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SHIKIMATE PATHWAY IN YEAST CELLS: ENZYMES, FUNCTIONING, REGULATION – A REVIEW

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The shikimic acid pathway occurs in various groups of microorganisms, plants and parasites, while it does not occur in animal organisms. An interesting protein synthesized by the yeast is the product of *ARO1* gene known as AROM protein. The occurrence of AROM protein in cells of the yeast is the most important difference as compared to the functioning of the shikimate pathway in other organisms.

The shikimate pathway constitutes a good source for designing antimicrobial agents. The key industrial significance of shikimic acid pathway metabolites consists in shikimic acid application as a starter material for the synthesis of a neuraminidase inhibitor (GS4104) and agents used in the antitumor therapy. The knowledge on that pathway should be continuously extended in the aspect of potential acquisition of its metabolites.

INTRODUCTION

For the first time, shikimic acid was isolated in 1885 from fruits of aniseed – a plant originating from Japan – *Illicium anisatum* [Jiang & Singh, 1998]. The Japanese name of aniseed: *shikimi-no-ki*, has been the backbone to the acid's name. The acid, in turn, being simultaneously the key metabolite of a pathway leading to the formation of chorismate, has given rise to the name of the pathway. The shikimic acid pathway, also referred to as shikimate pathway, occurs in cells of various groups of microorganisms, plants [Braus, 1991; Herrmann & Weaver, 1999] and parasites [Campbell *et al.*, 2004]. It has not been detected in animal organisms and for this reason, the shikimate pathway constitutes a good source for designing antimicrobial agents.

The principle of "unity in biochemistry" assumes that biochemistry of all living organisms is generally the same. The shikimic pathway has been demonstrated to function in the same mode in cells of bacteria, yeast, moulds, parasites or plants. Differences observed refer to the structure of enzymes of the pathway and mode of its regulation.

THE COURSE OF THE SHIKIMIC ACID PATHWAY IN CELLS OF *SACCHAROMYCES CEREVISIAE* YEAST

The shikimic acid pathway proceeds in 7 catalytic steps and combines carbohydrate metabolism with synthesis of aromatic amino acids. It begins with the condensation of phosphoenol pyruvate (PEP) and erythrose-4-phospahte (E4P), and ends with the synthesis of chorismate [Stryer, 1997; Herrmann & Weaver, 1999]. Chorismate is used in the synthesis of L-tryptophan, L-tyrosine, L-phenylalanine, and *p*-aminobenzoic acid. In eukaryotic microorganisms and bacteria the quinoid nucleus of ubiquinone (coenzyme Q) is derived from shikimate pathways. A precursor required for the biosynthesis of Q is 4-hydroxybenzoate derived directly from chorismate. The *S. cerevisiae* yeast may also form this metabolite from tyrosine [Meganathan, 2001]. The course of reactions in the shikimate pathway was presented in Figure 1. In the case of bacteria and fungi, enzymes of the shikimic pathway have been detected in their cytoplasm, whereas in the case of plants – in plastids.

A stage initiating the shikimic acid pathway is condensation of PEP with E4P, which results in the formation of 3-deoxy-D-arabino-heptulosonate-7-phosphate. PEP is a metabolite originating from glycolysis, whereas E4P is an intermediate compound of the pentose phosphate pathway [Floss *et al*., 1972]. The enzyme that catalyzes the first step of the shikimic pathway is 3-deoxy-arabino-heptulosonate-7-phospahte synthase (DAHP synthase, EC 4.1.2.15). At the second stage of the pathway, the formed chain of C_7 saccharide (DAHP) loses its phosphoryl group and is subject to cyclization to 3-dehydroquinone (DHQ) by means of 3-dehydroquinone synthase (DHQ synthase, EC 4.6.1.3) [Bender, 1998]. The DHQ synthase requires NAD^+. The third enzyme that introduces the first double bond to the aromatic ring and, consequently, activates the formation of an intermediate referred to as 3-dehydroshikimate (DHS), is 3-dehydroquinone dehydratase (EC 4.2.1.10). Through reduction with NADP, the DHS is transformed to the shikimic acid (SA), and that stage of the shikimate pathway is catalyzed by shikimate dehydrogenase (EC 1.1.1.25). The next stage of the shikimic acid pathway is phosphorylation of shikimate to 3-phosphoshikimate [Braus, 1991] catalyzed by shikimate kinase (EC 2.7.1.71). Afterwards, 3-phosphoshikimate is subject to condensation with the second molecule of PEP, thus

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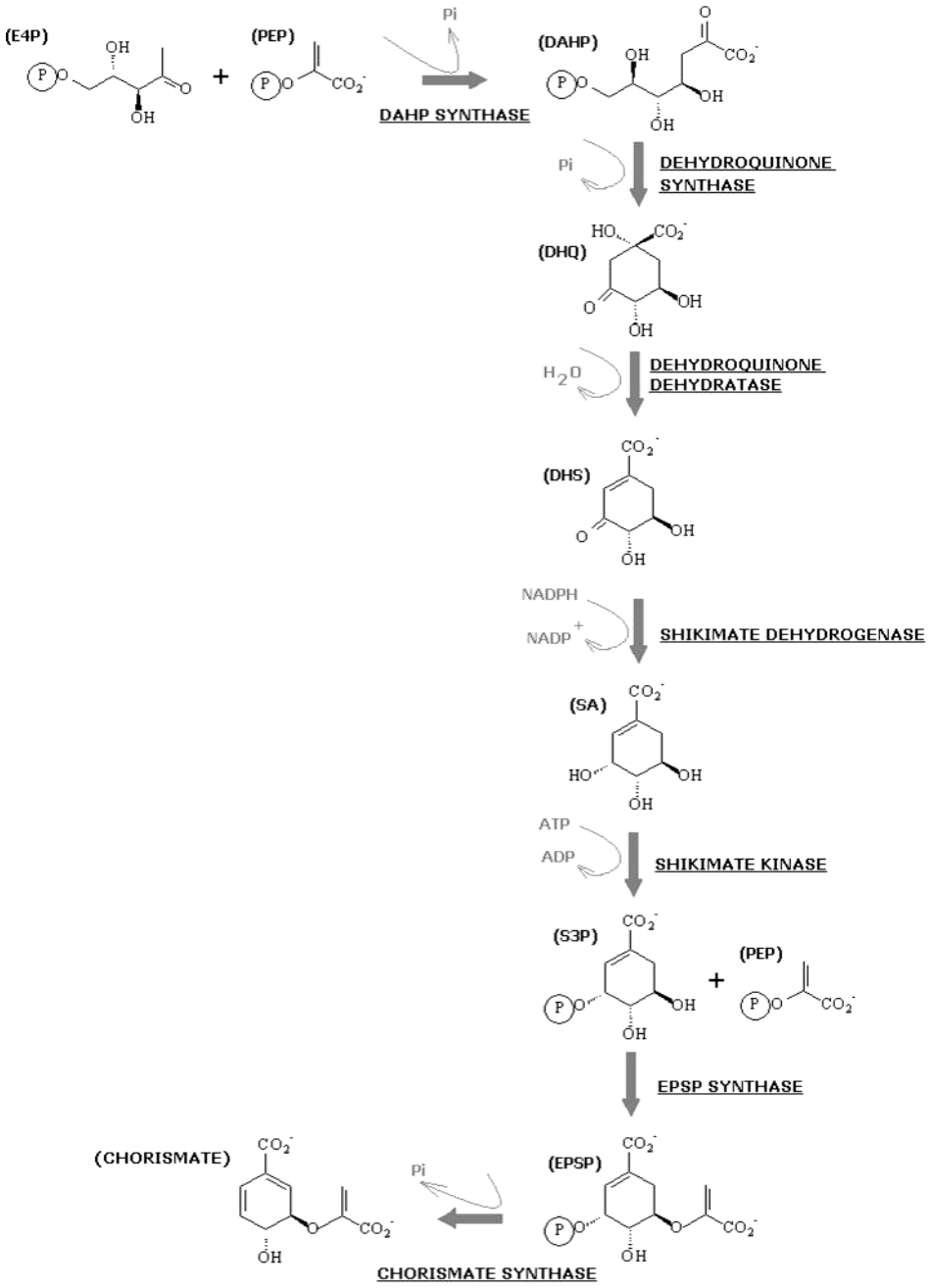

FIGURE 1. The shikimic acid pathway in cell of yeast [Boocock & Coggins, 1983].

producing 5-enolpyruvylshikimate-3-phosphate (EPSP) owing to catalytic properties of EPSP synthase (EC 2.5.1.19). In the next stage, the produced intermediate compound loses its phosphoryl group and is subject to the reduction to chorismate. The final stage of the shikimic pathway is synthase of chorismate (EC 4.2.3.5) used subsequently in the branch of aromatic amino acids and *p*-aminobenzoic acid synthesis.

Chorismate synthase is the only enzyme of the shikimate pathway isolated from cells of *S. cerevisiae* with determined spatial structure [Quevillon-Cheruel *et al.*, 2004]. That enzyme is encoded by *ARO2* gene located on chromosome VII.

An extremely interesting protein synthesized by *S. cerevisiae* is the product of *ARO1* gene. It is a vast (M_r 174555) and complex multidomain known as AROM protein [Duncan *et al.*, 1987]. It is an enzymatic complex constituting a mosaic of monofunctional domains. Since it possesses active centres of: 3-dehydroquinone synthase, 3-dehydroquinone dehydratase, shikimate dehydrogenase, shikimate kinase and EPSP synthase, that protein serves functions of five enzymes. Its active centres are encoded by the respective genes: *ARO1C, ARO1E, ARO1D, ARO1B* and *ARO1A*, located on chromosome IV [Duncan *et al.*, 1988]. The activity of AROM protein was depicted schematically (thick line) in Figure 2. AROM complexes have also been studied from *Schizosaccharomyces pombe* and moulds *Neurospora crassa, Aspergillus nidulans* [Herrmann & Weaver, 1999].

Although a few other multifunctional proteins of yeast cells have already been known, the AROM protein is unique

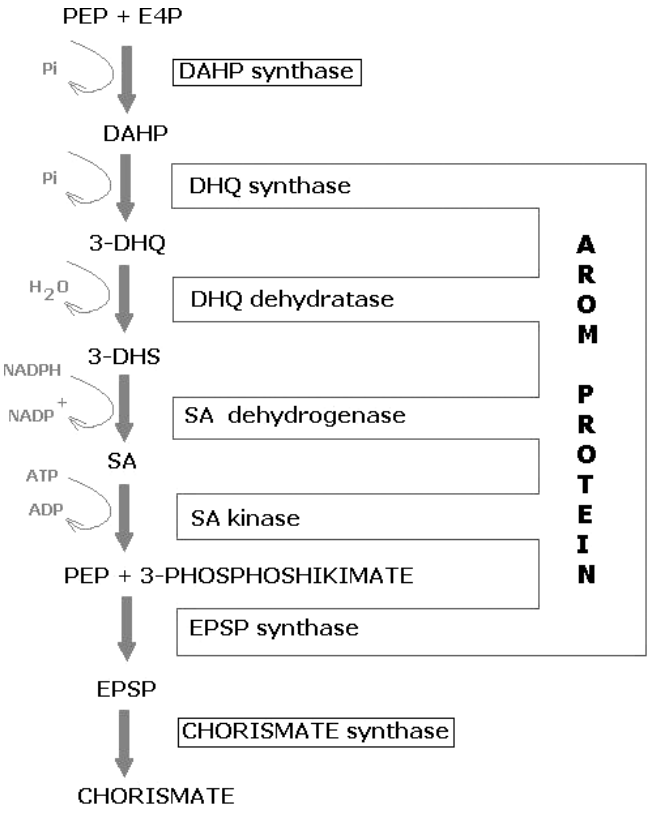

FIGURE 2. Scheme of the course of the shikimic acid pathway in cells of *S.cerevisiae* with consideration given to the activity of AROM protein (thick line). (PEP – phosphoenol pyruvate, E4P – erythrose-4-phospahte, DAHP – 3-deoxy-arabino-heptulosonate-7-phosphate, DHQ – 3-dehydroquinone, DHS – 3-dehydroshikimate, SA – shikimate, EPSP – 5-enol puryvate-3-phosphoshikimate).

for it catalyzes as many as five out of seven subsequent reactions ensuing on one pathway of biosynthesis. In wild strains of *S. cerevisiae* the expression of AROM protein is at a low level. As reported in 1993, the overexpression of that pentafunctional protein is feasible [Graham *et al.*, 1993].

MECHANISMS AND FACTORS REGULATING THE SHIKIMIC ACID PATHWAY

The shikimic acid pathway leads to the synthesis of aromatic compounds and thus its regulation is strictly connected with the need of regulating the synthesis of aromatic amino acids which are an indispensable substrate for proteins formation. In cells of *Saccharomyces cerevisiae* yeast the regulation of amino acids biosynthesis is linked with the so-called "mechanism of general control" being a response to conditions of amino acids starvation.

If yeast occur in a medium rich in nutrients, including amino acids, then the genes responsible for the synthesis of amino acids remain in the so-called dormancy. Already under conditions of amino acid starvation there occurs induction of synthesis of Gcn4p – a transcript activator of genes of amino acids biosynthesis in multiple pathways [Hinnebusch, 1988]. Such a response of the organism of a yeast cell is known under the name of "general control of amino acids". Synthesis of Gcn4p activator occurs also in the case of purines unavailability and glucose shortage [Hinnebusch & Natarajan, 2002]. A yeast strain deficient in the general control transcriptional regulatory system of amino acids biosynthesis is unable to live in the presence of high amounts of phenylalanine and tyrosine [Helmstaedt, 2005].

As postulated by Arndt & Fink [1986], the Gcn4p protein regulates the expression of genes encoding enzymes of the shikimic pathway: *ARO3* and *ARO4* (encoding phenylalanine-dependent DAHP synthase and tyrosine-dependent DAHP synthase) as well as *ARO1* – encoding the AROM protein. The Gcn4p protein binds to promoter fragments TGACTC present in genes and in this way regulates their expression. Gcn4p-dependent genes have been referred to as "Gcn4p targets" and their number in a *Saccharomyces cerevisiae* cell determined during histidine starvation accounts for 539. All those four genes encoding enzymes necessary for chorismate synthesis (*ARO1, ARO2, ARO3* and *ARO4*), as well as three out of four genes encoding enzymes that transform chorismate into tryptophan (*TRP2, TRP3, TRP4* and *TRP5*) are targets of the *Gcn4P* gene. In addition, the Gcn4p factor induces genes of 11 kinases and 26 transcription factors. Gcn4p has also been found to induce transcription of 10 members of the so-called "MCF family", *i.e.* a family of mitochondrial carriers that encode proteins responsible for transport between cytoplasm and mitochondria, namely: *ARG11/ORT1, YHM1, OAC1, YMC1, YMC2, CRC1, YER053C, YOR222W, YPR021C* and *YPR128C*. A yeast genome encodes *ca.* 35 members of the MCF family [Hinnebusch, 1988].

Factors that regulate the shikimic acid pathway in yeast include aromatic amino acids: L-phenylalanine and L-tyrosine. Both these amino acids are synthesized from chorismate. Each synthesis requires energy expenditure from a cell, especially that of so complex compounds as aromatic substances. If during culture the yeast have access to aromatic amino acids, synthesis of some enzymes of the shikimic acid pathway is subject to inhibition. That phenomenon is commonly known and referred to as "negative feedback", *i.e.* regulation of the synthesis of enzymes in a metabolic chain by its end product [Teshiba *et al.*, 1986].

In cells of *Saccharomyces cerevisiae*, the enzyme subjected to regulation is DAHP synthase. Cells contain two isoenzymes of DAHP synthase [Braus, 1991; Teshiba *et al.*, 1986; Paravicini *et al.*, 1989; Künzler *et al*., 1992; Schnappauf *et al*., 1998]. One of the isoenzymes is encoded by *ARO3* gene and the second by *ARO4* gene. The genes are located on chromosome I [Paravicini *et al.*, 1989] and II [Künzler *et al.*, 1992], respectively. The ARO3 and ARO4 izoenzymes consist of 370 amino acids with high sequence homology including 224 identical and 62 different amino acids residues [Schnappauf *et al.*, 1998]. As a result of the feedback both forms are subject to regulation with the product. L-phenylalanine is an inhibitor targeting the first (phenylalanine-dependent DAHP synthase, ARO3), whereas L-tyrosine in the case of the second isoenzyme (tyrosine-dependent DAHP synthase, ARO4) [Braus, 1991; Schnappauf *et al.*, 1998; Herrmann, 1995]. According to the above-cited authors, the phenomenon of inhibition is reversible.

In the year 2002, Hartman *et al.* [2003] published the crystal structure and kinetic studies on chimera and mutant pro-

teins DAHP synthase inhibited by phenylalanine (ARO3) and by tyrosine (ARO4). The DAHP syntase isoenzymes of yeast represent an extended β/α barrels with the catalytic site on the C-terminal face. The important region for regulation in the enzyme is the loop connecting the two half-barrels. Also the structural elements added to the barrel and the form a cavity on the N-terminal side of the β/α barrel are prerequisites for regulation. In the cavity of ARO4 at position 226 there is glycine residue and serine residue at this position in ARO3 [Hartmann *et al.*, 2003]. It was shown [Helmstaedt *et al*., 2005] that the substitution of a single amino acid S195A turns ARO4 into tryptophan-sensitive DAHP enzyme, which is very similar to the homologous from *E. coli* cells.

Known inhibitors of enzymes of the shikimic pathway are analogs of their substrates. The first enzyme of the shikimic pathway – DAHP synthase – is susceptible to fluorinated analogs of phosphoenol pyruvate (PEP), substituting themselves as substrates [Pilch & Somerville, 1976]. The maximum efficiency of transformation of those competitive inhibitors to fluorine-containing DAHP analogs by means of DAHP synthase is negligible. Similar DAHP analogs are competitive inhibitors to DHQ synthase – the second enzyme in the shikimic pathway [Widlanski *et al.*, 1989].

A number of factors have been found to inhibit the activity of EPSP synthase. Although some of those substances are capable of inhibition at a low concentration (milimole or even nanomole), their role as potential antimicrobial agents has not been sufficiently elucidated so far.

One of the EPSP synthase inhibitors with confirmed antimicrobial and herbicidal activity is glyphosate (N-(phosphonomethyl)glycine). It is widely applied herbicide produced by Monsato company. Glyphosate is a growth inhibitor of Gram-negative and Gram-positive bacteria *in vitro* [Amrhein *et al.*, 1983]. It displays bactericidal properties against *Bacillus subtilis* [Fisher & Rubin, 1987], *Klebsiella pneumoniae* [Steinrucken & Amrhein, 1984], *Escherichia coli*, *Salmonella* Typhimurium, as well as antimycotic properties against *Neurospora crassa* and various yeast strains [Bode *et al.*, 1986]. Glyphosate has also appeared to be a growth inhibitor to pathogenic parasites: *Plasmodium falciparum*, *Toxoplasma gonidii* and *Cryptosporidium parvum* [Roberts *et al*., 2002].

In the case of yeast of the genus *Candida maltosa*, glyphosate turned out to be an inhibitor of as many as three enzymes of the shikimic pathway [Bode *et al*., 1984]. Its micromole concentrations inhibited the activity of EPSP synthase, whereas the milimole ones inhibited tyrosine-dependent DAHP and dehydroquinone synthase. The herbicide has also elicited growth inhibition of the discussed yeast strains. According to Bode *et al.* [1986], the most susceptible yeast strain appeared to be *Yarrowia lipolitica,* and elongation of the generation period was reached with as little as 0.05 mmol/L concentration of glyphosate. A 20 mmol/L dose of glyphosate doubled the generation time of *Candida* sp. yeast. This author reports also that no inhibition of yeast growth was observed at simultaneous presence of aromatic amino acids (at a concentration of 1 mmol/L) in the medium containing glyphosate [Bode *et al*., 1984].

In addition, shikimic acid secretion to the medium has been observed during cultures of yeast of the genera: *Saccharomyces, Candida, Hansenula* or *Rhodotorula* in a glyphosate-containing medium [Bode *et al*., 1986]. It is speculated, thus, that glyphosate (being an inhibitor of EPSP synthase) present in the medium is blocking the shikimic pathway at a level of the intermediate, *i.e.* S3P. Simultaneously, pH of the medium decreases during yeast culture and under such conditions S3P is transformed into shikimic acid.

No secretion of shikimic acid to medium was observed in the case of yeast culture at simultaneous presence of glyphosate and aromatic amino acids [Bode *et al*., 1984]. Being inhibitors of DAHP synthase, the amino acids blocked the shikimic pathway at an early stage. In such a case, synthesis of any of the subsequent intermediates, including the shikimic acid, proved impossible [Bode *et al*., 1986].

The phenomenon of shikimic acid secretion to medium has also been observed during culture of yeast with *p*-aminobenzoic acid (PABA) [Reed *et al*., 1959; Surovtseva, 1970]. The quantity of secreted shikimic acid was proportional to PABA addition in the medium. In 2007, earlier reports were confirmed and new observations were made using an HPLC method, which indicated that once applied in high doses the *p*-aminobenzoic acid is an inhibitor of *Saccharomyces cerevisiae* growth [Gientka, 2007].

The key industrial significance of shikimic acid consists in its application as a starter material for the synthesis of a neuraminidase inhibitor (GS4104). It is an antiviral preparation – oseltamivir – produced by the Roche concern under commercial name Tamiflu®. It has been elaborated largely based on surveys of strains of human influenza virus, thus it prevents and alleviates symptoms of that diseases principally [Karpf & Trussardi, 2001]. Scientists and pharmaceutical companies have suggested, however, that it is also likely to be effective in fighting avian influenza virus [Krämer *et al*., 2003]. In 2006, a manuscript was published that describes the application of the shikimic acid pathway for stereoselective synthesis of (-)-zeylenone, *i.e.* an agent used in the anti-tumor therapy [Zhang *et al*., 2006].

Some derivatives of shikimic acid diminish the activity of the shikimic pathway. Thus, studies have been undertaken [Davies *et al*., 1994] to investigate the possibility of applying fluorinated derivatives of shikimic acid as a factor inhibiting the growth of *E. coli*. It has been speculated that (6S)-6-flouroshikimate and (6R)-6-fluoroshikimate, being substrates for shikimate kinase, are transformed to (6S)-6--flouro-EPSP and to (6R)-6-flouro-EPSP, respectively, and are potential inhibitors of the subsequent enzymes. (6R)-6--flouro-EPSP is an inhibitor of chorismate synthase and (6S)-6-fluoro-EPSP is transformed, with a very low efficiency, by chorismate synthase to 6-fluorochorismate [Ramjee & Balsubramanian, 1992]. The 6-fluorochorismate may inhibit the growth of *Neurospora crassa,* and seems to be an inhibitor of PABA synthase, as well as provides a possibility of inhibiting the growth of *E. coli in vitro* [Davies *et al*., 1994; Bornemann *et al*., 1995]. It has been found that (6S)-6-fluoroshikimate protects mice against infections induced by *E. coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*, yet spontaneous immunity has been reaching such a high frequency that the possibility of the clinical application of that compound has been

excluded [Davies *et al*., 1994]. It has been proved explicitly in the year 2004 that a target of (6S)-6-fluoroshikimate is 4-amino-4-deoxychorismate synthase [Bulloch *et al*., 2004].

SUMMARY

The recognition of all aspects of shikimic acid pathway functioning in organisms in which it proceeds is extremely valuable due to the possibility of effective designing of substances with antimicrobial potential that would, simultaneously, be safe to humans. Determination of the spatial structure of enzymes of that pathway facilitates, to a substantial extent, the search for their effective inhibitors, which in turn enables obtaining substances that inhibit the growth of pathogenic bacteria and parasites. Likewise, of outmost significance is further search for herbicides.

It seems that, in the case of yeast, extending knowledge on that pathway should be continued in the aspect of potential acquisition of its metabolites. Recent publications have pointed to ever increasing potential of their application. Due to their structure, which is difficult to obtain in chemical synthesis, individual intermediates of the shikimic pathway may constitute and interesting library of polycyclic compounds. The most promising, however, are manuscripts addressing the application of the shikimic acid in the pharmaceutical industry.

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