CROSS - LINKED ENZYME CRYSTALS (CLECs) – CATALYSTS FOR THE FUTURE

Murali Rajaa R., Ajay Galdhar, Praveen Kumar (*S.Y.Chem.Engg.*)

Catalysts are broadly classified as: (1) Homogeneous catalysts and (2) Heterogeneous catalysts.

In Homogeneous catalysis the catalyst is in the same phase as the reactants and is evenly distributed throughout. Heterogeneous catalysts are more widely used in industrially important reactions e. g. Platinum, Palladium. etc. are used in the oxidation and reduction reactions in industries on a large scale. Enzymes form a very integral and important part in the heterogeneous catalytic reaction field. The most important heterogeneous catalyzed reactions occurring in solution are those in which the catalysts are the enzymes derived from living organisms, the reason being that they can be used at ambient conditions, have low cost etc. Enzymes are proteins and so are macromolecular in nature. Enzymes are valuable tools in the preparation of optically pure compounds because of their ability to discriminate between enantiomers or enantiotopic groups in prochiral compounds. Crude enzyme preparations have certain advantages like availability, high operational stability and low cost over highly purified enzymes albeit they have low stereoselectivity. Hence they are used as biocatalysts. The ability to make enzymes more robust gives them tremendous potential as environmentally friendly catalysts in industrial applications.

Disadvantages of crude enzymes

A serious disadvantage of crude enzyme preparations is the presence of several competing enzymes or hydrolases.

Cell debris, nucleic acids, inactive proteins and pigments are normally present as impurities. Their presence contaminates the final product and makes the workup expensive and less feasible.

On a weight basis they have low acidity.

They have been used in traditional applications only in water solutions at near-ambient temperatures.

They have poor operational stability

They have inadequate selectivity for "unnatural" synthetic substrates

They have low volumetric productivity

CLEC History

Research on a type of bacteria that live in hot water near volcanic vents on the sea floor has led to the discovery of a class of enzymes that can function at temperatures close to 100°C, opening up a wide range of new applications for enzymes. This would mean that the traditional disadvantage of crude enzymes of use only near ambient temperatures would no longer be an obstacle. The assiduous work undertaken by the scientists having in front of them a new horizon led to the subsequent development of Cross-Linked Enzyme Crystals (CLECs) which involves cross linking of crystallized enzymes to form a robust product. These crystals have properties that exactly towered above the disadvantages of their crude counterparts. This mitigation above the limiting factors has broadened the potential for use of enzymes even further.

Structure of CLEC

The protein molecules are linked together in a three-dimensional lattice with ordered microscopic channels between them in the range of 20 to 50 A°. The lattice provides structural strength and durability since the energy of crystallization as well as the cross-links is added to the energy needed to denature the protein. In the case of cross-linked-enzyme-crystals, the microscopic channels allow the substrate to readily pass through the crystal lattice and access the active sites of the enzyme molecules.

CRL-CLECs (*Candida Rugosa Lipase – CLECs*) They contain about 50% solvent by volume and are macro-porous. To permit diffusion of solvents, substrates and products there are channels that traverse the body of the crystal. Large molecules should be rapidly diffused into the protein crystal and for this purpose CLECs have large pore diameter.

Preparation of CLECs

The catalyst (enzyme) that is used for the preparation for the CLEC should be the one with:

1. Well defined composition and performance.
2. High operational stability and activity.
3. Recycle and reuse value/ability.

This breakthrough technology involves the conversion of crude enzyme mixtures into pure, stable heterogeneous catalysts with high stability. The general methods of preparation of the CLECs is using microcrystals grown from aqueous solution (i.e. crystallization) and cross-linked with bifunctional agent such as glutaraldehyde which has high specific activity at elevated temperatures.

Properties of CLECs

A. High operational stability:

(1) CLEC catalysts are heterogeneous and can be readily isolated, recycled and reused many times (Fig.1)

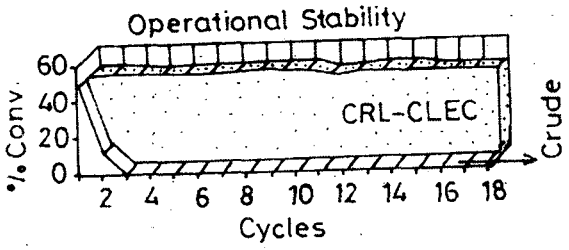

Fig. 1: Multicycle resolution of (S)-Ketoprofen. 10% Chloroethyl Ketoprofen in 50/50 PEG 1000/ pH5 ammonium acetate buffer, 40°C, 5 mg/ml CRL-CLEC.

(2) CLECs' high operational stability allows the chemist to conduct reactions at higher temperatures, and in aqueous organic solvent mixtures or neat organics, thus increasing the substrate solubility. Normally, one to two orders of magnitude in stabilization against heat and more than three orders of magnitude in stabilization against water-miscible organic solvents over soluble enzymes can be achieved (Fig. 2).

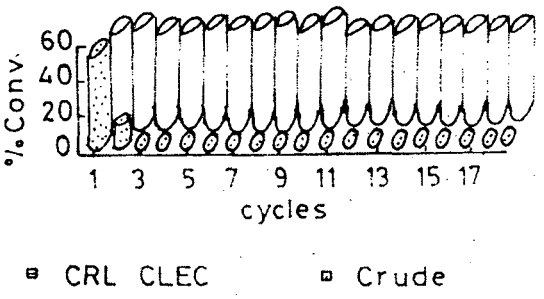

Fig. 2: Stability at 25°C of crude CRL and ChiroCLEC-CR in water-miscible organic solvents. Aqueous component was 10mM Tris, 10mM $CaCl_2$, pH 7.0. Activity was measured by triacetin assay.

(3) CLECs' stability against proteolysis and autolysis makes possible the use of high concentrations of enzymes in hydrolytic reactions. CLECs will not only allow a high enzyme concentration, but also, by preventing proteolysis by exogenous proteases, they increase the shelf-stability of enzymes. Long shelf-stability will solve storage problems and make it easier for chemists to handle enzymes as ordinary chemicals (Fig.3).

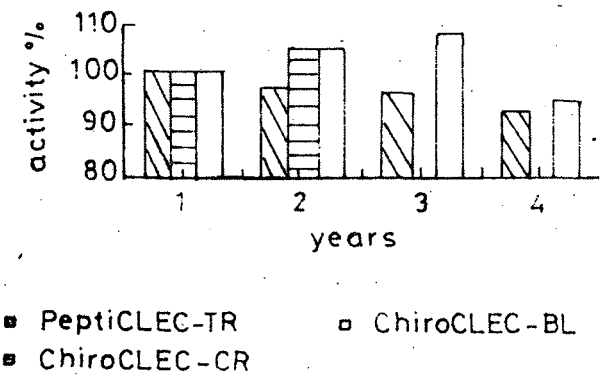

Fig. 3: Shelf stability of three CLEC products.

B. High selectivity: CLEC catalysts are highly purified enzymes. While pure enzymes have maximal selectivity, often it is the crude enzyme preparations that are used commercially today. Their high cost and low stability limit the use of pure, soluble enzymes in synthesis. The enzyme content in commercial lipase samples used in the synthesis of pharmaceuticals is normally less than 10% and in some cases less than 1%. Contamination of the reaction mixtures by the proteins and the products of protein self-digestion is a serious problem in the synthesis of peptides or other pharmaceuticals since these impurities can cause anaphylactic shock. In these situations, a thorough purification of the product is required. CLEC catalysts eliminate these problems since they are heterogeneous and do not contaminate the product

with unwanted protein. Another serious drawback of crude enzyme preparations is the presence in the mixture of several competing enzymes. These enzymes may have different stereo- and regioselectivities and thus reduce the yield and optical purity of the final product. ChiroCLEC-Candida Rugosa demonstrates more than a ten-fold enhancement in the optical purity of the pharmaceutically important products such as Ketoprofen, Ibuprofen, Fluorbiprofen and others because the competing enzyme activities present in crude Candida rugosa lipase were removed in the crystallization process to make the CLEC.

C. High volumetric activity: CLECs have high specific activity. The entire volume of a CLEC consists of active catalyst and not, as in the case of immobilized enzymes, inert carrier. Enzyme concentrations within the crystal approach the theoretical packing limit for molecules of a given size. Thus, the volumetric activity of CLECs is two to four orders of magnitude higher than that of conventional or immobilized enzymes, which serves to reduce both reaction times and volume of enzyme required, and to maximize volumetric productivity. In addition, CLECs do not require expensive supports, such as carrier beads, glasses, gels and films, or complex immobilization procedures.

Storage and Recovery of CLECs

CLECs can be easily recovered by filtration or centrifugation and be reused, as they are stable, insoluble and mechanically strong particles. In contrast their enzyme counterparts cannot be recycled. Immobilized enzymes have prohibitively low activity albeit they can be recovered and reused.

Example - For the reaction of repetitive batch resolution of Ketoprofen

Statistics of immobilized *Candida Rugosa Lipase (CRL)* used as catalyst

Reaction weight -35%

Reaction volume -90%

Statistics of *Candida Rugosa Lipase* when cross-linked i.e. *(CRL-CLEC)*

Reaction weight -0.27%

By comparison it was found that the reaction weight of immobilized CRL was very high as compared to that of CRL-CLEC. Also in case of CRL, after one cycle followed by extraction of the product, virtually all activity was lost. The CRL-CLEC was cycled VIII times before I immobilized enzyme cycle was completed. The high *enantioselectivity* and *activity* of CRL-CLEC was maintained throughout cycling. Chemical crosslinking of enzyme crystals stabilizes the crystalline lattice and it's constituent enzyme molecules, forming highly concentrated immobilized enzyme particles, which can be lyophilized and stored indefinitely at room temperature.

Catalytic Activity and Stability of CLECs

CLECs are 300-3000 times more stable in water and water miscible solvents, particularly in case of CRL-CLEC. Stability is due to combination of the crystallinity of the material and covalent cross-linking between enzyme molecules. In crystal lattice when concentration of enzyme is close to theoretical packing limit many protein-protein interactions (both polar and hydrophobic) are realized. These multipoint contacts significantly enhance their stability against heat and denaturants by preventing unfolding, aggregation or dissociation.

Conventional immobilized enzymes are principally used to facilitate catalytic recovery but they can also provide modest stability enhancements. Formulated as beads or particles, however, these systems consist mostly of inert carrier material. CLECs provide their own support, and so achieve enzyme concentrations close to theoretical packing limit-in excess of even highly concentrated enzyme solutions. Thus CLECs are particularly attractive in biosensor applications, where the largest possible signal per unit volume is often critical.

One of the most essential features of a synthetically useful biocatalyst is high specific activity. When a protein is held in a crystal lattice by chemical crosslinking, the active conformation of the protein is maintained and therefore the enzyme is nearly fully active relative to the soluble protein. The accessibility of the entire CLEC crystal is made up of 30-65% solvent filled channels that are 20-55A° in diameter. The crystals are arranged like zeolites but with larger pores, and it is obvious that these uniform solvent-filled channels can facilitate the transport of substrates and products into and out of the crystal easily.

Even though the basic catalytic properties of the CLEC form of the protein differ very little from those of the soluble form, the highly purified CLEC catalysts can be many times more enantioselective compared with crude commercial enzyme preparations. This increased enantioselectivity is not a consequence of the CLEC form itself, but rather a result of the removal of competing enzymes. Crude enzyme preparations

typically contain less than 10% of active enzyme, the remainder being other proteins, protein fragments, carbohydrates, cell debris, salts, stabilizers and, as a major source of irreproducibility, competing enzymes. Such enzyme mixtures can in certain cases, catalyze competing reactions, interfere with purification of the final product and make the work-up difficult and expensive. Competing enzymes may have different stero- and regioselectivities and reduce the yield and optical purity of the final product.

Example : Data are presented comparing free enzyme versus CLECs of thermolysin, which is used in the manufacture of the artificial sweetener *Aspartame*. The activity of CLECs is due to absence of competing enzymes.The data illustrate the superiority of CLECs with respect to activity over their counterpart enzymes.

SOLVENT	% MAXIMUM ACTIVITY, FREE THERMOLYSIN	% MAXIMUM ACTIVITY, CLECs OF THERMOLYSIN
Acetonitrile	42	102
Dioxane	66	97
Acetone	75	99
THF	36	96

Uses

A. Low cost of catalysis using CLEC catalysts:

1. CLECs are environmentally benign and easy to dispose of as compared to traditional chemical catalysts (precious metals), resolving agents or coupling agents. The cost savings due to the simplified product work-up and disposal of catalysts alone can be very significant.
2. CLECs can be recycled and reused many times in a batch configuration or over long period of time in a column configuration.
3. High stability of CLECs at elevated temperature and in the presence of organic solvents, as well as their increased selectivity significantly broadens the synthetic potential of enzyme catalysts.

B. In peptide synthesis: An important group of modern drugs is the peptide and peptide mimic groups. Peptides are typically prepared by either the stepwise coupling of the individual amino acids where the growing peptide is bound to a solid support or by the condensation of amino acids and or peptide fragments in solution. The solid phase approach is generally only suited to smaller quantities of peptides and suffers from the fact that considerable impurities can build up over the course of a lengthy synthesis making purification of the final product very difficult. Solution phase coupling allows for easier purification of the intermediate products as well as production of larger quantities. However there can be problems with solubility and removal of by-products such as dicyclohexyl urea. Finally, both methods suffer from partial racemization and the need to protect side-chain functionality.

Enzymes have been explored as a possible answer to the problems of peptide synthesis since 1938. This approach offers many advantages over the traditional methods. The reactions are catalytic, occur under extremely mild conditions, require little or no side-chain protection and most importantly are racemization free. However, the proteases that are useful for peptide coupling are generally unstable in the presence of organic solvents, contain peptide and protein fragments that are difficult to separate from product and in purified form suffer from autodigestion. PeptiCLEC-BL(Bacillus licheniformis) is derived from subtilisin and PeptiCLEC-TR(Bacillus thermproteolyticus) is prepared from thermolysin. Together these catalysts couple a broad range of amino acids (including non-biogenic amino acids), peptide fragments, peptide derivatives and mimics via ester aminolysis under mild conditions and free of racemization, thus proving useful in making peptide mimics. The carboxy moiety and amine donor used can be either optically pure or racemic. In the case of racemic starting materials, generally only one enantiomer of the compounds will react to form the peptide or peptide derivative, while the other will remain unreacted and can be separated at the end of the reaction.

Compound name	Chemical utility
PeptiCLEC-TR	A catalyst which is used to couple amino acids to amines and amino acids or their derivative to produce peptides, which can be used, for example, to make certain anti-cancer drugs
PeptiCLEC-BL	A catalyst, which is used to couple esters and amino acids or their derivatives to amines to produce peptides, which can be used, for example, to prepare certain drugs used to treat AIDS.

C. In synthetic chemistry: Other than chiral resolution and peptide coupling, CLECs are used in synthetic chemistry. SynthaCLEC-PA (Penicillin

acylase or Penicillin amidase), is used in the preparation of many widely used antibiotics, such as Amoxicillin(tm) and Ceclor(tm).

Compound name	Chemical utility
SynthaCLEC-PA	A catalyst which can be used to produce penicillin and cephalosporin-derived antibiotics.

D. Resolution of reacemates to obtain enantiomerially pure compounds : One of the major uses of enzymes in chemistry is for the resolution of racemates to obtain enantiomerically pure compounds. Several enzymes have been developed for this purpose and form the ChiroCLEC family of catalysts. ChiroCLEC-CR (Candida Rugosa), ChiroCLEC-PC (Pseudomonas cepacia), ChiroCLEC-BL (Bacillus licheniformis) and ChiroCLEC-EC (E. coli) can be used to resolve alcohols, acids and amines in a wide aware of structural types.

Compound name	Chemical utility
ChiroCLEC-CR	A catalyst used to perform chiral resolutions on molecules containing alcohols, acids and esters and can be used, for example, to manufacture certain anti-inflammatory drugs, such as Ibuprofen and Ketoprofen.
ChiroCLEC-EC	A catalyst used to perform chiral resolutions on molecules containing amines and amino acids and can be used, for example, to manufacture certain central nervous system drugs.
ChiroCLEC-PC	A catalyst used to perform chiral resolutions on molecules containing alcohols, acids and esters an can be used, for example, to manufacture certain Steroids.
ChiroCLEC-BL	A catalyst used to perform chiral resolutions on molecules containing amino acids, peptides and amino acid analogs which can be used, for example, to manufacture certain anti-hypertensives and analgesics.

Future Prospects

According to the publisher of 'Technical Insights', Peter Katz, "The total annual world enzyme market is worth about $1.5 billion and cross-linked enzyme crystals may soon account for a substantial share of that market. CLECs have the advantage of being able to withstand extremes of temperature and pH. Protease enzymes that destroy other enzymes don't affect them, and they can withstand an extensive range of solvents. This means that enzyme crystals function more effectively than conventional enzyme products, and at a lower cost."

So far only a few enzymes like Luciferase, Penicillin Acylase , Asparaginase etc. have been produced in CLEC form. CLEC technology can be implemented for other enzymes as well. *CLECs hold the key to a diamond vault in catalytic chemistry.*

Conclusion

Thus, the resultant enzyme obtained by chemically cross-linking enzyme crystals of the major component of corresponding protein (enzyme) is recoverable, active and dramatically stable. It's a novel form of catalyst, which combines high activity and productivity. This technology although in its nascent stage holds great promise and is asking to be tapped.

References

1. Jim J. Lalonde, Chandrika Govardhan, Nazer Khalaf, Aldo G. Martinez, Kalevi Visuri and Alexey L. Margolin, *J. Am. Chem. Soc.*, 1995, 117, 6845-6852.
2. Nancy L. Clair and Manuel A. Navia, *J. Am. Chem. Soc.*, 1992, 114, 7314-7316.
3. Nazer Khalaf, Chandrika P. Gavardhan, Jim J. Lalonde, Rose A. Persichetti, Yi –Fong Wang and Alexey L. Margolin, *J. Am. Chem. Soc.*, 1996, 118, 5494-5495.
4. Martin Baust-Timpson and Peter Seufer-Wasserhal, *Speciality Chemicals*, 1998, July/Aug. ,pg. 248.
5. Martin Baust-Timpson and Peter Seufer-Wasserthal, *Speciality Chemicals*, 1998, Sept., pg. 300.
6. http://www.altus.com/technology.html.
7. http://www.wiley.com/technical_insights/englewood.doc.
8. http://www.altus.com/chiral.html.
9. http://www.altus.com/pepti.html.
10. http://www.altus.com/syntha.html.