SHORT COMMUNICATION

Some Properties of Aspartate and Alanine Aminotransferases from *Trichoderma viride*

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Aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2) were extracted from mycelia of *Trichoderma viride*, partially purified and characterized. The pH optima for the enzyme activities were 9.0 and 8.6, respectively. Both enzymes were relatively stable at 37 °C but rapidly denatured at 60 °C. They were inducible by both L-aspartate and DL-alanine, but aspartate was the better inducer. The apparent Michaelis constants for aspartate aminotransferase when L-aspartate and 2-oxoglutarate were used were 7.14 mM and 1.32 mM, respectively. In the case of alanine aminotransferase, using DL-alanine and 2-oxoglutarate the corresponding values were 8.33 mM and 1.85 mM, respectively. The enzymes were competitively inhibited by succinate with K_i values of 21 mM and 17 mM for aspartate aminotransferase and alanine aminotransferase, respectively. Both enzymes could use L-methionine whereas only aspartate aminotransferase could use L-tyrosine as amino donor.

INTRODUCTION

Aminotransferases play an important role in nitrogen metabolism (Saier & Jenkins, 1967). Aspartate aminotransferase [glutamic-oxaloacetic transaminase (GOT), EC 2.6.1.1] and alanine aminotransferase [glutamic-pyruvic transaminase (GPT), EC 2.6.1.2] have been studied in animal tissues (De Rosa & Swick, 1975; Arnone *et al.*, 1977), in plants (Ellis & Davies, 1961; Forest & Wightman, 1972) and in micro-organisms (Meister & Rudman, 1953; Fincham & Boulter, 1956; Mavrides & Orr, 1974; Cazzulo *et al.*, 1977). These enzymes seem to have opposite functions in the mitochondria and cytosol of eukaryotic cells (Shrawder & Martino-Martinez, 1972; De Rosa & Swick, 1975). The mitochondrial GPT is involved in conversion of alanine to pyruvate, while cytosolic GPT takes part in the formation of alanine from pyruvate. Similarly, cytoplasmic GOT furnishes aspartate from oxaloacetate, while mitochondrial GOT converts oxaloacetate to aspartate. In fungi, these enzymes are believed to be responsible for synthesis of the so-called secondary amino acids from the primary amino acids (Bekker, 1963).

Trichoderma viride, a soil fungus, produces the antifungal substances gliotoxin and viridin. These compounds are being tested for controlling cassava root fungal diseases. Cassava is one of the important staple foods in West Africa and an understanding of the amino acid metabolism of *T. viride* could assist in the control of root diseases.

This paper describes the isolation and characterization of aminotransferases from *T. viride*.

METHODS

Organism. The strain of *Trichoderma viride* used was originally isolated from a wooden mortar and identified by standard procedures (Ainsworth, 1971; Clements & Shear, 1973). Stock cultures were grown on slants of Sabouraud agar for 7 d at room temperature and subsequently maintained at 4 °C. Subcultures were made at monthly intervals.

Growth medium. *Trichoderma viride* was grown in modified Frie's medium containing (% w/v): KH_2PO_4 , 0.15; NaNO_3 , 0.6; KCl, 0.05; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; and traces (about 0.003 %) of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. This salt solution was adjusted to pH 6.5 with 0.1 M-HCl, sterilized at 121 °C for 10 min and mixed with a solution of glucose (sterilized at 115 °C for 5 min) to give a final concentration of 4 % (w/v) glucose. After adding chloramphenicol (500 $\mu\text{g ml}^{-1}$), the medium was dispensed in 200 ml volumes into sterile 500 ml flasks.

Growth of fungus. Six flasks were each inoculated with three loopfuls of conidium-bearing mycelium from a 7 d culture of *T. viride* and incubated at room temperature (28 to 30 °C) for 7 d without agitation. Good growth was indicated by the formation of a dense mat of green mycelium on the medium. The medium was aseptically decanted, and the mycelium was used for enzyme induction studies.

Enzyme induction. The induction medium was of the same composition as the Frie's medium but with glucose replaced by the inducing amino acids L-aspartate or DL-alanine (1 $\mu\text{mol ml}^{-1}$ final concentration). Two of the six cultures were induced for aminotransferase with L-aspartate and two others were induced with DL-alanine. (The amino acids were the main sources of carbon and nitrogen in the medium.) The remaining two cultures were grown in Frie's glucose medium as controls.

Induction was commenced by aseptically decanting Frie's glucose medium from the cultures, washing the mycelia with 0.9 % (w/v) sterile NaCl at room temperature and then adding the induction medium. Control mycelia were similarly decanted and placed in fresh Frie's glucose medium. All the mixtures were incubated at room temperature without shaking and aminotransferase activity in the culture medium was measured every 24 h for 12 d.

Extraction and partial purification. All operations were performed at 0 to 4 °C unless otherwise stated. Mycelium (15.2 g wet wt) obtained by filtration using Whatman no. 2 paper, after induction with L-aspartic acid, was washed three times with ice-cold 0.9 % (w/v) NaCl and homogenized in 50 ml 5 mM-Tris/HCl buffer pH 7.6 in a Virtis '45' homogenizer (6 $\times$ 30 s bursts). Disintegration was checked microscopically until complete. The homogenate was centrifuged at 20000 g for 15 min and the supernatant was dialysed overnight against 100 vol. 5 mM-Tris/HCl buffer pH 7.6. The diffusate was treated with 2 vol. prechilled (−20 °C) acetone added at intervals over 30 min with gentle stirring. After standing for another 30 min the precipitate was collected by centrifugation at 20000 g for 10 min. The deposit was dissolved in 5 ml 5 mM-Tris/HCl buffer pH 7.6 and dialysed overnight against 50 vol. of the same buffer.

Gel chromatography. Sephadex G-150 was swollen in 5 mM-Tris/HCl buffer pH 7.6 containing 0.4 M-NaCl, and the slurry was used for preparing a column (50 $\times$ 2.5 cm) which was equilibrated by washing with an excess of the same buffer. A sample (3 ml) of the partially purified extract was layered on top of the column and eluted with the same buffer. Fractions (5 ml), collected at a flow rate of 0.1 ml min^{−1}, were analysed for GOT and GPT activities and assayed for protein concentrations by measuring the absorbance at 280 nm. Fractions showing enzyme activities were pooled and used for kinetic studies. Enzyme peaks for GPT were found in fractions 17 to 24 and fractions 21 to 27 showed GOT activity.

Enzyme assays. GOT and GPT activities were assayed by the method of Mohun & Cook (1957). In this assay the amounts of oxaloacetate and pyruvate formed are measured by means of the 2,4-dinitrophenyl-hydrazone derivative of pyruvic acid, determined spectrophotometrically by its absorbance at 520 nm. One unit of enzyme activity was defined as that amount liberating 1 μmol pyruvate min^{−1} (ml sample)^{−1}.

Effect of pH. pH optima of GOT and GPT were determined in Tris/HCl buffer pH 7.0 to 10. The assay mixtures contained 10 mM and 20 mM aspartate and alanine (final concentrations), respectively, and were incubated at 37 °C for 30 min, before enzyme activity was determined.

Effect of temperature. Enzyme solutions (2 ml) were exposed to 37, 40, 50, 60 and 70 °C. At zero time and at 5 min intervals for 30 min, 0.2 ml samples were removed and assayed.

Substrate specificity. Eight amino acids were tested for ability to act as amino donor for GOT and GPT. In each case the reaction mixture (final volume 1.2 ml) consisted of 20 mM donor amino acid, 2 mM-2-oxoglutarate, 0.2 ml enzyme solution and 5 mM-Tris/HCl buffer (pH 9.0 for GOT, pH 8.6 for GPT). Enzyme activities were determined by the standard method.

Effect of substrate concentration. Enzyme activities were determined with various concentrations of the amino donor and constant amino acceptor concentration, or vice-versa.

Inhibition by succinate. Inhibition of GOT and GPT by succinate (10 and 20 mM) was determined using various concentrations (10 to 30 mM) of L-aspartate for GOT and DL-alanine for GPT at the optimum pH

Fig. 1. Induction of GOT and GPT activities in *T. viride* by L-aspartate and DL-alanine. The amino acids ($1 \mu\text{mol ml}^{-1}$, final concentration) were added to Frie's medium and acted as the main sources of carbon and nitrogen. The control consisted of a culture grown in Frie's glucose medium without either of the amino acids. ○, GOT induction with L-aspartate; ●, GOT induction with DL-alanine; △ GPT induction with L-aspartate; □, GPT induction with DL-alanine; —, control.

values of 9.0 and 8.6, respectively, using Tris/HCl buffer. The reaction mixtures were incubated at 37°C for 30 min before enzyme activity was determined.

Effect of Hg^{2+} . The reaction mixture (final volume 1.2 ml) consisted of 20 mM donor amino acid and 2 mM acceptor amino acid in 5 mM-Tris/HCl buffer (pH 9.0 for GOT, pH 8.6 for GPT), 5 mM- HgCl_2 and 0.2 ml enzyme solution. After incubation as usual, enzyme activities were determined.

RESULTS

Enzyme induction. Extracellular GOT and GPT were both inducible by L-aspartate and DL-alanine, L-aspartate being the better inducer of both enzymes (Fig. 1). With L-aspartate the culture medium contained 77 units of GOT activity and 50 units of GPT activity after 12 d, whereas with DL-alanine the activities were 57 and 47 units, respectively.

Isolation and partial purification. Partial purification of the mycelial enzymes by acetone precipitation and gel filtration resulted in a 10-fold increase in specific activity of GPT and a 5-fold increase in GOT. The elution profiles indicated that GPT had a slightly higher molecular weight than GOT. The crude mycelial extract contained 235.2 units of GOT activity and 136.8 units of GPT activity per g mycelium.

Properties. The pH optimum was 9.0 for GOT and 8.6 for GPT.

When GOT and GPT were pre-incubated at various temperatures before assay, GOT lost 20 % of its activity at 50°C , 75 % at 60°C and about 80 % at 70°C , whereas GPT lost about 50 % of its activity at 50°C , 75 % at 60°C and 83 % at 70°C . Both enzymes showed biphasic denaturation kinetics at 60 and 70°C .

The apparent Michaelis constants were 7.14 mM for GOT and 8.33 mM for GPT. When the concentration of the amino acceptor was varied and the respective substrate concentration kept constant, the Michaelis constants were 1.32 mM for GOT and 1.85 mM for GPT.

Table 1 lists the relative activities of the two aminotransferases with different amino donors as substrates. For GOT, L-aspartate was the best amino donor followed by methionine and tyrosine. DL-Alanine was the best amino donor for GPT, followed by methionine. Tyrosine, glycine and serine were poor substrates for this enzyme.

Succinate was a competitive inhibitor of both GOT and GPT. The respective inhibitor constants (K_i) of 21 mM and 17 mM for succinate were calculated from Dixon plots (Dixon, 1953). Hg^{2+} also inhibited both enzymes; the relative inhibition was 93 % in both cases.

Table 1. *Trichoderma viride* GOT and GPT activities using various amino acids as amino donors and 2-oxoglutarate as amino acceptor

Relative activities are expressed in terms of activity with aspartate in the case of GOT and activity with alanine in the case of GPT.

Amino donor (20 mM)	GOT activity (units)	Relative GOT activity (%)	GPT activity (units)	Relative GPT activity (%)
L-Aspartic acid	130	100	18	18.8
DL-Alanine	10	7.7	96	100
L-Methionine	120	93	72	75
L-Tyrosine	110	84.6	35	36.5
L-Phenylalanine	10	7.7	11	11.5
L-Cysteine	34	26.2	18	18.8
L-Glycine	10	7.7	24	24
L-Serine	10	7.7	21	21.9

DISCUSSION

In this study it was assumed that the extracellular aminotransferase activities found in the *T. viride* culture medium resulted from leakage or secretion from the mycelium. Enzyme synthesis by the organism was therefore monitored by measuring enzyme activities in the medium. GPT and GOT were both inducible with L-aspartate and DL-alanine, although L-aspartate was a better inducer. Our observation is similar to that of Urm *et al.* (1973) in their study of GOT from *E. coli*. Our findings differ from those of Fincham & Boulter (1956), but these workers did not use exogenous amino acids as the main source of carbon and nitrogen.

Trichoderma viride GOT and GPT differed slightly in their amino donor specificities. The specificity pattern may be explained in two ways. Firstly, it is possible that the enzymes exist in more than one form. Two subforms of GOT have been reported to occur in *E. coli* (Mavrides & Orr, 1974; Chesne & Pelmont, 1974). The second explanation is based on the chain length of the various substrates. Saier & Jenkins (1967) have shown that as substrate chain lengths increase transamination rates decrease. This supports our observation of the high activity for GOT with methionine which resembles glutamate in chain length. Similarly, the low GOT activities observed with serine, glycine and cysteine may also be due to chain length differences.

The observed competitive inhibition of *T. viride* GOT and GPT by succinate is consistent with the findings for these enzymes from other sources (Jenkins *et al.*, 1959; Dybel *et al.*, 1972). The biphasic behaviour of the enzymes when subjected to higher than optimum temperatures may also suggest the existence of two distinct enzyme forms. The pH optima of 9.0 and 8.6 observed for GOT and GPT, respectively, were similar to those observed for these enzymes from other sources (Saier & Jenkins, 1967; Mavrides & Orr, 1974; Cazzulo *et al.*, 1977; Orlicky *et al.*, 1977). The Michaelis constants observed for these enzymes using L-aspartate and DL-alanine as amino donors suggest that transamination, though important, is not the primary pathway for glutamic acid synthesis or for replenishing pyruvic acid and oxaloacetic acid in this organism.

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