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Purines oxidation by immobilized xanthine oxidase on magnetic polysiloxane–polyvinyl alcohol composite

David F.M. Neri[a,b,*], Débora P.B. Bernardino[c,b], Eduardo I.C. Beltrão[c,b], Luiz B. Carvalho Jr.[c,b]

[a] Universidade Federal do Vale do São Francisco, Centro, Petrolina-PE CEP 56304-205, Brazil
[b] Laboratório de Imunopatologia Keizo Asami, Departamento de Bioquímica, Brazil
[c] Universidade Federal de Pernambuco, Cidade Universitária, Recife-PE CEP 50670-901, Brazil

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ABSTRACT

Bovine milk xanthine oxidase (XOD, E.C. 1.17.3.2) was covalently immobilized, via glutaraldehyde, on magnetic polysiloxane–polyvinyl alcohol (mPOS–PVA) particles yielding a preparation containing 9.5 ± 0.5 μg of protein per mg of support and specific activity of 36.3 ± 7.8 mU/mg of protein (55.0 ± 11.7% of the free enzyme). Optimal pH (8.8) and temperature (60 °C) were slightly higher than those established for the free enzyme (8.2 and 55 °C, respectively). No decrease of activity was observed after five reuses and only 17% was lost at the tenth reuse. The apparent Michaelis constant estimated for the mPOS–PVA–XOD (8.86 ± 0.88 μM) was not statistically different from the free enzyme (7.48 ± 1.01 μM). The 6-mercaptopurine oxidation catalyzed by the mPOS–PVA–XOD followed the same pathway described for the free enzyme, namely, 6-mercaptopurine → 6-mercapto-8-hydroxypurine → 6-thiouric acid, and no 6-thioxanthine was formed.

1. Introduction

Xanthine oxidase (XOD; E.C. 1.17.3.2) is one of the most studied enzymes [1]. It is widely distributed in mammalian tissues and has long been known to be a major constituent of the milk fat globule membrane, which surrounds fat globules in cow's milk [2]. Despite of the variety of possible substrates its main function is to oxidize hypoxanthine to xanthine and xanthine to uric acid [3].

The immobilization of enzymes onto polymeric supports offers several advantages over use of soluble enzyme preparations. These include increased storage stability, facilitated separation of products from the incubation mixture, the ability to recover and reuse the enzyme catalyst, and in many cases, stabilization of the tertiary structure of the water insoluble enzymatic derivatives. XOD has already been immobilized on innumerous supports such as: cellulose, sepharose, hornblende, enzacryl-TIO, and controlled-pore glass [4], polyacrylamide gel beads, polyamide-11 and Dacron [5]; polyaniline silicone support [6]; modified carbon paste electrode [7]; nanocrystal gold-carbon paste electrode [8]. These immobilized preparations have been applied in many fields, mainly in chemical analysis as the sensitive biological element of biosensors.

In our laboratory silica glass beads have been synthesized by alkoxide sol–gel process based on polyvinyl alcohol and tetraethylorthosilicate (TEOS) and were used as solid phase for antigen immobilization in immunodiagnosis [9,10]. Simultaneously, the structure and permeation characteristics of membranes composed of these organic–inorganic hybrid components were extensively studied by other research group [11]. These polysiloxane/polyvinyl alcohol (POS–PVA) beads were also converted in powder, magnetized and used in chemiluminescent assays [12]. In previous studies, this support was used for β-galactosidase immobilization and applied in lactose hydrolysis [13] and galactooligosaccharides synthesis [14]. Hybrid material composed of organic and inorganic species can be easily synthesized by sol–gel process, in which the starting materials are in solution and can be carried out at a low temperature [15]. Such materials combine the chemical and physical properties of the guest with an excellent optical, thermal and chemical stability of the host silicon oxide matrices.

The thiopurine antimetabolite 6-mercaptopurine (6MP) is an important chemotherapeutic drug used in the conventional treatment of childhood acute lymphoblastic leukemia (ALL). 6MP is mainly catabolized by both hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and xanthine oxidase (XOD) to form thioinosinic monophosphate (TIMP) (therapeutically active metabolite) and 6-thiouric acid (6TUA) (inactive metabolite), respectively [16].

* Corresponding author at: Universidade Federal do Vale do São Francisco, Colegiado de Ciências Farmacêuticas, Av. José de Sá Maniçoba, S/N, Cx. Postal 252, CEP: 56304-205, Campus Petrolina–Centro, Petrolina, Brazil. Tel.: +55 87 21016862; fax: +55 87 21016757.
E-mail address: davidfmneri@yahoo.com.br (D.F.M. Neri).

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Here, magnetic POS–PVA (mPOS–PVA) particles were used as a matrix for XOD immobilization. Some properties of the immobilized enzyme were investigated (apparent K_m using xanthine as substrate; optimal pH and temperature and reuse) and the catalytic action on the 6-mercaptopurine oxidation pathway.

2. Experimental procedures

2.1. Xanthine oxidase purification

Fresh bovine milk (1 L without any preservative) was unstirred kept at 4 °C overnight. The cream milk was separated from the fresh milk and then was hand churned for 3 h at 4 °C. Afterwards, the overlaid layer was discarded and the buttermilk was filtered by using cheesecloth. Then casein was precipitated at pH 4.8 by dropping 1 M HCl, followed by centrifugation at 27,000 × *g* for 15 min and immediately increasing pH value up to pH 7.0 by adding 1 M NaOH. The casein-free buttermilk (65 mL) was 38% saturated by adding ammonium sulfate. The suspension was centrifuged at 12,000 × *g* for 15 min and the precipitated was discarded. Further ammonium sulfate was added to the 0–38% ammonium sulfate supernatant to 50% saturation. The precipitate (38–50% fraction) was collected by centrifugation at 27,000 × *g* for 30 min and dissolved in 5 mL of 10 mM Tris–HCl, pH 8.0, containing 5 mM 2-mercaptoethanol and 100 μM EDTA. This suspension was dialyzed twice, first against the buffer containing 100 μM EDTA and later against buffer containing 30 μM EDTA. The dialyzed material was finally centrifuged at 35,000 × *g* and the supernatant stored at 4 °C and used throughout this work.

2.2. POS–PVA synthesis, magnetization and XOD immobilization

The magnetic POS–PVA synthesis and enzyme immobilization were carried out according to Neri et al. [13,14], except that XOD preparation (127 μg/mL) replaced the β-galactosidase and the used buffer was 10 mM Tris–HCl, pH 8.0, containing 100 μM EDTA. The particles were collected and the supernatant used for protein determination according to Sedmak and Grossberg [17] using bovine serum albumin as the standard. The bound protein was calculated by the difference between the offered protein and that estimated in the supernatant.

2.3. Enzyme activity measurements

The soluble XOD (50 μL) was incubated with 1000 μL of 100 μM xanthine, prepared in 50 mM of Tris–HCl, pH 8.2 (from now on called the buffer) and following the uric acid production at 295 nm (ε_{293} = 9.5 mM^{-1} cm^{-1}). The immobilized enzyme (10 mg) was mildly stirred with 1000 μL of 100 μM xanthine prepared in the buffer and at time intervals of 5 min the magnetic enzymatic preparation collected by a magnetic field of 0.6 T; the supernatant absorbance read at 295 nm and immediately reincubated with the immobilized enzyme allowing the reaction to proceed for another 5 min. Immobilized XOD activity on 6-mercaptopurine was also evaluated by incubating a 650 μM solution prepared in the buffer at 25 °C. Then the supernatant of the enzymatic reaction was simultaneously analyzed at 240 nm, 256 nm, 308 nm and 340 nm in 10 min intervals.

2.4. Optimal pH and temperature

The effect of pH and temperature on the enzyme activity were investigated in the ranges of 7.6–9.0 and 30–65 °C, respectively, using the above described activity determination procedures and xanthine as substrate.

2.5. Reuse of immobilized XOD on POS-PVA magnetic support

The reuse of the immobilized XOD activity was evaluated incubating the same preparation with fresh 1 mL of xanthine (100 μM) every 10 min for 10 times. The uric acid production was spectrophotometrically evaluated as described above.

3. Results and discussion

Xanthine oxidase was extracted from bovine milk and partially purified by ammonium sulfate precipitation. The fraction precipitated at 38–50% of ammonium sulfate presented the highest specific activity (69 mU/mg of protein with purification and yield of 1.57 and 66%, respectively). Previous report demonstrated that immobilized XOD using highly purified preparations lost activity during reuses [6]. Probably, this deleterious effect is due to the release of free radicals (O_2^-) and H_2O_2 produced during XOD activity. Addition of catalase and superoxidase dismutase prevented this effect [18]. Therefore, this partially purified enzyme preparation was used throughout this work.

The scheme of the chemical basis of this immobilization procedure is described elsewhere [13]. The POS–PVA composite degree of swelling has been described to decrease remarkably with increasing TEOS content [11]. The POS–PVA ratio used in this work allows a high degree of PVA swelling and a hydrophilic microenvironment for the enzyme catalytic action. Those hybrid beads were smashed into small particles (powder) to increase surface area for immobilization. The inconvenience of using small particles was overcome by magnetization, co-precipitating Fe^{2+} and Fe^{3+} ions in ammonia solution at 100 °C, which allowed the particles to be easily collected under a magnetic field.

The immobilization of XOD on mPOS–PVA yielded preparations containing 9.5 ± 0.5 μg of protein per mg of support. This would be equivalent to 63.5 ± 3.3% of the offered XOD for the 10 mg of the composite. Furthermore, these immobilized XOD POS-PVA derivatives presented specific activity of 36.3 ± 7.8 mU/mg of protein, retaining 55.0 ± 11.7% of that found for the free enzyme. The homogeneity of the magnetic particles sizes was demonstrated by the linear relationship achieved between the volume of the magnetic particles suspension *versus* their weight and specific enzymatic activities (data not shown).

The optimum pH (8.8) and temperature (60 °C) found for the immobilized XOD on the mPOS–PVA particles were slightly higher than those established for the free enzyme (8.2 and 55 °C, respectively), as one can see in Fig. 1. Shift to the right for the immobilized enzyme pH profile compared to the free enzyme one is usually caused by negatively charged support. Thus the enzyme microenvironment would presents higher hydroxonium concentration attracted by the support demanding for higher hydroxide ions in the bulk of the reaction, where de enzyme activity is measured. Here, the immobilized enzymatic preparation is composed of XOD, a semi-interpenetrated network of polysiloxane and polyvinyl alcohol strongly and magnetite (Fe_3O_4). Further studies should be carried out to evidence the presence of negative charge on the support surface. However, its presence does not seem to change optimum pH significantly.

The increase in the optimum temperature activity of the immobilized enzyme has been attributed to the more rigid conformation generated by bonds linking the enzyme to the water insoluble matrix [19]. The optimal pH values widely vary in the literature reports: 6–8 for XOD immobilized on $CuPtCl_6$/glassy carbon chemically modified electrode [20]; 7.3 on poly (mercapto-p-benzoquinone) [21]; 8.4 on polypyrrole film [22] and 6.0–7.5 on nanocrystal gold–carbon paste electrode [8]. Lower optimal temperatures have been reported for immobilized XOD such as 42 °C

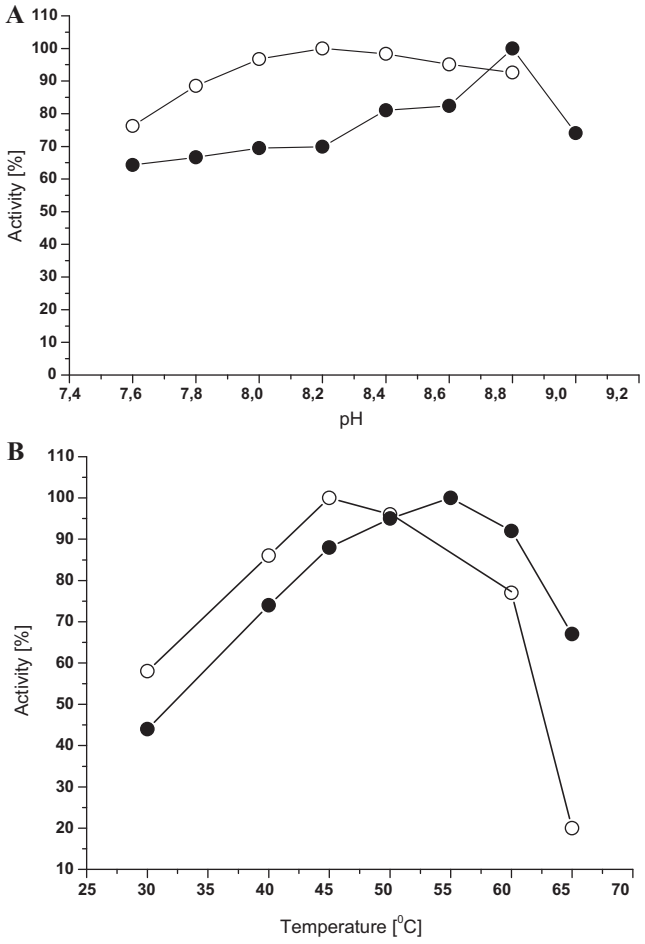

Fig. 1. Effect of the pH (A) and the temperature (B) on soluble (○) and immobilized XOD (●) on mPOS–PVA activity.

and 38 °C on CuPtCl6 chemically modified electrode [20] and in the bulk of a silica-graphite matrix by sol–gel techniques [23], respectively.

The apparent Michaelis constant (K_m^{app}) calculated for the immobilized and free XOD were $8.86 \pm 0.88\,\mu M$ and $7.48 \pm 1.01\,\mu M$, respectively, and this difference showed to be not statistically different. This result suggests that the events (partitioning, diffusional or mass transfer, conformational and steric effects) involving the immobilized enzyme-substrate interaction and its microenvironment do not markedly disturb the enzyme action. The K_m^{app} for the immobilized β-galactosidase on the same support was also found to be not significantly different from the free enzyme [13]. Higher K_m^{app} has been reported for other immobilized XOD derivatives: 24.5 μM for polyvinyl alcohol glutaraldehyde beads [24]; 2.8 mM for polypyrrole electrode [22]; 27 μM for carbon past electrode [25] and 80 μM for poly (mercaptobenzoquinone) gold glass carbon electrode [21]. XOD immobilized onto Sepharose 6B-conjugate heparin by noncovalent electrostatic interactions also presented higher K_m^{app} (15 μM) than that found for the free enzyme (3 μM) [26]. The K_m^{app} value of this work cannot be compared with the immobilized XOD in the bulk of a silica–graphite–benzyl viologen composite, also synthesized by sol–gel technique, because hypoxanthine was used as the substrate [21].

Among several reasons to use immobilized enzymes two main targeted benefits are: easy separation of enzyme from the product and its reuse. Enzyme reuse provides a number of cost advantages that are often an essential prerequisite for establishing an economically viable enzyme-catalyzed process. The rapid recovery from the reaction mixture and quick washing procedures of the magnetic XOD–POS–PVA composite by a magnetic field makes this derivative economically attractive. The stability of this immobilized XOD preparation during this uncomplicated reuse is shown in Fig. 2. There was no decrease of activity after five reuses and only 17% was lost at the tenth reuse. Similar behavior has been reported for immobilized β-galactosidase on the same support [13].

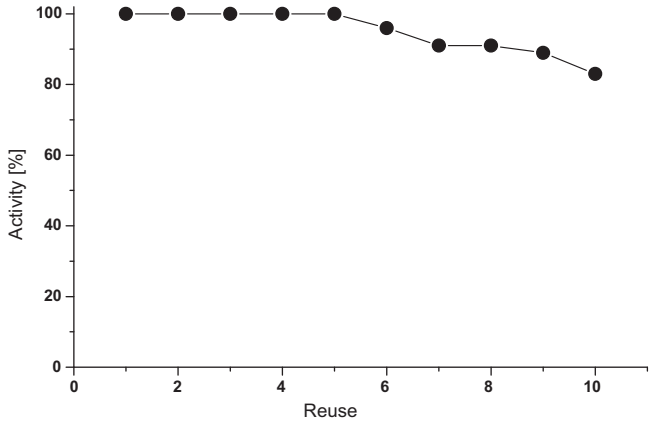

Fig. 2. Reuse of the immobilized XOD on mPOS–PVA.

XOD is recognized to have a large specificity so that can catalyze the oxidation of about one hundred compounds [3]. The conversion of hypoxanthine to xanthine and from xanthine to uric acid is physiologically catalyzed by XOD. This pathway belongs to the purines catabolism and is shown in Fig. 3(A). It is worthwhile to notice that the enzyme firstly attacks the carbon atom number two of the hypoxanthine and afterwards the number 8. Another oxidative pathway catalyzed by XOD is that related to the 6-mercaptopurine catabolism, important chemical used in leukemia treatment. Fig. 3(B) depicts the two possible ways, namely, XOD firstly recognizing the carbon atom either number two or number eight of the 6-mercaptopurine. The appearance of either 6-thioxanthine or 6-mercapto-8-hydroxypurine (intermediates) can be spectrophotometrically detected based on the best points of the spectra of all involved compounds. Therefore, the appearance of 6-mercapto-8-hydroxypurine, the disappearance of 6-mercatopurine, the formation of 6-thioxanthine and the conversion of 6-mercapto-8-hydroxypurine into 6-thiouric acid can be followed at 240 nm, 256 nm, 308 nm and 340 nm, respectively. The wavelengths referred are the isosbestic points [27]. However, the alternative way through the 6-thioxanthine does not seem to occur. Differently from the hypoxanthine pathway, XOD firstly recognizes the carbon atom number 8 of 6-mercaptopurine, forming 6-mercapto-8-hydroxypurine, and then attacks the carbon number 2, producing the 6-thiouric acid. This behavior may be ascribed to the specific properties of the sulfur atom, which exhibits only a weak tendency to form hydrogen bonds and thus resists transformation into a tautomeric form, analogous to the activated form of hypoxanthine. In the absence of such an activation process, the polarity of the molecule in the ground state becomes the decisive factor for orienting the enzymatic attack, namely, for the primary attachment of a hydroxyl ion.

In our laboratory we have demonstrated that the 6-mercaptopurine oxidative pathway catalyzed by the immobilized xanthine oxidase occurs according to the matrix. Immobilized XOD acts similarly to the free enzyme for the derivatives synthesized with polyacrylamide, polyamide and polyaniline [5]. On the other hand, the derivatives synthesized with chitosan, carboxymethyl cellulose and Dacron (polyethyleneterephthalate) does not recognize the carbon atom number 8 so that the intermediate compound,

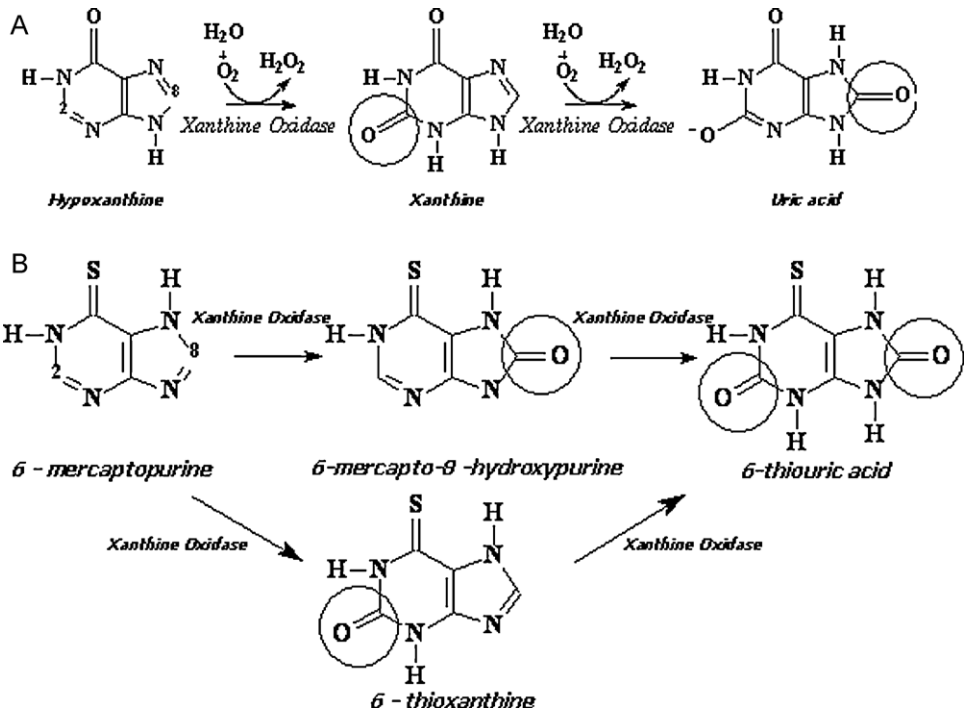

Fig. 3. Oxidative pathway of hypoxanthine (A) and 6-mercaptopurine (B) catalyzed by XOD.

6-mercapto-8-hydroxypurine, becomes the final product and 6-thiouric acid is not formed [5].

Fig. 4 displays the time course of the immobilized XOD on mPOS-PVA followed at the wavelengths 240 nm, 256 nm, 308 nm and 340 nm. These results suggest that the 6-mercaptopurine oxidation pathway is as follows: 6-mercaptopurine → 6-mercapto-8-hydroxypurine → 6-thiouric acid. There is no 6-thioxanthine formation because no absorbance decrease was detected at 308 nm. Therefore, one can assume that the immobilized XOD on mPOS–PVA catalyzes the oxidation 6-mercaptopurine as the free enzyme, and the immobilized preparations synthesized on polyacrylamide, polyamide and polyaniline. This result is also and additional evidence that the events surrounding the immobilized enzyme-substrate microenvironment do not strikingly disturb the enzyme action.

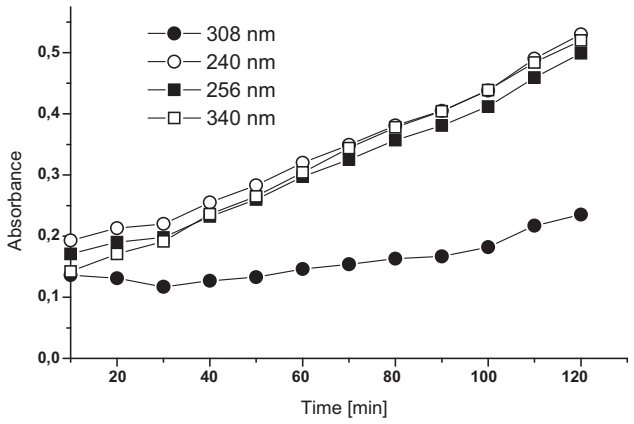

Fig. 4. Time course of the 6-mercaptopurine oxidation catalyzed by immobilized XOD on magnetic POS–PVA. The oxidative pathway was spectrophotometrically followed at 256 nm (consumption of 6-mercaptopurine-■); at 240 nm (production of 6-mercapto-8-hydroxypurine-○); at 340 nm (production of 6-thiouric acid-□) and 308 nm (eventual production of 6-thioxanthine-●).

4. Conclusions

From the above results one can conclude that an attractive water insoluble enzymatic derivative can be synthesized by using a magnetic semi-interpenetrated network composed of polysiloxane and polyvinyl alcohol as a matrix. mPOS–PVA has been already proved to act as a good matrix for antigen immobilization and β-galactosidase. Here, XOD preparation retained approximately half of the free enzyme specific activity; and its apparent Michaelis constant, optimal pH and temperature values were not very different from those of the free enzyme. This preparation also presents the following advantages: easy synthesis, low cost, reusable and can be easily removed from the reaction medium by a magnetic field.

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