Chapter 4

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INTRODUCTION

Studies on protein purification and characterization provide a basic understanding of metabolism in organisms. Despite the advent of molecular biology, the biological problems need a thorough characterization first at the protein level. Many investigations have stopped at the demonstration of a physiological effect of any particular phenomenon, needing thereby to back-track to the studies at the level of proteins. It is for this very reason that protein purification and characterization becomes very important. A study of the changes at the protein level is crucial to understand any physiological response(s). For accurate analysis, it is imperative to purify an enzyme from a mixture, so that no other enzyme can be detected except the one of interest. Once the enzyme is purified, a number of investigations can be taken up which include:

i) its catalytic activities,
ii) mechanism of catalysis including the active site and active domains or residues crucial for the enzyme functionality,
iii) responsiveness of the enzyme to regulatory molecules that raise or lower its activity.

The present study on characterization of the enzymes, DAHP synthase-Co (DS-Co) and chorismate mutase (CM) was attempted with the aim of understanding their role in the basic regulatory mechanisms during development in Brassica juncea.

DAHP synthase and CM from Brassica

A. DAHP synthase: We have earlier characterized two isozymes of DAHP synthase from leaves of Brassica juncea viz. DAHP synthase-Co (DS-Co) and DAHP synthase-Mn (DS-Mn) [Sharma et al., 1993]. These two isozymes have been characterized based on:
i) divalent cation requirement - DS-Co from *B. juncea* required 1 mM Co\(^{2+}\) for optimal activity, while DS-Mn functions best with 1 mM Mn\(^{2+}\) in the assay mixtures.

ii) end-product regulation - Whereas DS-Co was relatively unaffected by the end-product amino acids - phenylalanine (phe), tyrosine (tyr) or tryptophan (trp), DS-Mn was activated more than 2-folds by tyr; strongly inhibited by trp and weakly inhibited by phe.

Thus when the two isozyme peaks were eluted on an anion exchanger like DE-52, the two isozymes were easily characterized by performing the above tests on each peak.

B. *Chorismate mutase*: end-product regulation - CM from leaves of *B. juncea* was also seen to be present in two isozymic forms - CM-1 and CM-2. The CM-1 form was activated by tryptophan and inhibited by tyrosine. CM-2 was found to be insensitive to aromatic amino acids - phe, tyr or trp. Thus by performing an end-product regulation test on isozymes, it could be ascertained whether it was CM-1 or CM-2.

These properties of the isozymes of DS and CM served to characterize the enzymes during the present investigation.
Purification of DAHP Synthase-Co

Plant Material: Leaves of *B. juncea* (along with a part of the stem portion) were used for enzyme purification.

Preliminary studies revealed that the levels of DS-Co isozyme are slightly higher than DS-Mn in leaves (Fig.6) and DS-Co was found to be more stable to purification steps during initial characterization. Hence, an attempt was made to purify DAHP synthase-Co from *B. juncea*.

Methodology

Leaves of *B. juncea* cv. Pusa bold weighing ca. 500 gm were frozen in liquid nitrogen, ground to a very fine powder and extracted in 1000 ml extraction buffer (100 mM EPPS, pH 7.8, containing 10 mM β-mercaptoethanol, 1 mM PMSF, 5 mM sodium metabisulfite, 1 mM MnCl₂, 1 μM trp and 100 g PVP). The extract was filtered through four layers of cheese cloth. One-tenth (of filtrate) volume of 2% protamine sulphate in extraction buffer was slowly added to the crude extract while stirring. The mixture thus obtained was centrifuged at 10,000 rpm for 35 min. The supernatant obtained after centrifugation was desalted on a sephadex G-25 column, pre-equilibrated with extraction buffer, and further subjected to 0-65% ammonium sulphate precipitation. The resulting precipitate was collected by centrifugation at 10,000 rpm for 40 min. The precipitate was resuspended in extraction buffer and dialysed overnight against 2 changes of the same buffer.

The dialysate was cleared by centrifugation at 8,000 rpm for 15 min. Finely ground (NH₄)₂SO₄ was added to the dialysate to give 25% saturation. The pH of the suspension was maintained at 7.8 during the precipitation. The precipitate was discarded, and to the supernatant, (NH₄)₂SO₄ was added to further give 40% saturation. The precipitate was collected and resuspended in buffer A (50 mM...
Fig. 6  Comparative levels of DS-Co and DS-Mn from leaves of *B. juncea*. Activity is expressed in nM min$^{-1}$ mg$^{-1}$ protein.
EPPS, pH 7.8, containing 10 mM β-mercaptoethanol, 1 mM PMSF, 5 mM sodium metabisulphite, 0.5 mM MgCl₂, 0.5 mM MnCl₂, 100 μM trp and 10% glycerol) and dialysed against two changes of buffer A. The dialysate was cleared by centrifugation and the suspension was dialysed again for 10 hrs against buffer B (50 mM K-PO₄ buffer, pH 7.5, containing 5 mM sodium metabisulfite, 1 mM DTT, 1 mM PMSF, 0.1 mM MgCl₂, 0.1 mM MnCl₂, 0.5 mM PEP, 100 μM trp and 10% glycerol). The dialysate was again cleared by centrifugation and applied on a DE-52 column (2.8 x 45 cm), pre-equilibrated with buffer B. After loading the extract, the column was washed with two bed volumes of the same buffer till the O.D₂₈₀ was found to be less than 0.05. DAHP synthase was eluted with linear gradient of 0 - 0.4 M NaCl at a flow rate of 30 ml hr⁻¹ and 3 ml fractions collected. Fractions containing DAHP synthase-Co were pooled and precipitated with 75% ammonium sulphate. The precipitate was collected by centrifugation, resuspended in buffer B and dialysed against buffer C for 5 hrs x 1 change. Buffer C contained, 20 mM K-PO₄ buffer pH 7.5 and all other components of buffer B.

The dialysate was cleared by centrifugation and applied to a hydroxylapatite column (1.5 x 12 cm) pre-equilibrated with buffer D (0.1 M K-PO₄ buffer, pH 7.5 containing 1 mM DTT, 0.1 mM each MgCl₂ and MnCl₂, 0.5 mM PEP, 100 μM trp and 10% glycerol). The elution was performed with a linear gradient of 0.1 - 0.5 M K-PO₄ buffer, pH 7.5, containing 1 mM DTT, 0.1 mM each MgCl₂ and MnCl₂, 0.1 mM trp and 10% glycerol, at a flow rate of 15 ml hr⁻¹ and 2 ml fractions collected. Fractions containing DAHP synthase activity were pooled and fractionated with (NH₄)₂ SO₄ (35-40%) DAHP synthase was precipitated and this precipitate after dialysis against buffer D was further run on a sephacryl S-200 column (1.25 x 50 cm) at a flow rate of 15 ml hr⁻¹ and 2 ml fractions were collected for native molecular
weight determination. The enzyme was analysed on SDS-PAGE for subunit molecular weight determination.

Results and Discussion

For purification, field grown material was preferred since a large quantity of plant material was required. The tissue used for purification was always taken fresh since stored tissue lost considerable amount of activity (upto 30% after 2-3 days of storage). Liquid nitrogen was always used during grinding of the tissue since grinding was much better with it. Moreover, the use of liquid N\textsubscript{2} has been recommended by most workers for avoiding the loss in enzyme activity. The crude extract was prepared by using 1:2 (w/v) ratio of tissue to buffer as this was found to give optimal yield. Use of buffer in 1:1 ratio resulted in the enzyme slurry being thick and difficult to filter.

Various buffers used for extraction of DS and CM are mentioned at each step. It was important to include glycerol at 10\% or 5\% (v/v) in the buffers used for purification for the stability of the enzyme, especially during elution through columns (during the purification steps). In the initial steps of purification, \(\beta\)-mercaptoethanol was preferred over DTT since \(\beta\)-mercaptoethanol is commonly used for extracts prepared from plant origin. PVP (100 g per 1000 ml buffer) was used during extraction to prevent interference by phenolics in \textit{Brassica}.

On elution from DE-52, the main peak fractions were pooled. The enzyme eluted between a gradient of 0.2 - 0.25 M NaCl. The elution profile from DE-52 column is shown in Fig.7. The DS-Co presence was through all the steps of purification by SDS-PAGE. Three different steps are shown in Fig.8a. The enzyme preparation was checked on SDS-PAGE. A typical result of 10\% SDS gel stained
Fig. 7  Elution profile of DS-Co from DE-52 column. The loading of the column was done with buffer B and the elution was carried out with a linear gradient of 0 - 0.4 M NaCl in buffer B. $A_{549}$ represents DS-Co activity and $A_{280}$ the protein profile.
Fig. 8a  SDS-PAGE for DS-Co through different stage of purification.
Lane a: standard molecular weight protein markers,
Lane b-d: DS-Co through step 2-4 of Table I. Equal amount of protein was loaded in lanes b-d.

8b  Coomassie Brilliant Blue stained SDS gel (10%)
Lane a: standard protein molecular weight markers used are carbonic anhydrase (29 KD), ovalbumin (45 KD), bovine albumin (66 KD), phosphorylase b (97.4 KD), β-galactosidase (116 KD) and myosin (205 KD).
Lane b: purified DS-Co, 20 μl of the protein was loaded.

Fig. 9  Silver staining of purified DS-Co on a SDS gel (10%)
Lane a&b: 20 μl of the purified protein.
with CBB is shown in Fig. 8b, where a single band of 52.5 KD was observed in the lane containing DS-Co. Silver staining of DS-Co preparation showed a homogeneous preparation of the protein (Fig. 9).

Thus a homogeneous preparation of DAHP synthase-Co was obtained by following the steps outlined in Table 1 with an enrichment of 898-folds at the final step.

**Molecular Weight**

Molecular weight of the native enzyme was determined using gel filtration column (sephacryl S-200) as described in Materials and methods. From log molecular weight vs relative mobility \((V_e/V_0)\) plot (Fig. 10a) apparent native molecular weight of DS-Co was found to be 100 KD. Subunit molecular weight of DS-Co was determined by running the enzyme on a denaturing gel. The \(R_f\) values of standard proteins and DS-Co were measured. A plot of log molecular weight vs \(R_f\) values is shown in Fig. 10b. From the plot, the subunit molecular weight of DS-Co was estimated to be 52.5 KD. These results indicate that *Brassica* DS-Co exists as a dimeric protein. A homogeneous preparation of DS-Co obtained for the first time from *B. juncea* was used for kinetic studies to further characterize the enzyme. Earlier reports have indicated molecular weight of the native enzyme to be 110 KD from *Solanum tuberosum* (Pinto et al., 1986), 103 KD from roots of *Daucus carota* (Suzich et al., 1985), with subunit MW of 54 KD and 53 KD respectively.

During this study, it was observed that buffers for purification of DAHP synthase-Co required a careful formulation to maximise the recovery of enzyme activity. Following precautions were taken:

a) during the ammonium sulphate precipitation steps, frothing was avoided.

b) adjustment of proper flow rates was very crucial for elution of the enzyme.
Table 1: Purification of DS-Co from *B. juncea* leaves (scaled up for 1 Kg)

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Enrichment (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract</td>
<td>17,600</td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>II. Desalted &amp; 0-65% (NH$_4$)$_2$SO$_4$ (p)</td>
<td>5,892</td>
<td>110.5</td>
<td>0.018</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>III. 25-40% (NH$_4$)$_2$SO$_4$</td>
<td>819</td>
<td>86.8</td>
<td>0.105</td>
<td>5.83</td>
<td>78.55</td>
</tr>
<tr>
<td>IV. DE-52</td>
<td>21</td>
<td>39.5</td>
<td>1.88</td>
<td>104</td>
<td>35.7</td>
</tr>
<tr>
<td>V. Hydroxylapatite</td>
<td>0.99</td>
<td>13.2</td>
<td>13.3</td>
<td>740</td>
<td>11.94</td>
</tr>
<tr>
<td>VI. 35-40% (NH$_4$)$_2$SO$_4$</td>
<td>0.6</td>
<td>0.7</td>
<td>16.1</td>
<td>898</td>
<td>8.7</td>
</tr>
</tbody>
</table>
Fig. 10a Log molecular weight vs R_f plot for native molecular weight determination of DS-Co. The R_f values of the standards and the purified DS-Co are indicated. The standards used were cytochrome C (12.4 KD), carbonic anhydrase (29 KD), bovine albumin (66 KD), alcohol dehydrogenase (150 KD) and β-amylase (200 KD).

Fig. 10b Log molecular weight vs R_f plot for subunit molecular weight determination of DS-Co. The R_f values of the standards and DS-Co are indicated. The standard protein molecular weight markers used are the same as indicated in Fig. 8.
Carbonic Anhydrase

Relative Mobility ($R_f$)

Molecular weight $\times 1000$

Amylase
ADH
DS-Co
BSA
Cyt C

Relative Mobility ($R_f$)

Myosin
β-Gal
Phosphorylase b
BSA
DS-Co
Albumin Egg
Carbonic Anhydrase

Molecular weight $\times 1000$

Relative Mobility ($R_f$)
c) all the steps were carried out at 4°C.

d) inclusion of PEP in buffers was not very crucial in the initial stages, but 0.5 mM PEP was extremely important for maintenance of enzyme activity during the later stages of column running and long term enzyme storage. Therefore, PEP (0.5 mM) was invariably included in buffer B and buffer C.

e) metabisulfite (5 mM), helped to maximize enzyme recovery and PVP prevented browning of the extract. When metabisulfite was included in the extraction buffer extractable enzyme activity increased by 30%. Hence, sodium metabisulphite was included in all the buffers used for purification till step 3 (Table 1). After DE cellulose chromatography step, it was not necessary to include metabisulfite since DS-Co was quite stable at this stage. The DS-Co activity was stabilized by including DTT and β-mercaptoethanol.

**Kinetic Properties**

Various parameters were analysed to study the kinetic behaviour of the enzyme.

**Effect of different concentrations of the substrates, E4P and PEP on DS-Co activity:**

\( K_m \) values: DS-Co requires E4-P and PEP as substrates. To find out the \( K_m \) values for each substrate, different concentrations of E4-P and PEP were used.

**E4-P**: Enzyme assays were carried out with increasing concentrations of E4-P (0 to 5 mM) using 150 µl of purified enzyme keeping PEP concentration constant at 2 mM. There was a linear increase in the enzyme activity with increasing concentration of E4-P. Beyond 3 mM E4-P, enzyme saturation was recorded and the activity plateaued off (Fig.11a). A similar enzyme saturation at higher concentration of E4-P has been reported in *V. radiata* (Rubin and Jensen, 1985), where the substrate saturation curve was linear in the range of 0.5 - 2 mM E4-P.
Fig. 11 Substrate saturation curves and Lineweaver Burk plot for DS-Co.

a: Lineweaver-Burk plot of reciprocal of the initial velocity of DS-Co vs reciprocal of concentration of E4-P. Values of E4-P vs are taken from inset. The plot is at two different PEP concentrations (2 and 4 mM).

a (inset): DS-Co activity with varying concentration of E4-P with PEP concentration constant at 2 mM.

b: Lineweaver-Burk plot of reciprocal of the initial velocity of DS-Co vs reciprocal of concentration of PEP. Values of PEP are taken from inset. The concentration of E4-P was fixed at 4 mM.

b (inset): DS-Co activity with varying concentration of PEP with E4-P concentration fixed at 4 mM.
PEP: Enzyme assays were carried out with increasing concentrations of PEP (0-6 mM) using 150 μl of purified enzyme, keeping E4-P concentration constant at 4 mM. The PEP vs [v]^{-1} is shown in Fig. 11b. A hyperbolic saturation curve was obtained. A marginal inhibition was seen with PEP at concentrations higher than 2.5 mM.

From the Lineweaver Burk plots, the K_{m} calculated for E4-P was ≈ 1.5 mM (Fig. 11a, inset) and that for PEP, 0.78 mM (Fig. 11b, inset). Though the enzyme kinetics with purified DS-Co from plant systems have not been worked out earlier in detail, DS-Co from *Vigna* was reported to reach saturation with at least 4 mM E4-P and 2 mM PEP (Rubin and Jensen, 1985). Besides this, three peaks of enzyme activity, viz. I, II and III were isolated for DS from carrot roots (Suzich *et al.*, 1985). The activity peak III which was purified to homogeneity showed a K_{m} value of as low as 0.03 and 0.07 mM with PEP and E4P respectively. These K_{m} values were presumed to be for DS-Mn.

Effect of pH on DS-Co activity: The enzyme activity was observed in a pH range of 5 - 8.5 at 37°C on using different buffers: MES (pH 5 - 6.6), MOPS (pH 6.6 - 7.5), sodium phosphate buffer (pH 6.8 - 7.8) and EPPS (pH 7 - 8.5). A steep rise in the enzyme activity was observed till pH 7.8. The optimal pH for the enzyme activity was 7.8 (Fig. 12a) although considerable activity was observed even at pH 8.0. Thus DS-Co has a very narrow pH optima. For the crude extracts also, pH 7.8 was found to be the optimum, hence EPPS buffer was used for crude extractions. pH optima of 8.8 has been reported for DS-Co from *Vigna radiata* (Rubin and Jensen, 1985), 8.6 from *Nicotiana silvestris* (Ganson *et al.*, 1986) and 9.6 from *Spinacia oleracea* (Doong *et al.*, 1992), but these were only partially purified preparations. However, homogeneous preparation of DS-Co from
Fig. 12a Effect of pH on DS-Co activity. Activity is expressed in nM min$^{-1}$ mg$^{-1}$ protein.

b: Effect of divalent cations (Co$^{2+}$ and Mg$^{2+}$) on DS-Co activity. Maximal activities were assigned relative values of 100, 75, 50 and 25.

c: Effect of DTT on DS-Co activity. The enzyme was pre-incubated with DTT for indicated time prior to enzyme assay. Activity is expressed as nM min$^{-1}$ mg$^{-1}$ protein.
potato tubers showed a narrow pH optima for enzyme activity around 7.0 (Pinto et al., 1986).

In microbial systems, the pH optima reported for DS is generally between 6.5 - 7.0 (Bentley, 1990).

Effect of divalent ions on enzyme activity: DS-Co essentially requires divalent cations for its optimal activity as otherwise low enzyme activities were noticed (Bentley, 1990; Sharma et al., 1993). DS from higher plants has been shown to function in presence of cobalt or manganese and based upon the cation requirement it is characterized as DS-Co isozyme or DS-Mn isozyme respectively (see Literature Review). However, a report by Doong et al. (1992) indicated that DS-Co can function equally effectively with MgCl₂, but at a higher concentration of 10 mM.

In our earlier work on Brassica (Sharma et al., 1993), it was shown that DS-Co functions optimally in presence of 1 mM cobalt (as CoCl₂). In the present study also, very low activity was detected in the absence of cobalt, thereby confirming absolute requirement of Co²⁺ for DS-Co activity (Table 2). Amongst the various other ions tested at 1 mM concentration, Mg²⁺, Mn²⁺, Fe²⁺, Ni²⁺ and Ca²⁺ (Table 2), some activity could be detected in presence of Mg²⁺ and Mn² but Co²⁺ requirement was found to be mandatory. The optimal cation concentration for DS-Co activity was 0.6 mM Co²⁺ (Fig.12b). Since the activity optima vs divalent cation concentration curve is steep with respect to Co²⁺ (Fig. 12b), it implies that the availability of small molecules (like Co²⁺) to DS-Co produces significant effect on enzyme activity. Varying cation requirement has been reported from different systems among higher plants, for instance, in V. radiata 0.5 mM Co²⁺ and to a lesser extent 0.5 mM Mn²⁺ was found effective (Rubin and Jensen, 1985). Higher concentration of Mg²⁺ was reported to be quite effective in tobacco by Ganson et al. (1986).
Table 2: Effect of various divalent cations (at 0.5 mM) on DS-Co activity. The values represent the mean of three experiments. Activity is expressed as nM min⁻¹ mg⁻¹ protein.

<table>
<thead>
<tr>
<th>Divalent cation (0.5 mM)</th>
<th>DS-Co activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.54</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>15.40</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>5.46</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>4.90</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>0.70</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>0.70</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Table 3: Effect of L-trp on DS-Co activity. The concentration of trp used was 1 mM. The values represent the mean of three experiments. Activity is expressed as nM min⁻¹ mg⁻¹ protein.

<table>
<thead>
<tr>
<th>Effector added</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>14.24 ± 0.5</td>
</tr>
<tr>
<td>+ Trp</td>
<td>14.30 ± 0.2</td>
</tr>
</tbody>
</table>

Table 4: Effect of DTT on DS-Co activity. The enzyme was pre-incubated for 20 min with DTT (1 mM) prior to addition of E4P and PEP to the assay mixture. Final volume was made up with water, where no DTT was added. This served as the control. The values represent the mean of three experiments. Activity is expressed as nM min⁻¹ mg⁻¹ protein.

<table>
<thead>
<tr>
<th>Effector added</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>12.48 ± 0.30</td>
</tr>
<tr>
<td>+ DTT</td>
<td>12.0 ± 0.35</td>
</tr>
</tbody>
</table>
Taking a cue from these observations, and from Doong et al. (1992) in spinach, the enzyme activity was checked in presence of higher concentration of \( \text{Mg}^{2+} \) (Fig. 12b). At 15 mM \( \text{MgCl}_2 \), high enzyme activity was noted in \textit{Brassica}, unlike in spinach where 20 mM was found to yield the maximal activity. Doong et al. (1992) observed 10 mM \( \text{MgCl}_2 \) to be the most effective concentration for DS-Co in plants such as, pea squash and corn. The requirement for divalent cations in \textit{Brassica} can be explained on the basis of the role of metal ions in catalysis of PEP-utilizing enzymes eg. enolase as suggested by Brewer (1985) and PEP-carboxylase (O'Leary et al., 1981). These ions are required for reaction catalysis by DS-Co since it also utilizes PEP as a substrate. Hence the reaction mechanism is essentially similar to that described for enolase and other PEP utilizing enzymes.

Effect of L-trp and DTT on enzyme activity: L-trp has been shown to activate DAHP synthase activity and is essential for enzyme stability in some of the higher plants (Bentley, 1990). In the present study, trp was included in the enzyme reaction mixture to study its effect on the enzyme activity.

Addition of trp did not have any pronounced effect on DS-Co isozyme (Table 3). This is in contrast to its effect on DS-Mn in \textit{Brassica} (Sharma, 1991) which was considerably activated by trp. Eventhough L-trp does not have a significant effect on DS-Co activity of higher plants in general, there are cases where enzyme activity was affected by the presence of trp for eg. in \textit{N. silvestris}, where weak pH dependent inhibition by trp was observed.

Hysteresis: Certain enzymes have a lag phase before attainment of full activation, and are referred to as hysteretic enzymes. This lag phase can be overcome or reduced to a considerable extent by certain effectors like DTT, which activate the sulphydryl
groups of the enzyme. *Brassica* DS-Co did not exhibit a hysteretic response with DTT. Pre-incubation of enzyme in presence of DTT (1 mM) did not result in an enhanced activity of DS-Co (Table 4; Fig.12c). Progress curve of the enzyme did not show a characteristic slow activation phase, leading to the attainment of a linear rate of product formation. These results indicate that the regulatory sulfhydryl groups are probably not required for activation. However, conclusive evidence for this needs further study with specific modifiers like - iodoacetamide etc. In contrast to the non-hysteretic behaviour of DS-Co from *B. juncea*, earlier work in the same system showed that DS-Mn exhibits hysteretic properties, and the lag in the enzyme activation could be reduced by pre-incubation with reducing agent such as - DTT (Sharma, 1991). DS-Mn enzyme (feed back regulated isozyme) has also been reported to be the hysteretic isozyme - form in carrot roots (Suzich *et al.*, 1985).

**Effect of pathway intermediate - chorismate:** The effect of shikimate pathway intermediate - chorismate was checked on enzyme activity at two different concentrations (0.5 mM and 1 mM). As seen from Table 5, no significant effect was observed. In contrast, DS-Mn activity from *Brassica* was found to be markedly enhanced in presence of chorismate as observed earlier (Sharma, 1991) and in mung bean seedlings (Rubin and Jensen, 1985). Effect of intermediates, besides chorismate, viz., L-arogenate and prephenate has also been studied in tobacco (Rubin and Jensen, 1985) wherein these inhibited the DS-Mn activity. However, there is no data on the regulation of DS-Co by these metabolites.

From the present investigations in *Brassica*, it is concluded that DS-Co is not regulated by the aromatic amino acid, trp and pathway intermediate - chorismate.

**Thermostability of DS-Co:** The enzyme activity was assayed after storing it at different temperatures, viz. -20°C, 4°C and 25°C (room temperature) for varying
Table 5: Effect of chorismate on DS-Co activity. Chorismate was included in the enzyme reaction mixture at the indicated concentrations. The values represent the mean of three experiments. Enzyme activity is expressed as nM min⁻¹ mg⁻¹ protein.

<table>
<thead>
<tr>
<th>Effector added</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>12.97 ± 0.5</td>
</tr>
<tr>
<td>+ chorismate (0.5 mM)</td>
<td>13.10 ± 0.4</td>
</tr>
<tr>
<td>+ chorismate (1 mM)</td>
<td>13.08 ± 0.4</td>
</tr>
</tbody>
</table>

Table 6: Effect of different temperatures on stability of DS-Co. The activity was measured after storing the enzyme at three different temperatures for different intervals of time. Equal volume of enzyme was stored in all experiments. Activity is expressed in nM min⁻¹ mg⁻¹ protein.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>-20°C</th>
<th>4°C</th>
<th>25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.7</td>
<td>14.7</td>
<td>14.7</td>
</tr>
<tr>
<td>12</td>
<td>13.3</td>
<td>12.95</td>
<td>2.94</td>
</tr>
<tr>
<td>24</td>
<td>13.09</td>
<td>12.32</td>
<td>2.1</td>
</tr>
<tr>
<td>48</td>
<td>13.3</td>
<td>11.9</td>
<td>0.22</td>
</tr>
<tr>
<td>96</td>
<td>12.6</td>
<td>11.9</td>
<td>0.21</td>
</tr>
</tbody>
</table>
periods (Table 6). Within 24 hrs of storage, the enzyme activity decreased by 6.6%, 11.9% and 80% at -20°C, 4°C and 25°C respectively. Considerable loss in activity (ca. 99%) was observed on further storage at 25°C and only 1% of the original activity was retained after 96 hrs and beyond. DS-Co was found to be quite stable at -20°C and 4°C, but considerable enzyme activity was lost upon prolonged storage i.e., beyond 96 hrs at 4°C. Other workers have reported upto 30% loss in enzyme activity from carrot roots on storage for 12 d at 4°C, whereas at -20°C, most of the activity was lost (Suzich et al., 1985). In mung bean, upto 11% of the original activity was lost at 0°C after 24 hrs (Rubin and Jensen, 1985). These studies indicate varying degree of thermostability of DS-Co from different systems. Since at 4°C, 19% activity was lost on storage for 96 hrs, care was taken not to store the enzyme for prolonged period at this temperature.

In the present study, an addition of 0.05 mM PEP was able to confer protection from inactivation at 4°C to some extent. The temperature optima for catalysis, used for enzyme assay for DS-Co was in the range of 35-37°C in Brassica.

Table 7 summaries the properties of DS-Co from B. juncea.
Table 7: Summary of the characteristics of the isozyme DS-Co from *B. juncea*

<table>
<thead>
<tr>
<th>DS-Co</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Native MW</td>
<td>100 KD</td>
</tr>
<tr>
<td>Subunit MW</td>
<td>52.5 KD</td>
</tr>
<tr>
<td><strong>For catalysis</strong></td>
<td></td>
</tr>
<tr>
<td>pH optima</td>
<td>7.8</td>
</tr>
<tr>
<td>Temperature optima</td>
<td>35-37°C</td>
</tr>
<tr>
<td>Divalent cation requirement</td>
<td>Co²⁺ &gt; Mg²⁺ (in order of preference)</td>
</tr>
<tr>
<td>No effect of phe, tyr or trp</td>
<td></td>
</tr>
</tbody>
</table>
Purification and characterization of *Chorismate mutase* from *Brassica juncea*

The enzyme chorismate mutase, CM, (E.C. 5.4.99.5) is one of the crucial branch-point enzymes of shikimate pathway and growing knowledge of its control patterns has helped to understand the partitioned flow of carbon to divergent end products. CM has been shown to exist in two isozymic forms: CM-1 identified as an aromatic amino acid-sensitive form and CM-2 as an aromatic amino acid-insensitive isozyme. This demarcation has been characterized in a number of higher plants such as *Vigna radiata* (Gilchrist and Kosuge, 1974; 1975), *Nicotiana silvestris* (Goers and Jensen, 1984a,b; d’Amato et al., 1984), *Sorghum bicolor* (Singh et al., 1985), *Solanum tuberosum* (Morris et al., 1989a; Kuroki and Conn, 1989) and *Brassica rapa* (Schmidt et al., 1991). The isoenzyme ratio has also been shown to be controlled by a variety of external factors like plant age, wounding, etc. (see Literature Review).

Two isoforms of CM have also been characterised in *B. juncea* from our laboratory (Sharma et al., 1993), wherein CM-1 was regulated by the end product amino acids — phe, tyr and trp and CM-2 was insensitive to regulation by these amino acids. In this chapter, results on the purification of CM-1 and CM-2 from *Brassica juncea* along with their kinetic properties, viz. affinity for the substrates, pH and temperature optima, thermostability etc. are discussed.

**Methodology**

The entire purification procedure was carried out at 4°C. For CM purification, field grown plants were used. Plant material (250 gm) excluding root portion was frozen in liquid N₂, homogenized and extracted in 500 ml of extraction buffer (100 mM EPPS, pH 7.5, containing 10 mM β-mercaptoethanol, 1 mM DTT, 1 mM...
PMSF, 1 mM benzamidine HCl, 0.1 mM trp and 50 g PVP). The extract after homogenization was filtered through four layers of cheese cloth and centrifuged at 10,000 rpm for 35 min at 4°C. At this stage 0-70% and 40-70% ammonium sulphate precipitation of the crude extract was tried out. However, ammonium sulphate precipitation at this stage decreased the enzyme yield during the next step of dialysis. When the enzyme activity was checked after precipitation and dialysis, upto 50% loss in total CM activity was observed. Hence, ammonium sulphate precipitation was not carried out at this stage and the supernatant after centrifugation was desalted on a sephadex G-25 column (3.5 x 50 cm) pre-equilibrated with buffer B (100 mM EPPS pH 7.5, 10 mM β-mercaptoethanol, 1 mM DTT, 1 mM benzamidine HCl, 1 mM PMSF and 0.1 mM tryptophan).

After desalting, three different gel matrices were tried. The enzyme extract was loaded on:

(i) sephadex G-75 - 50 column or
(ii) DEAE sepharose CL-6 B or
(iii) DEAE-52 anion exchanger.

i) The sephadex G-75-50 column (2.0 x 80 cm) was pre-equilibrated with buffer B. After loading the protein, the column was eluted with the same buffer at a flow rate of 15 ml hr$^{-1}$. Fractions (6 ml each) containing the enzyme (ca. 60 ml) showed no distinct separation of CM-1 and CM-2.

ii) The desalted protein from the sephadex G-25 column was subjected to anion exchange chromatography using DEAE sepharose CL-6B. The desalted extract was loaded onto the sepharose column (1.0 cm x 40 cm) pre-equilibrated with buffer C (100 mM EPPS, pH 7.5, containing 1 mM benzamidine HCl, 1 mM PMSF, 10 mM β-mercaptoethanol, 0.1 mM tryptophan and 5%
glycerol). The column was washed with two bed volumes of the buffer C, followed by second wash with half the bed volume (≈ 15 ml) of the same buffer containing 0.1 M KCl. The enzyme was eluted with 0.1 - 0.45 M KCl in 350 ml of buffer C at a flow rate of 20 ml hr⁻¹ and 4 - 4.5 ml fractions were collected. Both the isozymes - CM-1 and CM-2 eluted in the gradient, CM-2 at ≈ 0.12 M KCl, and CM-1 at 0.14 M KCl. However, the two peaks were too close to allow an easy separation of the isoenzymes. Therefore, sepharose CL-6B was not preferred for purification of isozymes.

iii) DEAE cellulose chromatography gave the best separation of isozymes, hence it was used for purification of CM-1 and CM-2.

Lyophilization of some of the samples before loading on anion exchanger was also carried out. However, ultracentrifugation of an aliquot of crude enzyme preparation for 20 hr at 4°C at 20,000 g proved better than lyophilization, though either of these procedures resulted in some loss of enzyme activity. Hence, these steps were not utilized in purification protocol.

The extract was directly loaded on a DE-52 (Whatman) column without lyophilization or ultracentrifugation. The column (2.5 cm x 40 cm) was pre-equilibrated with running buffer D (50 mM EPPS, pH 7.5, containing 10 mM β-mercaptoethanol, 1 mM PMSF, 0.1 mM tryptophan and 5% glycerol) and washed with 350 ml of equilibration buffer D prior to application of 300 ml of linear salt gradient (40 mM to 280 mM NaCl) in the same buffer. Fractions (3 ml) containing CM-1 and CM-2 activities respectively were pooled separately and subjected to 40-75% ammonium sulphate fractionation. The fractions which precipitated between 40 -75% saturation with (NH₄)₂ SO₄, represented fairly good recovery of the total enzyme. The precipitate thus obtained was resuspended
in buffer E (50 mM K-PO₄, pH 7.5, containing 0.5 mM PMSF, 10 mM β-mercaptoethanol, 0.1 mM trp and 5% glycerol). Concentrated preparations of CM-1 and CM-2 after dialysis were subjected separately to hydroxylapatite chromatography. CM-2 was first adsorbed onto a hydroxylapatite (Biogel HTP) column (1.0 x 12 cm) pre-equilibrated with buffer E. The column was washed with 50 ml of buffer E, and a 300 ml linear gradient of 50’ - 250 mM K-phosphate buffer, pH 7.5 containing 0.5 mM PMSF, 10 mM β-mercaptoethanol, 0.1 mM trp and 5% glycerol. Fractions (2 ml) were collected at a flow rate of 40 ml hr⁻¹. The isozyme CM-2 eluted off the hydroxylapatite as a single peak with small residual activity and was used for molecular weight analysis and kinetic studies. The isoenzyme CM-1 was subsequently applied to the same hydroxylapatite column as used for CM-2 after thorough washing and equilibrating with the same equilibration buffer as above. Washing and elution of CM-1 was carried out in a similar way as for CM-2. The purified enzyme was further used for MW determination and kinetic studies.

Molecular Weight Determination

Gel filtration: The CM-1 and CM-2 isozymes were subjected to sephacryl S-200 column for native MW determinations. The column was calibrated with the molecular weight standards, viz., cytochrome C (12,400 D), carbonic anhydrase (29,000 D), albumin (66,000 D), alcohol dehydrogenase (150,000 D) and β-amylase (200,000 D). Void volume was determined by blue dextran (MW 2,000,000 D). sephacryl S-200 column was run as described for DAHP synthase-Co calibration.

Subunit MW Determination: Subunit MW was calculated by running the isoenzymes on SDS-PAGE as described for DS-Co.
Results and Discussion

Separation and purification of isoenzymes of Chorismate mutase

Two distinct peaks of CM were observed when enzyme extract was fractionated by DEAE-cellulose chromatography. A gradient of 40-280 mM NaCl facilitated peak separation of both the isozymes. Though the CM-2 isozyme was eluted out in the wash fractions, a much cleaner preparation was obtained if the starting gradient was adjusted to 40 mM NaCl. A linear gradient of 40 mM - 280 mM NaCl gave the elution profile as depicted in Fig. 13a. CM-2 isozyme could be washed through the column without retardation and CM-1 isozyme eluted at 0.13 M NaCl salt gradient.

Various steps of ammonium sulphate fractionation were carried out and CM activity was assayed in the supernatant and pellet fractions respectively. After several trials, 40-75% saturation was found to be the most effective and considerable enzyme activity was retained in the pellet. Inclusion of trp in resuspension and dialysis buffer was found to be crucial. Buffer lacking trp at this stage resulted in about 30% loss of CM-1 activity which further declined if the enzyme preparation was stored in the absence of trp.

Preparations carried through the second fractionation step of hydroxylapatite chromatography showed an elution profile as depicted in Fig. 13b. Application of CM-1 and CM-2 to the hydroxylapatite resulted in 82.96 and 48.46-folds purification of the isozymes, respectively (Table 8) and the respective yields were 9% and 7%. The CM-2 isozyme eluted first, followed by CM-1 at higher molarity of K-PO₄ buffer. Table 8 shows the enzyme activity at different steps of purification. Further studies were carried out using these CM-1 and CM-2 isozymic preparations. It was important to include trp in the buffer at various stages of
Fig. 13a Elution of CM-1 and CM-2 from DE-52 column by using a 40-280 mM NaCl gradient. $A_{320}$ represents CM activity and $A_{280}$ the protein profile.

Fig. 13b Elution profile of CM-1 and CM-2 from hydroxylapatite column. Preparations of CM-1 and CM-2 obtained after DEAE-cellulose chromatography were applied to hydroxylapatite as specified under Materials and methods. The leading peak is for CM-2 and the trailing peak is for CM-1. $A_{320}$ represents CM activity and $A_{280}$ the protein profile. The vertical dashed line represents starting of gradient elution. For convenience, CM-1 and CM-2 are represented in the same figure.
Table 8: Summary of the steps involved in purification of CM isozymes (CM-1 and CM-2) from *B. juncea* leaves.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (nM/min)</th>
<th>Specific Activity</th>
<th>Yield (%)</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1,250</td>
<td>6,875</td>
<td>110,311</td>
<td>16.04</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>G-25</td>
<td>1,538</td>
<td>5,520</td>
<td>79,298</td>
<td>14.36</td>
<td>71.9</td>
<td>0.89</td>
</tr>
<tr>
<td>DE-52</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM-1</td>
<td>35</td>
<td>139.65</td>
<td>27,866.5</td>
<td>199.54</td>
<td>25.3</td>
<td>12.4</td>
</tr>
<tr>
<td>CM-2</td>
<td>40</td>
<td>162</td>
<td>23,333.3</td>
<td>144.03</td>
<td>21.2</td>
<td>8.98</td>
</tr>
<tr>
<td>40-75% ammonium sulphate precipitation (P)</td>
<td>4.5</td>
<td>18.45</td>
<td>19,733</td>
<td>1069.5</td>
<td>17.88</td>
<td>66.68</td>
</tr>
<tr>
<td>CM-1</td>
<td>6</td>
<td>25.3</td>
<td>16,032</td>
<td>633.6</td>
<td>14.5</td>
<td>39.5</td>
</tr>
<tr>
<td>CM-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM-1</td>
<td>10</td>
<td>7.5</td>
<td>9,981</td>
<td>1330.8</td>
<td>9.05</td>
<td>82.96</td>
</tr>
<tr>
<td>CM-2</td>
<td>12.5</td>
<td>10.62</td>
<td>8,255</td>
<td>777.3</td>
<td>7.48</td>
<td>48.46</td>
</tr>
</tbody>
</table>
purification, otherwise an irreversible loss in the activity of enzyme was observed. Very little activity of CM-1 was detected on storage at 0°C after two weeks if trp was not included in the buffer. CM-2 activity was relatively unaffected by this effector molecule.

**Molecular Weight Determination**

Native molecular weight of the isozymes was determined by gel filtration (sephacryl S-200). Elution of standard proteins used for calibration is shown in Fig. 14a (calibration proteins were the same as for DAHP synthase-Co). A plot of molecular weight vs relative mobility (Ve/Vo), gave an apparent molecular weight of 58 KD for CM-1 and 50 KD for CM-2. Isozymes CM-1 and CM-2 were further run on a denaturing gel along with standard molecular weight markers. Relative mobility (Rf) of the standard proteins and the isozymes was measured. A plot of log molecular weight vs Rf gave a subunit molecular weight of 37 KD for CM-1 and 35 KD for CM-2 (Fig.14b).

These results indicated that the CM-1 and CM-2 are not made up of a single unit of 58 or 50 KD, but possess a subunit conformation. However, the results of native and subunit forms do not strictly correspond to a dimeric form. The MW of the subunits of CM isozymes from higher plants varies from system to system. For CM-1 in *Sorghum bicolor*, where this regulated isozyme was suggested to be a dimer, native MW was determined to be 56,000 D and subunit MW 36,0000 D. CM-2 was the minor component on electrophoresis and showed MW of 48,000 D (Singh et al., 1985). However, in *Nicotiana*, CM-1 had a native MW of 52,000 D and CM-2 65,000 D (Goers and Jensen, 1984a). More recently, CM-1 has been characterized from etiolated shoots of *Sorghum vulgare* and shown to have a native MW of 56,000 D [as reported earlier by Singh et al. (1985) for *S. bicolor*]. The subunit
Fig. 14a Log molecular weight vs $R_f$ plot for native molecular weight determination of CM-1 and CM-2. The $R_f$ values of the standards and CM-1 and CM-2 are indicated. The standards used are the same as indicated in Fig. 10a.

14b Log molecular weight vs $R_f$ plot for subunit molecular weight determination of CM-1 and CM-2. The $R_f$ values of the standards and CM-1 and CM-2 are indicated. The standard protein molecular weight markers are the same as indicated in Fig. 8. C. anhydrase represents carbonic anhydrase.
Fig. 14c Coomassie Brilliant Blue stained SDS gel (10%)

Lane a: Standard protein molecular weight markers used are carbonic anhydrase (29 KD), ovalbumin (45 KD), bovine albumin (66 KD), phosphorylase b (97.4 KD), β-galactosidase (116 KD) and myosin (205 KD).

Lane b&c: CM-1 and CM-2 preparations after DE-52 chromatography (step 3) of purification. 10 μl of the protein is used for loading.

Lane d&e: CM-1 and CM-2 preparations after 40-75% ammonium sulphate precipitation (step 4) of purification. 10 μl of the protein is used for loading.
Fig. 14c
MW, 32,000 D was, however, different (Siehl et al., 1992). The MW of CM-1 from oak has been reported to be 45,000 D (Gadal and Bouyssou, 1973).

Kinetic Properties of CM-1 and CM-2

Earlier work from our laboratory (Sharma, 1991) indicated that CM-1 and CM-2 from B. juncea vary in their regulatory properties. CM-1 is under stringent feedback regulation by end product amino acids. Whereas L-trp exerted a strong stimulatory effect on the CM-1 activity, L-phe showed slight inhibition. L-tyr, however, caused a strong allosteric inhibition. When these amino acids were used in combination in equimolar concentrations, characteristic regulatory patterns were noticed, eg. L-trp reversed the inhibitory effect of L-phe and L-tyr. When phe and tyr were used in combination, a non-cumulative inhibition was seen, suggesting a common binding effector site for phe and tyr.

In contrast to CM-1, CM-2 isozyme was found to be insensitive to phe, tyr or trp. The aromatic amino acids when used alone or in combination in equimolar concentrations did not activate or inhibit the enzyme activity. These discriminatory properties of CM-1 and CM-2 were used to distinguish between the isozyme pair in the present study.

Effect of different concentrations of the substrate - chorismate: $K_m$ value: The CM activity was assayed using 300 μl purified protein and increasing concentrations (0-10 mM) of chorismate. The $K_m$ values for CM-1 and CM-2 were determined by first drawing the [S] vs [V] plot which showed Michaelis-Menten kinetics and then calculating on the basis of Lineweaver - Burk scheme. From the data, the $K_m$ value of CM-1 was found to be 1.09 mM and that of CM-2 was 0.2 mM at pH 7.5 (Fig.15a and b). Thus the isozyme CM-2 has a much greater affinity for the substrate.
Fig. 15 Initial velocity and Lineweaver Burk plot for CM-1 and CM-2.
a (inset) CM-1 activity with varying concentrations of chorismate.
a: Lineweaver-Burk plot of reciprocal of the initial velocity of CM-1 vs reciprocal of concentration of chorismate.
b (inset) CM-2 activity with varying concentrations of chorismate.
b: Lineweaver-Burk plot of the reciprocal of the initial velocity of CM-2 vs reciprocal of concentration of chorismate.
chorismate as compared to CM-1 and gets saturated faster. This could be explained by the fact that the isoenzyme CM-2, which is present in greater amount in cytosol as seen in a few plant systems (Jensen, 1985a), might receive a low-concentration flow of chorismate that is reflective of low channelling of carbon to CM-2 but is enough to saturate the enzyme. On the other hand, the isoenzyme CM-1 (mainly localized in chloroplasts, as seen in a few plant systems) might get a high concentration of chorismate reflective of a high-flux of carbon, which is exclusively channelized for protein synthesis (since it is not involved in the secondary metabolism). This explanation could also justify the greater sensitivity of CM-1 to regulation by end product amino acids for stringent control of protein synthesis.

Effect of Trp, Phe and Tyr on enzyme activity: Inclusion of aromatic amino acid, trp was found to be essential during the purification steps of CM. The effect of addition of phe, tyr or trp on the enzyme extract was also studied. Fig.16a shows the effect of L-trp (0-1.5 mM) on CM-1 and CM-2 activities. CM-1 activity was found to be extremely sensitive to the addition of trp. Increased concentration of the allosteric effector trp, caused an increase in the enzyme activity and 0.75 mM trp resulted in the maximal activation. A further increase in the concentration of trp caused a slight decline in CM-1 activity. That trp is an allosteric activator of CM-1 was further confirmed by the [S] vs [V] curve plotted in the presence of L-trp with increasing concentrations (0 to 4 mM) of chorismate (Fig. 16b). Trp activation of CM-1 was most evident as compared to the control (without the addition of trp) where the velocity of reaction was much lower at an equivalent concentration of the substrate and followed a non-linear kinetic behaviour. CM-2 activity remained unaffected by trp. These results gave a cue that trp affinity can be made use of as a
Fig. 16a  Effect of tryptophan on CM-1 and CM-2 activity. The concentration of tryptophan included in the assay mixture varied from 0-1.5 mM. The concentration of chorismate used in the assay was 2 mM. The specific activity of the control reaction has been used to calculate % activity.

b:  Effect of tryptophan on CM-1 activity with increasing concentrations (0-4 mM) of chorismate. Tryptophan was included in the assay mixture at a concentration of 0.75 mM.

c:  v versus [S] plot showing effect of phenylalanine and tyrosine on CM-1 activity. The concentration of chorismate used in the assay was 2 mM and the reaction was initiated by addition of CM-1 isoenzyme. The specific activities of the control reaction has been used to calculate % inhibition.
Figure a: Activity (%) of CM-2 and CM-1 as a function of tryptophan concentration.

Figure b: CM-1 specific activity with and without tryptophan (Trp) as an effect.

Figure c: CM-1 inhibition (%) by phenylalanine (Phe) and tyrosine (Tyr) as a function of effector concentration.
Fig. 16d Inhibition curves for CM-1 in presence of phenylalanine. Lineweaver-Burk plot of reciprocal of the initial velocity of CM-1 vs reciprocal of concentration of chorismate. Four different concentrations of phe used are 0.5, 1.0, 1.5 and 2 mM.

d(inset) Slope vs [phenylalanine] plot for determination of $K_i$ value (phe).

Fig. 16e Inhibition curve for CM-1 in presence of tyrosine. Lineweaver-Burk plot of reciprocal of the initial velocity of CM-1 vs reciprocal of concentration of chorismate. Four different concentrations of tyr used are 0.5, 1.0, 1.5 and 2 mM.

e(inset) Slope vs [tyrosine] plot for the determination of $K_i$ value (tyr).
ligand in affinity chromatography purification, exclusively of CM-1 from Brassica, since CM-2 activity remains unaffected in the presence or absence of trp.

Effect of L-phe and L-tyr on the enzyme activity was also tested in the range of 0 - 1.5 mM, (Fig. 16c). Both phe and tyr caused an inhibition of CM-1 isoenzyme, whereas CM-2 was unaffected by these amino acids. Tyr was found to be a more potent inhibitor of CM-1 activity as compared to phe. At 2 mM substrate concentration, 87.5%, & 37.5% inhibition was obtained for CM-1 with 1.25 mM tyr and phe respectively. The inhibition followed almost linear kinetics with maximal inhibition at 1.25 mM phe and tyr respectively, though a lower concentration (0.12 mM) of tyr or phe was also found to be inhibitory. At pH 7.5, 50% inhibition of CM-1 was obtained by including 0.5 mM tyr in the assay mixture. The Lineweaver Burk plots indicate that both phenylalanine and tyrosine follow competitive inhibition kinetics. The $K_i$ values of CM-1 were obtained by plotting the kinetics on a double reciprocal plot (Fig. 16d and e). $K_i$ (phe) was 0.6 mM (Fig. 16d, inset) whereas $K_i$ (tyr) was 0.138 mM (Fig. 16e, inset).

Effect of pH: CM-1 and CM-2 activity was measured in various buffers (as described for DS-Co) in a pH range of 5-9. A rise in enzyme activity was observed till pH 7. The activity was constant between 7-8. On raising the pH upto 8, no further increase in enzyme activity was observed. The optimal pH range for CM-1 was between 7.0-8.0 in EPPS buffer with highest activity recorded at pH 7.5. The optimal pH range for CM-2 was 7.0-7.7 (Fig. 17).

The pH optima reported for plants other than Brassica varies depending on the system, eg. in potato, CM-1 and CM-2 have a pH optima of 7.5 - 8 (Kuroki and Conn, 1989). In Sorghum bicolor, Singh et al. (1985) observed a broad pH optima for CM-1 and CM-2. Whereas for CM-1 it was between 6 - 10, for CM-2 it was
Fig. 17 Effect of pH on CM-1 and CM-2 activity. The activity is measured in the range of pH 5-9 and is expressed as nM min$^{-1}$ mg$^{-1}$ protein.

Fig. 18 Thermal stability and prevention of thermal denaturation of CM-1 by addition of phenylalanine, tyrosine and tryptophan. Enzyme with or without the effector (phe, tyr or trp) was incubated at 50°C for indicated time intervals prior to assaying. The effectors were presented 1 mM.
found to be 7.5 - 9.5. Both the isozymes of *Nicotiana silvestris* too had a broad pH optima for catalysis between 6-8 (Goers and Jensen, 1984a).

**Effect of metal ions (divalent cation) on CM-1 and CM-2 activity:** Various metal ions (Co$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Cu$^{2+}$, Zn$^{2+}$ and Ni$^{2+}$) were included in the assay mixtures at a final concentration of 1 mM each (Table 9). None of the divalent cations had any effect on CM-1 or CM-2 activities thereby indicating that these ions do not have any regulatory role on the branch point enzyme, chorismate mutase. This finding is in contrast to that observed for DAHP synthase-Co in the present study where divalent cations ions are found to be necessary for activity.

**Thermostability of CM isozymes:** CM-1 and CM-2 were found to be very stable at $-20^\circ$C. There was negligible loss in enzyme activity at this temperature even if the isozymes were stored for up to one month (Table 10).

At $4^\circ$C, the enzyme activity showed a decline with time, and up to 23% decline in CM-1 activity was seen by day 4. However, the decline was not so sharp between day 4 and day 7. The loss in activity of CM-2 when stored at $4^\circ$C was 11.4% by day 4. Thus, CM-1 was comparatively less stable than CM-2 at $4^\circ$C. The enzyme activity was also checked after storage at $25^\circ$C. By day 4, about 96% CM-1 and 97% of CM-2 activity was lost. This loss of activity could be seen as early as 12 hrs when 42.3% and 38.5% of CM-1 and CM-2 activities, respectively were lost.

The optimal temperature required for reaction catalysis by isoenzymes CM-1 and CM-2 was between 35-37°C for a 30 min reaction time. Increase in the activity was observed with an increase in temperature (from $25^\circ$C to $37^\circ$C) during preliminary standardization of enzyme assay conditions (data not shown). The activity of isozyme CM-1 and CM-2 was stable throughout 30 min reaction even at $40^\circ$C,
Table 9: Effect of various divalent cations on CM-1 and CM-2 activity. The divalent cations were included in the assay mixture at a final concentration of 1 mM. Specific activity is expressed in μM min⁻¹ mg⁻¹ protein. Chloride salts were used.

<table>
<thead>
<tr>
<th>Effector added</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CM-1</td>
</tr>
<tr>
<td>None (Control)</td>
<td>1.29</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>1.30</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1.25</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>1.24</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>1.26</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>1.26</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Table 10: Effect of different temperatures on the stability of CM isozymes. The activity was measured at three different temperatures after storing for indicated time. Equal volume of enzyme was stored in all experiments. Activity is expressed in μM min⁻¹ mg⁻¹ protein. Activity at 0 time for CM-1 is 1.35 and for CM-2 is 0.7.

<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>12 hrs</td>
<td>1.3</td>
<td>0.7</td>
<td>1.15</td>
<td>0.67</td>
<td>0.75</td>
<td>0.43</td>
</tr>
<tr>
<td>24 hrs</td>
<td>1.25</td>
<td>0.68</td>
<td>1.07</td>
<td>0.65</td>
<td>0.15</td>
<td>0.09</td>
</tr>
<tr>
<td>48 hrs</td>
<td>1.25</td>
<td>0.67</td>
<td>1.05</td>
<td>0.63</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>96 hrs</td>
<td>1.24</td>
<td>0.67</td>
<td>1.0</td>
<td>0.62</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>1 month</td>
<td>1.15</td>
<td>0.67</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
</tbody>
</table>

n.d. not determined
thereby showing that there is no thermal inactivation at this temperature. However, beyond 40°C, thermal inactivation was observed.

Prevention of thermal denaturation of CM-1 and CM-2: Taking a cue from the fact that trp is an allosteric activator of CM-1, a study was undertaken to find out if it could prevent CM-1 from thermal inactivation. The CM-1 isozyme was incubated for varying time intervals (5, 10, 15 min) at 50°C in presence of phe, tyr or trp. The samples without any of these molecules served as control (Fig. 18).

About 77.4% of the initial activity was lost when isoenzyme CM-1 was kept at 50°C for 20 min prior to assay at 37°C. When tryptophan was present during thermal treatment at 50°C for 20 min, subsequent assay showed only about 36% loss from the initial activity of CM-1 as compared to 77.4% loss without the addition of trp. In contrast, the presence of phenylalanine or tyrosine during thermal treatment at 50°C for 20 min at pH 7.5, failed to protect against thermal denaturation. CM-2 was unaffected by presence or absence of any amino acid at 50°C (at pH 7.5) and its thermal denaturation was observed. At 50°C, phe, tyr or trp did not protect CM-2 from denaturation. Thermal denaturation of CM-1 and CM-2 in *Nicotiana* does not take place at least up to 45°C (Goers and Jensen, 1984a).

Table 11, summarizes the characteristics of CM-1 and CM-2 from *B. juncea.*
Table 11: Summary of characteristics of CM from *B. juncea*

<table>
<thead>
<tr>
<th></th>
<th>CM-1</th>
<th>CM-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native MW</td>
<td>58 KD</td>
<td>50 KD</td>
</tr>
<tr>
<td>pH optima</td>
<td>7 - 8</td>
<td>7 - 7.7</td>
</tr>
<tr>
<td>Temp. optima</td>
<td>37°C</td>
<td>37°C</td>
</tr>
<tr>
<td>$K_a$ (Trp)</td>
<td>1 mM</td>
<td>$K_m$ (Chm)</td>
</tr>
<tr>
<td>$K_m$ (Chm)</td>
<td>1.09 mM</td>
<td>No effects of Phe, Tyr or Trp</td>
</tr>
</tbody>
</table>