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Purification and Characterization of a Recombinant Pea Chloroplastic Fructose-1, 6-bisphosphatase

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A cDNA fragment encoding the chloroplastic fructose-1, 6-bisphosphatase (FBPase) was cloned via PCR from the cDNA library of pea leaves. The cloned cDNA, about 1.05 kbp without signal sequence, was introduced into a pET-28a vector for expression in *E. coli* strain BL21(DE3)pLysS. The recombinant FBPase was purified through Ni^+-NTA affinity chromatography and characterized. Molecular mass of the monomer was about 42,000. Enzymatic activity of the purified enzyme as the native pea chloroplastic FBPase was the highest at alkaline pH (pH 9.0). The recombinant enzyme was activated by a reducing agent DTT and was insensitive to AMP. The activation energy (Ea) and Arrehenius frequency factor were 42.67 kcal/mol and 2.65×10^{14}/s, respectively, slightly higher than those of the native enzyme. K_M and V_{max} were 99.98 μM and 52.9 μM/min, respectively, which were comparable with the native enzyme.

Key words: *recombinant chloroplast FBPase.*

Fructose-1, 6-bisphosphatase (FBPase) catalyzes fructose-1, 6-bisphosphate into fructose-6-phosphate and inorganic phosphate through dephosphoric reaction. In higher plants, the enzyme exists as two isoforms, one localized in chloroplasts and the other in cytoplasm,[1,2] showing different kinetics and regulatory properties.

The chloroplastic FBPase, essential for CO_2 fixation in Calvin cycle, is activated by increased pH and Mg^{2+} induced by light within stroma. The enzyme is also activated by light-dependent reduction of essential disulfide groups via a ferredoxin-thioredoxin f system.[3,4] Light-regulated expression of the chloroplastic FBPase has been reported in wheats,[5] peas[6] and soybeans.[7] In peas, the expression of chloroplastic FBPase was regulated by phytochrome system, whereby red light induces, while far-red light negates, the enzyme expression.[8] The light-mediated expression of the enzyme was regulated at the level of transcription.[8]

In contrast with the chloroplastic enzyme, the cytoplasmic FBPase is involved in sucrose synthesis. It has similar properties to animal FBPases, in which the enzyme activity was the highest at neutral pH and the enzyme was inhibited by increased AMP and fructose-2, 6-bisphosphate.[9,10]

The cDNA and deduced amino acid sequences of the chloroplastic FBPase have been reported in spinach,[11] wheat,[12] Arabidopsis,[13] potato,[14] rapeseed,[15] pea,[6,16] and soybean.[7] The amino acid sequence homology of the chloroplastic FBPases is high (80~90%) among several different species. However, the homology between the chloroplastic and cytoplasmic enzymes shows about 40%. The eight amino acid residues containing Lys(299) are highly conserved in both chloroplastic and cytoplasmic enzymes, an indication that this region might be an active site.[12] In particular, Lys299 in pea chloroplastic FBPase appears to play an essential role in substrate binding.[17] Involvement of a redox regulatory cysteine cluster (170's loop) in the light-regulated activation of chloroplastic FBPase is well-known.[18] It is investigated that the preceding region of the redox regulatory cysteine cluster (C-I-V-N-V-C) is binding site of thioredoxin f[19,20] and Cys-153 is essential residue for the redox regulation of the pea chloroplastic FBPase. In the rapeseed chloroplastic FBPase, Cys-53, Cys-191, Cys-157, Cys-174, and Cys-179 are involved in the structural stability and light-dependent redox activation.[21] However, the other regulation mechanisms of the chloroplastic FBPase activities such as activation by alkaline pH and Mg^{2+} have yet to be examined.

In this research, a cDNA fragment encoding a pea chloroplastic FBPase was cloned and expressed in *E.coli.* The purified recombinant protein was characterized, as a preliminary work for the elucidation of the regulatory mechanism of the chloroplastic FBPase *in vitro.*

Materials and Methods

Materials. Ex taq DNA polymerase was purchased from Takara Shuzo Co. pET28a expression vaetor and *Escherichia coli* strain BL21(DE3)pLysS were purchased from Novagen. Restriction endonuclease (NcoI, XhoI, EcoRI) and T4 DNA

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

A

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  1 atg gct gtg aag gaa gca act agt gag aca aag aaa aga agt gga tat gag att ata aca
  1 M  A  V  K  E  A  T  S  E  T  K  K  R  S  G  Y  E  I  I  T
 61 ctg act agt tgg ttg ttg cag caa gaa caa aaa ggg att att gat gca gaa ctt act att
 21 L  T  S  W  L  L  Q  Q  E  Q  K  G  I  I  D  A  E  L  T  I
121 gta ctt tct agt att tct atg gca tgt aaa caa att gct tct ttg gtt caa aga gcc aat
 41 V  L  S  S  I  S  M  A  C  K  Q  I  A  S  L  V  Q  R  A  N
181 att tct aac ctc act ggt act caa ggt gct gta aat att caa ggg gaa gac cag aaa aaa
 61 I  S  N  L  T  G  T  Q  G  A  V  N  I  Q  G  E  D  Q  K  K
241 ctt gat gtt atc tca aat gag gta ttc tca aat tgc ttg agg tca agt ggg agg aca ggg
 81 L  D  V  I  S  N  E  V  F  S  N  C  L  R  S  S  G  R  T  G
301 ata att gcg tcg gag gaa gag gat gtg gcg gtg gca gta gaa gag agt tat tca gga aac
101 I  I  A  S  E  E  E  D  V  P  V  A  V  E  E  S  Y  S  G  N
361 tac att gtt gta ttt gat cca ctt gat ggt tca tcc aat ctt gat gct gca gtc tca acc
121 Y  I  V  V  F  D  P  L  D  G  S  S  N  L  D  A  A  V  S  T
421 ggt tcc att ttc ggg att tac agc ccc aat gac gag tgt ctt cct gat ttt ggt gat gac
141 G  S  I  F  G  I  Y  S  P  N  D  E  C  L  P  D  F  G  D  D
481 tct gat gac aac aca ctt ggc aca gaa gaa caa agg tgc att gtg aat gtg tgt caa cca
161 S  D  D  N  T  L  G  T  E  E  Q  R  C  I  V  N  V  C  Q  P
541 gga agc aac ctt cta gca gct ggc tac tgc atg tat tct agt tca gta att ttt gtt ctt
181 G  S  N  L  L  A  A  G  Y  C  M  Y  S  S  S  V  I  F  V  L
601 acc ata ggc aaa gga gtg ttt gta ttc aca tta gat cca ttg tac gga gaa ttc gtt ttg
201 T  I  G  K  G  V  F  V  F  T  L  D  P  L  Y  G  E  F  V  L
661 act caa gag aat ctc caa ata ccg aaa tca ggg aaa atc tat tct ttc aat gaa ggg aat
221 T  Q  E  N  L  Q  I  P  K  S  G  L  I  Y  S  F  N  E  G  N
721 tac aag ttg tgg gat gaa aac ttg aag aaa tat att gat gat ctt aag gaa cca ggt cct
241 Y  K  L  W  D  E  N  L  K  K  Y  I  D  D  L  K  E  P  G  P
781 agt ggc aag cct tat tca gca agg tat att ggt agt ttg gtt ggt gat ttt cac agg aca
261 S  G  K  P  Y  S  A  R  Y  I  G  S  L  V  G  D  F  H  R  T
841 ctg tta tat ggt ggc att tat gga tac cct agg gac aag aaa agt aag aat ggg aag ctt
281 L  L  Y  G  G  I  Y  G  Y  P  R  D  K  K  S  K  N  G  K  L
901 agg ctt tta tat gaa tgt gct cca atg agc ttc att gtt gaa cag gct ggt gga aaa ggt
301 R  L  L  Y  E  C  A  P  M  S  F  I  V  E  Q  A  G  G  K  G
961 tca gat ggt cat caa aga gta ctt gac att caa ccc aca gaa att cat caa cgt gtt cca
321 S  D  G  H  Q  R  V  L  D  I  Q  P  T  E  I  H  Q  R  V  P
1021 ctt tac att ggg agc aca gaa gag gtg gag aag gtt gaa aag tac tta gct taa
341 L  Y  I  G  S  T  E  E  V  E  K  V  E  K  Y  L  A  *
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B

primer 1 : 5'- ATC CAT GGC TGT GAA GGA AGC AAC T-3'
NcoI
primer 2 : 5'- CAC TCG AGA GCT AAG TAC TTT T-3'
XhoI

Fig. 1. Nucleotide sequence of cDNA encoding a pea chloroplastic FBPase (A) and sequences of primers used for cloning a cDNA of the recombinant pea FBPase (B). The discrepancy between the cloned pea chloroplastic FBPase gene and previously reported sequences (Accessional No. L34806) is underlined. Two conserved cysteins of the redox regulatory cluster are indicated in bold letters. Two boxes indicate annealing sites of the primers.

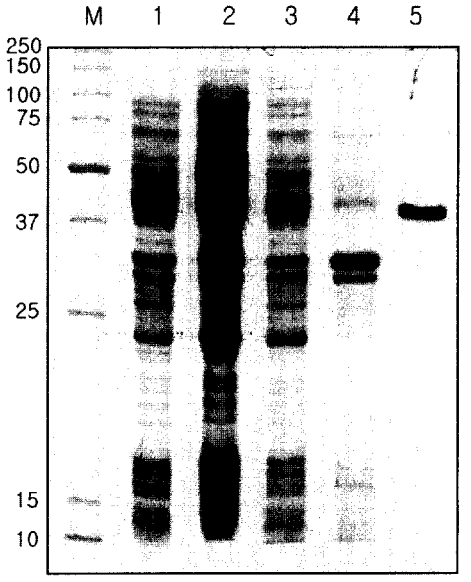

Fig. 2. SDS-PAGE patterns of the purified recombinant pea chloroplastic FBPase. Lane M, molecular weight standards; lane 1, (-IPTG) cell total extract fraction; lane 2, (+IPTG) cell total extract fraction; lane 3, (+IPTG) soluble fraction; lane 4, (+IPTG) insoluble fraction; lane 5, (+IPTG) purified FBPase.

ligase were purchased from Korea Steel Chemical Co. and primers for cDNA cloning were from Bioneer Co. Protein size marker and 1-kb DNA ladder was purchased from Bio-Rad and MBI, respectively. Gel extraction kit, plasmid purification kit, and Ni^+-NTA affinity resin were purchased from QIAGEN. Trypton, yeast extract, and agar were purchased from Duchefa. Reagents for enzyme activity assay (fructose-1, 6-bisphosphate, $NADP^+$, phosphoglucose isomerase, and glucose 6-phosphate dehydrogenase) and SDS-PAGE [acrylamide, N-N'-methylene-bisacrylamide, ammonium persulfate, TEMED, and sodium dodecyl sulfate (SDS)] were purchased from Sigma Chemical Co.

Cloning of cDNA encoding pea chloroplastic fructose-1, 6-bisphosphatase. To obtain a cDNA encoding the pea chloroplastic FBPase, we carried out PCR amplification using a pea cDNA library as a template and two end-specific oligonucleotides as primers. The primers were designed to have NcoI (5') restriction enzyme site as forward primer and XhoI (3') site as reverse primer. The sequence of the primers is showed in Fig. 1.

PCR products were purified through gel electrophoresis and digested with the restriction endonuclease NcoI/XhoI. Digested PCR fragment was introduced into pET-28a vector, which was predigested with the same restriction enzymes. pET-28a has a kanamycin-resistant gene as a selective marker and His-tag sequence in the C-terminus. The His-tag (6 × His) sequence was used for the purification of the expressed FBPase. The ligated mixtures were used to transform the DH5 *E. coli* strain. The recombinant plasmid was verified through agarose gel electrophoresis and DNA sequencing.

Expression and purification of recombinant pea chloroplastic FBPase. The recombinant plasmid was transformed into the *E.coli* strain BL21(DE3)pLysS. Kanamycin-resistant clones were cultured in LB medium supplemented with kanamycin (30 μg/m*l*). When OD_{600} of the culture reached approximately 0.6, IPTG was added to attain a final concentration of 0.3 mM, and the culture was incubated for 3 h at 37°C. The cells were harvested by centrifugation, and the pellet was kept frozen at −20°C.

All purification steps were carried out at 4°C. The cells were resuspended in small volume of cold buffer A (10 mM Tris-HCl, pH 8.5). To extract total proteins, the cells were lysed by sonication then centrifuged. Total protein extracts were applied to Ni^+-NTA column equilibrated with buffer A. The column was washed with a buffer (20 mM immidazole in buffer A), and then eluted stepwise with 50 mM to 250 mM immidazole of buffer A.

Activity assay of the recombinant pea chloroplastic FBPase. FBPase activity was assayed using the method of Kelly *et al.*[2] FBPase activity was determined by measuring the increase in absorbance at 340 nm at 25°C. Enzyme reac-

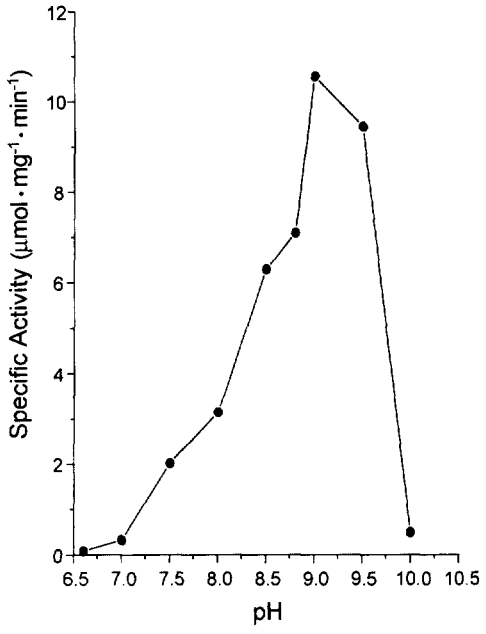

Fig. 3. Effect of pH on the activities of the recombinant pea chloroplastic FBPase. MES 0.1 M (pH 6.5), Tris-Cl 0.1 M (pH 7.0~9.0), Glycine 0.1 M (pH 9.5~10.0) buffer were used.

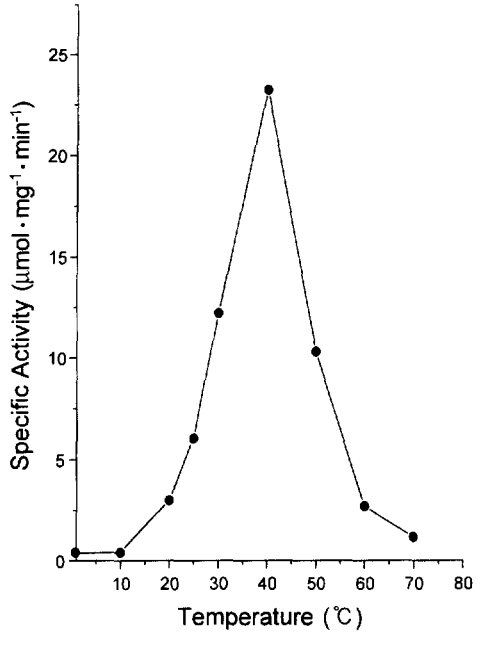

Fig. 4. Effect of temperature on the activities of the recombinant pea chloroplastic FBPase. Temperature was adjusted using a water circulation bath. The pre-mixture was incubated for 5 min at each temperature, and the reaction was started by adding recombinant enzyme.

tion was initiated by mixing 100 mM Tris-HCl, pH 8.8, containing 0.6 mM fructose-1, 6-bisphosphate, 0.5 mM EDTA, 10 mM $MgCl_2$, 0.3 mM $NADP^+$, 0.6 unit glucose 6-phosphate dehydrogenase, 1.2 unit phosphoglucose isomerase, and the purified enzyme (2 μg) (Fig. 5).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out as

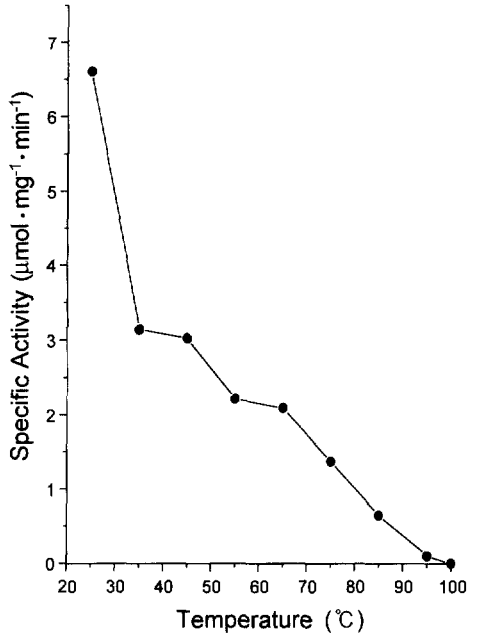

Fig. 5. Heat stability of the recombinant pea chloroplastic FBPase. The enzyme was incubated in a water bath at temperature ranges from 35 to 100°C and was added to an assay mixture into measure enzyme activity.

described by Laemmli.[22] A slap gel system consisting of 12% acrylamide running gel and 5% stacking gel was used. Before electrophoresis, the samples were denatured by boiling for 3 min, and the gel was stained with Coomassie Brilliant Blue (CBB).

Effect of pH on recombinant pea chloroplastic FBPase activity. The activity of the recombinant enzyme was measured at various pH ranges. MES 0.1 M (pH 6.5), Tris-HCl 0.1 M (pH 7.0~9.0), and glycine 0.1 M (pH 9.5~10.0) buffers were used.

Effect of temperature on recombinant pea chloroplastic FBPase activity. The activity of the recombinant enzyme was measured at different temperatures (0~70°C). The temperature was adjusted using a water circulation bath. Before activity assay, the premixture was incubated for 5 min at each temperature (0~70°C). The reaction was initiated by adding 2 g of the purified recombinant FBPase.

Heat stability of recombinant pea chloroplastic FBPase. The recombinant enzyme was incubated in a water bath at various temperature ranges from 35 to 100°C for 10 min. After rapid cooling in an ice bath, the heat-treated protein was added to an assay mixture to measure the activity.

DTT activation on the activity of recombinant pea chloroplastic FBPase. The enzyme activities were measured in terms of the increase in DTT concentration. Various concentration ranges of DTT up to 1 5mM were used.

$MgCl_2$ effect on the activity of recombinant pea chloroplastic FBPase. The enzyme reaction was carried out at different Mg^{2+} concentrations. Various concentration ranges of $MgCl_2$ up to 1 5mM were used.

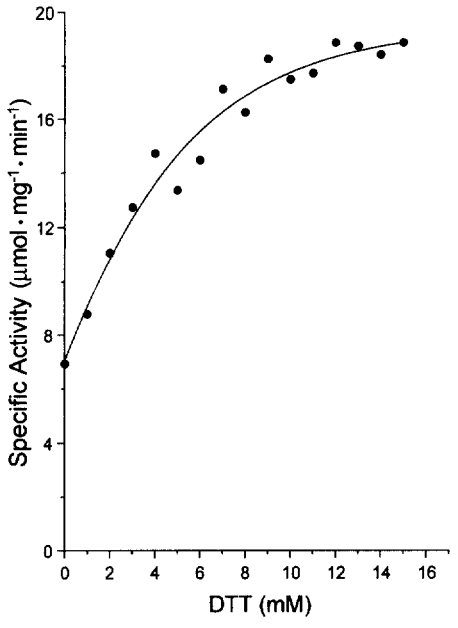

Fig. 6. DTT activation of the recombinant pea chloroplastic FBPase activities. Enzyme activities were determined based on increase in DTT concentration.

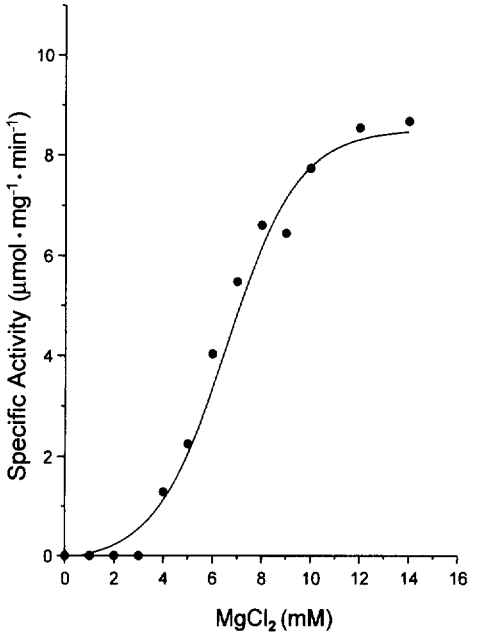

Fig. 7. Effect of $MgCl_2$ on the activities of the recombinant pea chloroplastic FBPase. Enzyme reaction was carried out at various Mg^{2+} concentrations.

Results and Discussion

Fructose-1, 6-bisphosphatase (FBPase) is a key enzyme in carbon metabolism. In higher plants, FBPase exists as two isozymes respectively in chloroplasts and cytoplasm. Both enzymes catalyze the hydrolysis reaction from fructose-1, 6-bisphosphate to fructose-6-phosphate and inorganic phosphate. However, the two isozymes differ in regulation mechanisms and kinetics.[22,23] Chloroplastic FBPase is essential for CO_2 fixation and regeneration of carbon source in Calvin cycle. Its activity is increased in alkaline pH and by increase of Mg^{2+} concentration in stroma upon light illumination.[3] The enzyme is also activated by light-dependent reduction of ferredoxin-thioredoxin f system.[3,4]

In this study, a cDNA fragment encoding chloroplastic FBPase was amplified via PCR from a cDNA library of pea leaves. The cloned cDNA fragment was 1073 bp and was inserted to pET28a vector for expression. The recombinant pea chloroplastic FBPase was purified via Ni^+-NTA affinity chromatography and characterized.

Cloning of cDNA fragment encoding FBPase from pea cDNA library. To obtain a cDNA fragment encoding the pea chloroplastic FBPase, we carried out PCR using total pea cDNA as a template and specific primers (Fig. 1B). The PCR product was about 1073 bp and confirmed by DNA sequencing as the full-length pea chloroplastic FBPase cDNA without signal sequence. The nucleotide sequence was compared with that of the reported pea chloroplastic FBPase cDNA.[6,16] Cloned sequence showd several discrepancies from the previously published sequence (Fig. 1A).[6] The nucleotide-327 and -328 had been determined as C and G, respectively. However, cloned sequence showed G and C instead, changing the protein sequence of Ala-110 into Pro-110. Nucleotide-588 was changed from G to A and nucleotide-589 from C to T, which resulted in the change of Val-197 to Ile-197. Nucleotide-694 changed from G to A resulting in the change of Glu-232 to Leu-232. Upon comparing with the amino acid sequence of Carrasco *et al.*,[16] Pro-32 and Arg-95 were found to have changed into Gly-32 and Ser-95 in present work.

Expression and purification of recombinant pea chloroplastic FBPase. The recombinant plasmid was transformed into *E. coli* strain BL21(DE3)pLysS, and the recombinant FBPase was induced to be produced by the addition of IPTG. From preliminary experiments, the expression was optimized in terms of cell density, IPTG concentration, expression temperature, and duration. Maximum expression of the recombinant FBPase was induced with 0.3 mM IPTG when the cell concentration was attained to 0.6 OD_{600}. The cells were incubated for 3 h at 37°C after addition of IPTG. The recombinant pea chloroplastic FBPase was purified through Ni^+-NTA affinity chromatography from soluble fractions of the total cell extracts. Monomeric molecular weight of the purified enzyme was about 42,000 (Fig. 2). The purified enzyme had slightly higher mass than the native pea chloroplastic FBPase (40KDa)[24] due to its His-tag (6 × His) fusion on the C-terminal end of the recombinant enzyme.

Effect of pH on the activity of recombinant pea chloroplastic FBPase. To study the pH effect on the recombinant enzyme activity, the activity was measured at various pH ranges. The recombinant enzyme was inactive at neutral pH, but active at alkaline pH as a native pea chloroplastic FBPase. The optimal pH was 9.0, and the activity was significantly decreased at pH 10.0 (Fig. 3). This result was almost same

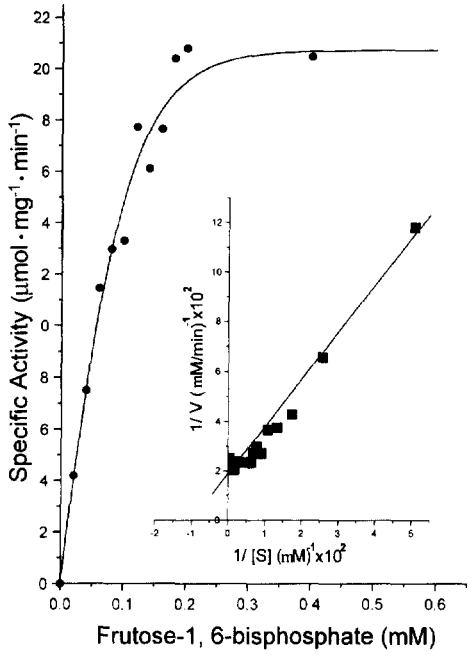

Fig. 8. Effect of substrate concentrations on the activities of the recombinant pea chloroplastic FBPase. Enzyme activities were measured in the presence of 10 mM DTT and $MgCl_2$. Inset shows Lineweaver-Berk plot of the recombinant pea chloroplastic FBPase activities. K_M and V_{max} of the recombinant enzyme were 99.98 μM and 52.9 μM/min, respectively.

with that of the pea chloroplastic FBPase, which was inactive in neutral pH and active in alkaline.[24] The optimal pH of the recombinant and native enzymes were 9.0 and 8.8, respectively.[24]

Effect of temperature on the activity of recombinant pea chloroplastic FBPase. To study the effect of temperature on the recombinant enzyme activity, enzyme reaction was carried out at various temperature ranged from 0 to 70°C. The enzyme activity gradually increased when the temperature was increased up to 40°C, but decreased at temperatures higher than 40°C (Fig. 4). In the case of pea chloroplastic FBPase, activities were increased by increasing the temperature up to 50°C but decreased at temperatures above 50°C. The calculated activation energy (Ea) and Arrenius frequency factor of the recombinant enzyme were 42.67 kcal/mol and 2.6 5× 10^{14}/s, respectively. This value is twofold higher than that of the native enzyme (21.46 kcal/mol),[24] an indication that catalytic efficiency of the recombinant FBPase is lower than that of the native enzyme.

Heat stability of recombinant pea chloroplastic FBPase. To test the heat stability of the recombinant FBPase, the enzyme was incubated at various temperatures for 10 min and the enzyme activity was measured at 25°C. The recombinant enzyme was relatively heat-unstable. The activity of the recombinant enzyme decreased about 50% at 35°C (Fig. 5). The native pea chloroplastic FBPase was very stable below 50, but the activity rapidly decreased at temperatures above 60°C.[24]

Effect of DTT on recombinant pea chloroplastic FBPase. To study the activity changes of the recombinant enzyme via reduction, DTT as a reducing reagent was used. The enzyme activity gradually increased with the increase of DTT. The enzyme was activated over twofold at DTT concentrations higher than 10 mM (Fig. 6). It was known that chloroplastic FBPase is activated by light-dependent reduction via a ferredoxin-thioredoxin f system *in vivo* and reducing agents such as DTT *in vitro*.[3,4] From this experiment, the recombinant enzyme was activated as the native pea chloroplastic FBPase.

Effect of $MgCl_2$ on the activity of recombinant pea chloroplastic FBPase. To study the effect of Mg^{2+} concentrations on the activity of the recombinant enzyme, the enzyme activity was measured at increasing $MgCl_2$ concentrations. The enzyme was inactive without Mg^{2+}, but was activated with sigmoidal curve-shaped increases of Mg^{2+}, an indication of a cooperative interaction of the recombinant enzyme with Mg^{2+} (Fig. 7).

It is also known that the native chloroplastic FBPase consists of four identical subunits. Mg^{2+} is an essential cofactor for the chloroplastic FBPase activity.[10,25] When it is bound to the enzyme, the enzyme shows allosteric effect.[26] The pea chloroplastic FBPase activity increased with increase in Mg^{2+} concentration.[24] The result which the recombinant enzyme was cooperatively activated by addition of $MgCl_2$ (Fig. 7) makes to suggest that the enzyme plays as homotetramer like the native chloroplastic FBPase.

Kinetic parameters of recombinant pea chloroplastic FBPase. To understand the kinetics of the recombinant enzyme, enzyme reactions were carried out in the presence of 10 mM DTT and Mg^{2+} by increasing the substrate (fructose-1, 6-bisphosphate) concentrations. The results followed the Michaelis-Menten kinetics. Lineweaver-Berk plot showed that K_M and V_{max} of the recombinant enzyme were 99.98 μM and 52.9 μM/min, respectively (Fig. 8). K_M value of the recombinant enzyme was some higher than that of the native pea chloroplastic FBPase (128 μM).

In conclusion, the purified recombinant pea chloroplastic FBPase showed similar properties to the pea chloroplastic FBPase. We suggested that the recombinant pea chloroplastic FBPase is also composed of homotetramer with monomeric molecular mass of 42 kDa like the pea chloroplastic FBPase. The enzyme was active in alkaline pH and activated in the presence of DTT and Mg^{2+}. For studing the regulatory mechanism of the pea chloroplastic FBPase, site-directed mutagenesis is in progress using the recombinant pea chloroplastic FBPase.

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