

Antigenic Relationships between Chloroplast and Cytosolic Fructose-1,6-Bisphosphatases¹

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Cytosolic fructose-1,6-bisphosphatases (FBPase, EC 3.1.3.11) from pea (*Pisum sativum* L. cv Lincoln) and spinach (*Spinacia oleracea* L. cv Winter Giant) did not cross-react by double immunodiffusion and western blotting with either of the antisera raised against the chloroplast enzyme of both species; similarly, pea and spinach chloroplast FBPases did not react with the spinach cytosolic FBPase antiserum. On the other hand, spinach and pea chloroplast FBPases showed strong cross-reactions against the antisera to chloroplast FBPases, in the same way that the pea and spinach cytosolic enzymes displayed good cross-reactions against the antiserum to spinach cytosolic FBPase. Crude extracts from spinach and pea leaves, as well as the corresponding purified chloroplast enzymes, showed by western blotting only one band (44 and 43 kD, respectively) in reaction with either of the antisera against the chloroplast enzymes. A unique fraction of molecular mass 38 kD appeared when either of the crude extracts or the purified spinach cytosolic FBPase were analyzed against the spinach cytosolic FBPase antiserum. These molecular sizes are in accordance with those reported for the subunits of the photosynthetic and gluconeogenic FBPases. Chloroplast and cytosolic FBPases underwent increasing inactivation when increasing concentrations of chloroplast or cytosolic anti-FBPase immunoglobulin G (IgG), respectively, were added to the reaction mixture. However, inactivations were not observed when the photosynthetic enzyme was incubated with the IgG to cytosolic FBPase, or vice versa. Quantitative results obtained by enzyme-linked immunosorbent assays (ELISA) showed 77% common antigenic determinants between the two chloroplast enzymes when tested against the spinach photosynthetic FBPase antiserum, which shifted to 64% when assayed against the pea antiserum. In contrast, common antigenic determinants between the spinach cytosolic FBPase and the two chloroplast enzymes were less than 10% when the ELISA test was carried out with either of the photosynthetic FBPase antisera, and only 5% when the assay was performed with the antiserum to the spinach cytosolic FBPase. These results were supported by sequencing data: the deduced amino acid sequence of a chloroplast FBPase clone isolated from a pea cDNA library indicated a 39,253 molecular weight protein, with a homology of 85% with the spinach chloroplast FBPase but only 48.5% with the cytosolic enzyme from spinach.

FBPase (EC 3.1.3.11) catalyzes the breakdown of Fru-1,6-bisP into Fru-6-P and Pi. Two FBPases co-exist in the photosynthetic cell: chloroplast FBPase is involved in the reductive pentose-phosphate pathway, whereas the cytosolic FBPase is concerned with gluconeogenesis and Suc synthesis (Cséke and Buchanan, 1986). Both enzymes play key regulatory roles in their corresponding pathways but show different kinetic and regulatory features. The photosynthetic enzyme appears saturated only at millimolar concentration of substrate, is AMP insensitive, and shows a light regulation through the Fd-thioredoxin system (Jacquot, 1984; Cséke and Buchanan, 1986). In contrast, the cytosolic FBPase shows characteristics similar to those of the gluconeogenic enzyme from yeast and mammals; it is inhibited by excess substrate, AMP, and Fru-2,6-bisP, and is also modulated by Mg²⁺ and Ca²⁺ (Zimmermann et al., 1978; Prado et al., 1991).

In spite of these differences, accurate determinations of both activities in leaf extracts are not possible, due to the overlapping pH optima and to the lack of a quantitative inhibition of the cytosolic enzyme by the above-mentioned modulators. Moreover, it is sometimes necessary to determine cytosolic or chloroplastic FBPases as protein entities despite their enzyme activities. This is the case for cytological localization of the enzymes in leaf sections, the determination of FBPase synthesis during the ontogeny of the plant, and the analysis of the import process into the chloroplast of the photosynthetic FBPase precursor form. These determinations can be carried out by immunological methods, but for this purpose a key point is to have antibodies that are specific to either the chloroplast or cytosolic FBPase.

Comparative sequence analyses of spinach (*Spinacia oleracea* L.) cytosolic FBPase with the chloroplast enzyme from spinach and wheat have shown some structural identities, with 52 and 50% homology, respectively (Raines et al., 1988; Ladrón et al., 1990; Hur et al., 1992). These similarities are lower than the 80% homology existing between the chloroplast FBPases from wheat and spinach (Marcus and Harsch, 1990) and seem to show a clear divergence between the photosynthetic and gluconeogenic enzymes. However, Rother et al. (1988) prepared a polyclonal antiserum against homogeneous spinach chloroplast FBPase, which in addition to recognizing the leaf chloroplast enzyme, also recognized the cytosolic enzyme in both spinach leaf and root extracts.

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Abbreviation: FBPase, fructose-1,6-bisphosphatase.

On the contrary, using antibodies prepared in our laboratory, we have found only one band, corresponding to the photosynthetic enzyme, in western blot experiments with pea (*Pisum sativum* L.) leaf extracts against pea chloroplast FBPase IgG (Hermoso et al., 1989). To clarify this subject we have carried out a systematic study of the cross-reactions between polyclonal antibodies raised against the chloroplast and cytosolic FBPases from pea and spinach. The results are discussed from a molecular biology point of view.

MATERIALS AND METHODS

Plant Material, Chemicals, and Biologicals

We have used young leaves of 20-d-old pea (*Pisum sativum* L. cv Lincoln) seedlings grown in vermiculite under $220 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, with 14-h photoperiod and a light/dark temperature range of 25°/20°C. Spinach (*Spinacia oleracea* L. cv Winter Giant) leaves were obtained from the local market.

Auxiliary enzymes for determination of FBPase activity, as well as vectors, restriction enzymes, and other reagents for cloning and sequencing were provided by Boehringer Mannheim. Reagents for ELISA and western blotting were purchased from Sigma, Millipore, and Dynatech Laboratory, Inc. Chloroplast FBPases from both pea and spinach were purified to homogeneity according to Plá et al. (1981), and the spinach cytosolic FBPase was purified as described by Prado et al. (1991). The final preparation of pea cytosolic enzyme, obtained by the latter procedure, showed some impurities that were difficult to remove and therefore could not be used for antiserum preparation.

Assays of FBPase Activity and Enzyme Inhibition

Chloroplast FBPase activity was measured at 25°C as the increase in A_{340} in 100 mM Tris-HCl (pH 8.8) containing 0.6 mM Fru-1,6-bisP, 10 mM MgCl_2 , 0.3 mM NADP^+ , 0.5 mM EDTA, 1.4 units of phosphohexose isomerase, 0.7 units of Glc-6-P dehydrogenase, and the solution to be tested, in a final volume of 1 mL. Cytosolic FBPase activity was measured in the same way, but in 100 mM imidazole-HCl (pH 7.5) containing 0.06 mM Fru-1,6-bisP, 5 mM MgCl_2 , 0.3 mM NADP^+ , and the auxiliary enzymes. In both cases one enzyme unit is the amount of FBPase that promotes the hydrolysis of 1 μmol of substrate/min under the assay conditions.

For inhibition experiments increasing concentrations of the corresponding antiserum were preincubated for 10 min at 37°C, with the same quantity of the FBPase solution used in the standard assay, in a final volume of 100 μL made up to 5 mM in MgCl_2 ; the preincubated solution was then added to the above assay mixture, and the remaining FBPase activity was determined as above. Parallel controls were run without antiserum and with a preimmune one.

Preparation of Antisera and Immunological Assays

Polyclonal antibodies against spinach cytosolic FBPase and against the chloroplast FBPase from spinach and pea were obtained by immunization of hybrid New Zealand rabbits with 400 μg of the purified protein, in four subcutaneous injections of 100 μg at 14-d intervals. Additional steps, as

well as determinations of antibody titer, were carried out as described by Hermoso et al. (1987). IgG-enriched fractions were obtained from the corresponding immune serum by precipitation with 40% saturated $(\text{NH}_4)_2\text{SO}_4$, followed by DEAE-cellulose chromatography.

Western blotting was performed by SDS-PAGE (Laemmli, 1970) of purified FBPases or crude leaf extracts, transfer to nitrocellulose sheets, and development of the immune-detected bands with horseradish peroxidase-conjugate goat antirabbit, followed by staining with the $\text{H}_2\text{O}_2/4\text{-Cl-1-naphthol}$ system (Towbin et al., 1979). Ouchterlony double immunodiffusion was carried out by conventional methods. Quantitative estimation of immunoreactions was performed by the ELISA noncompetitive method of Hermoso et al. (1987), coating the wells with 200 μL of increasing concentrations of the enzyme (0.3–1000 ng mL^{-1}) in 50 mM Na_2CO_3 buffer (pH 9.6), followed by a constant and suitable antibody solution at 25 $\mu\text{g mL}^{-1}$. In this case color was developed with peroxidase-conjugate goat anti-rabbit coupled to the $\text{H}_2\text{O}_2/o\text{-phenylenediamine}$ staining, and measured at 492 nm in a Titertek Multiskan Plus (Flow Laboratories, Lugano, Switzerland). Controls were carried out in parallel in which antibodies were replaced with similar concentrations of a preimmune serum. The determination of the antibody concentration for optimal cross-immunoreaction was performed by coating the wells with 200 μL of a 50 ng mL^{-1} FBPase solution in the above buffer and then with increasing concentrations of the corresponding antibody.

Sequencing of Pea Chloroplast FBPase

A pea cDNA library in the *EcoRI* site of $\lambda\text{gt}11$ (Clontech, Palo Alto, CA) was screened for photosynthetic FBPase production in *Escherichia coli* Y1090 cultures by assay with an FBPase antiserum. The DNA of a positive clone was purified and a 3.1-kb fragment was isolated by agarose gel electrophoresis after *KpnI-SacI* digestion. This fragment was subcloned in similar restriction sites of the vector pTZ18R, and the FBPase recombinant was isolated by CsCl gradient centrifugation. This plasmid subclone was digested with appropriate restriction enzymes, treated with exonuclease III, and subjected to nucleotide sequence analysis by the chain-termination method (Sequenase Version 2.0 kit, United States Biochemical). The primary structure of pea chloroplast FBPase was deduced from the nucleotide sequence of the DNA fragment and aligned with the aid of a computer program with those of the chloroplast and cytosolic enzymes from spinach (Marcus and Harsch, 1990; Hur et al., 1992) and with that of the photosynthetic FBPase from wheat (Raines et al., 1988).

RESULTS AND DISCUSSION

Ouchterlony and Western Blotting

Some Calvin cycle enzymes have cytosolic counterparts that are engaged in processes not directly related to photosynthesis. This is the case of FBPase, which shows a chloroplast activity involved in photosynthesis and a cytosolic activity engaged in gluconeogenesis and Suc synthesis. Comparative studies on the homology of these pairs of chloroplast

and cytosolic enzymes, carried out by sequence analysis and immunological cross-reactions, have provided contrasting results. Double-immunodiffusion experiments showed the absence of any precipitation band when cytosolic and chloroplastic FBPases, both from spinach and pea, were cross-reacted, respectively, with antisera raised against the photosynthetic and gluconeogenic FBPases (Fig. 1, A and B). The good titers of sera were corroborated by the strong immunoprecipitating bands obtained when sera against chloroplast FBPases were tested against both enzymes, as well as when the spinach cytosolic antiserum was checked against both cytosolic enzymes (Fig. 1, C and D). No precipitating bands were obtained with a preimmune serum.

Since the Ouchterlony test requires precipitating antibodies, which are normally a subset of total antibodies, we have studied cross-reactions by other techniques. Western blotting of spinach or pea crude extracts showed only one 43- and 44-kD band, respectively, using the antisera raised against the photosynthetic enzymes of both species (Fig. 2). Similarly, only one band of 38 kD molecular mass was obtained when crude extracts from spinach or pea were western blotted against a spinach cytosolic FBPase antiserum (Fig. 2). These molecular sizes are in accordance with the previous values

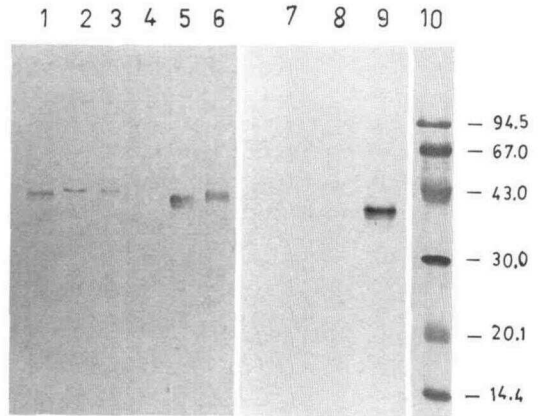


Figure 2. Western blotting of spinach and pea FBPases, against pea chloroplast (lanes 1–6) and spinach cytosol (lanes 7–9) FBPase antisera. Lane 1, Pea leaf extract; lanes 2 and 3, spinach leaf extract; lanes 4 and 9, spinach cytosol FBPase; lanes 5 and 8, pea chloroplast FBPase; lanes 6 and 7, spinach chloroplast FBPase; lane 10, molecular mass standards.

reported for the subunit of the chloroplast (Kelly et al., 1982; Sahrawy et al., 1990) and cytosolic (Zimmermann et al., 1978; Kelly et al., 1982; Ladror et al., 1990) FBPases. Similar results were found when crude extracts were substituted for by purified FBPases from pea and spinach, and they show the absence of any visible cross-reaction between both isozymes. These results are in contrast to those reported by Rother et al. (1988), who detected two bands by western blotting of spinach leaf extracts against a spinach chloroplast FBPase antiserum. These conflicting results can be explained only by a lower specificity of this antiserum, which is probably a consequence of insufficient purity of the enzyme preparation used for rabbit immunization.

Immunological Enzyme Inhibition

Figure 3 shows the inhibition pattern of chloroplast and cytosolic FBPase activities, both from spinach and pea, after reaction with increasing concentrations of pea and spinach chloroplast FBPase antisera and with the FBPase antiserum obtained against the spinach cytosolic enzyme. Chloroplast and cytosolic enzymes were increasingly inactivated by the chloroplast anti-FBPase IgGs and the spinach cytosolic IgGs, respectively, as the IgG concentration was increased. About 50 μ g of purified chloroplast anti-FBPase IgGs from either spinach or pea was sufficient to bring about more than 90% inhibition of pea or spinach chloroplast enzymes, indicating a high homology between both photosynthetic FBPases, at least concerning the catalytic site. On the contrary, chloroplast FBPases remained fully active when the assay was carried out with spinach cytosolic anti-FBPase antibodies, even at a 625- μ g IgG concentration. The spinach cytosolic FBPase activity was not inhibited by the pea or spinach chloroplast FBPase antibodies, whereas about 200 and 600 μ g of the spinach cytosolic anti-FBPase IgG was necessary for 50% inhibition of the spinach and pea cytosolic FBPase activities, respectively. These immunotitration assays support those of double immunodiffusion and western blotting on

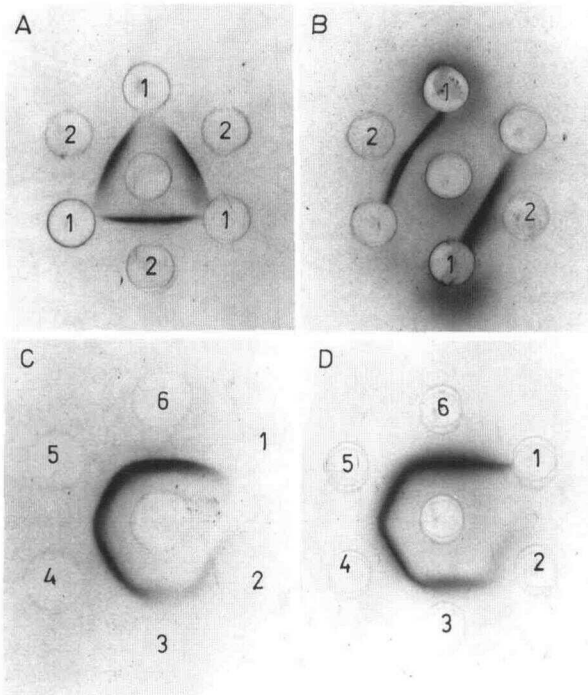


Figure 1. Double immunodiffusion of chloroplast and cytosolic FBPases against FBPase antisera. A, Spinach chloroplast anti-FBPase in the central well against the spinach cytosol (position 1) and pea chloroplast (position 2) enzymes. B, Pea chloroplast anti-FBPase in the central well against the spinach cytosol (position 1) and spinach chloroplast (position 2) enzymes. C, Pea chloroplast anti-FBPase in the central well against increasing concentrations (positions 1–6) of the pea chloroplast enzyme. D, Spinach cytosol anti-FBPase in the central well against increasing concentrations (positions 1–6) of the spinach cytosolic enzyme.

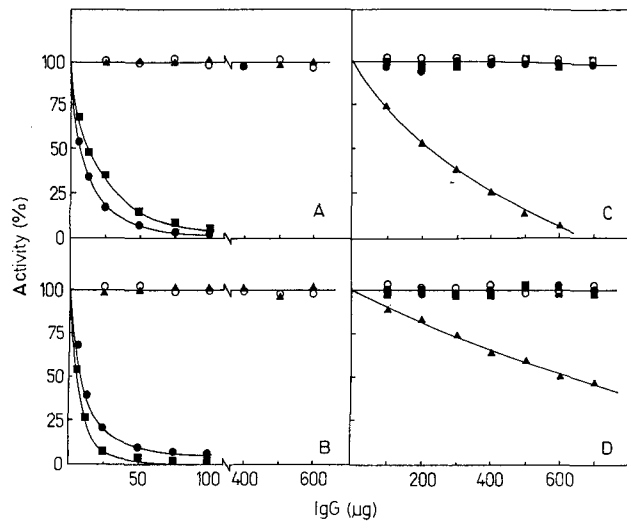


Figure 3. Inhibition pattern of FBPase activity by anti-FBPase IgG. Inhibition of spinach (A) and pea (B) chloroplast FBPases and of spinach (C) and pea (D) cytosolic FBPases by increasing concentrations of spinach chloroplast anti-FBPase (●), pea chloroplast anti-FBPase (■), and spinach cytosol anti-FBPase (▲) IgGs. O, Inhibitions of the corresponding FBPases by a preimmune serum.

the existence of poor antigenic relationships between the cytosolic and chloroplast FBPases.

From the nucleotide sequences of the corresponding genes, Longstaff et al. (1989) found 82% homology between chloroplast and cytosolic phosphoglycerate kinases from wheat, which decreased to 45 to 60% when the chloroplast enzyme was compared with the glycolytic enzyme from heterotrophic organisms. These results are in accordance with the strong immunological cross-reaction by immunotitration found between both isozymes from barley leaves (McMorrow and Bradbeer, 1990), but are in sharp contrast to the finding of Köpke-Secundo et al. (1990), who reported a scanty 4.6% cross-reaction when an antiserum raised against spinach chloroplast phosphoglycerate kinase was immunotitrated with the purified cytosolic enzyme.

ELISA

In contrast to the Ouchterlony double immunodiffusion and the immunotitration of the enzyme activity, the ELISA detects other antigenic determinants in addition to those concerned with enzyme activity and precipitation. Constant amounts of the different anti-FBPase IgGs were assayed with increasing concentrations of purified chloroplast and cytosolic FBPases from pea and spinach, and the formed IgG-enzyme complexes were determined with anti-IgG peroxidase. As displayed in Figure 4, chloroplast anti-FBPases from spinach and pea showed a high binding with both photosynthetic enzymes, even though binding was slightly higher with the homologous FBPase. In contrast, cross-reactions with the spinach cytosolic enzyme were practically nil. A similar behavior occurred with the cytosolic anti-FBPase from spinach, which showed a high binding with the homologous cytosolic

enzyme but did not cross-react with either chloroplast enzyme.

Because of the Michaelis-Menten kinetics of the above saturation curves, we have quantified the cross-reactions by comparing the maximum binding of the homologous and heterologous enzyme-antibody pairs. This was carried out using ELISA assays between a constant amount of enzyme and increasing concentrations of IgG antibodies. The maximum binding (V_{max}) was graphically determined from the reciprocal Lineweaver-Burk plots, and the percentage cross-reactivity was computed from each pair value (Fig. 5). These results show that 77% of the antibody population against spinach chloroplast FBPase reacts well with the pea chloroplast enzyme, whereas 64% of those against the pea photosynthetic enzyme cross-react with the spinach chloroplast FBPase (Table I). This means that the existence in both proteins of an antigenic reciprocity was derived from the existence of common epitopes and also the presence of some specific ones. On the contrary, less than 10% of the antibody population against both chloroplast FBPases cross-reacts with the cytosolic spinach enzyme, whereas less than 5% of the antibodies raised against the cytosolic FBPase from spinach

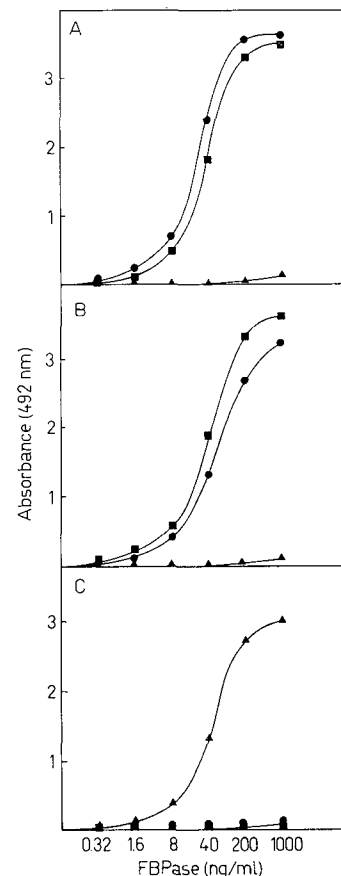


Figure 4. Immunological cross-reactivity (ELISA) of FBPases against anti-FBPase IgGs. Spinach (A) and pea (B) chloroplast anti-FBPase IgGs and spinach cytosol anti-FBPase IgG (C) were cross-reacted with increasing concentrations of spinach chloroplast (●), pea chloroplast (■), and spinach cytosol (▲) FBPases.

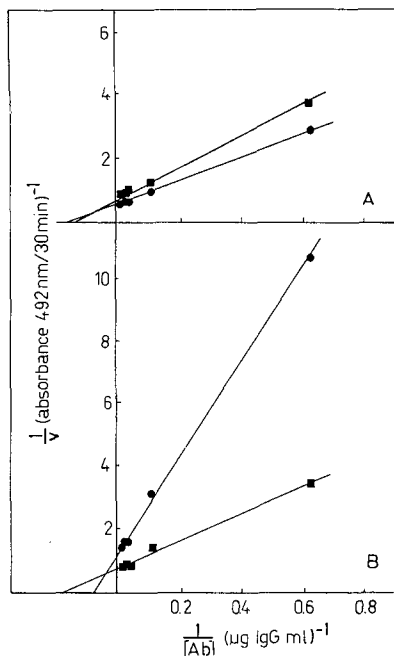


Figure 5. Lineweaver-Burk plots of immunotitration (ELISA) of spinach (●) and pea (■) chloroplast FBPsases with increasing concentrations of spinach (A) and pea (B) chloroplast anti-FBPase IgGs.

show cross-reactivity with the pea and spinach photosynthetic enzymes. These results show the strong antigenic divergence between chloroplast and cytosolic FBPsases.

Sequence-Related Antigenic Relationship

The absence of, or poor, cross-reactions between some isozymes with high sequence homology is noteworthy. In spite of the high sequence homology of cytosolic and chloroplast triose-phosphate isomerases from lettuce, they cross-react very differently with an antiserum raised against the cytosolic enzyme from spinach: 80 and 10%, respectively (Pichersky and Gottlieb, 1984). Concerning FBPsases, the reported 52% homology between the cytosolic and chloroplast enzymes from spinach (Ladror et al., 1990; Hur et al., 1992) appears to be substantiated in 175 homologous amino acids among the 341 residues of the cytosolic enzyme subunit

(Ladror et al., 1990; Marcus and Harrsch, 1990). The scope is similar when we compare the cytosolic FBPsase from spinach with the chloroplast FBPsase from pea. Computer identification of translational start and stop codons of the isolated 3.1-kb DNA fragment predicts an open reading frame encoding a 381-amino acid polypeptide. In comparison to the sequences of spinach (Marcus and Harrsch, 1990) and wheat (Raines et al., 1988) chloroplast FBPsases, the first amino acid of the pea enzyme was identified as the Met at position 25; accordingly, the 24-amino acid upstream sequence from this point is a portion of the transit peptide necessary for the chloroplast import of this nuclear-coded photosynthetic enzyme (Keegstra and Olsen, 1989). The subunit of the pea chloroplast FBPsase, then, appears to have 357 amino acids, with 39,253 mol wt, which is in the range of values earlier reported for other chloroplast FBPsases (Raines et al., 1988; Marcus and Harrsch, 1990). It shows a 48.5% exact homology with the spinach cytosolic enzyme, but the homology goes up to 85% when compared to the photosynthetic FBPsase from spinach (Fig. 6).

This broad difference in the level of homology between the cytosolic and chloroplast FBPsases can explain the absence of cross-reactions between both isozymes. Moreover, the longest noninterrupted homology within the primary structure of chloroplast and cytosolic FBPsases is only 13 amino acids, from position 122 to 139, which is in contrast to the 38-amino acid continuous homology located between positions 95 and 134 of both chloroplast enzymes (Fig. 6). This means there is a higher possibility for the latter to support sequence-related common antigenic determinants. Nevertheless, an additional feature of the chloroplast and cytosolic FBPsases must be the absence of antigenic-relevant exposure clusters within the zones with sequence homology. Spinach and cytosolic aldolases, which have about 50% homology, exhibit a 50% cross-reaction, probably due to the high level of homology between the exposed 29 NH₂-terminal amino acids of both enzymes (Marsh et al., 1989). An additional piece of evidence supporting the high homology between both chloroplast FBPsases and between both cytosolic enzymes from spinach and pea, as well as the lesser degree of identity between the chloroplast and cytosolic enzymes, has been discovered by comparing the peptide patterns obtained after SDS-PAGE of the tryptic and chymotryptic digests of the enzymes (data not shown).

We conclude that, in addition to different structural, ki-

Table I. Immunological saturation levels (ELISA) of chloroplast and cytosolic FBPsases from spinach and pea, with homologous and heterologous anti-FBPase IgGs

Constant amounts of FBPsase were immunotitrated with increasing concentrations of anti-FBPase IgG. The conventional values of saturation levels (V_{max}) were graphically determined from the reciprocal Lineweaver-Burk plots. Values in parentheses are the saturation percentages with respect to each homologous FBPsase-IgG pair.

Enzyme	Spinach Chloroplast Anti-FBPase IgG	Pea Chloroplast Anti-FBPase IgG	Spinach Cytosolic Anti-FBPase IgG
Spinach chloroplast FBPsase	1.76 (100)	0.83 (64)	<0.05 (<5)
Pea chloroplast FBPsase	1.36 (77)	1.30 (100)	<0.05 (<5)
Spinach cytosol FBPsase	<0.15 (<10)	<0.10 (<10)	1.06 (100)

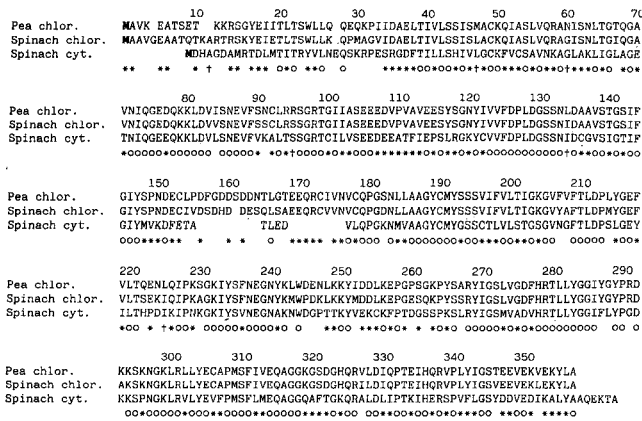


Figure 6. Amino acid sequence of the basic subunit of pea chloroplast FBPAse deduced from the nucleotide sequence of a pea cDNA clone. Amino acids have been aligned with the sequences of spinach chloroplast (Marcus and Harrsch, 1990) and spinach cytosolic (Hur et al., 1992) FBPAases. Open spaces are introduced for optimal alignment. Amino acid exact homologies are indicated as (O) between all three FBPAases, (*) between pea and spinach chloroplast FBPAases, and (+) between chloroplast and cytosolic FBPAases from spinach. Amino acids of the pea chloroplast enzyme are numbered from the amino terminal end. Bold type **M** corresponds to the initiation Mets.

netic, regulatory, and coding features of chloroplast and cytosolic FBPAases, both enzymes from spinach and pea show very poor, if any, antigenic similarities. No cross-reactivity was detected between the chloroplast and cytosolic FBPAases from either pea or spinach by immunoprecipitation, western blotting, immunotitration, and ELISA. On the contrary, chloroplast FBPAase from both species, and to a minor extent both cytosolic FBPAases as well, show clear cross-reactivity. These facts, which are dependent on the low sequence homology between the chloroplast and cytosolic enzymes, support the existence of a strong divergency between the genes coding for both FBPAases and endorse the endosymbiotic origin of chloroplasts.

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