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Stabilisation of oxygen-labile nitrilases via co-aggregation with poly(ethyleneimine)

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Abstract

Three different nitrilases lost 50–100% of their activity upon exposure to oxygen for 40 h, whereas their activity was fully retained under an argon atmosphere. This effect is ascribed to a reaction of oxygen, presumably with the catalytic cysteine residue.

Co-aggregates of the nitrilases and high MW poly(ethyleneimine) were prepared by precipitation; these were physically very stable and protein release was not observed. The PEI co-aggregates of the nitrilases were much more oxygen-tolerant than the freely dissolved enzymes. The nitrilase from *Pseudomonas fluorescens* EBC 191, in particular, retained its full activity upon exposure to oxygen for 40 h. This result is ascribed to a low local oxygen concentration in the biocatalyst, due to the salting-out effect of the polycationic PEI.
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1. Introduction

Nitrilases are very promising enzymes from a synthetic point of view because they selectively hydrolyse nitriles into the corresponding acids. Nitrilases with very different substrate specificities have been isolated from plants, fungi and bacteria. All nitrilases possess several conserved sequence motifs and invariably contain a cysteine residue in the catalytic center [1–5]. Nitrilases are well-known to be oxygen-sensitive, which is commonly ascribed to autoxidation of these cysteine residues.

The inactivation of nitrilases by oxygen can be avoided, in principle, by using an oxygen-free atmosphere, although this would restrict the reactor design. The addition of bio-compatible electrolytes in high ionic strengths could also lower the oxygen concentration in the reaction medium [6–9] and, hence, reduce the rate of enzyme inactivation. Such an approach may cause difficulties with hydrophobic substrates, however.

Another possible solution would be to surround the enzyme molecules with a hydrophilic shell, to reduce the oxygen concentration in the enzyme environment only. Thus, adsorption of enzymes on poly(ethyleneimine) (PEI) coated supports has been proposed to reduce the inactivation of enzymes, on the basis of the salting-out effect of these cationic polymers [10]. The creation of a highly hydrophilic shell around immobilised enzymes has proven to be quite effective in the stabilisation of enzymes against the deleterious effects of organic solvents. It has been suggested that this effect is caused by the decrease of the organic solvent concentration around the enzyme molecules [11,12]. Although the system was very effective, its preparation required many steps and was fairly complex.

Recently, the preparation of cross-linked enzyme aggregates (CLEAs) has been proposed as a very simple and effective method to prepare immobilised biocatalysts [13–15]. This latter method does not require the use of pre-existing supports or pure protein samples [16,17]. Furthermore, the method permits the co-immobilisation of proteins and polymers, such as PEI [12,16]. Coprecipitation has previously been used to co-immobilise enzyme molecules, penicillin G acylase and glutaryl acylase, for example, with “polymeric salts” (PEI–dextran sulphate) and has greatly improved the enzymes’ resistance to the action of organic solvents [18,19].

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In the present paper, we report the effects of oxygen on the operational stability of three different nitrilases and we will demonstrate their stabilisation by co-aggregation with PEI.

2. Experimental

2.1. Materials

The nitrilase from *Pseudomonas fluorescens* EBC191 was heterologously produced in *Escherichia coli* JM109 (piK9) and a cell-free abstract containing 20 mg protein/mL was obtained as described [20]. The nitrilases 104 and 106 were from Biocatalytics Inc. (Pasadena, CA). Dextran 100–200 kDa was from Serva Feinbiochemica and was oxidized as described previously [21]. Poly(ethyleneimine) 60 kDa was from Sigma Chemical Co. Sodium metaperiodate was from Janssen Chimica. All other reagents were of analytical purity.

2.2. Assay of nitrilase activity

The activity of the nitrilase preparations was assayed in the hydrolysis of mandelonitrile. The reaction mixture consisted of 1 mL of 0.5 M sodium phosphate (pH 7.5), 100 μL of a 0.2 M racemic mandelonitrile solution in methanol and an appropriate amount of the enzyme preparations. The mixtures were stirred at 20 °C (*P. fluorescens* nitrilase) or 32 °C (nitrilases 104 and 106). After 30 min, a sample of 200 μL was taken and added to 200 μL of 100 mM HCl to stop the reaction.

Samples were analysed by HPLC on a 4.6 mm × 50 mm Merck Chromolith RP-18e column, mobile phase ACN–water 10:90 containing 0.1% of trifluoroacetic acid at 1 mL/min, with UV detection at 225 nm. One unit (U) will hydrolyse 1 μmol of mandelonitrile per min.

The specific activities of the nitrilase preparations were as follows: *P. fluorescens* nitrilase, 4.25 U/mg protein; nitrilase 104, 0.05 U/mg; nitrilase 106, 0.51 U/mg.

2.3. Preparation of the aggregates

A 2 mL of 0.5 M sodium phosphate (pH 7.5) was mixed with 2 mL of polyaldehyde dextran or polyethyleneimine solution (10% w/v in water at pH 7). The nitrilase preparations were added 1 mL of a cell extract containing the *P. fluorescens* EBC 191 nitrilase (85 U/mL) or 1 mL of a 50 mg/mL solution of nitrilase 104 or 106. Finally, 5 mL of 1,2-dimethoxyethane were added. The suspensions were gently stirred for 16 h at 4 °C, centrifuged and washed five times with 40 mL water. The polyaldehyde dextran CLEAs were reduced with 40 mL of sodium borohydride (1 mg/mL) in 0.1 M sodium hydrogen carbonate at pH 8.5 for 45 min while stirring at 4 °C [21].

A leak test was performed on the polyaldehyde dextran-crosslinked and PEI aggregates as follows: the preparation (1 g) was suspended in 3 mL of 25 mM sodium phosphate at pH 7, stirred at the reaction conditions during 15 min and the activity of the supernatant and the particles was measured.

2.4. Enzyme stability under different atmospheres

The experiments were performed using a controlled atmosphere of oxygen or argon. The enzyme preparations were suspended in 0.1 M sodium phosphate at pH 7. In a typical experiment 5 mL of the different preparations were magnetically stirred at 1200 rpm. At this speed the aggregate particles remained stable and no activity was detected in the supernatant. At different time intervals, samples were taken and the enzyme activities measured. All the experiments were performed at 20 °C.

3. Results and discussion

3.1. Effects of oxygen on the stability of soluble nitrilases

The freely dissolved nitrilases were incubated under argon or oxygen atmospheres (Fig. 1). The enzymes hardly lost any activity during 40 h of incubation under argon atmosphere. In an oxygen atmosphere under otherwise similar conditions, in con-

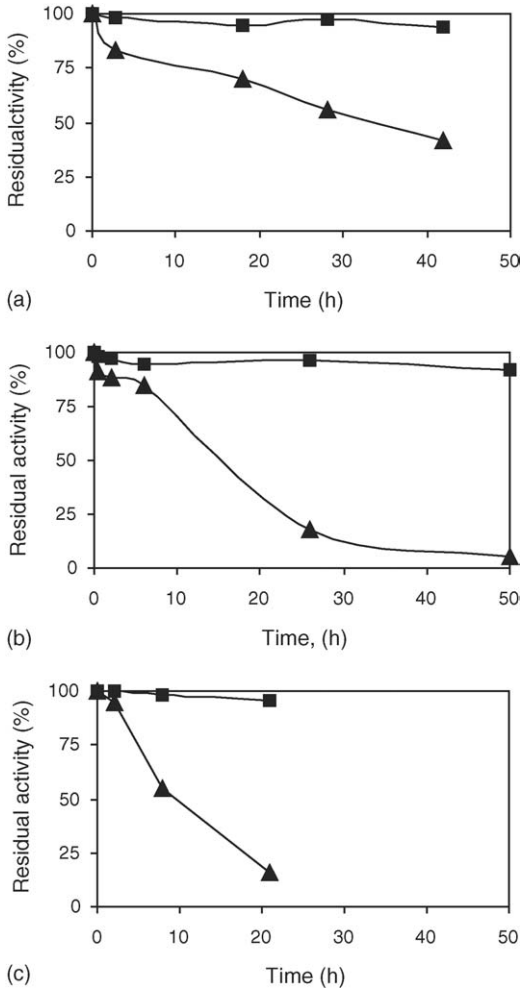

Fig. 1. Effects of oxygen (▲) and argon (■) atmosphere on the stability of dissolved nitrilases: (a) nitrilase from *Pseudomonas fluorescens* EBC 191; (b) Biocatalytics nitrilase 104; (c) Biocatalytics nitrilase 106. Conditions: 0.1 M sodium phosphate pH 7 and 20 °C.

trast, all three nitrilases were rapidly inactivated. The nitrilase from *P. fluorescens* EBC191 exhibited the highest stability and retained approximately 50% of its original activity after 40 h of incubation. The Biocatalytics nitrilases 104 and 106, in contrast, were almost completely inactivated within 24 h. This outcome confirms that the presence of oxygen is highly detrimental for these nitrilases. It is to be expected that the activity loss will be approximately five times slower upon exposure to air (21% oxygen), which still would be an obstacle to enzyme recycling and reuse.

3.2. Immobilisation of nitrilases as stable aggregates

The three nitrilases were immobilised as aggregates, which either were stabilised as CLEAs by cross-linking with dextran polyaldehyde [21] or, alternatively, by coprecipitation with PEI.

With the nitrilase from *P. fluorescens* EBC 191, 50% (dextran polyaldehyde CLEA) and 55% (PEI co-aggregate) of the initial activity was recovered in the immobilisate (see Table 1). With the nitrilase 104 the activity recovery was 60% and 64% with polyaldehyde and PEI, respectively; immobilisation of nitrilase 106 resulted in significantly lower activity yields (28% and 33%, respectively). Remarkably, both procedures resulted in very similar activity recoveries in these unoptimised experiments.

The PEI co-aggregates, when subjected to a leak test, did not release enzyme activity into the supernatant and no activity loss was found upon repeated use in hydrolysis experiments, leading us to conclude that no protein is released. Hence, co-aggregation with PEI effectively entraps the nitrilase molecules in the PEI network, without covalent attachment being necessary.

3.3. Effects of oxygen on the immobilised nitrilases

The dextran polyaldehyde CLEAs of the three nitrilases rapidly lost activity when exposed to oxygen, similar to the freely dissolved preparations (see Fig. 2); only the nitrilase 106 CLEA showed a modest gain in stability. The PEI co-aggregates, in contrast, were much more oxygen-resistant. The preparation of the nitrilase from *P. fluorescens* hardly lost activity upon exposure to oxygen for 48 h (Fig. 2a). Nitrilase 104, which is inherently more oxygen-sensitive, lost approximately 30% of its starting activity over 24 h and maintained this level for the next 24 h (Fig. 2b). The PEI co-aggregate of nitrilase 106 also showed an oxygen resistance that was much improved in comparison with the dissolved enzyme as well as the CLEA (Fig. 2c).

Table 1
Recovery of nitrilase activity in the aggregates with different nitrilases and stabilisers[a]

Nitrilase	Aggregate stabiliser, activity recovery in %	
	Dextran polyaldehyde	PEI
Pseudomonas fluorescens EBC 191	50	55
Biocatalytics 104	60	64
Biocatalytics 106	28	33

[a] Activity recoveries are relative to the initial activity of the soluble enzyme.

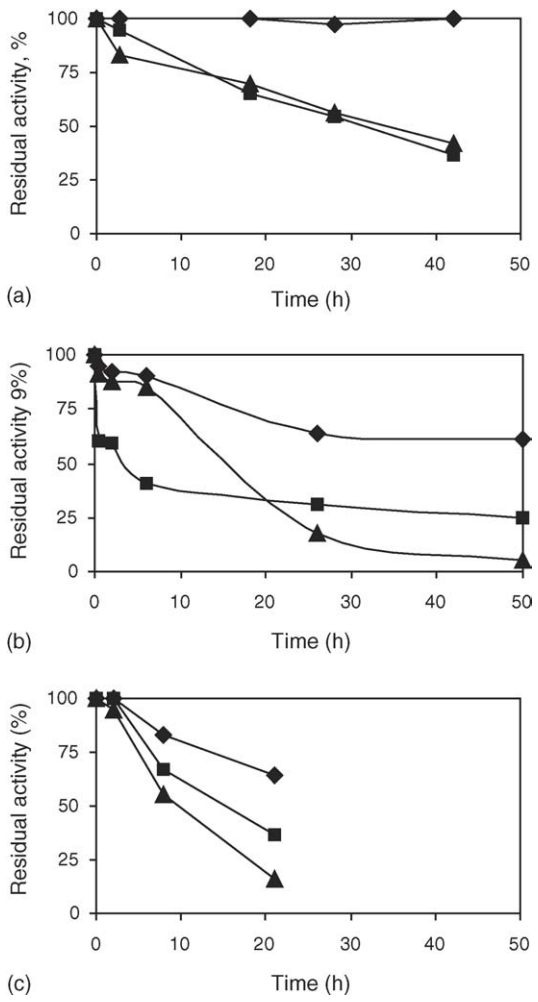

Fig. 2. Activity loss of dissolved nitrilase (▲, data from Fig. 1), polyaldehyde dextran CLEA (■), co-aggregate with PEI (♦) in oxygen atmosphere: (a) nitrilase from *P. fluorescens* EBC 191; (b) Biocatalytics nitrilase 104; (c) Biocatalytics nitrilase 106; conditions as in Fig. 1.

Presumably, the effect of PEI on the oxygen resistance is caused by the high local ionic strength in the biocatalyst particles, which locally reduces the oxygen concentration due to the salting-out effect. Employing the salting-out effect of PEI may be a general strategy for protecting enzymes against deactivation by oxygen or other hydrophobic deactivating agents.

4. Conclusion

We have shown that three nitrilases, when co-aggregated with poly(ethyleneimine), form stable preparations that do not require any additional cross-linking. The PEI co-aggregates also showed a much better oxygen resistance than the dissolved enzymes or cross-linked enzyme aggregates.

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