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Purification and Characterization of Aspartate Aminotransferase Isoenzymes from Rice Bran

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Isoenzymes of aspartate aminotransferase (EC 2.6.1.1, AAT) were purified to homogeneity and crystallized from bran of rice (*Oryza sativa* cv. Koganemasari). The native molecular weights of AAT-1 and AAT-2 isoenzymes were 88,000 and 94,000 with the subunit molecular weights of 44,000 and 47,000, respectively, indicating that the holoenzymes of the isoencymes are dimers. The isoelectric points of AAT-1 and AAT-2 were pH 6.5 and 5.0, respectively: both isoenzymes have no subform. The isoenzymes showed similar K_ms for four natural substrates, with the exception that AAT-1 had a higher affinity for L-glutamate than AAT-2. Amino donor and acceptor specificities of the isoenzymes were almost identical and fairly high. Amino acid compositions of the isoenzymes were similar but not the same. The isoenzymes contained one mole of pyridoxal 5′-phosphate (PLP) per subunit and showed characteristic absorption spectra of PLP enzymes. Polyclonal antibodies raised against AAT-1 selectively cross-reacted with AAT-1 but not with AAT-2. Conversely, the antibody raised against AAT-2 selectively cross-reacted with AAT-2 but not with AAT-1.

Aspartate aminotransferase (AAT, EC 2.6.1.1) catalyzes the reversible transfer of an amino group from aspartate to 2-oxoglutarate to form oxalacetate and glutamate. AAT plays a key role in the metabolism of amino acids and Krebs-cycle-related organic acids in organisms. In plants, AAT is involved in transferring CO_2 from the mesophyll to the bundle sheath cells in some C_4 plants[1] and reducing equivalents between the cytoplasm and organelles *via* a malate-aspartate shuttle,[2] and in assimilation of ammonia produced by the endosymbiotic rhizobia.[3] Plants contain several isoenzymes of AAT. Two forms of AAT have been identified in alfalfa[4] and *Eleusine coracana*[5] and purified partially or homogeneously. Amino acid sequences of AAT isoenzymes from *Panicum miliaceum*,[6] alfalfa,[7] and lupin root nodules[8] were deduced from cDNAs. The amino acid sequences of plant AATs have substantial homology to those of animal and bacterial AATs and a characteristic pyridoxal 5′-phosphate attachment site motif. The amino acid sequences predicted from cDNAs for mitochondrial AAT showed that mitochondrial AAT(s) are synthesized as precursor proteins that have a 28-amino-acid presequence.[6]

In spite of recent progress in studies of plant AATs, detailed comparative studies on enzymatic properties of the isoenzymes of plant AAT from a single plant source with homogeneously purified preparations have not been done because of difficulty in the purification of the isoenzymes of plant AAT in a large enough amount to characterize them.

AAT isoenzymes of rice plant also have not been purified to homogeneity or characterized by their enzymatic properties. Kanamori and Matsumoto[9] detected two isoenzymes in the roots of rice plant seedlings and 3 isoenzymes in the shoots and showed that one of the putative mitochondrial isoenzymes was increased by the supply of ammonia to the cultivation medium. However, the exact number and distribution of the AAT isoenzymes in rice plant cells still remains to be elucidated. We have found that rice bran is a good source for preparation of rice AAT isoenzymes. Thus, the objectives of these studies were to: (a) purify the isoenzymes of rice AAT from rice bran, (b) compare some physical and kinetic characteristics of the isoenzymes, and (c) produce antisera to the AAT isoenzymes.

Materials and Methods

Plant materials. Commercially available rice (*Oryza sativa* [L] cv. Koganemasari) bran freshly prepared from the grains of the rice was used. The tissues of rice plants were obtained from the plants grown for 20 days on our farm.

Reagents. L-Cysteine sulfinate and 2-oxo acids were purchased from Sigma Chemical Co., St. Louis, U.S.A.; methoxyphenazine methosulfate and nitroblue tetrazolium from Dojindo Lab., Kumamoto; NADH, pig heart mitochondrial malate dehydrogenase, and beef liver glutamate dehydrogenase from Oriental Yeast, Osaka; and Bio-Lyte 3/10 from Bio-Rad Lab., Tokyo.

Enzyme extraction and purification. All extraction and purification procedures were done at 0–5°C, unless otherwise stated. Tissue (5 grams) of rice plant was cut into small pieces and then suspended in 10 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM 2-oxoglutarate, 0.01 mM pyridoxal 5′-phosphate, and 0.01% 2-mercaptoethanol. The suspension was homogenized with a Polytron homogenizer. The homogenate was centrifuged at $10,000 \times g$ for 10 min. The supernatant solution was used as the crude extract of rice tissue.

Rice grain was extracted (5 g/10 ml buffer) with 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM 2-oxoglutarate, 0.01 mM pyridoxal 5′-phosphate, and 0.01% 2-mercaptoethanol using sea sand and a mortar and pestle. Rice bran (5 g) was defatted with 50 ml of cold (−20°C)

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Abbreviations: AAT, aspartate aminotransferase, EC 2.6.1.1; MDH, malate dehydrogenase, EC 1.1.1.37.

petroleum ether and dried. The dried bran was extracted with the buffer as described above.

For purification of isoenzymes from rice bran, the rice bran (1 kg) was mixed with 3 liters of cold (−20°C) petroleum ether. The mixture was filtered and washed with 1 liter of cold petroleum ether. The defatted rice bran was dried overnight at room temperature. To the dried rice bran was added 3 liters of Buffer A (10 mM potassium phosphate buffer, pH 7.0, containing 1 mM 2-oxoglutarate, 2 mM potassium hydroxide, 0.01 mM pyridoxal 5′-phosphate, and 0.01% 2-mercaptoethanol). The mixture was incubated at 37°C for 30 min under vigorous agitation, and then an extract was pressed through double-layered gauze. The extract was centrifuged at 10,000 × *g* for 10 min. The precipitate obtained was re-extracted with 3 liters of Buffer A. To the supernatant solution (5.5 liters) obtained by two extractions was added 972 g of ammonium sulfate (30% saturation). After being mixed and left for 1 h, the mixture was centrifuged at 10,000 × *g* for 10 min. Ammonium sulfate (720 g, 50% saturation) was added to the supernatant solution (5.7 liters). After standing overnight, the mixture was centrifuged at 10,000 × *g* for 10 min. The precipitated protein was dissolved in a minute amount of Buffer B (10 mM Tris–HCl, pH 8.0, containing 1 mM 2-oxoglutarate, 2 mM KOH, 0.01 mM pyridoxal 5′-phosphate, and 0.01% 2-mercaptoethanol), and then thoroughly dialyzed against Buffer B. The enzyme solution (600 ml) obtained after centrifugation of the dialysate was put on a DEAE-cellulose (DE-52, Whatman) column (3.5 × 30 cm) equilibrated with Buffer B. After the column was thoroughly washed with Buffer B until absorbance at 280 nm of the eluted buffer became lower than 0.1, the elution was done with a linear gradient of NaCl (0–0.3 M), in a total volume of 4 liters: one form of AAT isoenzymes (AAT-1) was eluted with the buffer containing 0.1 M NaCl and the other form (AAT-2) with the buffer containing 0.2 M NaCl. The AAT-1 fraction was concentrated 10-fold by ammonium sulfate precipitation (70% saturation). About one-tenth of the concentrated solution, which contained about 200 mg of protein, was chromatographed by FPLC on Mono Q (HR 10/10) equilibrated with Buffer B. The FPLC was repeated ten times. The AAT-1 activity fractions pooled were concentrated to 70 ml by ultrafiltration with an Amicon DH2 concentrator with a H1-type Diaflo hollow fiber cartridge. AAT-2 fraction was also concentrated 10-fold by the ammonium sulfate precipitation, and then was chromatographed by FPLC on Mono Q. The AAT-2 activity fraction eluted was concentrated to 50 ml by ultrafiltration.

The concentrated AAT-1 fraction was precipitated by ammonium sulfate, and the precipitate obtained was dissolved in a minute amount of 0.1 M potassium phosphate buffer (pH 6.0). To the enzyme solution was added 5 mM (final concentration) of L-cysteine sulfinate, and the enzyme solution was incubated at 30°C for 10 min. An equivolume of 1.0 M potassium phosphate buffer (pH 6.0) was added to the mixture. Then, the mixture was precipitated by ammonium sulfate (70% saturation). The precipitate was dissolved in 10 ml of 10 mM Tris–HCl (pH 7.5). The enzyme solution was dialyzed thoroughly against 20 mM Tris–acetate buffer (pH 7.0) and then chromatographed on a pyridoxamine affinity column (1.2 × 10 cm) as described previously.[10] The AAT-1 fraction eluted was dialyzed against 2 liters of Buffer A, and then concentrated to 0.5 ml with a Cetriflo CF25. To the concentrated AAT-1 solution was added polyethylene glycol 6000 (8%, w/v, final concentration). After this was left at 4°C for 6 days, the crystalline preparation of AAT-1 was obtained.

The concentrated AAT-2 fraction was precipitated by ammonium sulfate, and the precipitate was dissolved in 5 ml of 0.1 M potassium phosphate buffer (pH 6.0). After an addition of 5 mM L-cysteine sulfinate and incubation at 30°C for 10 min, the enzyme solution was precipitated by ammonium sulfate. The precipitate obtained was dissolved in 0.5 M potassium phosphate buffer (pH 5.5) containing 0.3 M sodium malonate. The enzyme solution was precipitated by ammonium sulfate. The precipitate obtained was dissolved in a minute amount of 2 mM of sodium malonate (pH 5.5) and dialyzed against 2 liters of the same buffer followed by 2 liters of 20 mM Tris–acetate buffer (pH 7.0). The dialysate was affinity-chromatographed as described above. The AAT-2 fraction eluted was thoroughly dialyzed against Buffer A, and then concentrated to 0.5 ml. The concentrated solution was dialyzed against 2 liters of distilled water at 4°C for 3 days. Crystalline AAT-2 was precipitated on the bottom of the dialysis bag.

AAT activity and protein measurement. AAT activity was routinely assayed by the coupled malate dehydrogenase method.[11] One unit of enzyme was defined as the amount that catalyzed the formation of 1 μmol of oxalacetate from L-aspartate per min. Amino donor specificity was measured by the method described previously using glutamate dehydrogenase.[11] Amino acceptor specificity, with L-glutamate or L-aspartate as the amino donor substrate, was measured by the method described previously.[12]

Protein was measured by the method of Lowry *et al.*[13] with bovine serum albumin as the standard. Protein concentrations of the homogeneous AATs were measured by the method of Kalckar.[14]

Electrophoresis and activity staining. PAGE using a modified Davis system (native-PAGE) and staining of AAT activity on the gel were done as described previously.[11] SDS–PAGE was done by the method of Laemmli.[15]

Molecular weight measurement. The molecular weights of native AATs were measured by exclusion–diffusion chromatography on a column (1.6 × 115 cm) of Ultrogel AcA34 as described previously.[11] The molecular weights of subunits were measured by the SDS–PAGE using standard proteins.[11]

Antiserum production. Antisera against isoenzymes of rice AAT were produced and prepared by the method described previously.[16] Japanese white rabbits were initially immunized by a subcutaneous injection of a total of 120 μg of the purified isoenzyme in Freund's complete adjuvant. Four booster injections of 200 μg of purified isoenzyme in incomplete adjuvant were given at monthly intervals.

Immunoprecipitation test of AAT activity. Serological properties of AAT-1 and AAT-2 was analyzed by an Ouchterlony double diffusion test as described previously.[11]

Other procedures. Polyacrylamide gel isoelectric focusing was done by the method of Wrigley[17] using Bio-Lyte 3/10. Michaelis constants and maximum reaction velocities were measured by the method of Velick and Vavra.[18] Amino acid analysis was done by the method of Moor and Stein[19] with a Hitachi 835 amino acid analyzer. Tryptophan was measured spectrophotometrically.[20]

Results

AAT specific activity and number of AAT activity band

Rice bran showed higher AAT specific activity than either leaves, stems, or roots, whether expressed on a protein or fresh weight basis (Table I). The cultivar Mineyutaka had the highest specific activity among the cultivars tested. The specific activity of the rice grain was higher than the tissues of rice plant, suggesting AAT is one of the storage proteins in rice seeds.

Native-PAGE of equal amounts of AAT activity from crude extracts from leaves, roots, bran, and grains showed two activity bands, AAT-1 (slow moving) and AAT-2 (fast moving). The crude extract from stems showed an additional

Table I. Rice Aspartate Aminotransferase Activity in Leaf, Stem, Root, Whole Grain, and Bran of cv. Koganemasari, and in Whole Grains of Other Cultivars

Source	AAT activity (units/g, wet material)	Specific activity (units/mg, protein)	AAT activity bands
Leaf	2.96[a]	0.12	2
Stem	1.88	0.24	3
Root	0.16	0.07	2
Bran	14.80	0.52	2
Grain			
Koganemasari	4.45	0.33	2
Koshihikari	3.15	0.44	2
Minaminishiki	3.40	0.45	2
Mineyutaka	4.35	0.68	2
Nihonbare	3.22	0.47	2
Nihonmasari	3.25	0.46	2
Minenishiki	4.07	0.54	2

[a] Each value represents the mean of three replicates.

Table II. Purification of Isoenzymes of Aspartate Aminotransferase from Rice Bran

Enzyme	Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Both	Crude extract	53,570	30,000	0.56	100
	Ammonium sulfate fractionation	12,700	28,000	2.2	93.9
	DEAE-cellulose				
	AAT-1	2,080	21,000	10.1	70.0
	AAT-2	3,580	8,600	2.4	29.0
AAT-1					
	FPLC (Mono Q)	202.0	9,780	48.3	32.6
	Affinity chromatography	7.5	2,780	380.0	9.0
	Crystallization	4.0	1,560	390.0	5.2
AAT-2					
	FPLC (Mono Q)	338.0	4,050	12.0	13.5
	Affinity chromatography	1.3	540	415.0	1.8
	Crystallization	1.2	540	450.0	1.8

faint AAT activity band between AAT-1 and AAT-2. The crude extract from stems and roots showed intense staining for both AAT-1 and AAT-2, but the bran and grains stained more intensely for AAT-1 and less so for AAT-2. Leaves stained more intensely for AAT-2 and less so for AAT-1. Zymograms of rice tissue coincided well with that reported by Kanamori and Matsumoto.[9]

Purification of AAT isoenzymes from rice bran

Since rice bran contained the highest amount of AAT activity, the isoenzymes of rice AAT were purified from rice bran (Table II). The petroleum ether extraction was essential to extract the AAT activity from rice bran. Fractionation with ammonium sulfate gave a 4-fold purification and removed a large amount of low molecular weight contaminants with high absorption at 280 nm. Anion exchange chromatography with DE-52 allowed the separation of the isoenzymes, AAT-1 and AAT-2 (Fig. 1). In the DEAE-cellulose column chromatography, the column was washed thoroughly to elute non-protein contaminants, which show high absorbance at 280 nm, before the gradient-elution was started. The elution patterns of FPLC on a Mono Q column are shown in Fig. 2. AAT-1 and AAT-2 activity fractions were eluted with the elution buffer containing 0.08 M and 0.25 M NaCl, respectively. The yields of the affinity chromatography step were low, but the step was essential to purify AAT isoencymes to near homogeneity: the difficulty in making apo forms of the AAT isoenzymes with limited denaturation caused such low yields. The crystallization step removed minor contaminants from the AAT isoencymes, which migrated just below AAT proteins on the SDS–PAGE.

The crystalline preparations showed a single band on the SDS–PAGE (Fig. 3, C). The crystals of AAT-1 and AAT-2 took the form of rods and rhombi, respectively (Fig. 3, A and B).

Molecular weight measurement

Molecular weights of native AAT-1 and AAT-2 were estimated by exclusion-diffusion chromatography on a column of Ultrogel AcA 34 (Fig. 4, A). Comparison of their elution volumes with those of standard proteins gave molecular weights of 88,000 and 94,000 for AAT-1 and AAT-2, respectively. By SDS–PAGE, the molecular weights of AAT-1 and AAT-2 were 44,000 and 47,000, respectively (Fig. 4, B).

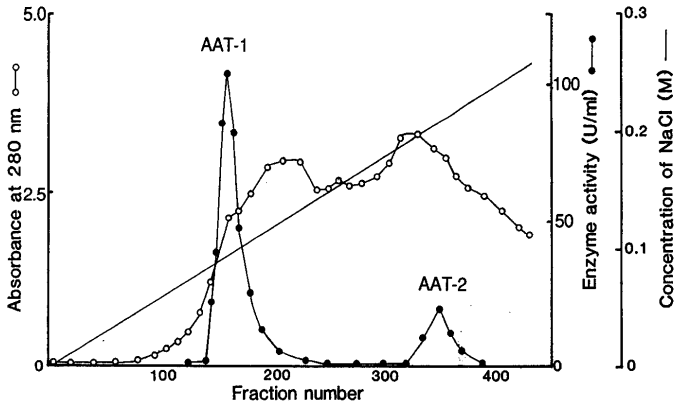

Fig. 1. Separation of the Isoenzymes of Rice Bran Aspartate Aminotransferase by DEAE-Cellulose Column Chromatography.
See the text for details.

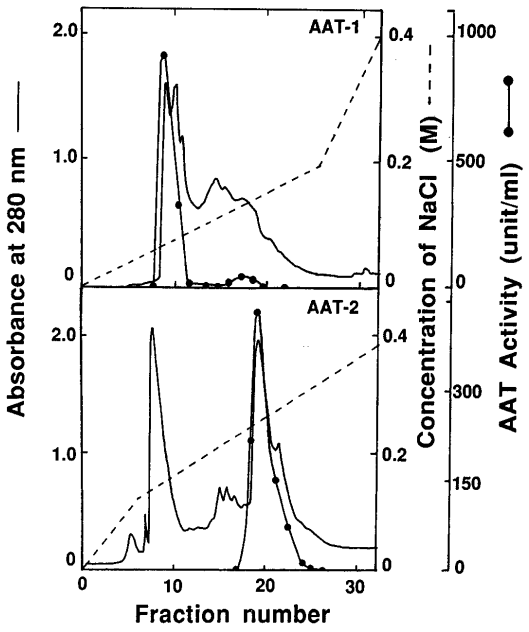

Fig. 2. Elution Patterns of Rice Bran AAT-1 and AAT-2 in FPLC on Mono Q (10/10).
See the text for details.

pH Optimum and substrate kinetics

The pH optima for AAT-1 and AAT-2 in forward and reverse reactions were identified (Fig. 5). In the forward reaction, in which L-aspartate and 2-oxoglutarate are substrates, pH optimum for both the isoenzymes was pH 7.0–8.0. At pH 6.5, AAT-2 activity was reduced by 20%, while AAT-1 was reduced by only 2%. In the reverse reaction, in which L-glutamate and oxalacetate are substrates, pH optima for AAT-1 and AAT-2 were pH 8.0–8.5 and pH 8.0–8.8, respectively.

The substrate kinetics for AAT-1 and AAT-2 were analyzed (Table III). Both forms of rice AAT isoenzymes showed high affinities for keto acid substrates. AAT-1 had a higher affinity for L-glutamate than AAT-2.

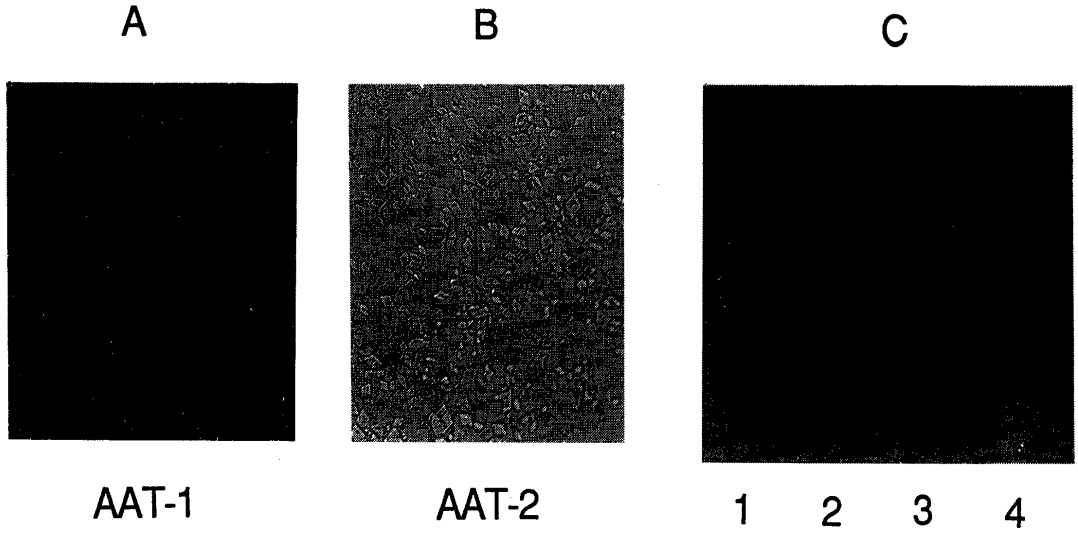

Fig. 3. Crystals of Rice Bran Isoenzymes of Aspartate Aminotransferase and SDS–PAGE Patterns of the Isoenzymes.

Lanes 1 and 4, 2, and 3 in the SDS–PAGE shows electrophoretic patterns of standard protein mixtures, AAT-1, and AAT-2, respectively. Standard proteins used were: phosphorylase (M_r 97,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soy bean trypsin inhibitor (21,500), and lysozyme (14,400).

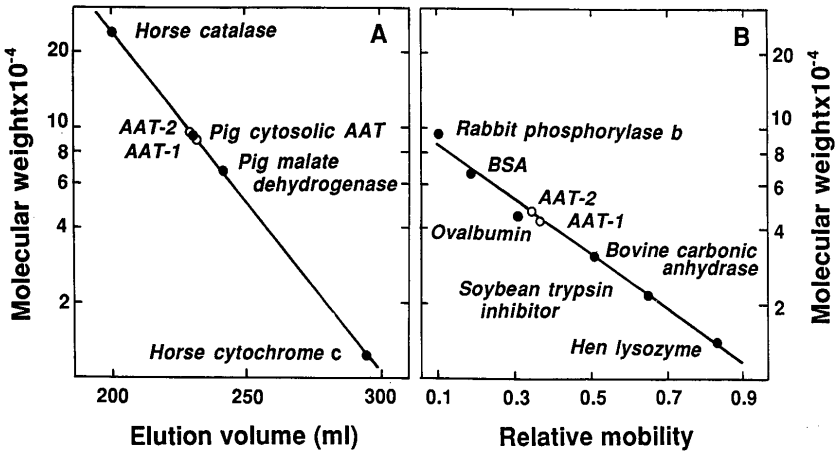

Fig. 4. Molecular Weight of Rice Bran AAT-1 and AAT-2.

A, gel filtration of native enzymes and standard proteins; B, comparative electric mobilities of denatured enzymes and standard proteins. See the text for details.

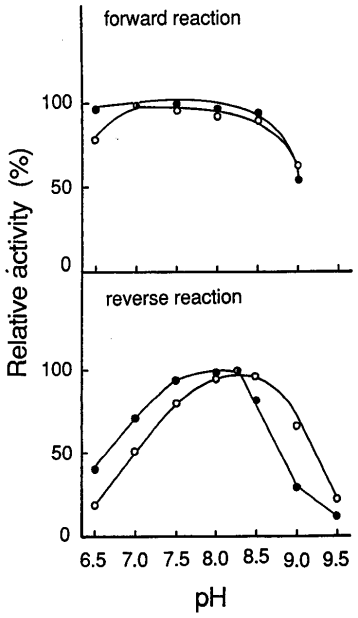

Fig. 5. Effects of pH on the Activity of Homogeneous AAT-1 and AAT-2 Isoenzymes of Rice Bran.

The buffer used was 100 mM Tris–HCl. Final concentrations of L-aspartate and 2-oxoglutarate in the forward reaction were 50 mM and 5 mM, respectively. The reaction mixture of the reverse reaction contained 50 mM L-glutamate and 5 mM oxalacetate as the substrates. The maximum activity at the optimum pH was shown as 100%. ●—● AAT-1, ○—○ AAT-2.

Table III. Kinetic Values for Rice Bran AAT-1 and AAT-2

Isoenzyme	K_m			
	L-Aspartate	2-Oxoglutarate	L-Glutamate	Oxalacetate
	mM			
AAT-1	2.40	0.112	5.00	0.043
AAT-2	3.70	0.105	13.20	0.048

Substrate specificity

The amino donor specificities of rice AAT isoenzymes were identified with 2-oxoglutarate as an amino acceptor (Table IV). L-Aspartate and L-cysteine sulfinate served as good substrates: AAT-1 had higher L-cysteine sulfinate transamination activity than AAT-2. The transamination of other amino acids, including aromatic amino acid, was also catalyzed at a very low but measurable rate. AAT-1 showed higher reactivity toward L-glutamate than AAT-2.

The amino acceptor specificities, with L-aspartate and L-glutamate as amino donors, of the rice AAT isoenzymes are shown in Table V. Both isoenzymes showed high reactivities toward 2-oxoglutarate and oxalacetate. Other 2-oxo acids, especially aromatic amino acid-related ones,

Table IV. Amino Donor Specificities of AAT Isoenzymes of Rice Bran

Amino donor	Conc. (mM)	Relative activity (%)	
		AAT-1	AAT-2
L-Aspartate	50	100	100
L-Glutamate	50	162	127
L-Cysteine sulfinate	50	128	63
L-Phenylalanine	40	0.21	0.32
L-Leucine	50	0.03	0.04
L-Valine	50	0.03	0.00
L-Methionine	50	0.00	0.00
L-Alanine	50	0.00	0.00
L-Ornithine	50	0.00	0.00
L-Lysine	50	0.00	0.00

Table V. Amino Acceptor Specificities of AAT Isoenzymes of Rice Bran

Amino acceptor	Relative activity (%)			
	AAT-1		AAT-2	
	L-Asp	L-Glu	L-Asp	L-Glu
2-Oxoglutarate	100	—	100	—
Oxalacetate	—	100	—	100
p-Hydroxyphenylpyruvate	0.32	0.14	0.53	0.54
Phenylpyruvate	0.07	0.11	0.31	0.14
Pyruvate	0.01	0.01	0.01	0.01
2-Oxomethionine	0.00	0.00	0.01	0.00
2-Oxoisocaproate	0.00	0.00	0.00	0.00
2-Oxobutyrate	0.00	0.00	0.00	0.00

The concentrations of the amino acceptor and donor (L-aspartate or L-glutamate) were 5 and 50 mM, respectively.

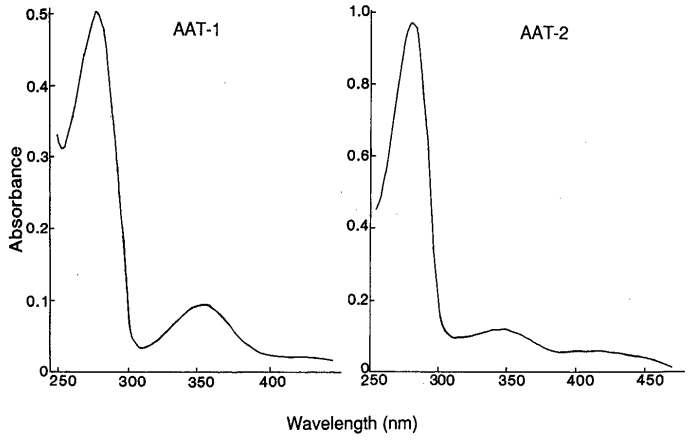

Fig. 6. Absorption Spectra of AAT-1 and AAT-2 Isoenzymes of Rice Bran.

AAT-1 (0.5 mg/ml) and AAT-2 (1.0 mg/ml) isoenzymes were dissolved in 10 mM potassium phosphate buffer (pH 7.0) containing 0.01 mM pyridoxal 5′-phosphate, 1 mM 2-oxoglutarate, and 2 mM KOH.

acted as amino acceptors, the rates being low but measurable. AAT-2 showed higher reactivity toward aromatic amino acid-related 2-oxo acid than AAT-1. AAT-2 showed reactivity toward 2-oxomethionine.

Absorption spectra

The absorption spectra, measured at pH 7.0, of the AAT-1 and AAT-2 are shown in Fig. 6. 2-Oxoglutarate was added to the enzyme solutions to stabilize the AAT isoenzymes, especially AAT-2. AAT-1 had absorption maxima at 280, 355, and 425 nm, showing the absorption of a typical Schiff base formed between the enzyme protein and pyridoxal 5′-phosphate. AAT-2 had absorption maxima at 280, 350, and 420 nm, also showing the presence of a Schiff base.

The contents of pyridoxal 5′-phosphate of AAT-1 and AAT-2 were 0.87 and 0.93 mol, respectively, per mole of subunit by the fluorometric method involving cyanide.[11]

Table VI. Amino Acid Compositions of AAT Isoenzymes of Rice Bran

Amino acid	Residues[a]	
	AAT-1 (M_r 44,000)	AAT-2 (M_r 47,000)
Aspartic acid	32.6	41.9
Threonine	20.4	15.6
Serine	27.8	27.2
Glutamic acid	36.6	42.1
Proline	22.6	23.0
Glycine	34.9	32.3
Alanine	39.6	40.0
Valine	27.4	28.8
Cysteine	4.1	4.0
Methionine	13.4	11.1
Isoleucine	20.4	20.5
Leucine	40.2	38.2
Tyrosine	14.2	15.3
Phenylalanine	15.4	19.1
Lysine	15.9	27.0
Histidine	9.0	6.7
Arginine	23.8	18.7
Tryptophan	4.0	8.0

[a] The average or extrapolated (threonine and serine) values for 24, 48, and 72 h hydrolysis.

Isoelectric point

The isoelectric points of AAT-1 and AAT-2 by the polyacrylamide gel isoelectric focusing were pH 6.5 and 5.0, respectively. The result coincided with the mobility of the isoenzymes in native PAGE and with the elution profile of DEAE-cellulose column chromatography described above.

Amino acid compositions

The amino acid compositions of AAT-1 and AAT-2 are shown in Table VI. The two AAT isoenzymes had homologous but different amino acids compositions. AAT-2 contained higher amounts of lysine and tryptophan residues than AAT-1.

Serological properties

Rabbit antisera against the rice AAT isoenzymes were prepared. In an Ouchterlony double diffusion test, the antiserum against AAT-1 formed a precipitin band with AAT-1 but not with AAT-2. Conversely, the antiserum against AAT-2 formed a precipitin band with AAT-2 but not with AAT-1. Both the antisera formed no precipitin band with AATs from *E. coli* B, and cytosol and mitochondria of *Rhodotorula minuta* and pig heart.

The antisera against the AATs from *E. coli* B,[16] bakers' yeast,[21] and *R. minuta*,[11] which were prepared as described

previously, formed a precipitin band with neither of the rice AAT isoenzymes.

Discussion

Two isoenzymes of rice AAT were crystallized for the first time from a single plant source. Primary structures of plant AAT isoenzymes have been deduced by cloning and sequence analysis of cDNA encoding these isoenzymes,[6-8] and these works showed that functionally and/or structurally important residues are conserved in plant AAT isoenzymes as well as AATs of animals and microorganisms. The results showed that physiological and kinetic properties of plant AAT isoenzymes resemble those of AATs of other organisms. However, the content of pyridoxal 5′-phosphate, and the absorption spectrum showing the presence of the coenzyme has not been shown in plant AATs: Numazawa *et al.*[22] only described the presence of an absorption maximum at 340 nm in the AAT-1 of *Panicum maximum* leaves. Our study showed that the two isoenzymes of rice bran AAT have absorption maximum of pyridoxal 5′-phosphate around 350 and 425 nm in the buffer containing 2-oxoglutarate as a stabilizing reagent, and the isoenzymes contained one pyridoxal 5′-phosphate per subunit.

Two or more isoenzymes of AAT have been found in various plants. Zymograms that depend on the activity-staining on the polyacrylamide gels showed even more AAT activity bands.[23,24] Carrot (a C_3 plant) suspension culture cells contain three isoenzymes of AAT with different molecular weights.[25] *Eleusine coracana* (a C_4 plant) also contained three isoenzymes of AAT.[5] *Planicum maximum* (a C_4 plant)[22] and alfalfa (a C_3 plant) root nodules[4] contain two isoenzymes of AAT. Rice bran, root, and leaf contained two isoenzymes. Rice stems showed another activity band. However, this activity was very low compared to that of AAT-1 and AAT-2, and was not detected in the crude extract of top tissues of rice plant seedlings (data not shown). Thus, the third AAT activity seemed to be inducible and tissue-specific. Since several aminotransferases catalyze the transamination of L-aspartate as a side reaction, it is possible that the third AAT activity may be catalyzed by a transaminase other than AAT. Purification and characterization of the component enzyme of the third AAT activity bands are required to identify it.

AAT isoenzymes from a single plant, so far studied, showed two types of relative activities toward oxalacetate. In one type such as AATs of lupin[26] and pea,[27] either one of the AAT isoenzymes shows a higher affinity for oxalacetate than the other AAT isoenzymes. In the other type such as AATs of seedling oat leaves,[28] alfalfa,[4] and *E. coracana*,[5] both of the AAT isoenzymes have almost the same affinity for oxalacetate. AAT isoenzymes of rice bran are classified as the latter type. Few studies have been done on substrate specificities of plant AAT isoenzymes with highly purified preparations. It has been shown that two AAT isoenzymes of bush bean shoots have 0.68 and 3.2% of the reactivity toward L-phenylalanine with 100% toward L-aspartate.[29] The reactivity of rice AAT isoenzymes toward L-phenylalanine was much lower than that of bush bean shoots isoenzymes, and similar to that of pig mitochondrial isoenzyme of AAT.

The amino acid compositional relatedness of both the isoenzymes of rice bran to animal cytosolic and mitochondrial isoenzymes of AAT was estimated by the compositional difference index ($S\Delta n$) defined by Cornish-Bowden.[30] $S\Delta n$ values calculated for rice AAT-1 *vs.* AAT-2, rice AAT-1 *vs.* pig mitochondrial AAT, rice AAT-1 *vs.* pig cytosolic AAT, rice AAT-1 *vs.* chicken mitochondrial AAT, and rice AAT-1 *vs.* chicken cytosolic AAT were 169, 213, 205, 175, and 143, respectively. $S\Delta n$ values calculated for rice AAT-2 *vs.* pig mitochondrial AAT, rice AAT-2 *vs.* pig cytosolic AAT, rice AAT-2 *vs.* chicken mitochondrial AAT, and rice AAT-2 *vs* chicken cytosolic AAT were 102, 177, 112, and 108, respectively. The results showed that rice AAT-2 isoenzyme has higher similarity to pig mitochondrial AAT. Preliminary protein sequence analysis showed that rice AAT-1 isoenzyme has the VNLG (33–36 from N-terminal) sequence that is specific for pig cytosolic AAT, and that rice AAT-2 isoenzyme has the Y(V)DP (161–164 from N-terminal) sequence that is specific for mitochondrial isoenzyme of pig AAT (data not shown). The results suggested that rice AAT-1 is the cytosolic isoenzyme and AAT-2 the mitochondrial one. Further studies, including an immunotitration of theAAT isoenzymes in the organelles are required to locate the AAT isoenzymes in the rice cells.

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