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ON THE DETERMINATION OF ISOZYME LEVELS IN PREPARATIONS CONTAINING CYTOPLASMIC AND MITOCHONDRIAL ASPARTATE AMINOTRANSFERASE

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Summary

A spectrophotometric assay has been developed for the determination of the content of each isozyme of aspartate transaminase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) in physiological fluids or tissue extracts. The methods relies on the ability of adipate, at low pH and ionic strength to inhibit the cytoplasmic isozyme but not the one from mitochondria. Two assays are necessary, one at pH 8.0 which measures the content of both isozymes and another at low pH which measures primarily the amount of mitochondrial isozyme. Results obtained by this simple procedure match those in which each isozyme is inhibited by its antibody. The validity of the results obtained by the new method was tested at different ratios of cytoplasmic:mitochondrial isozyme and with tissue extracts. Since the amounts of each isozyme determined by radial immunodiffusion match those values gathered by following enzymatic activity, it is concluded that the quantity of each isozyme obtained from its respective catalytic activity must represent the total protein content of each isozyme in a given sample.

Introduction

Mammalian glutamate aspartate transaminase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) exists in two electrophoretically distinct forms [1], the cationic isozyme associated with mitochondria and the anionic associated with cytoplasm [2,3]. Measurements of this transaminase activity in blood is a widely used discriminant of myocardial infarction and its activity level in

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physiological fluids has been used in the diagnosis of several other ailments including several liver and skeletal muscle diseases and hemolytic anemia [4].

Methods in use in the determination of aspartate transaminase in standard clinical practice rely on the measurement of enzyme activity of the sample and detect the total (both isozymes) transaminase concentrations [4]. The increasing discriminatory power of determination of each isozyme's level, as an aid to clinical diagnosis and research, is best illustrated by the successes with lactate dehydrogenase and creatine phosphokinase isozymes [4—9]. In aspartate transaminase, on the other hand, due to insufficient clinical data, it is not known how helpful it would be to measure the level of each transaminase in physiological fluids. Yet, limited studies which rely on the tedious, and costly, physical separation of the isozymes have indicated that testing for each isozyme's level would be of diagnostic value and of assistance as a research tool [10—13]. Previous work showed the discriminating inhibitory effect of anions, various dicarboxylic acids and pH levels on the two isozymes of aspartate transaminase [1,14,15]. Adipic acid was the best inhibitor of the cytoplasmic enzyme, but was found to exert little effect on the activity of the mitochondrial enzyme [14]. Lower pH also inhibits the cytoplasmic enzyme preferentially [1,14], while high anion concentration affected the mitochondrial isozyme [15] most severely.

In this work we show that it is possible to select conditions in which the supernatant isozyme is mostly inhibited while the mitochondrial isozyme retains most of its activity. Under these conditions it is possible to determine the composition of a mixture of the two enzymes.

Materials and Methods

Chemicals. Adipic acid was Baker's reagent grade; Tris, NAHD, malate dehydrogenase, L-aspartic acid and α-ketoglutaric acid were obtained from Sigma and cacodylic acid from Fisher.

Spectrophotometric studies. Absorption studies were carried out using a Gilford 2000 recording spectrophotometer and a Cary Model 15 recording spectrophotometer with standard 1-cm light path cuvettes. All studies were done at 25°C.

Preparation of enzyme. The mitochondrial and supernatant isozymes of aspartate transaminase were isolated from pig hearts as previously described [16,17]. Enzyme concentration was measured spectrophotometrically at 280 nm using a molecular weight value of 94 000 [18].

Enzyme activity. Aspartate transaminase catalyses the following reaction:

aspartate + α-ketoglutarate ⇌ glutamate + oxaloacetate

Enzyme activity was measured by coupling oxaloacetate production with NADH and malate dehydrogenase in which the disappearance of NADH can be measured spectrophotometrically by a decrease in absorption of 340 nm. Total assay volume was 3 ml and containing 50 mM Tris/cacodylate buffer, 8 mM α-ketoglutarate, 30 mM aspartate, excess malate dehydrogenase and $3 \cdot 10^{-4}$ g NADH. Adipate, when present was 50 mM, and the pH values of the assay solutions were adjusted to pH 8.0 and 6.2, as required. All experimental results are

the average of at least 6 determinations.

Preparation of anti-aspartate transaminase antibodies. Equal volumes of Freund's complete adjuvant and antigen solution (in saline) were mixed, emulsified and injected into the footpads of white female New Zealand rabbits. At the first injection each animal received approx. 3 mg protein and 3 weeks later received an additional 1-mg booster in incomplete Freund's adjuvant. After a few weeks the animals were bled by heart puncture. The freshly drawn blood was allowed to stand at room temperature for several hours and then in the refrigerator, to aid clot formation. After centrifuging $1000 \times g$ for 30 min the serum was decanted, the centrifugation repeated and the serum frozen until needed.

Tissue extracts. Each tissue was homogenized in twice its volume of potassium phosphate buffer, pH 6.5, using a Virtis blender. Total volume of homogenate was 200 ml and it contained $5 \cdot 10^{-6}$ M α-ketoglutarate, $2 \cdot 10^{-8}$ M pyridoxal-5'-phosphate, 0.2% Triton X-100 and antifoam. The tissue was homogenized for 2 min, and heated to 55°C for 5 min in a water bath. After centrifuging for 30 min at $15\,000 \times g$ the supernatant was collected and used as the source of enzymes.

Radial immunodiffusion. Agar gels were prepared using 1% Nobel agar (Difco) in 0.01 M TES (*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid), 0.5% NaCl, 0.01% NaN_3, pH 7.3. The agar was dissolved in buffer by heating in a water bath and was then cooled to 60°C before adding antibody. 15 ml of agar-antibody mixture was pipetted into standard petri dishes. Optional amounts of antibody were predetermined by trials of serial dilution of the antibody and comparison of ring sizes produced. The gel was allowed to solidify and holes were punched in it using a thin-walled hollow metal cylinder of outside diameter 3 mm. Gentle suction with a pasteur pipet removed the gel from the well, 5 μl of antigen (2 mg/ml) could then be pipeted into each well using a micropipet. The petri dishes were covered and incubated at room temperature in a humid atmosphere and observed after several hours (days). Plates were fixed with 7% acetic acid and ring diameter measured using a caliper.

Results and Discussion

Activity of the isozymes

Beef liver cytoplasmic and mitochondrial aspartate transaminase possess identical specific activity [19]. We find that in pig heart both isozymes, when assayed at pH 8.0, also have the same specific activity which is fortunate as the number units of enzymatic activity reflect a direct addition of the amount of each isozyme present in the sample. Varying the experimental conditions, however, one isozyme is mostly inactivated while the other remains essentially fully active. Using the Tris/cacodylate buffer in which the cacodylate anion concentration is inucuous to the mitochondrial isozyme's activity, the presence of adipate and low pH increases the inhibition values of the supernatant's enzyme activity (Table I). On the other hand, under the same experimental conditions the mitochondrial enzyme retains over 90% of its activity.

Standard curves

Mixtures containing varying proportions of the two isozymes were made

TABLE I

SELECTIVE INHIBITION OF THE CYTOPLASMIC ISOZYME OF ASPARTATE TRANSAMINASE

Enzyme activity determined as indicated in the text and the extent of inhibition related to the maximum activity at pH 8.0. The + and — signs refer to the presence or absence of 50 mM adipate, respectively, neutralized to the pH to be studied.

pH	Isozyme			
	Cytoplasmic Inhibition (%)		Mitochondrial Inhibition (%)	
	+	—	+	—
6.2	92	68	7	0
8.0	58	0	18	0

from homogeneous mitochondrial and cytoplasmic aspartate transaminases and the activity of the mixtures in the presence or absence of adipate was measured at pH 8.0 and 6.2. The percent of inhibition was determined for each mixture at constant total value of enzyme units and a theoretical curve drawn with which the experimental results were compared. Excellent correlation between theoretical and experimental values were observed for the complete range of mixtures. The separate activity values of the isozymes were not additive when the two proteins were mixed. This was probably due to the binding of the two isozymes together. Previous work [20] had shown that the mitochondrial enzyme can be bound to α_2-macroglobulin. Subsequent work was, therefore, carried out in the presence of plasma which eliminated the apparent isozyme-isozyme interaction and made the activities additive.

Antibodies directed against each isozyme inhibit the catalytic activity of the respective isozyme and there is no cross-reactivity between the antibodies against one protein and the other [19,21].

In order to test the validity of the assay method a more conventional approach, that of selective inhibition of each enzyme by its antibody, was also used. Therefore, another standard curve was drawn, this time using anti-cytoplasmic isozyme antibodies, instead of adipate, to inhibit that isozyme. Mixtures of the isozymes were made as before and rabbit anti-cytoplasmic serum added to each tube. Each mixture was assayed at pH 8.0 and produced a linear relationship of inhibition dependent on the amount of serum added.

A series of unknown mixtures could then be assayed at pH 8.0 and 6.2 and the percent inhibition calculated in each case. By comparison with a standard curve the cytoplasmic:mitochondrial isozyme ratio was determined by either method (Table II).

With tissue extracts the similarity of results obtained by our new procedure and that of the inhibition of a given isozyme by its antibody is striking. A greater degree of accuracy could be obtained if a complete standard curve were run at the same time as that of the unknown samples.

In all cases the experiments were carried out with both isozymes and the results agree with those reported in Table II for one isozyme.

TABLE II

CORRELATION OF VALUES OBTAINED BY VARIOUS METHODS OF DETERMINING THE PROPORTION OF EACH ISOZYME OF ASPARTATE TRANSAMINASE IN TISSUE EXTRACTS

Tissue	Percentage * of cytoplasmic isozyme obtained by		
	New assay procedure reported in this publication	Inhibition by by anti-cytoplasmic antibody	Radial immuno-diffusion
Heart	55	52	50
Kidney	48	—	46
Liver	49	44	50
Skeletal muscle	47	48	—

* Error in these determinations is ±6%.

Radial immunodiffusion

Measurements of enzymatic activity in physiological fluids or tissue extracts can be misleading as a true representation of the total enzyme concentration; among other reasons, variations in the presence of regulatory metabolites or inhibitors or interaction with other biological components in the medium can effect the catalytic activity of a given enzyme. These reasons can further complicate the picture in isozyme determination if the above variations affect each isozyme in opposite or diverse fashion.

Detection of the total immunochemical reactivity of an enzyme with its antibody can in many ways circumvent most of the problems outlined above. To quantitate antigen-antibody complex formation we chose radial immunodiffusion because of its sensitivity, ease of implementation and possibilities for the handling of many samples at the time [22,24]. It is also a technique that can be performed simultaneously on the same samples where the determination of activity are being carried out.

Tissues were extracted as described in Materials and Methods but using a buffer : tissue ratio of 1 : 1. Known serial dilutions of purified mitochondrial and supernatant isozymes used as standards; pure enzyme internal standards and crude tissue extract were then applied to the wells. (Mitochondrial isozyme was applied to the gel containing anti-mitochondrial isozyme antibodies; cytoplasmic isozyme to that containing anti-cytoplasmic isozyme antibodies). After 3 days the plates were fixed, ring diameter measured, and a graph of $(\text{diameter})^2$ vs. concentration of antigen (enzyme units) [24] plotted (Fig. 1). The $(\text{diameter})^2$ of the precipitin ring formed by the tissue extract was compared directly to the standard curve and the percent of cytoplasmic and mitochondrial isozymes in each extract assessed. The results obtained (Table II) by this method are very similar to those found by the assay method or the antibody inhibition procedure.

The total concentration of a given isozyme or tissue extract, is however, a function of the age of the tissue after the death of the animal, extraction procedure and storage of the extracted material. Because of these reasons the values reported here as concentrations of isozymes for a given tissue may not be the absolute value to be present in those tissues. They do, however, represent

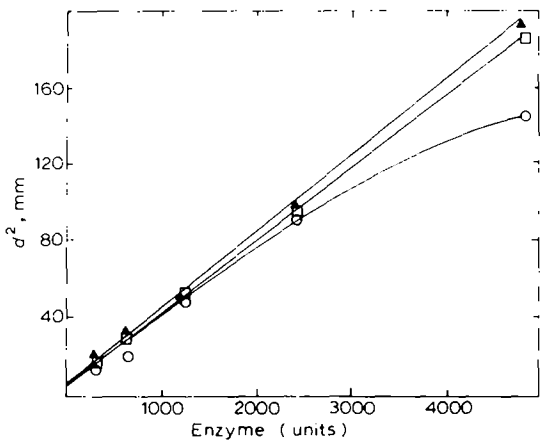

Fig. 1. Experimental results of several immunodiffusion plates are shown plotted as cytoplasmic isozyme concentration vs. the square of the precipitin ring diameter at different times of diffusion. Similar results were obtained in immunodiffusion studies of the mitochondrial isozyme against anti-mitochondrial serum. All unknowns are calculated by reference to similar graphs. Each unknown diffusion petri dish contained at least three internal standards of known concentrations of the isozyme under under study. Results shown were obtained after 24 h (○——○), 47 h (□——□) and 69 h (▲——▲) of diffusion.

the total amount of solubilized material obtained at any given time and are an example of the good correlation obtained by all methods. Kidney tissue in particular was cumbersome in this regard as some tissue samples contained 70 : 30 ratios of cytoplasmic to mitochondrial isozyme. Since these were commercially obtained kidneys, the cause of this variation could be due to any of the above reasons as well as to the age or diet of the animals.

Conclusion

The method reported here allows for the calculation of the concentration of each isozyme with results comparable to those obtained for serum or tissue extracts by more complex, costly, and time-consuming methods. The new spectrophotometric method can rely on two simple activity determinations and does not require the use of specialized equipment or reagents and it can therefore be adopted to any standard manual or automatic procedure for the determination of aspartate transaminase in clinical practice. This procedure, if incorporated into the routine testing of human samples, will help in ascertaining claims for the validity of aspartate isozyme determinations as an aid in clinical diagnosis.

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