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PURIFICATION AND PROPERTIES OF LEUCINE AMINOTRANSFERASE FROM SOYBEAN SEEDLINGS*

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Key Word Index—*Glycine max*, Leguminosae, leucine aminotransferase, soybean, isoenzymes.

Abstract—Two isoenzymes of leucine aminotransferase (LAT I and LAT II) were extracted and partially purified from etiolated soybean seedlings. LAT I accounted for about 87% and LAT II about 13% of the total LAT activity. LAT I was eluted from a DEAE-cellulose column with a buffer having lower ionic strength than LAT II. Both isoenzymes gave pH optima of 8.9. Kinetic data for the forward reaction were consistent with the accepted ping-pong bi-bi mechanism for aminotransferases. Isoenzymes were inhibited by excess of substrate and product. Inhibition by the substrate analogue maleate suggested that both substrates utilized the same catalytic site of the enzyme. Hydroxylamine inhibited the aldehyde form of the LAT while the amino form was found to be inert.

INTRODUCTION

Transamination is one of the key reactions of amino acid metabolism and all the amino acids are metabolized by this route, involving different transaminases. Branched chain amino acids valine, isoleucine and leucine are synthesized by branched chain aminotransferases (BCAT- E.C. 2.6.1.42). BCAT is ubiquitous in distribution and has been very well investigated in micro-organisms and animals [1–5]. In the case of higher plants, the activity of BCAT has been reported in maize embryo [6], peas [7] spinach [8], beans [9], tomato fruits [10] and barley [11]. However, detailed investigations, concerning purification and properties of BCATs from plants have not been reported.

In view of the inadequate information on BCAT from plants, detailed studies on leucine amino transferase (LAT)- one of the BCAT enzymes isolated from soybeans were carried out and are reported in this communication.

RESULTS

Purification of LAT isoenzymes

LAT isoenzymes were purified from 5-day-old etiolated soybean seedlings. At this stage of germination all tissues show the presence of two isoenzymes [12]. The presence of 5 mM α-ketoglutarate (pH 7.5) and 1 mM BME offered considerable protection against inactivation during purification. The globulin and other reserve proteins from the crude extract were removed by precipitation with 5 mM $MgCl_2$. The protein precipitated on addition of 60% ammonium sulphate contained LAT which was desalted on a Sephadex G-100 column. This gave considerable purification in addition to complete removal of the salt. Two isoenzymes of LAT were separated on DEAE-sepharose by elution with a stepwise gradient. Thus LAT I was eluted with 60 mM potassium phosphate buffer, pH 7.5 and LAT II was eluted with 100 mM potassium phosphate buffer, pH 7.5 (see Experimental). Table 1 gives information on the purification and yield of the enzyme at different stages. The data show that final purification obtained was 47- and 59-fold for LAT I and II, respectively, with yields of 50% and 13%.

Molecular properties

The M_rs and Stoke's radii of the isoenzymes were determined by gel filtration on standardized Sephadex G-200 column [13, 14]. The M_r of LAT I was found to be 68000 and that of LAT II was 93300. The Stoke's radius of LAT I was estimated to be 35 Å and that for LAT II it was 37.5 Å.

Kinetic studies

The dependence of the initial velocity of the enzyme reaction upon substrate concentration was studied with leucine and α-ketoglutarate. Concentrations of the substrates were varied systematically over a range, suitable to obtain kinetic constants. To illustrate the results, double reciprocal plots of reaction rates against varying concentration of one substrate were obtained at constant concentrations of the other substrate (Fig. 1). The complete matrix of the concentrations shown was run on two separate occasions and the same pattern of plots was obtained. The results fitted a pattern of linear, parallel plots. This pattern is unique to the ping-pong bi-bi mechanism for the interaction of the substrate with the enzyme as described in ref. [15]. Table 2 shows the K_m values for keto and amino substrates for LAT I and II. The isoenzymes did not differ much in this respect. Both isoenzymes showed more affinity towards amino substrate than for keto substrate. In the case of purified bacterial transaminase, a greater affinity for branched

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Abbreviations: BME, β-mercaptoethanol; LAT, leucine aminotransferase; PLP. pyridoxal phosphate.

Table 1. Purification of LAT isoenzymes from germinating soybean seeds

Step	Total activity units	Total protein mg	Specific activity (units/ mg × 10^{-2}	Yield %	Purification fold
1. Crude enzyme extract	54.4	35700	0.15	100	1
2. $MgCl_2$ precipitate	51.2	32100	0.17	94	1.1
3. $(NH_4)_2SO_4$ precipitate	42.9	15950	0.26	78	1.8
4. Sephadex G-100	49.9	2520	1.8	84	12
Purification of LAT I after Sephadex G-100 chromatography					
5(A). DEAE-sepharose (0.06 M eluant LAT I)	28.0	376	7.4	51	47
Purification of LAT II after Sephadex G-100 chromatography					
5(B). DEAE-sepharose (0.11 M eluant LAT II)	7.2	85.5	8.7	13.2	59

1 unit activity = 1 μmol of α-ketoisocaproate produced/min. The enzyme activity was assayed as described in Experimental.

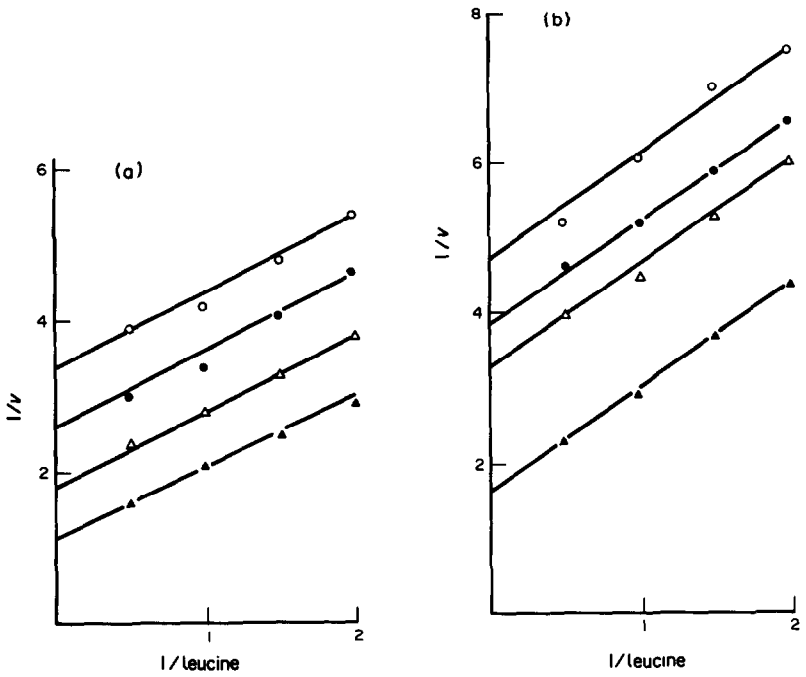

Fig. 1. Initial velocity pattern for the LAT isoenzymes with leucine as variable substrate. (a): LAT I, (b): LAT II, α-ketoglutarate concentrations: O——O, 0.5 mM, ●——●, 0.66 mM, △——△ 1.00 mM, ▲——▲ 2.00 mM.

Table 2. Kinetic properties of LAT I and LAT II from soybean

Substrate	Apparent K_m values mM	
	LAT I	LAT II
Leucine	2.5	1.67
α-Ketoglutarate	3.3	2.3
V_{max} α-ketoisocaproate formation: units/mg	5.0	1.25

chain keto acids than for branched chain amino acids was observed [15].

The mechanism of transaminase which appeared to be ping-pong bi-bi type was further confirmed by substrate and product inhibition studies. High concentrations of α-ketoglutarate produced linear competititve inhibition of LAT I while glutamate inhibited the enzyme noncompetitively with respect to α-ketoglutarate (Data not shown).

Inhibition by substrate analogues

The aminotransferase is inhibited by substrate analogues that are incapable of undergoing transamination.

The inhibition of aspartate aminotransferase by aliphatic dicarboxylic acids is well documented [16]. In the present studies a series of dicarboxylic acids were tested as inhibitors of transamination from L-leucine to α-ketoglutarate. Each acid was included in the assay mixture at a fixed concentration of 100 mM. The results are summarized in Table 3. All dicarboxylic acids were found to inhibit LAT, however, the extent of inhibition varied.

The nature of this inhibition was studied with maleate using LAT I preparation by the method of ref. [17]. At different concentrations of leucine and α-ketoglutarate, maleate inhibition of the enzyme activity is presented in Figs 2A and 2B. The dissociation constant obtained from the intercept yielded a value of 200 mM irrespective of whether the pyridoxal or pyridoxamine form of the enzyme was used (compare Figs 1A and 1B). This suggests that the interaction of maleate with the enzyme is associated with the protein moiety of the enzyme rather than the coenzyme.

Inhibition by carbonyl reagent

BCATs display a sensitivity to carbonyl binding reagents which is typical of PLP enzymes [18]. Inhibitory effects of these compounds are summarized in Table 4. The concentrations indicated refer to those used during prior incubation of the enzyme with the carbonyl reagent alone. The kinetics of inhibition were studied using hydroxylamine as shown in Fig. 3.

Hydroxylamine shows competitive inhibition of LAT when a series of concentrations of leucine was used. At a fixed concentration of leucine and at a series of α-ketoglutarate concentrations, hydroxylamine shows non competitive inhibition. These data suggest that hydroxylamine reacts exclusively with the aldehyde form of the enzyme.

Effect of pH

Both isoenzymes showed sharp pH activity curve with optimum pH at 8.9 (Fig. 4). In view of the sharp pH activity curve the rate controlling parameters were evaluated at different pH values. The initial velocity studies of LAT I at different pHs showed that the K_m of leucine remains constant in pH range 7–7.5 and thereafter increases to a maximum at pH 8.6 (Fig. 5). The ascending limb of the pH activity curve could be due to an increase in V_{max} only as K_ms continued to increase with pH. The declining limb, on the other hand, may reflect a large increase in the Michaelis constant for leucine and α-ketoglutarate. LAT is different from pig heart enzyme [19] where K_m of leucine decreased and that of α-ketoglutarate increased. Also it has been observed in the case of the pig heart enzyme that when α-ketoglutarate was replaced by α-ketoisovalerate, the BCAT activity remained constant even at a pH as high as 10.

The LAT from soybean in present studies, however, showed a similar pH activity curve, viz a drop beyond pH 8.9 even when α-ketoglutarate was replaced by α-ketoisocaproate (Fig. 6). This was expected because in both cases the K_ms continued to increase (Fig. 5), probably resulting in slower turnover of the enzyme.

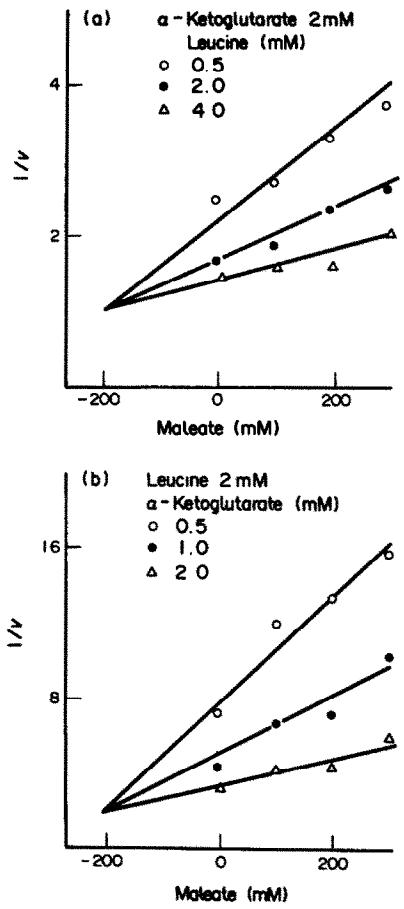

Fig. 2. Inhibition of LAT I by maleate. Plot of the data for maleate inhibition of the leucine, α-ketoglutarate transamination to yield, at the intersection point the dissociation constant of the complex between. (a) maleate and pyridoxamine phosphate form of the enzyme and (b) maleate and the pyridoxal phosphate form of the enzyme.

Table 3. Inhibition of LAT II from soybean by 100 mM dicarboxylic acids

Acid	% Inhibition
Glutaric	39
Maleic	45
Adipic	45
Succinic	56
Fumaric	64

DISCUSSION

Two isoenzymes of LAT were partially purified from soybean seedlings. The complete separation of two isoenzymes could be achieved on DEAE-sepharose. The two isoenzymes showed different apparant M_rs. This observation is at variance with that of Aarnes [11] who found both the isoenzymes of barley to have a M_r weight of 95 000. However, these isoenzymes differed in terms of charge distribution which was also shown by isoelectric focussing. Isoenzymes of BCAT from microorganisms and animal tissues have been reported [1–5] to have different M_rs.

Table 4. Inhibition of LAT isoenzymes from soybean by carbonyl reagents

Compound	Concentration (preincubation mixture)	% of Inhibition LAT I	LAT II
Hydroxylamine	1×10^{-6}	40	39
	1×10^{-7}	30	26
	1×10^{-8}	10	19
Phenylhydrazine	1×10^{-6}	43	32
	1×10^{-7}	13	23
	1×10^{-8}	9	22
Thiosemicarbazide	1×10^{-3}	70	62
	1×10^{-4}	20	19
	1×10^{-5}	12	16
Sodium bisulphite	1×10^{-1}	84	90
	1×10^{-2}	11	88
	1×10^{-3}	7	16
Potassium cyanide	1×10^{-1}	82	70
	1×10^{-2}	53	12

LAT isoenzymes were preincubated with carbonyl reagents in 50 mM Tris–HCl, pH 9 for 15 min at 37°. Transamination was then initiated by the addition of 40 mM leucine and 8 mM α-ketoglutarate, pH 7 and the activity was determined as given in the Experimental.

The kinetic analysis of the two isoenzymes showed characteristics that are typical of other transaminases. The mechanism of catalysis could be confirmed to be ping-pong bi-bi. The predicted inhibition by excess substrate and product was also observed. The two isoenzymes did show minor differences in apparent K_ms. The typical inhibition of isoenzymes by carbonyl binding reagents and maleate inhibition of LAT showed that both the substrates utilize the same catalytic site of the enzyme. The pH curve clearly shows that the LAT from soybean seedlings is different from that of animal system. In the case of pig heart enzyme [19] it has been suggested that at high pH the NH_2 group exchanged between branched chain carbon skeletons only, preventing the synthesis of glutamate, an active metabolite. This will result in the conservation of essential amino acids (leucine, isoleucine and valine) for the organism. The absence of such mechanisms in plants can be easily reconciled. It could be proposed that since the essential amino acids are not synthesized in animal systems, the above mechanism may be needed for the conservation of these amino acids. However, in plants essential amino acids are synthesized, and there is no need for such conservation through restricted interconversion.

EXPERIMENTAL

Materials. Soybean (*Glycine max* var. Kali tur) seeds were obtained from G. B. Pant University of Agriculture and Technology. Marker proteins and column materials were obtained from Pharmacia. All other chemicals used were of highest chemical grade.

Enzyme assay. The activity of LAT in the forward direction was assayed by measuring the amount of α-ketoisocaproate formed by the method of refs [1, 20]. The reaction in a total vol. of 0.9 ml contained 100 mM Tris–HCl buffer, pH 9, 40 mM leucine, 8 mM α-ketoglutarate and 0.2 mM pyridoxal phosphate, pH 7. After

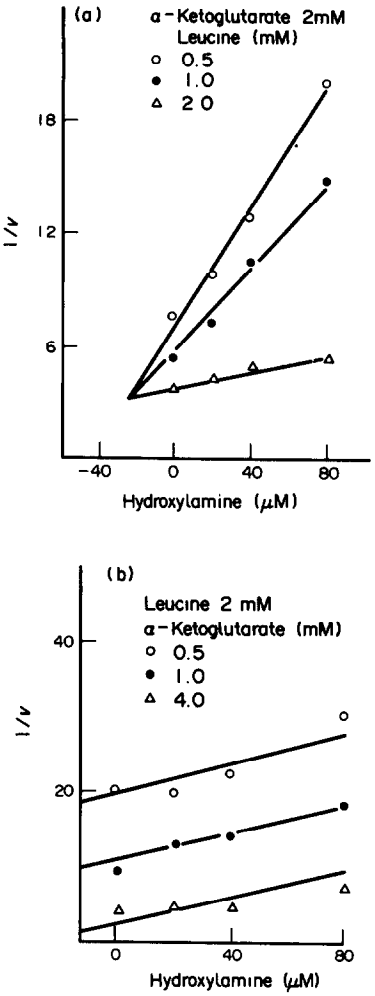

Fig. 3. Inhibition of LAT I by hydroxylamine (a): Competition of hydroxylamine with leucine for the bound PLP of the enzyme at 2 mM α-ketoglutarate pH 8.6, the intersection point projected on the abscissa corresponds to a dissociation constant of 20 μM for the enzyme inhibitor complex. (b): The non-competitive inhibition of the enzyme by hydroxylamine with respect to α-ketoglutarate concentration.

5 min equilibration at 37° the reaction was initiated by the addition of 100 μl of enzyme. The reaction was terminated after 30 min by the addition of 1 ml of 0.3% dinitrophenyl hydrazine and incubated for 5 min. Cyclohexane (5 ml) was then added and the mixture vortexed for 1 min and centrifuged for 5 min at 2000 *g*. From the upper layer, 4 ml was removed and mixed with 1.5 ml of 10% Na_2CO_3. The mixture was again vortexed for 20 sec and allowed to stand for 5 min. One ml of the lower layer was removed and mixed with 2 ml of 1 M NaOH. After 5 min the reddish brown colour was measured at 440 nm against blank.

Purification of LAT isoenzymes. All steps were carried out at 4–6° unless otherwise stated and all centrifugations were carried out at 16000 *g* for 45 min. All the buffers used for purification contained 5 mM α-ketoglutarate, pH 7.5, 1 mM EDTA and 1 mM BME. The etiolated soybean seedlings (4–5-days-old) were washed with H_2O and homogenized in two vols of 50 mM Tris–HCl, pH 7.5. The homogenate was filtered through 4 layers of cheese cloth. The filtrate was centrifuged and the supernatent was made 5 mM with respect to $MgCl_2$. After stirring for 5 min, it

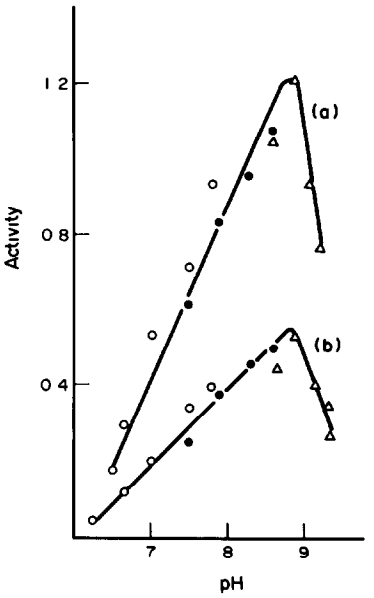

Fig. 4. pH dependence of LAT isoenzymes. LAT activity assayed as described in Experimental. (a): LAT I; (b): LAT II, Buffers used were O——O, 0.05 M potassium phosphate; ●——●, 0.05 M Tris–HCl; △——△ 0.05 M glycine–NaOH.

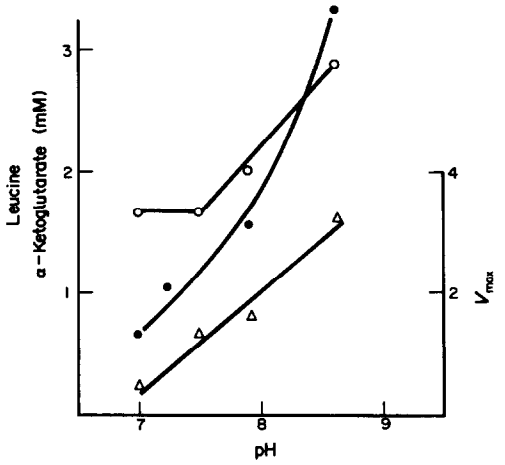

Fig. 5. pH dependence of the kinetic parameters of LAT I. Buffers used were 100 mM potassium phosphate or 100 mM Tris–HCl. O——O, K_m for leucine; ●——●, K_m for α-ketoglutarate; △——△, curve for V_{max}.

was allowed to stand for 20 min. The ppt. formed was removed by centrifugation and discarded. The supernatent was made 60% with respect to $(NH_4)_2SO_4$. The ppt. formed was collected by centrifugation and dissolved in a minimal vol. of elution buffer containing 20 mM K-Pi buffer, pH 7.5. The undissolved inactive protein was removed by centrifugation and discarded. The clear supernatent was desalted on Sephadex G-100 column (2.6 × 95 cm) which was equilibrated with the elution buffer. The column was eluted with the same buffer at a flow rate of 20 ml/hr. The active fractions were pooled and applied to DEAE-Sepharose column (2.4 × 12 cm) which was equilibrated with elution buffer. The column was washed with 100 ml of elution buffer. The protein was then eluted with stepwise gradient, first with soln A (60 mM K-Pi buffer, pH 7.5) till the A of eluate at 280 nm was below 0.01. The column was then eluted with soln B (110 mM K-Pi buffer, pH 7.5). The activity eluting with soln A was due to LAT I whereas the activity eluting with soln B was due to LAT II.

The separated isoenzymes were concd by dialysis against sucrose. This concd enzyme was stored at 4°. Under these conditions the activity was stable for 2 months.

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