J. prakt. Chem. 339 (1997) 394–396

Journal für praktische Chemie
Chemiker-Zeitung

Das Reagenz · The Reagent

Cross-Linked Enzyme Crystals (CLECs) – Powerful Biocatalysts for Synthetic Chemistry

Thomas Zelinski and Herbert Waldmann

Karlsruhe, Institut für Organische Chemie

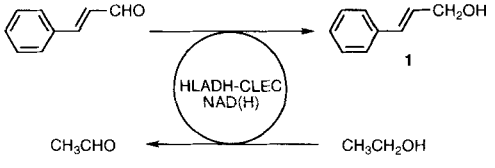
Received February 19th, 1997

Dedicated to Prof. Dr. Ekkehard Winterfeldt on the Occasion of his 65th Birthday

The use of enzymes as highly selective catalysts in organic synthesis is often an advantageous goal for preparative chemistry. The ca. 3000 known enzymes efficiently catalyse a broad variety of reactions under mild conditions and often with high regio- and stereoselectivity [1]. Unfortunately, in many large scale applications the stability of the biocatalysts is often a limiting factor. Thus, immobilisation methods for enzyme stabilization are of great interest in biotechnology [1]. Recently, an alternative technique was introduced, the use of exceptionally stable cross-linked enzyme crystals (CLECs) [2].

The finding that enzymes are catalytically active in the crystalline state was an early proof for the assumption that the conformation of proteins in solution and in the crystal are comparable [3]. CLECs were synthesized by careful crystallization of protein solutions in such a way that microcrystals were formed (crystals which are smaller than 100 μm). A subsequent cross-linking with bifunctional reagents (*e.g.* glutardialdehyde) delivers protein crystals which are no longer water soluble and mechanically stable, i.e. which are easy to handle. This leads to CLECs which retain their catalytic activity without changes of their x-ray reflexes [4]. In contrast to enzymes immobilized by classical methods, the high purity and stability of CLECs leads to high catalytic activity also at higher temperature and in organic solvents. This is due to the fact that by fixing the proteins in the crystal lattice and by the additional cross-linking the denaturation of the enzymes *via* unfolding, dissociation or aggregation is efficiently prevented [2]. In addition, CLECs are very stable towards degradation by proteases, since the protein–protein interactions which are necessary for this process are hindered and since the access of the proteases to the crystal lattice is limited. On the other hand, CLECs exhibit a macroporosive structure formed by long, solvent-filled channels which may be penetrated by small substrates and products. In the case of thermolysin for instance the pores have a diameter of ca. 25 Å big enough for compounds of a molecular weight up to ca. 3000 Da [5]. A rate limitation by pore diffusion was excluded by the use of microcrystals [6].

One of the early examples of CLEC applications in organic syntheses were the cofactor-dependent enzymes horse liver alcohol dehydrogenase (HLADH) and actually rabbit muscle lactate dehydrogenase (LDH). Crystallization and cross-linking of HLADH was carried out in the presence of the cofactor NADH incorporating HLADH as holoenzyme into the crystal lattice. The cofactor was bound so tightly to the crystal that it couldn't be released from HLADH-CLECs even by extensive washing with buffer indicating a tight binding to the enzyme. The synthesis of *E*-cinnamyl alcohol **1** from the corresponding aldehyde was carried out in the presence of dimethoxyethane and ethanol as cosolvents, whereby ethanol also served as second substrate for cofactor regeneration (Scheme 1) [7]. The immobilization as CLEC

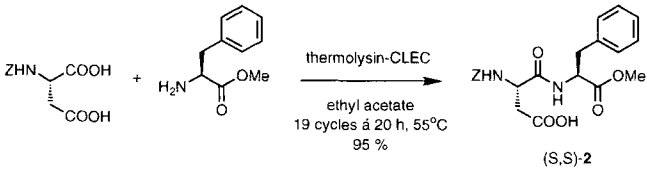


Scheme 1 Chemoselective reduction of cinnamyl aldehyde with HLADH-CLEC.

increased the stability of HLADH so that in the presence of 40% organic cosolvent its activity was fully retained. The use of LDH-CLECs for enantioselective synthesis of lactate solved a major problem of electroenzymatic cofactor regeneration. The complete deactivation observed for homogeneously soluble LDH in an electrolytic cell did not occur with LDH-CLECs. After an initial decline of the activity by 10%, the LDH-CLECs retained constant activity over 25 days. In addition the optimal pH range of LDH was broadened by the

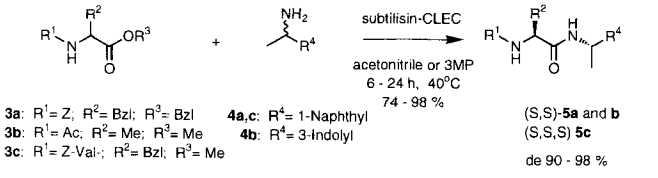
immobilization, thus widening the application range of the enzyme [8].

The interest in CLECs rose when thermolysin-CLECs were introduced which exhibited high protease activity under extreme conditions, for example at increased temperatures, in 50% mixtures of water and organic solvents, and in nearly anhydrous organic solvents suggesting that the stabilized biocatalyst might be used in a variety of syntheses. The synthesis of the aspartame precursor **2** was catalyzed by the thermolysin-CLEC in neat ethyl acetate at 55 °C. The dipeptide ester **2** was obtained in high yields and without deactivation of the enzyme during 19 repetitive reaction cycles (Scheme 2) [5].



Scheme 2 Thermolysin-CLEC catalyzed amide formation in the synthesis of an aspartame precursor (Z=benzyloxycarbonyl).

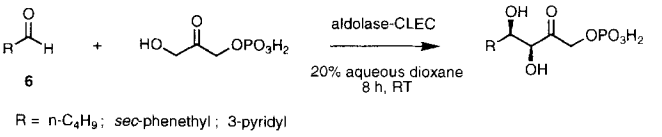
The synthesis of chiral alkylamides of amino acids and peptides was achieved by means of subtilisin-CLECs. A variety of acyl donors **3** was coupled with nucleophiles **4** to give di- and tripeptide amides **5** at 40 °C in acetonitrile (Scheme 3) [9].



Scheme 3 Synthesis of amino acid *N*-alkylamides with subtilisin-CLEC (3MP = 3-methyl-3-pentanol).

Since subtilisin catalyses the reaction with high stereoselectivity the racemic substrates **3a** and **4a** are converted to (*S,S*)-**5a** with high diastereoselectivity. As solvent 3-methyl-3-pentanol was used since the acyl donors **3** which were used as methyl- and benzyl esters were not transesterified by the enzyme in this alcohol.

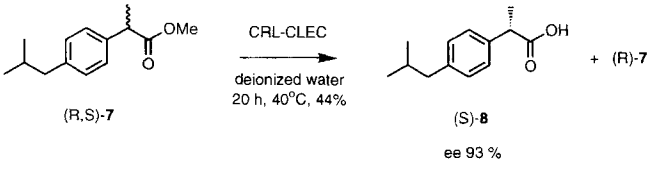
CLECs of the C–C bond forming enzyme fructose-1,6-diphosphate aldolase from rabbit muscle developed by Sobolov *et al.* are much more stable than the usually used lyophilized enzyme preparations [10]. After 5 days in buffered solutions or 1 hour in mixtures of water and organic solvents the homogeneously soluble enzyme retains less than 50% of its original activity. Under both conditions the aldolase-CLECs showed more than 90% activity. The synthetic potential of the aldolase-CLEC was demonstrated for poorly water-soluble non-natural substrates **6** (Scheme 4). The enzyme catalyzed reactions with dihydroxyacetone phosphate in 20% aqueous dioxane proceeded with results which are comparable to those of the natural substrate D-glyceraldehyde-3-phosphate (GA 3P). In the presence of the CLECs aldehydes **6** react with



Scheme 4 Asymmetric enzymatic C–C bond formation with aldolase-CLECs.

velocities of 68 to 116% relative to GA3P. The values are ca. 10 to 100 times higher than in the respective transformations in the presence of the lyophilized enzyme. Therefore, aldolase-CLECs offer a possibility for the execution of inefficient transformations with significantly higher velocities.

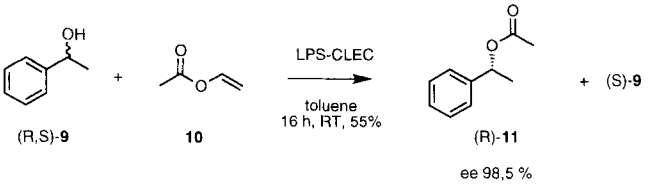
The capacity of *Candida rugosa* lipase (CRL)-CLECs was demonstrated in kinetic resolutions of the esters of arylpropionic acids (Scheme 5) [11]. (*R,S*)-Ibuprofen methyl ester



Scheme 5 Enantioselective ester hydrolysis with CLECs from *Candida rugosa* lipase.

7 was hydrolyzed to (*S*)-ibuprofen **8** with an enantioselectivity of 93% which was 3 times higher than that recorded for the crude enzyme preparations. This was attributed to the removal of contaminating hydrolases with opposite stereospecificity upon crystallization and the resulting high purity of enzyme crystals (>98%). The CRL-CLECs displayed a high degree of thermostability and cosolvent tolerance. Thus, after an incubation of 10 days in 50% THF the CRL-activity was completely retained.

The reaction rates of lipase- and protease-CLEC catalyzed transesterifications in neat organic solvents (Scheme 6) are



Scheme 6 Kinetic racemate resolution by LPS-CLEC catalyzed transesterfication.

several orders of magnitude lower than those recorded for the respective transformations with homogeneously soluble enzymes in aqueous solutions. For sublilisin CLECs this was explained by a shift of the pH-optimum upon crystallization, an unfavourable desolvatation energy of the substrates and a low flexibility of proteins in the crystal lattice mediated by water [6]. Upon addition of denaturing solvents (DMSO and formamide) to the organic reaction medium the transesterification activity of sublilisin-CLEC increased up to 100 fold,

a finding which supports the flexibility theory [12]. The activity of the sublilisin-CLECs could also be raised by two orders of magnitude by addition of an organic buffer system, which protonates the catalytic triad of the protein in an optimal manner [13]. Drying CLECs in the presence of detergents led to CLEC preparations which show high activity in nearly anhydrous organic solvents. For instance CLECs of the lipase from *Pseudomonas cepacia* (LPS) were applied advantageously for the enzymatic resolution of racemic 1-phenylethanol **9** with vinyl acetate **10**. After 16 h the acetate **11** was obtained at a conversion of 50% in pure toluene with an ee of 98.5% (Scheme 6) [14]. The substrate enzyme ratio was 4700: 1, i.e. also under these unusual conditions the enzymatic catalysis was fairly efficient. In the racemate resolution of (+, −)-menthol the use of detergent treated CRL-CLECs instead of purified and lyophilized CRL resulted in a higher enantioselectivity and an increase of the activity by five orders of magnitude [15]. The activation of the biocatalyst by the detergents reflected in these results has not been explained conclusively. An influence on the flexibility of the enzyme, an improved transition of the substrate from the solvent through the tightly bound water in the crystal to the active site and a regulation of the optimal water activity are being discussed.

The newly developed CLECs are highly selective and active biocatalysts with an exceptional stability in aqueous and organic solvents. Their easy handling makes CLECs valuable tools for organic synthesis, in particular if larger amounts of a given compound have to be synthesized. As an example for the high expectations which they have raised the announcement may serve that Ciba–Geigy's specialty chemical branch and Altus Biologics Inc. the manufacturer of CLECs will develop new additives for laundry detergents based on the CLEC technology.

Today many enzymes have been crystallized, but the quality of the obtained crystals is often not sufficient for an x-ray analysis [2]. Thus, only a small fraction of the known biocatalysts has been analyzed by x-ray structure determination. However, the know-how gained in performing these crystallizations now might be used advantageously for the development of a variety of new CLECs which may serve as efficient biocatalysts for organic synthesis.

References

[1] a) K. Drauz, H. Waldmann, Enzyme Catalysis in Organic Synthesis, VCH, Weinheim **1995**; b) K. Faber, Biotransformation in Organic Chemistry, Springer-Verlag, Berlin **1992**; c) C. H. Wong, G. M. Whitesides, Enzymes in synthetic organic chemistry, Elsevier Science Ltd., London **1994**

[2] N. L. St. Clair, M. A. Navia, J. Am. Chem. Soc., **114** (1992) 7314

[3] F. A. Quiocho, F. M. Richards, Proc. Natl. Acad. Sci. U.S. **52** (1964) 833

[4] F. A. Quiocho, F. M. Richards, Biochemistry, **5** (1966) 4062

[5] R. A. Persichetti, N. L. S. Clair, J. P. Griffith, M. A. Navia, A. L. Margolin, J. Am. Chem. Soc., **117** (1995) 2732

[6] J. L. Schmitke, C. R. Wescott, A. M. Klibanov, J. Am. Chem. Soc. **118** (1996) 3360

[7] K. M. Lee, M. Blaghen, J. P. Samama, J. F. Biellmann, Bioorg. Chem. **14** (1986) 202

[8] S. B. Sobolov, M. D. Leonida, A. Bartoszko–Malik, K. I. Voivodov, F. McKinney, J. Kim, A. J. Fry, J. Org. Chem. **61** (1996) 2125

[9] Y.–F. Wang, K. Yakovlevsky, A. L. Margolin, Tetrahedron Lett. **37** (1996) 5317

[10] S. B. Sobolov, A. Bartoszko–Malik, T. R. Oeschger, M. M. Montelbano, Tetrahedron Lett. **35** (1994) 7751

[11] J. J. Lalonde, C. P. Govardhan, N. Khalaf, A. G. Martinez, K. Visuri, A. L. Margolin, J. Am. Chem. Soc. **117** (1995) 6845

[12] O. Almarsson, A. M. Klibanov, Biotechnol. Bioeng. **49** (1996) 87

[13] K. Xu, A. M. Klibanov, J. Am. Chem. Soc. **118** (1996) 9815

[14] N. Khalaf, C. P. Govardhan, J. J. Lalonde, R. A. Persichetti, Y.–F. Wang, A. L. Margolin, J. Am. Chem. Soc. **118** (1996) 5494

[15] R. A. Persichetti, J. J. Lalonde, C. P. Govardhan, N. Khalaf, A. L. Margolin, Tetrahedron Lett. **37** (1996) 6507

Address for correspondence:
Prof. Dr. H. Waldmann
Institut für Organische Chemie der Universität Karlsruhe
Richard–Willstätter-Allee 2
D-76128 Karlsruhe
Telefax: +721-608-4825
e-mail: waldmann@ochhades.chemie.uni-karlsruhe.de