Note

A Simple Colorimetric Assay for Aspartate Aminotransferase

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Various colorimetric end point assays for aspartate aminotransferase (AAT, EC 2.6.1.1) have been devised, since the content of the enzyme in serum has important clinical diagnostic value.[1] The phenylhydrazine methods of Reitman-Frankel[2] or Tonhazy[3] are commonly used. These colorimetric assays, however, have some disadvantages in simplicity and reliability compared with rate assays[4]; the defects result mainly from the difficulty in preventing interference from the keto acid added as an amino acceptor substrate. Recently, we have found that AAT activity on polyacrylamide gels can be determined by following the increase in SO_3^{2-} produced from CSA, toward which AAT show high reactivity.[5] This method could sensitively assay mitochondrial and cytosolic AATs after electrophoretic separation, since the transamination reaction between CSA and 2-oxoglutarate proceeds completely.[6,7] Thus, we further attempted to devise a simple and sensitive colorimetric assay for AAT in aqueous solutions based on the same principle, and found that SO_3^{2-} forms a yellow product from INT in an alcoholic reaction mixture without an intermediate electron carrier. We report here a simple and sensitive colorimetric assay for AAT in aqueous samples including human serum.

CSA was purchased from Sigma, MDH from Boehringer-Mannheim, INT, NBT from Dojin Laboratories, Kumamoto, Japan, 2-oxoglutarate from Nakarai Chemicals, Kyoto, and ethanol (99.5%, v/v, special grade) from Wako Pure Chemical Industries, Osaka. All other reagents were analytical reagent grade. Homogeneous pig and rat AATs were gifts from Drs. M. Tanase and Y. Morino, Kumamoto University Medical School and Dr. H. Kagamiyama, Osaka Medical School, respectively. Bacterial AAT was purified as described previously.[8]

The new assay was performed at 30°C for 10 min in a reaction mixture consisted of 10 μmol of Tris-HCl (pH 8.5), 10 μmol of CSA, 5 μmol of 2-oxoglutarate, and enzyme in a final volume of 0.1 ml. The pH of the CSA and 2-oxoglutarate solutions was made 7.0 with NaOH prior to their addition to the assay mixture. The reaction was started by the addition of CSA. The blank experiment was performed with a reaction mixture in which 2-oxoglutarate was replaced by water. The reaction was stopped by the addition of 1.9 ml of ethanol. After this had been left for 5 min, 1.0 ml of 0.2 M Tris-HCl buffer (pH 8.5) and 0.1 ml of color reagent solution were added successively. The solution was prepared as follows: 90 ml of ethanol was added to 1 g of INT dissolved in 10 ml of benzyl alcohol. After the reaction mixture was incubated at 37°C for 60 min in the dark, its absorbance at 410 nm was determined. One unit of enzyme was defined as the amount of enzyme that catalyzed an increase of 1.0 μmol of SO_3^{2-} per min. Assay for AAT by the coupled MDH method was carried out as described previously[8] with the exception that the reaction was performed at 30°C. Bisulfite was standardized from its UV absorbance.[9]

Sodium bisulfite gave a yellow color with absorption maxima near 350 nm and 410 nm in the visible region when incubated with INT and NBT in an alcoholic solution, respectively (Fig. 1). The product from INT showed a higher net absorption than that from NBT at the wavelength of zero blank absorption. Thus, the SO_3^{2-} concentration was determined with INT by measuring absorbance at 410 nm. A linear relationship was obtained between SO_3^{2-} concentration and the increase in absorbance at 410 nm (Fig. 2, (A)). The absorbance increased linearly up to 1.5. The absorbance at 410 nm developed into a maximum in 1 hr and remained constant at least for another 1 hr (Fig. 2, (B)). It was preferable to determine the absorbance immediately after the maximum development, since a faint pink color developed in the blank sample, especially under light.

Highly purified ethanol should be used throughout the experiment to obtain reproducible development of the yellow color: ethanol stored in a can strongly inhibited the color developtment.

AAT activity could be determined in the reaction mixture containing CSA and 2-oxoglutarate by following the production of SO_3^{2-} under the conditions described above. The absorbance at 410 nm increased linearly and proportionally with reaction times (Fig. 3, (B)) and the enzyme concentrations in the reaction mixture (Fig. 3, (A)), respectively. The absorbance increased linearly in the range of 0～1.4. The concentration of 2-oxoglutarate in the reaction mixture needed to be sufficiently high to keep linearity in high absorbance range: the reaction curve was linear only in the absorbance range of 0～0.6 when the reaction mixture contained 1 μmol of 2-oxoglutarate.

This assay was applied to determine the activity of homogeneous AATs from various sources and AAT activity in human sera (Table I). The same samples were also

Abbreviations: AAT, aspartate aminotransferase; CSA, L-cysteine sulfinate; INT, *p*-iodonitrotetrazolium violet; MDH, malate dehydrogenase; NBT, nitroblue tetrazolium; PMS, phenazine methosulfate.

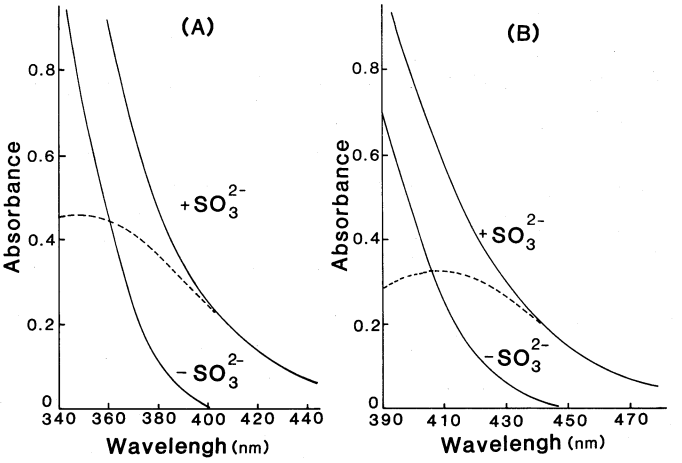

FIG. 1. Absorption Change of Tetrazolium Salts Caused by Incubation with Sodium Bisulfite.

Ethanol (1.9 ml), Tris-HCl buffer (1.0 ml), and 0.1 ml of 10 mM INT (A) or NBT (B) solution dissolved in ethanol were added to a mixture which contained Tris-HCl (pH 8.5) (10 μmol), CSA (10 μmol), 2-oxoglutarate (5 μmol) and sodium bisulfite (0.15 μmol) in 0.1 ml. The absorption spectra were measured with a Union Giken SM-401 spectrophotometer, after the solution was incubated at 37°C for 1 hr. Blank experiments were performed with the reaction mixture without sodium bisulfite. Dashed lines indicate the difference spectra.

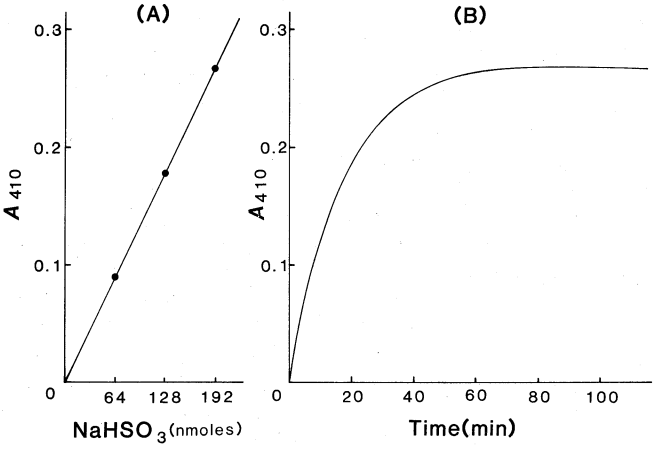

FIG. 2. Relationships between Absorbance at 410 nm and the Amount of Sodium Bisulfite and the Incubation Times.

(A): Various amount of sodium bisulfite was added to the reaction mixture, and the mixture was incubated with INT as described in Fig. 1.

(B): Increase in absorbance at 410 nm was followed in a cuvette maintained at 37°C with a Shimadzu UV-210 A spectrophotometer following the addition of color reagent solution to the reaction mixture containing 192 nmol of sodium bisulfite. The absorbance was corrected by subtracting the base line absorbance.

assayed by the coupled MDH method. Mitochondrial AATs from pig and rat and bacterial AAT showed a higher value in the present assay than that in the coupled MDH method. On the other hand, cytosolic AATs from both animals showed very low values in the present assay: only about 20% of the value that was obtained in the coupled MDH method. This assay could be applied for the determination of AAT activity in human sera, though the reaction time had to be extended up to 1 hr.

Tetrazolium salts (INT and NBT) formed yellow products when they were incubated with SO_3^{2-}. On the other hand, these tetrazolium salts produced red formazans

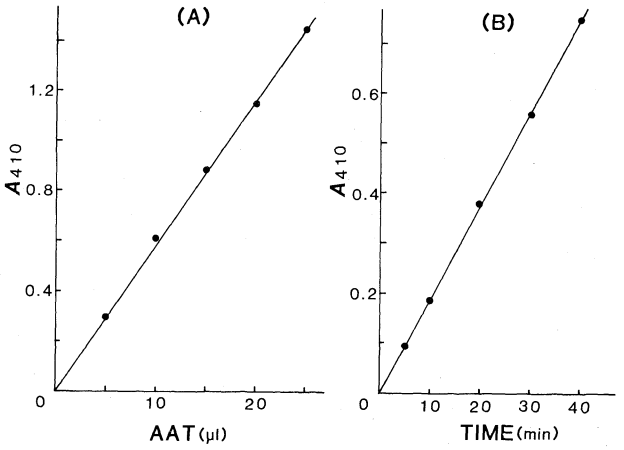

FIG. 3. Linear Relationships between Absorbance at 410 nm and AAT Concentrations and Reaction Times.
(A) The enzyme reaction was performed for 10 min with various amount of *Escherichia* AAT (4.35 U/ml).
(B) The reaction was performed in 1 ml of the reaction mixture, and aliquots of 0.1 ml were removed at various intervals and mixed with 1.9 ml of ethanol.

TABLE I. COMPARISON OF ASSAY METHODS WITH VARIOUS ASPARTATE AMINOTRANSFERASES[a]

AAT	MDH[b]	CSA[b]	CSA/MDH
Pig mitochondrial AAT	0.365	0.656	1.78
Pig cytosolic AAT	0.560	0.130	0.23
Rat mitochondrial AAT	0.413	1.090	2.64
Rat cytosolic AAT	0.482	0.094	0.20
Escherichia AAT	0.638	1.387	2.17
Human serum[c]	0.013	0.006	0.46
	0.008	0.006	0.75

[a] Results are expressed as units per milliliter of each sample.
[b] MDH and CSA indicate the coupled MDH method and the present method, respectively.
[c] The enzyme reaction was performed for 1 hr with 30 μl of serum from a healthy human. After ethanol addition, the precipitate produced was centrifuged off (3,000 rpm, 10 min). To the supernatant filtered through a Millex-GS filter unit was added the color reagent solution.

when they were incubated with NADH and PMS under the same condition (data not shown). It has been shown that homogeneous tetrazolium salts including monotetrazolium salts like INT produce two or several formazans with different colors depending on interconversion of the geometrical isomers of the formazan.[10] Thus, it is suggested that SO_3^{2-} and tetrazolium salts produce formazans with other structures than formed from NADH, PMS, and tetrazolium salts, though the structure of these products is unknown.

Comparison of activities determined by the coupled MDH method with those obtained by the present method showed a difference of characteristic AATs from various sources. Cytosolic AATs are markedly different from mitochondrial and bacterial AATs in this respect. The result that mitochondrial AATs show higher activity value in the present method is compatible with the fact that mitochondrial AAT utilizes CSA as a better substrate than L-aspartate.[5] The low value of cytosolic AAT in the present assay may result from the fact that 2-oxoglutarate added to the reaction mixture in excess is highly inhibitory to the action of cytosolic AAT.[11]

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