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CROSS-LINKED PROTEIN CRYSTAL TECHNOLOGY IN BIOSEPARATION AND BIOCATALYTIC APPLICATIONS

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Abstract

Chemical cross-linking of protein crystals form an insoluble and active protein matrix. Cross-linked protein crystals (CLPCs) have many excellent properties including high volumetric activity and stability. In this thesis CLPC technology was studied in bioseparation and biocatalytic applications.

A novel immunoaffinity separation material, cross-linked antibody crystals (CLAC), was developed in this thesis for enantiospecific separation of a chiral drug, finrozole. Previously, the preparation of an antibody Fab fragment ENA5His capable of enantiospecific affinity separation of the chiral drug has been described. However, in a carrier-immobilized form ENA5His suffered from poor stability in the presence of a high concentration of organic solvents needed to release the bound drug. In this study crystallization conditions for ENA5His were found by means of small-scale vapor diffusion experiments. Crystallization was further scaled up to 10 ml batch crystallization with a 70% protein yield. Glutaraldehyde cross-linking modified the ENA5His crystals into an insoluble form producing a CLAC matrix. The CLAC matrix packed in a column separated pure enantiomers from the racemic mixture of the drug. The CLAC matrix was totally stable at the elution conditions enabling reuse of the immunoaffinity column. However, the specific drug enantiomer binding capacity of CLAC was only 50% of the corresponding capacity of carrier-immobilized ENA5His. Also a cross-linked carrier-immobilized ENA5His column was prepared to study the effect of bare chemical cross-linking. Surprisingly, the cross-linked immobilized ENA5His was as active as the native immobilized ENA5His and simultaneously stable against the denaturing effect of methanol.

Xylose isomerase (XI) is a widely used enzyme in industry as a result of its ability to catalyze isomerization of D-glucose to D-fructose. Previously it has been shown that XI accepts all of the pentose sugars and many hexose sugars as isomerization substrates. In the present study novel tetrose isomerizations and C-2 epimerizations with both the D- and L-forms of the sugars by an industrial xylose isomerase (XI) from *Streptomyces rubiginosus* were described. Furthermore, the results showed that the real equilibrium of XI catalyzed reactions is not between two isomers but between a ketose and its two aldose isomers. These findings together with previous results show that XI can be used as a catalyst for production of a variety of sugars.

Obtaining homogeneous enzyme crystals is critical to their application as catalysts in a cross-linked form. In this study a method for the production of homogeneous crystals of XI was developed. Firstly, the XI crystal solubility was measured with respect to precipitant salt concentration, temperature, and pH. Secondly, based on the results of the solubility study, a process for the production of uniform XI crystals of different size classes was developed. Crystals with average crystal sizes between 12 μm and 360 μm were produced. XI crystals were further cross-linked by glutaraldehyde and L-lysine to prepare insoluble cross-linked xylose isomerase crystals (CLXIC).

The cross-linking of XI crystals improved their stability towards organic solvents. This can be exploited in the production of D-fructose and possibly also other sugars. Adding acetone to the reaction mixture enhanced the production of D-fructose from D-glucose catalyzed by CLXIC in terms of product yield and reaction rate. The D-fructose equilibrium concentration increased from 49% to 64% when increasing the acetone concentration from 0% to 90% in the reaction mixture at 50 °C. In 50% acetone the fructose production rate was more than doubled compared with that in a pure buffer solvent. Acetone seems to have only a minor role in the inactivation of cross-linked crystals. The residual activity of CLXIC was 70–80% of the initial activity after a 24-h incubation at 50 ^{o}C in a buffer solution (pH 7.2) containing 10–90% acetone. In the soluble form, the activity of XI decreased by 75% after incubation in 50% acetone for 24 h at 50 °C.

Preface

This work was carried out in the Laboratory of Bioprocess Engineering, Department of Chemical Technology, Helsinki University of Technology, during the years 2000–2004.

First, I must thank Professor Matti Leisola for his advice, interest and enthusiasm, and for providing me the opportunity to work in his research group. I warmly thank Dr. Jouni Jokela for helping me throughout the work, for always being there, and for many fruitful discussions. Kalevi Visuri and Sinikka Uotila are thanked for their excellent co-operation. I gratefully acknowledge also all the other co-authors Kristiina Kiviharju, Prof. Outi Krause, Tarja Nevanen, Dr. Ossi Pastinen, Dr. Hans Shoemaker, and Kati Vilonen for their contribution to this work. Dr. Jarkko Valjakka is thanked for many fruitful discussions about protein crystallization.

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I would like to thank my parents, brothers, and friends for their support over the years. Finally, my deepest gratitude belongs to my wife Tarja and our children Emma and Aarne for showing me what really is important in life.

Kirkkonummi, June 15, 2004

Antti Vuolanto

List of publications

This thesis is based on the following five publications, which are, throughout the summary, referred to as Roman numerals.

I Vuolanto, A.; Kiviharju, K.; Nevanen, T.K.; Leisola, M.; Jokela, J. Development of cross-linked antibody Fab fragment crystals for enantioselective separation of a drug enantiomer, *Cryst. Growth Des.* **3** (2003) 777–782.

II Vuolanto, A.; Leisola, M.; Jokela, J. Enantioselective affinity chromatography of a chiral drug by crystalline and carrier-bound antibody Fab fragment, *Biotechnol. Prog.* **20** (2004) 771–776.

III Vuolanto, A.; Uotila, S.; Leisola, M.; Visuri, K. Solubility and crystallization of xylose isomerase from *Streptomyces rubiginosus, J. Cryst. Growth*. **257** (2003) 403–411.

IV Vuolanto, A.; Pastinen, O.; Schoemaker, H.E.; Leisola, M. C-2 epimer formation of tetrose, pentose and hexose sugars by xylose isomerase, *Biocatal. Biotransform.* **20** (2002) 235–240.

V Vilonen, K.; Vuolanto, A.; Jokela, J.; Leisola, M.; Krause, A.O.I. Enhanced glucose to fructose conversion in acetone with xylose isomerase stabilized by crystallization and cross-linking, *Biotechnol. Prog.* (2004) In press.

The author's contribution in the appended publications

Publication I: Antti Vuolanto was responsible of the research plan, carried out most of the experimental work, carried out the interpretation of the results, and produced the manuscript jointly with Jouni Jokela.

Publication II: Antti Vuolanto designed and carried out the experimental work, and produced the manuscript together with Jouni Jokela.

Publication III: Kalevi Visuri and Sinikka Uotila designed and carried out the experimental work jointly with Antti Vuolanto. Antti Vuolanto carried out the interpretation of experimental results and produced the manuscript with Kalevi Visuri.

Publication IV: Antti Vuolanto designed and carried out the experimental work. Antti Vuolanto carried out the interpretation of experimental results and produced the manuscript with Matti Leisola who wrote the first version of the manuscript.

Publication V: Antti Vuolanto and Kati Vilonen jointly designed the experiments, interpreted the results, and produced the manuscript. Kati Vilonen carried out the experimental work.

Abbreviations

CLPC	Cross-linked protein crystal
CLAC	Cross-linked antibody fragment ENA5His crystal
CLXIC	Cross-linked xylose isomerase crystal
D_e	Effective diffusivity
DMF	*N*,*N*-dimethylformamide
DMSO	Trimethyl sulfoxide
DPDPB	1,4-di-[3'-(2'-pyridyldithio)propionamido]butane
GC	Gas chromatography
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLADH	Horse liver alcohol dehydrogenase
HPLC	High performance liquid chromatography
kDa	Kilodalton
MβCD	Methyl-β-cyclodextrin
MPD	2-methyl-2,4-pentane-diol
MW	Molecular weight
MWCO	Molecular weight cut-off
NADH	Reduced nicotinamide adenine dinucleotide
PBS	Phosphate buffered saline
PEG	Polyethyleneglycol
PMSF	Phenylmethylsulfonylfluoride
R	Radius of spherical catalyst particle
r_{actual}	Actual reaction rate
r_{ideal}	Ideal reaction rate
Sc-fluids	Supercritical fluids
TAME	Tosyl-L-arginine methylester
THF	Tetrahydrofuran
Tris	Tris(hydroxymethyl)aminomethane
XI	Xylose isomerase
Å	Ångström, 10^{-10} m
ε_{max}	Maximum energy dissipation rate
Φ	Thiele modulus
η	Effectiveness factor
ρ_c	Density of catalyst

Contents

1 Introduction

Enzymes catalyze virtually all reactions in biological systems. They are highly active and specific catalysts. The specificity is the result of the active site in enzymes, which specifically binds the substrate that has a precise size and shape that only fits to the active site. The active site in enzymes can even distinguish between optical isomers and enantiotopic groups of chiral or prochiral molecules. There is a growing interest in application of proteins as catalysts in fine chemical production, as biosensors, as pharmaceuticals, and in food and detergent applications. Lalonde summarized [1] the main advantages in using enzymes in practical applications: they are highly selective, they are efficient, they work under ambient conditions at neutral pH, they are environmentally benign, a broad range of reactions are possible, and they are "green" catalysts producing less waste and consuming less energy. However, there are drawbacks in enzyme-catalyzed reactions. They can exhibit a slow reaction rate, they can be unstable in practical operational conditions, the storage stability can be poor, the downstream processing can be complex, the scale-up of enzymatic processes is often difficult, and the bulk supply of proteins is often limited [1, 2]. The slow reaction rate can be a result of the addition of organic solvents or reagents in the reaction mixture or the inhibition of enzymes at high concentrations of substrates or products, for example. Enzyme catalysts are inactivated by a variety of commonly encountered process conditions, including organic solvents, proteolysis, elevated temperatures, and a high concentration of organic reagents. Moreover, biocatalysts are commonly used as impure water-soluble protein preparations that contain only a small fraction of the active catalyst (usually 0.5–10%). In catalysis it is even possible that more catalyst is added than reactant [3]!

The properties of enzymes have been improved by protein engineering or by immobilization, for example. Protein engineering can be used in order to enhance both stability and activity. However, protein engineering is laborious and only one property of one protein can be altered at one time. Furthermore, expression systems and an economically viable production process have to be developed for the new protein. Enzyme immobilization is used to facilitate the down-stream processing and to improve the stability of enzymes. Immobilized enzymes can be easily removed from the reaction mixture and, thus, can be recycled and reused. However, the stability enhancement is usually negligible. Moreover, the use of an inert solid support leads to a low overall activity of an immobilized protein matrix.

Chemical cross-linking of enzyme crystals was first developed in the 1960's to stabilize enzyme crystals in order to facilitate the manipulation and the collection of crystallographic data by X-ray crystallography [4]. However, it was soon recognized that cross-linked crystalline enzymes were catalytically active [5]. Cross-linked enzyme crystals, or more generally cross-linked protein crystals (CLPCs) are created by crystallization of protein followed by a chemical cross-linking that "locks" the crystalline state outside the crystallization conditions. CLPCs form an active, insoluble, mechanically robust, and microporous protein matrix. CLPCs have been used as catalysts in various enzymatic production processes, as separation matrices, and in some other applications. However, the development of CLPC technology is still under way and the number of proteins commercially available as cross-linked crystals is very limited. Protein crystallization and CLPC technology are described below in greater detail.

1.1 Crystallization of proteins

A solution system remains at equilibrium until the point is reached where there is insufficient solvent to maintain full hydration of the solute molecules. If more solute is added to the solution, the system is no longer at equilibrium under this so-called supersaturated state. The system will be thermodynamically driven toward a new equilibrium corresponding free energy minimum by forming a solid phase. The solid phase can appear as either amorphous precipitate or crystals. The principle of crystallization is similar for small molecules (e.g., salts and small organic compounds) and macromolecules (e.g., proteins, DNA, and RNA). Three stages are common to all crystallizations: nucleation, crystal growth, and cessation of crystal growth. In nucleation molecules or non-crystalline aggregates (dimers, trimers, etc.) produce a stable aggregate with a repeating structure. The nucleus must first exceed a specific size, called the critical size, before it is capable of further growth. However, the formation of crystal nuclei from supersaturated solution does not necessarily result in the subsequent formation of macroscopic crystals. Cessation of crystal growth can occur for many reasons. The most obvious reason is that the equilibrium between the crystalline and soluble form is achieved.

The crystallization of proteins is usually more difficult than small molecule crystallization. This is probably a result of the difficulty to obtain a high quality protein sample for crystallization. Because precise protein–protein contacts are needed for the crystallization of proteins, any factor that introduce heterogeneity into the protein sample may have interfering effects on the crystallization. There are numerous factors that can introduce heterogeneity into a protein sample including differential glycosylation on the protein molecules, different conformations of the protein can be present, contamination in the form of other proteins, protein denaturation, and protein degradation [6].

The basic strategy to crystallize proteins is to bring the system into a state of limited degree of supersaturation. This can be done, for example, by removing the solvent, adding a precipitating agent, or by altering some physical properties such as temperature. However, there are dozens of other parameters that are involved in protein crystallization [6]. Protein crystallization is presented in detail in numerous handbooks (e.g., [6, 7]).

1.2 Applications of protein crystallization

1.2.1 Structure determination

Protein crystallization is mainly used for structure determination by X-ray crystallography where large, high quality crystals are needed. Obtaining high quality diffractive crystals is the bottleneck in protein structure determination. For structure determination purposes protein crystals are most often crystallized by small-scale microdiffusion methods (for a review of these methods, see Refs [6, 7]). Very often crystallization conditions are found after setting up hundreds or thousands of individual crystallization conditions employing the “trial and error” approach, as crystallization is often considered as a necessary evil in the structure determination work.

The structural analysis is based on the principle that in a perfect crystal all the molecules have the same conformation and orientation. When X-rays are focused through protein (or any other pure substance) crystal, individual atoms diffract the rays. The number of electrons in each atom determines the intensity of the scattering of X-rays and, thus, the X-rays striking particular atoms will all be diffracted in the same way. A diffraction

pattern is generated by the interference of individual X-rays. A series of diffraction patterns are taken from several angles and these patterns represent the way atoms are arranged in the molecule and can be used to determine the structure of the protein. There are currently more than 18000 protein structures in the Protein Data Bank (PDB) [8], which have been determined by X-ray diffraction analysis.

1.2.2 Protein purification

Crystallization is an efficient protein purification method. However, purification by crystallization is relatively rare even though the quality of the crystals is not crucial. Chromatographic methods have replaced protein crystallization in protein purification although the latter was commonly used earlier. However, protein purification by crystallization has many advantages: high yield, high purity in one step, unlimited scale-up possibilities, and the product is a highly concentrated protein crystal slurry ready for further formulation. Some industrial proteins, like xylose isomerase (EC 5.3.1.5) [9], cellulase (EC 3.2.1.4) [10], and protease (EC 3.4.-.-.) [11], have been purified by crystallization in large-scale.

1.2.3 Medical applications

A majority of small molecular weight drugs are produced in crystalline form because of the high storage stability, purity, and reproducibility of the drug properties [12]. There are hundreds of macromolecular therapeutic agents used in clinical trials or approved as drugs [13]. However, only insulin is produced and administered in a crystalline form [14]. According to Margolin and Navia [15] the crystallization of macromolecular pharmaceuticals can offer significant advantages, such as: a) protein purification by crystallization as presented above; b) high stability of the protein product compared with soluble or amorphous forms; c) crystals are the most concentrated form of proteins, which is a benefit for storage, formulation, and for drugs that are needed in high doses (e.g., antibiotics); d) the rate of crystal dissolution depends on the morphology, size, and additives; thus, crystalline proteins may be used as a carrier-free dosage form.

1.2.4 Cross-linked protein crystals (CLPCs)

In many applications crystallized proteins are not suitable for use as such as a result of their fragility and solubility. In order to produce a crystalline protein matrix that is insoluble also in other conditions than those used in crystallization, the crystals have to be chemically cross-linked. In general, chemical cross-linking of protein crystals creates an active and microporous protein matrix that can be used in catalytic and separation applications.

1.3 Chemical cross-linking of protein crystals

1.3.1 Glutaraldehyde

Protein crystals dissolve in other conditions than those used in the crystallization. Chemical cross-linking of protein crystals “locks” the crystalline state and, thus, the cross-linked protein crystals are insoluble also outside the conditions that initially led to crystallization. The most commonly used cross-linking reagent is a bifunctional aldehyde, glutaraldehyde. Most often, the cross-linking reaction is carried out near neutral pH by adding 0.1–2% (w/v) glutaraldehyde to the crystallization mother liquor containing the native protein crystals. This mixture is then incubated in cool conditions

(0–25 °C) for 0.5–2 h. The resulting chemical cross-linking takes place throughout the crystalline lattice and is intermolecular but intracrystalline.

It is well established that glutaraldehyde reacts with the free ε-amino groups of lysine residues in proteins. In numerous studies the amino acid composition of cross-linked protein crystals has been analyzed and it was clearly shown that only lysine residues of proteins reacted in the cross-linking reaction by glutaraldehyde [5, 16, 17, 18]. As an exception, an amino acid analysis of cross-linked chloroperoxidase (EC 1.11.1.10) crystals suggested that also serines and arginines were modified in the glutaraldehyde cross-linking reaction [19]. Crystallographic studies show that glutaraldehyde cross-linking reaction had only minor effects on the structure of lysozyme (EC 3.2.1.17) [20], carboxypeptidase A (EC 3.4.17.1) [4], subtilisin (EC 3.4.21.62) [21], an *N*-cadherin fragment [22], and a gp120 ternary complex [22]. The X-ray crystallographic data from triclinic lysozyme crystals indicate that only lysine residues were modified in glutaraldehyde cross-linking [20].

In principle, the carbonyl function in aldehydes reacts with free amino groups and the reaction product is a Schiff base. A general characteristic of Shiff bases is that they are readily hydrolyzed in acidic conditions. However, numerous examples show that the cross-linking reaction between glutaraldehyde and proteins is irreversible, shown by incubating cross-linked protein crystals in acidic solution [23], for example. This rules out a simple Shiff base formation.

Glutaraldehyde is known to form polymers in solution [24, 25, 26]. It is speculated that the ability of glutaraldehyde to stabilize protein crystals stems from the fact that in aqueous solution glutaraldehyde forms a mixture of oligomers of different lengths and structures [27], and therefore the most appropriate cross-linking species reacts with crystalline protein [17]. At acidic pH, the oligomers are cyclic hemiacetals (Figure 1a) [25, 26] that form Shiff bases in analogy with the mechanism of Cordes and Jencks [28]. At neutral or slightly alkaline pH, where protein cross-linking is carried out, α,β-unsaturated glutaraldehyde polymers are formed (Figure 1b) [25, 26]. Basing his conclusions on earlier studies [24, 29], Wong [30] proposed a reaction mechanism where the unsaturated glutaraldehyde polymer cross-links the amino groups (lysine residues) of proteins as shown in Figure 1b. Rydon and co-workers have proposed a different reaction mechanism, where glutaraldehyde cross-linking of proteins leads mainly to formation of stable quaternary pyridinium compounds [25, 26]. As a conclusion, the mechanism of glutaraldehyde cross-linking of proteins is not fully understood.

Visuri [31] showed in his patent that glutaraldehyde cross-linking alone didn't provide insolubility of xylose isomerase (XI) crystals. However, when an amino group containing compound was added to the cross-linking reaction mixture, the resulting cross-linked XI crystals were insoluble. Visuri used ammonium salts, amines, and amino acids as the source of the additional amino groups. The reason for the improved cross-linking in the presence of exogenous amino groups remained unknown.

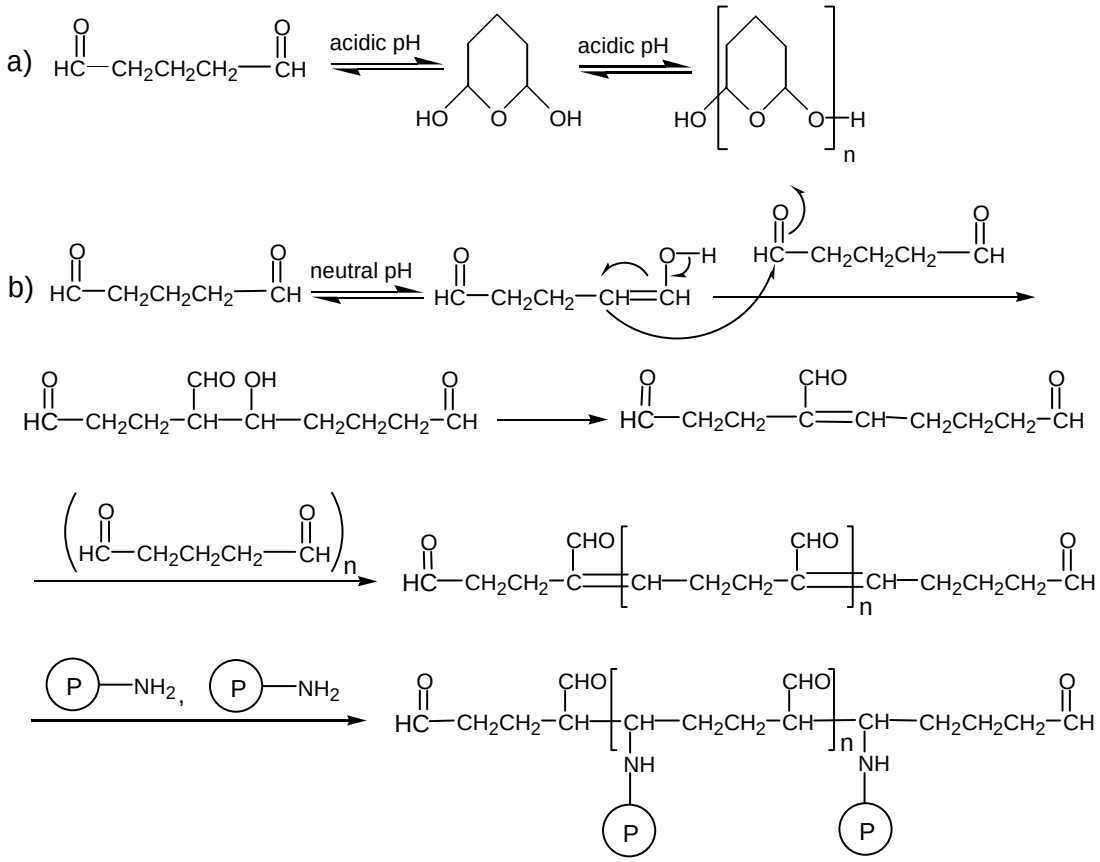

Figure 1. Polymerization of glutaraldehyde at a) acidic pH (redrawn from [32]) and b) at neutral pH and its cross-linking reaction with proteins (redrawn from [30]).

1.3.2 Other cross-linking reagents

A great number of chemical cross-linking reagents capable of reacting with the side chains of amino acids of proteins have been identified (for a thorough review, see [30]). However, in addition to glutaraldehyde, only a few of them have been used in the preparation of cross-linked protein crystals. Dialdehydes analogous to glutaraldehyde have been used to a minor extent. Margolin *et al.* [33] used succinaldehyde, glyoxal, and octanaldehyde to cross-link subtilisin crystals. However, the properties of these cross-linked products were not reported in detail.

Quiocho and Richards [5] studied the cross-linking of carboxypeptidase A crystals also with cross-linking reagents other than glutaraldehyde. Crystals cross-linked with 1,3-difluoro-4,6-dinitrobenzene in resulted in insoluble crystals that were as active as glutaraldehyde cross-linked crystals. Also biphenyl-4,4'-bisdiazonium chloride was reported to cross-link carboxypeptidase crystals successfully, but no properties of the cross-linked crystals were provided. In their review Margolin and Navia [15] briefly described that they have used more than ten cross-linking agents. However, they don't provide any information about what proteins they have cross-linked and the properties of the cross-linked products. Margolin and Navia [15] described also a reversible cross-linking method. They modified *Candida rugosa* lipase (EC 3.1.1.3) by Traut's reagent in order to introduce additional SH groups to the protein. Then the SH groups were further cross-linked with DPDPB. When the water-insoluble cross-linked product was incubated with exogenous cysteine, the crystals dissolved completely and released 85% of the initial lipase activity.

Margolin *et al.* [34] described the cross-linking of glycoprotein crystals based on chemical carbohydrate-to-carbohydrate cross-linking. Carbohydrates in glucose oxidase (EC 1.1.3.4) crystals were first oxidized by sodium periodate and then cross-linked by 1,8-diaminooctane. Thereafter $NaCHBH_3$ was used to reduce the imino groups in the product. The resulting cross-linked product was slightly soluble in water and exhibited specific activity similar to the soluble counterpart. These crystals were used to produce gluconic acid. Margolin *et al*. also prepared carbohydrate cross-linked crystals of *C. rugosa* lipase and several vaccine antigens.

1.4 Porous structure of CLPCs

In a crystalline form proteins are in a highly ordered three-dimensional array where the protein molecules are bound to each other with specific intermolecular interactions. Protein crystals contain not only protein molecules but also uniform solvent-filled pores that constitute 30–78% of the crystal volume [35, 36, 37] depending on the protein and the crystallization conditions. The solvent flows almost freely in the pores of crystalline proteins. Morozov *et al*. [38] measured that the self-diffusion coefficient of intracrystalline water in the tetragonal lysozyme crystals was reduced by about 30–40% compared with that of bulk water. Table 1 shows that the average diameter of the pores in cross-linked crystals varies between 25–80 Å, the porosity is 0.5–0.8, and the pore surface area is 600–1800 $m^2\ g^{-1}$ depending on the protein in question. The pore surface area of cross-linked crystals is comparable with that of inorganic porous materials, such as zeolites, silica gels, and activated carbon [15]. The pore sizes in Table 1 have been calculated by using a cylindrical pore model and symmetrical pore distribution. However, the pores within protein crystals are complicated in structure and cannot, thus, be described by a simple cylindrical model [36, 39, 40]. Morozova *et al.* [40] presented that the pore size in cross-linked tetragonal lysozyme crystals varied between 6–40 Å measured from crystallographic data. Figure 2 illustrates the pore structure in a native xylanase (EC 3.2.1.8) crystal lattice. The pore sizes measured from crystallographic data [36, 38] are systematically smaller than presented in table 1.

Table 1. Characteristics of CLPCs.

Protein	**Solvent content**	**Porosity**	**Pore surface area ($m^2\ g^{-1}$)**	**Pore diameter (Å)**	**Ref**
Thermolysin (EC 3.4.24.27)	50%	0.51	800	46	[36]
Lipase (*Pseudomonas cepacia*) (EC 3.1.1.3)	74%	0.80	1800	82	[36]
Lipase (*C. rugosa*)	49%	0.50	900	38	[36]
Human serum albumin	–	0.65	1500	54	[36]
Xylose isomerase	41–53%	0.47	600	29	[39]

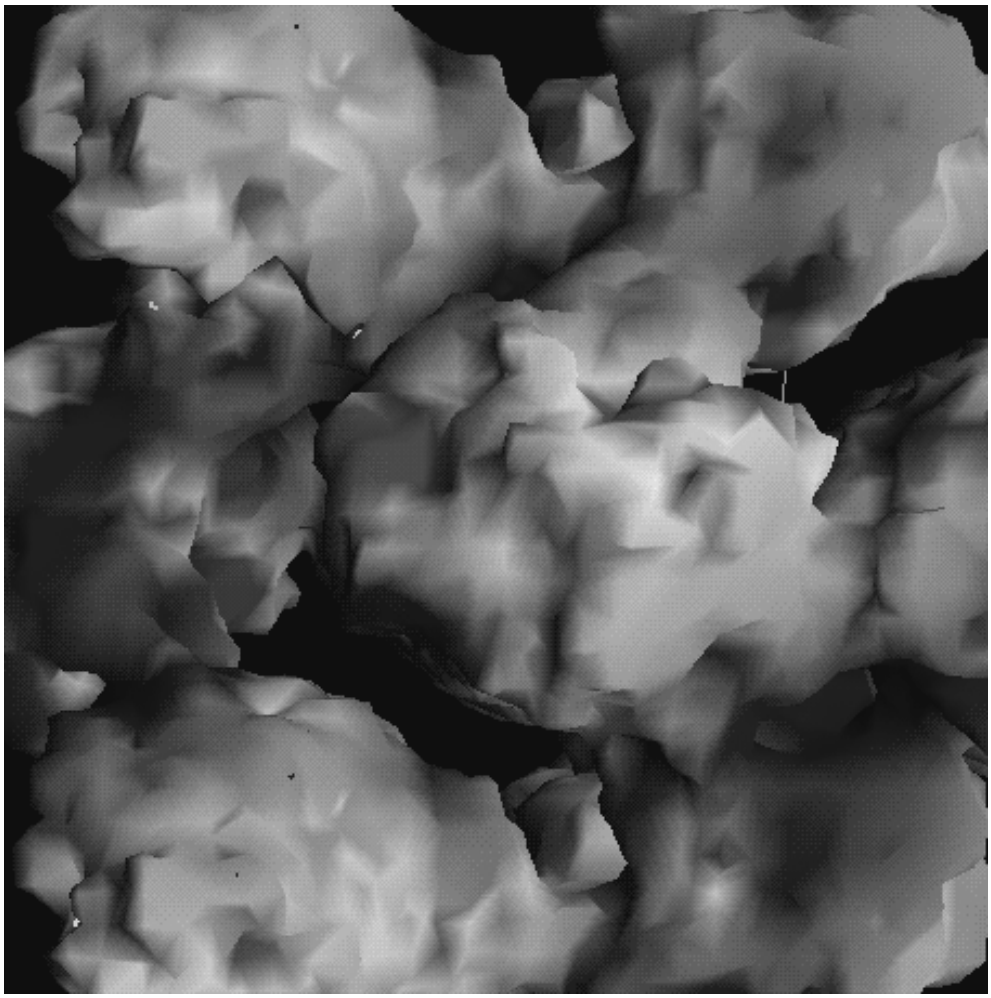

Figure 2. Crystal lattice structure of *Trichoderma reesei* xylanase II crystal. The solvent-filled channels are shown in black. The maximum cross section of the pore opening in the crystal is approximately 7×25 Å [41]. The surface representation was computed from 1.5 Å resolution X-ray diffraction data [42].

1.5 Activity of CLPCs

In aqueous solvent the activity of cross-linked protein crystals is most often reduced compared with that of soluble proteins. Table 2 shows the activity of soluble proteins, crystalline proteins, and cross-linked crystalline proteins. As shown in the table the lower activity may a result from crystallinity or the chemical modification of proteins, or a combination of both.

Mass-transfer limitations have been recognized in numerous publications to decrease the activity of CLPCs compared with the soluble counterparts. There are three factors affecting the mass-transfer limitations: the size of the crystalline particle [5, 15, 43], the size of the substrate [15, 17, 21, 36], and the pore size of the crystal. As shown above, the sizes of the open channels in protein crystals are 10–80 Å in diameter. As a consequence, large molecules having a molecular weight higher than 1000–10000 g mol^{-1}, depending on the protein crystal in question, cannot penetrate the crystals at all [36, 39]. Smaller molecules can diffuse through the pores. However, the diffusion rate can be considerably decreased compared with the diffusion rate in water. The effective diffusion coefficients of surfactants (MW 246–316 g mol^{-1}) inside tetragonal lysozyme crystals were 3–4 orders of magnitude lower than those of the corresponding free molecules in water [44].

Table 2. Activity of some cross-linked protein crystals (CLPC) compared to the activity of the protein in soluble or un-cross-linked crystalline form in aqueous solvent. Note that the units of activity are not comparable with each other as they are presented here in the same form as in the original publications.

Protein	**Reaction**	**Activity**			**Crystal size* (μm)**	**Activity ratio ($V_{CLPC}/V_{soluble}$)**	**Ref**
		Soluble	Crystalline	Cross-linked crystals (CLPC)			
Subtilisin	Hydrolysis of *N*-trans-cinnamoylimidazol	0.094 s^{-1}	N.D.	0.115 s^{-1}	<5	1.2	[18]
Subtilisin	Hydrolysis of TAME at pH 8.0	136 s^{-1}	80 s^{-1}	30 s^{-1}	<5	0.2	[18]
Subtilisin	Hydrolysis of *N*-acetyl- L-phenylalanine ethyl ester in 15% $(NH_4)_2SO_4$	N.D.**	2.3 mmol min^{-1} mg^{-1}	1.3 mmol min^{-1} mg^{-1}	N.D.	–	[21]
Subtilisin	Hydrolysis of *N*-acetyl- L-phenylalanine ethyl ester in 0.1% NaCl	10 mmol min^{-1} mg^{-1}	N.D.	6 mmol min^{-1} mg^{-1}	N.D.	0.6	[21]
Carboxypeptidase A	Hydrolysis of carbobenzoxyglycyl-L-phenylalanine	140 ΔA_{570} min^{-1} mg^{-1}	0.5 ΔA_{570} min^{-1} mg^{-1}	0.3 ΔA_{570} min^{-1} mg^{-1}	<5	2×10^{-3}	[5]
Carboxypeptidase A	Hydrolysis of carbobenzoxyglycyl-L-phenylalanine	10000 min^{-1}	N.D.	364 min^{-1}	0.5	4×10^{-2}	[43]
Carboxypeptidase B (EC 3.4.17.2)	Hydrolysis of benzoxyglycylarginine	3600 min^{-1}	N.D.	220 min^{-1}	1	6×10^{-2}	[45]
Lipase (*C. antarctica*) (EC 3.1.1.3)	Hydrolysis of *p*-nitrophenyl butyrate	1	N.D.	0.013	<1	1×10^{-2}	[46]
Lipase (*C. rugosa*)	Hydrolysis of chloroethyl ester of 2-(3-benzoylphenyl)propionic acid	1.2 mmol h^{-1} mg^{-1}	N.D.	1.4 mmol h^{-1} mg^{-1}	2	1.2	[47]
Thermolysin	Hydrolysis of furylacryloylglycyl- L-leucine amide	1	N.D.	0.8	N.D.	0.8	[48]
Chloroperoxidase	Oxidation of thianthrene	555 s^{-1}	N.D.	0.85 s^{-1}	10	2×10^{-3}	[19]
Chloroperoxidase	Oxidation of thioanisole	4170 s^{-1}	N.D.	28 s^{-1}	10	7×10^{-3}	[19]
Catalase (EC 1.11.1.6)	Oxygen release from hydrogen peroxide	1	0.55	0.55	N.D.	0.6	[16]
Fructose diphosphate aldolase (EC 4.1.2.13)	Synthesis of tagetitoxin	1	0.65	0.82	pulverized	0.8	[49]
Lactate dehydrogenase (EC 1.1.1.27)	Lactate production from pyruvate	1	0.7	0.3	N.D.	0.3	[50]

*shortest dimension; **N.D. = not determined

Morozova *et al.* [40] calculated that the mobility of cations was 4–50 fold lower and that of anions 100–300 fold lower in the tetragonal lysozyme crystals compared with the mobilities in solution. Even the self-diffusion coefficient of intracrystalline water in the tetragonal lysozyme crystal is reduced by about 30–40% compared to that of bulk water [38].

The crystal size plays a crucial role in the activity of the crystals. Most of the catalytic reactions are taking place inside the crystals, as even with very small crystals (e.g., $1\times1\times2$ μm^3) more than 99% of the protein molecules are located in the interior of the crystal [15]. As a consequence, the substrate and the product have to diffuse from the bulk to the catalytic site and from the catalytic site to bulk solution, respectively. Table 3 shows the relative activity of cross-linked crystalline enzymes in respect to the crystal size. The effect of the crystal size on the activity depends on the kinetics of the reaction, the size of the pores, and the size of the substrate and product [5, 15]. The crystal size has the most dramatic effect on reactions involving large molecules that can diffuse only partially or cannot diffuse at all to the pores of crystal [15, 17, 21, 36].

Table 3. The effect of particle size on the relative activity of cross-linked protein crystals.

Protein	Particle size in the smallest dimension (μm)	Relative activity* (per unit weight of enzyme)	Ref.
Carboxypeptidase A	58	1	[5]
	28	1.6	
	6	5.6	
Alcalase	40	1	[15]
(EC 3.4.21.-)	1.2	3.2	
Penicillin acylase	142	1	[15]
(EC 3.5.1.11)	57	3.4	

*Activity relative to the activity of the biggest crystals

Limited conformational flexibility may cause dramatically reduced protein activities [51]. In the crystal lattice proteins have multiple specific intermolecular interactions. These interactions may stabilize proteins but can also reduce the flexibility of protein molecules. Another source of limited flexibility is the chemical cross-linking. In recent reports Ayala *et al*. [19] and Costes *et al*. [52] showed that the specific activity of cross-linked crystals of chloroperoxidase and hydroxynitrile lyase (EC 4.1.2.37), respectively, decreased with increasing the glutaraldehyde concentration used in cross-linking. However, maximal stability and total insolubility was only achieved in conditions using high concentration of glutaraldehyde in both cases. Similar results were obtained with subtilisin [18]. However, Sobolov *et al.* [49] reported that the use of *increasing* amounts of glutaraldehyde increased also the specific activity of cross-linked fructose diphosphate aldolase crystals. The reason for this phenomenon is not known.

However, the reduced protein flexibility caused by crystallization and cross-linking can also be exploited. Grochulski *et al.* [53, 54] showed that the properties of cross-linked *C. rugosa* lipase crystal preparations were affected by the conformation of lipase during crystallization and cross-linking. In lipase molecule there is a certain “lid” that is “open” enabling substrate binding to the active site when the active site is unoccupied. However, when the “lid” is “closed” the substrate is enclosed to the active site of lipase. If lipase was crystallized and cross-linked “lid open”, the activity was three times higher than if

crystallized “lid closed”. The crystallization and cross-linking of lipase in “lid open” form allowed a rapid binding of the substrate to the active site and a rapid removal of the product from the active site thus increasing the specific activity.

In addition to the reduced flexibility the cross-linking can also have other effects on the activity of protein crystals. It is possible that glutaraldehyde cross-linking creates steric hindrances in the crystal lattice, which may occlude some active sites [51]. Chemical cross-linking may also modify catalytically important residues at or near the active site and, thus, affect the catalytic properties of the protein.

1.6 Stability of cross-linked protein crystals

The chemical cross-linking of protein crystals significantly enhances the stability against heat denaturation, proteolytic enzymes, and organic solvents compared with corresponding soluble proteins as shown in Table 4. The enhanced stability can be a result of crystallinity, chemical cross-linking of the proteins, or a combination of both. In the crystalline form proteins maintain better their native conformation in conditions that would lead to an unfolding of soluble protein [15]. This is a result of the highly ordered three-dimensional array of protein molecules in crystal where protein molecules are bound to each other with specific intermolecular interactions. The crystalline environment enhances the stability of proteins against heat and other denaturants by preventing unfolding, aggregation, or dissociation of proteins [15, 18, 30]. The chemical cross-linking is another source of stabilization. It has been shown that long glutaraldehyde cross-linking times yielding a high degree of cross-linking is needed to achieve maximal stability of cross-linked protein preparates [18, 19, 51]. It seems that cross-linked crystals are universally resistant to digestion by proteolytic enzymes [18, 20, 23, 48, 55]. Possibly, proteases can, at least partly, digest the proteins locating on the protein crystal surface. However, these digested surface proteins remain attached to the surface of the crystal preventing the proteases to enter the crystals. Because only a small fraction of the protein molecules are located on the crystal surface, the activity of cross-linked crystal remains at the initial level.

Table 4. Stability of soluble proteins and cross-linked protein crystals.

Protein	Incubation conditions	Half-life		Ref.
		Soluble	Cross-linked crystals	
Stability at high temperatures				
Horse liver alcohol dehydrogenase (EC 1.1.1.1)	HEPES buffer, 30 °C, pH 8	14 d	No loss in activity after 3 months	[56]
Subtilisin	55 °C, 0.1 M acetate, pH 5.7	2 h	No loss in activity after 20 h	[18]
Yeast alcohol dehydrogenase (EC 1.1.1.1)	40 °C, 0.1 M Tris-HCl buffer, pH 7	<1d	No loss in activity after 4 d	[23]
Lactate dehydrogenase	25 °C, 0.1 M phosphate buffer, pH 7.5	<4 d	10% loss in activity after 25 d	[50]
Lipase (*C. antarctica*)	40 °C, 0.02 M phosphate buffer, pH 7	<4 d	No loss in activity after 6 d	[46]
Lipase (*C. rugosa*)	40 °C, 0.01 M Tris-HCl buffer, pH 7	5 h	>13 d	[47]
Thermolysin	65 °C, Tris-HCl buffer, pH 7	6 h	No loss in activity after 5 d	[48]
Xylose isomerase	77.5 °C, 45% (w/v) glucose/fructose solution, pH 7	71 min	407 min	[57]
Stability in the presence of proteolytic enzymes				
α-Chymotrypsin (EC 3.4.21.1)	5 mg ml^{-1} unspecific bacterial protease, 0.1 M citrate buffer, pH 4	4 min	11 h	[23]
Thermolysin	Mixture of *Streptomyces griseus* proteases, 65 °C, Tris-HCl buffer, pH 7	<5 min	5% loss in activity after 4 d	[48]
Stability in the presence of organic solvents				
Horse liver alcohol dehydrogenase	90% 2-propanol, 25 °C	<1 min	2 d	[56]
α-Chymotrypsin	50% (v/v) dioxane, 0.1 M citrate buffer, pH 4	0.5 h	2.3 h	[23]
α-Chymotrypsin	50% (v/v) hexadecane, 0.1 M citrate buffer, pH 4	16 h	3500 h	[23]
Subtilisin	Acetonitrile, 45 °C	5 d	200 d	[58]
Subtilisin	50% acetone, 40 °C	4 d	No loss in activity after 7 d	[55]
Lipase (*C. rugosa*)	50% DMSO, 25 °C, 0.01 M Tris-HCl buffer, pH 7	0.7 h	300 h	[47]

1.7 Mechanical stability of CLPCs

Native protein crystals are known to be fragile and they are easily cracked or chipped even by gentle mechanical forces. In contrast, cross-linked protein crystals are considerably more stable towards mechanical stresses than native crystals. The source of this mechanical stabilization is clearly the chemical cross-linking. Quite surprisingly, only Lye and co-workers have actually measured the mechanical stability of cross-linked protein crystals using *C. rugosa* lipase and yeast alcohol dehydrogenase as model crystals. Flat plate (thickness <2 μm) crystals of lipase [23] cross-linked in 0.5%

glutaraldehyde and yeast alcohol dehydrogenase [59] cross-linked in 0.05% glutaraldehyde remained intact in conditions equivalent to those expected in the high-shear regions of stirred-tank reactors (ε_{max} = 0.34 $\times 10^5$ W kg^{-1}). The native crystals of yeast alcohol dehydrogenase were significantly fragmented in the same conditions [59]. However, at high shear stress (ε_{max} = 6.8 $\times 10^5$ W kg^{-1}) both lipase and yeast alcohol dehydrogenase crystals were broken, and the median crystal size was decreased from an initial value of 13 μm to 7 μm and 12 μm to 7 μm for lipase and yeast alcohol dehydrogenase, respectively [23, 59]. The lipase crystals that were cross-linked in 5% glutaraldehyde, and small cross-linked rod-shaped yeast alcohol dehydrogenase crystals having an average size of 5 μm, were totally resistant even to the high shear stress (ε_{max} = 6.8 $\times 10^5$ W kg^{-1}). The visual inspection of the broken crystals showed significant rounding of the crystal edges. This suggests that the mechanism of breakage is primarily a result of attrition by crystal-crystal collisions [59].

The mechanical integrity of cross-linked yeast alcohol dehydrogenase crystals was studied under compressive and abrasive forces found in dead-end filtration. The cake resistance was constant over five cycles of mixing and filtration at a pressure of 100 kPa indicating no breakage of the crystals. Rod-shaped crystals had a lower cake resistance than the hexagonal crystals. This was explained by the size and shape of the crystals. Rod-shaped crystals are cylindrical and allow more voidage within the cake. The hexagonal crystals are flat plates that can stack on each other forming a very dense cake increasing the cake resistance. As a conclusion, Vaghjiani *et al.* [23] stated that the control of the size and shape of the crystals is important in relation to the downstream processing of cross-linked crystal suspensions.

1.8 Cross-linked enzyme crystals as catalysts

The reason for the choice to use an enzyme in the form of cross-linked crystals is most often based on the stability of the cross-linked crystals in both aqueous and organic media. The stability is achieved without a considerable loss of specific activity. Cross-linked crystals are insoluble throughout the production process. They are mechanically robust and can be easily removed from the reaction mixture by filtration or centrifugation and, thus, they can be recycled, which increases the productivity of the catalyst [e.g., 17, 23, 47, 59, 60]. The purity of the catalyst leads to a high enantioselectivity of the reaction [55]. Cross-linked crystals are, in addition, an extremely stable storage formula compared to soluble enzymes, for example [16, 18, 48, 49, 50, 56]. Table 5 summarizes the catalytic applications where cross-linked enzyme crystals have been used.

Table 5. Catalytic applications where cross-linked enzyme crystals have been used.

Enzyme	Reaction	Ref.
Subtilisin	Synthesis of peptides and peptidomimetics	[17, 55]
Subtilisin	Regioselective hydrolysis	[55]
Subtilisin	Transesterification	[55]
Subtilisin	Catalysis in supercrirical fluids	[61, 62, 63, 64]
Thermolysin	Synthesis of peptides	[17]
Lipase	Enantioselective ester hydrolysis	[47, 60, 65]
Lipase	Enantioselective transesterification	[65, 66]
Lipase	Enantioselective acylation	[65]
Hydroxynitrile lyase	Asymmetric syntheses	[52]
Fructose diphosphate aldolase	Synthesis of tagetitoxin	[49]
Xylose isomerase	Carbohydrate conversions	[68, 69]
Xylanase	Hydrolysis of xylo-oligomers	[41]
Alcohol dehydrogenase	Reduction of ketones with cofactor regeneration	[70]
Lactate dehydrogenase	Production of lactate	[50]
Organophosphorous hydrolase (EC 3.1.8.1)	Hydrolysis of pesticide	[71]

1.8.1 Proteases

The protease subtilisin is used in synthetic chemistry in both aqueous and organic solvents for regio- and enantiospecific reactions, amide bond syntheses, and hydrolyses. Two major problems have limited the use of subtilisin: low stability in water and water-miscible solvents, and low activity in neat organic solvents [55]. The reason for low stability in water is the autodigestion, which reduces the half-life of the catalyst and makes the product purification difficult.

Tüchsen and Ottesen [18] demonstrated that cross-linked subtilisin crystals were resistant to the autodigestion, which rapidly decreased the activity of soluble subtilisin in aqueous solvent. Wang *et al.* [55] showed that cross-linked subtilisin crystals retained full activity after 7 d incubation in aqueous buffer at 60 °C, 50% acetone at 40 °C, and in 70% DMF at 25 °C. Crude enzyme extract lost half of its activity after 11 h in aqueous buffer and after 4 d in 50% acetone. Cross-linked subtilisin crystals were highly active in various organic solvents. However, cross-linked crystals required a small amount of water in neat organic solvents, especially in a polar solvent such as acetone or DMF [55]. The total absence of water resulted in the loss of catalyst selectivity and ultimately in the complete loss of activity [55]. Xu and Klibanov [72] showed that the use of the cross-linked crystalline form of subtilisin in neat organic solvents enhances the enzyme activity compared with lyophilized subtilisin precipitate. The activity of lyophilized subtilisin in nonaqueous media is affected by the pH of the solution, where the lyophilization is conducted (so called “pH memory”). When lyophilized enzymes are suspended in non-aqueous media, the protonation status of these groups does not change. In the case of cross-linked crystals, water can be removed by repeated washing with a water-miscible solvent. The solvent diffuses through the channels in the cross-linked crystals and the

interstitial water diffuses out. Since this occurs while there is still water present, the protonation state adjusts accordingly, as the protons are not kinetically trapped [72]. However, Montaneñez-Clemente *et al.* [73] showed that the activity of subtilisin Carlsberg was enhanced by lyophilization with methyl-β-cyclodextrin (MβCD). This method yielded a catalyst that was more active than cross-linked crystals of subtilisin. In all demonstrated reactions the MβCD-co-lyophilized enzyme was more active and as enantioselective as the cross-linked crystalline preparation.

In the synthesis of peptides and peptidomimetics, the use of polar, water miscible organic solvents is useful since it improves the substrate solubility and shifts the equilibrium towards products [74,75,76]. Wang *et al.* [55] synthesized peptides with cross-linked subtilisin crystals. Acetonitrile containing 1% water was found to be the best solvent for the synthesis. In the same conditions the soluble enzyme was inactivated rapidly. Cross-linked subtilisin crystals accepted both L- and D-amino acid amides as nucleophiles and showed a similar reaction rate for both enantiomers. One major benefit with the cross-linked subtilisin crystals is the lack of autodigestion. A peptide synthesis catalyzed by soluble subtilisin leads to product purification problems as a result of the contaminating peptides produced by subtilisin autodigestion. Persichetti *et al.* [17] showed that cross-linked thermolysin crystals catalyzed several peptide syntheses. The specific activities of soluble and cross-linked crystalline thermolysin were very close to each other. Also a 30 amino acid peptide, an oxidized insulin B chain, was a suitable substrate for cross-linked thermolysin crystals with an initial rate of 4% of that observed for a soluble catalyst. However, in the cross-linked crystalline form thermolysin was considerably more stable than the soluble enzyme in ethyl acetate and in mixtures of water-miscible solvents (DMF, THF, acetone) and water [17]. St. Clair and Navia [48] also reported a greatly enhanced stability of thermolysin in the cross-linked crystalline form.

Wang *et al.* [55] studied the regioselective hydrolysis of the terminal amide of peptide amides by cross-linked subtilisin crystals. The catalyst accepted a broad range of substrates and the reaction yields were quantitative. This hydrolysis method renders it possible to carry out the reactions in mild conditions. Normally harsh conditions are needed for regioselective hydrolysis leading to product racemication. Cross-linked subtilisin crystals have also been used for the hydrolysis of proteins, but they suffered from severe mass-transfer limitations of the high molecular weight substrates [18]. Wang *et al.* [55] used cross-linked subtilisin crystals also for a variety of reactions: enantioselective synthesis of a variety of molecules, regioselective reactions of many polyfunctional molecules, and for transesterification of amino acid and peptide esters. The cross-linked subtilisin catalyst was found to be excellent as a result of the high activity and stability in organic solvents, high regio- and enantioselectivity, and mild reaction conditions. The reusability of cross-linked subtilisin crystals was demonstrated by the resolution of a certain amine. After seven resolution cycles cross-linked crystals retained full activity without loss in enantioselectivity [55]. The crude enzyme extract lost 50% of its activity after two cycles.

1.8.2 Lipases

Lipases can be used for stereoselective ester conversions, regioselective and chemoselective acylations and deacylations, and simple hydrolysis of esters under mild reaction conditions [47]. Most often crude enzyme preparations have been used as biocatalysts because of their availability and low cost. However, crude lipase mixtures suffer from stability problems in organic solvents and they contain contaminating side

activities that have unexpected synthetic properties affecting the enzyme catalysis [47, 77, 66, 65]. Lalonde *et al.* [47] identified competing hydrolase activities and protease activity in a crude extract of *C. rugosa* lipase. Cross-linked lipase crystals are considerably more stable in the presence of organic solvents than the soluble crude lipase preparations [46, 47, 77, 66, 65]. Neither cross-linked soluble lipase nor its precipitate lacking crystalline structure exhibit stability beyond the level of soluble enzyme [47]. Soluble pure lipases cannot be used in synthesis as a result of low stability against high temperatures and organic solvents [47, 78, 79]. The stabilization effect in organic solvents is extremely important, since many organic compounds are poorly soluble in an aqueous solvent. Both substrate specificity and enantioselectivity of cross-linked lipase crystals are markedly different from those of crude enzyme extract [47, 66, 65], which shows the benefit of using pure protein catalysts. In the preparation of cross-linked lipase crystals the enzyme is simultaneously purified in the crystallization.

The use of cross-linked *C. rugosa* lipase crystals in organic solvents greatly improved enantioselectivities of several reactions including the resolution of acids by esterification [65], acylation of secondary alcohols with different carboxylic acids and with activated esters [65], and the ester hydrolysis [47]. The increased enantioselectivity was attributed to the purity of the protein. Persichetti *et al.* [65] showed that cross-linked lipase crystals were six orders of magnitude more active than lyophilized lipase in the resolution of menthol with vinyl butyrate in toluene [65]. Lalonde *et al.* conducted preparative resolutions (100 g scale) of methyl-Ibuprofen and Ketoprofen by cross-linked lipase [47]. The crystalline catalyst was recycled 18 times. During this time the specific activity dropped by 44%. However, immobilized lipase preparation lost virtually all of its activity after the first reaction cycle. Browne *et al*. [66] studied the production of inherently chiral calix[4]arenes by cross-linked crystals of lipases from *Mucor miehei* (EC 3.1.1.3) and *Aspergillus niger* (EC 3.1.1.3). The use of *A. niger* lipase in a cross-linked crystalline form improved the enantioselectivity of the reaction over chemical catalysis and free *C. rugosa* lipase catalysed synthesis.

Collins *et al*. [60] reported the scale-up of chiral resolution using cross-linked crystalline lipase from *Pseudomonas cepacia*. The reaction studied was the chiral resolution of *R-sec*-phenethyl alcohol from racemic *R/S-sec*-phenethyl acetate. The reaction parameters were first studied in small-scale (0.2–1 l) in order to determine the optimum conditions for a 100 l reactor. The reaction mixture contained two phases: aqueous phase and organic phase consisting of the substrate *R/S-sec*-phenethyl acetate. Cross-linked lipase catalyst was not detrimentally affected by a high shear in the reactor needed to obtain homogenous mixing in the two-phase system even in large-scale. In small-scale, the reaction yield was 48% and the product was formed with 99% enantiomeric excess. These values were not changed in the 100 l scale. A total number of nine runs were made with the same batch of cross-linked lipase crystals. In the first run, 50 g of crystals were added to the reaction mixture (0.5 g l^{-1}). After nine runs about 12.5 g of lipase crystals were collected from the reactor. This decrease in the catalyst amount was a result of the problems in the catalyst recovery process after each run. Also the specific activity of the cross-linked catalyst decreased to one third of the initial value.

In addition to catalytic applications, the cross-linked crystalline form of lipase can be used to determine the reaction kinetics and the parameters controlling the enantioselectivity in non-aqueous media, which cannot be studied by pure native enzymes as a result of their instability [65]. Cross-linked crystals are the only pure form stable and active enough for mechanistic studies [46, 47, 77, 66, 65].

1.8.3 Dehydrogenases

The use of dehydrogenases in synthetic applications is often limited by the instability of both the enzymes and their nicotinamide cofactors [70]. St. Clair *et al.* [56] crystallized and glutaraldehyde cross-linked horse liver alcohol dehydrogenase (HLADH) in the presence of the reduced nicotinamide adenine dinucleotide (NADH), which was tightly bound to the enzyme by cross-linking. No leakage of cofactor from cross-linked horse liver alcohol dehydrogenase crystallized with NADH was detected in a prolonged incubation of concentrated protein crystal slurry. This was expected, as in the presence of the cofactor, horse liver alcohol dehydrogenase undergoes a large conformational change enclosing the cofactor [80] and, thus, preventing its dissociation from holoenzyme after crystals were cross-linked. Cross-linked crystals were active catalysts without any cofactor addition and catalyzed a reduction of several ketones. When HLADH was crystallized in the absence of NADH, the activity of the resulting cross-linked crystals was low even in the presence of exogenous NADH. The crystallization and cross-linking of HLADH in the presence of NADH greatly improved the stability of the enzyme and the cofactor. The half-lives of soluble HLADH and NADH were about 14 d and 3 d in a buffer solution at 30 °C, respectively. The cross-linked HLADH-NADH crystals maintained full activity after three months of incubation. Especially the stability of HLADH in the presence of organic solvents is important, because high concentrations of isopropanol, ethanol, and butanediol can be used for cofactor regeneration. The half-life of cross-linked HLADH-NADH crystals in 90% isopropanol was 2 d at 25 °C, for example. Soluble HLADH precipitated and lost activity immediately in these conditions. The cofactor recycle inside cross-linked HLADH-NADH crystals was demonstrated by the reduction of cinnamaldehyde in the presence of 200 mM of 1,4-butanediol to regenerate the cofactor. A column packed with approximately 50 mg of HLADH-NADH containing about 1.2 μmol NADH reduced 15 mmol cinnamaldehyde to cinnamyl alcohol.

Sobolov *et al.* [50] studied the production of lactate from pyruvate in an electrolytic cell catalyzed by cross-linked lactate dehydrogenase crystals. The cross-linked crystalline form of the enzyme was used in the study solely because of the improved stability. Lactate dehydrogenase produces lactate from pyruvate and NADH. Only 10% of the activity of cross-linked crystalline lactate dehydrogenase was lost during 25 days of operation, while soluble lactate dehydrogenase lost its activity totally after 5 days. Furthermore, the cross-linked lactate dehydrogenase crystals could be recycled. The yield of lactate from pyruvate was 70% with cross-linked lactate dehydrogenase crystals. The yield with soluble enzyme was 50%, even though an aliquot containing the same amount of fresh enzyme that was present in the corresponding experiment in the form of cross-linked crystals was added daily to the reaction mixture.

1.8.4 Other enzymes

Leisola and co-workers have studied cross-linked xylose isomerase (CLXIC) and xylanase crystals in packed bed reactors [41, 68, 69]. Because of the high enzyme to substrate ratio, they were able to identify new substrates for xylose isomerase (XI) [69]. They used a CLXIC reactor for isomerizations of a variety of pentose and hexose sugars [69], such as for the production of an unnatural sugar, L-ribose, from L-arabinose [69]. However, the reported efficiency of this conversion was low compared with chemical synthesis [81] or catalysis by a specific enzyme [82, 83]. They further showed that the packed bed reactor was capable of catalysis and simultaneous product separation [68].

The reason for this was that each of the sugars (substrates and products) had a different reaction rate and binding characteristics to the CLXIC matrix. Similar product separation was observed in the hydrolysis of xylotetraose to xylose by cross-linked xylanase crystals in a packed-bed reactor [68].

Sobolov *et al.* [49] characterized cross-linked fructose diphosphate aldolase crystals for the synthesis of tagetitoxin [67]. Soluble fructose diphosphate aldolase was an effective synthetic catalyst but suffered from instability, water insolubility of unnatural substrates, and high cost. However, the cross-linked crystalline form was highly stable without any observable loss of activity after 6 months of incubation in room temperature. In the same conditions the soluble enzyme lost its activity after 3–10 days. The specific activity of cross-linked crystals was 65% of the activity obtained with the soluble enzyme. In organic solvents (e.g., DMSO, acetonitrile, dioxane) the soluble form of fructose diphosphate aldolase lost 75–97% of the initial specific activity after 1 h incubation. However, with cross-linked crystals no significant loss of activity was observed.

Hydroxynitrile lyase is used for asymmetric syntheses of intermediates for pharmaceuticals and agrochemicals. Hydroxynitrile lyase have been used both in soluble and carrier-immobilized forms. However, the stability of these enzyme preparations is relatively low both in aqueous and organic media [84, 85]. Costes *et al.* [52] prepared cross-linked hydroxynitrile lyase crystals and compared this with immobilized preparates. They used dry acetone in the cross-linking reaction in order to reduce the cross-linking time and glutaraldehyde concentration. They found that by increasing the concentration of glutaraldehyde in cross-linking the resulting crystals were more stable. The specific activity of cross-linked crystals was two orders of magnitude lower than the activity of Celite-immobilized hydroxynitrile lyase. However, the cross-linked crystals were much more stable in the presence of organic solvents. After seven one-hour reactions in a dibutyl ether solution, only 1% of the initial activity of Celite-immobilized enzyme was left. In contrast, more than 70% of the initial activity of the cross-linked crystals was retained in the same conditions.

An organophosphorous hydrolase has been recognized to hydrolyze P–O, P–F, P–S, and P–CN bonds making a variety of chemical agents nonhazardous, including chemical warfare nerve gas VX [15, 71]. However, for practical applications the activity and stability has to be enhanced. Hoskin *et al.* [71] showed that cross-linked crystals of the organophosphorous hydrolase hydrolyzed the common pesticide Demeton-S. The authors assumpted that in the form of cross-linked crystals organophosphorous hydrolase could be economically used in practical applications. However, experimental data supporting this assumption was not shown.

1.8.5 Enzyme catalysis in supercritical fluids

Supercritical fluids (sc-fluids) are liquids that are heated under pressure until the vapor phase is as dense as the liquid phase. Sc-fluids have liquid-like densities and gas-like diffusivities. They are an environmentally friendly alternative to organic solvents for biocatalysis, because the solvent can be completely removed after depressurization. The most common sc-fluids are sc-CO_2 and sc-ethane. Barreiros and co-workers have studied the properties of cross-linked subtilisin crystals in sc-fluids [61, 62, 63, 64]. The model system was cross-linked crystalline subtilisin catalyzing transesterification reactions. Cross-linked crystals in sc-ethane were 2–10-fold more active than in hexane or acetonitrile under similar conditions [61]. The specific activity of cross-linked crystals in

sc-ethane was 5–100-fold higher than the specific activity of lyophilized subtilisin. Especially with low water activities cross-linked crystals were superior [61]. The activity of cross-linked subtilisin was further enhanced by addition of salt hydrates [63] or zeolites [64] to the reaction media. This was probably a result of acid-base buffer effect of these additives in low water-activity media. The enzymatic catalysis by cross-linked crystalline enzymes in sc-carbon dioxide has been patented recently [86].

1.9 Cross-linked protein crystals as separation media

Protein crystals have many properties that make them interesting separation matrices. They are made of L-amino acids that create an asymmetric environment. The interior of many globular proteins is hydrophobic. Several amino acid residues are either negatively or positively charged leading to ion exchange properties of proteins. Enzymes have also specific binding sites for their substrates and cofactors. In addition, protein crystals are microporous materials.

Immobilized proteins have been used in chiral separations (for a review, see [87]). Vilenhick *et al*. [36] showed for the first time that cross-linked protein crystals can also be used in chiral separations. They described the chiral separations of phenylglycine and phenyllactic acid by cross-linked thermolysin crystals. These compounds are not substrates for thermolysin. Moreover, they showed that the cross-linked precipitate of human serum albumin did not separate the two enantiomers of folinic acid, while cross-linked human serum albumin crystals gave good chiral resolution. This result suggests that the crystallinity is needed for good separation characteristics. The high surface area of crystalline proteins explained this result. Pastinen *et al.* [39] used cross-linked xylose isomerase crystals CLXIC in a chiral separation of a racemic mixture of D/L-arabitol. However, arabitol is also a substrate analog of the enzyme and the separation can be a result of the chiral nature of the protein, the specific binding of arabitol to the active site, or a combination of both. CLXIC showed also weak chiral separation ability for some D/L pairs of some amino acids [39].

CLXIC separated n-alcohols from C_1 to C_8 based on the hydrophobic interaction between the carbon chain of the alcohols and the protein [39]. The retention time of 1-octanol was approximately doubled compared with the retention time of methanol. CLXIC separated L-forms of tryptophane, histidine, tyrosine, and phenylalanine from other natural amino acids [39]. Vilenchik *et al*. [36] showed that cross-linked thermolysin crystals separated a mixture of Ibuprofen and phenyllactic acid. The mechanism of these separations is not known.

Pastinen *et al.* [88] used CLXIC as an affinity separation matrix for sugar alcohols xylitol and sorbitol, which are substrate analogs to xylose isomerase. Sugar alcohols were specifically bound to CLXIC even from impure solutions, and were eluted by dilute $CaCl_2$. Only the active form of the enzyme had the separation ability. However, only one molecule of xylitol or sorbitol was bound to each active center of xylose isomerase, which led to a low overall separation capacity of 1 g xylitol per 500 g CLXIC.

Both Pastinen *et al.* [39] and Vilenchik *et al.* [36] pointed out the good mechanical stability of a cross-linked protein crystal stationary phase. A cross-linked xylose isomerase column was packed with 30 MPa pressure, and thermolysin and human serum albumin columns with 10 MPa pressure, which shows the rigidity of the crystals [36, 39].

Vilenchik *et al.* [36] used a single column of both thermolysin and human serum albumin crystals for more than 500 injection cycles without any loss of separation efficiency.

1.10 X-ray crystallography

All protein crystals are sensitive to radiation used in X-ray crystallography, which is an essential tool in the structure determination of proteins. In some cases the crystal sensitivity is unmanageable and the diffraction quality of crystals deteriorates rapidly. Quiocho and Richards initially described the concept of cross-linked protein crystals in 1964 [4]. They stabilized carboxypeptidase A crystals by glutaraldehyde treatment for X-ray structure analysis. There are only minor changes in the diffraction pattern of cross-linked protein crystals compared with that of the native crystals [4, 20, 21, 22]. Cross-linked crystals render it possible to study the protein structure in organic solvents, which cannot be done with native crystals [21]. Recently Lusty [22] described the use of crystal cross-linking together with cryocrystallography, which is a common way to increase the stability of crystals towards the radiation. In cryocrystallography, crystals are treated with a cryoprotectant (e.g., sugar solutions, ethylene glycol, PEG, glycerol) and cooled rapidly to cryogenic temperatures (e.g., with nitrogen gas around 100 K). However, the crystals often suffer damage on cooling. Lusty showed that glutaraldehyde cross-linking protected the crystals from the damage on cooling without any effect on the diffraction properties.

1.11 Design of new protein materials

Dotan *et al.* [89] described the production of a pre-designed protein crystal lattice. They chose a nearly tetrahedral lectin concavalin A as a model building block. This lectin is a tetramer with a fully characterized three-dimensional structure. The lectin tetramer contains four binding sites for α-D-mannopyranoside or α-D-glucopyranoside. They used computer modeling, which suggested that the cross-linking of soluble lectin tetramers by bismannoside would result in the formation of a diamond-like three-dimensional protein lattice. Indeed, the addition of bismannopyranoside to the lectin solution resulted in a rapid formation of lectin crystals, which were simultaneously cross-linked. A chemical analysis of the bismannopyranoside content in the crystals supported the hypothesis that each lectin molecule was cross-linked through four molecules to its neighbors. The size of the resulting crystals was 0.1–100 μm depending on the pH in the crystallization. The cross-linked crystalline product was insoluble and resistant to pH changes.

Häring and Shreier [90] combined the methods of chemical modification of the enzymatic framework as well as the enzymatic active site. First they prepared cross-linked crystals of protease subtilisin. Then they activated the active site serine 221 by PMSF and subsequently PMSF was substituted by sodium hydrogen selenide. The selenol group was oxidized with hydrogen peroxide to yield a stable seleninic acid form of cross-linked subtilisin crystals. As a result, the semisynthetic seleno-subtilisin showed glutathione peroxidase activity, and catalyzed kinetic resolution of several racemic hydroperoxides. The preparation of seleno-subtilisin has been described earlier [91]. However, the crystalline seleno-subtilisin showed a high specific activity, and stability against organic solvents and high temperatures compared to amorphous seleno-subtilisin [90].

1.12 Medical applications

St. Clair *et al.* [92] investigated the possibility to use cross-linked protein crystals as antigens. Currently the progress is toward subunit vaccines, which are composed of a purified antigenic determinant separated from the disease-causing organism. To enhance the immune response, subunit vaccines universally require adjuvants and delivery vehicles. Human serum albumin in a cross-linked crystalline form showed up to 30-fold increased immunogenity and increased stability over the soluble albumin that was formulated with a commercial aluminium adjuvant. Additionally, Margolin *et al.* [34] crystallized and carbohydrate cross-linked several vaccine antigens to formulate vaccines. However, no details of the function of these antigens as vaccines were provided.

Margolin and Navia described in their review [15] that cross-linked protein crystals could be used in oral lumenal therapy. Metabolic or gastrointestinal diseases are often the result of impaired or missing enzymes or protein function in the biochemical pathway. However, the conditions in the gastrointestinal track are challenging for protein drug delivery. The pH is acidic (pH < 2) and there is a high concentration of proteases in the stomach and gut. Because of the excellent stability and good activity of proteins in the cross-linked crystalline form, the patient can orally administer a cross-linked crystalline therapeutic protein to perform the desired reactions in the gut lumen. According to Margolin and Navia [15] preliminary results indicate that in a cross-linked crystalline form lipases are at least 100-fold more active and significantly more stable than commercial lipase products. These principles are also described in a recent patent application [93].

1.13 Biosensor applications

De Mattos *et al.* [94] compared glucose biosensors prepared from commercial lyophilized glucose oxidase, crystalline glucose oxidase, and cross-linked crystalline glucose oxidase. The best performance in terms of sensitivity, detection limit, and operational stability was obtained with biosensors based on cross-linked protein crystal technology. Probably, the enzyme stability during immobilization, which includes drying the enzyme layer and contact between dried glucose oxidase and ethanol, is critical. The cross-linked crystals seemed more resistant to denaturation upon the preparation of the sensor.

2 Aims of the work

Cross-linked protein crystals (CLPC's) possess many excellent properties including high volumetric activity and stability. Thus, CLPC technology shows great potential as a novel protein formulation for use in various practical applications. The aim of this work was to study CLPC technology in bioseparation and biocatalytic applications. Two model systems were used in the study.

An antibody Fab fragment ENA5His specifically recognizes one enantiomer of a racemic mixture of a chiral drug [95]. Previously, ENA5His has been used in a carrier-immobilized form as an immunoaffinity chromatography material in order to prepare pure enantiomers from a synthetic racemic drug. However, the carrier-immobilized ENA5His suffered from poor stability in the presence of a high concentration of organic solvents needed to release the bound drug [95]. This means that simple carrier immobilization of ENA5His produces a workable but disposable separation matrix. The aim of the work with ENA5His was to stabilize the antibody molecule towards the harsh conditions used in the separation application and to develop a reusable immunoaffinity chromatography matrix by using CLPC technology. More specifically the aims were:

- To create crystallization and cross-linking procedures for the production of a sufficient amount of active crystalline ENAHis material for immunoaffinity studies

- To develop a reuseable immunoaffinity chromatography matrix for the separation of drug enantiomers

Xylose isomerase (XI) is a widely used enzyme in industry as a result of its ability to catalyze the isomerization of D-glucose to D-fructose. Previously it has been shown that XI accepts all of the pentose sugars and many hexose sugars as isomerization substrates [96, 97, 98]. The aim of this study was to use cross-linked XI crystals to improve the production process of D-fructose or other sugars. More specifically the aims were:

- To develop a crystallization process for the production of homogenous XI crystals of different size classes, which can be further used in cross-linked form for catalytic studies

- To study reactions catalyzed by XI for the production of sugars

- To investigate the possibility to use organic solvents in the sugar reactions catalyzed by cross-linked XI crystals to enhance the product yield

3 Materials and methods

The materials and methods used in this study are described in detail in the original publications I–V. Here only essential materials and methods are briefly described.

3.1 Antibody Fab fragment ENA5His

3.1.1 Purification of ENA5His [I]

The recombinant antibody Fab fragment ENA5His (molecular weight of 50.1 kD based on the amino acid sequence) was provided by VTT Biotechnology (Finland). The His-tagged ENA5His was cloned and produced by *Escherichia coli* in a bioreactor as described by Nevanen *et al.* [95]. ENA5His was purified by metal-affinity chromatography. After a buffer change to 0.1 M Na-acetate buffer at pH 5.0, the purified Fab fragment was concentrated to 8 mg ml^{-1} by ultrafiltration and used further for crystallization.

3.1.2 Crystallization and cross-linking of ENA5His [I]

Vapor diffusion crystallization experiments were set up in hanging drops by the standard procedure. Batch crystallization was first set up in 0.2 ml scale in 0.5 ml Eppendorf tubes and then in 10 ml scale in 15 ml plastic test tubes in conditions derived from the hanging drop experiments. The ENA5His crystals produced in the batch crystallization were cross-linked by incubation in 0.5% (w/v) glutaraldehyde for 30 min.

3.1.3 Preparation of native and cross-linked carrier-immobilized ENA5His [II]

Chelating Sepharose Fast Flow (Amersham-Pharmacia, Sweden) was loaded with copper ions to prepare Cu-IMAC carrier. Sedimented carrier was mixed with ENA5His solution and the mixture was incubated for 30 minutes with a gentle mixing at 20 °C. The slurry was packed in a small glass capillary with an inner diameter of 2.5 mm. Cross-linked carrier-immobilized ENA5His was prepared by adding 1 ml of 0.5% glutaraldehyde solution in to the column at room temperature. The column was washed with fresh PBS (phosphate buffered saline) before further use.

3.1.4 Drug racemate [I,II]

Finrozole, 4-[3-(4-fluorophenyl)-2-hydroxy-1-(1,2,4-triazol-1-yl)-propyl]benzonitrile ($C_{18}H_{15}FN_4O$), was provided by Hormos Medical Corp. (Finland). The two drug enantiomers studied are designated here as **a** and **d.**

3.1.5 Immunoaffinity chromatography of the drug racemate [II]

The immunoaffinity columns containing CLAC matrix, carrier-immobilized ENA5His, or cross-linked carrier-immobilized ENA5His were equilibrated with 20 bed volumes of PBS. Drug racemate (4 mg ml^{-1} in 100% DMSO) was diluted with 2% DMSO in PBS to the injection volume of 100 µl prior to the application into the column. The unbound drug enantiomer was washed from the column by 2% DMSO in PBS. The bound drug enantiomer was eluted by 60% methanol in PBS. The columns were operated by gravity. The drug enantiomers were analyzed from the fractions by HPLC.

3.2 Xylose isomerase (XI)

3.2.1 Purification of XI [III, IV, V]

D-xylose isomerase (XI) produced by a strain of *Streptomyces rubiginosus* was a courtesy from Genencor International Inc. (Hanko plant, Finland). The enzyme was diafiltered by a hollow-fiber ultrafiltration (MWCO 50 kDa) to 100-fold dilution with distilled water. After diafiltration, XI was purified by 3- or 4-fold crystallization from 0.76 M ammonium sulfate at pH 7.2 as described previously [99].

3.2.2 Solubility of XI crystals [III]

Wet XI crystals were washed into the desired salt solution by repeated centrifugation–suspension cycles. The suspension containing XI crystals in a salt solution was incubated with a rocking mixer placed inside an incubator at a desired temperature. The soluble protein concentration was analyzed by measuring the absorbance of the liquid phase at 280 nm.

3.2.3 Crystallization of XI [III]

Small-scale crystallization experiments were made in plastic test tubes. XI crystals were dialyzed against fresh water to dissolve the protein crystals and to remove the ammonium sulfate used in the purification of the protein. The protein concentration was adjusted to 40 g l^{-1} by adding water. A proper amount of a concentrated salt solution was added to a 4–8-ml aliquot of the protein solution to reach the desired final salt concentration. The solution was incubated with a rocking mixer placed inside an incubator at a desired temperature. The soluble protein concentration in the supernatant phase was analyzed measuring the absorbance of the liquid phase at 280 nm, and the presence of crystals was confirmed by light microscopy.

Large-scale cooling crystallization experiments were made in 0.1–4.0-liter volumes. The protein concentration was adjusted to 40 g l^{-1}. The XI solution was tempered to 28 °C in a water bath with impeller agitator using a stirring rate high enough for preventing sedimentation. Ammonium sulfate or magnesium sulfate was used for the crystallization. The temperature was lowered gradually to 4 °C.

Uniform XI crystals were produced by cooling crystallization from 0.17 M magnesium sulfate. An appropriate amount of seed crystals was added with a stirring rate high enough for preventing sedimentation. The crystallization was continued at 25 °C until the soluble protein concentration was no more decreasing. After this, the temperature was lowered gradually to 5 °C within 48 h to maintain small supersaturation in the solution for preventing spontaneous nucleation but enabling the seed crystal growth. Seed crystals were prepared by crushing XI crystals through mixing them vigorously with glass beads (d = 3 mm) for 2 h.

3.2.4 Cross-linking of XI crystals [V]

XI crystals were cross-linked by glutaraldehyde and L-lysine hydrochloride. Cross-linked XI crystals (CLXIC) were stored at 4 °C in water.

3.2.5 Measurement of initial reaction rates catalyzed by CLXIC (unpublished)

The measurements were conducted in a 100 ml batch reactor equipped with impeller agitator. The stirring rate was 100 rpm to exclude external mass-transfer limitations. All reactions were carried out in substrate solution containing 360 g l^{-1} D-fructose in Na-HEPES buffer (pH 7.5) supplemented with 20 mM $MgSO_4$. Adding about 50 mg of cross-linked XI crystals (CLXIC) to 50 ml of substrate solution initiated the isomerization reaction of D-fructose to D-glucose. The exact amount of the enzyme added to the reactor was measured afterwards. Samples of 1 ml were taken intermittently from the reaction mixture. The D-glucose concentration of the samples was measured by an enzymatic analyzer YSI 2700 (YSI Instruments, USA). Measurements were made at temperatures of 40 °C, 50 °C, 60 °C, and 67 °C. After the reaction, CLXIC was filtered from the reaction mixture and washed several times with reverse osmosis treated water. After vacuum drying for 24 h at 20 °C the crystal samples were weighed. The initial reaction rate ($mol_{glu}\ g_{CLXIC}^{-1}\ s^{-1}$) of D-fructose isomerization to D-glucose was calculated from samples where the conversion of the substrate was less than 5%. The initial reaction rates were measured for CLXIC of different size classes. All measurements were run in duplicates.

The effective diffusivity of the isomerization reaction, D_e, was estimated from the experimental results assuming first-order kinetics. Thiele modulus, Φ, is given by:

$$\Phi = R\sqrt{\frac{r_{ideal}\rho_c}{D_e C}} \qquad (1)$$

in which R is the radius of a spherical catalyst pellet, r_{ideal} is the ideal reaction rate obtained when mass transfer limitations can be excluded (moles product per mass of catalyst), ρ_c is the density of catalyst (1160 g 1^{-1} for CLXIC) [39], and C is the concentration of the substrate (2 mol l^{-1}). XI crystal shape is polyhedral and, thus, very close to spherical.

The value of Thiele modulus can be calculated from the internal effectiveness factor, η, which is given for spherical catalyst pellets and first-order kinetics by:

$$\eta = \frac{r_{actual}}{r_{ideal}} = \frac{3}{\Phi}(\Phi \coth \Phi - 1) \qquad (2)$$

in which r_{actual} is the actual reaction rate.

3.2.6 C-2 epimerization reactions catalyzed by XI [IV]

The reactions were carried out at 25–60 ^{o}C in 50 mM maleate buffer containing 20 mM $MgSO_4$ at pH 7.2. Unless otherwise stated, substrate concentration in the reactions was 5 mg ml^{-1} and soluble XI concentration was 1 mg ml^{-1}. Adding the enzyme solution to the tempered substrate solution started the reaction. Adding 1 M sulphuric acid to lower the pH below 2 stopped the reaction. Some reactions were done with cross-linked XI crystals (CLXIC) packed in a 1.6 cm × 38 cm column. Sugar compositions were measured by HPLC.

3.2.7 XI catalysis in the presence of organic solvents [V]

All the enzyme reactions were conducted in a 25 ml batch reactor equipped with impeller agitator. The reactions were carried out in a 50 mM maleate buffer (pH 7.2) containing 4 mM Mg^{2+} or in a mixture of buffer solution and acetone or ethanol. The enzyme concentration was approximately 3 mg ml^{-1} and the exact amount of the enzyme was measured afterwards. The sugar composition of the samples was measured by GC. The initial rates of glucose isomerization (g_{fru} g_{CLXIC}^{-1} min^{-1}) were determined from samples where the conversion of the starting sugar glucose was below 5%.

The deactivation of XI was measured as follows. First XI was incubated in aqueous ethanol or acetone supplemented with 50 mg ml^{-1} D-glucose. After incubation for 24 h, XI was separated from the incubation medium by filtration (CLXIC) or by ultrafiltration (soluble XI) and washed with a fresh maleate buffer solution. Then the initial rate of isomerization with the washed enzyme was measured in pure aqueous maleate buffer containing 50 mg ml^{-1} D-glucose as the substrate. The sugar composition of the samples was measured by GC.

4 Results and discussion

4.1 Model system for bioseparation applications

4.1.1 Crystallization of antibody fragment ENA5His [I]

A preliminary screening of crystallization conditions for ENA5His was conducted employing hanging drop vapor diffusion experiments. After optimization good crystallization behavior was obtained in hanging drops containing 20–28% PEG 3350 with 0.1–0.6 M KH_2PO_4 or NaH_2PO_4 at 5 °C and 20 °C. Figure 3a shows typical ENA5His crystals obtained in hanging drops. Harris *et al.* [100] were the first to describe the use of PEG 3350 in low ionic strength conditions for the crystallization of intact antibodies. Since then PEG 3350 has been used successfully in numerous crystallizations of antibodies and their fragments and, thus, it seems to be an especially useful crystallization agent for antibodies. Also in this work PEG 3350 proved to be the only precipitant producing ENA5His crystals. All the other precipitants including PEGs with molecular weights of 600, 4000, 6000, and 12000 failed to crystallize ENA5His.

Once the conditions leading to good crystallization were identified by the hanging drop method, the crystallization was transferred to larger scale batch crystallization. A 10 ml crystallization batch containing 3.5 mg ml^{-1} protein, 20% PEG 3350 and 0.2 M NaH_2PO_4 at pH 4.7 and 20 °C produced 25 mg of ENA5His crystals with a 70% yield. Figure 3b shows typical ENA5His crystals obtained in batch crystallization. Most of the crystals were relatively large being >50 μm in the smallest dimension but also small crystals and crystal fragments were present.The results obtained here together with the results of Lee *et al*. [101] show that a batch crystallization procedure can be relatively easily developed and scaled up based on crystallization conditions found by routinely used microdiffusion methods. Batch crystallization is the only realistic method for the production of protein crystals for practical applications. The ENA5His crystals produced in batch crystallization were cross-linked by the most commonly used protein cross-linking agent, glutaraldehyde. The resulting cross-linked antibody fragment crystals (CLAC) were insoluble in all the solutions that were used in the drug enantiomer separation application. This is a pre-requisite for the use of protein crystals in applications.

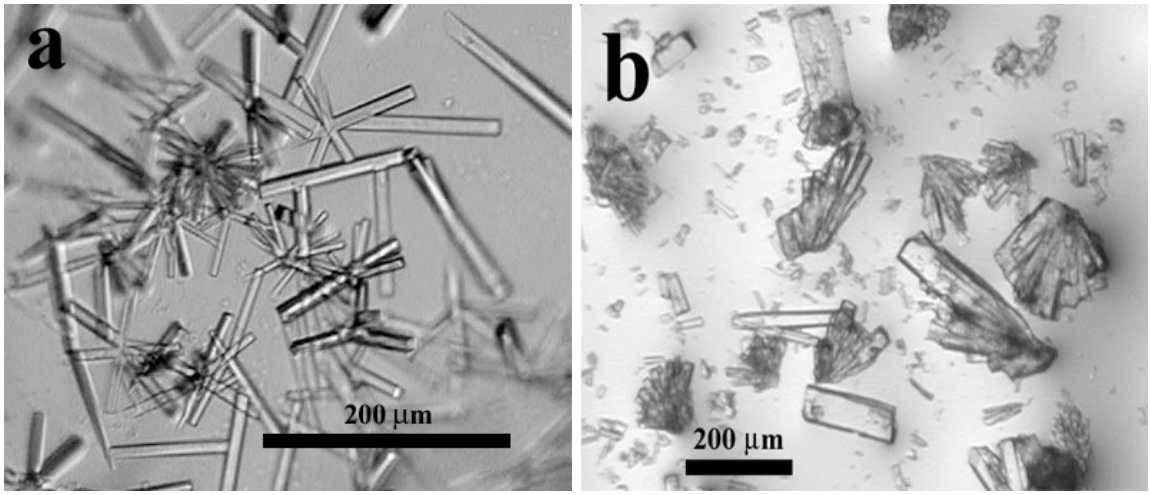

Figure 3. Crystals of an antibody Fab fragment ENA5His obtained a) by hanging drop method and b) in batch crystallization.

4.1.2 Chiral separation of drug racemates by re-usable ENA5His columns [II]

Immunoaffinity columns packed with cross-linked ENA5His crystals (CLAC) separated pure drug enantiomers from the racemic mixture. However, the large particle size of intact CLAC crystals (about 50×100×300 μm^3) resulted in ineffective separation of the drug enantiomers probably as a result of mass-transfer limitations (publication [II], Figure 2). To enhance mass transfer, CLAC was crushed with glass beads to a size of about 1×5×20 μm^3 or smaller. The crushing of CLAC considerably enhanced the separation of drug enantiomers. Figure 4 shows a typical drug separation chromatogram obtained with crushed CLAC. The drug **d**-enantiomer binding capacity was 2.3 μg per 1 mg of crushed CLAC, which is about 35% of the theoretical binding capacity based on 1:1 molar binding ratio. With carrier-immobilized ENA5His, the binding capacity corresponded to 70% of the theoretical binding capacity.

Reduced enzyme activities of cross-linked enzyme crystals have been frequently reported in the literature. The reduced activities are usually explained either by mass-transfer limitations inside the crystals or by the reaction between the protein and the cross-linking reagent, which limits the flexibility of protein molecule [15]. In this case, mass-transfer limitations fail to explain the reduced binding capacity of CLAC, as the mass-transfer limitations were seen as prolonged binding and elution times when big crystals were used. The high specific binding activity of the cross-linked carrier-immobilized ENA5His column showed that the reaction between the immobilized antibody and glutaraldehyde did not reduce the binding capacity (publication [II], Table 1). One reasonable hypothesis for the reduced binding activity of CLAC is that the extreme closeness of the protein molecules in the crystalline structures could easily lead to steric hindrances keeping a certain amount of binding sites not available for substrate binding. However, the reason for the reduced binding capacity of CLAC remained unknown. CLAC was resistant to the denaturing effect of methanol. During and after 25 binding-elution (with 60% methanol) cycles the binding capacity and elution profiles of an immunoaffinity CLAC column were unchanged. The improved protein stability obtained by the use of cross-linked protein crystal technology has been explained by the crystallinity of the material, the chemical modification of the protein, or a combination of both [15].

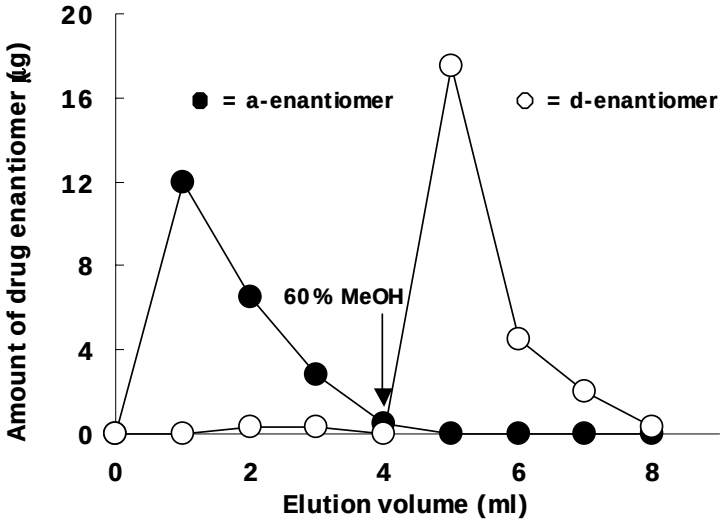

Figure 4. Typical chiral separation chromatogram of a drug racemate by an immunoaffinity column packed with crushed CLAC. About 13 mg of crushed CLAC was packed in the column; 50 µg of drug racemate was injected to the column; the bound drug was eluted from the column by changing the eluent to 60% methanol.

Because of the outstanding stability of the CLAC column, the role of cross-linking in the stabilization of ENA5His was studied in more detail with carrier-immobilized ENA5His, which was glutaraldehyde cross-linked. Glutaraldehyde cross-linking stabilized also immobilized ENA5His. After five times repeated enantioseparation cycles no loss in the drug binding capacity was observed. This result shows that glutaraldehyde cross-linking alone was enough to stabilize the ENA5His molecule against 60% methanol needed in the elution of the antibody bound drug. The glutaraldehyde cross-linking had no effect on the maximal **d**-enantiomer binding capacity, which was statistically identical to the value measured with native immobilized ENA5His (publication [II], Table 1). Simple glutaraldehyde cross-linking of carrier-immobilized ENA5His produced a highly stable and active protein matrix capable of producing pure enantiomers of the chiral drug.

4.2 Model system for biocatalytic applications

4.2.1 Solubility and crystallization of XI [III]

XI crystal solubility in ammonium sulfate solution followed the traditional salting-out behavior. The solubility decreased logarithmically when increasing the ionic strength in the solution (Figure 5a). Surprisingly, in a magnesium sulfate solution XI crystals had a solubility minimum at approximately 0.17 M $MgSO_4$ (Figure 5b). The reason for the specific effect of Mg^{2+} is not known. The effect of cations on protein solubility has been considered to be small compared with the effect of anions [102, 103, 104]. However, recently Bénas *et al*. [105] showed that also cations have an important role in the solubility of lysozyme.

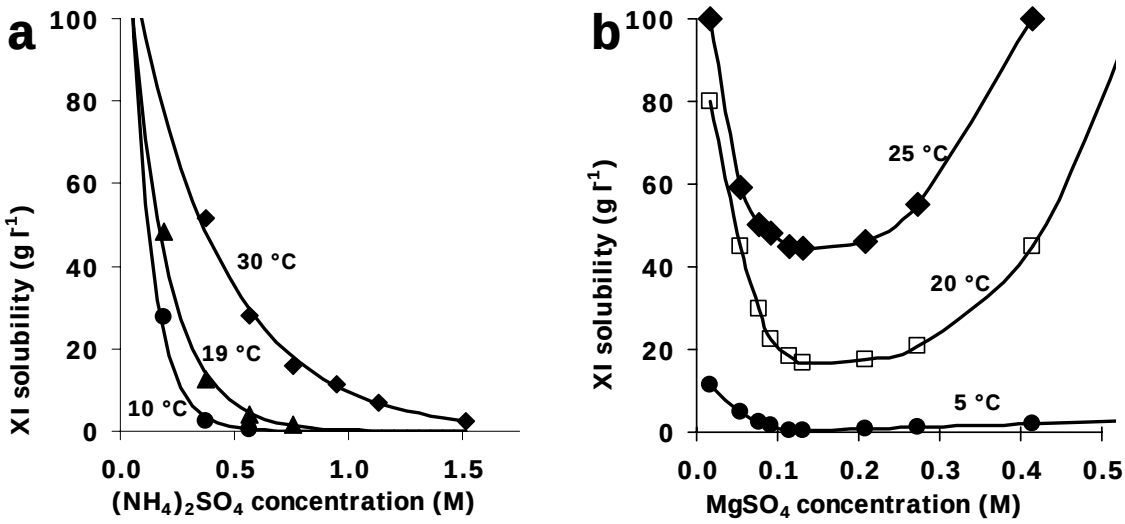

Figure 5. Solubility of XI crystals in a) ammonium sulfate solution, b) magnesium sulfate solution.

XI crystal solubility in both ammonium sulfate and magnesium sulfate increased logarithmically when increasing the temperature (publication [III], Figure 3). The strong temperature dependency of the solubility of the XI suggested that XI could be crystallized effectively by simple cooling crystallization. Indeed, XI was crystallized from concentrated ammonium sulfate solution and from 0.17 M magnesium salt solutions using cooling crystallization. The crystalline protein yield in the cooling crystallizations was very high, always over 95%. Magnesium sulfate was chosen for further crystallization optimization since ammonium sulfate must be used in large amounts compared to the amount of magnesium sulfate.

4.2.2 Preparation of uniform XI crystals (III)

The crystals produced by spontaneous nucleation resulted in heterogeneous crystal sizes of 5–150 μm even if a very gentle temperature gradient was used. However, the control of the size and shape of enzyme crystals is critical to their application as catalysts [15, 23]. Large uniform crystals are needed for catalytic applications and small uniform crystals for analytical/chromatographic applications [15]. A well-known method for the production of uniform crystals of small molecular weight compounds is the use of seed crystals in crystallization. However, no reports of such a technique being used in large-scale batch crystallization of proteins are available in the literature.

On the basis of the solubility study, we were able to develop a simple and reproducible procedure to prepare homogeneous XI crystals. The preparation of uniform XI crystals was based on cooling crystallization from 0.17 M $MgSO_4$. At 28 °C no spontaneous nucleation occurred in a 40 g l^{-1} XI solution supplemented with 0.17 M $MgSO_4$. Seed crystals were added to this solution to initiate the crystallization process. The temperature of the crystallization was then lowered gradually to 5 °C within 48 h. During this time the seed crystals were grown to their final size. The crystalline protein yield was typically 95–98%. As expected, the final crystal size was bigger when the amount of seeds was decreased. Figure 6 shows XI crystals of different sizes prepared by using crushed crystals as seeds.

XI crystals were further cross-linked by glutaraldehyde and L-lysine for catalytic studies. The use of L-lysine was obligatory in obtaining insoluble crystals. When the cross-

linking was made by glutaraldehyde alone, the crystals completely dissolved in water within 30 min at 60 °C. Cross-linked XI crystals (CLXIC) were insoluble apart from strong alkaline conditions. CLXIC dissolved in 0.1 M NaOH within 24 h at 30 °C. Previously it has been shown that CLXIC is more thermostable in the presence of substrate than the soluble protein [57].

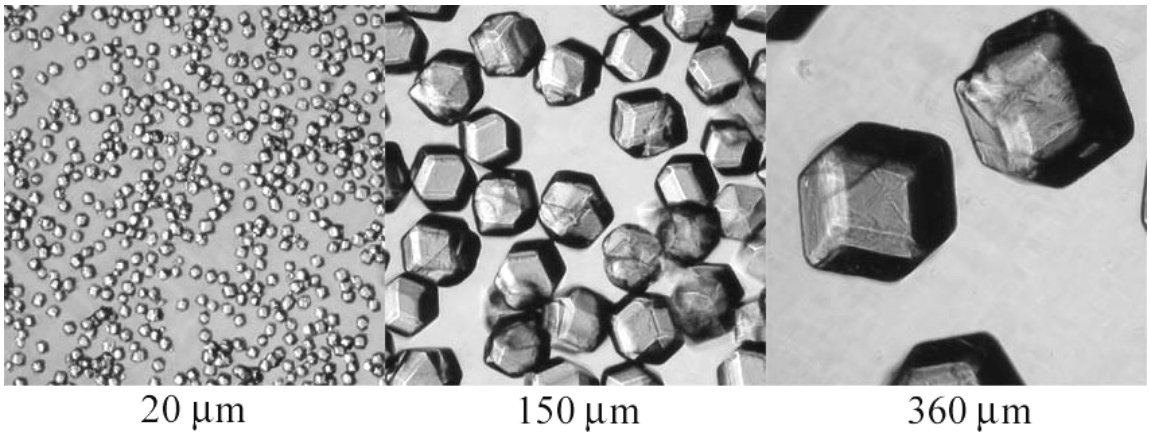

Figure 6. Uniform XI crystals produced by seeding crystallization from 0.17 $MgSO_4$. Note that the crystals are in the same scale.

4.2.3 Intracrystalline mass-transfer limitations (unpublished)

The initial D-fructose isomerization rate catalyzed by cross-linked XI crystals (CLXIC) of different crystal sizes was measured in order to study the effect of crystal size on the actual activity of CLXIC. Figure 7 shows that the actual activity of CLXIC decreased when increasing the crystal size. The reason for this is clearly the mass-transfer limitations inside the crystals. The activity of the smallest crystals having a particle size of <1 µm was even higher than the corresponding activity of the soluble enzyme. This result shows that the mass-transfer limitations can be excluded when these crystals are used. These results indicate that it would be reasonable to use the smallest crystals in applications in order to obtain maximal enzyme activity. However, small crystals are difficult to handle in practice. The removal of small particles is difficult from the reaction mixture, for example. However, from Figure 7 it can be seen that relatively high activities are obtained also with big crystals suitable for use in practical applications.

The effective diffusivity of the isomerization reaction calculated from experimental data was in the range of $(0.5–1.2)\times10^{-11}$ m^2 s^{-1}. This value was independent of the reaction temperature (40–67°C). The value of effective diffusivity is only estimation, as the assumption of first-order kinetics is inaccurate when high substrate concentrations are used. The reaction rate measurements were made at a high substrate concentration in order to discover the values of the overall effectiveness factor for different catalyst particle sizes in conditions close to those found in real processes. The calculated effective diffusivity was two orders of magnitude lower than the diffusivity of D-glucose or D-fructose (molecular weight 180 g mol^{-1}) in water at 25 °C [106]. The value of effective diffusivity was in good agreement with diffusivity values reported for lysozyme crystals. The diffusivities of fluorescent surfactants (molecular weight 246–316 g mol^{-1}) inside tetragonal lysozyme crystals were 3–4 orders of magnitude lower compared with the diffusivities of corresponding free molecules in water [44]. The values of diffusivities were measured by following the penetration of light-emitting surfactants into the pores of lysozyme crystals. Morozova *et al.* [40] calculated that the mobility of cations was 4–50

fold lower and the mobility of anions 100–300 fold lower in the tetragonal lysozyme crystals compared with their mobility in solution. The calculation was based on the experimentally determined crystal conductivity and transference number of ions.

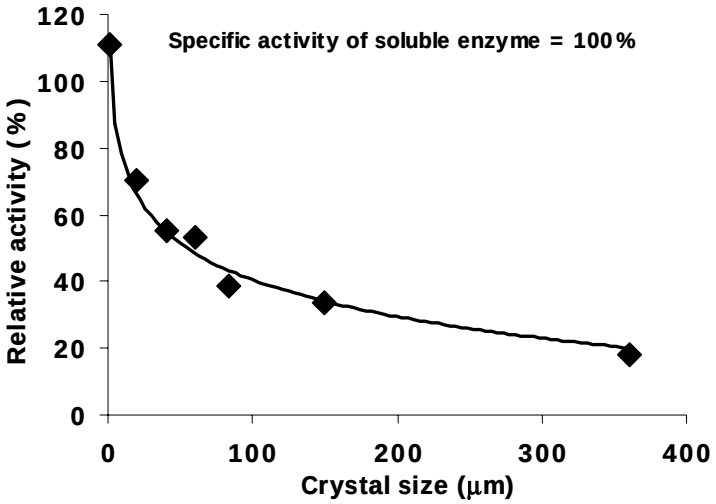

Figure 7. Relative activity of CLXIC decreases when increasing the crystal size.

4.2.4 XI catalyzes a C-2 epimerization of sugars (IV)

The reactions catalyzed by XI have been studied extensively. In addition to an isomerization, it has been observed that also a C-2 epimerization occurs with some pentose and hexose substrates [97]. Nevertheless, in the most extensively studied isomerization of D-glucose to D-fructose, formation of the C-2 epimer (D-mannose) has not been observed previously. However, while studying D-glucose isomerization to D-fructose catalyzed by a packed bed reactor containing CLXIC as the catalyst, in addition to D-fructose also D-mannose was detected from the out coming stream. This was a result of the extremely high enzyme to substrate ratio of the CLXIC column, which enables the detection of weak activities [68]. The finding of D-mannose, which is a C-2 epimer of D-glucose, led to a more detailed study of the C-2 epimerization catalyzed by XI. Both D- and L-forms of tetrose (erythrose, erythrulose, threose), pentose (arabinose, ribulose, ribose) and hexose (glucose, fructose, mannose) sugars were chosen as the substrates.

The C-2 epimer formation rate was highest with tetroses and lowest with hexoses. Lengthening the sugar carbon chain decreased the C-2 epimer formation rate roughly by one order of magnitude per one carbon atom. The C-2 epimer formation from D-mannose is an exception to this rule as a result of the fast isomerization rate between D-fructose (an isomer) and D-glucose (a C-2 epimer). The results of this study show that the real equilibrium in XI catalysed reactions is not between two isomers but between a ketose and its two aldose isomers.

4.2.5 Acetone enhances CLXIC catalyzed D-glucose isomerization to D-fructose (V)

Immobilized XI is used industrially for the conversion of D-glucose to D-fructose. At equilibrium at 60 °C, the isomerization reaction mixture contains 51% D-fructose and 49% D-glucose [107]. The thermodynamic equilibrium of the reaction favors D-fructose at high temperatures. In high fructose syrups used to sweeten soft drinks, for example, a D-fructose content of 55% is preferred to achieve the same sweetening effect as with sucrose and to prevent the crystallization of D-glucose [108]. However, the production of

a 55% D-fructose solution would require a continuous operation at 85–100 °C, which is too high a temperature for even the most thermostable XIs [109]. It has been shown previously that the addition of high concentration of ethanol favors the production of D-fructose [110, 111]. However, as a result of the low productivity and poor stability of XI in the presence of ethanol, the use of ethanol was not applicable to real processes.

The stability of CLXIC was studied in the presence of ethanol and acetone. After a 24-h treatment in 50% ethanol only 2% of the isomerase activity was retained. At the same conditions the soluble enzyme lost its activity totally. However, the 24-h treatment decreased the activity of CLXIC only by 20–30% in solutions containing 10–90% acetone (publication [V], Figure 1). Surprisingly, acetone seems to have only a minor role in the inactivation of cross-linked crystals. The soluble enzyme was much more sensitive to acetone. In 50% acetone the activity of the soluble enzyme decreased by 75%. This result shows that XI cannot be used in high ethanol concentration at relevant isomerization temperature. By contrast, XI in the form of CLXIC was stable even at very high acetone concentrations.

Figure 8 shows the initial D-glucose isomerization rates in pure aqueous solvent, 50% ethanol, and 50% acetone containing maleate buffer at different reaction temperatures when CLXIC was used as the catalyst. The reaction rate in 50% ethanol was similar to that in pure aqueous solvent, except at the highest temperature (50 ^{o}C) measured, where the reaction rate was clearly smaller in ethanol containing solvent as a result of the inactivation of CLXIC. In 50% acetone the reaction rate was more than doubled compared with the rate in pure aqueous solvent at all temperatures. A higher temperature could not be used, as the boiling point of acetone is 56.5 °C at atmospheric pressure. Excluding the highest temperature with ethanol the activation energy of 59±2 kJ mol^{-1} for the isomerization reaction can be calculated from the slopes in Figure 8. The activation energy is not affected by the addition of organic solvents. This shows that the addition of organic solvent does not have an effect on the active site of XI.

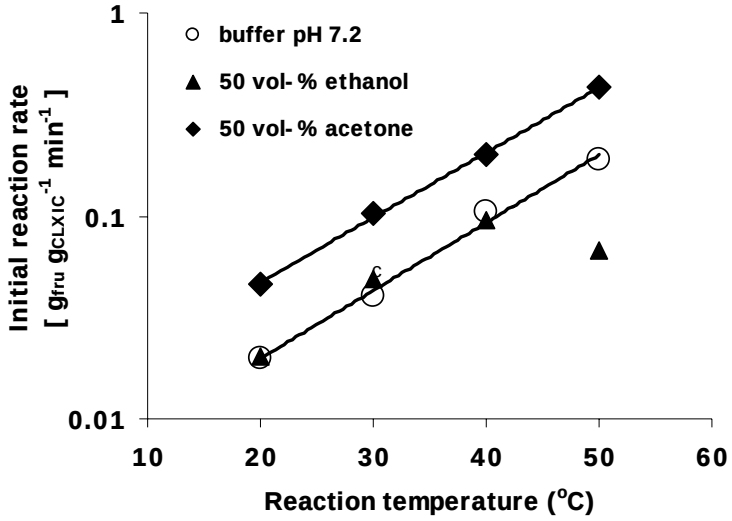

Figure 8. Initial rate of D-glucose isomerization catalyzed by 65 µm CLXIC in pure buffer solvent, in 50% ethanol and in 50% acetone as a function of temperature.

The actual enzyme activity of CLXIC increased in a linear manner when the acetone content in the reaction mixture was increased at 50 °C as seen in Figure 9. The initial D-fructose production rate was more than doubled when high concentrations of acetone

were used. A higher concentration than 70% acetone could not be used as a result of the low solubility of D-glucose. No similar effect with ethanol was observed, as the initial D-fructose production rate was lower than the rate in buffer solution. The increased activity of CLXIC can be partly explained by a shift in the concentration of the α-pyranose form of D-glucose present in the solution. In water, about 39% of the soluble D-glucose is present as α-pyranose. However, the α-pyranose content increased exponentially when the acetone content was increased (publication [V], Figure 3). In 90% acetone, the α-pyranose content was 46%. The active center of XI accepts only α-pyranose form of D-glucose as the substrate [112]. Lee and Hong [113] measured a 43% increased initial isomerization rate with pure α-D-glucose compared with buffer equilibrated glucose. Thus, the difference in the glucose composition accounts for the increased reaction rate only partly.

The addition of acetone to the isomerization reaction mixture increased the concentration of D-fructose at the equilibrium in an exponential manner (publication [V], Figure 2). At equilibrium at 50 °C in buffer solution the reaction mixture contained 49±1% D-fructose. However, in 90% acetone the equilibrium D-fructose concentration was 63±1%. The reason for this increase may be the increase of the α-pyranose form of D-glucose in acetone solutions.

As a conclusion CLXIC is stable even in high acetone concentrations. The use of a high concentration of acetone in the isomerization of D-glucose to D-fructose was beneficial in two ways, by increasing the reaction rate and the product yield. However, D-glucose is poorly soluble in acetone and, thus, high acetone concentrations (>70%) cannot be used in practical applications.

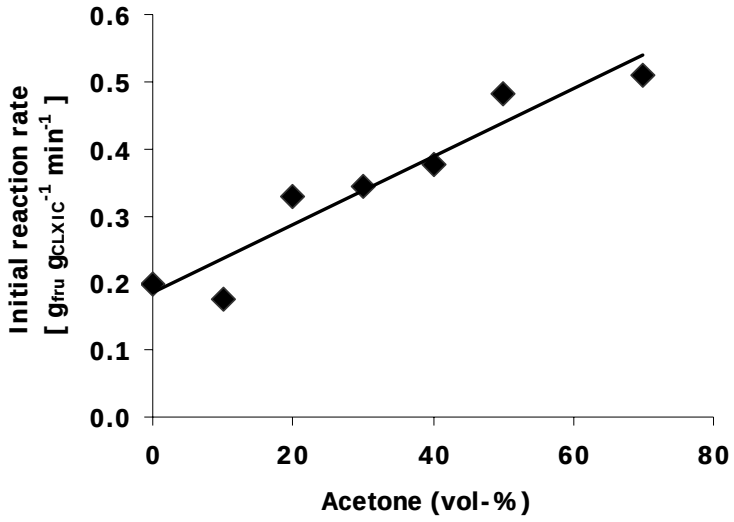

Figure 9. The effect of the acetone concentration on the initial rate of glucose isomerization at 50 °C.

5 Conclusions

Protein crystallization is widely considered troublesome because of the often assumed unpredictability and irreproducibility of crystallization. However, the data presented in this thesis show that protein crystallization procedures can be developed systematically. The starting point for the crystallization development for xylose isomerase and the antibody Fab fragment ENA5His was quite different. Xylose isomerase is a widely used

enzyme that was available in kilogram quantities for the development of crystallization, crystal cross-linking, and biocatalytic applications. The crystallization process development for xylose isomerase was based on a systematic solubility study. Based on the results of the solubility study, a crystallization procedure yielding homogeneous xylose isomerase crystals with a high crystal yield was developed.

The antibody Fab fragment ENA5His was only produced for research purposes in milligram scale. Thus, crystallization conditions had to be screened by using small-scale vapor diffusion experiments consuming only small amounts of ENA5His. The initial crystallization conditions found were analogous to conditions previously reported to be especially suitable for antibodies. The scale-up of the crystallization of ENA5His employing batch crystallization was straightforward.

The results showed that the common cross-linking agent, glutaraldehyde, worked well in practice. ENA5His crystals became insoluble after glutaraldehyde treatment. However, the addition of L-lysine to the glutaraldehyde cross-linking reaction mixture was needed to obtain an insoluble form of cross-linked XI crystals.

The cross-linking increased the stabilities of ENA5His and XI towards the denaturing effects of organic solvents. The improved stability enabled the use of both studied proteins effectively in applications, where high concentrations of organic solvents were needed.

Crystallization and cross-linking affected the activity of the protein samples. When the mass transfer limitation was excluded by studying the activity of small crushed XI crystals, the specific activity of XI remained at the same level compared with the activity of soluble XI. However, when bigger XI crystals were used the activity decreased as a result of intracrystalline mass transfer limitations. Also cross-linked crystals of ENA5His suffered from mass transfer limitations. Decreasing the crystal size by crushing decreased the effects of the mass transfer limitations. However, crystallization and cross-linking decreased the specific drug binding activity of ENA5His by about 50% compared with carrier immobilized ENA5His.

As a result of the stability and high activity of cross-linked XI crystals new reactions catalyzed by XI were found. Furthermore, the increased stability of the cross-linked crystalline form enabled addition of acetone to XI catalyzed D-fructose production from D-glucose, which increased the reaction rate and the product yield.

The increased stability of ENA5His in cross-linked crystalline form enabled the reuse of ENA5His in the drug racemate separation application. The results of this thesis show that the source of the increased stability was the chemical cross-linking by glutaraldehyde shown by the high stability of cross-linked immobilized ENA5His column. Cross-linking of immobilized protein column is a simple and economic process and, thus, very attractive way to increase the stability of proteins.

The results of this thesis together with the results obtained previously show that cross-linked protein crystal technology can improve the applicability of proteins. Especially, the regularly increased stability may open new applications for proteins using cross-linked protein crystal technology. Moreover, the results show that cross-linked protein crystal materials can be designed systematically. Still, a future challenge is to apply the cross-linked protein crystal technology to commercial applications.

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