Immunological characteristics of PEP carboxylase from leaves of C₃-, C₄- and C₃-C₄ intermediate species of *Alternanthera* - Comparison with selected C₃- and C₄- plants

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Immunological cross-reactivity of phosphoenolpyruvate carboxylase (PEPC) in leaf extracts of C₃, C₄ and C₃-C₄ intermediate species of *Alternanthera* (along with a few other C₃- and C₄-plants) was studied using anti-PEPC antibodies raised against PEPC of *Amaranthus hypochondriacus* (belonging to the same family as that of *Alternanthera*, namely Amaranthaceae). Antibodies were also raised in rabbits against the purified PEPC from *Zea mays* (C₄-monocot - Poaceae) as well as *Alternanthera pungens* (C₃-dicot - Amaranthaceae). Monospecificity of PEPC-antisera was confirmed by immunoprecipitation. Amount of PEPC protein in leaf extracts of *A. hypochondriacus* could be quantified by single radial immunodiffusion. Cross-reactivity of PEPC in leaf extracts from selected C₃, C₄, and C₃-C₄ intermediate species (including those of *Alternanthera*) was examined using Ouchterlony double diffusion and Western blots. Anti-PEPC antisera raised against *A. hypochondriacus* enzyme showed high cross-reactivity with PEPC in leaf extracts of *A. hypochondriacus* or *Amaranthus viridis* or *Alternanthera pungens* (all C₄ dicots), but limited cross-reactivity with that of *Zea mays, Sorghum* or *Pennisetum* (all C₃ monocots). Interestingly, PEPC in leaf extracts of *Alternanthera tenella, A. ficoides*, *Punarnemia hysterophora* (C₃-C₄ intermediates) exhibited stronger cross-reactivity (with anti-serum raised against PEPC from *Amaranthus hypochondriacus*) than that of *Pisum sativum, Convolvulus baugelensis, Alternanthera sessilis* (C₃-plants).

Further studies on cross-reactivities of PEPC in leaf extracts of these plants with anti-PEPC antisera raised against PEPC from leaves of *Zea mays* or *Alternanthera pungens* confirmed two points - (i) PEPC of C₃-C₄ intermediate is distinct from C₃ species and intermediate between those of C₃ and C₄-species; and (ii) PEPC of C₄-dicots was closer to that of C₃-species or C₃-C₄ intermediates (dicots) than to that of C₄-monocots.

Being a key enzyme, properties of PEPC can be used as an interesting tool to assess the evolutionary relationships between C₃ and C₄ plants and C₃-C₄ intermediates. Our studies on biochemical properties of the enzyme demonstrated that PEPC from C₃-C₄ intermediates of *Alternanthera* (*A. tenella* and *ficoides*) exhibits an intermediate status between those of C₃- (*A. sessilis*) and C₄-species (*A. pungens*).

A rapid conventional method for purification of PEPC from the leaves of *Amaranthus hypochondriacus* (another C₄-dicot and belonging to the same family of *Alternanthera*, namely Amaranthaceae) has been developed in our lab. We have raised anti-PEPC (*Amaranthus*) antibodies in rabbit and used anti-PEPC antiserum to check the cross-reactivity with PEPC of *Alternanthera* species.

In contrast to the extensive literature on purification and storage of PEPC, the studies on immunological properties of PEPC are quite limited. Since the topic is of general interest, a comprehensive study has been attempted, by including a few more C₃ and C₄ plants. The photosynthetic status of C₃- and C₄-plants and C₃-C₄ intermediates used in this study has already been established. It is well known that there is a significant difference between PEPC of C₄-dicots and C₄-monocots. We have therefore included in the test plants, a few monocots as well. Anti-serum prepared against PEPC of maize (a monocot) was also used to confirm the differences, if any, between monocots and dicots. Finally, the cross-reactivity was confirmed by using anti-PEPC antibodies raised against the enzyme from *Alternanthera pungens* (a C₄-species).

**Materials and Methods**

**Plant material**— The details of the plant material and their mode of multiplication/growth have been reported earlier.

**References**

Experimental methods—Assay and purification of PEPC from leaves of *Amaranthus* or *Alternanthera* were done as per the procedure described elsewhere.

Anti-PEPC antiserum was raised in 6 month-old white rabbits, as per the principles described by Nimmo *et al.* Pre-immune serum was collected from ear-vein of the rabbit. Then, 0.5 mg of purified PEPC in 500 μL, emulsified in equal volume (500 μL) of 50% Freund’s complete adjuvant, was injected subcutaneously at about 10 sites. Four weeks later, the animal was given (through subcutaneous injections) a booster dose of 0.25 mg in 250 μL of purified enzyme, emulsified with equal volume (250 μL) of 50% Freund’s incomplete adjuvant. After 2 weeks, blood was collected from the ear vein. The blood was allowed to coagulate and the antiserum was collected by centrifugation at 10,000 g for 30 min. The antiserum was split into several small aliquots and stored at -20 °C.

The animal was again injected with 0.25 mg in 250 μL of enzyme emulsified with equal volume (250 μL) of 50% Freund’s incomplete adjuvant. Blood was collected after 6 to 8 weeks. Anti-PEPC antiserum was collected as described above by centrifugation and stored in multiple aliquots.

Ouchterlony double-dimensional diffusion—Specificity of PEPC antiserum was checked by using 1% (w/v) agarose gels prepared on glass slides. Five wells (0.5 cm diam) were punched in agarose gels, with the help of a sharp gel-puncher. One well was in the center and was surrounded by four wells located symmetrically in the outer ring. A graph sheet was placed below the glass slide, so as to achieve symmetry and precise distance between wells.

In the center-well, the purified PEPC of leaf extract from *A. hypochondriacus* (or other plants) was loaded. Different dilutions of crude anti-PEPC antiserum (1/10 to 1/100) was loaded into other four wells in the outer ring. The precipitin band was observed within 24 hr of incubation at 10°-12 °C. The reaction was stopped by washing the gel several times with 0.9% (w/v) NaCl, to remove unbound proteins. Pre-immune serum showed no cross-reaction (i.e., no precipitation) with either purified PEPC or leaf extracts of *A. hypochondriacus*. The cross-reactivity of PEPC from *A. hypochondriacus* with other species was examined by loading purified enzyme/crude extracts of various C₃, C₃-C₄ and C₄ dicot and C₄ monocot species in the outer wells and center well was filled with anti-PEPC antiserum.

Western blotting—Cross-reactivity of PEPC between C₃, C₃-C₄ and C₄ plants was evaluated through Western blots, after transferring electrophoretically the proteins from the gel onto the polyvinylidene difluoride (PVDF) membranes.

Proteins in the leaf extracts were separated by 10% SDS-PAGE. The proteins were transferred on to PVDF membranes (immobilon-PC from Millipore, procured from Sigma Chemicals Co., USA). The gel, PVDF membranes and chromatography papers were soaked in transfer buffer (25 mM, Tris-HCl/192 mM, glycine; pH 8.3 and 20% (v/v) methanol) for 30 min. The gel and membranes were sandwiched between filter papers (three on each side) saturated with the buffer and blotted using a semi-dry blotted (LKB 2117 Multiphor) for 3 hr. A constant power of 90 volts was supplied (through Atto Digi-power SJ-1081).

The transfer of proteins was confirmed by Ponceau’s staining [0.2% (w/v) Ponceau’s stain and 3% (v/v) TCA]. Ponceau’s stain was removed by repeated washing with distilled water. The membranes were blocked with 5% of non-fat milk powder in Tris-buffered saline (TBS) containing 25 mM, Tris-HCl, pH 7.5 and 150 mM, NaCl. Blocking was necessary to saturate the non-specific binding sites. The blocking was allowed for 1 hr at room temperature with constant shaking.

The blocked membranes were probed for 1 hr with antiserum of anti-PEPC (from *A. hypochondriacus* or *Alternanthera pingens* or *Zea mays*) antiserum, diluted 1:500 and 1:400 respectively, in blocking solution. The blotted membranes were washed three times (15 min each wash) with TBS and incubated with anti-IgG alkaline phosphatase conjugate (1:7500) for 1 hr and washed three times. The washed blot was developed with 33 μL of 5-bromo-4-chloro-3-indoly phosphate (BCIP; 50 mg mL⁻¹ stock solution) and 60 μL of p-nitroblue tetrazolium chloride (NBT) (50 mg mL⁻¹ stock solution in 10 mL of 16 mM Tris-HCl, pH 9.5), 4 mM NaCl and 0.2 mM MgCl₂.

Results and Discussion

In contrast to the extensive literature on purification and storage of PEPC, the studies on immunological properties of PEPC are quite limited. An attempt has, therefore, been made to study comprehensively the immunological properties of PEPC purified from *A. hypochondriacus*, a NAD-ME type C₃-dicot plant and assessed its cross-reactivity with a few other C₃- and C₄-type dicots and C₄-monocots.

Antibodies were raised in rabbits against PEPC purified from the leaves of *Amaranthus hypochon-
The antibody showed a titre value of 1/100 against purified PEPC from leaves of A. hypocondriacus as well as PEPC in leaf extracts of A. hypocondriacus or Alternanthera pungens (data not shown).

The specificity of PEPC-antisem was checked initially by immunoprecipitation. The leaf extracts were incubated with anti-PEPC antisem and incubated overnight to precipitate down the PEPC-protein. There was a marked decrease in PEPC activity on treatment with anti-PEPC antisem, presumably due to the precipitation of PEPC from the supernatants (Fig. 1). In controls, when leaf extracts were treated with non-immunised rabbit serum, there was no change in PEPC activity in the supernatants, and obviously PEPC was not precipitated from the supernatants. An examination on SDS-PAGE (10%) confirmed the presence of PEPC in the precipitate. Level of PEPC-protein in the precipitate increased as the amount of PEPC antisem increased (Fig. 2). This indicated the antisem prepared was fairly specific to PEPC protein even in crude leaf extracts. Sugiyama et al. have shown the specificity of the antisem prepared against purified maize PEPC by immunoprecipitation with crude extracts of maize PEPC.

Single radial immunodiffusion was performed to quantitate the amount of PEPC protein in crude leaf extracts using anti-PEPC antisem of A. hypocondriacus. There was a proportionate increase in diameter of the ring with the increase in antigen concentration.

Using ELISA, it was possible to quantitate low levels (ng) of PEPC protein in crude leaf extracts of A. hypocondriacus, as indicated by linear relationship between immuno-precipitate-linked absorbance and PEPC protein in leaf crude extracts. In contrast, μg amounts of PEPC protein was required for detecting PEPC in pea leaf extracts indicating that the cross-reactivity of C₃ dicot PEPC with C₄-PEPC was less than that in C₄ dicot PEPC.

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**Fig. 1**—Immunoprecipitation of PEPC extracted from illuminated leaves. To a fixed volume of leaf extract, containing 0.2 units of PEPC, variable volumes of anti-PEPC antisem or preimmune serum (0-100μL) were added and the mixtures were left overnight at 4°C. After centrifugation the supernatant was checked for PEPC activity. The control samples were treated with non-immunised serum.

**Fig. 2**—PEPC from leaf extracts of Amaranthus hypocondriacus after precipitation by the addition of 20-100μL of anti-PEPC antisem. The pellet was washed and examined by SDS-PAGE for the presence of PEPC. PEPC bands were observed only on precipitation with anti-PEPC antisem (A). In controls (preimmune serum, set B), no PEPC band was observed indicating that precipitation of the enzyme did not occur.

**Fig. 3**—Ouchterlony double-diffusion, to assess the cross-reactivity of PEPC in leaf extracts of selected C₄, C₃ and C₄-C₃ intermediate species (including those of Alternanthera). The centre wells contained the anti-PEPC antisem (raised against purified PEPC from Amaranthus hypocondriacus, C₄-plant, Amaranthaceae). The outer wells 1, 2, 3 contained the leaf extracts of A. hypocondriacus. The outer well No.1 contained the leaf extracts of A. hypochondriacus. The outer wells 2 to 4 contained in the given order. (A)—Leaf extracts of C₃ plants—Alternanthera pungens (dicot), Zea mays (monocot) and Amaranthus viridis (dicot); (B)—Leaf extracts of C₄ plants—Alternanthera sessilis (dicot), Commelina benghalensis (monocot), Pisum sativum (dicot); (C)—Leaf
Cross reactivity of PEPC from *A. hypochondriacus*, a C₄ dicot, with PEPC in leaf extracts of C₃ or C₄ monocots and dicots was examined by Ouchterlony double-diffusion (Fig. 3). The anti-PEPC antiserum, raised against PEPC of *A. hypochondriacus*, showed very strong reaction (as indicated by the precipitin band) with *Amaranthus viridis* and *Alternanthera pungens* (C₄-dicots). On the other hand, the same anti-PEPC antiserum, showed a faint reaction with PEPC from *Zea mays*, a C₄ monocot (Fig. 3). This suggested that PEPC from *A. hypochondriacus* had only limited identity with the tertiary structure of PEPC from either *Zea mays* (C₄-monocot) or C₄-dicot or C₃-C₄ intermediates. Iglesias et al. have observed through the Ouchterlony technique, that PEPC of *Zea mays* exhibited only limited cross-reaction and thereby depicted partial identity with anti-PEPC antiserum raised against PEPC purified from *A. viridis*. Distinctness of C₄ dicot-PEPC from that of C₃ monocot species was further confirmed by Western blot analysis.

Although, Ouchterlony double diffusion did not show much cross-activity of PEPC from *A. hypochondriacus*, a C₄-dicot with C₃ plants or C₃-C₄ intermediates, it could be also because of limited sensitivity of Ouchterlony technique. Since the immune reaction can be amplified with Western blot, we checked the cross-reactivity with C₃ and C₃-C₄ intermediate species with that of PEPC from either (*Amaranthus*) a C₃-dicot and maize (C₃-monocot) plants using anti-PEPC antiserum. When probed with the anti-PEPC antiserum against PEPC of *A. hypochondriacus*, significantly positive cross-reactivity was obtained (i.e., intensely stained blots) with PEPC in leaf extracts of three C₃-dicot plants, *A. hypochondriacus*, *A. viridis* and *Alternanthera pungens* (Fig. 4a). In case of these C₃-dicots, the leaf extracts containing protein (20 µg) were loaded on the gel for visual appearance of blot. PEPC in the leaf extracts of three monocots (*Zea mays*, *Sorghum bicolor*, and *Pennisetum*) could also be visualized by Western blot using anti-PEPC antiserum against C₄-dicot PEPC (Fig. 4b). However, the protein to be loaded onto the gel in case of C₃-monocots (30 µg of protein) was more than that (20 µg protein) needed in case of C₄ dicots. Such need for higher levels of C₃-monocot protein than that of C₄-dicot protein for similar intensity on Western blots, suggested that the PEPC of C₄-dicot was immunologically similar yet distinct from that of C₃-monocot.

Similarly, probing with PEPC-antiserum (against of PEPC of *Amaranthus*) could detect the PEPC in leaf extracts of C₃-C₄ intermediates (*Parthenium hysterophorus*, *Alternanthera ficoideas*, *Alternanthera tenellia*; Fig. 4c) as well as that of C₃ species (*Pisum sativum*, *Commelina benghalensis*, *Alternanthera sessilis*). However, the intensity of protein bands in case of C₃-C₄ intermediate species was stronger than that of C₃ species (Fig. 4d). PEPC-protein in leaf extracts of

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**Fig. 4**—Western blots of PEPC in leaf extracts of selected C₄ and C₃-plants and C₃-C₄ intermediates. The blots were probed with anti-PEPC antiserum (raised against PEPC of *Amaranthus hypochondriacus*, a C₄-dicot). (A)- C₄-dicots (Lanes 1 to 3 in the given order: *A. hypochondriacus, Amaranthus viridis, Alternanthera pungens*); (B)- C₄-monocot (Lanes 1 to 3: *Zea mays, Sorghum bicolor, Pennisetum glaucum*); (C)- C₃-C₄ intermediate dicots (Lanes 1 to 3: *Parthenium hysterophorus, Alternanthera ficoideas, Alternanthera tenellia*); (D)-C₃-plants (Lanes 1 to 3: *Pisum sativum, Alternanthera sessilis* (both dicots), *Commelina benghalensis* (monocot)). The location of PEPC is indicated by an arrow. The amount of protein loaded in each lane was 20 µg in case of C₄ dicots and 30 µg in all other cases.
C₄-dicot species was evident when probed with only antiserum against PEPC of A. hypochondriacus, but not of maize (Figs. 4a, 5a). Antiserum against maize PEPC could react and showed up only the maize enzyme on the gels (Fig. 5a), but not of C₄-dicots nor of C₃-C₄ intermediates (Fig. 5b,c).

Immunological distinctness of PEPC from C₃-C₄ intermediates and C₄-dicots was further confirmed in the experiments involving the use of anti-PEPC antiserum raised against the enzyme of Alternanthera pungens (Fig. 6). When probed with this anti-PEPC (of Alternanthera) anti-serum, the strongest reaction was with PEPC of Amaranthus, or A. pungens (C₄-dicots) followed by A. tenella and A. ficoides (C₃-C₄ intermediates), and then A. sessilis (C₃ species). Least immunoreaction was with PEPC of maize (C₄-monocot).

We noticed that at least two bands of PEPC appeared on Western blots with leaf extracts of C₃ and C₃-C₄ intermediate species indicating the existence of different isoforms (100 and 110 kD) of PEPC. In C₃ species, only a single band was obtained on 10% of SDS-PAGE gels or immunoblots, particularly when loaded with 30 µg or more of protein. Pattern of double-bands (with leaf extracts C₃ or C₃-C₄ intermediates) after immunoblotting remained unaltered even in presence of protease inhibitor like PMSF during extraction.

![Fig. 5](image)

Fig. 5—Western blots of PEPC in leaf extracts of selected C₄-, C₃-species and C₃-C₄ intermediates. The blots were probed with anti-PEPC antiserum (raised against PEPC of Zea mays, a C₄-monocot). (A) C₄-plants [Lanes 1 to 3 in the given order: A. hypochondriacus, Alternanthera pungens, Zea mays]; (B) C₃-C₄ intermediate dicots [Lanes 1 to 3: Parthenium hysterophorum, Alternanthera ficoides, Alternanthera tenella]; and (C) C₃-plants [Lanes 1 to 3: Pismum sativum, Alternanthera sessilis (both dicots), Commelina benghalensis (monocot)]. Further details are as in Fig. 4.

![Fig. 6](image)

Fig. 6—Western blots of PEPC in leaf extracts of C₃ and C₄-plants and C₃-C₄ intermediate species of Alternanthera, compared to the reactivity of pea, maize or Amaranthus. The blots were probed with anti-PEPC antiserum (raised against PEPC of Alternanthera pungens, a C₄-dicot). [Lanes 1 to 7, in the given order: Pismum sativum (C₄-dicot), Alternanthera sessilis (C₃ dicot), Alternanthera ficoides (C₃-C₄ intermediate dicot), Alternanthera tenella (C₃-C₄ intermediate dicot), Alternanthera pungens (C₃ dicot). Amaranthus hypochondriacus (C₃ dicot) and Zea mays (C₄ monocot).] The location of PEPC is indicated by an arrow. The amount of protein loaded in each lane was 20 µg. Further details are as in Fig. 4.

**Table 1**—A summary of the observations on the cross-reactivity of PEPC in leaf extracts of C₃, C₄ and C₃-C₄ intermediate plants against the antibodies raised against PEPC of Amaranthus hypochondriacus or maize.

<table>
<thead>
<tr>
<th>Species/Photosynthetic type</th>
<th>Antibodies raised against PEPC from Amaranthus hypochondriacus</th>
<th>Zea mays</th>
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<tbody>
<tr>
<td>Amaranthus hypochondriacus (C₄)</td>
<td>++++++</td>
<td>&lt;&lt;&lt;&lt;</td>
</tr>
<tr>
<td>Zea mays (C₄)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Sorghum bicolor (C₄)</td>
<td>+</td>
<td>+=</td>
</tr>
<tr>
<td>Alternanthera pungens (C₃)</td>
<td>+++++</td>
<td>&lt;&lt;&lt;&lt;</td>
</tr>
<tr>
<td>A. tenella (C₃-C₄)</td>
<td>+</td>
<td>&lt;</td>
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<tr>
<td>A. ficoides (C₃-C₄)</td>
<td>+</td>
<td>&lt;</td>
</tr>
<tr>
<td>A. sessilis (C₃)</td>
<td>+</td>
<td>&lt;</td>
</tr>
<tr>
<td>Pismum sativum (C₃)</td>
<td>+</td>
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*"++" indicates a positive reaction. The sensitivity of reaction is arbitrarily represented by the number of ‘+’ signs.*
Appearance of two or three PEPC bands on Western blots indicating the presence of isoforms in C3 have been noticed in extracts from C3 plants and CAM plants. Wiegend and Hincha (1992) have observed only one PEPC band with C3 and CAM species while two bands appear on immunoblots of extracts from C3 plants using antiserum against maize PEPC. During Western blotting, only one PEPC band in mesophyll cell protoplasts, two PEPC bands in mesophyll tissue preparations, and three in guard cell protoplasts have been found earlier. Epidermal tissue of Vicia faba has shown two bands of PEPC cross-reacting with antibodies against K. daigremontiana. Slocombe et al. have also shown two or three bands appearing in C3 performing M. crystallinum using antiserum directed against PEPC from CAM-performing K. daigremontiana. Similarly, presence of two PEPC bands in germinating cotyledons of Ricinus communis on probing with antiserum against maize leaf PEPC have also been observed earlier.

Based on the Ouchterlony double diffusion and Western blot analysis (summarized in Table 1), possibly PEPC from C3 dicots was distinct from C4 monocots (or C3-C4 intermediates or C3 plants), as indicated by cross reactivity. Similar suggestion has been made earlier based on comparison of cDNA sequences of PEPC. Similarly PEPC of C3-C4 intermediates exhibited a distinct intermediary in terms of immunological cross-reactivity between C3 and C4 species. This is the first report on such immunological distinctness of PEPC from C3-C4 intermediates within the genus of Alternanthera. It has been suggested that Alternanthera can be another good model to study the molecular nature of PEPC, like Flaveria.

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References
