Flavin-Grafted Poly(vinyl alcohol): Preparation and Properties

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Received 30 April 2001; accepted 16 December 2001

ABSTRACT: An NAD(P)H oxidase-like activity was found in semisynthetic flavoadducts, prepared from an aldehyde derivative of riboflavin and commercial poly(vinylalcohol) (PVA), previously grafted with aminopropyl side chains. Both water-soluble and water-insoluble beaded preparations were obtained. The products showed a noticeable NAD(P)H oxidase-like activity, converting the nucleotide substrate to its oxidized counterpart $NAD(P)^+$ at the expense of molecular oxygen, the latter being reduced to hydrogen peroxide. In contrast to some "true" β-NAD(P)H oxidases, the PVA adducts do not require the presence of additional flavin adenine dinucleotide (FAD) to work; some properties of the flavoadducts were studied that make these flavoadducts good candidates for technological applications. © 2002 Wiley Periodicals, Inc. J Appl Polym Sci 85: 2471–2477, 2002

Key words: poly(vinylalcohol); flavoenzymes; flavopolymers; β-NAD(P)H oxidase; recycling

INTRODUCTION

The use of enzymes in both soluble and insolubilized forms is widespread in technological processes, and a further growth of enzyme applications in agrofood, pharmaceutical, and biomedical industries can well be expected. Unfortunately, enzymes requiring coenzymes and/or cofactors are often expensive and not very stable, thereby limiting their application in technological processes. This limitation is particularly true with redox enzymes, which use expensive and unstable redox cofactors. Moreover, the regeneration of such cofactors after the redox reaction, to recycle them, is seldom achieved with conventional and inexpensive organic reactions. However, the problem of $NAD(P)^+$ regeneration[1,2] in systems that produce NAD(P)H as the result of dehydrogenation reactions has been solved in part by means of certain coupled redox enzymes, which consume NAD(P)H by reducing a suitable inexpensive substrate. The main problem encountered with these coupled enzymic dehydrogenations resides in the increased complication of the reacting system, taking into account the different operative requirements of the regenerating systems, and

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Journal of Applied Polymer Science, Vol. 85, 2471–2477 (2002)
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eventually the difficult separation of the desired products from large amounts of α-hydroxyacid by-products.

A completely different approach is based on the oxidation of NAD(P)H at the expense of molecular oxygen. Unfortunately, no simple chemical methods are known, leading to a sharp regeneration of the reduced pyridine cofactors, and also under the best conditions a noticeable extent of irreversible cofactor alteration is unavoidable. Some enzymes are known[3,4] to be able to catalyze the reaction

$$NAD(P)H + H^+ + O_2 \rightarrow NAD(P)^+ + H_2O_2$$

Among these enzymes, the β-NAD(P)H oxidase extracted from the extreme thermophilic bacterium *Thermus aquaticus*[5] shows a good specific activity and a very high thermoresistance, being still highly active after a prolonged incubation at 80°C. The enzyme is also active in the covalently immobilized state.[6] On the other hand, the enzyme is not really an oxidase, but rather a NAD(P)H : FAD transhydrogenase, compulsorily requiring the very expensive flavin adenine dinucleotide (FAD) to work. Unfortunately, the comparatively inexpensive flavin mono nucleotide (FMN) cannot be substituted for FAD.

The direct oxidation of NAD(P)H at the expense of molecular oxygen could be achieved with the aid of flavins, properly modified to enhance their catalytic ability. In fact, some flavin derivatives, containing the isoalloxazine ring, grafted on suitable macromolecules, both natural or synthetic, have been described,[7] showing a NAD(P)H oxidase activity. These macromolecular flavoadducts show a certain degree of catalytic activity, more efficient than that of their physiological coenzyme counterparts, i.e., riboflavin, flavin mono nucleotide (FMN) and flavin adenine dinucleotide (FAD).

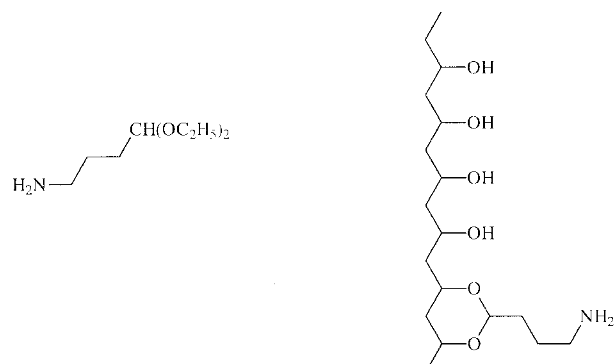

Figure 1 The structures of 4-amino-butyraldehyde diethylacetal, ABA (left), and of a chain portion of 3-aminopropyl–PVA (right).

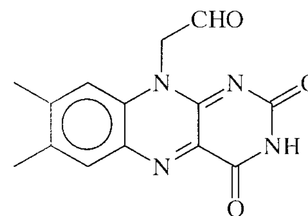

Figure 2 The structure of 7,8-dimethyl-10-formylmethyl-isoalloxazine (flavoaldehyde).

Among the wide variety of macromolecules, potentially useful for isoalloxazine derivatives attachment, poly(vinylalcohol) (PVA) has been chosen as it is a readily available and inexpensive polymer, useful in a wide range of applications owing to its sharp hydrophilic character, high solubility in water, and negligible toxicity. PVA is reactive enough to undergo a number of topochemical modifications, leading to new products with widely varying physicochemical properties. In particular, PVA undergoes acetal formation with several aldehydes, under acidic catalysis. So, insoluble beads of PVA can be obtained with dialdehydes such as terephtaldehyde and glutaraldehyde. Also, upon acetal formation with 4-amino-butyraldehyde diethylacetal (ABA) it leads to a grafted polymer, bearing aminopropyl side chains, which can in turn undergo several further reactions (Fig. 1). So, from reductive alkylation with 7,8-dimethyl-10-formylmethyl-isoalloxazine (flavoaldehyde, Fig. 2), basic flavopolymers can be obtained, bearing the same substituted isoalloxazine nucleus of riboflavin and its coenzyme derivatives FMN and FAD.

The present work shows that a simple isoalloxazine derivative, easily obtainable from riboflavin and quite inexpensive, could be covalently bound to PVA-based synthetic polymers, and the resultant adducts (flavo-PVA preparations) are catalytically active and potentially suitable for practical industrial applications. Moreover, some results concerning the mechanism of activity of the adducts, compared with the behavior of a "true" NAD(P)H oxidase, are reported.

EXPERIMENTAL

Reagents

α-NADH, β-NADH, β-NADPH, FMN, FAD, PVA (average MW 70,000), bovine liver catalase, bovine

plasma superoxide dismutase (SOD), horseradish peroxidase, and yeast alcohol dehydrogenase (ADH), were from Sigma Aldrich Italia, Milan. Sodium cyanoborohydride, sodium (meta)periodate, glutaraldehyde 50% aqueous solution were from Fluka, Buchs, Switzerland. Riboflavin and ABA were from Merck, Darmstadt, Germany. All other chemicals were of the best grade available and were used without further purification.

Quantitation of Acetal Formation Degree

The amount of the acetal formation reaction between PVA and ABA was determined by means of a photometric assay, based on the reaction with a ninhydrin/hydrindantin reagent.[8] In the case of soluble aminopropyl PVA, the polymer was lyophilized and dissolved in water to a suitable concentration prior to the reaction. The crosslinked preparations were directly subjected to the chromogenic reaction, provided that the beads quickly precipitate and do not interfere with the photometric estimation. A standard curve was drawn by using ABA as the reference amine.

Quantitation of β-NAD(P)H Oxidase Activity

Oxidase activity was measured by following the initial rate of NAD(P)H consumption at 340 nm and 20°C, using a Cary 219 spectrophotometer (Varian, Milan, Italy). The assay mixture contained, in a final volume of 1 mL, 0.200 mM NAD(P)H in 20 mM potassium phosphate buffer, pH 7.0. The nature and/or the concentration of the buffer could be modified when required to study the dependence of the oxidase activity on pH, ionic strength, or presence of organic solvents. The same ϵ of $6220M^{-1}$ cm^{-1} was assumed for α-NADH and β-NAD(P)H. For certain experiments, a modified cuvette, bearing a sidearm and a stopcock, was used, to exclude molecular oxygen from the reaction mixtures. The mixtures were therefore subjected to some cycles of degassing and fluxing with pure nitrogen, prior to the addition of flavo-PVA (this latter separately degassed).

The same reagent concentrations were adopted for the polarographic assay, which was carried out by means of an oxygraph equipped with a Clark electrode (Gilson, Villiers-le-Bel, France). For certain polarographic experiments, bovine liver catalase (50 enzyme units) and/or SOD (25 enzyme units) were incorporated into reaction mixtures. For all kinds of measurements, the reaction was initiated by the addition of a suitable amount of enzyme or flavoadduct, as appropriate. Obviously, only the polarographic assay was used in the case of the insoluble flavoadduct prepared starting from the crosslinked PVA.

PVA–Flavoadduct Determinations

This was performed photometrically, by assuming an unchanged ϵ of $11{,}300M^{-1}cm^{-1}$ at 450 nm for riboflavin, FMN, FAD, and both free and coupled flavoaldehyde-9. In the case of the crosslinked adduct, flavoaldehyde loadings were quantitated by difference.

Preparation of 7,8-Dimethyl-10-Formylmethyl-Isoalloxazine (Flavoaldehyde)

This preparation was performed by strictly following a published procedure.[10] The orange microcrystalline product was lyophilized and stored at −20°C until use. All the subsequent operations involving the flavoaldehyde were carried out with exclusion of direct sunlight and with aluminum foil protection whenever applicable.

Preparation of 3-Aminopropyl-Grafted PVA

Some water-insoluble polymer were obtained, grafted with 3-aminopropyl side chains and crosslinked by means of glutaraldehyde (T:C = 10:5), as already described[11] with minor modifications. Other polymers were also prepared, but with omission of the crosslinking agent, glutaraldehyde, and therefore water-soluble materials resulted. In a typical procedure, 10 g of PVA were dissolved with warming in 100 mL water, under magnetic stirring. Then the chosen amount of ABA was added, followed by enough 6 N HCl to bring the pH around 0. The solution was maintained warm for 30 min, then made slightly alkaline by means of a rapid addition of concentrate ammonia solution, and thoroughly dialyzed against water until no chloride was found in the dialyzate (assay with dilute $AgNO_3$ in dilute HNO_3). Various polymers were prepared, both crosslinked and soluble, by varying the ratio between PVA and ABA, i.e., with PVA:ABA of 10:1, 10:0.5, 10:0.2, 10:0.1 (w/v). All the preparations were finally lyophilized.

Coupling of Flavoaldehyde to the Polymers

A common procedure for both soluble and crosslinked aminopropyl PVA preparations was

Table I Yields of 3-Aminopropyl Moieties Grafted on Soluble and Crosslinked PVA

PVA/ABA Ratio	Theoretical Loading (μmol —NH_2/g)	Found Loading (μmol —NH_2/g)	% Yield
Soluble polymer			
10:1.0	533	346	65
10:0.5	274	186	68
10:0.2	112	77	69
10:0.1	57	39	69
Crosslinked polymer			
10:1.0	519	402	77
10:0.5	266	215	81
10:0.2	110	90	82
10:0.1	55	43	81

followed to carry out the covalent coupling: 100 mg of flavoaldehyde were suspended in 5 mL of 0.1*M* potassium phosphate buffer, pH 6.0. To the mixtures, 250 mg of aminopropyl–PVA were added with mixing; the pH was adjusted with 1*M* HCl when required, and 100 mg of sodium cyanoborohydride were then added, followed by a drop of 1-octanol to avoid excessive foaming. The mixtures were gently inverted end-over-end for 20 h in the dark and at 4°C, then centrifuged at 10,000 rpm for 10 min. In the case of the soluble flavopolymers, the supernatants were recovered and passed through a Sephadex G-25 column, 20 $\times$ 2.5 cm, equilibrated with deionized water. The polymer-containing fractions were collected and lyophilized. In the case of the flavoadducts, prepared starting from crosslinked aminopropyl PVA, the supernatants were stored for residual flavoaldehyde quantitation and the polymers washed thoroughly with 0.5*M* NaCl, and then with large volumes of water. The yellow pastes so obtained were dried with suction and lyophilized.

RESULTS AND DISCUSSION

Covalent Coupling of Flavoaldehyde to Aminopropyl PVA

Acetal formation reactions, also comprising crosslinking, are well known for PVA.[11,12] In particular, the grafting with ABA proceeds well, provided that the reaction medium is made sharply acidic to ensure the rapid hydrolysis of the diethylacetal while preventing any undesired condensation side reaction. At the same time, the very low pH promotes the highly efficient condensation between 4-aminobutyraldehyde (as the hydrochloride) and PVA. Glutaraldehyde, when present, does not hinder ABA attachment on PVA; on the contrary, higher loadings were seen, most probably as a result of the insoluble nature of the product, preventing the reverse of acetal formation. The amounts of grafted aminopropyl side chains for the different kinds of PVA are summarized in Table I.

The condensation products between aldehydes and primary amines, known as Schiff bases, are readily hydrolyzed under a variety of experimental conditions, so their usefulness for preparation of conjugates is usually poor. However, they could be readily and irreversibly reduced to the corresponding secondary amines, that are quite stable.

Also, flavoaldehyde reacts with primary amines leading to Schiff bases, which in turn can be reduced to stable nonhydrolyzable derivatives; the method has been successfully applied to the preparation of some catalytically active flavopolymers.[13] The reduction is best accomplished with sodium cyanoborohydride, which operates rather selectively and at pH values neutral or also slightly acidic, whereas the lower cost sodium borohydride is not selective (therefore leading to flavoaldehyde carbonyl reduction rather than Schiff base reduction) and requires high pH values, incompatible with the integrity of the isoalloxazine nucleus.

Also, when operating with a large stoichiometric excess of flavoaldehyde, reductive alkylation of amino groups on PVA is never complete, perhaps for steric reasons. As expected, the more hindered crosslinked polymers could bind lower amounts of flavoaldehyde than could their soluble counterparts (Table II). For the subsequent experiments, only the flavopolymers, grafted with the highest

Table II Flavoaldehyde Loadings on the Different Kinds of 3-Aminopropyl PVA

PVA/ABA Ratio	Theoretical Flavoaldehyde Loadings (μmol/g PVA)	Found Flavoaldehyde Loadings (μmol/g PVA)	% Yield
Soluble PVA			
10:1.0	346	269	77
10:0.5	186	158	85
10:0.2	77	68	88
10:0.1	39	35	89
Crosslinked PVA			
10:1.0	402	289	72
10:0.5	215	163	76
10:0.2	90	70	78
10:0.1	43	34	79

amount of 3-aminopropyl side chains, and therefore bearing the highest concentrations of isoalloxazine derivatives, were used.

The ultraviolet-visible (UV-Vis) spectra of the flavoadducts are essentially identical to those of the original flavoaldehyde as well as to those of the most common flavins, i.e., riboflavin, FMN, and FAD. The reduction of the Schiff base is therefore without consequence as regards the electronic arrangement of the isoalloxazine nucleus, which maintains its typical adsorption maximum at 450 nm (Fig. 3).

Both soluble and crosslinked flavo-PVA, owing to the inert covalent linkages between the polymer backbone and the isoalloxazine moiety, are quite stable under hard conditions such as prolonged boiling in water, washing, or dialysis against water or concentrate salts or buffers. As expected, organic solvents are unable to break the linkage between PVA and the isoalloxazine moiety. On the other hand, the preparations were rather light sensitive, and should be keep well protected against direct sunlight and/or intense artificial lighting. They are also sensitive to even moderate alkaline conditions, whereas they are reasonably stable under neutral or moderately acidic conditions. Dried and frozen preparations can be kept at − 20°C for months without significant changes in their spectroscopic and catalytic properties.

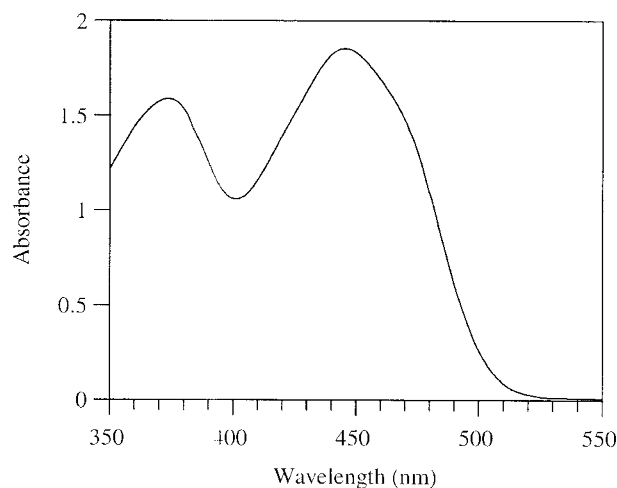

Figure 3 UV/Vis absorption spectrum of soluble flavo-PVA in 20 m*M* potassium phosphate buffer, pH 6.

Both types of flavo-PVA, soluble and crosslinked, show a noticeable NAD(P)H oxidase activity, leading to $NAD(P)^+$ at the expenses of molecular oxygen, which is reduced to H_2O_2. The reduction of the isoalloxazine moiety is independent on the presence of molecular oxygen, as found when working *in vacuo*: the yellow color of the flavopolymers was lost, whereas a drop in NAD(P)H concentration was seen at 340 nm (data not shown). Upon introduction of air, the yellow color was instantaneously restored, indicating a quick reoxidation to the oxidized (resting) isoalloxazine nucleus. Several cycles of degassing, addition of NAD(P)H, and reoxidation with air could be conducted without significant alteration of the polymer, as judged by the complete restoration of the original UV/Vis spectrum. The isoalloxazine reduction is direct, in other words “external” flavin coenzymes are not required for the reaction (difference form the true β-NADH oxidase from *T. aquaticus*, which compulsorily needs additional FAD to work). Moreover, addition of FAD to the reaction mixture adversely affects the reaction, so that in the presence of 1 m*M* FAD the catalytic activity of the polymers is almost abolished (data

Table III Kinetic Parameters for the Flavopolymers[a]

	K_M (μM Reduced Pyridine Nucleotide)	k_{cat} (min^{-1})
Soluble polymer, NADH	61	13.9
Soluble polymer, NADPH	43	1.9
Crosslinked polymer, NADH	75	8.5
Crosslinked polymer, NADPH	58	1.5

[a] These values are based on photometric measurements, performed as described under Experimental.

not shown). This could be explained in terms of electrostatic competition between NAD(P)H and FAD.

The formation of hydrogen peroxide is well demonstrated when the oxidation is conducted in the presence of horseradish peroxidase and a suitable chromogenic substrate of the latter; however, a careful determination of the stoichiometry is prevented by the nonenzymic side reaction of still unreacted NAD(P)H with the quinonoid product of peroxidase action. When NAD(P)H oxidase-like activity is assayed polarographically, in the presence of catalase the rate of oxygen consumption is reduced by 50% with respect to the same assay, when carried out in the absence of catalase. These observations lead to the proposal of a two-step reaction mechanism, formally identical to that of true NAD(P)H oxidases: first, a transhydrogenation reaction takes place between the reduced pyridine nucleotide and the isoalloxazine nucleus; second, the reduced form of the latter is very rapidly reoxidized by molecular oxygen, with stoichiometric release of hydrogen peroxide.

A huge body of experimental and speculative work has been carried out in the last decades to elucidate the reoxidation mechanism of dihydroflavins by molecular oxygen[14]; the effective participation of superoxide as a transient intermediate under physiological conditions is still *sub judice* but its involvement at least to a slight extent is generally accepted. Somewhat surprisingly, in the case of the described flavo-PVA preparations the addition of excess SOD to the reaction mixtures is without action toward the activity patterns, so the participation of superoxide into the reoxidation mechanism could be reasonably excluded.

The kinetic characterization of the flavoadducts (Table III) has shown surprisingly high affinities for NAD(P)H, comparable to those found for *T. aquaticus* β-NAD(P)H oxidase, whereas the reaction rates are sharply lower. In particular, NADPH has a higher affinity but a much lower reaction rate than NADH. This is probably due to the sharper anionic character of the former, which is more easily bound by the flavopolymers, but with a bonding geometry less favorable to the transhydrogenation. Both the electrostatic nature of the interaction and the lack of intervention of the polymer backbone in the NAD(P)H recognition are revealed by the finding that both the flavopolymers cannot distinguish between α- and β-NADH (data not shown).

CONCLUSIONS

Preliminary observations have revealed that the nature as well as the ionic strength of the buffer, chosen for an experimental set, noticeably affects the efficiency of the reaction. It has been also noted that relatively high concentrations of water-miscible organic solvents do not hinder the reaction, but on the contrary, in certain cases enhance the catalytic performances of the polymers. In conclusion, the described method allows the preparation of both water-soluble and insoluble flavin-grafted PVA, which show a noticeable β-NAD(P)H oxidase activity, and are remarkably stable under a wide range of operative conditions. They have a useful combination of high catalytic activity and low price, which render them very suitable for future technological applications.

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