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Inhibition of Aspartate Aminotransferase by Glycation *In Vitro* Under Various Conditions

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Incubation of 50 mM D-glucose with aspartate aminotransferase (AST, EC 2.6.1.1) preparations (purified pig heart enzyme or a rat liver 20,000 × g supernatant) at 25°C had no effect on enzyme activity. 50 mM D-fructose or D-ribose gradually inhibited pig heart AST under the same conditions to zero activity after 14 days. 50 mM DL-glyceraldehyde decreased enzyme activity to zero after 6 days of incubation. The inhibition of pig heart AST by 50 mM D-fructose or D-ribose was marked even at a temperature of 4°C but it was less pronounced than at 25°C. There was no effect of 0.5 mM 2-oxoglutarate on AST activity during incubation, while the presence of 25 mM L-aspartate decreased it rapidly. 0.5 mM 2-oxoglutarate partly prevented inhibition of AST by D-ribose or D-fructose, while an analogous experiment with 25 mM aspartate resulted in a rapid decline similar to that in the absence of sugars.

Keywords: Aspartate aminotransferase; Transaminase; Inhibition; Glycation; Monosaccharides

INTRODUCTION

Non-enzymatic glycation is a common modification of proteins, which can impair their functions in living organisms. The first product of protein glycation is a Shiff base, formed over a period of hours in a reaction between the free amino groups of lysine and the N-terminal amino acid residues in proteins and the carbonyl groups of reducing sugars. The glycation reaction between amino acids and sugars was first described by Maillard as early as 1912.[1] The Shiff base is unstable and dissociates readily but in several days it may be converted into an Amadori product (AP), the first stable glycation product. The process is accelerated during hyperglycemia in diabetes, later yielding advanced glycation end products (AGEs), which are thought to be involved in the pathogenesis of diabetic complications.[2,3,4]

Glycation of proteins is considered also to contribute to the pathophysiology of normal aging.[5,6] The extent of the reaction depends on the number of reactive ε-amino groups of lysine residues in the protein, the type and concentration of sugars, and the biological half-life of the protein.[7]

Aspartate aminotransferase (AST, L-Aspartate: 2-oxoglutarate aminotransaminase, EC 2.6.1.1) is an enzyme which catalyzes the conversion of aspartate and 2-oxoglutarate to oxalacetate and glutamate, or the reaction in the reverse direction. In metabolism, AST activity mediates the connection between the metabolism of amino acids and saccharides and participates in the transportation of reduced equivalents across the membrane of mitochondria as a part of the malate–aspartate shuttle. The catalytic activities of AST in cells are implemented by two isoenzymes—the cytosolic and the mitochondrial one, which differ in primary structure and in some properties.[8,9] Regardless of its metabolic role, the enzyme could represent a good model for protein glycation studies. The enzyme is very stable *in vitro*.[10] The protein part of the molecule, consisting of two identical, non-covalently bound subunits, binds one molecule of the coenzyme pyridoxal-5′-phosphate (PLP) to lysine (Lys258) of each subunit.[8,9] About 20 other lysine residues are present in the molecule, with some differences according to species and intracellular localization.[11,12]

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TABLE I Influence of monosaccharides on rat liver AST[a] after 4 days of incubation at 25°C

Compound added (50 mM)	0 (Control)	D-glucose	D-fructose	D-ribose	DL-glyceraldehyde
Relative AST activity (%)[c]	100[b]	100	84.6	69.2	61.5

[a] Supernatant 20 000 × g of the tissue homogenate (25 mg/ml), diluted 1:100 by 0.1 M phosphate buffer, pH 7.4 before the assay, so as to obtain enzyme activities within the analytical range of the method. [b] The mean of three samples. [c] Absolute control activities of AST decreased from 0.28 ± 0.089 μkal/l at day 0 to 0.13 ± 0.046 μkal/l at day 4. For calculation of relative AST activity, the 4th day control value was considered 100%.

Early stages in glycation of aspartate aminotransferase from the rat liver by carbohydrates were investigated by Okada *et al.*[13] Fitzgerald *et al.*[14] found out that pig heart cytosolic AST was inhibited by 5 mM glyceraldehyde 3-phosphate within 24 hours at 23–50°C, while glucose-6-phosphate and ribose-5-phosphate at the same concentration did not affect the enzyme. We recently described glycation *in vitro* of a similar enzyme—alanine aminotransferase.[4] The present work aims to investigate the influence of several carbohydrates on the pig heart AST activity in a complete glycation course of 3–4 weeks and under various conditions with the aim of elaborating a model for the study of factors that might influence the process of glycation. Such a model should show a stable catalytic activity in the control samples and a distinct gradual decrease in enzyme activity during the course of the weeks of its incubation with glycating sugars.

MATERIALS AND METHODS

Chemicals

Two different sources of AST for the study were used. In the first experiments, rat liver enzyme from the supernatant 20,000 × g of tissue homogenate (25 mg tissue/1 ml) was used as described elsewhere.[15] Purified aspartate aminotransferase from the porcine heart (cytosolic enzyme suspended in 3 M ammonium sulfate) was then used in most of the other experiments. The preparations obtained from Serva had a stated activity of 200–290 units per mg protein whereas those purchased from Sigma (used in experiments with various concentrations of D-fructose) were of 470 units per mg protein. All other chemicals used were of analytical grade. Monosaccharides and their phosphates (Lachema Brno, Boehringer Ingelheim and Reanal Budapest), L-aspartate (Reanal, Budapest), 2-oxoglutarate (Fluka, Buchs) and sodium azide (Merck, Darmstadt) were purchased.

Preparation and Incubation of Samples

In the experiments with purified enzyme, an AST suspension was centrifuged at 1600 × g for 20 min. The supernatant was removed and the enzyme pellet dissolved in 0.1 M sodium phosphate buffer (pH 7.4) with 0.05% sodium azide to obtain an enzyme solution at a concentration of about 10^{-5} M. The enzyme was distributed into samples containing buffer, individual monosaccharides, and/or substrates. The final concentration of sugars (D-fructose D-glucose, DL-glyceraldehyde or D-ribose) in samples was 50 mM. Concentrations of the enzyme substrates, L-aspartate and 2-oxoglutarate were 25 mM and 0.5 mM, respectively. The final enzyme protein concentration was 0.3–1.0 mg/ml and the total volume of samples was 1.0–3.2 ml according to the experiment. Samples were incubated for 14 to 21 days at 25°C or for 28 days at 4°C. AST in phosphate buffer without sugar and substrates was used as the control.

Determination of AST Activity

An automated UV kinetic method was used for measurements of the catalytic activity of AST.

Sample aliquots (100 μl) were removed at time zero and at various intervals up to 21 days of incubation. After collection, the aliquots were diluted with 0.1 M phosphate buffer, pH 7.4, according to the experiment, in order to obtain enzyme activities within the analytical range of the used method. AST activity was assayed on a Hitachi 917 analyzer using the standard clinical laboratory method (Roche Diagnostics, Mannheim, Germany). All experiments were performed in triplicate. AST activities in Precinorm U and Precipath U (both from Roche Diagnostics) were measured before and after each assay. The between-run coefficient of variation of the assay for Precinorm U was 1.8%; for Precipath U it was 1.5%.

The results for the final assay samples are presented in μkat/l or as % of the respective control value.

Statistical Analysis

Values of enzymatic activity, taking into account diluting factors for different experiments, are given as means ± standard deviation (SD).

RESULTS AND DISCUSSION

The results of incubation of AST from the rat liver 20 000 × g supernatant suggested some influence of

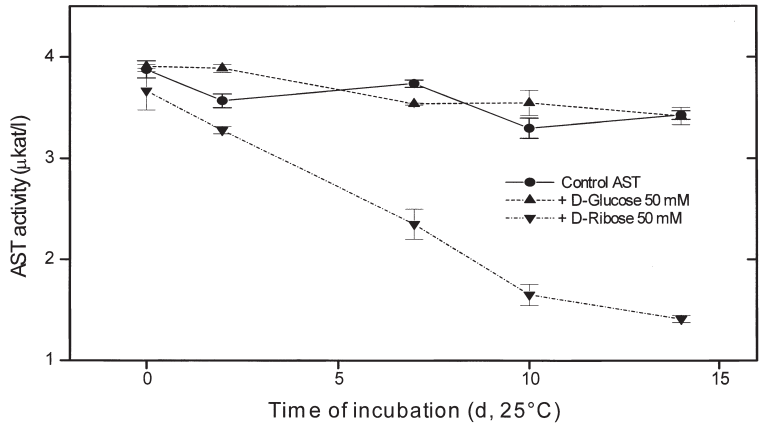

FIGURE 1 Influence of D-glucose and D-ribose on pig heart AST activity *in vitro* with incubation at 25°C. Concentration of the enzyme (Serva) in the incubation mixture: 1.04 mg/ml, final catalytic concentration in the mixture 3.53 μkat/ml. The incubation mixture was diluted before the assay in order to obtain activities within the analytical range of the method. Values are mean ± SD of three independent samples. Each sample was measured three-times and the mean was used to calculate the value presented.

D-ribose, D-fructose and DL-glyceraldehyde after 4 days of incubation at 25°C (Table I). At later intervals, AST activity rapidly declined in the control samples as well as in the presence of sugars. This diminishing enzyme activity in all samples was probably due to the presence of proteolytic enzymes and it made any further investigation of AST activity in the rat liver supernatant impossible. Nevertheless, it can be concluded that while the effects of D-fructose and D-ribose are comparable with those found in the experiments using purified pig heart AST, the inhibition of rat liver AST by DL-glyceraldehyde is smaller in comparison with the later experiments using the purified pig enzyme.

With purified AST the time course of glycation was examined using pig heart enzyme for as long as 14 to 28 days. The influence of D-glucose and D-ribose was firstly compared. With the enzyme from Serva at a concentration of about 1 mg/ml (stated activity 200–290 units per mg protein), no effect of 50 mM D-glucose was found up to 14 days of incubation, while AST activity was declining markedly in the presence of the same concentration of D-ribose (Figure 1). D-glucose did not affect AST activity even if a lower concentration of enzyme protein (0.41 mg/ml, Sigma, 470 units per mg protein, about 200 units per ml) was used (Figure 2). Under these conditions, the influence of D-ribose on pig heart AST was confirmed. The effect of D-fructose was similar to that of D-ribose, while DL-glyceraldehyde had a much stronger effect, with AST activity decreasing to zero after 6 days of incubation with DL-glyceraldehyde. The results presented in Figure 2 are expressed as a % of the respective control because of some day-to-day fading of enzyme activities.

It is difficult to compare our results with those of Fitzgerald *et al.*[14] because the latter authors used a too short interval of incubation (24 h) and probably a too low concentration of ribose-5-phosphate (5 mM).

The influence of D-ribose and D-fructose on AST was also investigated at 4°C. The effect of both

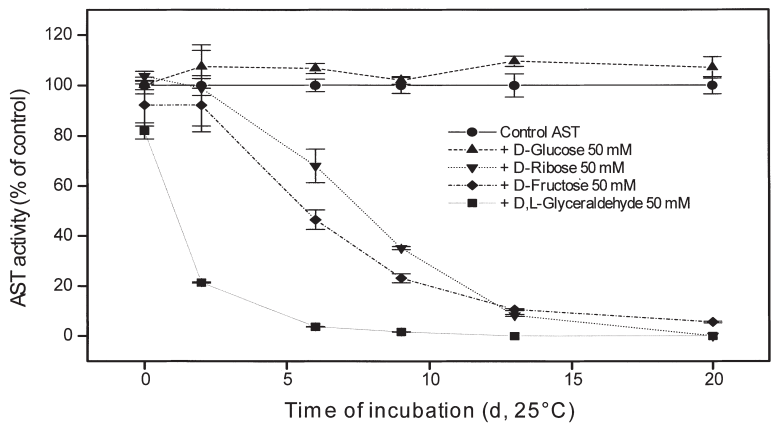

FIGURE 2 Influence of D-glucose, D-ribose, D-fructose, and DL-glyceraldehyde (50 mM) on pig heart AST activity *in vitro*. Concentration of the enzyme (Sigma) in the incubation mixture: 0.413 mg/ml, catalytic activity 470 U/mg (7.83 μkat/mg). Values are mean ± SD of 6 (control) or 3 (with individual monosaccharides) independent samples. Each sample was measured three-times and the mean was used to calculate the value presented.

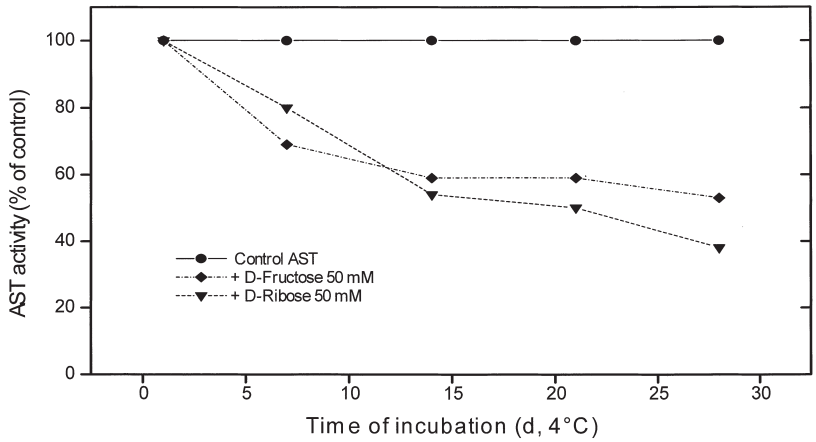

FIGURE 3 Influence of D-glucose and D-ribose on pig heart AST activity *in vitro* with incubation at 4°C. Concentration of the enzyme (Serva) in the incubation mixture: 0.278 mg/ml, catalytic activity in the mixture 0.94 μkat/ml. Values are mean ± SD of 9 (control) or 3 (with individual monosaccharides) independent samples. Each sample was measured three-times and the mean was used to calculate the value presented.

monosaccharides was marked even at this temperature but it was less pronounced than at 25°C (Figure 3). In comparison, Okada *et al.*[13] studied the influence of glucose and fructose at a 50 mM concentration and glyceraldehyde at a 1 mM concentration on rat liver AST for a maximal period of up to five days. These authors found some small decrease in AST activity in the presence of 50 mM glucose. Our results with pig heart enzyme exclude such an effect of glucose at this concentration up to 28 days and up to four days with the rat liver enzyme.

Another part of the study dealt with the effects of the enzyme substrates L-aspartate and 2-oxoglutarate on AST activity and its inhibition during glycation reaction. It is well known that addition of L-aspartate causes a change in the coenzyme from its pyridoxal form to pyridoxamine and liberation of the Lys-258 amino group from its binding to the coenzyme. On the other hand, addition of 2-oxoglutarate supports the pyridoxal form and binding of the active site lysine to the coenzyme. We believe that liberation of Lys-258 could increase the probability of glycation of Lys-258 in the active site of AST, since we recently obtained similar results in an analogous study of glycation of alanine aminotransferase.[4] The effect of substrates on AST and its glycation was investigated at an enzyme concentration of 0.41 mg/ml (Sigma) and with a 50 mM concentration of D-ribose or D-fructose. There was no effect of 2-oxoglutarate (OG) on the control AST activity throughout the experiment, which demonstrates a high stability of the pyridoxal form both in the presence and absence of this substrate. On the other hand, the presence of 25 mmol/l L-aspartate caused a rapid decrease in AST activity even in the control reaction (Figure 4). The results with D-ribose and D-fructose (Figures 5 and 6) suggest that glycation of AST can be partly prevented by addition of 0.5 mmol/l

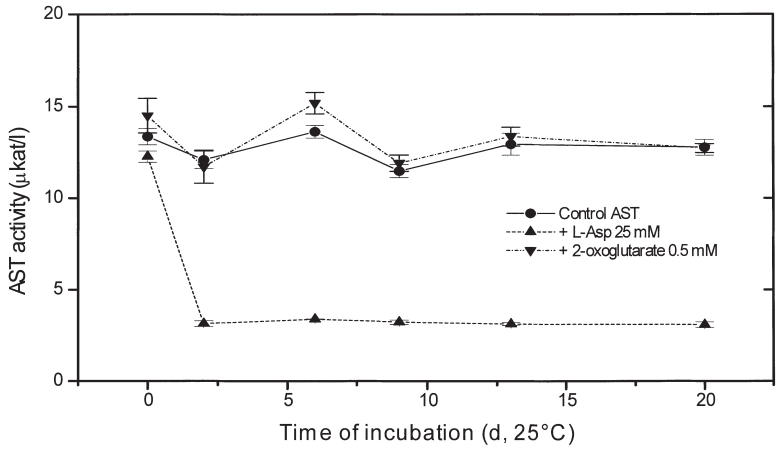

FIGURE 4 Influence of substrates on control AST activity during incubation *in vitro*. Enzyme concentration as stated for Figure 2. Values are mean ± SD of 6 (control) or 3 (with individual monosaccharides) independent samples. Each sample was measured three-times and the mean was used to calculate the value presented.

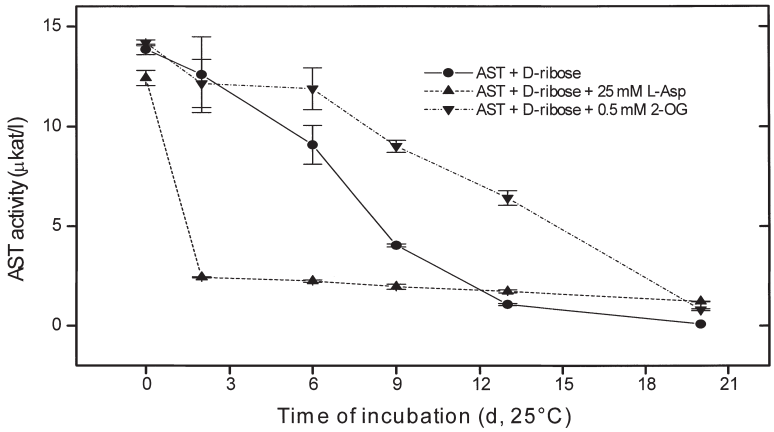

FIGURE 5 Influence of substrates on AST activity during glycation by D-ribose *in vitro*. Enzyme concentration as stated for Figure 2. Values are mean ± SD of 6 (control) or 3 (with individual monosaccharides) independent samples. Each sample was measured three-times and the mean was used to calculate the value presented.

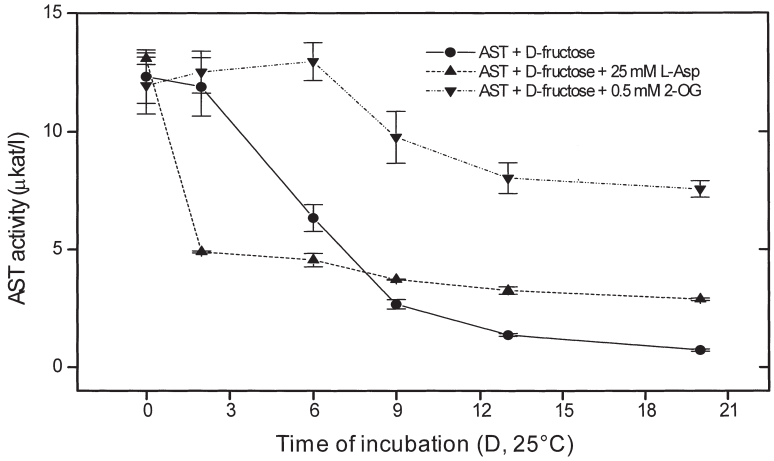

FIGURE 6 Influence of substrates on AST activity during glycation by D-fructose *in vitro*. Enzyme concentration as stated for Figure 2. Values are mean ± SD of 6 (control) or 3 (with individual monosaccharides) independent samples. Each sample was measured three-times and the mean was used to calculate the value presented.

2-oxoglutarate. This supports the idea that the active site lysine is involved in glycation of free enzyme. However, an analogous experiment with addition of 25 mmol/l L-aspartate did not help in supporting this view because AST activity in the presence of Asp and sugars showed a similar rapid decline as in the above-mentioned control reaction with Asp.

It may be concluded that D-glucose (50 mM) is not able to decrease AST activity even after 3 weeks of incubation at 25°C. D-ribose and D-fructose show a marked effect on decreasing AST activity during their incubation with the enzyme under such conditions, with final AST activity close to zero. DL-glyceraldehyde (50 mM) inactivates the enzyme at 25°C within 6 days.

The results presented in this paper suggest that AST would be a prospective model for studies of enzyme inhibition by glycation, provided more reactive glycating agents than glucose are used. The aim of the present authors is to use this model in future investigations of compounds that may interfere with glycation of proteins.

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