

Stability of alcohol oxidase entrapped into AOT-isooctane reverse micelles

Received for publication, October 20, 2012

Accepted, January 15, 2013

MELANIA-LILIANA ARSENE, MIHAELA DONI*, MARIA-LUIZA JECU

National Institute for Research & Development in Chemistry and Petrochemistry – ICECHIM,
202 Spl. Independentei 060021, Bucharest, Romania

*Corresponding author Mihaela Badea Doni: e-mail: m_badea@yahoo.it

Abstract

Stability of alcohol oxidase from Hansenula polymorpha, during and after the process of entrapment in AOT-isooctane reverse micelles, has been investigated. The study of relationship between pre-micellar components of reverse micelles and catalytic activity/stability of enzyme has revealed a major influence of the apolar solvent upon enzyme stability in time. Even the decrease of enzyme relative activity has been up to about 50% after 2 days of incubation in the presence of isooctane, a prolonged incubation time of 7 days didn't totally inactivate the enzyme. An insignificant influence of the anionic surfactant AOT has been detected, either in aqueous media or apolar media, for the experimental concentrations (maximum 0.7mM, respectively 50 mM). After the complete entrapment of enzyme in reverse micelles, as global system, it has been determined a similar stability profile of alcohol oxidase as in pre-micellar experimental conditions, suggesting a partial inclusion of large octameric molecule of alcohol oxidase in the AOT-consisting micellar interface. For the experimental conditions, solubilization of enzyme molecule in the aggregates of RMs was possible in the range of $w_0 = 10-30$, but a proper enzyme stability has been obtained at the relative high w_0 -values of the range, $w_0 = 20-30$.

Key words: oligomeric enzyme, micellar effects, enzyme inactivation.

Introduction

Reverse micelles (RMs), a representing of the colloidal chemistry, are based on the self-assembling capacity of amphiphilic molecules of surfactants in organic solvent. The RMs comprise orderly and dynamic nano-meter sized aqueous compartments ("water pools") [1] delimited by the head group region of the surfactant, while its alkyl tail(s) are immersed in a nonpolar solvent [2-3]. In large reverse micelles, most of the water resides in a bulk like core and a smaller fraction is influenced by the interface [4]. In small reverse micelles, the interface can perturb the entire water nanopool so that all water molecules deviate from bulklike behavior [5].

The water pools can safely and homogenously accommodate hydrophilic solutes, as biomolecules, due to the surfactant protection from direct contact with the organic solvents [6]. In fact, the concept of enzyme solubilization in reverse micelles is referring to the increase of its solubility in the apolar phase when surfactants are added, at higher concentration than the critical micellar concentration [7].

The solubilization dynamics of molecules by reverse micelles contributes to the general understanding of transport processes through more complex membrane structures [8]. The controlling parameters of the reverse micelles size is the water content, expressed as the ratio between molar concentrations of water and surfactant [6, 9-11].

The micelles swell with water is causing a significant micellar growth; i.e. 0.3% water incorporated reverse micelles were found to be respectively 53%, and 28% larger than the empty micelles respecting maximum size and core radius [12].

Many proteins/enzymes have been studied in reverse micelles, always keeping or even increasing their biological activity. The ability of RMs to entrap proteins and enzymes [13-16]

provides the opportunity for the development of new bio-synthetic reaction, protein purification, etc., in the organic phase. Despite the advantages of enzymatic reaction in reverse micellar system, the denaturation of the enzyme during or after the entrapment in this medium is still a severe problem for practical application. Consequently, the study of the process of (bio)molecules entrapment in micellar aggregates plays an important role in biological [17,18] and industrial processes, being the first step in order to determine the opportunity and feasibility for the use of RMs as media for different enzymes approaches.

In the present paper, we performed the study of time stability of alcohol oxidase (EC 1.1.3.13, alcohol: oxygen oxidoreductase) in reverse micellar system of Aerosol-OT (AOT) in isooctane. AOT, sodium bis(2-ethylhexyl) sulfosuccinate, is a charged surfactant molecule with branched alkyl tails and an anionic sulfonate head group, having a sodium counter ion. The ternary system consisting of AOT surfactant, water, and a nonpolar phase is often used in order to study enzyme activity in RMs [19–21].

Alcohol oxidase, a peroxisomal enzyme, plays a major role in the metabolism of methanol resulting in the formation of formaldehyde [22-24]. It has been detected in several genera of yeasts, such as *Candida*, *Pichia*, and *Hansenula*, that utilize methanol as a sole carbon and energy source. The enzyme has a significant practical role in analytical determination of alcohols and corresponding aldehydes [25-32], hydrogen peroxide [33], as well as in the biosynthesis of various heterologous proteins [34-39].

The major challenge of studying alcohol oxidase in reverse micelles of AOT in isooctane is given by its structure, the enzyme being a homooctameric flavoprotein with eight equal subunits of 83 kDa; each of which contains a flavin adenine dinucleotide (FAD) molecule.

Therefore, the most important objective of the present study was to evaluate the interactions which are responsible for the conservation of the integrity of the enzyme octameric structure in micellar system, the only active form from catalytic point of view. The current research was focused on the influence of pre-micellar effects on enzymatic activity during the entrapment process and the examination of the operational stability of alcohol oxidase, taking into account future applications in biotechnological industry, as alcohols oxidation.

2. Materials and methods

2.1. Reagents

AOT, sodium bis(2-ethylhexyl) sulfosuccinate or dioctyl sodium sulfosuccinate, was purchased from LOBA Feinchemie GmbH (CAS No:577-11-7). Alcohol oxidase (AO) from *Hansenula polymorpha*, catalase free, has been obtained in our laboratory by biosynthesis, followed by extraction, purification and catalase separation [40]. Isooctane and all other chemicals used in this study were of analytical grade. Deionized, distilled water was used throughout.

2.2. Reverse micellar solutions

The reverse micelle solutions were obtained by injecting, with a micro syringe, the reagents stock solutions, into 50 mM solution of the AOT surfactant into isooctane. The reagents stock solution were buffered at a given pH with 50 mM 0.05 M phosphate buffer. The final water content in reverse micelle solutions was calculated by summing all water volumes added in the form of reagent solutions and the desired hydration degree was set by additional injection of same buffer into the micellar solutions.

The mixtures were then vigorously shaken for a few seconds until a completely optically transparent solution was obtained, which confirms the formation of reverse micelles.

All reactions were performed in 20 ml-vials that were closed to ambient air and the reactions were conducted at a constant room temperature.

All concentrations of the reagents reported below (e.g. U/ml or mM) are expressed in terms of the total volume of the reverse micelle systems, except that of water content which is expressed in terms of w_0 .

2.3. Enzyme assay

The standard procedure for alcohol oxidase assay was performed according to Janssen and Ruelius. The initial rate of reaction was recorded at 415 nm [41].

2.4. Experimental conditions for the pre-micellar components

The individual effects of the anionic surfactant (AOT) and apolar organic solvent (isooctane) upon the enzyme activity in aqueous solution were studied in the presence of methanol as a substrate. All experiments have been done in triplicate.

The surfactant was introduced into the aqueous reaction medium as acetone solution due to its low water-solubility and pronounced opalescence of aqueous solution of AOT. Final concentration of acetone in the reaction medium did not exceed 0.33% v/v, and, in a separate experiment, it was verified that this concentration does not influence enzyme activity.

Experimental, the influence of the organic solvent on the alcohol oxidase activity was performed by bringing together 0.1 mL aqueous solution of enzyme with 1 mL isooctane, in such a manner to prevent dispersal of the aqueous solution into separate drops or mixing phases (the surface of contact between the two macroscopic phases was approximately 1 cm²). Samples of aqueous phase (20 μ L) were taken with a Hamilton syringe, without stirring the liquid – liquid biphasic mixture.

The effect of the mixture consisting of both components of the reverse micellar, the anionic surfactant and the apolar solvent, upon the alcohol oxidase activity was performed in a similar manner with the previous experiment, except the fact that the aqueous phase containing the enzyme was in contact with a solution of AOT in isooctane (50 mM). In the absence of a mechanical stirring, the two solutions achieved a two-phase liquid – liquid system, with well-defined phases, unlike reverse micelles which is characterized by pseudo-homogeneity.

Results and Discussions

The study of alcohol oxidase activity in the new reaction medium involves, in first stage, the inclusion of the enzyme in the structure of reverse micelles. In itself, the process of enzyme entrapment in the water-pools of the RMs is not occurring instantaneously, its duration, generally being of 5-10 s. In the period of time between the injection of enzyme solution into the RM solutions and the complete inclusion into the micellar aggregates, the enzyme is found in a medium with minimum 95% apolar solvent, also containing unaggregated molecules of surfactant.

Furthermore, the presence of ionic surfactants, like the anionic surfactant AOT, was suggested to affect the enzymatic activity and stability due to the electrostatic and hydrophobic interactions between the enzyme molecule and the ionic surfactant.

For this reason, there is, more or less, the possibility of an irreversible loss of the enzyme catalytic activity before the existence of aqueous phase as a separate phase and the enzyme inclusion into the internal water cavity of reverse micelles. The inactivation process may be exacerbated by the trend of enzyme aqueous phase to disperse into very fine drops due to the mechanical stirring, preceding the enzyme entrapment into reverse micelles. In this way, it is increased the total interfacial areas and, so, the surfactant accumulation into the aqueous phase is favoured.

Influence of pre-micellar components upon alcohol oxidase stability

The experiments were running in an aqueous medium containing 0.1-0.7 mM AOT, a concentration well above the estimated concentration of AOT remained in the organic solvent in further studies with 50 mM AOT-isoctane reverse micelles. The results have emphasized the insignificant influence of the presence of the anionic surfactant, in the noted range of concentration, regardless the time of incubation (0-4h) of enzyme with acetone solution of AOT. (Fig.1).

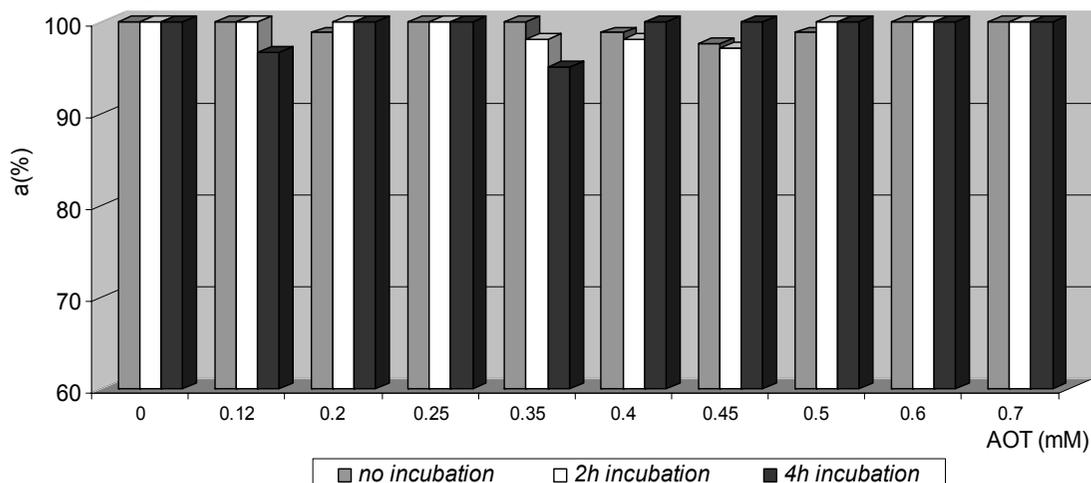


Fig.1. Time effect of AOT concentration on alcohol oxidase activity, during the incubation

In the case of the organic solvent, respectively isoctane, its effect on alcohol oxidase was more drastic, the decrease of enzyme relative activity being up to about 50% after 4-48 h of incubation (Fig.2), but without of total loss of the catalytic capacity even after a prolonged incubation (7 days). A similar influence upon alcohol oxidase activity it has the presence of a solution of 50mM AOT in isoctane, so it has not been identified any potentate influence of one component (anionic surfactant and organic solvent) in the presence of the other one, even at a high AOT concentration, comparable with those selected for working conditions.

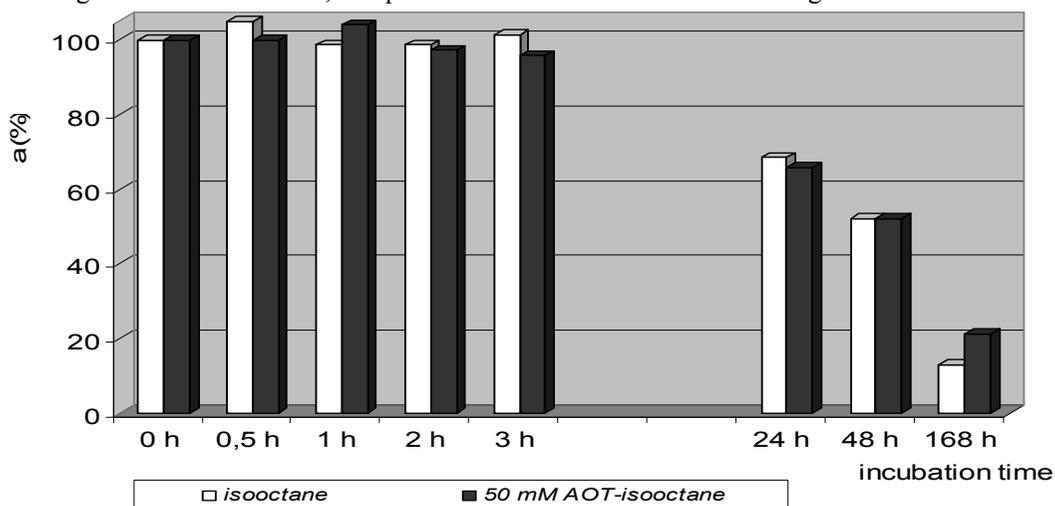


Fig.2. Time effect of isoctane, with or without AOT, on alcohol oxidase activity during the incubation

The similarity between the influence of the mixture composed of the two components of the reverse micelles and of the organic solvent on the enzyme activity leads to the conclusion that the predominant factor on alcohol oxidase inactivation is represented by the enzyme interaction with the apolar solvent and by the anionic surfactant, in an insignificantly extent.

Influence of reverse micellar aggregates upon alcohol oxidase stability

After complete enzyme accommodation inside RMs, the enzyme structure and activity depend on the protein localization inside the aggregates of RMs, mainly involving the size of the reverse micelles and the dimensions of the enzyme [42-43].

RM are characterized by the hydration degree, w_0 , defined as the molar ratio of water and surfactant. This parameter induce most of the structural and physico-chemical characteristics of the reverse micellar environment, being more important even then the absolute quantity of water or surfactant in the system.

In our experiments, the octameric enzyme molecules have been entrapped into small to moderate sizes RMs, corresponding to $w_0=10-30$ (Fig.3), fact experimentally proved by the optic homogeneity of the microheterogeneous system. At high w_0 -values, the pseudo-homogeneity is very labile, probably, due to the escape of the enzyme from the larger aggregates of RMs.

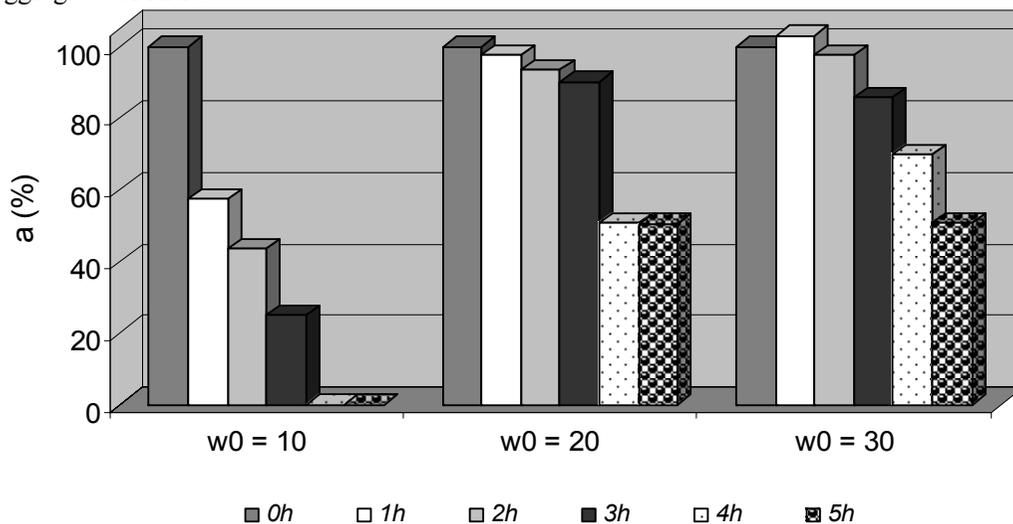


Fig 3. Time stability of alcohol oxidase solubilized in AOT-isooctane reverse micelles

The reverse micelles characterized by the smaller tested w_0 – value ($w_0 = 10$) have the most drastic effect on alcohol oxidase stability by reducing its catalytic activity to 50% after only one hour incubation and complete loss of the enzymatic activity after 4 hours incubation .

The accommodation of alcohol oxidase in much larger AOT-isooctane reverse micelles have evidenced a slower decrease of enzyme stability, the relative activity recovered after 4 h of incubation being, respectively, 50% for $w_0=20$ and 70% for $w_0=30$. During the 5 hours of monitoring of enzyme stability in moderate to large size RMs, alcohol oxidase is keeping its activity at least at the level corresponding to one hour of accommodation in $w_0=11$ RMs.

A comparison between the stability of alcohol oxidase during the entrapment process, through the effects of the individual components, and after the enzyme accommodation inside the aggregates of RMs has been done. The experimental data show that, after complete entrapment, enzyme stability is affected in the same way as in the presence of the apolar solvent, with or without AOT, suggesting that alcohol oxidase is not efficiently protected against solvent action by the aid of the well-defined structure of the micellar interface.

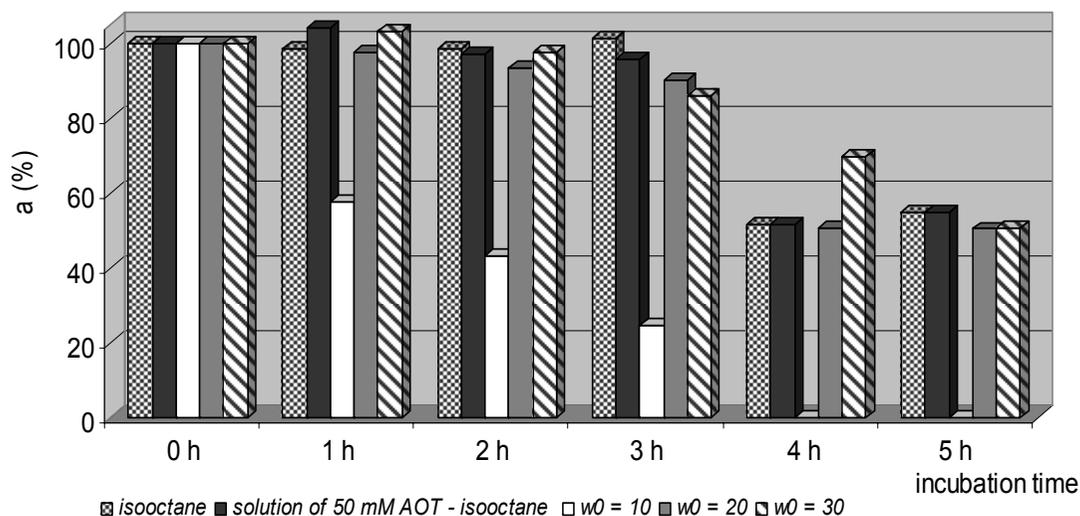


Fig.4. Comparison between premicellar and different micellar conditions upon time stability of alcohol oxidase

The similarity between the effects of prolonged solvent and AOT-aggregates of RMs action upon alcohol oxidase stability in time leads to an hypothetical model of enzyme localization in RMs, according to which this enzyme is partially included in the structure of AOT micellar interface of the RMs, probably the most significant part of enzyme molecule being exposed to the solvent action.

The experimental model proposed for the accommodation of alcohol oxidase inside AOT-isooctane RMs, based on the premicellar/micellar effects on enzyme activity, have been analyzed according to the literature data referring to the size of the enzyme and of the AOT-isooctane RMs.

In the convenient w_0 -range for enzyme stability ($w_0 = 20-30$), the inner cavity core radius (r_m) of RMs is 3.4-4.9 nm, established by the equation [20,44-46] for the direct relation between these two parameters:

$$r_m (\text{\AA}) = 4 + 1.5 \times w_0$$

As the reported size of the octameric molecule of alcohol oxidase is (12 x 12 x 12) nm, the mean protein radius (r_p) can be estimated to about 6 nm [47]. Taken together these data, the hypothesis of partial inclusion of alcohol oxidase into the structure of AOT micellar interface, in the w_0 -range =10-30, seems to be confirmed.

Conclusions

Unlike other oligomeric enzymes, the activity of alcohol oxidase is preserved even in the presence of relatively high concentrations of AOT in the environment, keeping intact its catalytic activity at a concentration which is over 100 times higher than the concentration, respectively 5.5 μM AOT, reported to total and irreversible inactivate lactate dehydrogenase [48]. As neither surfactant concentration, or its prolonged action does not affect enzyme activity, is obvious the lack of interaction between the AOT surfactant and the active catalytic centre of alcohol oxidase, although its complex quaternary structure.

Among the pre-micellar components, the significant influence upon alcohol oxidase stability has the apolar solvent, respectively isooctane. However, even in its presence, the

catalytic activity was kept for a significant period of time after the complete enzyme entrapment into reverse micelles.

The accommodation of the large alcohol oxidase molecule in the aggregates of RMs is possible for AOT-isooctane reverse micelles in the range of $w_0 = 10-30$, but based on these experimental results, it may be affirmed that a proper enzyme stability is realized at relative high w_0 -values of the range, $w_0 = 20-30$. Entrapment of the enzyme into small RMs ($w_0 = 10$) leads to a more rapid deterioration of its catalytic activity, probably caused by the unfolding of the active oligomeric protein into inactive monomers. Time stability of alcohol oxidase in RMs is enhanced along with the increase of the hydration degree, respectively the size of the reverse micelles, in the range of stability of this special type of water-in-oil microemulsion. .

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