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Crosslinking of Imprinted Proteases to Maintain a Tailor-Made Substrate Selectivity in Aqueous Solutions

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Abstract—A covalent method to keep imprinted properties of proteins stable in aqueous as well as in organic environment is described. To stabilize the ligand induced acceptance for D-configured substrates by α-chymotrypsin or subtilisin Carlsberg, each protein was first vinylated by acylation with itaconic anhydride. Then, the tailoring of the derivatized proteins by precipitation in the presence of *N*-acetyl-D-tryptophan from an aqueous medium with 1-propanol, and the subsequent crosslinking of the enzyme preparations with ethylene glycol dimethacrylate in cyclohexane was carried out. The crosslinked imprinted proteins (CLIPs) obtained catalyzed the hydrolysis of *N*-acetyl-D-tryptophan ethyl ester in phosphate buffer and the corresponding back reaction in cyclohexane, respectively. The repeated use of CLIP-α-chymotrypsin in D-ester hydrolysis was demonstrated. Furthermore, this particular CLIP-α-chymotrypsin showed no loss in activity when it subsequently was used in the synthesis of *N*-acetyl-D-tryptophan ethyl ester in cyclohexane again. In the case of D-ester hydrolysis the reaction rate acceleration (k_{enz}/k_{nonenz}) was in the same order of magnitude of about $10^4 - 10^5$ mM^{-1} for the two CLIP-proteases. The results suggest that enzymes tailored by imprinting technique do not lose their induced "new" property in the presence of water when they are prepared according to the described vinylation/crosslinking method (CLIP technique).

Introduction

To tailor the properties of proteins in order to match new desired abilities concerning stability, substrate selectivity or enantioselectivity, rational[1] or random[2] mutagenesis have emerged as powerful methods that modify the protein at its genetic level. However, for practical reasons it would be desirable to have methods available that allow a predictable alteration of protein properties directly at the protein level. One step in this direction is based on the ability of proteins to maintain a structural memory when transferred from aqueous into anhydrous environment.[3] So, if a native protein is precipitated[4] or lyophilized[5] from aqueous environment in the presence of a defined ligand, the ligand can cause a predictable alteration of the property of the protein in organic solvents. Generally, this phenomenon is called (bio)imprinting.[6] By this, the catalytic activity,[7] the substrate selectivity[8] or the enantioselectivity[4] of enzymes and, the binding properties[9] of proteins were manipulated. Also a "de novo" catalytic ability in existing mature proteins via imprinting with a transition state analogue was reported recently.[10] Unfortunately, all these examples of tailoring proteins by imprinting techniques are limited by the fact that the new protein properties are available in nearly anhydrous environment only since aqueous environment causes a renaturation of the protein and loss of the imprinting effect.[3] But, if the imprinting effect relies on any kinds of structural manipulation of the proteins,[11] it should be possible to maintain them by crosslinking the "rigid" protein in organic solvent. Here, first results of studies are reported which suggest that the imprinting effect can indeed be covalently stabilized by combining imprinting with a two step immobilization method and, the imprinted enzymes do permanently maintain their "new" property in aqueous as well as in organic environment.

Key words: Tailor-made enzymes; imprinting; bioimprinting; immobilization.

Abbreviations: CT, α-chymotrypsin; SUB, subtilisin carlsberg; NIP, native imprinted protein; DIP, derivatized imprinted protein; CLIP, crosslinked imprinted protein; L-ee, *N*-acetyl-L-tryptophan ethyl ester; D-ee, *N*-acetyl-D-tryptophan ethyl ester; *N*-Ac-D-Trp, *N*-acetyl-D-tryptophan.

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Results

Manipulation of the substrate selectivity of native α-chymotrypsin

As described recently,[4] the substrate selectivity of native α-chymotrypsin (CT) in organic solvents is altered when

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CT is "imprinted" with its competitive inhibitor *N*-acetyl-D-tryptophan (*N*-Ac-D-Trp) in a buffer solution. In comparison to non-imprinted CT, imprinted CT was then able to accept *N*-Ac-D-Trp as the substrate in ester synthesis.[4] In order to investigate this finding in more detail, solubilized CT was precipitated with 1-propanol in the presence of *N*-Ac-D-Trp at pH 3.6, 5.4 and 7.8. Then, the washed and dried protein precipitates were used for *N*-Ac-D-Trp ethyl ester synthesis in cyclohexane at various temperatures (15–35°C). The highest yields in D-ester synthesis were obtained when CT was precipitated at pH 5.4 and the reaction was carried out at 25°C. Another important parameter for ester syntheses in organic solvents is the free water activity.[12] Thus, supplementary water in the range from 0 to 1.7 M was added in the beginning of the D-ester synthesis reaction running in dry cyclohexane and, the yield of *N*-Ac-D-Trp ethyl ester was measured after 48h. For this reaction, the optimum of the water concentration turned out to be 440 mM. However, this value deviated significantly from the published data so far (1.2 mM).[4] The specific activities calculated in our experiments were 0.02 nkat/g imprinted CT when no supplementary water was added to the reaction medium, 0.17 nkat/g at a water concentration of 440 mM and no activity when 1.7 mM water was added. The latter probably is explained by the renaturation of the CT in the presence of too many water molecules and with that, CT's loss of the imprinted property.

Also the effect of the concentration of the additive *N*-Ac-D-Trp used in the buffer prior to the precipitation step was investigated. CT was co-solubilized with either 10 mM, 20 mM or 40 mM *N*-Ac-D-Trp. The yields in *N*-Ac-D-Trp ethyl ester syntheses obtained with the resulting CTs were estimated by HPLC after 24 h. The specific activities (nkat/g imprinted CT) were calculated to be 0.11, 0.162 and 0.174, respectively. In all further studies, an additive concentration of 20 mM *N*-Ac-D-Trp was used. This value corresponds to an approx. 5-fold concentration of the K_i of CT for *N*-Ac-D-Trp (4.16 mM).[13]

Aside from achieving the imprinting effect by precipitation, lyophilization of the mixture containing CT and *N*-Ac-D-Trp could also be used. After extensive washing of the lyophilizated powder with 1-propanol it was tested for *N*-Ac-D-Trp ethyl ester synthesis in cyclohexane and, approx. 83% of the specific activity measured with the precipitated imprinted CT (see above) was obtained.

Derivatization and crosslinking of α-chymotrypsin

To maintain the manipulated properties of CT caused by imprinting with *N*-Ac-D-Trp permanently, polymerizable vinyl groups were introduced on CT by acylation with itaconic anhydride. When itaconic anhydride is used to acylate proteins it mainly reacts with the amino groups of lysines and to some extent also with the hydroxyl groups of tyrosines and the thiol groups of cysteines.[14] A non-linear correlation between the degree of acylation of CT and its resulting residual hydrolytic activity towards benzoyl tyrosine ethyl ester (BTEE) was found (Fig. 1). In order to have enough attachment points per CT available for crosslinking but also an acceptable residual activity, a derivatization degree of about 60% was chosen empirically in further experiments. This derivatized CT was imprinted with *N*-Ac-D-Trp in buffer solution, precipitated, dried and either tested directly for *N*-Ac-D-Trp ethyl ester synthesis in organic solvent or, it was first crosslinked with ethylene glycol dimethacrylate by radical polymerization and then tested for D-ester synthesis. In Figure 2 the time

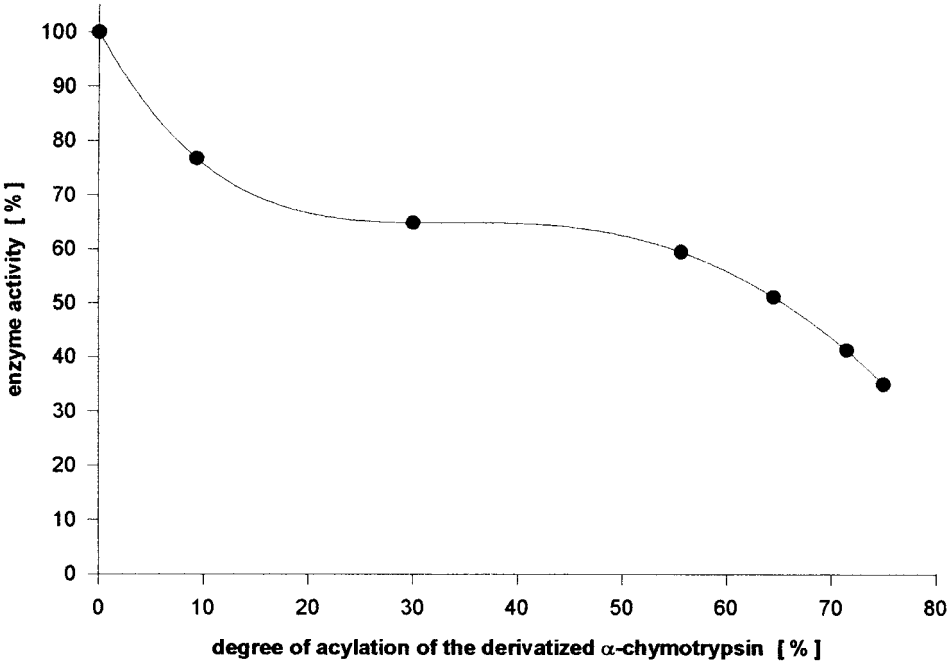


Figure 1. Activity of derivatized α-chymotrypsin dependent on the particular degree of enzyme acylation with itaconic anhydride.

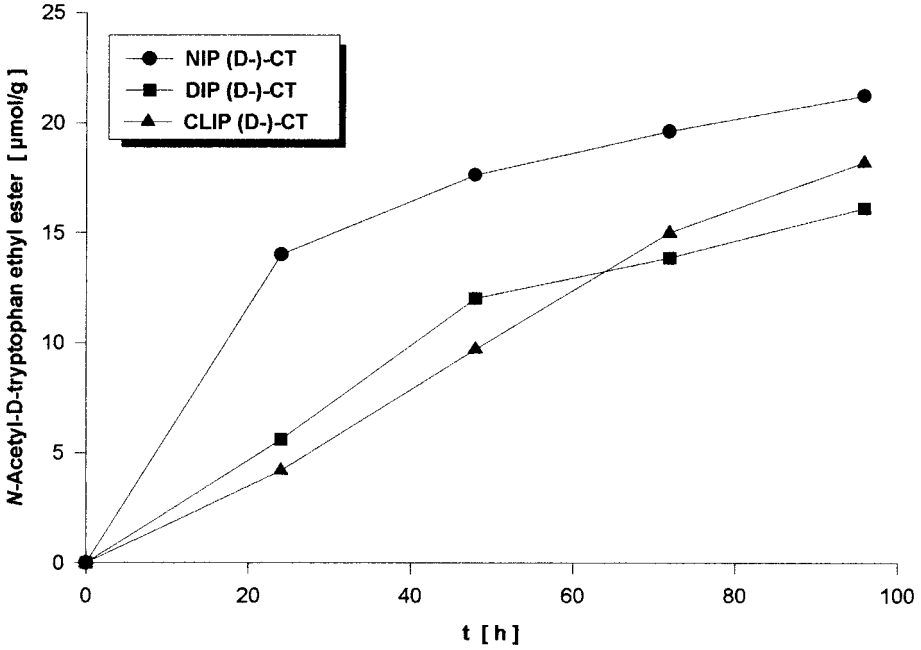


Figure 2. Synthesis of the *N*-acetyl-D-tryptophan ethyl ester in cyclohexane using various kinds of α-chymotrypsin [NIP (D-)-CT = native imprinted protein (α-chymotrypsin) that was imprinted with *N*-acetyl-D-tryptophan, DIP (D-)-CT = derivatized imprinted protein (α-chymotrypsin) that was imprinted with *N*-acetyl-D-tryptophan, CLIP (D-)-CT = crosslinked imprinted protein (α-chymotrypsin) that was imprinted with *N*-acetyl-D-tryptophan]. Reaction conditions: 10 mg of each biocatalyst preparation, *N*-acetyl-D-tryptophan = 20 mM, total reaction volume = 1.26 mL, 25°C; thermomixer 1200 rpm.

course of D-ester synthesis of the native, imprinted protein [NIP (D)-CT], the derivatized, imprinted protein [DIP (D)-CT] and the crosslinked, imprinted protein [CLIP (D)-CT] is shown. Clearly, all three kinds of imprinted CT kept their imprinted property to catalyze the *N*-Ac-D-Trp ethyl ester synthesis. After 96h the yields of D-ester product produced by one gram of DIP (D)-CT and CLIP (D)-CT, respectively, were only about 25% and 15% lower compared to NIP (D)-CT. For the CLIP (D)-CT mass transfer effects were recognizable. Nevertheless, its reaction rate was almost linear up to 96h. The average particle size of the CLIP (D)-CT was determined to be about 10 µm (Fig. 3) and the specific surface area was about 5.5 m^2/g. Figure 4 presents a picture of CLIP (D)-CT.

In addition, active site titration of the different CT species was done with *N-trans*-cinnamoyl imidazole. The value obtained for native CT was set to 100%. In comparison, NIP (D)-CT and CLIP (D)-CT resulted in 85 and 75% of accessible catalytically active sites. Thus, the derivatization and crosslinking procedures seemed to be quite gentle.

Maintenance of the imprinted property of proteases in aqueous solution

Subtilisin Carlsberg (SUB) was treated in the same manner as CT and the two CLIP D-proteases were able to hydrolyze *N*-Ac-D-Trp ethyl ester in buffer solution (Fig. 5). Neither the native enzymes nor the NIP (D)-CT/SUB nor the DIP (D)-CT/SUB enhanced the slow spontaneous hydrolysis of the substrate, that was calculated to be 1.9 10^{-5} min^{-1} at pH 7.8 (see Fig. 5). Based on the apparent k_{enz} for the enzymes, a relative reaction rate acceleration (k_{enz}/k_{nonenz}) of 5.3 10^4 mM^{-1} and 1.1 10^5 mM^{-1} for CLIP (D)-CT and CLIP (D)-SUB were estimated. The first time it was demonstrated that an imprinted property of an enzyme—in this case the manipulated enantioselectivity of CT and SUB—could be stabilized by covalent crosslinking and thus, this "tailor-made" property was maintained and usable in aqueous solution.

The operational stability of CLIP (D)-CT was tested in 4 consecutive cycles over a total reaction time of 408 h. No decrease in specific activity was observed during that time. Furthermore, when this particular preparation of CLIP (D)-CT was reused for D-ester synthesis in cyclohexane afterwards (after extensively washing and drying under vacuum) no loss in specific activity was observed after 96h in comparison to a CLIP (D)-CT that was not incubated in aqueous solution before.

Kinetic data of native CT, imprinted native CT and imprinted crosslinked CT

For comparison, imprinting of native CT and derivatized CT was done with *N*-Ac-D-Trp or N-Ac-L-Trp prior to precipitation. The derivatized CTs were crosslinked in the same manner as described above. Then, the various CT preparations were subjected to *N*-Ac-D-Trp ester syntheses (Table 1a) or hydrolyses (Table 1b), respectively. Concerning the "natural" L-ester synthesis reaction (Table 1a), the highest V_{max} was measured with the precipitated, non-imprinted CT. Imprinting of CT with *N*-Ac-L-Trp increased the affinity towards the substrate [K_M 8.4 mM of NIP (L)-CT)] but decreased V_{max}.

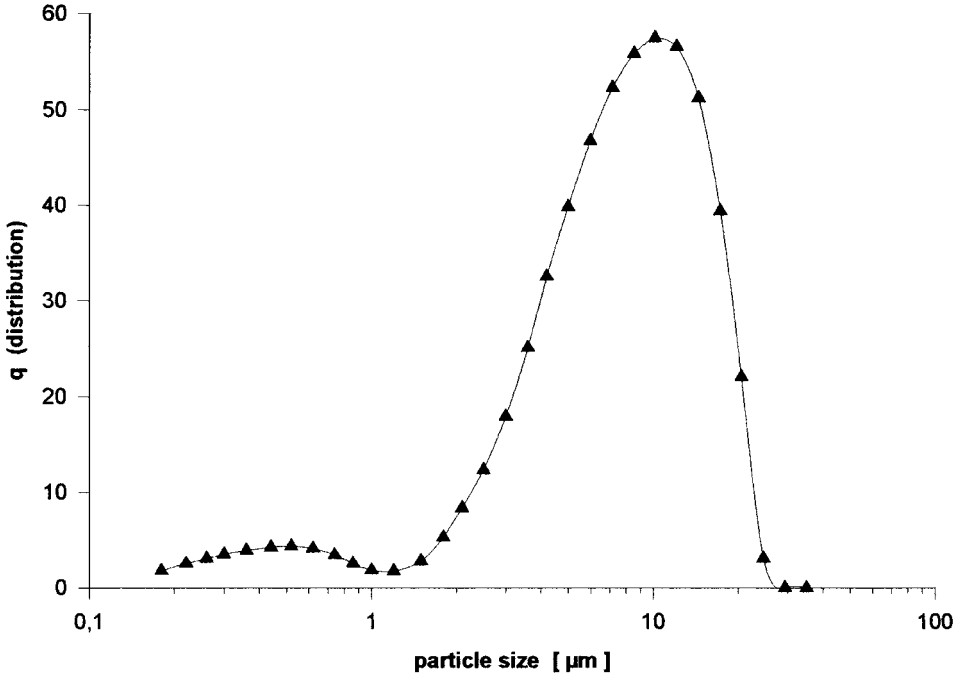


Figure 3. Distribution curve resulting from particle size analysis of CLIP (D)-CT.

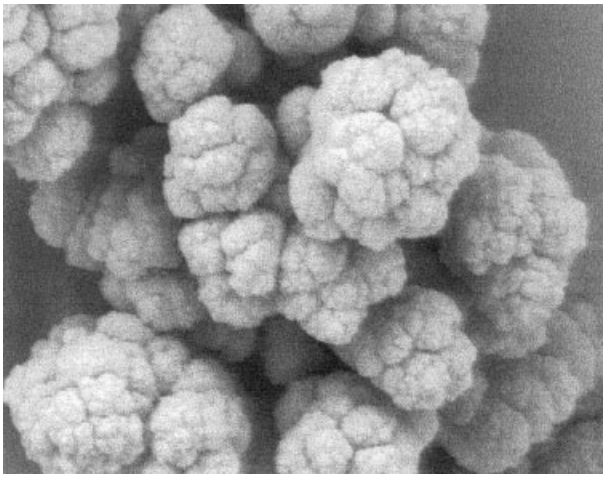


Figure 4. SEM picture of CLIP (magnification 20,000x).

The CLIP (L)-CT had the lowest K_M (3.7 mM) and V_{max}. The latter may be rationalized by a large mass transfer impact of the particles that is also recognized by comparing the V_{max} values of NIP (D)-CT (100%) and CLIP (D)-CT (20%) for D-ester synthesis.

In the case of the L-ester hydrolysis (Table 1b), native non-imprinted CT had a high affinity towards the substrate (K_M 0.08 mM). Crosslinked non-imprinted CT as well as CLIP (L)-CT possessed an about 100-fold lower affinity and astonishingly, the two were identical according to K_M and V_{max}. However, only CLIP (D)-CT was able to hydrolyze *N*-Ac-D-Trp ethyl ester and, its K_M versus the D-enantiomer (7.3 mM) was lower compared to the CLIP (L)-CT versus the L-enantiomer (12.8 mM). The Vmax cannot be directly compared to any other preparation of CT because none of them accepts the D-ester as a substrate. For that reason, the k_{enz} and k_{nonenz} values are mentioned here and the ratio k_{enz}/k_{nonenz} results in 5.3 10^4 mM^{-1}.

Discussion

The main interest of this work was to stabilize imprinted protein properties, which were up to now stable only in organic solvents,[3] also in aqueous surroundings. The results of the experiments demonstrated clearly that crosslinking the acylated and imprinted enzymes with ethylene glycol dimethacrylate in an organic solvent prevented the two proteases from losing their ligand induced D-amino acid acceptance in the aqueous medium.

Since lyophilization itself causes unwanted structural changes on proteins,[11,15] alcohol precipitation[4] seems to be the more advantageous method to dehydrate proteins for imprinting purposes. The vinylation of the proteases under controlled buffer conditions was intended to introduce selective groups for subsequently crosslinking in organic solvents and thus, not further disturbing the intramolecular interactions of the protein keeping its manipulated property. By using itaconic anhydride for this tight amide bonds were obtained,[14] and in the case of the β-glucosidase[14] from almonds nearly 100% of activity was retained after a complete derivatization of the enzyme as measured by TNBS method. However, also other vinylization agents, for instance acryloyl chloride, could be used in principle.[16] The amount of crosslinker used was high, taking into account the experiences in the field of synthetic imprinted polymers.[17] This caused mass transfer limitations

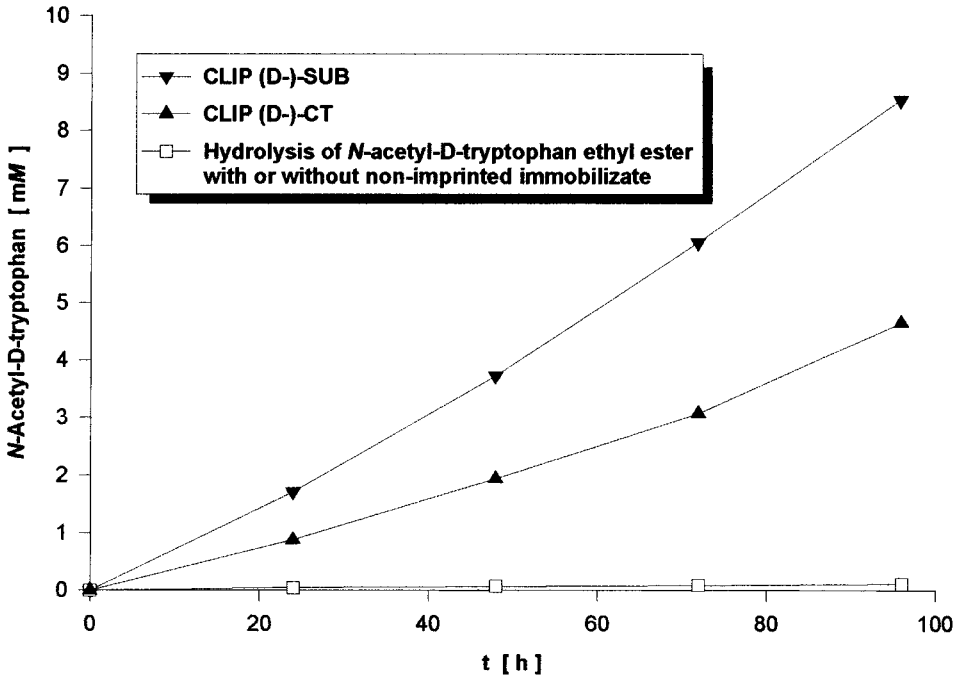


Figure 5. Hydrolysis of *N*-acetyl-D-tryptophan ethyl ester catalyzed by CLIP (D-) proteases [CLIP (D-)-CT/-SUB = crosslinked imprinted proteins (α-chymotrypsin/subtilisin) that were imprinted with *N*-acetyl-D-tryptophan] in comparison to the nonenzymatic hydrolysis of the D-ester in the absence as well as in the presence of the polymer containing the non-imprinted acylated proteases. Reaction conditions: 10 mg of each biocatalyst preparation, *N*-acetyl-D-tryptophan 0.03 M, 0.05 M phosphate buffer pH 7.8, 25°C; thermomixer 1200 rpm.

Table 1a. Kinetic data of the synthesis of *N*-acetyl-L-tryptophan ethyl ester (L-ee) and *N*-acetyl-D-tryptophan ethyl ester (D-ee) catalyzed by precipitated α-chymotrypsin (CT), NIP-CT (native, imprinted protein [α-chymotrypsin] and CLIP-CT (crosslinked imprinted protein [α-chymotrypsin]

Reaction	Biocatalyst preparation	K_M[a] [mM]	V_{max} [%]
L-ee synthesis	Native α-CT, precipitated	19.5	100
	NIP (L-)[b]-CT	8.4	63
	CLIP (L-)[b]-CT	3.7	21
D-ee synthesis	NIP (D-)[b]-CT	16.1	100
	CLIP (D-)[b]-CT	25.1	20

[a] K_M of the corresponding enantiomer of *N*-acetyl-Trp.
[b] The appendix (L-) means that the biocatalyst was imprinted with *N*-acetyl-L-tryptophan and (D-) means the imprinting with the corresponding D-enantiomer.

Table 1b. Kinetic data of the hydrolysis of *N*-acetyl-L-tryptophan ethyl ester (L-ee) and *N*-Acetyl-D-tryptophan ethyl ester (D-ee) catalyzed by CT (α-chymotrypsin) and CLIP-CT (crosslinked imprinted protein [α-chymotrypsin] [100% activity = 9.3×10^5 nkat/g (5.6×10^4 units/g)]

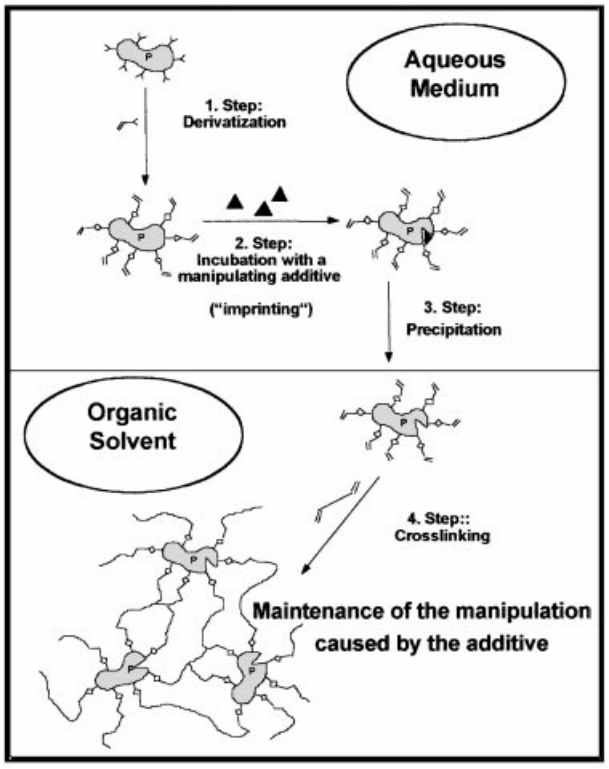
Reaction	Biocatalyst preparation	K_M[a] [mM]	V_{max} [%]
L-ee hydrolysis	Native	0.08	100
	Crosslinked, non-imprinted CT	12.8	12.6
	CLIP (L-)[b]-CT	12.8	12.6
D-ee hydrolysis	CLIP (D-)[b]-CT	7.3	1.02 mM^{-1} min^{-1} (k_{enz})
	Spontaneous non-enzymatic hydrolysis		$1.9\ 10^{-5}$ min^{-1} (k_{nonenz})

[a,b] See Table 1a.

and the rather low specific surface area of the polymer. Whether this high concentration of crosslinker is really necessary to maintain an imprinted property of proteins has to be investigated in the future. Also a comparison of the catalytic efficiency of optimized CLIPs with the glutaraldehyde crosslinked enzyme crystals[18,19] (CLECs) would be interesting since both methods make use of crosslinking enzymes in their conformational stable forms (CLIPs: enzymes are "rigid" due to non-aqueous environment; CLECs: enzymes are "rigid" due to crystallization).

If the D-ester hydrolysis activity of CLIP (D)-CT is compared with the activity of the free native CT catalyzing the "natural" L-ester hydrolysis, the latter was about 1000-fold higher. However, this has to be judged carefully since the CLIP (D)-CT preparation is just a very first example for the stabilization of an imprinted property by crosslinking. The fact that the "new" substrate acceptance of two evolutionary convergent proteases CLIP (D)-CT and CLIP (D)-SUB lead to k_{enz}/k_{nonenz} values for the enzymatic D-ester hydrolysis in the range of 10^4–10^5 mM^{-1} fold remains impressive. The data are already in the same order of magnitude as with the majority of catalytic antibodies.[20]

Though this was only a first step to make the use of imprinted proteins available and stable in aqueous



Scheme 1. Broadening the substrate selectivity through the combination of bioimprinting and a covalent immobilization technique.

phases, the principle of the method seems to allow the tailoring of biocatalysts by biochemical means (Scheme 1).[21] Its degree of freedom concerning the amounts and kinds of vinylization agents and crosslinking agents, the conditions for derivatization and crosslinking (e.g. adding stabilizing additives in each case) is promising to achieve higher specific activities towards creating imprinted "new" enzyme properties in the future.

Experimental

Chemicals. All chemicals used were commercially available and purchased in their highest grades. *N*-Acetyl-D-tryptophan was purchased from Bachem (Bubendorf, Switzerland, no. F-1090, lot 502198)

Enzymes. α-Chymotrypsin type II from bovine pancreas (EC 3.4.21.1) was purchased from SIGMA Chemical Co. (St. Louis, MO, no. C 4129) as a dialyzed, essentially salt-free, lyophilized powder. The enzyme preparation was 3× crystallized from 4× crystallized chymotrypsinogen (lot 91H7195). Subtilisin Carlsberg type VIII from *Bacillus licheniformis* (EC 3.4.21.62) was also ordered from SIGMA (No. P 5380, lot 46H0141) as a crystallized and lyophilized preparation.

α-chymotrypsin/subtilisin assay. α-Chymotrypsin/subtilisin activity was determined following the hydrolysis of benzoyl-L-tyrosin ethyl ester as described elsewhere.[22,23]

Enzyme vinylization. For functional group acylation with itaconic anhydride of about 60%, 50 mg of α-chymotrypsin/subtilisin were dissolved in 10 mL of potassium phosphate buffer (0.01 M; pH 7.8). 120 mg of itaconic anhydride were added slowly in small portions to the stirred enzyme solution. The pH value was maintained by titration with a NaOH solution (5 M). After all of the anhydride had been added the stirring was prolonged for 30 min. Gelfiltration using PD-10 columns (Pharmacia) with H_2O dist. as the eluent was applied in order to cut off low-molecular compounds from the enzyme solution. The eluents were combined and finally lyophilized. The extent of enzyme acylation was determined with 2,4,6,trinitrobenzenesulfonic acid (TNBS) as described elsewhere.[14] Since the TNBS assay is not selective for a particular protein amino acid composition, it was assumed that the obtained data correspond to the statistical average of all proteins in the sample and thus for CT or SUB.

Enzyme imprinting. Typically, 30 mg of α-chymotrypsin/subtilisin were dissolved in 1 mL potassium phosphate buffer (0.01 M, pH 7.8) containing *N*-acetyl-D-tryptophan or *N*-acetyl-L-tryptophan (0.02 M). The solution was cooled to 0°C for 30 min, 4 mL of 1-propanol were added at a temperature of −20°C and the mixture was mixed vigorously. After an additional 30 min the resulting precipitate was centrifuged and the supernatant was decanted. The precipitate was washed 3 times with each 3 mL of 1-propanol and finally dried under vacuum.

Synthesis of *N*-acetyl-D-/*N*-acetyl-L-tryptophan ethyl ester. In general 10 mg of each enzyme preparation (NIP, DIP, CLIP) were suspended in 1 mL dry cyclohexane puriss. in 1.5 mL organic solvent resistant tubes (Soerensen, Bioscience Inc.). After the suspension was sonified with a Sonorex DK 514 B for 5 min the reaction was started upon addition of 250 μL dry ethanol puriss. containing *N*-acetyl-D-tryptophan or the corresponding L-enantiomer in a concentration of 0.1 mol/L and 10 μL H_2O dist. at 25°C with the help of a thermomixer (Eppendorf 5436) at 1200 rpm.

Ester analysis. The rate of esterification was measured by determining the amount of *N*-acetyl-D-tryptophan- or *N*-acetyl-L-tryptophan ethyl ester formed during the reaction at certain intervals. A 50 μL aliquot of the reaction mixture, from which the enzyme had been removed by centrifugation, was diluted 1/40 and injected in a HPLC RP-18 column (LiChroCart 125-4, Merck). The elution was done with water:acetonitrile:acetic acid (65:30:5 v/v/v) at a flowrate of 1 mL/min. The D-, and respectively, the L-configurated ester were detected at 275 nm. The calibration was done with the pure *N*-acetyl amino acids or their ethyl esters as external standards. All analyses were run at least in duplicates. The resulting HPLC integrals deviated less than 8% from each other. The optical purity of the product was confirmed by measuring the specific rotation with a polarimeter.

Crosslinking of DIP. Ten milligrams of DIP were suspended in 0.5 mL cyclohexane and sonified until

homogeneity. To this suspension 0.23 mL of ethylene glycol dimethacrylate (EDMA) and 4 mg of AIBN were added. The radical copolymerization was initiated by UV irradiation at 366 nm at 4°C. After 5 h the resulting polymer underwent three consecutive washing steps each with 0.5 mL of cyclohexane. Finally the cross-linked imprinted biocatalyst was dried under vacuum.

Hydrolysis of *N*-acetyl-D-tryptophan ethyl ester. Ten milligrams of CLIP was suspended into 1 mL of potassium phosphate buffer (0.01 M; pH 7.8), containing *N*-acetyl-D-tryptophan ethyl ester (0.03 M). The reaction mixture was incubated at 25°C at 1200 rpm with the help of a thermomixer. At certain intervals 50 μL aliquots were taken, centrifuged and the supernatant was diluted 1/40 prior to HPLC analysis (s. ester analysis). To exclude any nonspecific, non-enzymatical hydrolysis, this reaction was conducted with the polymer alone, the substrate alone and the polymer, containing non-imprinted enzyme.

Operational stability of CLIP (D-)-α-CT. After the initial 120 h of CLIP (D-)-α-CT catalyzed D-ester hydrolysis, CLIP (D-)-α-CT was centrifuged and washing steps using potassium phosphate buffer were applied until there was no longer any substrate/product detectable in the washing solutions. After drying the CLIP (D-)-α-CT under vacuum "fresh" substrate solution was added and again the hydrolysis was conducted for another 96 h. This procedure was done 3 times, so that the total reaction time amounted to 408 h (17 days).

Reuse of CLIP (D-)-α-CT and CLIP (D-)-SUB. After the D-ester hydrolysis took place, the substrate and the product were removed with dry cyclohexane as the washing solution until these compounds were no longer detectable by HPLC analysis. Then CLIP (D-)-α-CT/CLIP (D)-SUB was dried under vacuum and the synthesis of *N*-acetyl-D-tryptophan ethyl ester was conducted again (see above).

Determination of the kinetic parameters V_{max} and K_M. The kinetic constants were determined following the initial velocities of the hydrolysis/synthesis reactions at 25°C with increasing substrate concentrations up to substrate saturation. The resulting hyperbolic saturation curves were linearized according to Hanes. All related transformations were conducted independently of each other in triplicates.

Calculation of reaction acceleration. The reaction acceleration was calculated by comparing two second-order rate constants, k_{nonenz} for the non-enzymatic reaction $\{v_{nonenz} = k_{nonenz}$ [S] [Solvent] $\}$ and k_{enz} for the enzymatic reaction $\{v_{enz} = (k_{cat} \cdot K_M^{-1})$ [S] [E] $\}$.

Active-site titration. The accessibility of low-molecular compounds to the active-site of α-chymotrypsin was determined by titrating the enzyme with *N-trans*-cinnyamoylimidazol as described elsewhere.[24]

Measurement of the CLIP's specific surface area. The specific surface area was determined according to the BET[13,25] method using a Mikromeritics DeSorb 2300 A (Mikromeritics, Georgia, USA).

Determination of the CLIP's average particle size (APS). The average particle size was measured with a Sympatec HELOS particle analyzer (Sympatec, Germany).

Scanning electron microscopy (SEM) of CLIP. SEM pictures of CLIP (D)-CT were taken with a Zeiss DSM 982 Gemini after the samples had been sputtered with Au.

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