 7.8), with collecting of eluate in 2-ml fractions. The presence of proteins in fractions was determined by measuring of absorbance at 280 nm. Additional measurements of activity of SDH (chapter 4.6.1, page 35) and LDH in individual fractions were performed. LDH catalyze formation of NAD^+ at 340 nm, causing decrease in absorbance. The LDH assay mixture contained 100mM Tris-HCl buffer (pH 8.1), 2mM sodium pyruvate and 0.5mM NADH, plus 50 µl of the enzyme extract for starting the reaction. The molecular weight was estimated by using an elution volume (V_e) plot against log M_r of standard proteins.

5. Results

5.1. Screening of plant extracts for SDH activity

The activity of SDH was determined in crude extracts of 15 types of vegetable (mainly in roots), using method described in chapter 4.4.1. (page 33) and chapter 4.6.1, (page 35). Each measurement was performed with a freshly prepared crude extracts of particular vegetable. Whereas onion, radish, and broccoli crude extracts had low SDH activity, the highest SDH activity was found in the crude extracts from parsley root (1.02 µmol.min^{-1}.g^{-1} F. W.) and zucchini fruit (0.76 µmol.min^{-1}.g^{-1} F. W.), as shown in Figure 13. Thus fresh parsley root was chosen as the most suitable vegetable for the isolation of SDH.

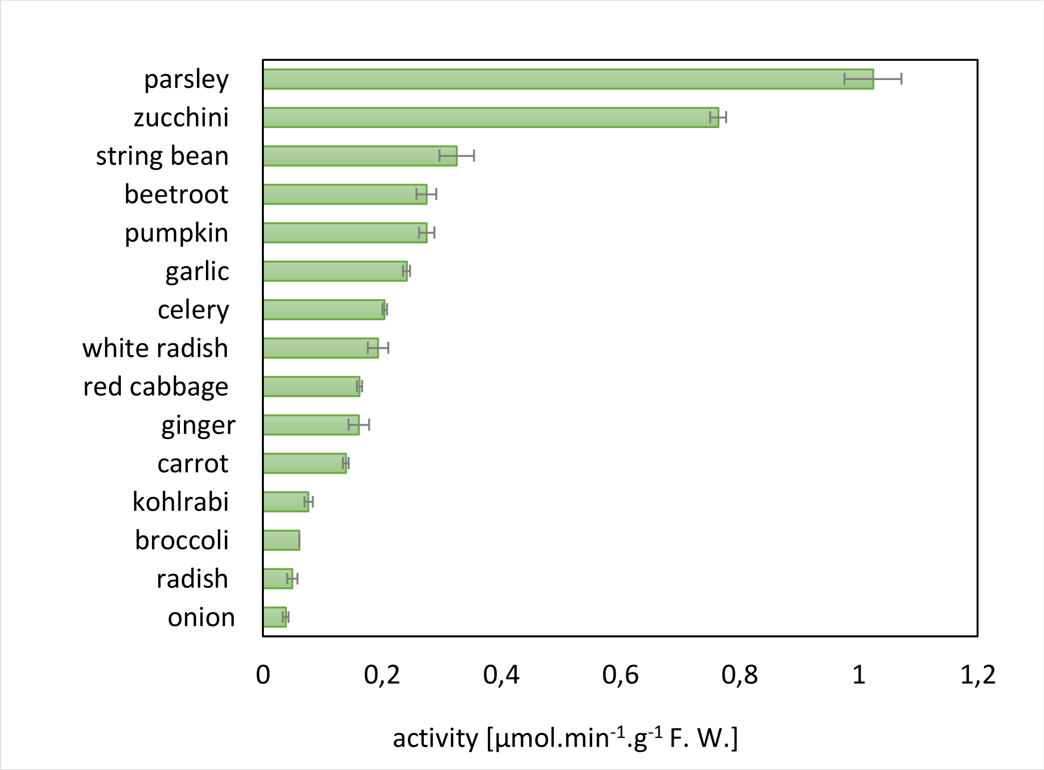


Figure 13 Fresh weight activity of SDH in various plant material described in detail in chapter 4.3, page 33.

5.2. Purification of SDH from parsley

The SDH was isolated from 100 g of parsley (*Petroselinum crispum*) roots and purified to a final specific activity of 470 μmol.min^{-1}.mg^{-1}. The purifiaction process comprised precipitation with ammonium sulfate and several chromatographic steps: chromatography on DEAE cellusose, gel filtration, and affinity chromatography (table 5).

Typical protein and activity data for the various stages of purification of parsley SDH are summarized in table 5. Results presented are representative of a number of separate purification processes. The specific activity increased approximately 20-fold by the purification procedure, with overall yield 17 %. Although the yield of purified enzyme afforded by the procedure was low, it provided sufficient material for characterization of the enzyme properties. The SDH was moderately stable in crude extracts, with the highest loss of total activity in the $(NH4)_2SO_4$ – precipitation step, with about 50% of the original activity being recovered. The dialysis against the buffer B at low temperature did not have significant negative effect on SDH activity, and thus was included in all purification procedures to eliminate salts from enzyme preparate.

Table 5 Procedures used for purification of SDH from parsley root

Procedure	Total activity [mmol.min^{-1}]	Total protein [mg]	Specific activity [μmol.min^{-1}.mg^{-1}]	Purification factor	Yield [%]
Crude extract	13.8	542.8	24.1	1	100
$(NH4)_2SO_4$	6.2	94.5	65.6	2.7	45
dialysis	6.1	93.5	65.3	2.7	44
DEAE-cellulose	3.9	10.9	359.2	14.9	28
Sephacryl S-300	2.3	4.8	479.2	19.9	17

Further purification step was done by DEAE-cellulose column. SDH was bound on DEAE cellulose column at pH 7.8 and had to be eluted with the gradient of 0 - 0.3 M NaCl. The fractions eluted with buffer B after absobtion of the enzyme did not show any activity of SDH, suggesting the majority of the SDH present in sample was bound to a column. Minimally 100 ml of elution buffer B had to be applied onto a column for washing out the residual amount of proteins present in absorbed extract. The SDH was eluted as a single peak (figure 14) with the highest SDH activity in fractions collected in the half of 250-ml linear gradient 0 - 0.3 M NaCl in buffer B, and about 70 % of the activity applied was recovered in the fraction with the highest activity.

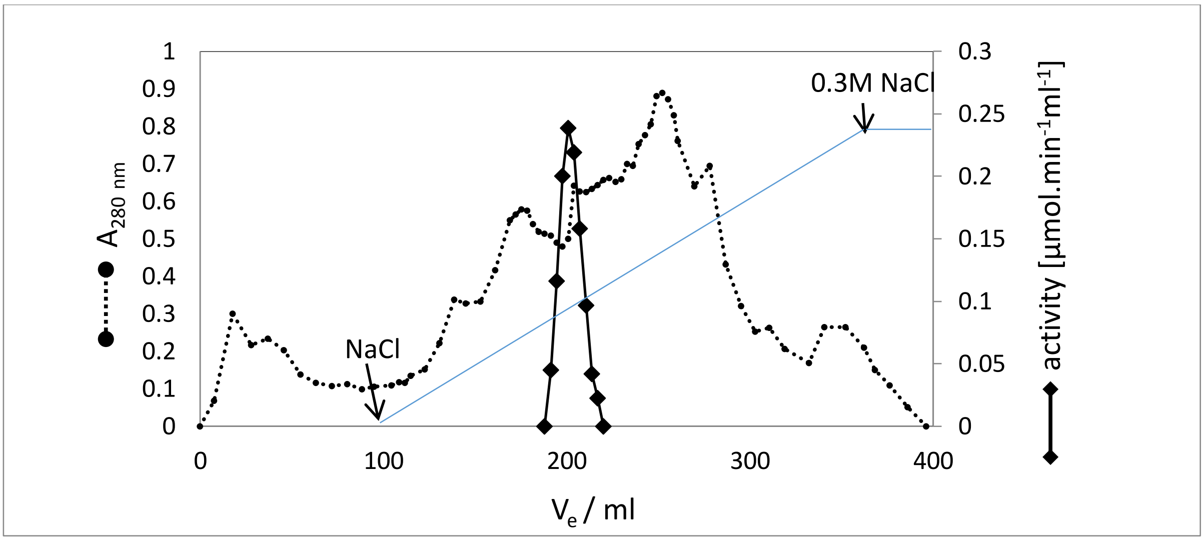


Figure 14 Separation of SDH from parsley root by chromatography on DEAE – cellulose. The column was eluted firstly with a buffer B (pH 7.8) and after that with a linear gradient of NaCl (0-0.3M) in the same buffer. (◆—◆) enzyme activity; (●···●) absorbance at 280 nm.

The most active fractions from DEAE-cellulose step of total volume appoximately 30 ml were precipitated with $(NH_4)_2SO_4$. The sediment resuspended in 200 µl of elution buffer B was applied to a Sephacryl S-300 column. Similarly to the elution from DEAE-cellulose column, the enzyme was eluted as a single peak (figure 15, page 46), and approximately 60% of total SDH activity applied was recovered in the final enzyme preparate. This fraction was used for studies of activity characteristics.

A portion of the final enzyme preparate was subjected to additional purification step by affinity chromatography, however the method used did not give sufficient results. The part of SDH activity applied on the column was detected in fractions collected after absorbtion step, suggesting the SDH passed through the 2’5’ ADP Sepharose 4B column without binding. Thus additional elution with $NADP^+$ of different concentration (1 – 5 mM) in buffer B did not provide any SDH activity in eluate. Consequently this step needs to be revised for the future enzyme purification.

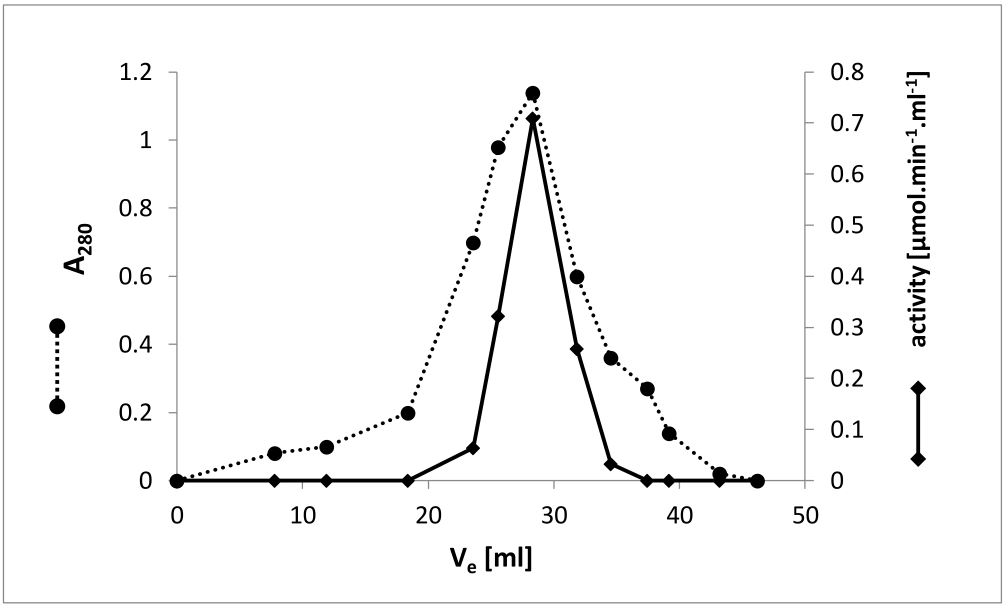


Figure 15 Separation of SDH from parsley root by gel chromatography on Sephycryl S-300 column. The column was eluted with a buffer B (pH 7.8). (◆—◆) enzyme activity; (●·····●) absorbance at 280 nm.

The purity of the samples collected during purification procedure (crude extract, preparate after dialysis and after ion exchange chromatography, and final enzyme preparate) was monitored by SDS electrophoresis (chapter 4.7.1., page 39). The protein bands after separation in 12% acrylamide/bis-acrylamide gel were visualized with CBB stain, as shown on figure 16, page 47. Due to few contaminant proteins present in the final enzyme preparate, the molecular weight determination by SDS-PAGE was not possible.

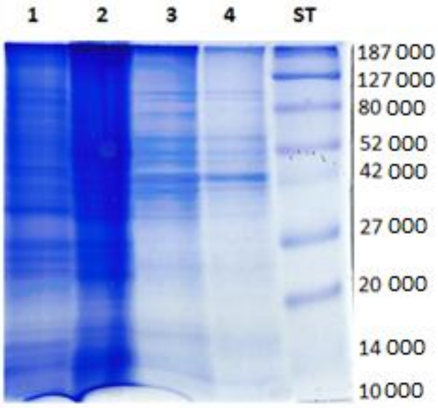


Figure 16 Electrophoretic separation in the presence of SDS documenting purification of the SDH: 1) crude extract, 2) sample after dialysis, 3) sample after ionex chromatography, 4) final enzyme preparate, ST = protein standard (10 – 187 000). Each line contained 60 µg of soluble proteins.

5.3. Characterization of SDH from parsley

5.3.1 pH optimum

The activity of SDH was pH-dependent, having pH optimum between 9.5 – 10.0 (figure 17). A significant decrease in the enzyme activity was found below pH 8.0 and above pH 10.5.

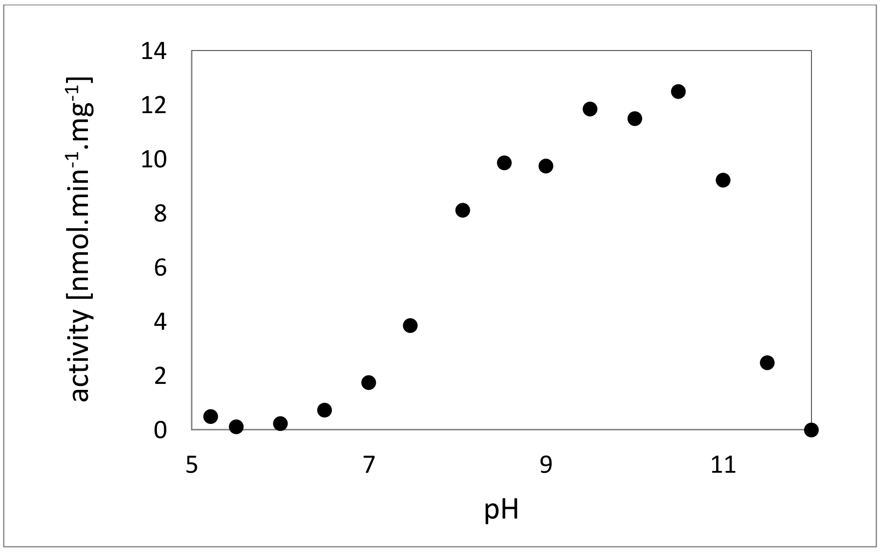


Figure 17 Effect of pH on activity of SDH from parsley.

5.3.2. Molecular weight of SDH

5.3.2.1. Molecular weight determination of SDH by gel chromatography

Molecular weight of purified SDH from parsley was determined by gel chromatography (Sephacryl S-300 column) (chapter 4.9., page 42). As standard proteins ovalubumin (OVA, 45 000), BSA (67 000) and LDH (140 000) were used. LDH and SDH fractions were confirmed by activity assay. The SDH enzyme was eluted as a single peak, indicating the only isoform of this enzyme present in parsley root, with native molecular weight 60 000, determinated from a plot of log M_r against elution volume (figure 18).

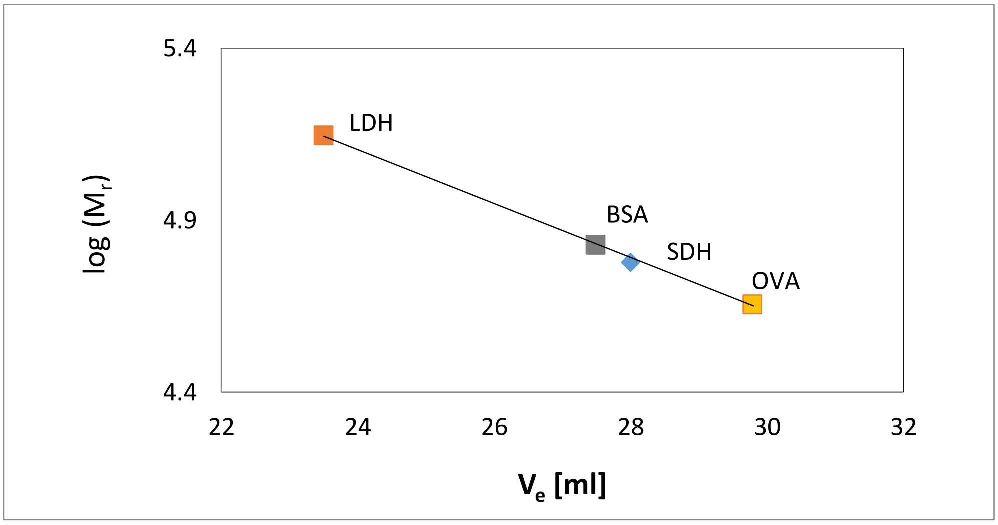


Figure 18 Molecular weight calibration – determination of M_r of SDH from parsley by gel permeation chromatography. Proteins eluted from a Sephacryl S-300 column: 1) LDH (140 000), 2) BSA (67 000), 3) shikimate dehydrogenase (60 000), 4) ovalbumin (OVA, 45 000).

5.3.2.2. Molecular weight determination of SDH by red native electrophoresis

The relative molecular weight of the SDH was determined also by the second method, RN-PAGE on 13% acrylamide/bis-acrylamide gel, as described in chapter 4.7.2. (page 40). As standard proteins myoglobine (17 800), chymotrypsinogen A (25 000), ovalbumin (45 000) and BSA (67 – 201 000) were used, with respect to the results obtained on gel chromatography column. The protein bands after separation in non-denaturating conditions were detected with CBB stain. Line of the gel with SDH was detected for enzyme activity, as described in chapter 4.7.2. (page 41). Only one protein band with the SDH activity was found (figure 19). The relative molecular weight of parsley SDH was determined as 63 000, deduced from a plot of log M_r against retention factor (R_f). To create this calibration curve (line) only protein standards detected in RN-PAGE gel by CBB staining could be used, i. e. BSA dimer, BSA monomer, ovalbumin. Other standards, such as chymotrypsinogen A and myoglobine were not detected by CBB staining, indicating their passing through the pores of gel during separation process.

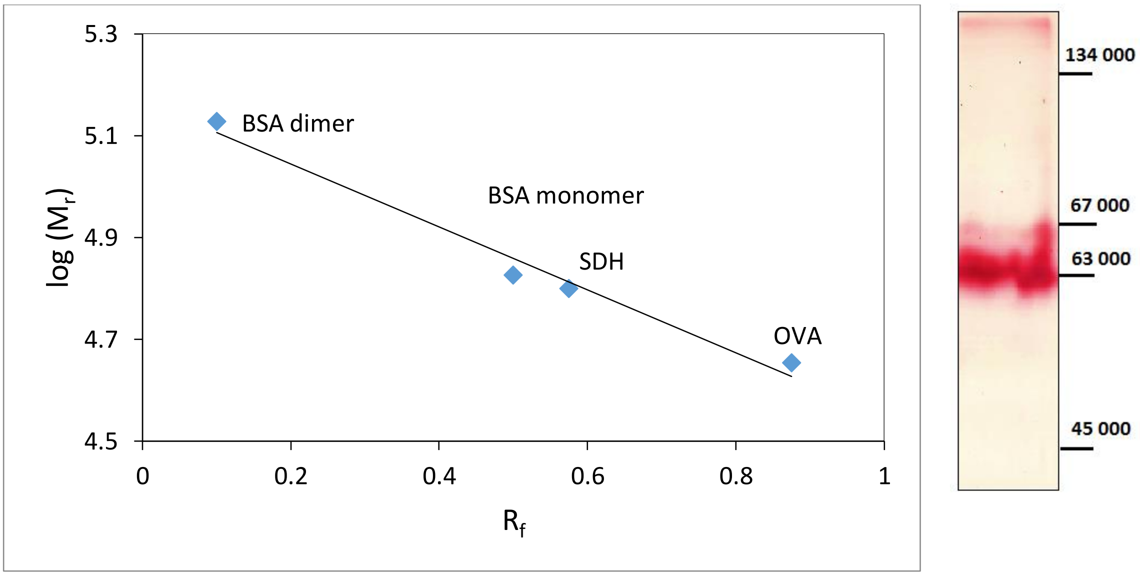


Figure 19 Molecular weight calibration - M_r determination of SDH from parsley by red native electrophoresis. Right-hand side: RN-PAGE gel stain-tested for SDH activity, with positions of molecular weight markers: 1) BSA dimer (134 000), 2) BSA monomer (67 000), 3) SDH (63 000), 4) ovalbumin (45 000).

5.3.3. Isoelectric point

The isoelectric point (pI) of SDH was detected by isoelectric focusing described in chapter 3.7.3. (p. 41). The final enzyme preparate was concentrated before the application on separating gel (chapter 4.4.2., page 33), and thus reducing the amount of salts that have effect on position of sample after IEF [112]. However, most likely a residual amount of buffer ions in sample caused slight concave pattern of SDH band present in gel assayed for SDH activity (chapter 4.6.1, p. 35). Only one form of SDH was detected, with the estimated isoelectric point pI 4.5 (figure 20).

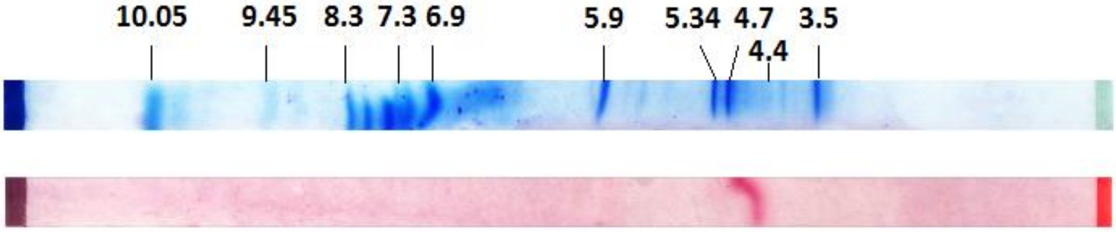


Figure 20 SDH from parsley separated by IEF detected for enzyme activity (red, down), with isolectric focusation markers stained with CBB, with indicated pI values (blue, up).

5.4. Kinetic properties of SDH

5.4.1. Mechanism of reaction catalyzed by SDH

The kinetic mechanism of the two – substrate reaction catalyzed by the SDH present in the parsley root was analyzed by initial rate studies with five various concentration of NADP$^+$, i. e. 0.05, 0.1, 0.15, 0.2 and 0.5 mM, at five fixed concentration of shikimic acid (SKA), i. e 0.2, 0.3, 0.5, 1.0 and 3.0 mM, as described in chapter 4.6.4., page 37. In order to determine the dissociation and Michaelis constants of SDH for both substrates (NADP$^+$, SKA), reciprocal velocities were plotted against reciprocal concentration of a first substrate at several constant conentrations of the second substrate (figure 21, p.51, figure 22, p.52).

The experimental data were fitted using the equations characterizing both types of two – substrate reactions: ping pong (Eq. 5, p. 37) and sequential (Eq. 6, page 37).

Sequential type of of two-substrate reaction is characterized by lines intercepting in one point at (above/below) the x-axis (abscissa) in a double reciprocal plot. The ping-pong mechanism is characterized by a set of parallel lines in a double reciprocal plot. The standard errors counted for both outputs were differing in a small number in all performed experiments, with a slightly lower value for the sequential mechanism of the reaction. The mechanism of reaction catalyzed by SDH will be probably sequential, however the ping-pong mechanism could not be excluded definitely (figure 21, p.51, figure 22, p.52:C, D).

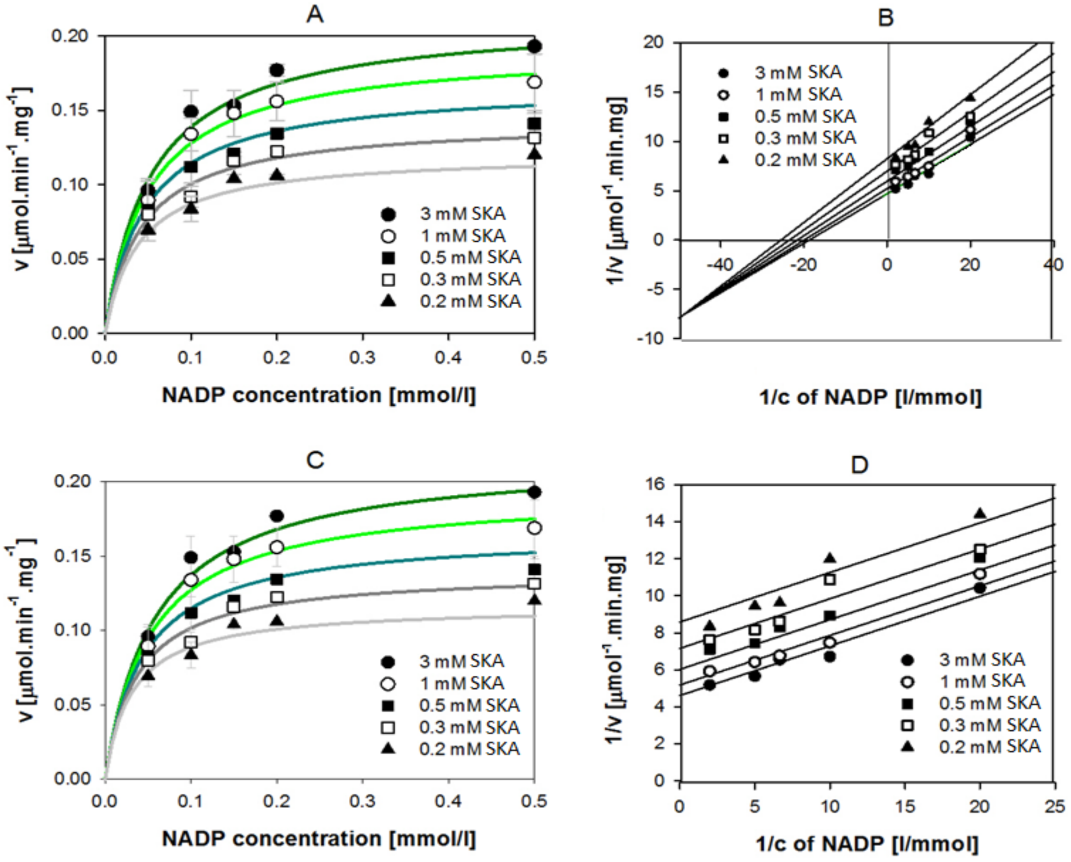


Figure 21 Effect of NADP$^+$ concentration on the reaction rate of SDH from parsley roots at 5 different concentration of shikimate fitted with equation corresponding to sequential mechanism (A) and with equation corresponding to ping-pong mechanism (C). Relevant double reciprocal plots are shown in (B) and (D). Concentration of the NADP$^+$: 0.05 – 0.5 mmol/l; concentration of shikimic acid (SKA): 0.2 – 3.0 mmol/l.

No inhibition by excess of the substrate was observed with any of the SDH substrates.

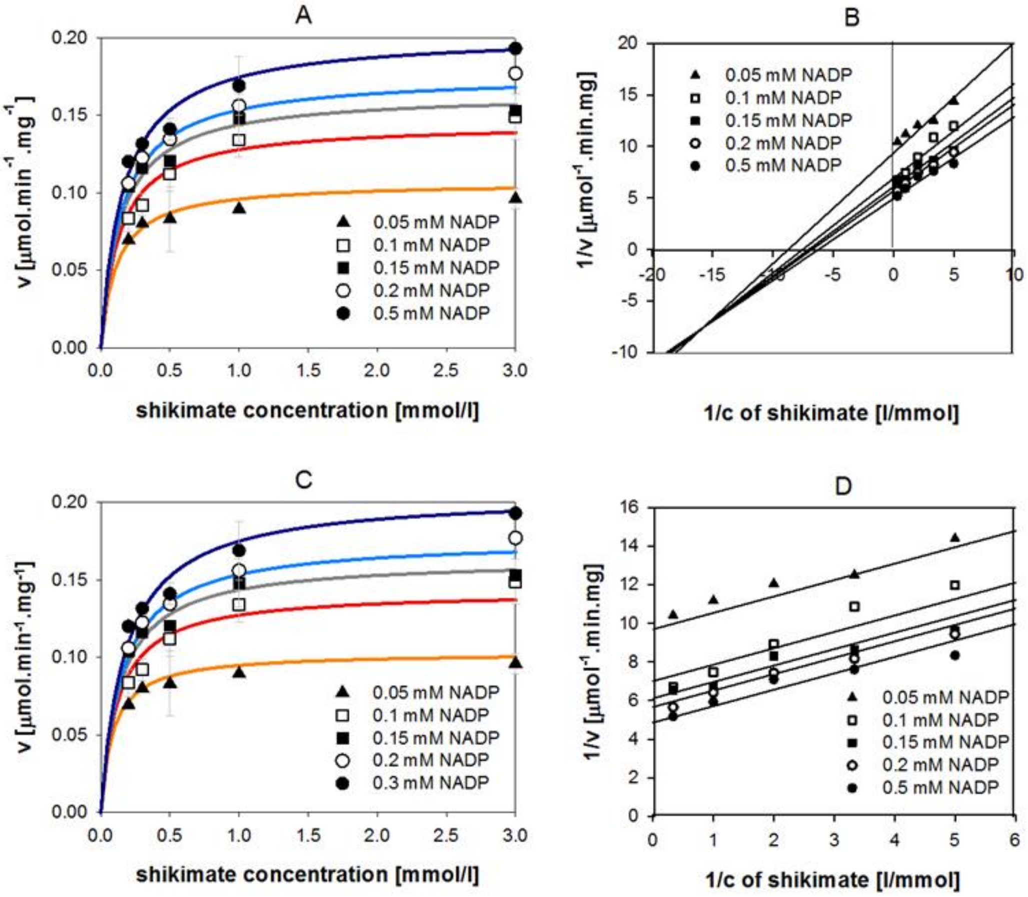


Figure 22 Effect of shikimate concentration on the reaction rate of SDH from parsley roots at 5 different concentration of NADP$^+$ fitted with equation corresponding to sequential mechanism (A) and with equation corresponding to ping-pong mechanism (C). Relevant double reciprocal plots are shown in (B) and (D). Concentration of the NADP$^+$: 0.05 – 0.5 mmol/l; concentration of shikimic acid (SKA): 0.2 – 3.0 mmol/l.

The obtained apparent Michaelis constants of parsley SDH are summarized intable 6. Values shown were calculated from at least 3 sets of data.

Table 6

Kinetic parameters of SDH characterizing the dependence of the reaction rate on the concentration of NADP$^+$ and shikimate – from the initial velocity studies.

	K_m SDH for NADP$^+$	K_m SDH for shikimate	K_A
Ping – pong	0.063 ± 0.012	0.194 ± 0.010	-
Sequential	0.054 ± 0.011	0.161 ± 0.009	0.030 ± 0.008

5.4.2. Effect of various secondary metabolites on SDH activity

The potential regulatory effect of 25 secondary metabolites (figure 23, p. 54) on SDH activity was tested, using method described in chapter 4.6.5., page 38. These substances were dissolved in 96% ethanol and added into the standard SDH activity assay mixture in a volume 100 µl and final concentration of 1 mmol/l, replacing the same amount of distilled water. Concentration of NADP$^+$ and shikimate were maintained at saturating levels (3 mM for SKA and 0.2 mM for NADP$^+$) during assay. In case of control the pure ethanol 96% was added to assay mixture instead of substance. The effect of ethanol on SDH activity was minimal at this concentration, as the additional measurements revealed (data not shown).

The highest inhibitory effect on SDH activity was detected in case of tannic acid and caffeic acid (both 1mM). Lowering their concentration in the reaction mixture 10-times did not change the inhibitory effect that was still very high, with SDH activity about 6 % of no-inhibited enzyme. Substances such as ampelopsin, coumaric acid, ferulic acid, quinnic acid or vanillin had significant inhibitory effect on SDH activity. The cinnamic acid was found as a slight activator of SDH. Simple phenylpropanoids – coumaric acid and ferulic acid were chosen for the detailed inhibition studies.

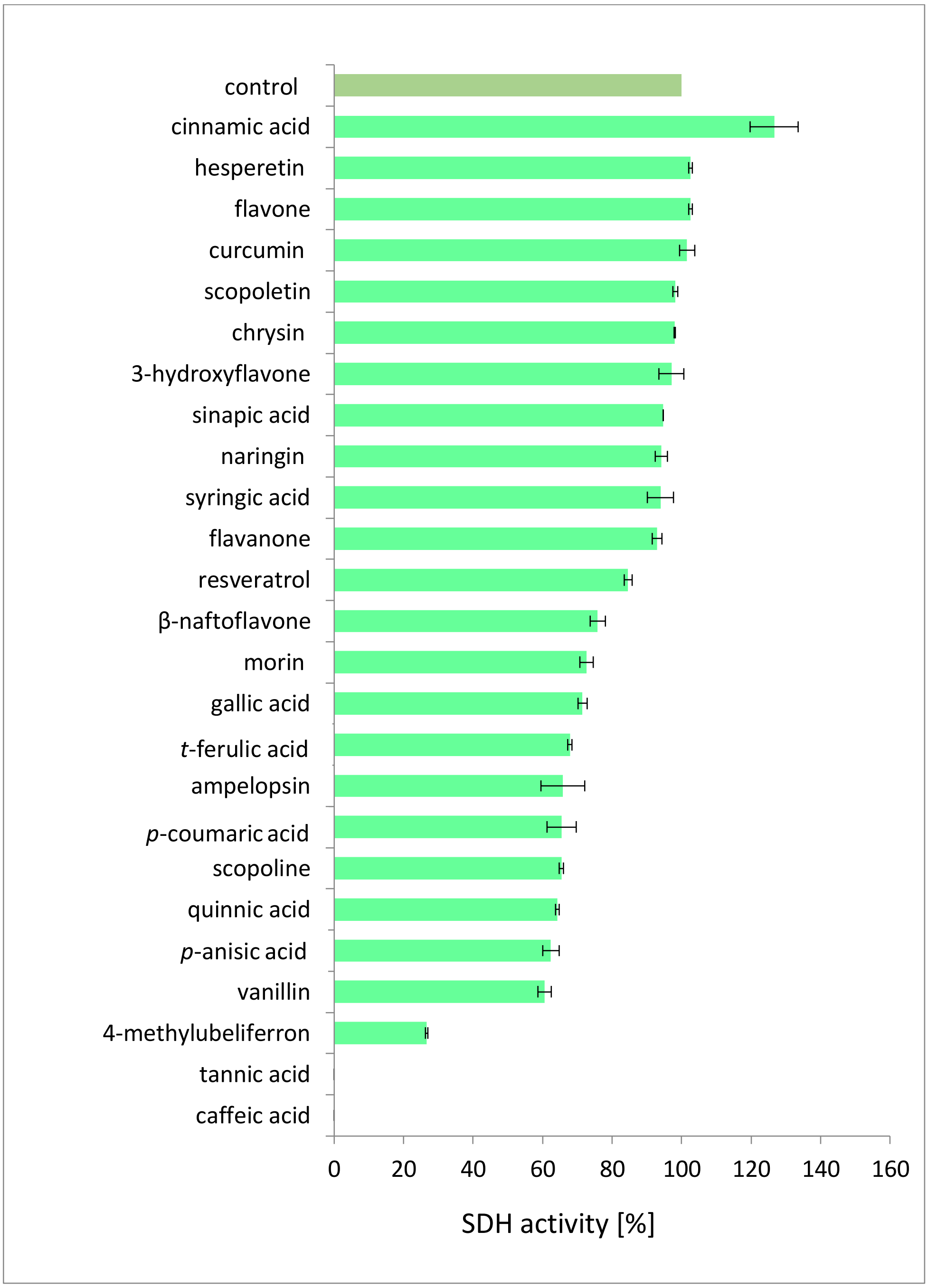


Figure 23 Effect of various phenolic compounds on the activity of SDH from parsley root in comparisson of control without modulator. The substances were present at 1mM concentration.

5.4.3. Inhibition of SDH by coumaric acid and ferulic acid

Detailed inhibition studies were performed with a simple phenylpropanoids, including coumaric acid (CA), ferulic acid (FA) using method described in chapter 4.6.7., page 38. The inhibition constants and type of inhibition for these secondary metabolites were established with 3 concentration of inhibitor, i. e. 0.1, 0.2, 0.3 mM, towards 5 various concentration of shikimate, i. e. 0.1, 0.3, 0.5, 1.0 and 3.0 mM, at room temperature and pH 9 (AMP-NaOH buffer). The experimental data were fitted to various diagnostic plots (double reciprocal plot (figure 24, p. 55, figure 25, p. 56), the Dixon plot, the Hanes-Woolf plot and the Woolf-Augustinsson-Hoffstee plot (data not shown)) and the inhibition constants calculated from Eq. 7 – 9 (page 39) for different types of inhibition.

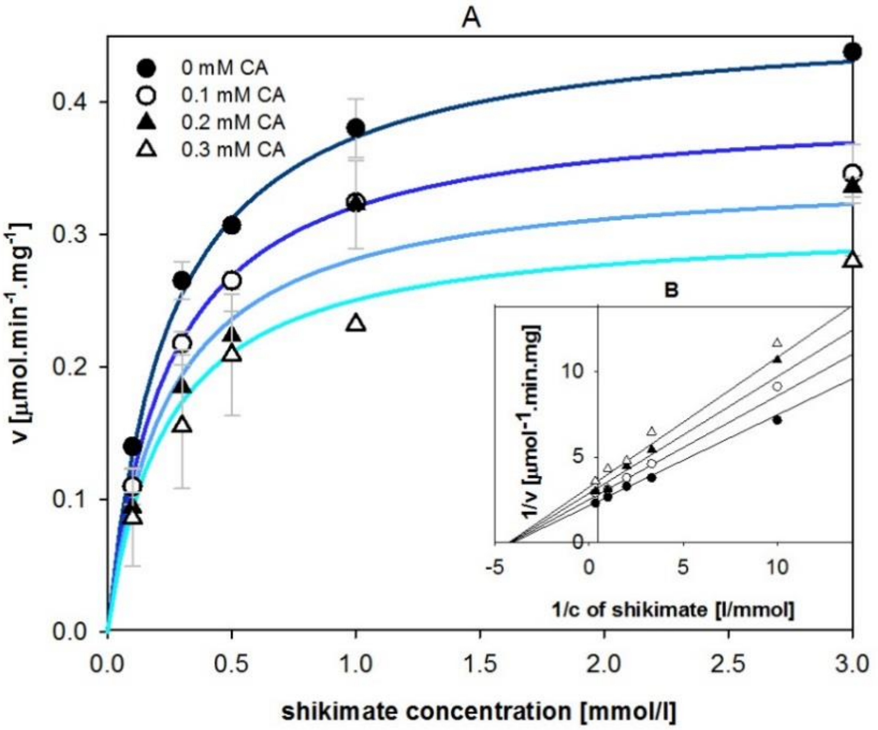


Figure 24 Mixed inhibition of SDH from parsley roots by *p*-coumaric acid (CA), with a double reciprocal plot (insert).

The most suitable results suggested mixed type of SDH inhibition for both coumaric acid and ferulic acid as inhibitors, with the lowest standard errors values compared to other mechanisms.

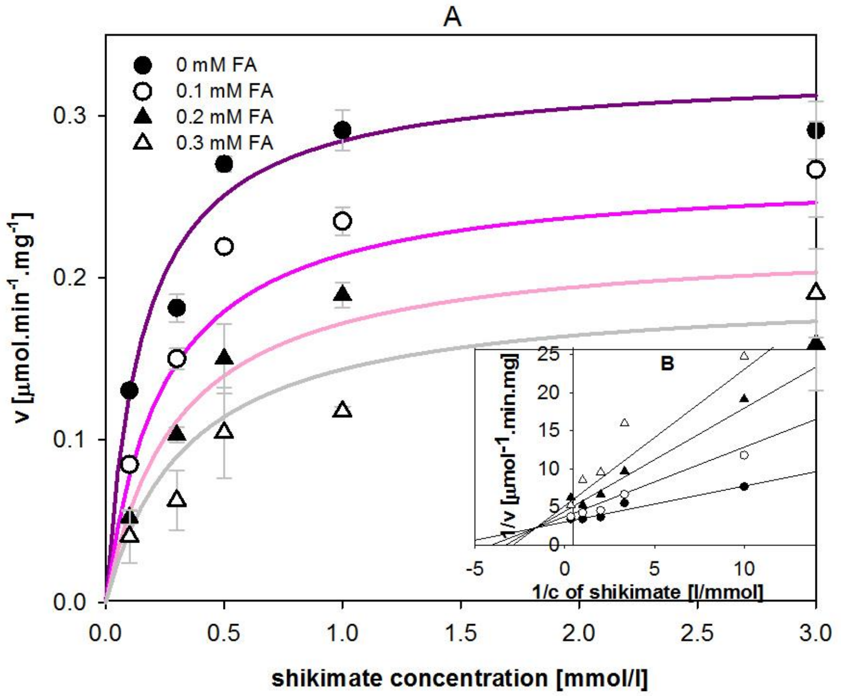


Figure 25 Mixed inhibition of SDH from parsley roots by *trans*-ferulic acid with a double reciprocal plot (insert).

The relevant inhibition constants for both inhibitors are summarized in the table 7.

Table 7

Inhibition constants (mmol/l) for mixed-type of inhibition of SDH from parsley. K_{ic} and K_{iu} inhibition constants for the inhibitor derived from slope and intercept, respectively, of Lineweaver-Burk plots, calculated from Eq. 7 – 9 (page 39).

	K_{ic} [mM]	K_{iu} [mM]
t - ferulic acid	0.08 ± 0.01	0.48 ± 0.07
p - coumaric acid	0.25 ± 0.11	0.65 ± 0.04

5.4.4. Effect of various ions on SDH activity

Figure 26 summarizes the effects on SDH activity when different ions were added to the reaction mixture, using method described in chapter 4.6.6., page 38. This mixture contained a small amount of NaOH, as part of the 100 mM AMP-NaOH buffer (pH 9) and $MgCl_2$ as part of the enzyme preparate in buffer B – considered as SDH with no extra added ions. Generally, none of the tested ions, except for Zn^{2+} and Cu^{2+} had significant effect on SDH activity. Na^+, K^+ and Cl^- were activating, suggesting no dependence of SDH activity on presence of some specific ion, as described in different scientific studies [94].

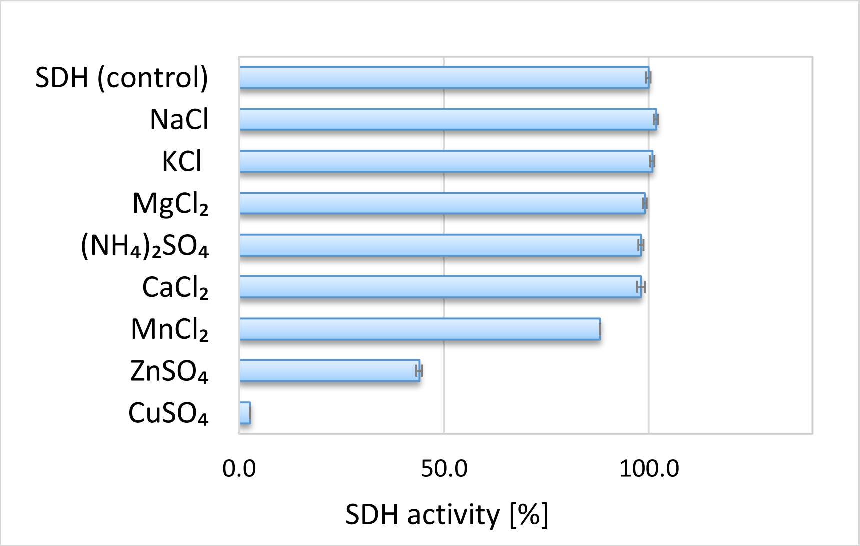


Figure 26 Effect of various ions (0.1mM $ZnCO_4$, $CuSO_4$; 5 mM other ions) on the activity of SDH from parsley. The standard deviation values did not exceed 1%.

6. Discussion

Although shikimate pathway in plants represents very important metabolic flow, which provides not only aromatic amino acids for protein biosynthesis but also it provides precursors for a large variety of other compounds formed by plant in large quantities (particularly phenylpropanoids) [28, 113], there is a little information about the regulation of the key enzyme - shikimate dehydrogenase by respective phenylpropanoid compounds (see table 4, p. 27). It is supposed that regulation of the shikimate pathway occurs mostly at gene expression level [14, 59] however how particular phenolic compounds affect plant SDH was studied less.

One of the reasons why the SDH activity could be low in plant crude extracts is the presence of phenolics. In general, when exposed to air, plant phenolics readily oxidize, generating products that form complexes with proteins and inhibit enzyme activity [28]. This fact was acknowledged also from the previous results of our laboratory. Even though compounds decreasing unwanted interactions of phenolics with proteins (i. e. polyvinylpolypyrrolidone, saccharose, and ascorbate) were used during the extraction procedure, the activity of SDH in herbal extracts usually did not get over 0.05 μmol.min^{-1}.g^{-1} FW. These extracts possess enormous quantities of the phenolics [114]. Therefore the group of vegetables (and preferentially the roots) was chosen for the searching of the source of high SDH activity as indicated in previous work [110]. From the group of 15 vegetable sources the parsley roots (*Petroselinum crispum*) exhibited the highest fresh weight activity of SDH (1 μmol.min^{-1}.g^{-1} FW) (figure 13, page 43) and therefore was chosen for SDH isolation.

SDH isolation procedure was taken from the literature and modified for parsley SDH [115]. It contains ammonium sulfate precipitation, ion exchange chromatography on DEAE-cellulose, gel filtration, and affinity chromatography on 2,´5´-ADP-Sepharose 4B, however the last step was not too much successful. SDH activity was either lost on 2,´5´-ADP-Sepharose 4B column or minor part of applied activity was found in unbounded fraction. Even after applying higher ionic strength on column (1M KCl added to elution buffer) activity of SDH was not eluted. This method has to be further optimized. Probably higher concentration of NADP$^+$ than 5 mM will be needed, for example SDH from *Pinus taeda* needles was eluted with 80-ml linear gradient of 0 – 8 mM NADP$^+$ [95] and up to 20 mM NADP$^+$ is allowed in product instructions. On the other hand, only 1 mM gradient of NADP$^+$ was enough for the 2,´5´-ADP-Sepharose 4B elution of the SDH isolated from *E. coli* [115]. However the final specific activity of parsley SDH (probably monomer) 470 µmol.min^{-1}.mg^{-1} (table 5, page 44) and the purity of the enzyme preparation (figure 16, p. 47) was sufficient for the enzyme kinetics and inhibition studies.

One elution peak with SDH activity after all chromatographic methods implied one isoform of this enzyme (figure 14, p. 45, figure 15, p.46). Also after red native electrophoresis and isoelectric focusation one significant band corresponding to SDH activity was detected (figure 19, p. 49, figure 20, p. 50). The very low number of genes for SDH in model plants (single gene in *Arabidopsis*, two genes in tobacco) [41] indicated the presence of only one isoform in parsley root extract.

Characteristic property of SDH is small size, molecular weight of SDH from other sources did not exceed 75 000 ([31], table 3, p. 25). The relative molecular mass of parsley SDH was determined using 2 different methods - RN-PAGE and gel chromatography. RN-PAGE is native electrophoresis, which employed Ponceau Red to impose uniform negative charge on proteins to enable their electrophoretic separation according to their relative molecular masses without enzyme denaturation and loss of the activity. From both methods it can be concluded that molecular weight of parsley SDH is between 60 000 and 63 000 (figure 18, p. 48, figure 19, p. 49).

The determined pH optimum 9.5 - 10.5 of parsley SDH is in agreement with pH optimum of SDH from other sources, from plants as well as from bacteria [29, 116]. Amino acids residues Lys385 and Asp423 (numbered with respect to the *Arabidopsis* protein), located in the SDH active site, which are conserved in DHD/SHD enzymes and which have been proposed to be involved in proton transfer during catalysis are very probably responsible for such high value [71]. Limited number of SDH studies presented pI. Isoelectric point of parsley SDH was 4.5. This value is little different from pI of SDH from *Spinacia oleracea* L. 6.4 [90].

Kinetic mechanism of 2-substrate reaction catalyzed by parsley root SDH was analyzed by initial rate studies with 5 various concentration of NADP$^+$ at 5 fixed concentration of shikimate and vice versa. Unfortunately, the experimental data are suitable to both types of the mechanism: sequential and ping-pong and it is difficult to distinguish, which mechanism is more appropriate (figure 21, p. 51, figure 22, p. 52). The set of lines obtained for sequential mechanism (figure 21, p. 51, figure 22, p.52: A, B) showed intercept in double-reciprocal plot under the abscissa in both cases, for $NADP^+$ and for SKA as variable substrate, suggesting some degree of interaction between SDH substrates, possibly, supporting each other in binding to the enzyme active center [117]. In ordered sequential reactions, one substrate is obligated to bind to the enzyme before a second substrate. In random sequential mechanisms there is no preference [118].

The value of dissociation constant K_A of binary complex is low indicating tight binding of substrates to enzyme. The sequential mechanism of 2-substrate reaction was found for SDH from other sources, e. g. *Pisum sativum* [94] or *Capsicum annuum* [29] or from *Mycobacterium tuberculosis*, where the sequential mechanism was further specified to ordered [119]. Interestingly, in the last mentioned study, the lines of a double reciprocal plot (specific activity vs. substrate concentration) also intersected below the x-axis (left to ordinate). Further determinations such as product inhibition and kinetic isotope effect studies would be needed to clarify the mechanism certainly. Obtained Michaelis constant of parsley SDH for $NADP^+$ (about 0.06 mM) and for shikimic acid (about 0.180 mM) are most comparable with SDH from *Spinacia oleracea* L. and *Nicotiana tabacum* L. (table 4, page 27).

A limited number of inhibition/activation studies was published for plant SDH (table 4, page 27). Mostly inhibition by divalent metal ions was found: e.g. SDH from *Solanum lycopersicum* was strongly inhibited by Zn^{2+} and Cu^{2+} [33] or by herbicides or by dihydroxybenzoic acid and its derivatives [88, 89, 90]. The SDH from parsley roots was also inhibited by Zn^{2+} and Cu^{2+}, with more pronounced effect with Cu^{2+} ions (figure 26, p. 57). Interestingly, a lot of various compounds of phenylpropanoid metabolism inhibit SDH from parsley roots: simple phenols (gallic acid, *t*-ferulic acid, *p*-coumaric acid, caffeic acid, *p*-anisic acid, and vanillin), cyclic polyol (quinnic acid), coumarine (4-methylumbelliferone), stilben (resveratrol), flavonoids (β-naftoflavone, morin), and polyfenols (tannic acid) as well (figure 23, page 54). From these compounds the strongest inhibitors were tannic acid and caffeic acids. Tannic acid, caffeic acid, and gallic acid tend towards irreversible inhibition with regard to fact that these compounds nearly exponentially reduced SDH activity. It was difficult to find out 3 suitable concentration for the double reciprocal plots. Effect of gallic acid on SDH activity was not stable, exhibiting both inhibitory and activation effects in different sets of experiments (data not shown). With respect to the recently presented information that gallic acid can be created by action of SDH [16], the overall effect on this substance need to be examined more in the following studies.

Since tannic acid contain a lot of hydroxyl groups on ring, gallic acid 3 and caffeic acid 2 hydroxyl groups, the interaction between hydroxyl group and amino acid residue in the enzyme active center is possible. The essential amino acids in SDH active site are Lys385 and Asp423 (numbered with respect to the *Arabidopsis* protein). On the other hand cinnamic acid (simple phenol without hydroxyl groups) activated parsley SDH. Detailed inhibition study with *p*-coumaric and *t*-ferulic acid (containing 1 hydroxyl group on the phenyl ring) were done. Mixed type of inhibition was found for both, *p*-coumaric acid and ferulic acid, however the inhibition of *p*-coumaric acid was near to non-competitive type of inhibition. It indicates the binding of the inhibitor to other site than substrate and that the inhibitor can interact with both, free enzyme and complex enzyme-substrate, respectively. The lower inhibition constants for *t*-ferulic acid, indicated that this compound is slightly stronger inhibitor than *p*-coumaric acid (table 7, page 56).

The regulation of plant shikimate pathway is more complex than in bacteria and yeast, in which 3 aromatic amino acids represents the end products and enzymes of shikimate dehydrogenase pathway can be regulated by feedback inhibiton. In plants aromatic amino acids are only starting point for many branches of this pathway (biosynthesis of individual phenylpropanoids and also other compounds such as phytoalexins, alkaloids, glucosinolates). From the results of this study it seems, that the branch leading to synthesis of precursors of monolignols (initiating with cinnamic acid, continuing with *p*-coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic and sinapic acid) could show the interesting case of feedback regulation of SDH from parsley roots by products. If this regulation of SDH occurs also in other plants (roots) has to be elucidated yet. For example, cinnamic acid, *p*-coumaric acid, vanillic acid, and caffeic acid have no effect on SDH from *Spinacia oleracea* L. and *Capsicum annuum* L. leaves [31, 92]. Therefore the origin of SDH may be important and the regulation of SDH between photosynthetic and non-photosynthetic tissue could be different.

It is known that the activity of SDH is changed developmentally and in response to stress. Enhanced activity of SDH during various types of stress (such as heavy metals, salt, cold) was associated with increased lignification or increased phenolic content [120 - 123]. An increased lignification seems to be defence response to stress (mechanical injuries, mineral defficiency, ultraviolet-B radiation, heavy metals. drought) in the case of many plants [123]. Phenolic compounds play not only an important role in reactive oxygen species removal during stress but they act as metal chelators as well and thus protect lipid against peroxidation [64].

It is possible that increased demand of phenylpropanoids leads to decrease of the concentration of particular phenolics, which under non-stress condition cause inhibition of SDH and therefore activity of SDH is enhanced. In turn higher SDH activity accelerates the shikimate pathway and production of respective phenylpropanoids.

7. Summary

- From the group of 15 vegetable sources the parsley root (*Petroselinum crispum*) exhibited the highest fresh weight activity of SDH (1 μmol.min^{-1}.g^{-1} FW) and therefore was chosen for SDH isolation.
- SDH extracted from parsley root has been partially purified to specific activity 470 μmol.min^{-1}.mg^{-1}, with ammonium sulfate precipitation, ion exchange chromatography on DEAE-cellulose, gel filtration.
- The enzyme exhibited one band after both isoelectric focusing and red native electrophoresis.
- Relative molecular mass of native enzyme was determined by gel chromatography (M_r 60 000) and red native electrophoresis (M_r 63 000).
- Isoelectric point pI 4.5 was determined by isoelectric focusing.
- Optimal pH for SDH action was determined in rage pH 9.5 – 10.0.
- The kinetic parameters of SDH (K_m, V_{max}) for both substrates (shikimate, NADP$^+$) were determined by the initial velocity studies.
- K_m of SDH for shikimate was 161 ± 9 µM and for NADP$^+$ 54 ± 11 µM.
- Excess of shikimate or NADP$^+$ did not inhibit SDH activity.
- Mechanism of two-substrate reaction catalysed by SDH was determined as sequential.
- Inhibitory effect of 25 secondary metabolites was tested and *t*-ferulic acid with *p*-coumaric acid have been chosen for detailed inhibition studies.

8. References

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Svoluji k zapůjčení této práce pro studijní účely a prosím, aby byla řádně vedena evidence vypůjčovatelů.

Jméno a příjmení	Adresa	Číslo OP	Datum půjčky	Poznámka