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Short communication

Determination of quality of pyridoxal-5′-phosphate enzyme preparations by spectroscopic methods

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Abstract

The present study evaluates purified aspartate transaminase (AST, EC 2.6.1.1) preparations from three commercial sources. The enzyme molecule contains pyridoxal-5′-phosphate coenzyme (PLP), which provides AST characteristic absorption spectra in the wavelength range of 300–500 nm. The coenzyme bound in the active site also shows circular dichroism (CD) spectra in the same range. Besides, AST like other proteins may be modified in vitro or in vivo by reactions with other molecules, e.g. reactive sugars, and may form fluorescent products (advanced glycation end products, AGE). Spectroscopic methods were used to assess the quality of AST preparations from three different sources, Serva, Roche, and Sigma. Absorption spectra showed that the peak 360 nm characteristic of the active PLP form of AST prevailed in the Serva and Sigma preparations, while 330 nm was the major peak in the Roche preparation. CD spectra demonstrated the major maximum at 360 nm in the Serva and Roche samples, thus suggesting the predominance of the active PLP form in both preparations. The Sigma sample showed a CD profile less characteristic of AST. Fluorescence measurements revealed formation of AGE in the case of the Roche preparation, while fluorescence of the other two preparations was low. In general, the Serva sample presented the most convenient properties of purified AST among the preparations tested. The results will be used for the selection of a commercial enzyme preparation applicable in our future spectroscopic studies of glycation of AST as a model protein and in our research of the influence of antioxidants on this process.
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Keywords: Aspartate transaminase; Absorption spectra; Circular dichroism; Pyridoxal-5′-phosphate; Fluorescence; Protein purity

1. Introduction

Aspartate transaminase (AST, EC 2.6.1.1) is an enzyme frequently assayed in clinical laboratories. AST belongs to a group of enzymes characterized by the presence of pyridoxal-5′-phosphate (PLP) and its participation in catalysis. As for the protein part of the molecule, AST is composed of two identical, non-covalently bound subunits with more than 400 amino acid residues and possesses a molecular mass of about 93,000 Da, with some differences according to the animal species [1].

The PLP coenzyme is covalently bound to the ε-amino group of lysine 258 in an active site of aminotransferase [1–3]. It provides the AST molecule characteristic absorption spectra that differ from that of free PLP and which in general are the same regardless of species or cellular fraction (see Fig. 1 for an example).

The PLP form of AST shows, depending on pH, a major absorption peak at 360 nm (an active, unprotonized form of the coenzyme, prevailing at lower pH values [2]), and/or a peak at 430 nm (an inactive, protonized form, increasing at lower pH values). After a reaction with L-aspartate

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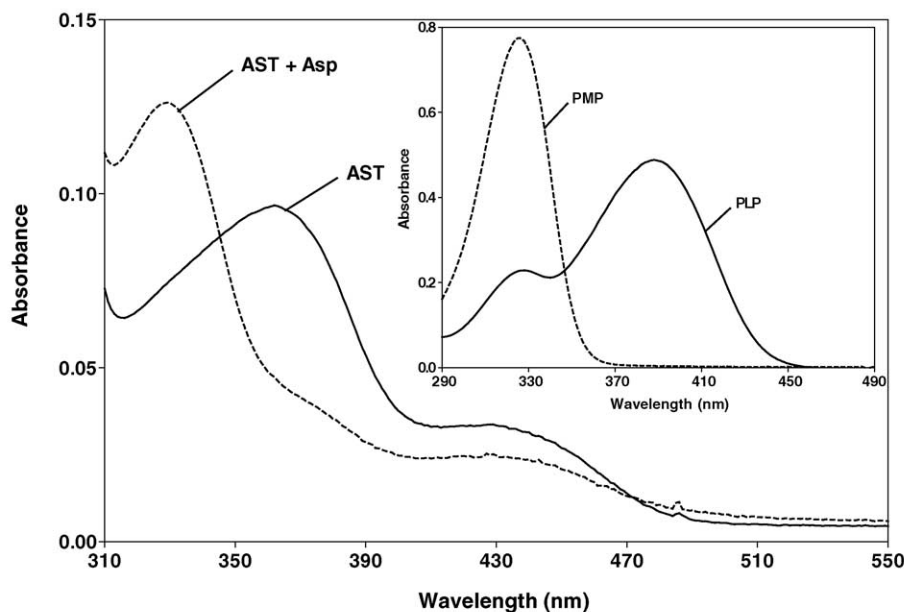

Fig. 1. Characteristic UV–vis absorption spectra of AST (Serva) and of the free coenzyme. Protein concentration 1.0 mg/ml of 0.1 M Na phosphate buffer pH 7.4), — PLP form (free AST); - - - PMP form (AST + Asp 1 mM). The insert shows the UV–vis spectra of free PLP (0.1 mM) and PMP (0.1 mM).

during the first part of a ping-pong transaminating reaction, the pyridoxamine-5′-phosphate (PMP) form of the coenzyme appears and the original absorption maxima shift to 325–330 nm.

While free PLP or PMP are not optically active substances, the coenzyme bound in the active site of AST shows circular dichroism (CD) spectra in the range of 300–500 nm, which are similar to absorption spectra. The CD effect is caused by the change in the electronic configuration of the molecule [4]. Circular dichroism clears away absorption characteristics of optically inactive components, which facilitates identification of the specific coenzyme signal and its changes, and also permits to uncover peaks of aromatic amino acids at 260–280 nm. Evaluation of intensity, characteristics of and changes in absorption and CD spectra facilitate, beside measurements of its catalytic activity, to assess the quality of enzyme preparations and help to understand the interactions of AST with other molecules.

Another property of proteins is their susceptibility to modify chemical reactions in vitro and in vivo. Such a typical reaction is the formation of advanced glycation end products (AGE), the process known in food chemistry for a century [5] and in medical biochemistry for decades [6]. AGE production accompanies diabetes mellitus and ageing and may indicate the history of interactions of individual protein molecules with sugars and other reactive compounds. Products of such protein modifications have more or less characteristic fluorescent properties. Fluorescence emission at 440 nm after excitation by light of 370 nm is considered to quantify total AGEs, while 335 nm excitation and 385 nm emission is partly specific for pentosidine as one of characteristic AGE products. The method is commonly used to evaluate haemoglobin glycation in the laboratory diagnosis and monitoring of diabetes.

We found out that the spectral as well as catalytical properties of AST make the enzyme a useful tool in drug–protein interaction [7] and in protein glycation studies [8]. It is advisable to have the enzyme as pure as possible for such research.

The enzyme is available in a relatively pure and stable form from several commercial sources and despite the fact that the commercial preparations are probably not intended for a special use in spectral studies, they appeared more or less convenient for our previous research [7]. The aim of the present study was to exploit the spectral properties and fluorescence of purified AST preparations from three sources for the evaluation and selection of preparations suitable for our future studies of protein glycation and the influence of antioxidants on this process.

2. Experimental

2.1. Chemicals

Cytosolic AST preparations from the porcine heart were obtained as suspensions in ammonium sulphate with properties partly declared:

(1) Serva Electrophoresis GmbH, Germany: Glutamate–oxaloacetate transaminase from the porcine heart in saturated ammonium sulphate, with 0.0025 M α-ketoglutarate, catalytic activity 291 U/mg;
(2) Roche Diagnostics GmbH, Germany: Glutamate–oxaloacetate transaminase (GOT) from the pig heart (catalytic activity not specified);

(3) Sigma–Aldrich GmbH, Steinheim, Germany, USA: Glutamate–oxaloacetate transaminase type I from the porcine heart. Suspension in 3.0 M $(NH_4)_2SO_4$ solution containing 0.05 M maleate and 2.5 mM α- ketoglutarate, pH 6.0, catalytic activity 284 U/mg.

Aspartic acid (p.a.) and sodium azide (p.a.) were obtained from Lachema, Brno, Czech Republic. Sodium phosphate buffer (0.1 M) pH 7.4 with 0.05% sodium azide (to prevent microbial contamination during experiments) was prepared from sodium hydrogen and dihydrogen phosphates and used for the preparation of all samples.

Protein contents of individual preparations and protein concentrations in samples prepared were checked by Bradford's method with Coomassie Brilliant Blue G-205 (CBBG) [9], [10] and calibrated by the bovine serum albumin (BSA) standard (Bio-Rad).

2.2. Sample preparation and incubation

All three suspensions of AST were centrifuged at 5000 rpm at 4 °C for 20 min. The supernatant was removed and enzyme pellets were reconstituted in an appropriate amount of 0.1 M sodium phosphate buffer pH 7.4 with 0.05% sodium azide in order to obtain solutions with protein concentration at 2.0 mg/ml for the preparation of final samples: AST 1 mg/ml in the buffer only (supposed PLP form of AST) and AST 1 mg/ml in buffer incubated 4 h with 1 mM L-aspartate (final concentrations). For some measurements, the samples prepared as above were dialyzed against sodium phosphate buffer and 0.05% sodium azide (three times for 1.5 h at 4 °C). All samples prepared were stored overnight in the dark at room temperature before the spectra were measured. In the case of dialysis, dialysis of selected samples was carried out against 200 ml of the standard buffer in a refrigerator (4 °C) three times for 90 min.

2.3. Absorption and circular dichroism spectra measurements

Absorption spectra were measured on a spectrophotometer HP 8453 in a 0.5 cm quartz cuvette against sodium phosphate buffer (pH 7.4; 0.1 M) with 0.05% sodium azide. Circular dichroism was measured on a dichrograph CD6 (Jobin Yvon, France, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague) in a 0.5 cm quartz cuvette against sodium phosphate buffer (pH 7.4; 0.1 M) with 0.05% sodium azide. The CD spectra were expressed as differential dichroic absorption $\Delta\varepsilon$.

2.4. Fluorescence of AST samples

The samples prepared by reconstitution of the enzyme solution from the precipitates were stored overnight at a dark room. Fluorescence was measured five times in separate samples (200 μl each) prepared from the same enzyme source on a luminescence spectrometer Perkin–Elmer LS 50B. Excitation and emission wavelengths: at 370/440 and 335/385 nm for total AGEs and pentosidine, respectively. The final results were expressed as relative fluorescence. Fluorescence of 200 μl of the BSA sample 1 mg/ml was taken as one arbitrary unit (AU).

3. Results and discussion

3.1. Absorption spectra of AST preparations

Fig. 2 shows the UV–vis absorption spectra of all three preparations. While the samples of Serva represent the typical absorption profile of present PLP coenzyme, and the Sigma profile is similar but flatter in the range of 310–470 nm, an absorption maximum about 330 nm prevails in the Roche preparation, reminding the PMP enzyme or free PMP.

Addition of L-aspartate to the Roche preparation caused a further increase in the maximum of 330 nm and disappearance of the shoulder at 360 nm, which confirmed formation of PMP form of the coenzyme from the original PLP form. On the other hand, the peak at 330 nm decreased after dialysis by one-third of its original height before dialysis, which demonstrates that at least a part of this peak was not caused by the firmly bound coenzyme.

3.2. Circular dichroism spectra of AST preparations

Fig. 3 represents characteristic CD spectra of the Serva preparation. Dialysis did not change the major positive peak, demonstrating thus the firmly bound PLP coenzyme. Addition of L-aspartate (1 mM) shifts this peak to 330 nm as a proof of transaminase activity.

An increase in CD signal at 260–290 nm suggests also a change in the aromatic amino acid region during the change in the active site. This change could not be observed on absorption spectra because absorption of aromatic amino acids of AST is drowned in the shoulder of the big peak caused by absorption of peptidic bonds of the protein.

Comparison of CD spectra of all three preparations (Fig. 4) demonstrates nearly identical properties of original PLP forms of the Serva and of Roche samples. This profile shows, above all, that the active PLP form prevails also in the Roche preparation.

Both preparations exhibit the same properties in the range of CD spectra of aromatic amino acids, too. The Sigma preparation of AST displays a remarkably lower positive peak of the active coenzyme at 360 nm, which agrees with the absorption profile of the sample in Fig. 2. An additional plateau between 440 and 510 nm is rather surprising and unrepresentative for CD spectra of relatively pure AST. The presence of 2-oxoglutarate in the preparation should, according to our experience [7], confirm the CD profile of PLP form of AST, an influence of maleic acid, declared in description of the preparation, should be demonstrated. The

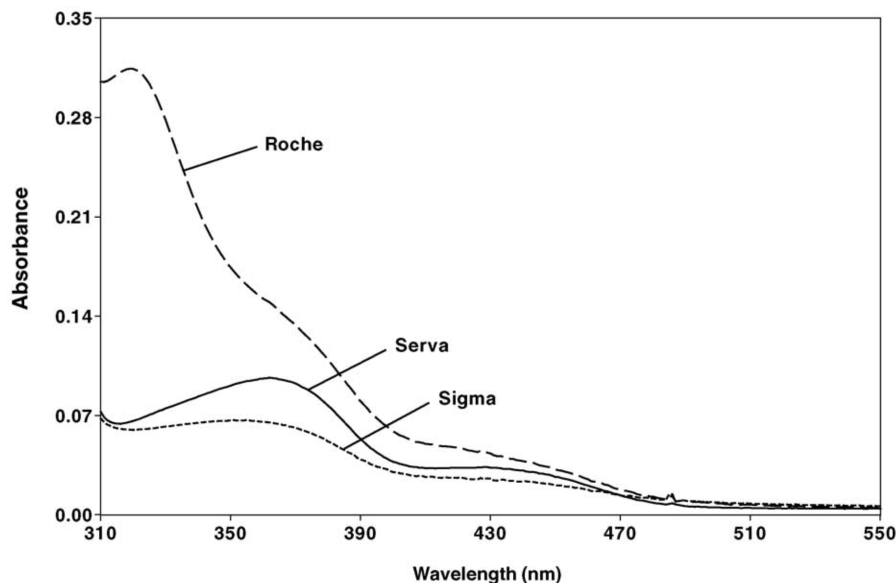

Fig. 2. UV–vis spectra of AST. Comparison of three preparations. Protein concentration 1.0 mg/ml of 0.1 M Na phosphate buffer pH 7.4. — Serva; – – Roche; - - - Sigma.

CD signal of aromatic amino acids is similar as for the profile but higher than with the Serva and Roche preparations.

3.3. Fluorescence

Table 1 summarizes the results of measurement of fluorescence of individual AST preparations.

While the fluorescence of the Sigma and Serva samples is similar to that of the standard BSA, the fluorescence of the Roche preparation is about twice of it. This suggests that the preparation contains molecules partly modified by reactive compounds like sugars. Dialysis had no remarkable influence on the fluorescence of the Serva and Sigma samples; the fluorescence of the preparation Roche partly decreased after dialysis, which suggests the presence of low-molecular fluorescence components beside the modified enzyme itself in the Roche preparation. We suppose that the cause of it might be either old animals as the source of the enzyme or a modifying step during processing of the product. The preparation will be convenient for measurements of catalytical activity but not for glycation studies.

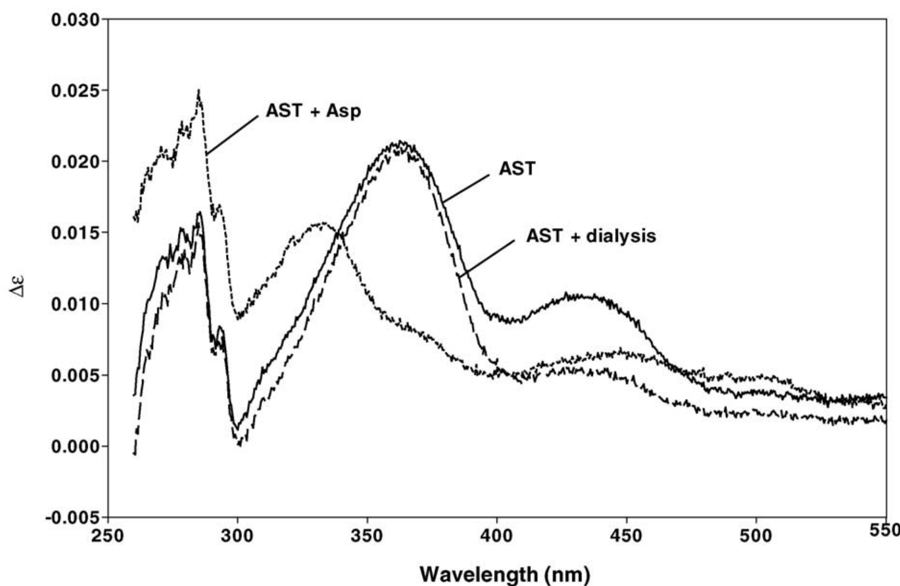

Fig. 3. Circular dichroism spectra of AST. (Serva Electrophoresis GmbH, Germany; protein concentration 1.0 mg/ml); protein concentration 1.0 mg/ml of 0.1 M Na phosphate buffer pH 7.4. — PLP form of AST; – – the same after dialysis; - - - PMP form (AST + Asp 1.0 mM).

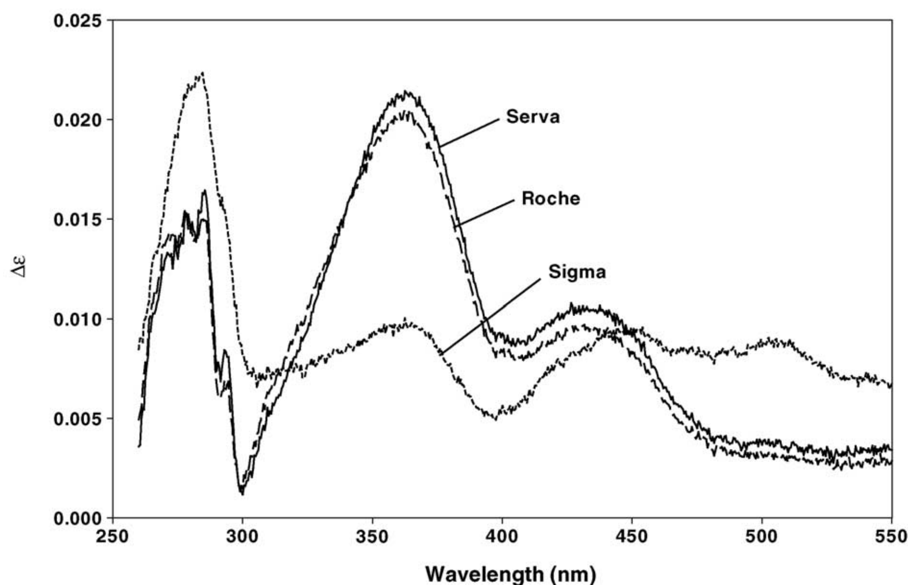

Fig. 4. Circular dichroism spectra of AST. Comparison of three preparations. Undialyzed samples. Protein concentration 1.0 mg/ml of 0.1 M Na phosphate buffer pH 7.4. — Serva; – – Roche; - - - Sigma.

Table 1
Fluorescence of three AST preparations

Preparation	Incubation sample[a]	Fluorescence (AU/mg)[b]	
		Total AGEs	Pentosidine
Serva	AST	0.83 ± 0.02	1.02 ± 0.13
	AST after dialysis	0.91 ± 0.01	1.13 ± 0.05
Sigma	AST	0.97 ± 0.05	1.16 ± 0.17
	AST after dialysis	0.94 ± 0.01	1.09 ± 0.05
Roche	AST	1.66 ± 0.03	1.81 ± 0.11
	AST after dialysis	1.12 ± 0.01	1.35 ± 0.10

[a] Each sample = 200 μl, protein concentration 1.0 mg/ml in 0.1 M Na phosphate buffer pH 7.4.
[b] Relative fluorescence of AST vs. BSA (BSA fluorescence = 1.0 AU). Each value = average ± S.D. of five separately processed and measured samples of the same preparation.

4. Conclusions

Among the AST preparations tested, Serva demonstrated the highest fidelity of absorption and CD spectra to the profiles described in the literature, and low fluorescence of the preparation. The preparation is suitable for application in any study of enzyme glycation or interactions with other molecules, e.g. drugs. The other two preparations differ more or less from the characteristic pattern, the Sigma sample having less characteristic CD spectra and the Roche preparation remarkable fluorescence.

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