Purification and Properties of Alanine aminotransferase from Germinating Castor Bean Endosperms

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Abstract

Alanine aminotransferase from germinating castor bean endosperms was purified about 1,500-fold by ammonium sulfate fractionation and chromatographies with DEAE-Sepharose CL-6B, TEAE-Cellulose, QAE-Sephadex A-50, and TSK-5PW (HPLC). The molecular weight of the enzyme was about 110 kDa, with two identical subunits. The purified enzyme was highly specific for alanine as an amino donor and 2-oxoglutarate as an acceptor, and it showed also glyoxylate transamination activities at low rate. The evidence indicates that alanine: 2-oxoglutarate aminotransferase and glyoxylate aminotransferase activities are associated with the same protein. Pyridoxal phosphate as a cofactor was so tightly bound to the enzyme that added pyridoxal phosphate was not required in the assays. The enzyme showed normal Michaelis-Menten kinetics for all substrates. Reactions proceeded by a Ping Pong Bi Bi mechanism. The K_m for L-alanine, 2-oxoglutarate were 3.4 mM and 0.26 mM, respectively. The optimum pH was 8.5 and pI was about 4.6.

Introduction

During germination of the castor bean, the storage-reserves of the endosperm are hydrolyzed and the metabolic products are transferred via the cotyledons into the growing embryo[1]. In germinating castor bean, sucrose and glutamine are transported from endosperm to axis as carbon and nitrogen carriers, respectively[2]. The protein nitrogen level of the endosperm decreases from the beginning of germination with a rapid decrease occurring after day 4. As a result of hydrolysis of the storage protein, soluble amino nitrogen in the endosperm increases initially, reaching a peak after 5 to 6 days, then decline to zero by about day 8 when the endosperm has complete disappeared.

The amino acids which on deamination can give rise to intermediates in the pathway of conversion of fat to sucrose are largely converted to sucrose and the nitrogen transported as glutamine. Some carbon from the gluconeogenic amino acid is also transported as glutamine. It has been reported that alanine was converted to sugars more than any of the acids studied with a castor bean seedlings[3].

Alanine aminotransferase (AlaAT, EC 2.6.1.2) acts a wide range of metabolic pathways, catalyzing the reversible transfer of an amino group from alanine to 2-oxoglutarate to form pyruvate and glutamate; alanine + 2-oxoglutarate $\rightleftarrows$ Pyruvate + glutamate. The enzyme in plants plays a pivotal role in (a) synthesis of alanine by transamination of a keto acid precursor,

2-oxoglutarate, (b) degradation of alanine, (c) and the intercellular carbon shuttle associated with C_4 photosynthesis[4]. Despite its important in nitrogen and carbon metabolism in all plants, transaminases other than aspartate aminotransferase have not yet been purified and characterized from plant tissue. Most plant aminotransferase preparations obtained so far have not been completely pure and this lack of purity seriously limits the conclusions that can be drown about catalytic properties such as substrate specificity[5]. The presence of an alanine aminotransferase in glyoxysomes from castor bean endosperm was first demonstrated Cooper and Beevers[6] and confirmed by Breidenbach with purified glyoxysome fraction[7]. We report the purification and characterizations of AlaAT from germinating castor bean endosperms.

Materials and Methods

Materials

Castor bean seeds (*Ricinus communis*, harvested in Thailand) were supplied by Ito Seiu Co.. TSK DEAE-5PW column was purchased from Tohso Co. Finepak SIL AF-102 column were purchased from Japan Spectroscopic Co.. Chemicals used were commercial preparations of the highest purity available.

Enzyme assay

AlaAT was measured spectrophotometrically at 340 nm by linking the reduction of the product, pyruvate, of the oxidation of NADH, as catalyzed by addition of excess lactate dehydrogenase according to the method of Grassal[8]. The standard reaction mixture contained 50 mM Tris-HCl buffer (pH 8.2), 5 mM 2-oxooglutarate, 50 mM L-alanine, 0.18 mM NADH, 40 μ M pyridoxal 5'-phosphate, 5.5 units lactate dehydrogenase and enzyme in a total volume of 2.5 ml. The reference cuvette contained all the reactants except α-ketoglutarate. One unit of the enzyme activity was defined as the amount of enzyme which produces 1 μ mole of NAD per 1 min at 25℃.

Glutamate : glyoxylate aminotransferase activity was measured by the method of Rowsell *et. al*[9].

Enzyme specificity

Substrate specificity was determined by Amino acid analyzer. The reaction mixture contained enzyme, 40 mM amino acid,10 mM oxo-acid, and 50 mM potassium phosphate buffer (pH 8.2) in a total volume of 2.5ml. The mixtures were incubated for 1 hour at 25℃. The reaction was stopped by placing in a boiling water bath for 2 min and was dried under reduced pressure. The residue was dissolved in 2 ml citrate buffer for amino acid analysis. The aqueous fraction was filtered through a Milipore filter and analyzed with an automatic amino acid analyzer.

Amino acid analysis

Purified enzyme was hydrolyzed in a sealed evacuated ampoule in 6N HCl at 110℃ for 20, 40, 70 and 96 hr. The number of amino acid residues per molecular of subunit of the enzyme was calculated on the basis of a subunit molecular weight of 58,000.

Other procedures

Protein contents were estimated by the method of Lowry et al. with bovine serum albumin as the standard[10]. For determination of the molecular weight of AlaAT, gel filtration chromatography was carried out on a Sephadex G-150 column (1.5 × 88.5 cm) calibrated with cytochrome C (12.5 kDa), chymotrypsin (25 kDa), ovalbumin (45 kDa), aldolase (158 kDa), catalase (240 kDa) and ferritin (45 kDa) as maker proteins. SDS-PAGE was performed using 10% gels according to the method of Laemmli[11]. Isoelectric point was estimated by gel electroforcusing according to the method of Wrigley[12].

Results and Discussion

Changes in the activity of AlaAT in germinating castor bean endosperms

When seeds of castor were germinated, the activity of AlaAT in the endosperm increased rapidly between the 3rd and 4th days of germination, reaching a peak after the 6th day (Fig. 1).

This change of activity may be reflected on the change of transport capacity of nitrogen transfer from endosperm to embryo[3]

Purification of AlaAT

All procedures were carried out at 1-5℃. Crude extract was obtained from6-day-old castor bean endosperm as described previously[13,14]. Crude extract was fractionationed with ammonium sulfate. The precipitate between 30 and 55 % saturation of ammonium salfate was dissolved in a 50mM Tris-HCl buffer pH 8.0, containing 5 mM β-mercaptoethanol and 3 mM EDTA (buffer A) and dialyzed against the same buffer overnight. The dialysate was centrifuged at 10,000 rpm for 20 min.

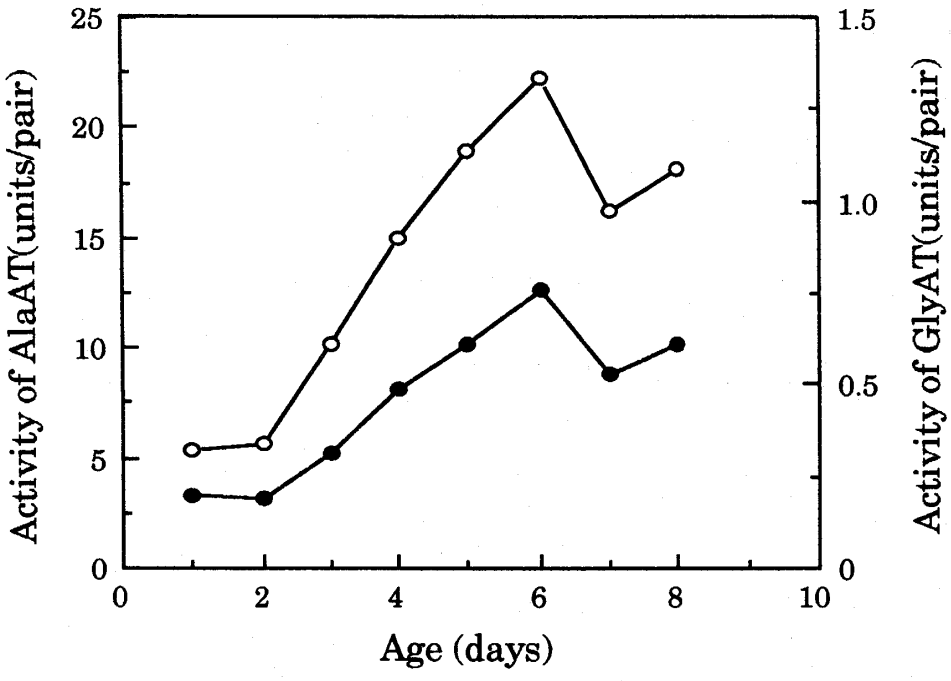

Fig. 1. Changes in the activity of AlaAT and GlyAT and GlyAT in geminating costor bean endosperm.
—o— : AlaAT, —●— : GlyAT (Glutamate : glyoxylate aminotransferase)

The supernatant was applied to a DEAE-Sephadex column equilibrated with buffer A. The elution was carried out with a linear gradient of potassium chloride (0-0.4N) in the same buffer. The active enzyme fraction was pooled and dialyzed against 50mM Tris-HCl buffer (pH 8.5), containing 5 mM β-mercaptoethanol (buffer B). The dialysate was centrifuged at 10,000 rpm for 20 min. The supernatant was applied to a TEAE-Cellulose column equilibrated with buffer B. The elution was carried out with a linear gradient of potassium chloride (0-0.4N) in the same buffer. The active enzyme fraction was pooled and concentrated with ultrafiltration (Ultrafilter UK-20), and then dialyzed against buffer B. The dialysate fraction was applied to a QAE-Sephadex A-50 column equilibrated with buffer B. The elution was carried out with a linear gradient of potassium chloride (0.2-0.5N) in the same buffer. The active enzyme fraction was pooled and then dialyzed against buffer B. The dialysate fraction was applied to a second DEAE-Sepharose CL-6B column equilibrated with buffer B. The elution was carried out with 0.2N potassium chloride in the same buffer. The active enzyme fraction was pooled and then dialyzed against buffer B. The dialysate fraction was concentrated with ultrafiltration (Ultrafilter UK-20) and Centricon-30 (Amicon). The concentrated enzyme solution was further purified with a TSK-DEAE-5PW column (Tosoh, mobile phase elution of 0.23M KCl in buffer B, flow rate, 2 ml/min) The active fraction at retention time of around 16 min was finally purified with Finepak SIL AF-102 column (Japan Spectroscopic Co.). AlaAT was obtained as the single peak at retention time of 9 min. and used for the characterization of the enzyme.

The results of the purification are summarized in Table 1. At the final stage of purification, the specific activity of AlaAT was 220 units/mg protein, representing 1,500-fold purification. Nondenaturing-PAGE of the purified AlaAT yielded a stained protein bond which coincided exactly with the active enzyme band (data not showm).

Properties of AlaAT

Molecular weight and Isoelectric point : The molecular size of purified enzyme, as estimated by gel filtration on a Sephadex G-150 column, was about 110 kDa, and the size of the subunit, as determined by SDS-PAGE, was 58 kDa. These results indicate that the enzyme consists of two identical monomers. The molecular size of castor bean endosperm is similar to those reported for

Table 1 Purification of AlaAT from castor bean endosperms

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Fold	AlaAT*) / GlyAT
Crude extract	16,500	2,500	0.15	100	1	42
30−55% $(NH_4)_2SO_4$	4,660	1,300	0.28	51	2	54
DEAE-Sepharose CL-6B	93	970	10	38	67	59
TEAE-Cellulose	34	680	20	27	130	53
QAE-Sephadex A-50	13	480	37	19	250	51
DEAE-Sepharose Cl-6B	3	370	120	15	800	55
TSK DEAE-5PW (HPLC)	0.5	110	220	4	1,500	55

*) The ratio of Alanine : 2-oxoglutarate aminotransferase (AalAT) activity to glutamate : glyoxylate aminotransferase (GlyAT) activity.

the enzyme from barley roots (97 kDa)[15], pig heart (100 kDa)[16], rat liver (114 kDa)[17], *Drosophila* (113 kDa)[18], tomato (100 kDa)[19], and *Panicum miliaceum* leaves (102 kDa)[4]. There is no available information on the subunits, except for *Drosophila* AlaAT (56 kDa)[18] and *P.miliaceum* AlaAT (50 kDa)[4], which is composed of two identical subunits, similar to castor bean endosperm AlaAT. The isoelectric point of purified enzyme was estimated at 4.6.

Pyridoxal phosphate requirement : The enzyme did not show any appreciable increase in activity when pyridoxal phosphate was added to the reaction mixture. It suggested that sufficient coenzyme was tightly bound to the apoenzyme moiety. Similar results were observed for other aminotransferases[20]

Effect of pH : The effect of pH on the catalytic activity was studied in the phosphate and Tris-HCl buffer in the range of 6.0-9.0 (Fig. 2). The optimum pH of the enzyme was 8.2. This value is comparable to that of AlaAT from other higher plants[20-22]. The enzyme was stored at various pH and then the remaining activity was then assayed. The enzyme was stable at pH 7.5, but

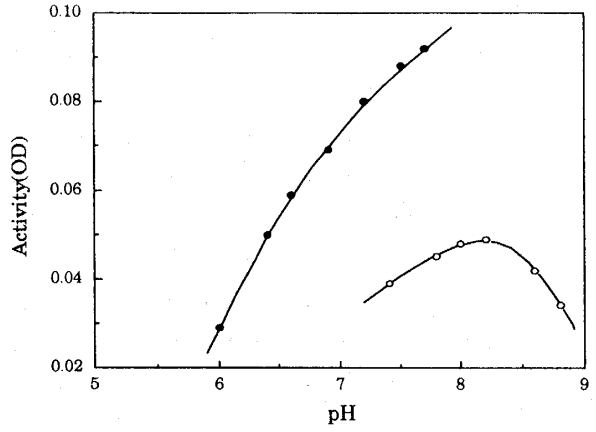

Fig. 2. Effect of pH on AlaAT activity.
—○— : 0.1M Tris-HCl buffer
—●— : 0.1M Phosphate buffer

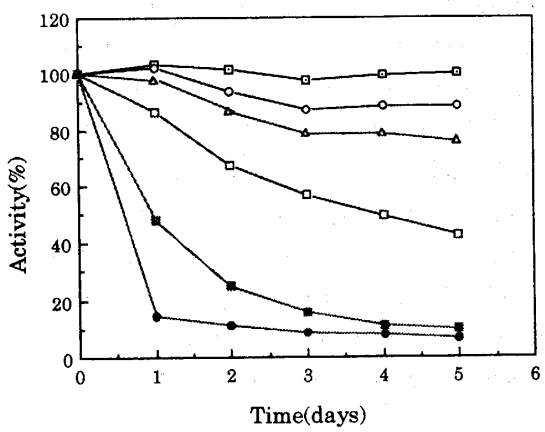

Fig. 3. pH stability of AlaAT.
—□— : pH 7.5, —○— : pH 7.0, —▲— : pH 6.5,
—▣— : pH 6.0, —■— : pH 5.5, —●— : pH 5.0,

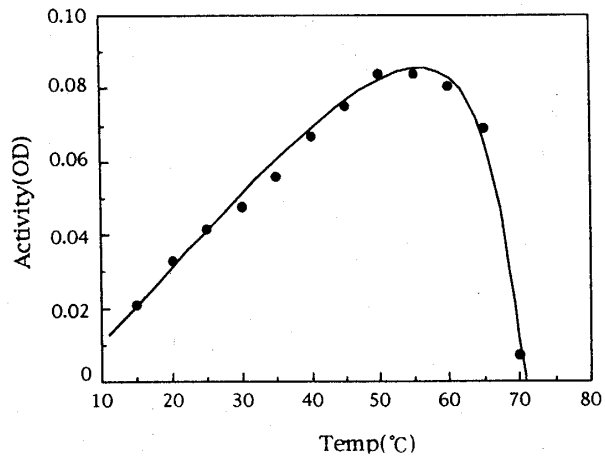

Fig. 4. Effect of temperature on AlaAT activity.

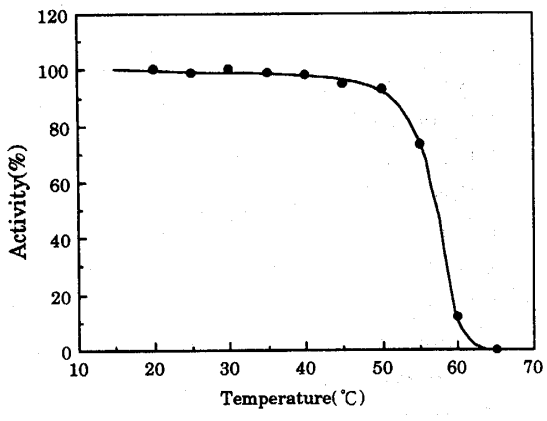

Fig. 5. Heat stability of AlaAT

it was rapidly inactivated at pH 5.0 (Fig. 3)

Effect of temperature : The effect of temperature on the catalytic activity of AlaAT are determined in the range of 15-70℃ (Fig. 4). The optimum temperature for catalysis was 55℃. Activation energy calculated from the Arrhenius Plots was 5.99 Kcal/mol.

Thermostability of the enzyme was investigated by incubating the enzyme in Tris-HCl buffer, pH 8.2, for 10 min at the temperature, and then the residual activity was assayed at 25℃ under standard conditions. The enzyme was stable at temperature up to 50℃, but above 55℃ gradually inactivated (Fig. 5).

Substrate specificity : The purified enzyme were tested against amino acids, each in presence of one of the 4 oxo-acids substrates normally involved in plant transamination reactions. The results are presented in Table 2. High activity was found for alanine-2-oxoglutarate and the low glyoxylate aminotransferase activities was shown by the enzyme catalyzing the alanine-glyoxylate, aspartate-glyoxylate, glutamate-glyoxylate, arginine-glyoxlyate and glutamine-glyoxylate transamination reactions. Glutamate-glyoxylate transamination was catalyzed by the purified enzyme at a rate more than 55 times slower than that of alanine-2-oxoglutarate transamination. The activity ratio of AlaAT and glutamate : glyoxylate aminotransferase (EC 2.6.1.4) remained constant throughout the purification from endosperm fraction. (Table 1). In elution profiles from a DEAE-Sepharose CL-6B and TEAE-cellulose column at purification process, an activity peak with AlaAT and glutamate : glyoxylate aminotransferase activities coincident was obtained. As shown as Fig. 1, the change of glyoxylate : glutamate aminotransferase activity in castor bean endosperms during germination was parallel to that of AlaAT activity. On polyacrylamidegel electrophoresis, the enzyme preparation migrated to the anode as a single protein band, which coincided with both alanine : 2-oxoglutarate aminotransferase and glutamate : glyoxylate aminotransferase activities. These results show that the two enzyme activities are derived from the same protein. A similar

Table 2 Substrate specificity of AlaAT

Amino acid	Activity (%)			
	Reaction with 2-oxoglutarate	Reaction with pyruvate	Reaction with oxaloacetate	Reaction with glyoxylate
L-Alanine	100	–	2.3	–
L-Aspartate	14	11	–	72
L-Glutamate	–	100	1.0	62
L-Orunitine	17	5	2.8	30
L-Glycine	11	–	0.27	–
L-Arginine	6	2	0.3	74
L-Glutamine	–	23	0	51
L-Serine	0	0	–	10
L-Threonine	0	0	–	4.9
γ-Aminobutylate	0.8	0	0	0.22
β-Alanine	0	0	0	0.44

result was obtained for an AlaAT of spinach leaf[21].

Kinetic properties : The effects of substrate concentrations on the catalytic rate of AlaAT were determined at optimum pH. The reactions showed Michaelis-Menten kinetics and the K_m, as estimated from the Lineweaver-Burk plot, was 3.4 mM for L-alanine and 0.26 mM for 2-oxoglutarate. These values compare favorably with those obtained for the enzymes from other plant sources[20,21,23,24]. Lineweaver-Burk plots for one substrate at fixed concentrations of the other substrate give a family of parallel lines. This kinetic evidence indicate that the reaction proceeds by formation of a binary complex in the manner designated as the Ping Pong Bi Bi mechanism[25]. Kinetic data presented in the following references support a Ping Pong mechanism for plant aminotransferase[19,21,26].

Effect of divalent ions : AlaAT was preincubated with different divalent ions and then assayed for the activity. All the added metal ions inhibited the enzyme activity except Mn^{2+} (Table 3). None of the divalent ion enhanced the activity, which shows that AlaAT requires no metal ion for its activity. Although the role of metal ions in transamination reactions is not clear, many reports are available on the effects of various cations[27].

Effect of SH-blocking reagent : The SH-reagents such as *p*-CMB and $HgCl_2$ inhibited the enzyme activity. Dithiothreitol completely protected this inhibition (Table 4). Thus, it seems possible that SH-groups are important for the activity and the retention of conformation of the enzyme molecule.

Amino acid composition : The amino acid composition of the AlaAT of castor bean is presented in Table 5. Values are the average of extrapolation of 4 detaminations (20 h, 40 h, 70 h, and 96 h

Table 3. Effect of various divalent ions on AlaAT activity

Divalent ion	Relative activity (%)
$MgSO_4$	112
$MnSO_4$	67
$CaCl_2$	56
$CuCl_2$	21
$ZnSO_4$	21
$FeSO_4$	0

Table 4. Effect of SH-reagents of AlaAT activity

SH-reagent	Relative activity (%)
1.0 mM DTT	100
0.1 mM *p*-CMB	107
0.2 mM *p*-CMB	7
0.1 mM *p*-CMB + 1 mM DTT	109

Table 5. Amino acid composition of AlaAT

Amino acid	Human [27]	Rat [28]	Castor bean *)
Asx	32	32	36
Thr	18	16	13
Ser	25	27	20
Glx	64	71	39
Pro	33	33	18
Gly	38	36	23
Ala	52	44	20
Val	41	39	17
Met	11	16	1
Ile	18	21	17
Leu	55	48	29
Tyr	15	16	24
Phe	21	22	13
His	8	7	10
Lys	16	19	10
Arg	37	33	25
Trp	1	1	–
Cys	10	14	–

*) The number of amino acid residues per molecule of subunit of enzyme was calculated on the basis of siubunit Mr of 58,000.

hydrolysis) in 6N HCl. The contents of Cys and Try were not determined. The major amino acid residues were Asp, Ser, Glu, Gly, Ala, Leu, Tyr and Arg. AalAT of castor bean was poor in Met, Lys and His residues. The amino acid composition of AlaAT as compared with those of animal AlaAT is given in Table 5 [20,29].

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