Chapter 2

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Fig. 1  A generalized overview of shikimate pathway, major enzymes and products leading to biosynthesis of phenylalanine, tyrosine and tryptophan.
\[
\text{CHO} + \text{CHO} \quad \text{Phosphoenol pyruvic acid} + \quad \text{CHO} \quad \text{Erythrose 4-phosphoric acid} \\
\text{c-o-PO} \quad \text{BCOB} \quad \text{BCOB} \\
\text{Hl.} \quad \text{CH}_4\text{PO}_3\text{H}_2 \\
\text{Phosphoenol pyruvic acid} + \text{Erythrose 4-phosphoric acid} \\
\text{BCOB} \quad \text{BCOB} \\
\text{CH}_4\text{PO}_3\text{H}_2 \\
\]

Deoxy-D-arabino heptulosonate 7-phosphate synthase

3-Deoxy-D-arabinoheptulosonic acid 7-phosphate

Dehydroquinate synthase

5-Dehydroquinic acid

5-dehydroquinate dehydratase

3-Dehydroshikimic acid
NAOPH → Shikimate dehydrogenase

\[ \text{COOH} \]

Shikimate

ATP → Shikimate kinase

\[ \text{COOH} \]

Shikimate acid 3-phosphate

Phosphoenol pyruvate → Pyruvylshikimate phosphate synthase

\[ \text{COOH} \]

5-Enolpyruvyl shikimate acid 3-phosphate

\[ \text{PI} \]

Chorismate synthetase

\[ \text{COOH} \]

Chorismic acid

Chorismate mutase

Prephenic acid

Glutamine → Glutamate

Anthranilate synthase

\[ \text{COOH} \]

Anthranilic acid
THE SHIKIMATE PATHWAY

The shikimate pathway, named after the first characterized intermediate - shikimic acid, was elucidated first in bacteria and fungi with the aid of tracer studies and using the auxotrophic mutants (Srinivasan et al., 1962; review Bentley, 1990). That a common reaction sequence is operative in algae and higher plants for the synthesis of aromatic amino acids was shown by isolation of pathway intermediates (Haslam, 1974). Final confirmation and stepwise deduction of the pathway in higher plants has been followed by isolating ‘nearly all’ the pathway enzymes. The comparison of biochemistry of regulation of these enzymes in bacteria, fungi and higher plants has resulted in molecular characterization of the genes involved and the analysis of the molecular evolution of plants (Crawford, 1975; Byng et al., 1982; Ahmad and Jensen, 1986).

The aromatic amino acids - phenylalanine (phe), tyrosine (tyr) and tryptophan (trp) are synthesized by this pathway as depicted in Fig.1. The basic pathway is the same in microorganisms (eg. E. coli, yeast etc.) and in plants. However, the mechanism of regulation seems to vary. Since this pathway is absent in animals, these amino acids need to be supplemented in human diet and are thus termed ‘essential amino acids.’

The first step in the shikimate pathway is the condensation of erythrose 4-phosphate (a pentose phosphate pathway intermediate) with phosphoenolpyruvate (a glycolytic pathway intermediate). The pathway bifurcates at chorismate and leads to two different routes resulting in the synthesis of either phe and tyr (one branch) or trp (other branch) as shown in Fig.2. Phenylalanine and tyrosine are synthesized from chorismate via a common intermediate, prephenate. Tyrosine can also be made by animals directly from phenylalanine using the enzyme, phenylalanine hydroxylase, which also participates in the degradation of phe. Tyrosine is thus sometimes
Erythrose 4-Phosphate
  +
Phosphoenolpyruvate
  ↓
Shikimate
  ↓
Chorismate
  ↓
Prephenate  Anthranilate
  ↓  ↓
Phenylalanine  Tyrosine  Tryptophan
  ↓
Tyrosine
considered a non-essential amino acid since it can be synthesized from an essential amino acid, phenylalanine.

The studies on the shikimate pathway in microbes have been quite exhaustive (Haslam, 1974; Crawford, 1975; Gibson and Pittard, 1968; Bentley, 1990). In the present review, emphasis has been laid on the findings regarding the enzymes of shikimate pathway with respect to DAHP synthase and chorismate mutase in higher plants.

THE IMPORTANCE OF SHIKIMATE PATHWAY IN HIGHER PLANTS

Aromatic amino acids are the precursors of a vast array of plant substances (Fig.3). There are numerous products derived from the shikimate pathway. Phenylalanine, tyrosine and tryptophan are converted into a number of important compounds in plants. These amino acids are directly or indirectly involved in the synthesis of primary metabolites like - proteins, purines, pyrimidines and secondary compounds like - quinate, phenolics, alkaloids, antibiotics, pigments etc. (Jensen, 1985a). Many nitrogenous compounds, apart from primary metabolites are derived from this pathway, which include folate, anthranilic acid important for trp biosynthesis, several bacterial pigments, antibiotics eg. streptonigrin (anticancerous antibiotic from Streptomyces flocculus) etc. The shikimate pathway serves as a precursor for phenylpropanoid pathway (Fig.3). Among the commercially significant natural products formed from phe and tyr include, alkaloids such as morphine and tannins that inhibit oxidation of wines and components that impart flavour to the spices. These have potent physiological effects and are the basis of many of the neurotransmitters eg.: tyrosine gives rise to a family of catecholamines that include dopamine, norepinephrine and epinephrine:
Fig. 3  Generalized relationship of shikimate acid pathway intermediates, aromatic amino acids and selected secondary metabolites in higher plants. [After D.G. Gilchrist and T. Kosuge. The biochemistry of plants. (1980). A comprehensive treatise: Amino acids and derivatives, vol.5. B.J. Miflin (ed.)].
Phosphoenolpyruvate + Erythrose 4-phosphate

Phosphoenolpyruvate + Erythrose 4-phosphate

Quinate

Dehydroquinate

m-Carboxyphenylalanine

Isochorismate

Shikimate

Chorismate

Prephenate

3,4-Dihydroxybenzoate

D-aminobenzoate

Folate

IAA

Tryptophan → Alkaloids

L-Dopa

Tyrosine

Lignin precursors → Substituted coumarins

Dihydroxyacetate

Dihydroxyacetate

Phenylalanine → Cinnamate

Isolavonoids

Dihydroxyacetate

Dihydroxyacetate

Coumarin
Tyrosine → dopa → dopamine → norepinephrine → epinephrin.

Tryptophan → 5-hydroxytryptophan → serotonin.

Tryptophan also gives rise to neurotransmitters like serotonin. Tryptophan is also the precursor of the growth regulator, indole-3-acetic acid (IAA), which is involved in the regulation of a wide array of biological and physiological processes in plants. The rigid polymer, lignin, is derived from phenylalanine and tyrosine. It is second only to cellulose in abundance in plant tissues (Lehninger, 1993).

Although all higher plants produce significant amounts of secondary metabolites, individual species may produce characteristic substances peculiar to that species for eg. dhurrin (a cyanogenic glycoside) in Sorghum (Singh et al., 1986). In many cases, the physiological and biochemical roles of these metabolites are yet to be unraveled.

Summarizing the importance of the shikimate pathway - basically the three amino acids phe, tyr and trp are the primary metabolites which serve as the precursors for many natural secondary products such as, vitamins, quinones (for electron transport chain), caffeoyl quinate (storage compound) flavonoids, phenolics, coumarins, alkaloids, glucosinolates and cyanogenic glycosides etc. (Kishore and Shah, 1988; McCue and Conn, 1990; Bentley, 1990). These metabolites play a very crucial role in plant metabolism. Other cellular components derived are chromagens in flowers and fruits, browning agents in fruits limiting agents in disease and insect attack (Gilchrist and Kosuge, 1980). Therefore, the products of the secondary pathway not only provide the structural components of plants but also a number of alkaloids as well as antimicrobial and antifungal compounds which help to overcome the various stresses.
ROLE OF SHIKIMATE PATHWAY IN STRESS RESPONSE(S)

Products of shikimate pathway have been reported to be involved in overcoming stress due to various environmental stimuli, such as, wounding, light and fungal attack. If a plant is subjected to high light intensities, particularly in the UV range, it responds by converting phenylalanine to flavonoids, which absorb UV light and prevent DNA damage (Hahlbrock and Ragg, 1975; Hahlbrock and Scheel, 1989). Amongst the various responses to external factors involving the aromatic amino acid pathway, wounding is the most well studied. Plants respond to wounding by increased production of compounds involved in the repair of wound damage and in defense against microbial invasion. While the repair processes require lignin and suberin (Sequeira, 1983), the defense against pathogenesis is associated with the synthesis of phytoalexins (Lamb et al., 1986), which are low molecular weight anti-microbial compounds (Deverall, 1982).

Wounding stimulates polysome formation and induces protein synthesis for several metabolic manifestations (Davies, 1987). The initiation of wound repair characteristically begins with increased accumulation of hydroxyproline-rich proteins in plant cell walls (Chrispeels et al., 1974). These proteins impart wall rigidity and site for lignin deposition (Hammerschmidt et al., 1984). Lignification appears to be ubiquitous in wounded tissues, and is accompanied by activation of enzymes of lignin biosynthesis, including phenylalanine ammonia lyase (Lamb and Rubery, 1976; Sacher et al., 1972), cinnamate 4-hydroxylase (Camm and Towers, 1973), p-hydroxycinnamate-Co A ligase (Rhodes and Wooltorton, 1975) chorismate mutase (Kuroki and Conn, 1988; Morris et al., 1989b); and DAHP synthase (Dyer et al., 1989). Genes encoding key enzymes of lignin biosynthesis have been found to be transcriptionally activated by wounding (Lawton and Lamb, 1987; Condit
and Meagher, 1987; Ryder et al., 1987). Involvement of some of the enzymes of shikimate pathway in overcoming stress due to light, wounding or fungal infection has been shown by McCue and Conn (1990) and Henstrand et al. (1992).

The multibranched shikimate pathway, a major route for the carbon metabolism, is of utmost importance in plants, considering the fact that about 20% of fixed carbon flows through this pathway, and that upto 60% or more of the ultimate plant mass (dry weight) is represented by molecules that once traversed through it (Herrmann et al., 1991).

The following part of the review will focus on two of the enzymes of the shikimate pathway, viz., DAHP synthase and chorismate mutase, which have been characterized in the present study from Brassica juncea.

DAHP SYNTHASE

3-Deoxy-D-arabino-heptulosonate-7 phosphate (DAHP) synthase or DS (E.C.4.1.2.15) is the first enzyme of the shikimate pathway and catalyses the first committed step of condensation of phosphoenolpyruvate and D-erythrose 4-phosphate with the formation of DAHP and Pi (Fig.1). The enzyme was discovered in 1959 by Srinivasan and Sprinson. The principle structure of the product of the enzyme, DAHP, was confirmed by chemical synthesis (Herrmann and Poling, 1975; Sprinson et al., 1962) and the fine structure was revealed by NMR spectroscopy (Garner & Herrmann, 1984). DAHP synthase was isolated and characterized for the first time from E. coli (Schoner and Herrmann, 1976; McCandliss et al., 1978; 1979).

Characterization of DAHP synthase in plants

The DAHP synthase activity has been shown to exist in several plant species. Two isozymes of DAHP synthase have been reported from Vigna radiata (Rubin
et al., 1982), Daucus carota (Suzich et al., 1985), Nicotiana silvestris, Spinacia oleracea (Ganson et al., 1986) and Solanum tuberosum (Morris et al., 1989a). A Mn$^{2+}$-stimulated form appears to be plastidic, whereas the second form which is activated specifically by Co$^{2+}$, Mg$^{2+}$ or Mn$^{2+}$ is considered cytosolic in N. silvestris, Spinacia oleracea and Petroselinum crispum (Ganson et al., 1986; McCue and Conn, 1989). The differential localization of two isozymes in two different subcellular compartments, chloroplast and cytosol respectively, led to the "Dual pathway hypothesis" for the synthesis of aromatic amino acids (Morris et al., 1989a) which is discussed later in this chapter.

Regulation of DAHP synthase by end product amino acids

Preliminary studies in plants suggested that DAHP synthase or DS was not subject to feedback inhibition. Later when regulatory interactions were detected in plants, the mode of regulation varied depending on the source of DAHP synthase, for eg. the enzyme from maize roots was not inhibited by tryptophan but the enzyme extracted from maize shoots was partially inhibited (Graziana and Boudet, 1980). Tyrosine and to a lesser extent tryptophan was reported to stimulate DAHP synthase from carrot suspension cultures (Suzich et al., 1984), whereas in pea leaves, tyrosine and phenylalanine inhibited the enzyme activity. This inhibition was relieved by tryptophan (Reinink and Borstlap, 1982). The purified DS preparation from carrot roots was activated by micromolar concentrations of trp or Mn$^{2+}$ at pH 7.0. Three multiple forms of DS were detected in pea seedlings but their individual roles in aromatic biosynthesis have not been elucidated (Rothe et al., 1976). Like the DAHP synthases from carrot root (Suzich et al., 1985) and cultured carrot cells (Suzich et al., 1984), potato DAHP synthase is activated by tryptophan (Pinto et al., 1986). In contrast, corn DAHP synthase is inhibited by tryptophan (Graziana and Boudet,
1980), one isozyme of DAHP synthase from bean is inhibited by arogenate (Rubin and Jensen, 1985) and the enzyme from tea (Saijo and Takeo, 1979) and cauliflower (Huisman and Kosuge, 1974) is not affected by amino acids. In other plant tissues too, effects of aromatic amino acids have been reported which vary from system to system.

**Existence of isozymes of DS in plants**

The differential regulation of DS by the aromatic amino acids could be explained on the basis of existence of multiple enzymes (isozymes) capable of catalysing the same reaction. Existence of multiple isozymic forms in higher plants, localized in different compartments of cell has been suggested by Gottlieb (1982). Two distinct isozymes of DAHP synthase have been reported from a number of plant systems (Hrazdina and Jensen, 1992). One isozyme was found to be activated by Mn$^{2+}$ ions and inhibited by excess concentration of the substrate (> 0.5 mM) erythrose-4-phosphate. Alternatively, the second activity was linked to ion requirement of 0.5 mM Co$^{2+}$ which could be replaced by 10 mM Mg$^{2+}$ and E4-P concentration of 4-6 mM. The former was referred to as the DAHP synthase - Mn type isozyme (abbreviated as DS-Mn) and the latter as DAHP synthase-Co type isozyme (abbreviated as DS-Co). Another amazing fact is that the ratios of the two isozymes were found to vary during growth (Ganson and Jensen, 1987), light response and other external factors (Gilchrist and Kosuge, 1980). In mung bean, preliminary investigation of Minimikawa and Uritani (1967) showed only one activity peak of DAHP synthase which was activated by light. Later work from Jensen’s laboratory indicated the presence of DS-Co and DS-Mn in the same system, where DS-Co was inhibited by caffeate (a secondary metabolite) but was unaffected by any of the aromatic amino acids or related compounds like p-hydroxycinnamate (Rubin
and Jensen, 1985). The isozyme, DS-Co from this system was also subject to
feedback inhibition by a herbicide N-phosphonomethyl glycine (glyphosate), a
phenomenon dependent on the availability of Co$^{2+}$ and complexing of the ion with
glyphosate. The second isozyme, DS-Mn from mung bean was not affected by this
herbicide (Ganson and Jensen, 1988). However, this isozyme was activated by
chorismate (pathway intermediate) and inhibited by arogenate, prephenate and
tryptophan (in that order). The regulatory properties of DS-Mn also depended on
several other factors, like pH and temperature. This led the workers to conclude that
the *in vivo* regulation of DS depended on several factors, such as, pH milieu,
temperature, metal ion concentration and presence of allosteric activators or
inhibitors. Therefore in subsequent investigations with plant DAHP syntheses, several
other properties which help to distinguish between DS-Co and DS-Mn were worked
out, such as, the pH and temperature optima for catalysis, $K_m$ for substrates,
hysteretic response, divalent cation requirement etc. These properties helped in
detection of one isozyme without masking the activity of the other. It could probably
be due to lack of proper detection assays that the earlier work by researchers led to
the characterization of only one isozyme from a few plants. However, extensive work
has resulted in the identification of second isozyme too, by standardizing the assay
conditions optimal for each isozyme from several higher plants.

One of the exceptional systems is pea, where three multiple forms of
the enzyme were detected in seedlings but their intracellular distribution
and individual roles in aromatic amino acid biosynthesis were not elucidated
(Rothe *et al.*, 1976).

Doong *et al.* (1992) have shown that cytosolic isoenzyme of DAHP synthase
(DS-Co) in *Spinacia oleracea, Solanum tuberosum* and many other higher plants could
use a diversity of substrates like, diose (glycolaldehyde), triose (D- or L-glyceraldehyde and DL-glyceraldehyde 3-phosphate), tetrose (D- or L-erythrose, D-erythrose 4-phosphate, DL-erythrose) and even pentose (D-ribose 5-phosphate and D-arabinose 5-phosphate). These substrates were utilized in combination with second substrate, phosphoenolpyruvate (PEP).

*E. coli* has three DS isoenzymes encoded by *aroF* (DAHP synthase-Tyr), *aroG* (DAHP synthase-Phe), and *aroH* (DAHP synthase-Trp). Other micro-organisms which have been reported to possess three isozymes include, *K. pneumoniae*, *Aeromonas hydrophila* and *Alteromonas putrefaciens* (Bentley, 1990). Compared to lower organisms, plant DAHP synthases appear to be less stable.

**Purification and stability**

Three isoforms of DS activity - I, II, III have been reported from carrot roots. While I & II remained relatively uncharacterized, isozyme III was purified 338 - folds to electrophoretic homogeneity. The native enzyme (MW 103,000 D) was a dimer of subunit MW 53,000 D. This enzyme showed hysteretic behaviour and was activated by physiological concentrations of trp and to a lesser extent by tyr (Suzich *et al.*, 1985). The DS-III preparation from carrot roots was activated by micromolar concentration of trp or Mn$^{2+}$ at pH 7. These observations on the activation of carrot enzymes are in contrast with those of highly purified (1000-fold) DS from cauliflower florets, in which the enzyme contained an essential - SH group, required Mn$^{2+}$, and was not influenced by aromatic amino acids or chorismate and prephenate (Huisman and Kosuge, 1974).

DS from *S. tuberosum* tubers has been purified to electrophoretic homogeneity (Pinto *et al.*, 1986) where two distinguishable forms with isoelectric points of 7.8 and 8.4 have been reported. Potato DS (MW 110,000 D) is a dimeric protein,
stabilized by dithiothreitol and stimulated by trp and Mn$^{2+}$. Other divalent cations such as Fe$^{2+}$, Zn$^{2+}$, Cu$^{2+}$ strongly inhibited the enzyme activity. This enzyme was used for raising polyclonal monospecific antibodies (Pinto et al., 1988). Goers and Jensen (1984a) reported partial purification of the enzyme from tobacco.

The activity of DAHP synthase from a number of plants has been shown to be partitioned between two isoenzymes-DS-Co (cytosol localized) and DS-Mn (plastidically localized) (Doong et al., 1992). Various plants which follow these trends are Vigna radiata (Rubin et al., 1982; Rubin and Jensen, 1985), Nicotiana silvestris, Brassica oleracea, Glycine max, Medicago sativa, Cucurbita pepo, Secale cerale, Triticum aestivum (Ganson et al., 1986), Spinacia oleracea (Jensen, 1986), Solanum tuberosum (Morris et al., 1989a), Petroselinum crispum (McCue and Conn, 1989) and Pisum sativum (Doong et al., 1992) etc.

Effect of external factors under in vivo and in vitro conditions

The enzyme activity of DS varies during cell growth in plants, like carrot and potato in suspension cultures (Suzich et al., 1984; Pinto et al., 1988) and in potato plants with organ age (Pinto, 1984). Wounding of potato tubers, leaves and also of tomato fruit elevated DS activity and also the mRNA levels (Dyer et al., 1989). Similar results have been obtained with potato suspension cultures treated with glyphosate (Pinto et al., 1988), a broad spectrum herbicide, the main target enzyme of which is EPSP synthase.

Molecular studies relating to DAHP synthase

Molecular studies on shikimate pathway enzyme have been undertaken in recent years in order to understand and characterize the regulation at molecular level (Coruzzi, 1991). cDNAs encoding both isozymes of DAHP synthase have been
isolated from some plants viz., potato (Dyer et al., 1990; Zhao and Herrmann, 1992),
tobacco (Wang et al., 1991), Arabidopsis (Keith et al., 1991), and tomato (Görlach
et al., 1993).

Characterization of cDNA encoding DAHP synthase from:

Potato: Characterization of cDNA encoding DAHP synthase from potato has been
carried out by Dyer et al. (1990) who constructed a cDNA expression library in
\(\lambda\text{gt}11\) from suspension cultures of potato cells. This clone represented the first cDNA
for DS from any eukaryotic source. The library was screened with the DS specific
antibodies. A full length cDNA encoding potato DS was cloned and its nucleotide
sequence determined. The cDNA clone referred to as \(shk\ A\), contained a 1527-bp
open reading frame that encoded a protein of MW 56,153 D and hybridized to a
2 kb mRNA. The amino terminus of the deduced polypeptide resembled a chloroplast
transit sequence. The primary structure of the pure enzyme was confirmed through
partial amino acid sequence analysis.

The deduced protein sequence of \(shk\ A\), contained 26% hydroxylated amino
acid residues with a net positive charge characteristic of chloroplast targeted proteins
(Keegstra et al., 1989). Amino acid homology between the potato enzyme and the
enzyme from \(E.\ coli\) (Davies and Davidson, 1982; Ray et al., 1988; Shultz et al.,
1984) was only about 22%. However, a plasmid bearing full-length potato cDNA
under a prokaryotic promoter, complemented DAHP synthase \(E.\ coli\) mutants
(Herrmann et al., 1991). This potato cDNA clone isolated for the first time served
as a probe for isolation of homologous sequences from tobacco (Wang et al., 1991),
tomato (Henstrand et al., 1990) and Arabidopsis (Keith et al., 1991). A second
cDNA, \(shk\ B\), encoding DAHP synthase was cloned and sequenced from potato (Zhao
and Herrmann, 1992). The 2014 bp shk B sequence contained an open reading frame of 511 codons which was 30 codons shorter than of shk A.

**Tobacco:** The shk A was used to isolate a tobacco homolog. The coding region of DS gene from tobacco (Wang et al., 1991) was found to be almost identical to the shk A coding sequence of potato (Dyer et al., 1990). The 1928 bp tobacco cDNA contained an open reading frame encoding a 542 - amino acid polypeptide and was suggested to encode a plastidic DAHP synthase.

**Tomato:** In *Lycopersicon esculentum*, DAHP synthase - specific cDNAs, DHS 1 and DHS 2 encoding both the isozymes respectively, have been cloned and characterized (Görlich et al., 1993). The two exhibited organ-specific differential expression. Steady state levels of DHS-1 specific transcripts were significantly higher in leaves, flowers and cotyledons as compared to the DHS-2 specific transcripts. Görlich et al. argued that the DAHP synthesized from DHS 1 may not be used in the synthesis of chorismate but rather for secondary metabolite production. Both the forms were reported to have transit peptides. However, no explanation as to why there are two plastidic forms of DAHP synthase could be given. Moreover, no cross-hybridization of the two forms was detected on southern blots suggesting that the sequenced genes do not share much homology. The authors have even suggested that the cytosolic enzyme might not be a DAHP synthase as it readily utilized aldehydes other than E4-P as a substrate (Doong et al., 1992). In comparison with tobacco, the shk A homolog from tomato showed a remarkable difference at the 5′ end.

**Arabidopsis**

Keith et al. (1991) have described the sequence and differential expression of two DAHP synthase genes -DHS-1 and DHS-2 from *A. thaliana*, where the DHS-1
transcript levels increased in response to wounding and pathogenic bacteria while DHS-2 transcript level did not show any significant change.

*Regulation of DAHP synthase-specific transcript levels*

The cDNA's that have been isolated for DAHP synthase may be the product of alternative splicing or transcription from different genes as suggested by Görlach et al. (1993). DAHP synthase is developmentally regulated in carrot suspension cells (Suzich et al., 1984), tomato pericap (Henstrand et al., 1990) and on wounding (Dyer et al., 1989) or glyphosate treatment in potato (Pinto et al., 1988).

In most of the systems where differential expression of two DAHP synthase clones encoding two different amino acids is observed, different roles have been assigned to the respective genes. The gene induced by wounding and pathogen attack is thought to play a role in supplying an increased amount of secondary metabolites, with increased levels of amino acids required under stress conditions. The second clone which is not induced by these treatments is thought to accommodate more constitutive needs like protein synthesis. In instances where both the genes show similar organ specific expression patterns, the two appear to be expressed in different cell types in a manner which may reflect their different physiological roles.

DAHP synthase mRNA was found to be induced upon wounding (Dyer et al., 1989; Muday and Herrmann, 1992), fungal elicitor treatment and light (Henstrand et al., 1992; McCue and Conn, 1989). This was attributed to an induced expression of the corresponding genes, since the mRNA induction could be inhibited by both cyclohexamide and actinomycin D (McCue and Conn, 1989). The transcript levels specific to plastidic form of DAHP synthase (DS-Mn) and also of CM (CM-1) are reported to be increased by light (McCue and Conn, 1990) or by fungal elicitors
(McCue and Conn, 1989) and wounding in potato (Dyer et al., 1989). In Arabidopsis the two DAHP synthase genes DHS-1 and DHS-2 were found to be differentially induced by wounding and pathogenic attack. The level of DHS-1 specific mRNA increased in leaves which were physically wounded or infiltrated with pathogen (Keith et al., 1991).

**CHORISMATE MUTASE**

The pivotal position occupied by "chorismate" in the shikimate pathway has been well established in microorganisms and higher plants (Edwards and Jackman, 1965; Cotton and Gibson, 1968; Schmidt and Zalkin, 1969; Gilchrist et al., 1972). The branch point metabolite chorismate leads to the synthesis of tryptophan, phenylalanine and tyrosine. One branch of the pathway leading to tryptophan synthesis is considered to be highly regulated by feedback inhibition of the first committed reaction, catalysed by anthranilate synthase (Artz and Holzschu, 1983). The second branch of the pathway leads to the formation of prephenate from chorismate using chorismate mutase and subsequently to the synthesis of phenylalanine and tyrosine by two independent pathways (Fig.2) (Balinsky and Davies, 1962; Belser et al., 1971).

Chorismate mutase (CM) (E.C. 5.4.99.5) is the branch point enzyme which was first characterized by Cotton and Gibson (1968) from Aerobacter aerogens and E. coli. Regulatory controls of the biosynthesis of phenylalanine and tyrosine in microorganisms and plants share several common features, whereas the mode of control of CM activity may differ, thereby preserving the unifying fact of stringent control. In bacteria, independent regulation of phenylalanine and tyrosine biosynthesis is found to occur by specific channelling of chorismate through enzyme complexes.
(Cotton and Gibson, 1964; Friedrich et al., 1976). Thus phe synthesis is regulated through chorismate mutase - prephenate dehydratase complex possessing two distinct catalytic sites for respective reactions (Duggleby et al., 1978). This complex is sensitive to simultaneous feedback inhibition and repression by phenylalanine (Schmidt and Zalkin, 1969). Similarly tyrosine synthesis is mediated by chorismate mutase - prephenate dehydrogenase complex which is repressed by tyrosine.

Characterization of CM in plants

CM has been well characterized from Solanum tuberosum, Nicotiana silvestris, Sorghum bicolor, Spinacia oleracea, Daucus carota, Vigna radiata and Sorghum vulgare as has been earlier reviewed (Siehl et al., 1992).

Existence of isozymes of CM and regulation by end product amino acids

Two distinct isozymes of CM have been isolated from many plant systems including Vigna radiata (Gilchrist and Kosuge, 1974; 1975), Nicotiana silvestris (Goers and Jensen, 1984a,b) and Sorghum bicolor (Singh et al., 1985). Chorismate mutase designated as CM-1, is subject to regulation by aromatic amino acids, whereas CM-2 is not specifically regulated. Though CM-1 is the amino acid-sensitive form but its mode of regulation by phe, tyr and trp varies from system to system, eg. CM-1 from N. silvestris (Goers and Jensen, 1984a,b) and that from S. bicolor (Singh et al., 1985) is activated by tryptophan and inhibited by tyrosine and phenylalanine. These activation or inhibition effects are, however, complex and seem to depend upon pH and the combination of effectors tested. The extent of activation or inhibition of CM-1 isolated from different species was found to vary significantly (Singh et al., 1986). Work from various laboratories has substantiated the fact that CM-2 is the amino acid-insensitive form. The only potential regulatory interaction which can be associated
with CM-2 is the inhibition by caffeate as in *Nicotiana* (Goers and Jensen, 1984b). Aromatic amino acid sensitive form of CM has been identified from alfalfa (Woodin and Nishioka, 1973; Woodin *et al.*, 1978), pea (Cotton and Gibson, 1968), oak (Gadal and Bouyssou, 1973), mung bean (Gilchrist *et al.*, 1972; 1974; 1975), rice (Chu and Widholm, 1972) and tissue cultures of tobacco and tomato (Widholm, 1974). In most of these plants, multiple forms of CM enzyme have been reported, some of which are insensitive to regulation by phe, tyr or trp. As regards the existence of different isoforms, it is believed by many workers (Jensen, 1986) that the role of the sensitive form CM-1 is probably directly related to protein synthesis, while physiological function of the unregulated form is the synthesis of secondary metabolites.

The two isoenzymes of chorismate mutase are distinct species differing in catalytic properties, molecular weight, subunit composition, isoelectric point, immunological properties, as well as subcellular localization. These isozymes not only differ in their relative ratio in different tissues but one or the other isozyme has been reported to be absent in some systems (Singh *et al.*, 1986). In the rhizogenic callus of *B. juncea*, Sharma *et al.* (1993) observed the absence of CM-1. A single species of CM i.e., the amino acid sensitive form (CM-1), inducible by wounding was isolated from *Solanum tuberosum* tubers (Kuroki and Conn, 1987). Subsequently, induction of both CM-1 and CM-2 and also of some other enzymes of the shikimate pathway upon wounding has been observed in potato tubers (Morris *et al.*, 1989b).

**Purification and properties of CM**

Purification of CM from mung bean seedlings and *Sorghum* was achieved as early as 1972. In mung bean seedlings, two readily separable forms were found which could further be distinguished by their response to allosteric effectors like phe, tyr or
trp (Gilchrist et al., 1972). CM-1 was highly purified and on the basis of its *in vitro* response to the end product amino acids, appeared to be directly involved in the synthesis of phenylalanine and tyrosine (Gilchrist and Kosuge, 1974). This isoenzyme shared similarities in its kinetic properties with partially characterized CM-1 from oak (Gadal and Bouyssou, 1973). CM-2 represented 50% of the total extractable activity and did not respond to any of the three aromatic amino acids under *in vitro* assays (Gilchrist and Kosuge, 1975). Mung bean CM-1, displayed a number of characteristics of a regulatory enzyme including, substrate activation, end product inhibition and cross-pathway metabolite activation. This isoform of the enzyme displayed pH sensitive homotropic cooperativity towards chorismate. The characteristic property of feedback inhibition of CM-1 by phe and tyr differed in mung bean and *Sorghum*. In mung bean, trp acted as an activator and at equimolar concentration reversed the feedback inhibition by phe and trp whereas it was not so in *Sorghum*. CM-2, from oak was insensitive to all the three aromatic amino acids and 26 other secondary metabolites tested, which included p-coumaric, o-coumaric, p- and o-coumaric acid, anthranilate, caffeate, ferulate, chlorogenate and indolacetate (Gilchrist and Kosuge, 1975).

CM-1 from *Sorghum* has been purified to electrophoretic homogeneity (1389-fold purification) and has an apparent MW of 56,000 D. This regulated isozyme was possibly a dimer having subunit MW of 36,5000 D. Purified CM-2 isozyme (1018-fold), has been reported to have a MW 48,000 D (Singh et al., 1985). These two isozymes from *Sorghum* were found to be immunologically distinct (Singh et al., 1986). Similarly in *Nicotiana*, two isozymes of CM were partially purified. CM-1 (MW 52,000 D) was the major fraction in proplastids (obtained from suspension cultured cells) and the chloroplasts of green tissues and was subject to feedback
inhibition by tyr. On the other hand CM-2 (MW 65,000 D) was speculated to be cytosolic in nature and was found to be unaffected by aromatic amino acids (d'Amato et al., 1984; Goers and Jensen, 1985b).

**Regulation of isozymes of CM during growth and development**

Activities of both the isozymes of CM have been shown to be regulated by a number of developmental and environmental factors, though a detailed study of the effect of these factors is lacking in higher plants. The enzyme activity has been shown to vary depending on the age of the plant and nature of the tissue as shown in tobacco (Goers and Jensen, 1984a). The ratio of potato CM-1 : CM-2 activity was 2:1 in fresh and 9:1 in aged potato tuber discs. In green leaves of potato, the ratio of CM-1 : CM-2 was found to be 1:4. These shifts in the ratio of isozyme activities in potato and other systems suggested an organ specific regulation of CM-1 and CM-2 (Kuroki and Conn, 1989). In contrast to the report in S. tuberosum, CM-1 : CM-2 ratio in various organs and tissues of Sorghum bicolor did not show any significant change with age or during development (Singh et al., 1985). In another study, the level of chorismate mutase did not show any change in transgenic tobacco plants overexpressing tryptophan decarboxylase from Catharanthus roseus (Poulsen et al., 1994). The significance of the shift in CM-1 : CM-2 ratio during different developmental stages of a plant still remains to be discovered.

**Molecular studies on CM**

During last few years molecular approaches for studying amino acid biosynthesis in plants have provided extensive new information on some of the old questions relating to aromatic amino acid biosynthesis and allied products derived from the shikimate pathway (Coruzzi, 1991). However, molecular studies of the
shikimate pathway enzymes in higher plants have been restricted mainly to the first and the fifth enzyme of the pathway viz., DAHP synthase and EPSP synthase and to some of the enzymes of the tryptophan biosynthesis branch. To the best of our knowledge, no information is available on cloning and sequencing of CM from higher plants, although advances have been made towards immunological characterization of CM, as in *Sorghum* (Singh *et al.*, 1986), where no immunological similarity between the two isoforms was found. The characterization of active site of CM-1 has been reported from the same system (Siehl *et al.*, 1992).

**COMPARTMENTALIZATION OF SHIKIMATE PATHWAY ISOZYMES:**

**DUAL PATHWAY HYPOTHESIS**

It has been suggested by Jensen (1985a) in the "Dual Pathway hypothesis" that there are likely to be two complete shikimate pathways; one plastidic and the other cytosolic and that the cytosolic pathway enzymes are used for production of compounds destined to become secondary metabolites. However, reports on the induction of DS-Mn by fungal elicitors (McCue and Conn, 1989) and wounding (Dyer *et al.*, 1989) indicate that the plastidic isozyme may also participate in the production of precursors for secondary metabolites.

A detailed consideration of the differential allosteric regulation of these two isoforms has indicated that major role of CM-1 (the chloroplast enzyme) is in the biosynthesis of aromatic amino acids, while the cytosolic isozyme CM-2 is probably involved in the secondary metabolite biosynthesis (Goers and Jensen, 1984b). "Dual pathway hypothesis" of Jensen (1985a,b) whereby two isoformic pairs are shown to exist in both compartments of the cell i.e. cytosol and the plastids seems to hold good for DAHP synthase, CM and for many of the other enzymes which form a part of the
shikimate pathway (Mousdale and Coggins, 1985). The observations that have led to the postulation of "Dual pathway hypothesis" and its characterization from various systems are discussed here:

Isolated chloroplasts from spinach have been shown to assimilate either $\text{CO}_2$ or shikimate into aromatic amino acids (Bickel et al., 1978; Buchholz et al. 1979; Leuschner and Schultz, 1991). This indicated that an intact aromatic pathway must be located within the chloroplast organelles. However, chloroplast-localized biosynthesis could not account for 100% aromatic amino acid biosynthesis (Buchholz et al., 1979). Hence, a spatially separate pathway having its own complement of enzymes localized in the cytosol was postulated to exist. A strong precedent for such duplicate pathways occupying separate intracellular niches (i.e., present in both the cytosol and the chloroplast) is known to exist for the glycolytic pathway and the oxidative pentose phosphate cycle (Gottlieb, 1982; Hrazdina and Jensen, 1992). Such a spatial organization of these two pathways suggested the existence of isozymic forms of aromatic-pathway enzymes too, that have different subcellular locations. A systematic search for separately compartmentalized isozymes of aromatic biosynthesis has been reported from a few higher plants (Hrazdina and Jensen, 1992). Progress in this direction included the characterization of two distinct isozymes of DAHP synthase from mung bean (Rubin and Jensen 1985). DAHP synthase-Mn (stimulated by Mn$^{2+}$ and requiring dithiothreitol) is located in the plastid compartment, while DAHP synthase-Co (requiring Co$^{2+}$ or Mg$^{2+}$ for activity) is located in the cytosol. Two isozymes of chorismate mutase have also been reported from $N.\ silvestris$. Chorismate mutase-1 (CM-1) located in chloroplasts of leaf tissue as well as in proplastids of suspension-cultured cells, and chorismate mutase-2 (CM-2) located in the cytosol (d'Amato et al., 1984). Since DAHP-synthase-Co and chorismate mutase-2 are
reported to be cytosolic enzymes in few other higher plants, it was suggested that the intervening six steps of the common pathway must also be present in the cytosol. Otherwise, at least two transport events between cellular compartments would be required (i.e., to shuttle a product derived from action of DAHP synthase-Co into the plastid and to shuttle a source of substrate for chorismate mutase-2 out of the plastid).

Work in recent years has added in the favour of "dual pathway hypothesis" (Jensen 1985a; Morris et al. 1989a). However, Keith et al. (1991) suggested that both isozymes of DAHP synthase could be chloroplast targetted, since clones picked for both isozymes of DS showed transit peptide sequences. Fig.4 represents the link between carbohydrate metabolism and aromatic amino acid biosynthesis.

**Differential regulation of the two shikimate-pathway systems**

Multibranched biochemical pathways such as shikimate pathway are commonly subject to a fine-tuned form of allosteric control. Enzymes located at metabolic branch points are known to bind molecules that are pathway intermediates or end products. Such effector molecules bind at sites that are spatially distinct from the catalytic site of the enzyme, either causing feedback inhibition or allosteric activation. Such an allosteric control is known to operate for the two shikimate-pathway enzymes DS and CM too.

The plastidial isozymes, DAHP synthase-Mn and chorismate mutase-1 are subject to allosteric control (Rubin and Jensen 1985, Goers and Jensen 1984a,b), for instance, the regulation of DAHP synthase-Mn by the pathway intermediate L-arogenate indicates a pattern of control called sequential feedback inhibition. In the presence of excess endproducts, the following events are known to promote elevation of arogenate levels: feedback inhibition of anthranilate synthase by L-tryptophan, inhibition of arogenate dehydrogenase by L-tyrosine, inhibition of arogenate dehydrogenase by L-tyrosine, inhibition of arogenate dehydrogenase by L-tyrosine, inhibition of arogenate dehydrogenase by L-tyrosine, inhibition of arogenate dehydrogenase by L-tyrosine
dehydratase by L-phenylalanine, and allosteric activation of chorismate mutase by L-tryptophan. Although L-tyrosine and L-phenylalanine inhibit chorismate mutase-1, L-tryptophan activation dominates in the presence of all three amino acids, as reported by Goers and Jensen (1984b). Elevation of L-arogenate levels by higher activity of latter pathway triggers the early-pathway circuit of feedback control, which then triggers the feedback inhibition of early-pathway, whereby DAHP synthase-Mn is shut off (sequential feedback inhibition). In contrast, the cytosolic enzymes, DAHP synthase-Co and chorismate mutase-2, are insensitive to inhibition by biosynthetic-pathway metabolites in N. silvestris (Goers and Jensen 1984b) and few other systems. Not only DS-Co and CM-2 but a feedback-insensitive isozyme of anthranilate synthase has also been described in potato (Carlson and Widholm, 1978). A minor isozyme of arogenate dehydrogenase (that is insensitive to feedback inhibition by L-tyrosine) has been detected in N. silvestris (C. Gaines and R.A. Jensen, unpublished data as in Hrazdina and Jensen, 1992). These systems exemplify a strong support in favour of the hypothesis, that an entire unregulated cytosolic array of shikimate-pathway enzymes supplies the connecting network of secondary metabolites. In this case the availability of starting substrates of secondary metabolism in the cytosol may primarily depend upon: (i) the supply of erythrose-4-phosphate and phosphoenolpyruvate generated by cytosolic carbohydrate metabolism, and (ii) competition between secondary metabolism and cytosolic protein synthesis from aromatic amino acids. The available literature strongly indicates a tight allosteric regulation and suggests a compartmentalization of shikimate pathway enzymes. Whether there are two sets of enzymes catalysing the eleven reactions of shikimate pathway is still an open question.
CONCLUSION

After conducting the literature survey on the shikimate pathway in plants, it is clearly evident that there are many control points of this pathway (Bentley, 1990), but two major ones are:

i) at the level of first enzyme of the pathway, i.e. DAHP synthase and

ii) at the branch point enzyme - chorismate mutase.

Regulation of biosynthesis of amino acids relies mainly on feedback mechanisms, some of which have been demonstrated both in vivo as well as in vitro plant systems. It seems from a number of plant studies, that adequate control measures are operative to account for the maintenance of pool sizes of various metabolites of shikimate pathway at reasonably low levels.

The levels of feedback regulation of branch point intermediate, chorismate are indicated in Fig.5.

Studies with isolated enzymes in vitro revealed feedback inhibition of DS and CM by one or the other aromatic amino acid. Tryptophan apparently controls its own biosynthesis by feedback inhibition of anthranilate synthase. It further exerts a control on the partitioning of chorismate between the two competing routes of chorismate metabolism by its ability, to both activate CM and relieve the inhibition imposed on this step by phe and tyr (Gilchrist and Kosuge, 1980; Bryan, 1990). In addition, carbon flux through chorismate to prephenate is also sensitive to the concentration of chorismate due to allosteric substrate activation of CM by chorismate.

In conclusion, aromatic amino acid biosynthesis in higher plants basically appears to follow the same sequence of events as previously elucidated in micro-organisms. The current information establishes a striking evolutionary conservation of shikimate pathway from prokaryotes to higher plants (Ahmad and
Fig. 5  Feed-back regulation of chorismate by end-product amino acids - phenylalanine, tyrosine and tryptophan. Bold lines indicate inhibition, dashed lines indicate activation of Chorismate mutase. [After D.G. Gilchrist and T. Kosugi (1980). The biochemistry of plants. A comprehensive treatise. Amino acids and derivatives (vol.5)]
Jensen, 1986). It is thus of utmost importance to understand the underlying mechanism of enzyme action and regulation of shikimate pathway in higher plants. However, detailed studies on the regulatory mechanisms of these enzymes in higher plants during different developmental stages and under various environmental conditions need to be strengthened. One of the ways to carry out these studies is to use an in vitro tissue culture system as shown by Sharma et al. (1993) or to study regulation at the molecular level. The emphasis also needs to be laid on a complete study in one plant system rather than isolated reports of the enzymes from different plants. This would enable a precise comparison of these two enzymes of shikimate pathway, viz. DS and CM in the experimental plant vs other plants. The role of these enzymes during development and under varied external stimuli needs to be established. Though a beginning has been made in this direction, due to the paucity of data on molecular aspects, much remains to be achieved.

At attempt has been made in the present investigation to answer some of the important questions regarding the characterization of DAHP synthase and chorismate mutase, as well as their regulation under different environmental conditions using an economically important oil species, *Brassica juncea*. 