Purification and Properties of Fructose-1, 6-Bisphosphatase from Germinating Castor Bean Endosperm

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Purification and Properties of Fructose-1,6-Bisphosphatase from Germinating Castor Bean Endosperm

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Abstract

Two FDPase isozymes have been purified by DEAE-Sephadex column chromatography and gel filtration, and then characterized from endosperms of germinating castor beans (*Ricinus communis*). One of the enzymes is localized in the cytosol and the other is confined to plastids. There are physical, kinetic and regulatory differences between the isoenzymes. The Km value of c-FBPase and p-FBPase for F-1, 6-BP was 8.2 μM and 23.6 μM, respectively. The optimum pH of c-FBPase was in the range 7.5-7.8, whereas the p-FBPase was 6.7. The p-FBPase being more negatively charged than the c-FBPase. The c-FBPase is regulated by AMP, and F-2<6-BP, whereas the p-FBPase is slightly regulated by AMP.

Introduction

During germination of castor bean seeds, the large lipid reserve in the endosperm is rapidly converted into sucrose[1]. Fructose-1, 6-bisphosphatase (FPBase, D-Fructose-1, 6-bisphosphate phosphohydrolylase, EC 3. 1. 3.11) catalyzes the irreversible conversion of F-1, 6-BP to F-6-P and Pi and is believed to be a key regulatory enzyme of the gluconeogenic pathway. A wide variety of nonplant FBPase have been highly purified and extensively characterized[1]. More recentfy, the molecular, immunological and/or kinetic properties of several purified plant cytosolic FBPase have been studied in detail[2-4]. In plants, the p-FBPase of photosynthetic tissues is reversibly light activated through the ferredoxin system and plays an important function in regulation of the reductive pentose phosphate cycle[5]. Plant c-FBPase does not appear to be light regulated but show potent allosteric inhibition by F-2, 6-BP and AMP[6-8], as does the enzyme from animals and yeast[1,9,10]. Mammalian and plant chloroplast FBPases have been indicated by various data an association, apparently physiologically relevant, between FBPase and the sequential enzyme aldolase in several tissues[11-14]. Recently, Moorhead and coworker reported that c-FBPase and c-aldolase of germinating castor bean endosperm might specifically associate *in vivo*[15]. Nishimura and Beevers reported the occurrence of plastid and cytosolic isozymes of FBPase in germinating castor bean endosperm[16]. The molecular, immunological, and/or kinetic properties of purified

Abbrevations: c-FBPase and p-FBPase, cytosolic and plastidic fructose 1,6-bisphosphatase, respectivly; ER, endoplasmic reticulum; F-1, 6-BP: fructose-1, 6-bisphosphate; F-2, 6-BP, fructose-2, 6-bisphosphate; F-1-P, furctose-1-phosphate; F-6-P, fructose-6-phosphate; G-1, 6-BP, Glucose-1, 6-bisphosphate; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; R-5-P, ribose-5-phosphate; PEP, phosphoenol pyruvate; p-CMB, p-chloromercurbenzoic acid

c-FBPase from germinating castor bean endosperm have been studied in detail[15,17–19]. In general the properties of c-FBPase from castor bean endosperm[17–19] are similar to those reported for the enzyme from spinach leaf[8,17,20–22]. In contrast, there have been a few studies on the p-FBPase of castor been endosperm[16]. This present communication described the purification to near homogeneity of p-FBPase and c-FBPase from germinating castor bean endosperm and reports some physical and kinetic properties of purified enzymes.

Materials and Methods

Materials

Castor bean seeds (*Ricinus comminis*) were supplied by Ito Seiyu Co. and germinated as described previously[23]. Standard for the estimation of molecular weight were obtained from Boehringer/Mannheim. Isoelectric point markers "pI Calibration Kits Electran" was obtained from BDH Limited. "2D-SILVER STAIN • II "DAIICHI" was obtained from Daiichi Pure Chemicals Co. LTD. Ohter chemicals used were commercial preparations of the highest purity available.

Enzyme assays

FBPase activity was assayed spectrophotometrically at 30℃ by following the rate of NADH formation at 340nm. The standard reaction mixture consisted of 100mM Hepes-KOH, pH7.5 for c-FBPase and pH7.0 for p-FBPase, 5mM $MgCl_2$, 0.1mM EDTA, 0.2mM NAD^+, 0.2mM Fructose-1, 6-bisphosphate, 2.1 units of phosphoglucose isomerase, 1 unit of glucose-6-phosphate dehydrogenase and enzyme in a total volume of 1ml. The reaction was started by the addition of fructose 1, 6-bisphosphate. One unit of enzyme activity is defined as the amount of enzyme resulting in the utilization of NADH/min.

Electrophoresis

Nondenaturing PAGE was performed according to the method of Maurer[24]. The final acrylamide monomer concentration was 7.5(w/v) in the separating gel and 2.5%(w/v) in the stacking gel. SDS-PAGE was performed using 10% gels according to the method of Laemmli[25]. FBPase on electrophoresis gel was detected according to the method described by Shaw[26], except for the use of polyacrylamide gel instead of starch gel.

Gel electrofocusing

Isoelectric point was estimated by gel electrofocusing with immobiline drystrip (Pharmacia biotech) and pI calibration kits electran(BDH Limited) as markers. After focusing, gel was stained with 2D-SILVER STAIN • II "DAIICHI"(Daiichi pure chemicals Co. LTD.)

Other procedures

Protein concentration were determined by the dye-binding method of Bradford[27] using the Bio-Rad-prepared reagent and bovine serum albumin as standard. For determination of the molecular weight of FBPase, HPLC was carried out on two Asahipak GS-520 column(7.6×500mm, Asahi Chemical Industry Co. LTD.) calibrated with cytochrome C(12.5kDa), chymotrypsinogen A(25kDa), ovalbumin(45kDa), aldolase(158kDa) and catalase(240kDa) as marker proteins.

Results and Discussion

Changes in the activity of FBPase in germinating castor bean endosperms

When seeds of castor been were germinated the total activity of FBPase in the endosperm increased rapidly from 3rd day of germination, reaching a peak after 5days, and then declined (Fig.1). Since gluconeogenesis in the endosperm start on the 3rd day[28], the increase of FBPase corresponded to gluconeogenesis.

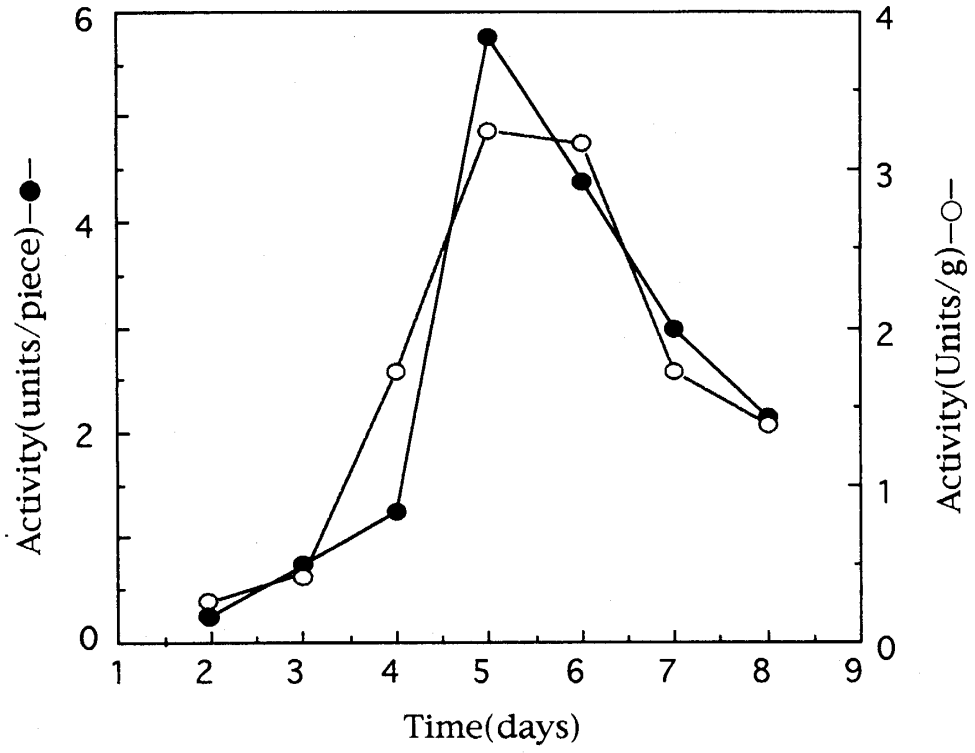

Fig. 1 Change in the activity of FBPase in germinating castor bean endosperm

Purification of EBPase

All procedures were carried out at 0-5℃. Crude extracts were obtained from 5-day-old castor bean endosperms as described previously[23].

Buffer used in enzyme purification: Buffer A: 100mM HEPESKOH(pH7.0), 1mM EDTA, 2mM $MgCl_2$, 13.5mM 2-mercaptoethanol, and 10%(v/v) glycerol; Buffer B: 10mM HEPES-KOH(pH7.0), 1mM EDTA, 2mM $MgCl_2$, 13.5mM 2-mercaptoethanol and 30%(v/v) glycerol; Buffer C: 10mM HEPES-KOH(pH7.0), 1mM EDTA, 2mM $MgCl_2$, 13.5mM 2-mercaptoethanol and 10% grycerol. The FBPases in castor bean endosperm are extremely instable but can be stabilized by 2-mercaptoethanol and grycerol.

Purification of c-FBPase: The extract was fractionation with ammonium sulfate. The precipi-

tate between 40-70% saturation of ammonium sulfate was dissolved in a small volume of Buffer A and dialyzed against the same buffer overnight at 4℃.

The dialysate was centrifugated and the supernatant was applied to a DEAE-Sephadex column equilibrated with the buffer B. The elution was carried out with a linear gradient of KCl (0-150mM) in the same buffer.

The active enzyme fraction was pooled and concentrated with ultrafiltration (Ultrafilter UK-20), and then chromatographed on a sepharose 6B column equilibrated with Buffer B. The active fraction was pooled and concentrated with ultrafiltration.

The enzyme solution was applied to a DEAE-Sephacel column equilibrated with buffer B and eluted with a linear gradient of KCl(50-150mM) in buffer B. Pooled active fraction was desalted and placed to buffer B containing 5mM K_3PO_4 with ultrafiltration.

The enzyme solution was applied to P-Cellulose column equilibrated with same buffer. The elution was carried out with Buffer B containing 5mMK_3PO_4 and successively with Buffer B containing 1mM Fru-1, 6-P. The FBPase was eluted with Buffer containing 1mM Fru-1, 6-P. Pooled active fraction were desalted and placed to buffer B with ultrafiltration.

The enzyme solution was applied to DEAE-Sephdex column equilibrated with buffer B and eluted a linear gradient of KCl(50-150mM) in buffer B. Pooled active fraction were concentration and desalted with ultrafiltration.

The results of the purification are summarized in Table 1. At the final purification, the specific activity of c-FBPase was 42.4units/mg protein, representing 283-fold purification. Nondenaturing-PAGE of the purified enzyme yielded a single stained protein band which coincided exactly with the activity band.

Table 1 Purification of FDPase from Castor Bean Endosperm

Fraction	Total Protein (mg)	Total Activity (Units)	Specific Activity (Units/mg)	Recovery (%)	Fold
Crude extract	1989	301.0	0.15	100	1
$(NH_4)_2SO_4$ (30-45%)	1197	209.4	0.17	70	1.1
DEAE-Sephadex	123.3	97.1	0.79	32	5.3
Sepharose CL-6B	49.9	14.1	0.28	5	1.9
DEAE-Sephacel	22.6	50.9	2.25	17	15
P-Cellulose	3.46	46.3	13.4	15	89
DEAE-Sephadex	0.18	7.64	42.4	2.5	283

Purification of p-FBPase: Crude extract was obtained from 5-day-old germinating castor bean endosperm. Extraction and ammonium sulfate fractionation were carried out by the same method of c-FBPase purification. The resulting precipitate was dissolved in a small volume of Buffer C containing 150mM KCl and dialyzed against the same buffer overnight at 4℃.

The dialysate was applied to a DEAE-Sephadex column equilibrated with the buffer B containing 150mM KCl. The first elution was carried out with the same buffer. After the unabsorbed protein was eluted, step elutions was successively carried out with buffer C containing 200

mM, 250mM, and 500mM KCl respectively(Fig.2). The pooled active fraction was concentrated and replacement to buffer C containing 150mM KCl. The active fraction was applied to a Sephadex G-100 column equilibrated with the buffer C containing 150mM KCl. The column was eluted the same buffer. The specific activity of final purified p-FBPase was 17.8units/mg protein, representing 70-fold purification. The p-FBPase activity was less than 1% of the total endosperm FBPase activity.

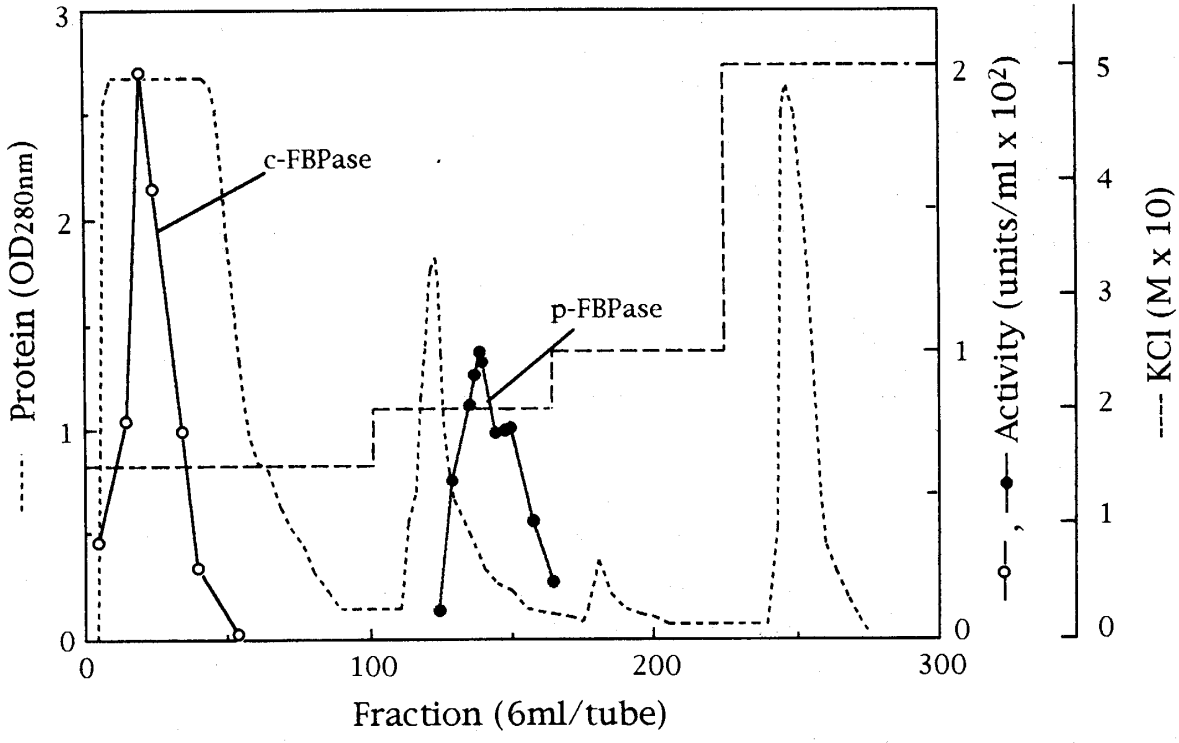

Fig. 2 Elution pattern of FBPase from DEAE-Sephadex Column
—○—; c-FBPase —●— ; p-FBPase

Properties of FBPase

Molecular weight and isoelectric point: The molecular size of purified c-FBPase, as estimated by HPLC, was about 135kDa, and the determined subunit size by SDS-PAGE, was 37.5kDa. The purified p-FBPase has also a about same molecular size. This value is close to that of cytosolic FBPase of spinach and sugar beet, which are tetrametrs of approximately 37kDa[2,4]. The isoelectric points of c-FBPase and p-FBPase were estimated to be 6.15 and 4.02, respectively, from gel electrofocusing data.

Effect of pH:The effect of pH on the activity was studied in the Mes-KOH, HEPES-KOH and Tris-HCl buffer in the range of 6.0-9.0(Fig.3). The p-DBPase is active over only a narrow pH range with an optimum at pH6.7, whereas the c-FBPase is active over a much wider pH range with an optimum at pH7.4-7.8, a pH at which the p-FBPase is almost inactive.

Effect of Mg^{2+}: The c-FBPase require Mg^{2+} or Mn^{2+} for its activity. The concentrations of Mg^{2+} and Mn^{2+} for the maximal activity were 2~5mM and 0.5mM, respectively, and that at high-

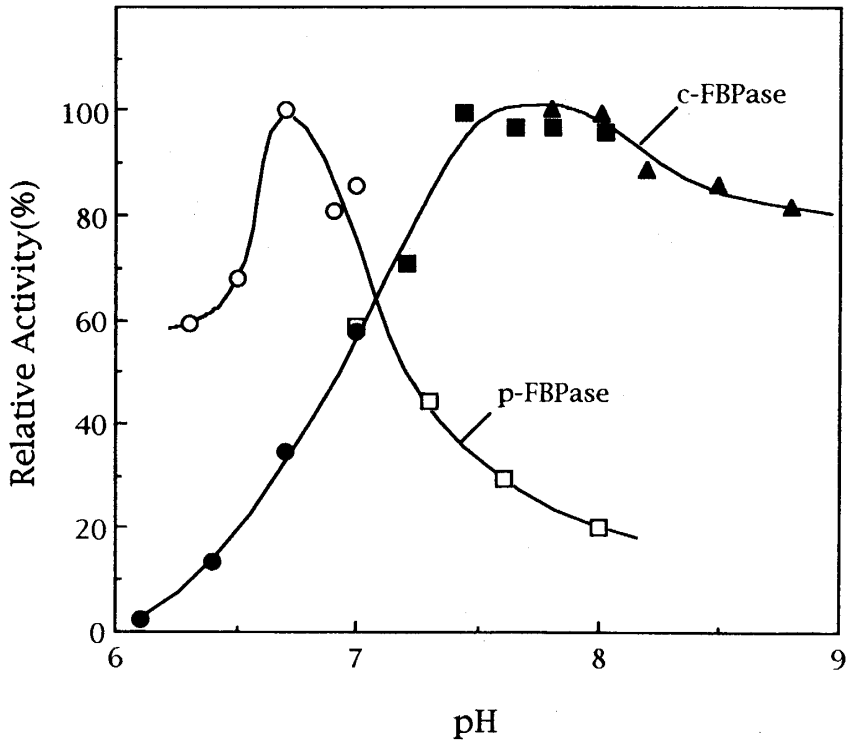

Fig. 3 Effect of pH on FBPase Activity

p-FBPase: ○ MES, □ HEPES
c-FBPase: ● MES, ■ HEPES, ▲ TRIS

er concentrations, particularly on Mn^{2+}, inhibition of enzyme activity occurs. p-FBpase was little affected by the addition of Mg^{2+}(Fig.4). Km value of c-FBPase for Mg^{2+} estimated from Lineweaver-Burk plots was 0.59mM.

Effect of substrate concentration: The effects of substrate concentrations on the catalytic rate of c-FBPase and p-FBPase were determined at pH7.5 and 7.0, respectively. Km value of c-FBPase determined by Lineweaver-Burk plots was 8.2μM, whereas its of p-FBPase was 26.3μM. The value of c-FBPase is close to that of c-FBPase from spinach leaves[3].

Substrate specificity: The following compounds were assayed for FBPase specificity; F-1, 6-BP, F-2, 6-BP, F-1-P, F-6-P, G-1, 6-BP, G-1-P, G-6-P, R-5-P and PEP. The c-FBPase appears fairly specific for F-1, 6-BP, and shows no activity for any other compounds tested.

Effect of SH-blocking reagent: The SH-blocking reagent such as p-CMB inhibited the FBPase activity, and dithiothreitol(DTT) protected this inhibition(Table 2). Thus, it seems possibly that SH-groups are important for the activity and retention of conformation of the enzyme molecules.

Effect of various compounds: The effects of several metabolites on activity of c-FBPase were estimated. The activities were inhibited by AMP, ADP, ATP, F-2, 6-BP. Of the amino acid, metabolites of TCA-cycle and glyoxylate cycle tested, only His, Gln and OAA showed some inhibition for the activity.

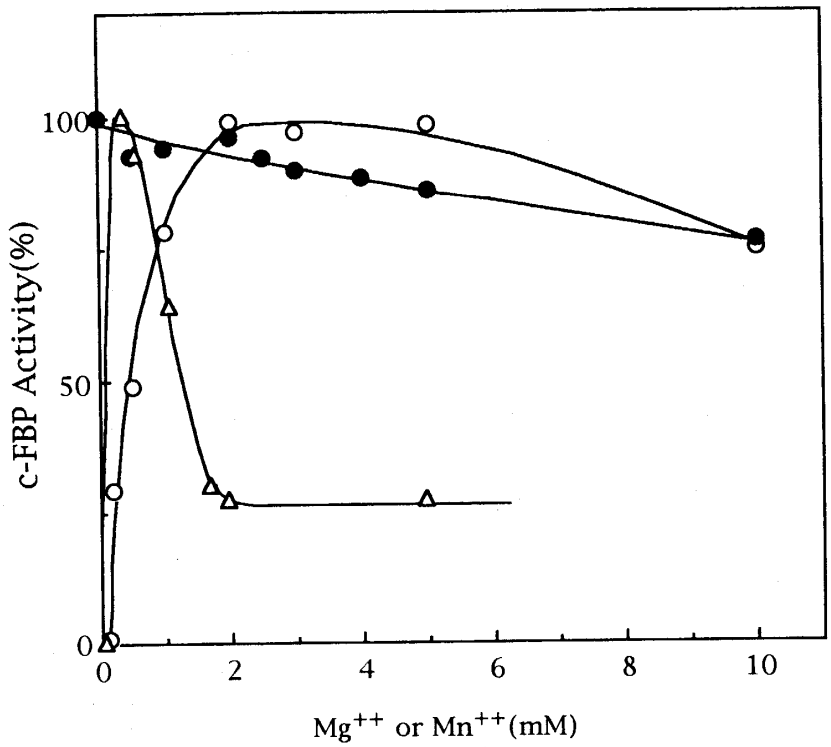

Fig. 4 Effect of Mg^{++}(Mn^{++}) on activity of FBPase

○ c-FBPase(Mg^{++}) △ c-FBPase(Mn^{++}) ● p-FBPase(Mg^{++})

Table 2 Effect of SH-reagent on FBPase Activity

SH-reagent	Activity(%)	
	C-FBPase	p-FBPase
none	100	100
0.2mM pCMB	9	11
1.0mM DTT	126	106
0.2mM pCMB+1.0mM DDT	72	96

pCMB: P-Chloromurcuribenzoic acid, DTT: Ditiothreitol

Effect of AMP: The purified c-FBPase potent allosteric inhibition by F-2, 6-BP, and AMP, as does the enzyme from animals, yeast, other plants[1,7]. In contrast, p-FBPase showed a slight inhibition by AMP(Table 3).

Effect of anions: The effect of anions on the activity of c-FBPase was carried out in assay mixtures containing 5mM Mg^{2+}. The concentration of the anions were used 3mM at pH 7.5. The enzyme activity was significantly inhibited by Ca^{2+}, Zn^{2+}, Ca^{2+}, Hg^{2+}, and slightly inhibited by Na^{+}, K^{+}, NH_4^{+}.

Table 3 Effect of AMP on FBPase Activity

AMP	Activity(%)	
	c-FBPase	p-FBPase
0mM	100	100
1.0mM	7	67

Role of FBPase in the germinating caster bean endosperm

Base on the present data and many investigations on germinating castor bean endosperm have been discussed the roles of FBPase isozymes in castor bean endosperm during germination.

In castor bean seedling, the reserve stored in the endosperm is enzymatically broken down to produce energy as well as carbon skeletones during the course of germination and eventually utilized for the growth of young plant tissues[28-30]. This transformation requires the presence of at least two other organelles, the glyoxsomes and the mitochondria. The glyoxsomes convert fatty acids to succinate and generate NADH, while the mitochondria convert succinate to oxaloacetate and generate ATP by coupled of NADH. The enzymes which synthesize sucrose from oxaloacetate occur in the cytosol. The glyoxsome and mitochondria increased dramatically during the first 5 days of germination[31]. The production of these new organelles requires the prior synthesis of membrane constituents, namely protein and phospholipids. Synthesis of the protein most likely occurs by the reassembly of amino acids from degraded protein bodies, while synthesis of most of the phospholipids[32-37] take place in the ER, beginning with phosphatidic acid synthesis by reaction of glycerol-3-phosphate with fatty acyl-CoA thioesters. The fatty acids for the synthesis of phospolipids were synthesized in the plastids and transported out of the plastids to ER. The phospholipids thus formed in the ER are available for incorporation into the membranes of the developing mitochondria and glyoxysomes, which then function in the conversion of storage lipid to carbohydrate as they do in other tissues. It seems clear that the plastid FBPase in germinating castor bean endosperm play a major role in the synthesis of fattyacids incorporated into the membranes of developing organelles, while the cytosolic isoenzyme plays an essential role in this tissues as are all other gluconeogenic tissues[6-8].

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