ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 11 (2001) 607–612

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Cross-linked crystals of hydroxynitrile lyase as catalyst for the synthesis of optically active cyanohydrins

David Costes *, Ernst Wehtje, Patrick Adlercreutz

Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, PO Box 124, S-221 00 Lund, Sweden

Abstract

Purified hydroxynitrile lyase (HNL) from *Manihot esculenta* was crystallized by the sitting-drop vapour-diffusion method. The bipyramidal crystals formed (10–20 μm) were cross-linked with different amounts of glutaraldehyde and used as biocatalyst for the synthesis of optically active cyanohydrins. The cross-linked crystals were more stable than Celite-immobilized enzymes when incubated in organic solvents, especially in polar solvents. After six consecutive batch reactions in dibutylether, the remaining activity of the cross-linked crystals was more than 70 times higher than for the immobilized enzymes. Nevertheless, the specific activity of the cross-linked crystals (per milligram protein) was reduced compared to the activity of immobilized enzymes. The product enantiopurity was independent of the type of enzyme preparation used.

Keywords: Hydroxynitrile lyase; Crystal; Cross-linking; Activity; Stability

1. Introduction

Hydroxynitrile lyases (HNLs) are used for the asymmetric synthesis of intermediates for pharmaceuticals and agrochemicals [1,2]. Successful processes have been developed and yield cyanohydrins of high enantiopurity [3,4]. Dissolved HNLs are usually used in water or two-phase systems while HNLs immobilized on solid supports are preferred when working in organic solvents [5,6]. The enzymatic activity is relatively high and a complete substrate conversion can be reached in most cases within a couple of hours [3–6]. Nevertheless, the stability of the enzyme preparation is relatively low, both in aqueous and organic media [7–9]. This reduced stability results in poor enzyme productivity [10] and is a major obstacle to the recycling or to the use of the enzyme in a continuous process. Therefore, the operational stability of the enzyme needs to be improved before HNLs become a fully competitive biocatalyst for industrial applications.

Cross-linked enzyme crystals appear to be an attractive alternative to the classical enzyme preparations [11]. They are described as more stable, active, and enantioselective than their respective solubilized form [12]. Unfortunately, the number of enzymes commercially available as cross-linked crystals is very limited [13].

We have developed the preparation of cross-linked crystals of HNL in an attempt to improve the enzyme stability under working conditions. The sta-

* Corresponding author. Tel.: +46-46-2224841; fax: +46-46-2224713.

E-mail address: david.costes@biotek.lu.se (D. Costes).

1381-1177/01/$ - see front matter
PII: S1381-1177(00)00057-6

bility, activity, and enantioselectivity of the cross-linked crystals in organic solvent are presented.

2. Materials and methods

2.1. Chemicals and enzyme

Purified recombinant HNL from *Mahinot esculenta* (cassava) (E.C. 4.1.2.37), 4840 u/ml, 46 mg protein/ml was donated by the Institute of Organic Chemistry, University of Stuttgart (Stuttgart, Germany) [14].

3-Phenylpropionaldehyde (hydrocinnamaldehyde) (95%) and acrolein (2-propenal) (90%) were obtained from ICN Biochemicals (Aurora, OH, USA), 2-methyl-2,4-pentanediol (MPD) (99%) from Acros (Geel, Belgium). Benzaldehyde (99%), *N,N*-dimethylformamide (DMF) (99.5%), and Celite (0.2–0.5 mm) were purchased from BDH Chemicals (Poole, England), polyethylene glycol (PEG) 8000 from Union Carbide (Antwerpen, Belgium). Potassium cyanide (97%), sodium citrate (99%), acetone (99%), and hexane (99%) came from Merck (Darmstadt, Germany). Glutaric dialdehyde (glutaraldehyde) 50 wt.% solution in water and dibutylether (99%) were purchased from Aldrich (Steinheim, Germany), tertbutyl-methylether (MTBE) (99.5%) and ethyl acetate (99.8%) from Lab scan (Dublin, Ireland). Hydrogen cyanide (HCN) was prepared in the laboratory as described in Ref. [6]. Other chemicals used were of analytical grade. The substrates were used without further purification.

2.2. Immobilization

The HNL solution was diluted with 50 mM citrate buffer, pH 5.4. The resulting enzyme solution (1.84 mg protein/ml) was then added to the solid support (Celite). The preparation was dried for at least 12 h under vacuum (20 mbar). The enzyme preparation contained 3.7 mg protein/g Celite.

2.3. Crystallization

The crystallization procedure was adapted from Lauble et al. [15]. The crystallization solution contained 5% PEG 8000 and 25% MPD in 100 mM sodium citrate pH 5.4. The sitting-drop vapour-diffusion method was used [16]. The enzyme solution (25 μl, 46 mg protein/ml) was mixed with the crystallization solution (25 μl) in a 1.5 ml open vial. The vial was placed to equilibrate at 23°C in a 10 ml closed reservoir containing 2 ml crystallization solution. The bipyramidal crystals appeared within 5 days and have a size of 10–20 μm.

2.4. Cross-linking

The crystals were mixed with 500 μl acetone solution with varying amount of 50% aqueous glutaraldehyde. The cross-linking was carried out by shaking the mixture (300 rpm) for 5 min. The acetone solution was removed by centrifugation. The cross-linked crystals were washed twice with 500 μl acetone and dried under vacuum for 10 min.

2.5. Activity assay

The Celite-immobilized enzymes (20 mg) added of 15 μl citrate buffer (50 mM, pH 5.4) or the cross-linked crystals (1.15 mg protein) added of 10 μl citrate buffer were mixed with 1.3 ml dibutylether solution containing 200 mM benzaldehyde. The reaction was started by the addition of 15 μl HCN (final concentration in the reaction mixture, 300 mM) and performed on a shaker (1000 rpm) at 25°C. Samples (50 μl) were withdrawn at different stages of conversion.

2.6. Stability measurement

The Celite-immobilized enzymes (20 mg) or the cross-linked crystals (1.15 mg protein) were incubated on a shaker (1000 rpm) for 24 h at 25°C in 1.3 ml buffer saturated solvents (hexane, MTBE, or ethyl acetate) added of 50 μl citrate buffer (50 mM, pH 5.4) or in 1.3 ml a DMF solution containing 50% buffer. After incubation, the solution was removed by centrifugation. The enzyme preparation was washed three times with 500 μl dry MTBE then dried under vacuum for 30 min.

The remaining activity was calculated by dividing the initial reaction rate of the incubated enzyme

preparation by the initial reaction rate of a non-used enzyme preparation measured under the same reaction conditions.

A non-used enzyme preparation was washed three times with 500 μl dry MTBE and dried under vacuum for 30 min. The activity of this preparation was the same (+/− 5%) than the activity of a same amount of non-washed enzyme. This indicates that the washing procedure used does not influence the activity measurement.

2.7. Batch reaction

The Celite-immobilized enzymes (20 mg) added of 15 μl citrate buffer (50 mM, pH 5.4) or the cross-linked crystals (9.2 mg protein, cross-linked with 1% glutaraldehyde) added of 10 μl citrate buffer were mixed with 1.3 ml dibutylether solution containing 200 mM benzaldehyde. The reaction was started by the addition of 15 μl HCN (final concentration in the reaction mixture, 300 mM) and performed on a shaker (1000 rm) at 25°C. Samples (50 μl) were withdrawn at different stages of conversion. The reaction was stopped after 1 h. The solution was removed by centrifugation. The enzyme preparation was washed three times with 500 μl dry acetone then dried under vacuum for 30 min. This procedure was repeated seven times using the same enzyme preparation.

2.8. Analysis

HCN excess was removed from the samples under reduced pressure (20 mbar) for 20 min. The cyanohydrin derivatisation procedure was adapted from Effenberger and Schwämmle [17]. Acetic anhydride (50 μl), pyridine (10 μl), and dichloromethane (500 μl) were added to the sample. After at least 3 h at room temperature, the enantiomeric excess and the conversion were determined directly from the reaction mixture by gas chromatography. A GC-14A from Shimadzu (Kyoto, Japan) equipped with a flame ionisation detector was used together with a FS-Cyclodex beta-I/P column (0.25 mm inner diameter, 50 m long) from CS-Chromatographie Service (Langerwehe, Germany). The carrier gas was helium. The column temperature was 115°C for 15 min then increased at a rate of 15°C/min to 160°C to separate the benzaldehyde cyanohydrin derivatives. Column temperatures of 160°C and 100°C were used to separate phenylpropanal and acrolein cyanohydrins, respectively. The degrees of conversion were calculated from the relative peak areas of aldehyde and cyanohydrin derivative.

3. Results and discussion

3.1. Influence of cross-linking

To the best of our knowledge, the cross-linking of dissolved or crystallized proteins has been, to date, exclusively performed in aqueous buffer [18–21]. A reaction time of 1 h with an aqueous solution of 1% glutaraldehyde is the usual requirement to obtain an optimal cross-linking [13]. By diluting the glutaraldehyde in dry acetone instead of aqueous buffer, the cross-linking time and cross-linking agent concentration needed to prepare stable cross-linked crystals can be significantly reduced. The cross-linking of the HNL-crystals took only 5 min in an acetone solution containing from 0.05% to 1% glutaraldehyde (Fig. 1). At 1% concentration, the cross-linked crystals

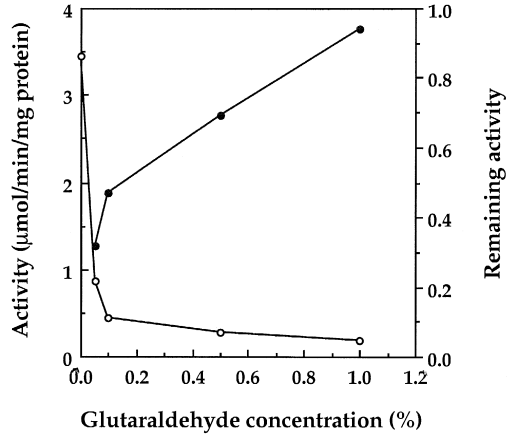

Fig. 1. Effect of the glutaraldehyde concentration used for cross-linking on the initial activity of the HNL cross-linked crystals prepared (○) and on their stability (●). The remaining activity was measured after 24 h incubation in ethyl acetate as described in Section 2.6.

obtained were yellow-brownish and most of the activity was lost. This means that the degree of cross-linking was already excessive after this short reaction time. On the other hand, these highly cross-linked crystals were extremely stable, more than 90% of their activity was retained after incubation in ethyl acetate for 24 h. Increasing the cross-linking agent concentration (or the reaction time) yield cross-linked crystals of improved stability. Unfortunately, the cross-linked crystal activity will decrease exponentially with the degree of cross-linking. In acetone, cross-linking with 0.1% glutaraldehyde for 5 min seems to be a good compromise between gain of stability and loss of activity.

3.2. Stability in solvents

Cross-linked crystals (0.1% cross-linked) and immobilized HNLs were incubated in different organic solvents. Both preparations were very stable in hydrophobic solvents such as hexane (log P: 3.5) (Fig. 2) [22]. However, the stability was decreased when moving toward polar solvents. In DMF (log P: -1.0), although the cross-linked crystals retained around 40% of their initial activity after incubation, the activity of the immobilized HNLs was almost completely lost. Similar behaviour was noticed when incubated in ethyl acetate (log P: 0.68). As expected, MTBE, a solvent of moderated polarity (log P: 1.4), had an intermediate deactivation effect. The gain of stability when using cross-linked crystals instead of immobilized enzymes in organic media is considerable in polar solvents. However, this gain is reduced in hydrophobic solvents, since immobilized enzymes there are also stable.

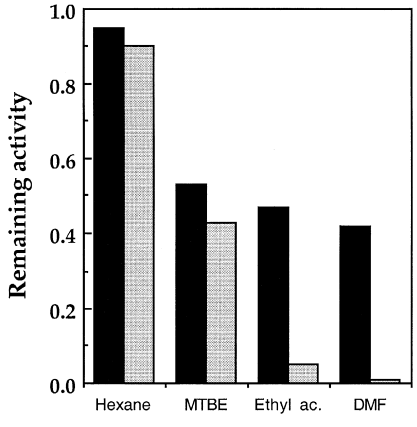

Fig. 2. Remaining activity of the HNL cross-linked crystals (■) (cross-linked with 0.1% glutaraldehyde) and Celite-immobilized HNLs (□) after incubation for 24 h in buffer saturated hexane, MTBE or ethyl acetate (ethyl acetate) added of 4% v/v buffer or in a DMF solution containing 50% buffer as described in Section 2.6

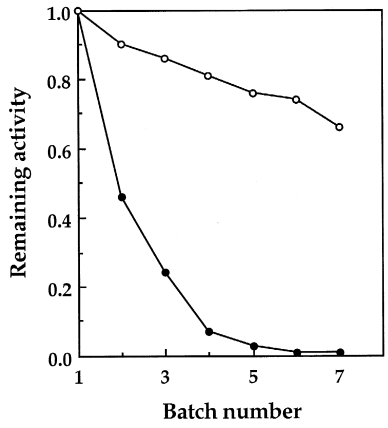

Fig. 3. Remaining activity of the HNL cross-linked crystals (○) (cross-linked with 1% glutaraldehyde) and Celite-immobilized HNLs (●) after batch reactions in a dibutylether solution containing 200 mM benzaldehyde and 300 mM HCN as described in Section 2.7. The reaction time in each batch was 1 h.

3.3. Operational stability

Cross-linked crystals (1% cross-linked) and immobilized HNLs were used under working conditions in seven successive batch reactions. Each reaction was run for 1 h. At the end of this reaction series, almost all the activities of the immobilized enzymes were lost (less than 1% activity left) while the cross-linked crystals retained around 70% of their initial activity (Fig. 3). It should be noticed that a non-negligible amount of cross-linked crystals might have been lost during the repeated washing procedures, consequently, the real remaining activity for the cross-linked crystals was certainly higher than the estimated. In an HNL-catalyzed reaction, the enzyme stability is affected by the organic solvents but is also sensitive to the substrates (aldehyde

and HCN) [9]. The cross-linked crystals were found more resistant to the substrates deactivation than the immobilized HNLs.

3.4. Activity

The initial specific activity of non-cross-linked HNL crystals was measured in dibutylether. The measurement, corresponding to the activity at 0% glutaraldehyde in Fig. 1, was possible since the non-cross-linked crystals did not dissolve immediately when introduced in the reaction mixture. This activity represents only 8% of the initial activity of immobilized HNLs. Diffusion limitation effects, which could have explained such a reduced activity, seem to be excluded in the case of microcrystals [10,11]. Margolin [11] suggests that depending of the conditions, the same enzyme can be crystallized under different conformations. In some of these conformations, the active site of the enzyme can be hardly accessed by the substrate. An inappropriate enzyme conformation in the cross-linked crystal could therefore result in a lower activity. Secundo et al. [23] have recently reported low activities for cross-linked lipase crystals used in transesterification reactions. The authors suggest that it is certainly due to reduced substrate accessibility to the active site. In their crystallographic studies of HNL from *M. esculenta*, Lauble et al. [15] have observed that the active site of the enzyme was obstructed. The crystals used in the present study were prepared with the same enzyme and from crystallization conditions similar to the one used by Lauble et al. [15]. Therefore, it is highly possible that a restricted access to the active site in the HNL-crystals formed is the cause to the low activity observed.

The specific activity of the cross-linked HNL-crystals in dibutylether was, depending of the substrate and the degree of cross-linking, from one to two orders of magnitude lower than the activity of immobilized HNLs. The cross-linking of the crystals with glutaraldehyde was obviously the cause of further activity decrease (Fig. 1). It is therefore necessary, in order to preserve the enzyme specific activity, to limit the cross-linking to the minimum needed to provide a reasonable stability to the crystals.

Margolin [11] suggests also that substrates with a considerable size could have difficulties to penetrate the crystal channels and thus would lead to a reduced activity. This hypothesis is ruled out in our case, since a very small substrate molecule like acrolein did not give, by comparison to its activity with immobilized enzymes, a better activity than bigger molecules such as phenylpropanal or benzaldehyde.

3.5. Enantioselectivity

The enantiomeric excess of the cyanohydrins produced with the cross-linked crystals was identical to the enantiomeric excess obtained with immobilized or dissolved HNLs. An enantiomeric excess higher than 99% was measured with benzaldehyde as substrate. However, to use cross-linked crystals did not allow improving the limited enantiopurities usually observed with certain substrates [6].

4. Conclusions

For the first time, cross-linked crystals of HNL have been used as catalyst for the asymmetric synthesis of cyanohydrins. The cross-linked crystals prepared were obviously more stable than the immobilized enzymes. The choice of cross-linked crystals as catalyst appears particularly interesting if the reaction has to be performed in polar solvent or under harsh conditions. Nevertheless, crystallization and cross-linking induce an important loss of specific activity. Therefore, a compromise between improving the enzyme stability and keeping a high specific activity has to be made when selecting the form of biocatalyst to be used.

Acknowledgements

We thank Dr. Hanspeter Lauble for the helpful discussion and Dr. S. Förster and H. Wajant from the Institute of Organic Chemistry, University of Stuttgart, for the gift of the enzymes. We are grateful to the European commission (projects BIO4-CT97-5126 and BIO4-CT96-0112) and the Swedish Research Council for Engineering Sciences (TFR) for financial support.

References

[1] F. Effenberger, Chimia 53 (1999) 3.
[2] M. Schmidt, H. Griengl, Top. Curr. Chem. 200 (1999) 193.
[3] F. Effenberger, H. Wajant, J. Roos, S. Foerster, Degussa, Frankfurt, Germany, 1999, US patent 5885809.
[4] H. Griengl, N. Shi, U. Pichler, N. Klempier, P. Pochlauer, DSM Chemie Linz, Linz, Austria, 1998, US patent 5714356.
[5] U. Niedermeyer, M.R. Kula, Angew. Chem., Int. Ed. Engl. 29 (1990) 386.
[6] D. Costes, E. Wehtje, P. Adlercreutz, Enzyme Microb. Technol. 25 (1999) 384.
[7] M. Bauer, R. Geyer, M. Boy, H. Griengl, W. Steiner, J. Mol. Catal. B: Enzym. 5 (1998) 343.
[8] M. Bauer, H. Griengl, W. Steiner, Enzyme Microb. Technol. 24 (1999) 514.
[9] D. Costes, E. Wehtje, P. Adlercreutz, Biocatal. Biotransform., submitted for publication.
[10] W. Tischer, V. Kasche, TIBTECH 17 (1999) 326.
[11] A.L. Margolin, TIBTECH 14 (1996) 223.
[12] T. Zelinski, H. Waldmann, Angew. Chem., Int. Ed. Engl. 36 (7) (1997) 722.
[13] M.A. Navia, N.L. St. Clair, Vertex Pharmaceuticals, Cambridge, MA, USA, 1997, US patent 5618710.
[14] H. Wajant, S. Förster, A. Sprauer, F. Effenberger, K. Pfizenmaier, Ann. N.Y. Acad. Sci. 799 (1996) 771.
[15] H. Lauble, K. Decanniere, H. Wajant, S. Förster, F. Effenberger, Acta Crystallogr. D55 (1999) 904.
[16] A. Ducruix, R. Giegé, Crystallization of Nucleic Acids and Proteins — A Practical Approach, Oxford Univ. Press, New York, 1992, p. 82.
[17] F. Effenberger, A. Schwämmle, Biocatal. Biotransform. 14 (1997) 167.
[18] S.C. Mohapatra, J.T. Hsu, Biotechnol. Tech. 8 (1) (1994) 13.
[19] S.B. Sobolov, A. Bartoszko-Malik, T.R. Oeschger, M.M. Montelbano, Tetrahedron Lett. 35 (42) (1994) 7751.
[20] D.R. Walt, V.I. Agayn, Trends Anal. Chem. 13 (10) (1994) 425.
[21] N. St.Clair, B. Shenoy, L.D. Jacob, A.L. Margolin, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 9469.
[22] C. Laane, S. Boeren, K. Vos, C. Veeger, Biotechnol. Bioeng. 30 (1987) 81.
[23] F. Secundo, S. Spadaro, G. Carrea, P.L.A. Overbeeke, Biotechnol. Bioeng. 62 (5) (1999) 554.