

Serum level of circulating xanthine oxidase in healthy and chronic hepatitis B patients

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Abstract

During severe liver damages, xanthine oxidase (XO) released into the blood, so a blood assay for XO is a way to determine if liver damage has happened. In this work we aimed to determine levels of XO in serum of healthy and hepatitis B subjects using an enzyme linked immunosorbent assay ELISA. In the first step, we purified XO from frozen human milk, then we assessed the purity of the enzyme through, SDS-PAGE and UV-visible spectrum. Then, we used this enzyme to immunize rabbits to get anti XO antibodies. After that, we optimized a sensitive sandwich ELISA test to determine levels of XO in serum of normal and hepatitis B subjects. After purification, the yield of XO enzyme was 5.7 ± 1.8 mg /L (n=4). Anti-XO antibodies were purified with affinity chromatography on XOR immobilized column. The obtained antibodies were from IgG class. The developed sandwich ELISA test allowed us to get a XOR levels of 5.11 ± 1.23 ng/ml for healthy subjects and 45.15 ± 15.61 ng/ml for hepatitis B patients. These results showed no significant correlation between XO level and transaminases. Based on the obtained results, the level of XO in serum of hepatitis B subject is significantly higher than its level in normal subjects.

Keywords: Xanthine Oxidase, Hepatitis B, ELISA sandwich, Anti- Xanthine Oxidase antibodies, Transaminases

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INTRODUCTION

Xanthine oxidoreductase is a flavomolybdo enzyme that present in milk and many other tissues; it is a homodimer of around 300 KDa (Parks & Granger, 1986). This enzyme has two forms; xanthine dehydrogenase (XDH; EC 1.1.1.204) and xanthine oxidase (XO; EC 1.1.3.22). The dehydrogenase form prefers to reduce NAD in contrast to the oxidase form, which preferring oxygen molecular. The main role of this enzyme is to catalyze the hydroxylation of hypoxanthine and xanthine to xanthine and uric acid respectively (Bray, 1975). Reduction of oxygen lead to the generation of superoxide anion and hydrogen peroxide and these reactive oxygen species (ROS) lead to the wide spread interest of the enzyme.

Last years, attention was directed toward the physiological and pathological significance of XO, which considered as an important source of ROS in Ischemia/Reperfusion injury (Mc Cord, 1985) and in other many pathological states. This ROS is use also in signal transduction as a physiological role (Behrend et al., 2003).

The increased level of XO was detected for the first time in patients with viral hepatitis (Shamma'a et al., 1965). After that, several investigations showed that in various pathological conditions, both in experimental animals and in humans, the level of XO was increased. After being released into plasma, XDH is converted to the oxidase form of the enzyme (Battelli et al., 1992), and it has been suggested that XO can cause damage at other sites (Yokoyama et al., 1990) through the production of active oxygen species.

The XO in human serum has also been studied, and an elevated level was found in several conditions of liver damage such as in viral hepatitis (Bruder et al., 1984; Yamamoto et al., 1996). The occurrence of XO enzymatic activity in human serum has been recognized for many years, while levels in normal subjects are accepted to be very low, significant increases have been reported in cases of liver damages (Battelli et al., 2001), rheumatic diseases (Benboubetra et al., 1997) and Ischemia/Reperfusion (Granger et al., 1981). Circulating XO with its increased levels in many liver damages is relevant to the diagnostic. For these reasons we now reported to determine the levels of circulating XO in normal and chronic hepatitis B human subjects.

The present research was undertaken to investigate the level of XO in the serum of patients with hepatitis B virus. Serum XO was determined by ELISA, as this test is highly sensitive and convenient for routine use in clinical laboratories (Battelli et al., 1999). Our aim was to ascertain whether XO levels in serum reflect liver disorder.

Materials And Methods

Subjects and blood samples

Blood samples were collected from 30 healthy human subjects from the CHU of Setif in Algeria; serum was immediately prepared and stored at -20 °C until use, while the 30 samples of chronic hepatitis B subjects were kindly supplied by Pr. Roger Harrison from the University of Bath in England.

Milk and reagent

Breast milk was kindly donated by mothers in the special care baby units of the following hospitals: Bristol Royal Infirmary, South mead Hospital, Bristol Princess Margaret Hospital, Swindon; and the royal united hospitals, Bath. Milk was stored at -20°C until use.

Anti-XO antibodies were obtained by rabbit immunization, using standard protocols, with purified XOR. The anti-XO antibodies were purified. All other reagents were from Sigma.

XOR Purification

XO purification was carried out according to the previously described protocol for human milk XOR (Benboubetra et al., 1997). Briefly, breast milk (1500 ml) was supplemented with EDTA, phenyl- methylsulphonyl fluoride (PMSF) and sodium salicylate. The mixture was then stirred at 4°C for 30 min and all the following steps were carried out at 4°C. The cream was collected by centrifugation at 3000 g for 30 min (Sigma 3K30C centrifuge), resuspended in a double volume of K₂HPO₄, stirred for 2 hours and centrifuged at 6000 g for 30 min. Ice cold butanol (15% v/v) and ammonium sulphate (15% w/v) were slowly added to the obtained filtrate, stirred and centrifuged at 11.000 g for 30 min. The supernatant was collected and 20% (w/v) of ammonium sulphate was added slowly under stirring, over a period of 30 minutes and centrifuged at 13.000 g for 30 min. The precipitate was collected and resuspended in heparin buffer and dialyzed overnight against the same buffer and centrifuged at 40.000 g. The supernatant was applied to a column of heparin (Sigma, type 1) equilibrated with heparin buffer. XO was eluted with NaCl in the same buffer. The enzyme obtained was dialyzed overnight against sodium bicine buffer, aliquoted and stored at -20°C.

Estimation of enzyme concentration and purity assessment

XO concentration was estimated at 450 nm using the FAD extinction coefficient of 36 000 M⁻¹cm¹ (Fhaolain and Coughlan, 1976). The purity of enzyme was assessed with protein/flavin ratio (PFR = A₂₈₀/A₄₅₀) and with 10% SDS-PAGE (Bray, 1975). Standard of six proteins were used as molecular weight markers in all gels.

Purification of anti-XO antibodies

White rabbits were immunized with 100g of XO, which was suspended in 500 µl of PBS and 500 µl of freud's complete adjuvant, the emulsion was injected into many subcutaneous sites on rabbits, a week later another XO prepared in 500 µl of PBS and 500 µl of freud's incomplete adjuvant was injected. Every week we made reminder injections and a week after the fourth reminder injection, animals were bled and sera were collected.

The IgG obtained by ammonium sulfate precipitation were purified by affinity chromatography on XO immobilized column,

the anti-XO antibodies were eluted with sodium sulfate buffer (Benboubetra et al., 1997). The concentration of the anti-XO antibodies was determined by Bradford method.

ELISA for determination of XO level

To determine XO concentration in normal sera and sera of hepatitis B subjects we used ELISA sandwich method, the specific antigen was determined as described previously (Benboubetra et al., 1997).

Purified anti-XO antibodies were absorbed in Na₂CO₃, 50 mM in each well of a 96 wells microtiter plate, and allowed to stand at 4 °C overnight. Wells were then washed by 300 µl per well three times with phosphate buffer solution (PBS) containing 0,05 tween 20 (PBS- Tween). The remaining binding sites were blocked by incubation 2h of PBS containing 2% of casein. Then, serum samples in different dilutions were added each in triplicate well, and the plates were incubated for 90 min, at 37 °C, washed twice with PBS-Tween and incubated twice, each for 5 min at room temperature with the same solution. Capture anti-XO antibodies were added and incubated for 90 min, at 37°C, and then the plate was washes twice with PBS- Tween. Immunoglobulin conjugated with horseradish peroxidase (Sigma) was then added to each well and the plate was incubated for one hour at room temperature before three washes with PBS-Tween. Substrate buffer was then added to each well (100 µl per well) and the yellow color was allowed to develop for 20 to 30 min. Finally the reaction was stopped by the addition of 50 µl of sulfuric acid 2N to each well and the absorbance was recorded at 492 nm.

The determination of XO concentration was based on standard graph of absorbance against concentration's logarithm, for each plate we draw a graph and we use just the linear part to calculate the concentrations.

Statistical analysis

The results are expressed on mean ± SD (standard deviation). The analysis is made by the t test of Student for the simple comparisons of the results between normal and damage subjects. The difference is considered statistically significant when the value of p is inferior of 0.05 (p< 0.05). The comparison of the averages and the variances is determined with the software Graphpad Prism version 5.0. We also use the test of box plot to determine the pertinent values from the aberrant one.

Results

Purification and characterization of XOR

Human milk XOR was purified by affinity chromatography on heparin, yielding up ± 1.8 mg of enzyme per liter of milk (table 1). Purified XO showed an UV/VIS spectrum with three major peaks at 280, 325 and 450 nm (Fig. 1). This enzyme runs on a gradient (5– 20%) SDS-PAGE showed one major band of approximately molecular wight of 145.3 KDa (Fig. 1)

Table 1. Characterization of XOR

XOR (mg/L)	RPF
5.7 ± 1.8	5.10 ± 0.22

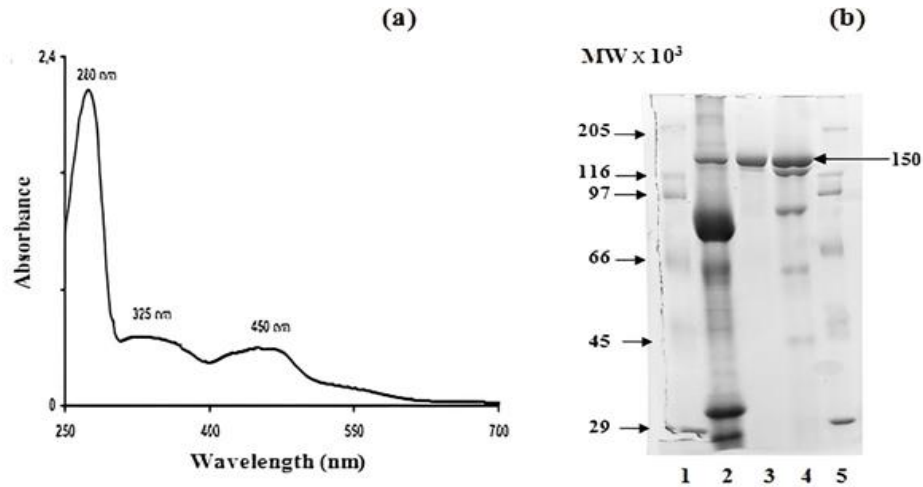


Fig 1.UV/VIS (250 – 700) spectrum of purified human milk XOR.B: SDS-PAGE (10%) of human milk XOR. Lines: 1,5 molecular weight markers; 2, Brut XOR; 3, XOR after chromatography; 4, XOR before chromatography.

Serum XO determination

In the present study we tried to optimize an ELISA sandwich assay to determine serum XO in healthy (30 samples) and hepatitis B subjects (30 samples). A 12% cut-off for the ELISA kit provided a 97% sensitivity and a 95% specificity.

Results of serum XO determination by sandwich ELISA in healthy subjects and hepatitis B patients is shown in figure 2. Values of XO serum levels in patients with hepatitis b ($45,15 \pm 15,61$ ng/ml) are significantly higher ($p < 0.05$) than those of healthy subjects ($5,11 \pm 1,23$ ng/ml). The corresponding comparison for XO is shown in Fig. 2.

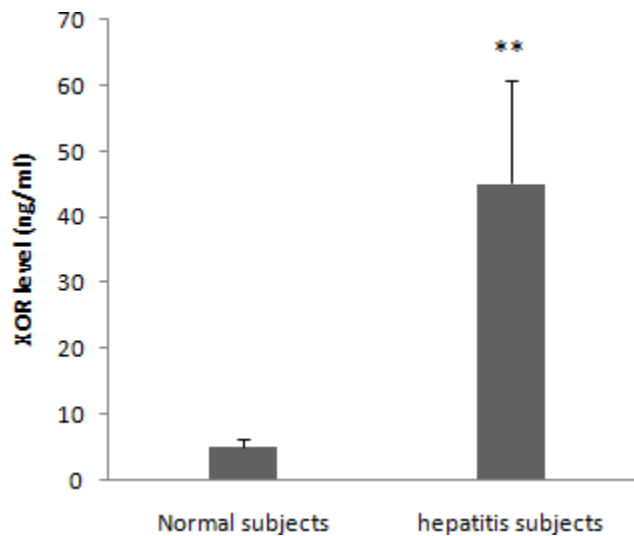


Fig. 2. Level of XOR in normal and hepatitis B subjects

Conventional blood markers of liver damage; Alanine aminotransferase (ALAT) and Aspartate aminotransferase (ASAT) were evaluated between patients and healthy subjects. Results indicated a significant differences ($p < 0.05$) as shown in table 2.

Table 2. Serum transaminases markers in healthy and patients with chronic hepatitis B

Normal subjects	Hepatitis B Subjects
ALAT (UI/l) < 35	130.8* ± 51.06
ASAT (UI/l) < 35	105.0* ± 37.95

The correlations between biochemical parameters and XO serum level were determined using Spearman test (Table 3). XO values showed a positive correlation with biochemical parameters ASAT and ALAT, and the correlation with ASAT is stronger than that of ALAT ($r = 0.25$; $p < 0.13$ and $r = 0.43$; $p < 0.04$ respectively)

Table 3. Correlation Between serum XO and liver related laboratory data for patients with chronic hepatitis B

Transaminases	r	p
ALAT (UI/l)	0.43	0.04
ASAT (UI/l)	0.25	0.13

The correlation coefficient (r) and statistical significance (p) were obtained by the Spearman test for combined patients and for each patient group. * Statistically significant correlation ($p, 0.05$).

Discussion

The present study aimed to determine the level of XOR in human healthy and hepatitis B subjects. Purification procedure of XOR was followed the method of Benboubetra and his collaborators (Benboubetra et al., 1997), this method generates enzyme of high purity and it is much better suited to the purification of large volumes of frozen breast milk.

We used ELISA sandwich method to determine XO serum levels in healthy subjects. The XO concentration was remarkably low (ng scale). Sarnesto and his collaborators were the first who determined the concentration of XO in human serum, they were detected the enzyme in the sera of 20 subjects with a maximal concentration of 5ng/ml (Sarnesto et al., 1996). Another study by Batteli and his collaborators, based on developed ELISA competitive assay determined XO concentration in human sera, showed that the minimal concentration of XO was 32 ng/ml (batteli et al., 2001). Our finding with ELISA sandwich assay for 30 subjects correlated with previous researches. To determine the concentration of the same enzyme in serum of human patients (chronic hepatitis B), we used the same test (ELISA sandwich), our results showed a high level of serum XO 45.15 ± 18.61 ng/ml, ($p < 0.05$). This finding was in agreement with previous reports, Battelli and her collaborators were observed a significant elevation of XO level in serum of liver damage subjects in comparison with healthy subjects (batteli et al., 2001; Martin et al., 2004).

The high level of XO enzyme in the liver could be explained by the naturally high distribution and activity of this enzyme in liver (Harrison, 2004; Kurosaki et al., 1995). XOR mRNA was detected in most tissues and the highest levels of transcription were detected in liver and intestine (Kurosaki et al., 1995). Also, the interferon production associated to viral infections could be responsible for the high level of XO in liver.

The weak correlation between XO serum concentration and that of transaminases should be pointed out. This finding could be interpreted as meaning that XO enzyme unlike transaminases enzymes is not released in plasma during hepatocytes necrosis but in the earliest stages of liver inflammation, during the very early phase of damage. It is possible that normal hepatic synthetic activity is a necessary prerequisite for XO elevation, as the severe reduction of hepatocyte function in cirrhosis may limit the expression of the enzyme in liver and, consequently, the availability of a serum XO source (batteli et al., 2001; Stripe et al., 2002).

The limited number and type of cases reported in the present study does not allow us to get interpretations about the physiopathological and clinical meaning of serum XO elevation.

Conclusion

Our results indicate that the significant increase of XO level in hepatitis B subjects makes this enzyme a sensitive biological parameter. These findings are consistent with the preliminary results with Professor Roger Harrison at the University of Bath in England which showed that serum XO level is a better parameter for monitoring liver function.

Disclaimer

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. This study was supported by grants from Ministry of higher education and scientific research in Algeria.

Ethical Approval

The experimentations carried out are in accordance with guidelines and rules of the international animal experimentation chart.

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Conflict Of Interest

The authors declare no conflict of interest, financial or otherwise

Consent For Publication

All authors gave consent to the publication.

Authors' Contributions

This work was carried out in collaboration between all authors. Authors MOSBAH Asma, SLIMANI Abdelkader, MAHROUK Abdelkader performed the work, and wrote the first draft of the manuscript. Authors KHITHER Hanane and MOSBAH Camélia and BOUCHERIT Hanane designed the study and managed the searches. All authors read and approved the final manuscript.

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