Properties of an Aminotransferase of Pea (*Pisum sativum* L.)[1]

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ABSTRACT

A transaminase (aminotransferase, EC 2.6.1) fraction was partially purified from shoot tips of pea (*Pisum sativum* L. cv. Alaska) seedlings. With α-ketoglutarate as co-substrate, the enzyme transaminated the following aromatic amino acids: D,L-tryptophan, D,L-tyrosine, and D,L-phenylalanine, as well as the following aliphatic amino acids: D,L-alanine, D,L-methionine, and D,L-leucine. Of other α-keto acids tested, pyruvate and oxalacetate were more active than α-ketoglutarate with D,L-tryptophan. Stoichiometric yields of indolepyruvate and glutamate were obtained with D,L-tryptophan and α-ketoglutarate as co-substrates. The specific activity was three times higher with D-tryptophan than with L-tryptophan.

Transaminases (aminotransferases, EC 2.6.1), particularly those of plants, are quite incompletely described in regard to their amino acid and keto acid specificities. Some partially purified preparations are highly specific for particular amino acids (1, 2, 5, 30). Other preparations reportedly transaminate a variety of amino acids (7, 8, 12, 15, 25, 29). Many workers have concentrated on transamination of aromatic amino acids. Two transaminase fractions have been isolated from rat brain, one of which showed highest activity toward tyrosine and phenylalanine and the other of which showed highest activity toward tryptophan and 5-hydroxytryptophan (4, 6, 26). Partially purified tryptophan transaminase from the bacterium *Clostridium sporogenes* catalyzed the transamination of tyrosine and phenylalanine, as well as tryptophan (23). A transaminase fraction from mung bean (*Phaseolus aureus*) also transaminated tryptophan, phenylalanine, and tyrosine, as well as several aliphatic amino acids (7, 8).

The purpose of these investigations was to purify partially and ascertain some properties of a transaminase fraction from pea shoots and to attempt to corroborate the evidence reported by Moore (20) and Moore and Shaner (21, 22) that transamination is an initial reaction in the conversion of tryptophan to IAA.

MATERIALS AND METHODS

Source and Purity of Reagents. D,L-Tryptophan, D-tryptophan, L-tryptophan, D,L-phenylalanine, D,L-tryosine, D,L-glutamate, α-ketoglutarate, pyridoxal phosphate, and dithiothreitol were purchased from Sigma Chemical Company. Chloramphenicol, oxalacetic acid, glyoxylic acid, and pyruvic acid were obtained from Cal-Biochem. D,L-Methionine, D,L-leucine, and D,L-alanine were acquired from Nutritional Biochemicals Corporation, Cleveland, Ohio. Hydroxylapatite was obtained from Bio-Rad Laboratories. Silica Gel G was obtained from Brinkmann Instruments Company, Westbury, N.Y. All other chemicals used were of reagent grade, and all organic solvents were redistilled prior to use.

Culture of Plants. The Alaska variety of pea (*Pisum sativum* L.) (from W. Atlee Burpee Company, Riverside, Calif.) was used in all experiments. Seeds were planted in vermiculite and kept under a 16-hr photoperiod at 20 ± 1 C with a light intensity of 900 to 1000 ft-c and an 8-hr dark period at 16 ± 1 C. After 10 to 14 days, 50 to 70 g of shoot tips were removed immediately above the fifth node and placed in liquid nitrogen for overnight storage prior to use.

Preparation of Enzyme Extract. Each sample of shoot tips was homogenized in a chilled mortar and pestle with 2 ml of 0.5 M borate buffer (pH 8.5), containing 150 μM chloramphenicol and 1 mM dithiothreitol, per g fresh weight of tissue. The homogenate then was centrifuged at 40,000g for 10 min at 4 C, and the resulting supernatant was the crude enzyme extract. The extract was then brought to 25% (v/v) acetone with the addition of ice-cold acetone. After setting in an ice bath for 10 min, the 25% (v/v) acetone-crude extract was centrifuged at 10,000g for 10 min. The supernatant was then brought to 60% (v/v) acetone, allowed to set for 10 min in an ice bath and then was centrifuged at 10,000g for 10 min. The resultant supernatant was discarded, and the pellet was resuspended in 0.5 M borate buffer (pH 8.5) in a total of one-sixth of the original volume of crude extract. This suspension was then centrifuged at 40,000g for 10 min, and the resulting gold-yellow colored supernatant was used as the acetone-precipitated enzyme fraction. In some studies, the acetone-precipitated enzyme was added to a column of hydroxylapatite (1.6 × 17 cm) which was previously equilibrated with 0.02 M KH$_2$PO$_4$-Na$_2$HPO$_4$ buffer at pH 8.5. The column was then washed with 70 ml of 0.05 M sodium-potassium phosphate buffer (pH 8.5) and 50 ml of 0.08 M sodium-potassium phosphate buffer (pH 8.5). The enzyme was then eluted with 0.15 M sodium-potassium phosphate buffer (pH 8.5) at a flow rate of 2 ml/min and collected in 5-ml fractions.

Reaction Conditions. Routine reaction mixtures contained 0.5 ml of enzyme extract and 2.5 ml of 0.5 M borate buffer (pH 8.5) containing 0.1 μmole of pyridoxal phosphate, 40 μmoles of amino acid (except 10 μmoles of the sparingly soluble tyrosine), and 20 μmoles of α-keto acid. The reaction mixtures were incubated for 60 min at 30 C in rubber-stoppered 10-ml test tubes. Reaction mixtures minus α-ketoglutarate were used as controls and as blanks for the spectrophotometric assay.

Measurement of Aromatic α-Keto Acid Formed. The enol-

borate buffer assay of Lin *et al.* (16) was employed to assay yields of aromatic α-keto acids. The enol tautomers of the aromatic α-keto acids combine instantaneously and reversibly with borate to form complexes of a mixed ester-anhydride structure, which in turn displaces the keto-enol equilibrium in favor of the enol tautomer. The enol tautomer and its borate complex absorb strongly in the 300 nm region (13). The indolepyruvate-borate complex has a maximum absorption at 328 nm; β-phenylpyruvate and p-hydroxyphenylpyruvate have absorption maxima at 310 nm (16). Therefore, after the reaction mixtures had been incubated for 60 min, absorbance was measured directly either at 328 or 310 nm to determine the amount of aromatic α-keto acid formed.

Isolation of Glutamate by Thin Layer Chromatography. The procedure described by Rowsell (24) for isolating and assaying amino acids by paper chromatography was modified for TLC and used to determine quantitatively the amount of glutamate formed when D,L-tryptophan and α-ketoglutarate were co-substrates. After the reaction had proceeded for 60 min, two drops of concentrated HCl were added to each reaction mixture to stop the reaction. The reaction mixtures were then placed in a boiling water bath for 5 min to precipitate protein. Following centrifugation for 5 min at top speed in a clinical centrifuge, the resulting supernatant was then applied to a thin layer plate coated with Silica Gel G and cochromatographed with authentic D,L-glutamate. Three solvent systems were used: 1-propyl alcohol-water (70:30, v/v), 1-butyl alcohol-glacial acetic acid-water (80:20:20, v/v/v), and 96% ethanol-34% ammonia solution (70:30, v/v). After the chromatograms were developed and dried in a current of warm air, they were sprayed with ninhydrin reagent, which was prepared according to Waldi (28). The plates were then heated to 110 C for 10 min, after which the spots corresponding to the authentic glutamate were scraped into 12-ml conical centrifuge tubes. Five ml of 70% (v/v) ethanol were then added to each tube. The tubes were stoppered, shaken vigorously for 1 min to elute the product from the silica gel, and the tubes were centrifuged. The resulting supernatants were decanted for absorbance measurement at 575 nm.

Protein Determination. Protein was determined by the method of Lowry *et al.* (17) using bovine serum albumin as standard.

RESULTS

Partial Purification of Transaminase. Before any purification steps were applied to the crude enzyme preparation, only low activity was observed. The specific activity of this crude enzyme was variable, but averaged approximately 0.028 units/mg protein, a unit of activity being defined as 1 μmole indolepyruvate formed/hr. Purification by 25 to 60% (v/v) acetone precipitation increased specific activity to an average value of 0.263 units/mg protein, resulting in a purification factor of about 9.4. Further purification by hydroxylapatite increased the average specific activity of the enzyme to 1.482 units/mg protein and yielded a purification factor of approximately 53.0. With tryptophan, phenylalanine or tyrosine as amino acid substrate, the hydroxylapatite column elution profiles were very similar, indicating no apparent fractionation of transaminase activity by this procedure. Further enzyme purification was attempted by using gel filtration chromatography on Bio-Gel P-150 and P-300 columns but without success. Often, an apparent decrease in purification was observed due to the relative instability of the enzyme. Ammonium sulfate precipitation was also attempted, but the time necessary for dialysis was a limiting factor and only slight activity was recovered.

Since the acetone precipitate purification step gave a high specific activity with good recovery, it was selected as the preparation to be used for all further studies. That preparation is relatively stable for at least 2 months when stored in liquid nitrogen.

Properties of the Enzyme Using Tryptophan and α-Ketoglutarate as Substrates. The pH optimum for this transaminase reaction was 8.5, with an abrupt decline at higher pH values (Fig. 1). The yield of indolepyruvate was directly proportional to enzyme concentration up to 0.75 ml (9.9 mg of protein) of enzyme per reaction mixture (18). Five-tenths milliliter of enzyme extract was chosen as the amount of enzyme extract to be used routinely in further experiments. The apparent velocity of the transaminase reaction became constant after a lag period of approximately 12 min (Fig. 2). The velocity of the reaction then remained constant beyond the 60-min incubation time used in these experiments. An unequivocal explanation for the apparent lag period cannot be presented; perhaps it can be attributed to the relative insensitivity of the assay procedure at low levels of activity. Using the preceding conditions for the transaminase reaction, it was observed that increasing amounts of D,L-tryptophan up to 40 μmoles per reaction mixture increased the amount of indolepyruvate formed (18). A saturating amount of α-ketoglutarate was approximately 20 μmoles per reaction mixture (18).

Conditions Affecting Enzyme Activity. The necessity of each ingredient of the routine reaction mixture and factors affecting product yield are shown in Table I. Evidence presented later will show that it was not the enzyme activity but the chemical stability of the indolepyruvate that was increased under anaerobic conditions.

The essentiality of pyridoxal phosphate could not be conclusively demonstrated except by the use of competing inhibitors such as semicarbazide and hydroxylamine (Table II). The coenzyme obviously was tightly bound to the apoenzyme, since significant enzyme activity was observed even without the addition of exogenous pyridoxal phosphate to the reaction mixture.
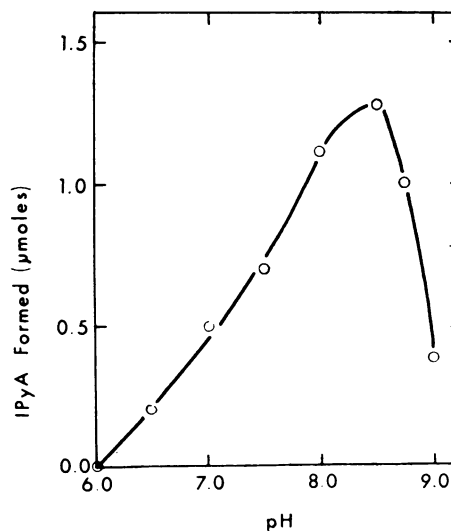

FIG. 1. Effect of pH on enzyme activity. Reaction mixtures each contained 1.0 μmole of pyridoxal phosphate, 40 μmoles of D,L-tryptophan, 10 μmoles of α-ketoglutarate, and 0.5 ml of enzyme extract (5.3 mg of protein) in a total of 3 ml of 0.5 M borate buffer. Each reaction mixture was adjusted to the appropriate pH prior to the addition of α-ketoglutarate. The pH was also measured after the reaction was complete to insure that no significant pH change had occurred. IPyA: indolepyruvate.
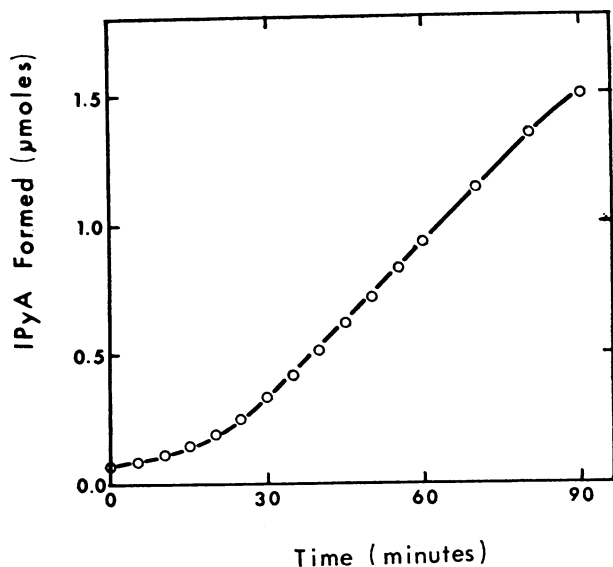
FIG. 2. Time course of the transaminase reaction. Each complete reaction mixture contained 0.5 ml of enzyme extract (5.8 mg of protein), 1.0 μmole of pyridoxal phosphate, 40 μmoles of D,L-tryptophan, and 20 μmoles of α-ketoglutarate in a total volume of 3.0 ml. Reactions were run in quartz cuvettes and incubated at 30 C. Absorbance measurements were taken every 5 min for the first 60 min, then every 10 min thereafter. IPyA: indolepyruvate.

Table I. *Requirements Using D,L-Tryptophan and α-Ketoglutarate as Co-substrates*

Complete reaction mixtures each contained 0.5 ml of enzyme extract (4.6 mg of protein), 40 μmoles of D,L-tryptophan, 20 μmoles of α-ketoglutarate, and 0.1 μmole of pyridoxal phosphate. All reactions were run in 0.5 M borate buffer at pH 8.5. Incubations were for 60 min at 30 C.

Treatment	Indolepyruvate Formed
	μmoles
Complete	0.99
Complete (anaerobic)	1.52
Complete (boiled enzyme)	0.09
Complete (zero time)	0.05
Minus enzyme	0.12
Minus pyridoxal phosphate	0.46
Minus D,L-tryptophan	0.15
Minus α-ketoglutarate	0.00

Substrate Specificity. The transaminase from pea utilizes more than one keto acid as substrate (Table III). This could indicate either the presence of more than one enzyme or the lack of specificity of one transaminase towards various keto acid substrates. The enzyme preparation also utilizes various aromatic and aliphatic amino acids as substrates (Fig. 3 and Table IV).

Stoichiometry of Indolepyruvate and Glutamate Formation. By cochromatographing authentic glutamate with reaction extracts in three different solvent systems and comparing the R_F values obtained and colors produced by ninhydrin reaction with the glutamate spots, the presence of glutamate in the reaction mixtures was shown. To show that indolepyruvate was being formed via transamination and not by some other process, it was necessary to show that equivalent amounts of indolepyruvate and glutamate were being formed. Equivalent amounts of indolepyruvate and glutamate, 1.07 and 1.06

μmoles respectively, were indeed formed under anerobic conditions. Similar amounts of glutamate were formed under both aerobic and anaerobic conditions (1.12 and 1.06 μmoles, respectively), but the yield of indolepyruvate was reduced to 0.62 μmole under aerobic conditions. These data support

Table II. *Effects of Pyridoxal Phosphate and Inhibitors on Activity*

Reaction mixtures each contained 0.5 ml of enzyme extract (3.7 mg of protein), 40 μmoles of D,L-tryptophan, 20 μmoles of α-ketoglutarate, and 0.1 μmole of pyridoxal phosphate, except where noted. All reactions were run in 0.5 M borate buffer at pH 8.5. Incubations were for 60 min at 30 C.

Treatment	Indolepyruvate Formed
	μmoles
Complete	0.94
Minus pyridoxal phosphate	0.46
Plus 5 μmoles semicarbazide	0.47
Plus 10 μmoles semicarbazide	0.32
Plus 20 μmoles semicarbazide	0.28
Plus 5 μmoles hydroxylamine	0.31
Plus 10 μmoles hydroxylamine	0.26
Plus 50 μmoles hydroxylamine	0.26
Minus pyridoxal phosphate plus 20 μmoles semicarbazide	0.24
Minus pyridoxal phosphate plus 50 μmoles hydroxylamine	0.11

Table III. *Comparative Activity with Selected Keto Acids*

The reaction mixtures each contained 0.5 ml of extract (2.8 mg of protein), 0.1 μmole of pyridoxal phosphate, 40 μmoles of D,L-tryptophan, and 20 μmoles of the appropriate keto acid. Reaction time was 60 min.

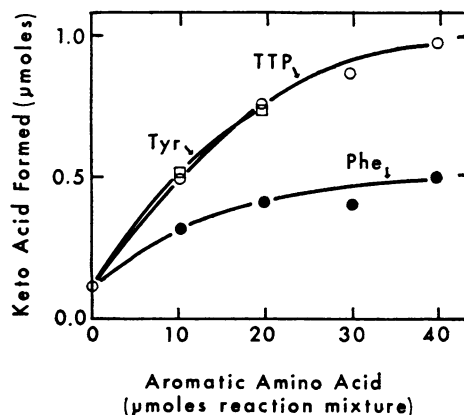| Keto Acid Substrate	Amino Acid Product	Indolepyruvate Formed
		μmoles
α-Ketoglutarate	Glutamate	0.72
Pyruvate	Alanine	1.18
Oxalacetate	Aspartate	1.08
Glyoxylate	Glycine	0.35

Aromatic Amino Acid
(μmoles reaction mixture)

FIG. 3. Comparative activity of selected aromatic amino acids with transaminase from pea. Reaction mixtures contained 0.5 ml of enzyme extract (7.2 mg of protein), 0.1 μmole of pyridoxal phosphate, 20 μmoles of α-ketoglutarate, and various concentrations of either D,L-tyrosine, D,L-phenylalanine, or D,L-tryptophan.

Table IV. *Comparative Activity with D,L-Tryptophan and Selected Aliphatic Amino Acids*

The analysis of glutamate was by TLC and ninhydrin reagent. Reaction mixtures each contained 0.5 ml of enzyme extract (3.3 mg of protein), 0.1 μmole of pyridoxal phosphate, 20 μmoles of α-ketoglutarate, and 40 μmoles of the appropriate amino acid.

Amino Acid Substrate	Glutamate Formed
	μmoles
D,L-Tryptophan	1.14
D,L-Leucine	0.89
D,L-Methionine	1.16
D,L-Alanine	3.75

Table V. *Comparative Activity with D- and L-Tryptophan*

Reaction mixtures each contained 0.5 ml of enzyme extract (4.5 mg of protein), 0.1 μmole of pyridoxal phosphate, 20 μmoles of α-ketoglutarate, and the indicated amounts of specific isomers of tryptophan.

Substrate	Indolepyruvate Formed
	μmoles
D-Tryptophan, 20 μmoles	0.70
L-Tryptophan, 20 μmoles	0.22
D,L-Tryptophan, 40 μmoles	0.87
D,L-Tryptophan, 20 μmoles	0.62

the idea that the increase in indolepyruvate observed under anaerobic conditions (Table I) was due to increased stability of indolepyruvate and not to increased enzyme activity.

Enzyme Activity with D- or L-Tryptophan. The pea enzyme preparation was more active with D-tryptophan as substrate than with L-tryptophan. Over a 3-fold increase in indolepyruvate yield was observed when 20 μmoles of D-tryptophan was used rather than 20 μmoles of L-tryptophan as substrate (Table V). Also, the combined indolepyruvate yield from 20 μmoles of D-tryptophan and 20 μmoles of L-tryptophan was nearly identical to the yield from a racemic mixture of 40 μmoles of D,L-tryptophan. Finally, nearly equivalent yields of indolepyruvate were observed when either 20 μmoles of D-tryptophan or 20 μmoles of a racemic mixture of D,L-tryptophan were utilized as substrate, suggesting the possible enzymic conversion of some L- to D-tryptophan via a racemase.

DISCUSSION

Approximately 53-fold purification of a transaminase from pea (*Pisum sativum* L.) was achieved by acetone precipitation followed by column chromatography on hydroxylapatite, but fractionation of activity analogous to that reported for rat brain (4, 6, 26) was not achieved. Hence, for convenience, the acetone-precipitated fraction was utilized routinely in other investigations. Several properties of the enzyme from pea are closely similar to transaminases from other plant sources.

With α-ketoglutarate as co-substrate, the enzyme fraction transaminated the following aromatic amino acids: D,L-tryptophan, D,L-tyrosine, and D,L-phenylalanine, as well as the following aliphatic amino acids: D,L-alanine, D,L-methionine, and D,L-leucine. Similar broad spectrum amino acid specificity has been reported for partially purified transaminases from mung bean (*Phaseolus aureus* Roxb.) (7, 8) and *Clostridium sporogenes* (23). Neither is the specificity of the pea transaminase for keto acid substrate very strict. Of the keto acids tested,

pyruvate and oxalacetate were more active than α-ketoglutarate, with D,L-tryptophan as amino acid. Gamborg and Wetter (8) found that pyruvate was superior to α-ketoglutarate as amino acceptor in mung bean, whereas glyoxylate and oxalacetate were relatively inactive as amino acceptors. The pH optimum for the pea transaminase was 8.5, which agrees closely with the value of 8.4 reported for a tryptophan transaminase from *Clostridium sporogenes* (23), 8.2 for glyoxylate transaminase from wheat leaves (12), 8.0 for aspartate aminotransferase from pea cotyledons (30), and 8.0 to 8.5 for glutamate-aspartate transaminase from wheat germ (1).

The essentiality of pyridoxal phosphate for the enzyme was difficult to demonstrate, as has often been found for plant transaminases (1, 2, 8, 30). Approximately a 50% decrease in activity was observed when pyridoxal phosphate was omitted from the reaction mixtures. By adding semicarbazide or hydroxylamine to the reaction mixture, further reduction in pea transaminase activity was observed. According to Fowden (5), these inhibitors bind with aldehydes and inhibit reactions known to be dependent on pyridoxal phosphate.

Particular attention was given to the formation of indolepyruvate from tryptophan via transamination because of the implication of that reaction in the biosynthesis of IAA in peas (20–22). The formation of equimolar yields of indolepyruvate and glutamate from tryptophan and α-ketoglutarate was readily demonstrable under anaerobic conditions, which prevented loss of indolepyruvate. Since no reduction in yield of indolepyruvate was observed in the absence of oxygen, oxidative deamination (10) apparently was not contributing to the production of indolepyruvate. Gordon and Paleg (11) reported that indolepyruvate and IAA also could be formed through the action of polyphenols on tryptophan in mung bean. Direct investigation revealed that neither was this reaction operative in the enzyme extracts prepared from peas.

The most unexpected result of these investigations was the fact that the pea transaminase was approximately three times as active with D-tryptophan as amino acid substrate as with L-tryptophan. There are other reported cases where D-tryptophan can be utilized by plant enzymes. Gordon (10) and others have noted that D-tryptophan is equal to or more effective than L-tryptophan as a precursor of IAA in several plant preparations. In systems in which tryptophan mimics the effects of IAA and is presumably converted to the auxin, D- and L-tryptophan both have been found to be equally effective. Examples are the retardation of abscission of debladed *Coleus* petioles (27) and the growth requirement for an excised wheat root culture (3). Additionally, the conversion of D-tryptophan to malonyl-D-tryptophan has been repeatedly observed in plant tissues (9, 19, 31).

Miura and Mills (19) recently described a tryptophan racemase in cell cultures of tobacco (*Nicotiana tabacum* L.) which converts, by a readily reversible reaction, D-tryptophan to L-tryptophan. Since the pool size of free tryptophan in pea seedlings is very small (14), it would appear that the synthesis and utilization of tryptophan is subject to rigid control. Perhaps conversion of L-tryptophan to D-tryptophan is a means of ensuring a pool of tryptophan for particular pathways including, but not restricted to, auxin biosynthesis. Whether, in fact, this process is operative instead of, or in addition to, a direct transamination of L-tryptophan to indolepyruvate currently is unknown.

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