Chapter 5

Selection of multiple mutated cephalosporin acylases with modified substrate specificity

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Part of this work has been submitted.

Cephalosporin acylases are important enzymes in the production of semi-synthetic β-lactam antibiotics. In the search for improved hydrolysis of cephalosporin C and adipyl-7-ADCA both rational and random mutagenesis approaches have been used. It was concluded that five residues in the active site of the enzyme are important for the binding of the substrate; Tyr178, Tyr231, Arg255, Asn266 and Phe375. Single mutants of residues Tyr178, Asn266 and Phe375 were shown to result in improved activity towards both adipyl-7-ADCA and cephalosporin C. It is, however, likely that a combination of mutations may improve this activity even further. Therefore, we integrated random and rational approaches to change the substrate specificity of a cephalosporin acylase.

We started with the combination of the best mutant in the α-subunit with known improved mutants in the β-subunit. Biochemical analysis of the combinants showed, however, that only one of the mutants, SY-77$^{Y178H+F375L}$, acquired an increased k_{cat} compared to both single parents on adipyl-7-ADCA. All other combinations resulted in a similar or worsened value for both k_{cat} and K_m resulting in a similar or lower catalytic efficiency.

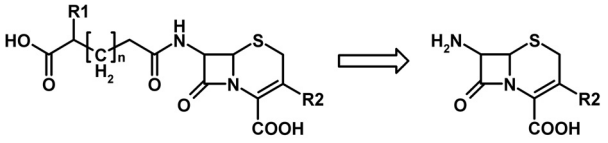
In a second experiment, we created a mutant library in which all possible amino acids at the hot spot positions 266 and 375 were combined. This library was cloned into a background sequence of wild type acylase and with the best α-subunit mutation, Y178H, respectively. In order to avoid extensive screening, the libraries were selected on minimal medium containing adipyl-leucine or amino adipyl-leucine as sole leucine source. No mutants could be selected from the selective plates with amino adipyl-leucine. From the adipyl-leucine plates, however, four different multiple mutants were selected showing a 4-fold improved hydrolysis ratio of adipyl-7-ADCA over glutaryl-7-ACA. They were purified and catalytic parameters towards glutaryl-7-ACA, adipyl-7-ADCA and cephalosporin C were determined. On adipyl-7-ADCA, k_{cat} values of the multiple mutants are equal or lower than those of the single mutants, while K_m values have improved. Remarkably, only multiple mutants comprising the N266Q mutation display a higher additive catalytic efficiency (k_{cat}/K_m) than the single mutants. Although the mutants were selected on adipyl-leucine, some of them showed improved hydrolytic activity of cephalosporin C compared to wild type acylase. Surprisingly, the single mutant SY-77^{Y178H} was found to have the highest cephalosporin hydrolysis activity. None of the selected combinations of this mutation with mutations at position 266 and/or 375 results in an increased activity towards cephalosporin C.

As a last approach, the randomisation of all five important substrate specificity residues was pursued. In order to prevent a bias towards the original codons, we randomised the amino acids Tyr178, Tyr231, Arg255 and Asn266 by building this part of the gene synthetically. We hybridised and ligated degenerate oligonucleotides containing NNS codons at the positions to be randomised. This synthetic part was cloned into a pool of vectors containing all amino acids on

position 375. The ligation and hybridisation of the synthetic gene resulted in a mutant library that comprises in theory $3*10^6$ different mutants, which covers statistically 94% of the total possible library. Unfortunately, cloning and transformation of the total fragment into the randomised pool of vectors did not yield sufficient transformants. It resulted only in a total of $1.3*10^5$ different colonies, which represents 0.13% of the possible library of the five totally randomised residues. Plating of this small portion of the total library on minimal medium containing amino adipyl-leucine did not result in any active mutants. A new cloning procedure may, however, result in some multiple mutants with an improved activity towards amino adipyl-leucine and consequently towards cephalosporin C.

Introduction

Cephalosporin acylases are important enzymes in the environmentally friendly production of the key intermediates of semi-synthetic cephalosporins, 7-amino-desacetoxycephalosporanic acid (7-ADCA) and 7-aminocephalosporanic acid (7-ACA) (Figure 1). The hydrolytic activity of cephalosporin acylases towards the substrates adipyl-7-ADCA and cephalosporin C (CPC) is, however, very small and industrially not exploitable. In former research we used directed evolution strategies in order to pinpoint important residues of the glutaryl acylase from *Pseudomonas* SY-77 for substrate specificity [28,116]. The results of the directed evolution experiments could be rationalised with the three-dimensional structure of the highly homologous glutaryl acylase from *Pseudomonas diminuta* KAC-1 [32]. Based on these findings, five residues in the active site of the enzyme are considered particularly important for the binding of the substrate; Tyr178, Tyr231, Arg255, Asn266 and Phe375. Four of these residues were also mentioned in rational mutagenesis approaches, but Asn266 was never indicated [24,32,115].



Compound	n	R1	R2
Glutaryl-7-ACA	1	-H	$-CH_2OCOCH_3$
Adipyl-7-ADCA	2	-H	$-CH_3$
CPC	2	$-NH_2$	$-CH_2OCOCH_3$
7-ACA	-	-	$-CH_2OCOCH_3$
7-ADCA	-	-	$-CH_3$

Figure 1: The production of 7-A(D)CA from β-lactam compounds.

In order to see the influence of the five amino acids on substrate specificity, saturation mutagenesis of single residues was performed previously. Within the α-subunit of SY-77 glutaryl acylase, the single mutant SY-77^{Y178H} was found to have the highest hydrolysis activity towards adipyl-7-ADCA [28]. In more detailed studies, positions Asn266 and Phe375 were changed into all 19 other amino acids by site-directed mutagenesis and the activity of the purified mutants was analysed [122,123]. It could be concluded that most amino acids are structurally allowed at either position and that every mutant has its own substrate specificity profile towards glutaryl-7-ACA, adipyl-7-ADCA and CPC. Although up to a 15-fold increase of the catalytic efficiency for hydrolysis of adipyl-7-ADCA was measured, it appeared likely that further improvement towards this compound and activity towards CPC is possible by additional mutations or different combinations of mutations. In spite of being part of the substrate binding site, no mutations were found at positions Tyr231 and Arg255 upon random mutagenesis of the β-subunit. Probably, single mutations at these positions deteriorate acylase activity as Tyr231 is conserved in most acylases and the positive charge of Arg255 is necessary to complement the carboxylic head of the substrate.

The crystal structure of the enzyme reveals a very tight and intricate pattern of interactions among the active site residues and the substrate. It seems logical that several mutations are necessary to significantly improve hydrolysis activity to a desired substrate by creating a new network of interactions with this substrate. Changing only one of the important amino acids will disturb interactions and can result in lower activities towards both the preferred substrate glutaryl-7-ACA and the desired substrates adipyl-7-ADCA and CPC [116,122,123]. Furthermore, the larger side chains of both adipyl-7-ADCA and CPC may need more space and interactions with different amino acids in order to be fully accommodated in the active site of the enzyme. Combinations of single mutations in an enzyme can result in no changes at all or may lead to antagonistic, (partially) additive or synergistic effects, giving insight in the nature and extent of interactions between specific amino acids.

We decided to start with the combination of different pairs of mutations and finish with the total randomisation of all five amino acids mentioned above. Firstly, the best mutation in the α-subunit was combined with mutations in the β-subunit that were known to improve the enzymatic activity. These combinations were screened by biochemical analyses of the purified enzyme. A next step was to randomly combine all amino acids at the two hot spot positions 266 and 375. In order to avoid extensive screening, a library, theoretically comprising all 400 possible mutants, was plated onto selective media containing (amino) adipyl-leucine as sole leucine source. This library was studied in the context of the wild type α-subunit sequence and combined with the Y178H mutation in the α-subunit. Finally, we started with the construction of the library of all five randomised amino acids. Site-saturation mutagenesis is the most straightforward process to create a total randomised library. Maximal randomness is particularly important for larger changes in enzyme activity, like the conversion of a glutaryl acylase into a CPC acylase. Most mutagenesis methods involve a step in which

a mutagenic oligonucleotide is annealed to the wild type template, which may result in a bias towards the original codon as we have observed before [122]. Different modified mutagenesis methods have been developed in order to prevent this bias [124]. The most successful approaches of preventing bias in directed evolution experiments involve the predominant use of synthetic oligonucleotides [65]. Therefore, we decided to randomise the four amino acids Tyr178, Tyr231, Arg255 and Asn266 completely by assembling it from synthetic oligonucleotides. In this way the NNS-codons are present on both strands avoiding bias towards the original codon as was also shown in a degenerate homoduplex shuffling method [95]. Cloning this synthetic part into a pool of vectors comprising all 20 amino acids at position 375 is likely to yield an unbiased library of five totally randomised amino acids, which is not too large to be selected by growth selection. All libraries were plated onto selective media containing adipyl-leucine or amino adipyl-leucine as sole leucine source in order to find better mutants towards both adipyl-7-ADCA and CPC, respectively.

Materials and Methods

Bacterial strains, chemicals, plasmids and DNA manipulations

The plasmid pMcSY-2 [116] and the leucine deficient *Escherichia coli* DH10B (Invitrogen) were used for the cloning of the libraries and the expression of the *Pseudomonas* SY-77 glutaryl acylase. Molecular DNA techniques were executed following standard protocols [41]. The enzymes used for DNA manipulations were purchased from New England Biolabs and Invitrogen and applied according to the instructions of the manufacturer. Isolation and purification of DNA was performed using the QIAprep and QIAquick kits of QIAGEN. The β-lactam substrates glutaryl-7-ACA, adipyl-7-ADCA and CPC (as sodium salt) were gifts from DSM, The Netherlands. Amino adipyl-leucine was synthesised by Syncom B.V., The Netherlands.

Construction and testing of the rationally combined mutants

Improved mutants found in the epPCR library of the β-subunit and saturation mutagenesis of position Phe375 were combined with mutation Y178H in de α-subunit. Initial experiments showed that normal cloning procedures resulted in a significant fraction of wild type sequence in the *Hin*dIII-*Nco*I portion of the gene, probably caused by self-ligation of linearised plasmid that could not be fully separated by gel electrophoresis. To avoid this, the plasmid containing mutation Y178H was truncated by elimination of a small *Stu*I fragment located in the 3' moiety of the gene, encoding part of the β-subunit. The resulting plasmid was named pMcYHtr-Stu. Plating *E. coli* DH10B with this plasmid on minimal plates with glutaryl-leucine, adipyl-leucine or amino adipyl-leucine showed no growth after 14 days of incubation at 30°C. The β-subunits of mutants SY-77^{F229L}, SY-77^{N266H}, SY-77^{N266S}, SY-77$^{M271V+Q291K+T374S}$, SY-77^{F375H} and SY-77^{F375L} were ligated into the truncated plasmid using the appropriate restriction enzymes. Resulting plasmids were sequenced to confirm the presence of the mutations. The mutants were tested for the production of soluble enzyme with a polyclonal antibody by spot blotting as described before [122]. Mutant acylases were purified up to 90% and catalytic parameters on glutaryl-7-ACA, adipyl-7-ADCA and CPC were determined as described before [116].

Construction of the double and triple libraries

To create the library in which both position 266 and 375 are totally randomised, 20 plasmids containing one of the 20 amino acids at position 266 were isolated from *E. coli* DH10B and mixed in equimolar amounts [123]. The same was performed for 20 plasmids containing all different amino acids at position 375 [122]. Fragments containing the 20 F375X mutations were cloned into the backbone of pMcSY-77^{N266X} using the restriction enzymes *Hin*dIII and *Nco*I. Ligation mixtures were transformed to *E. coli* DH10B and plated onto LB plates containing 0.4% glucose and 50 µg/ml chloramphenicol. Ten transformants were picked randomly to determine the DNA sequence of the plasmids. The rest of the transformants was scraped together for further use. This library was named the NF-library.
The fragments from the NF-library containing all 400 mutants were ligated into pMcYHtr-Stu using the restriction enzymes *Xma*I and *Kpn*I. This library was called the YNF-library.

Randomisation of 5 residues using synthetic oligonucleotides

In order to get an unbiased library of five totally randomised residues, synthetic oligonucleotides containing NNS-codons were used (Table 1). The part from the gene between restriction sites *Eco*O109I and *Nco*I was constructed in several steps (Figure 2). The first part, from base 283 until 525, was made by PCR using 0.5 mM of oligonucleotides A1 and B1 in the following program: 3 min at 95°C, 30 cycles of 45 s at 95°C, 1 min at 60°C, 1 min at 72°C and a final step of 10 min at 72°C. The resulting PCR product was purified from a 2% agarose gel. The second part of the gene from base 486 until 989 was assembled from synthetic oligonucleotides A2 until A11 and B1 until B10, containing NNS-codons at the codons for amino acids Tyr178, Tyr231, Arg255 and Asn266.
Since synthetic oligonucleotides do not have a phosphate group at the 5'-end, they cannot be joined together by ligase and need to be phosphorylated. In order to get the best possible phosphorylation of the oligonucleotides, they were heated to 100°C for 5 min and immediately put on ice, hereby losing any secondary structures that might block the end of the oligonucleotide from being phosphorylated. After cooling down they were phosphorylated using T4 PolyNucleotideKinase (PNK, Roche) according to the protocol of the supplier. Complementary pairs (e.g. A11 and B1, A2 and B2) were added together and allowed to hybridise by boiling for 5 min in a small water bath, followed by slow ON cooling down in this water bath. Hybridised pairs were ligated together one pair at a time by incubation for 5 min with Ampligase Thermostable DNA ligase (Epicentre). The optimum temperature for this enzyme of 45°C is favourable, since the minimum overhang of 10 bases between the synthetic oligonucleotides has a higher melting temperature then the normal overhang of two to four bases after restriction. The starting temperature of the hybridisation of two double stranded pairs was at least 3°C lower than the melting temperature of each individual pair to prevent the hybridised pairs from melting.

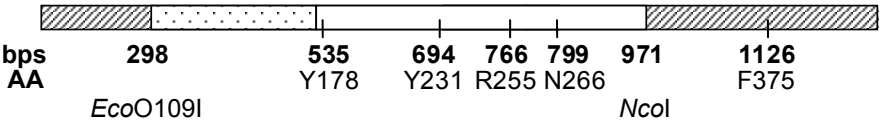


Figure 2. Graphical outline of the strategy in order to get 5 totally randomised residues.

See text for explanation. = vector, = 'normal' PCR, = synthetic gene.

Hybridisation and ligation was performed by lowering temperature 1°C per 2 minutes until 10°C below the hybridising part melting temperature, but not lower than 25°C because of the optimal temperature of Ampligase. The ligation mixtures were kept on ice during which the ligation was checked on a 2% agarose gel. Successfully ligated fragments were used in a following cycle of ligation with Ampligase. After joining all pairs together, the resulting DNA fragments encoding the four randomised amino acids were amplified by PCR using primers A11 and B11 in the following program: 3 min at 95°C, 30 cycles of 45 s at 95°C, 1 min at 65°C, 1 min at 72°C and a final step of 10 min at 72°C. In order to calculate the diversity of the amplified PCR product, control PCRs starting from known DNA concentrations were performed. Both parts of the acylase gene were assembled by PCR using 600 ng DNA of each part per 100 µl PCR mix and 0.5 mM of each of the primers A1 and B11 comprising the restriction sites *Eco*O109I and *Nco*I. The following PCR program was used: 3 min at 95°C, 30 cycles of 45 s at 95°C, 1 min at 56°C, 1.5 min at 72°C and a final step of 10 min at 72°C. 160 ng DNA of the resulting library of PCR fragment was ligated into a pool of vectors containing the rest of the acylase gene with codons for all 20 amino acids at position 375.

Table 1. Synthetic oligonucleotides used for randomising 5 residues.
Primers named A are forward primers, B primers are backward primers. NNS codons are given in bold, restriction sites are underlined.

Name	Length	Sequence (5'→3')
A11	29 bps	TTCCGGCGCCGACGTGGTGGCCCACGCCC
A2	32 bps	ACCGCCTGATGAACTTCCTC**NNS**GTCGCGTCG
A3	94 bps	CCCGGGCGCACCCTGGGCGAGGGCGACCCGCCGGACCTGGCATCAA GGATCCAACTCCTGGGCGGTGGCGCCGGGAAAGACGGCGAACGGGA
A4	43 bps	ACGCCCTGCTGCTGCAGAACCCGCACCTGTCCTGGACGACGGA
A5	33 bps	CTACTTCACC**NNS**TACGAGGCGCATCTCGTCAC
A6	39 bps	GCCGGACTTCGAGATCTATGGCGCGACCCAGATCGGCCT
A7	35 bps	GCCGGTCATC**NNS**TTCGCCTTCAACCAGCGGATGG
A8	32 bps	GCATCACC**NNS**ACCGTCAACGGCATGGTGGGG
A9	88 bps	GCCACCAACTATCGGCTGACGCTTCAGGACGGCGGCTATCTGTATGAC GGTCAGGTGCGGCCGTTCGAGCGGCCTCAGGCCTCGTATC
A10	77 bps	GCCTGCGTCAGGCGGACGGGACGACGGTCGACAAGCCGTTGGAGATC CGCTCCAGCGTCCATGGCCCGGTCTTCGAG
B1	39 bps	ATCAGGCGGTGGGCGTGGGCCACCACGTCGGCGCCGGAA
B2	35 bps	GGGTGCGCCCGGGCGACGCGAC**SNN**GAGGAAGTTC
B3	62 bps	CGGCGCCACCGCCCAGGAGTTGGATCCTTGATCGGCCAGGTCCGGCG GGTCGCCCTCGCCCA
B4	51 bps	GGACAGGTGCGGGTTCTGCAGCAGCAGGGCGTTCCCGTTCGCCGTCTT TCC
B5	34 bps	GCGCCTCGTA**SNN**GGTGAAGTAGTCCGTCGTCCA
B6	41 bps	CTGGGTCGCGCCATAGATCTCGAAGTCCGGCGTGACGAGAT
B7	33 bps	GTTGAAGGCGAA**SNN**GATGACCGGCAGGCCGAT
B8	32 bps	CCGTTGACGGT**SNN**GGTGATGCCCATCCGCTG
B9	85 bps	AGGCCGCTCGAACGGCCGCACCTGACCGTCATACAGATAGCCGCCGT CCTGAAGCGTCAGCCGATAGTTGGTGGCCCCCACCATG
B10	90 bps	CTCGAAGACCGGGCCATGGACGCTGGAGCGGATCTCCAACGGCTTGT CGACCGTCGTCCCGTCCGCCTGACGCAGGCGATACGAGGCCTG
A1	30 bps	GGGGGCCGAATACTGGGGCCCGGATTACG
B11	20 bps	CTCGAAGACCGGGCCATGGA

After transformation of these mutant plasmids into the leucine-deficient *E. coli* strain DH10B, transformants were scraped together from LB plates, containing 0.4% glucose and 50 µg/ml chloramphenicol, and frozen as -80°C stocks.

Selection, purification and catalytic parameter determination of mutant enzymes

E. coli DH10B containing the different libraries were taken from -80°C stocks, diluted 5 times in 2x YT-medium and grown at 37°C (250 rpm) for 1 hour, starved for 2 hours in 0.9% NaCl (37°C, 250 rpm), plated onto minimal medium containing 0.1 mg/ml adipyl-leucine or amino adipyl-leucine as sole leucine source and incubated at 30°C. *E. coli* DH10B expressing wild type SY-77 glutaryl acylase and mutant SY-77^{Y178H} were also plated onto the selection plates as a control for the different libraries. Plasmids of transformants that grew faster than control colonies were isolated, retransformed to *E. coli* DH10B and plated onto the same selection medium again. Single colonies appearing on the second plate were tested for the production of enzyme with a polyclonal antibody by spot blotting as described before [122]. Acylase producing mutants were used for an initial activity screen with crude cell extracts. Mutants with an improved hydrolysis ratio of adipyl-7-ADCA over glutaryl-7-ACA were purified to >90% purity by anion exchange and hydrophobic interaction column chromatography. The kinetic parameters on glutaryl-7-ACA and adipyl-7-ADCA were determined using different substrate concentrations in a fluorescamine assay as described before [116].

Results

Combining the best α- and β-subunit mutations

In previous research we obtained mutants in both the α- and β-subunit which had an improved hydrolysis activity towards adipyl-7-ADCA. Since the multiple mutations of one of the mutants (SY-77$^{M271V+Q291K+T374S}$) appeared to be additive [116], we constructed other combinations which could also have additive or even synergistic effects. Therefore, the best mutation in the α-subunit, Y178H, was combined with six improved enzymes having mutations in the β-subunit, SY-77^{F229L}, SY-77^{N266H}, SY-77^{N266S}, SY-77$^{M271V+Q291K+T374S}$, SY-77^{F375H} and SY-77^{F375L}. All combinations resulted in soluble enzymes and were purified (> 90% pure). Activity towards both glutaryl-7-ACA and adipyl-7-ADCA was measured using the fluorescamine assay. Hydrolysis of glutaryl-7-ACA was decreased in all mutants compared to their single parents (data not shown). On adipyl-7-ADCA only mutant SY-77$^{Y178H+F375L}$ showed an increased k_{cat} compared to both single parents. All other combinations showed similar or worsened k_{cat} and K_m values resulting in a similar or lower catalytic efficiency (Table 2). Remarkably, the high k_{cat} value of single mutant SY-77^{F375H} is reduced 2.6-fold when combined with mutation Y178H.

Construction and selection of libraries randomised at positions Asn266 and Phe375

A library was made by combining gene fragments encoding all 20 amino acids at position 266 with those that encode all 20 amino acids at position 375. Sequence

Table 2. Catalytic parameters of rationally constructed mutants and their parents on adipyl-7-ADCA.
The k_{cat} and K_m on adipyl-7-ADCA were determined by measuring the initial rate of hydrolysis on a range of substrate concentrations with a fixed amount of enzyme as described earlier. The catalytic parameters of mutant SY-77^{Y178H} are mentioned separately, since it is the parent of these mutants.

Mutation(s)	k_{cat} (s^{-1})		K_m (mM)	
	multiple	- Y178H	multiple	- Y178H
Y178H	0.66 ± 0.05	0.38 ± 0.02	0.47 ± 0.05	0.8 ± 0.1
Y178H+F229L	0.71 ± 0.05	0.47 ± 0.02[a]	1.1 ± 0.2	0.52 ± 0.04[a]
Y178H+N266H	0.95 ± 0.04	0.55 ± 0.02[a]	0.19 ± 0.07	0.14 ± 0.02[a]
Y178H+N266S	0.43 ± 0.06	0.34 ± 0.02[a]	0.9 ± 0.3	0.42 ± 0.04[a]
Y178H+M271V+Q291K+T374S	0.44 ± 0.09	0.61 ± 0.04[a]	0.8 ± 0.2	0.7 ± 0.1[a]
Y178H+F375H	0.44 ± 0.03	1.16 ± 0.08[b]	0.56 ± 0.04	0.9 ± 0.2[b]
Y178H+F375L	1.0 ± 0.2	0.67 ± 0.04[b]	0.7 ± 0.1	0.7 ± 0.1[b]

[a] Values were taken from ref. [116]
[b] Values were taken from ref. [122]

analysis of 10 randomly picked clones all revealed different combinations of amino acids at positions 266 and 375, indicating that the random combination of the two positions succeeded. After correction for self-ligation, the plasmids that contain the library randomised at positions Asn266 and Phe375 (NF-library) or this library combined with mutation Y178H (YNF-library) was transformed to *E. coli* DH10B. This resulted in approximately 2000 and 800 discrete colonies for the NF- and YNF-library, which statistically includes all 400 possible mutants with a certainty of 99% and 86%, respectively. The transformants were scraped together and frozen as -80°C stocks.
The frozen stocks of both libraries were thawed, diluted into 2x YT-medium and revived for one hour at 37°C. The cells were washed and starved in 0.9% NaCl in order to drain the internal leucine supply. After another washing step, cells were plated onto minimal medium containing adipyl-leucine or amino adipyl-leucine as a sole leucine source and incubated at 30°C. As a control for the NF- and YNF-library, respectively, both *E. coli* DH10B expressing wild type SY-77 glutaryl acylase and mutant SY-77^{Y178H} were plated. Plates were inspected daily for eight days until very small colonies started growing on both negative control plates and selection plates. At the 9th day, 35 of the largest and earliest spotted clones from both libraries on the adipyl-leucine plates were streaked onto fresh selection plates and incubated at 30°C (Table 3). At the same day the YNF-library showed some very small dots on the selective plates containing amino adipyl-leucine, from which the three largest were selected and streaked onto a fresh selection plate. The NF-library showed no colonies at all on amino adipyl-leucine selection plates. When equal amounts of bacteria of the libraries and wild type were plated with glutaryl-leucine as sole leucine source, a much smaller number of colonies was observed with the libraries.
Plasmids from selected transformants were retransformed to *E. coli* DH10B and production of acylase was tested. Unfortunately, 61% of the transformants did not produce any acylase as shown by spot blotting using a polyclonal rabbit antibody. Crude

extracts of the transformants that did show acylase production were tested for activity towards glutaryl-7-ACA and adipyl-7-ADCA (Table 3). Sequencing of the 15 mutants that produced acylase revealed that some mutants were selected more than once. The mutants SY-77$^{Y178H+N266H}$ and SY-77$^{Y178H+N266M}$ were each selected three times and mutant SY-77$^{N266Q+F375L}$ was selected twice. Of the 12 different mutants only four mutant enzymes showed an improved ratio of adipyl-7-ADCA over glutaryl-7-ACA compared to the wild type enzyme, the other mutants showed hardly any activity at all. All four active enzymes comprised multiple mutations and had an improved ratio of at least four times. Three of these mutants were the ones that were selected multiple times.

Table 3. Selection of transformants of the NF- and YNF-library.
AL, adipyl-leucine; AAL, amino adipyl-leucine; A-7-A, adipyl-7-ADCA; G-7-A, glutaryl-7ACA.

Library and selection medium	# selected mutants	# acylase producers	# different sequences	# better A-7-A/G-7-A	# better on CPC
NF on AL	20	8	7	1	0
NF on AAL	0	-	-	-	-
YNF on AL	15	7	3	3	2
YNF on AAL	3	0	-	-	-

Determining catalytic parameters of mutants found in the NF- and YNF-library

The kinetic parameters k_{cat} and K_m of the purified mutant proteins were determined on glutaryl-7-ACA and adipyl-7-ADCA. Since the hydrolysis of CPC is too low for wild type SY-77 to determine the catalytic parameters k_{cat} and K_m, the conversion rates of the mutants were calculated from the linear slope of hydrolysis in the first four hours (Table 4 and Figure 3). In order to be able to conclude whether the combination of single mutations have additive or even synergistic effects, values from former research of the respective single mutants were added to the table and figure. As seen before, hydrolysis activity of the preferred substrate glutaryl-7-ACA decreases upon improvement towards other substrates. The k_{cat} values of the multiple mutants have decreased compared to the single mutants, whereas the K_m values have increased, resulting in a much lower catalytic efficiency. On adipyl-7-ADCA, the k_{cat} values of multiple mutants are equal or lower than those of the single mutants. The K_m values, however, have improved compared to the associated single mutants. This results in a diverse spectrum of the catalytic efficiency values (Figure 3). Multiple mutants comprising mutations N266Q and F375L/M display a higher catalytic efficiency than the single mutants. The differences are, however, additive and not synergistic. The combination of Y178H with N266H/M does not lead to significantly different catalytic efficiencies on adipyl-7-ADCA than those of the single mutants SY-77^{N266H} or SY-77^{N266M}.

Surprisingly, the single mutant SY-77^{Y178H} shows the highest cephalosporin hydrolysis activity. None of the selected combinations of this mutation with mutations at positions 266 and/or 375 results in an increase of activity towards CPC. Adding N266H to mutation Y178H hardly affects the activity, but the double mutant SY-77$^{Y178H+N266M}$ has

a significantly lower hydrolysis activity than the single mutant. Single mutations at position 375 or combinations with Y178H and/or N266Q do not seem to be beneficial for the enzyme's cephalosporin hydrolysis activity.

Table 4. Catalytic parameters of selected mutants and their single parents on adipyl-7-ADCA and glutaryl-7-ACA.
The k_{cat} and K_m on adipyl-7-ADCA and glutaryl-7-ACA were determined by measuring the initial rate of hydrolysis on a range of substrate concentrations with a fixed amount of enzyme as described earlier. Mutated amino acids are in bold.

Amino Acid at pos.			adipyl-7-ADCA		glutaryl-7-ACA	
178	266	375	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)
Tyr	**Gln**	**Leu**	0.48 ± 0.06	0.17 ± 0.02	0.75 ± 0.07	0.17 ± 0.03
His	**His**	Phe	0.95 ± 0.04	0.19 ± 0.07	2.2 ± 0.1	0.08 ± 0.02
His	**Met**	Phe	1.2 ± 0.2	0.17 ± 0.02	0.89 ± 0.02	0.30 ± 0.05
His	**Gln**	**Met**	0.72 ± 0.03	0.28 ± 0.06	0.72 ± 0.06	0.62 ± 0.09
Tyr	Asn	Phe	0.38 ± 0.02	0.8 ± 0.1	4.1 ± 0.3	0.042 ± 0.009
His	Asn	Phe	0.66 ± 0.05	0.47 ± 0.05	2.5 ± 0.2	0.08 ± 0.01
Tyr	**Gln**	Phe[1]	0.46 ± 0.04	0.62 ± 0.13	2.4 ± 0.2	0.12 ± 0.03
Tyr	Asn	**Leu**[2]	0.67 ± 0.04	0.7 ± 0.1	1.10 ± 0.09	0.26 ± 0.07
Tyr	**His**	Phe[1]	0.47 ± 0.01	0.14 ± 0.01	3.1 ± 0.3	0.04 ± 0.01
Tyr	**Met**	Phe[1]	1.9 ± 0.1	0.33 ± 0.05	1.8 ± 0.1	0.17 ± 0.02
Tyr	Asn	**Met**[2]	0.65 ± 0.02	0.65 ± 0.04	2.02 ± 0.09	0.17 ± 0.02

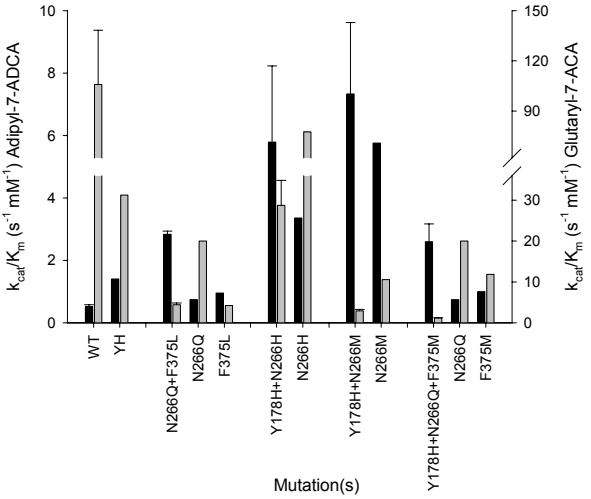
[1] Values were taken from ref. [123]
[2] Values were taken from ref. [122]

Construction and selection of the 5 randomised amino acids library

In order to randomise the four amino acids Tyr178, Tyr231, Arg255 and Asn266 completely, we assembled the part of the gene containing these residues from synthetic oligonucleotides (Table 1). After phosphorylation, complementary pairs were hybridised by heating them for five minutes and cooling down very slowly in order to cover the whole temperature range in which different randomised codons anneal to each other.

First it was tried to assemble all double stranded primer pairs in one tube, but this did not result in any band of the right size. Three PCR rounds of 25 cycles each with primers on the outer ends of the desired product were necessary to get a faint band of the right size. Calculation of the concentration of this band showed that it could only have originated from a pool of DNA that was too small to comprise all possible mutants. Therefore, we decided to connect the different double stranded pairs one at a time. To obtain the best possible hybridisation and ligation without separating the previously hybridised strands, a controlled ligation was performed, using the thermostable Ampligase to ligate the different pairs. Ligation of single and double pairs normally resulted in bands of the right size (Figure 4A and 4B). The ligation of larger fragments resulted, however, in very faint bands, which made it difficult to analyse the success of the experiment. Boosting of the final product with primers at the front and

A



B

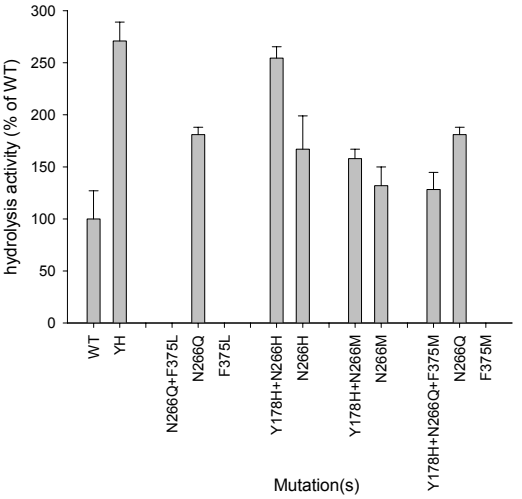


Figure 3. Catalytic efficiencies of selected mutants and their single parents on adipyl-7-ADCA and glutaryl-7-ACA (A) and conversion rates on CPC (B).
Values of earlier described parents were added to the graph in order to compare single and double mutants. Conversion of cephalosporin by mutant SY-77$^{N266Q/F375L}$ was not measurable.

back of the synthetic gene resulted in a band of the right size (Figure 4C). The concentration of this band was calculated and compared with similar PCR products from known start concentrations of DNA. This revealed that the PCR had started from $3*10^6$ molecules which is sufficient to cover all possible combinations ($32^4=1*10^6$). The first part of the synthetic gene was produced by a normal PCR and both PCR products were ligated together by a subsequent PCR step. This resulted in a clear band of the expected size that was cloned into a pool of vectors comprising all 20 amino acids at position 375 (Figure 2). Transformation of the ligation mixture resulted in a total of $1.3*10^5$ different colonies, which comprises only 0.13% of the possible library of five totally randomised residues with 99% certainty. Nonetheless, all transformants were scraped and frozen at -80°C.

The frozen stock of this library was thawed, starved and plated onto minimal medium containing amino adipyl-leucine as sole leucine source. After two weeks 27 colonies were picked, the plasmids were retransformed to *E. coli* DH10B and plated onto minimal medium again. After five days single colonies were picked and acylase production of the 25 remaining colonies was tested. Only eight colonies revealed acylase production, but none of them showed any activity towards glutaryl-7-ACA, adipyl-7-ADCA or CPC.

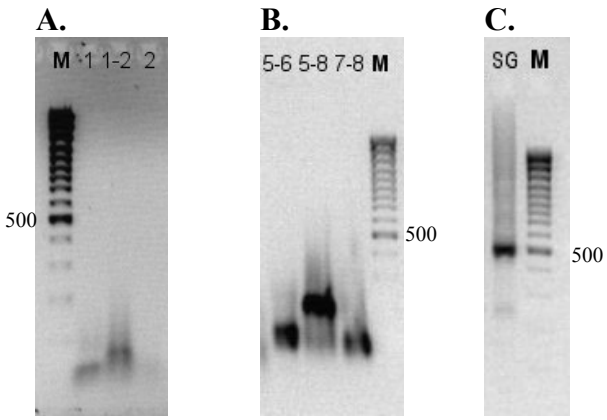


Figure 4. Typical examples of hybridisation of one or two oligonucleotide pairs using Ampligase (A, B) and the resulting synthetic gene (C).
M, Low Range 100 bps Marker, Fermentas MBI
A. Hybridisation of two single pairs: 1, pair A11-B1 (39 bps); 1-2, hybridised pairs 1 and 2 (74 bps); 2, pair A2-B2 (45 bps).
B. Hybridisation of two double pairs: 5-6, ligated oligos A5-B5 and A6-B6 (83 bps); 5-8, ligated pairs 5-6 and 7-8 (152 bps); 7-8, ligated oligos A7-B7 and A8-B8 (77 bps).
C. Synthetic gene (SG) after boosting hybridisation of all 10 pairs (504 bps).

Discussion

Protein engineering of cephalosporin acylases has been pursued for some time in order to find an industrially relevant activity towards adipyl-7-ADCA or CPC. Rational and directed evolution approaches have been used by us and other researchers to achieve this goal [24,28,115,116,122,123]. In this research, we used a combination of both strategies to explore the sequence space of the substrate binding pocket of the enzyme in depth. This was performed in several subsequent experiments.

Rational recombination of improved mutants

First, we rationally combined the mutants in the α- and β-subunit with the highest activity towards adipyl-7-ADCA and determined their catalytic parameters towards glutaryl-7-ACA and adipyl-7-ADCA. Only mutant SY-77$^{Y178H+F375L}$ showed an increased k_{cat} towards adipyl-7-ADCA when compared to both single parents. As described before, His178 may attract the carboxyl group of the adipyl-7-ADCA, forcing the longer adipic side chain to go further inside the active site [28]. The extra space created by mutation F375L can accommodate also part of the side chain, thereby positioning the amide bond even closer to the active site Ser199 [122]. Some other mutations seem to counteract each other, like Y178H and F375H, which both may attract the head of the adipic side chain and thereby decrease the hydrolysis activity towards both glutaryl-7-ACA and adipyl-7-ADCA. So, combinations of improved single mutants may very well distort the active site. Therefore, a more random approach of combining mutations at important positions followed by a selection may result in a better outcome.

Recombination of two site specific random libraries

In former studies it was indicated that amino acids at positions 178, 266 and 375 are important for substrate specificity [28,116]. Single mutations at these positions resulted in improved hydrolysis activity towards adipyl-7-ADCA and in some cases even towards CPC [123]. From the crystal structure it was clear that these amino acids have a direct or indirect interaction with the substrate [32,115]. It therefore seemed likely that combinations of mutations at these positions can increase hydrolysis activity towards the larger side chains of adipyl-7-ADCA and CPC even more than their single mutations. As a first approach in this rationalised random experiment, we decided to combine the 20 different amino acids at position 266 with all 20 amino acids on position 375, and to study this in the context of a wild type sequence and together with the Y178H mutation in the α-subunit. These two libraries were plated onto minimal selective medium containing adipyl-leucine or amino adipyl-leucine as sole leucine source. In this way, we merged the rational approach of changing specific active site residues with the selection method of a directed evolution experiment.

On the minimal medium containing amino adipyl-leucine only a few, very small colonies appeared that did not produce any acylase as determined by spot blotting. Apparently, the hydrolysis activity of the mutants was not adequate to liberate sufficient leucine to allow visible growth. The selection plates containing adipyl-leucine did show small colonies after eight days of incubation. In total 38 colonies were selected and checked for acylase activity (Table 3). Unfortunately, more than half of the colonies did not show any acylase production while amongst the rest only four different mutants were identified with a better hydrolysis ratio of adipyl-7-ADCA over glutaryl-7-ACA compared to the wild type cephalosporin acylase. The mutants that were selected all contained multiple mutations, confirming our hypothesis that multiple mutants do exhibit improved properties towards the desired substrates. Remarkably, single mutants SY-77^{Y178H}, SY-77^{N266H}, SY-77^{N266S} and SY-77^{F375L}, which were selected in the former random mutagenesis experiments, could not be identified in this selection. However, already in previous studies, these mutations could not be isolated in all random mutagenesis approaches, an observation that was assumed to be related to the relative large amount of dead colonies on the selection plates, which may form an alternative leucine source for bacteria that do not show activity towards adipyl-leucine [116]. This part of the selection still needs to be optimised.

The four mutants with an improved hydrolysis ratio of adipyl-7-ADCA over glutaryl-7-ACA were purified and characterised. These mutants expressed a decreased activity towards the preferred substrate glutaryl-7-ACA compared to the wild type enzyme. On adipyl-7-ADCA, the selected mutants show decreased k_{cat} values, but improved K_m values compared to the associated single mutants. Apparently, the optimisation of interactions from residues at the three positions 178, 266 and 375, combined with the used selection procedure, allows for further improvement of the K_m and not the k_{cat} value.

Remarkably, three of the four mutations found on position 266 comprise a more bulky amino acid than the original Asn. Obviously, the indirect interactions of Asn266 with the substrate and neighbouring amino acids make the effects of mutations difficult to predict as observed earlier [123]. Hydrogen bonding and other interactions that for instance may result in a displacement of Arg255, seem to be more important at this side of the active site than creating space for the larger (amino) adipyl side chain. It is noteworthy that in this randomised approach to isolate optimised adipyl-7-ADCA hydrolysing multiple mutants by combining single mutations at positions 178, 266 and 375, the combination of the best single mutants found in an earlier research (SY-77^{Y178H} and SY-77^{N266M} [28,123]) resulted in an improved double mutant displaying the highest catalytic efficiency.

The mutants picked from adipyl-leucine plates show a similar or improved conversion rate of CPC compared to wild type, but they were not found on minimal plates containing amino adipyl-leucine. Similarly, single mutant SY-77^{Y178H} was not isolated from the library, but did show some small colonies when plated as a control on selection plates with amino adipyl-leucine. This mutant displays an almost three times higher

in vitro conversion rate of CPC than wild type acylase, which may be the minimum activity of hydrolysis to allow growth on selective medium containing 0.2 mM amino adipyl-leucine. It should be realised, however, that the *in vitro* activity on CPC was tested at a high substrate concentration (10 mM). Consequently, improved *in vitro* hydrolysis activities are likely to represent changes in k_{cat} values and not so much K_m values, whereas both parameters are related to the capability to grow on selective medium.

None of the selected multiple mutants had a higher CPC hydrolysis rate than SY-77^{Y178H}. Remarkably, the combination with N266M lowers the CPC hydrolysis rate, in contrast to the positive effect on the hydrolysis activity towards adipyl-7-ADCA (Figure 3). The selected combinations with mutations at position 375 show a similar effect. They demonstrate an improved catalytic efficiency towards adipyl-7-ADCA, whereas they appear to negatively affect CPC hydrolysis activity (Figure 3). For further improvement of CPC hydrolysis activity, mutation Y178H may have to be combined to mutations of other (active site) residues than 266 and 375. Alternatively, in a multiple mutant with high CPC hydrolysis activity, an amino acid other than Tyr or His may be needed at position 178. In a recently published research on the cephalosporin acylase from *P. diminuta*, the most improved mutant of in the second round comprised mutations Y149K and Q50βM, which would be Y178K and Q248M in SY-77 acylase [24]. This mutant showed a 6-fold improved activity towards CPC compared to the wild type enzyme, which confirms that other mutations than Y178H may indeed improve hydrolysis activity towards CPC in multiple mutants.

Random mutagenesis of 5 important substrate specificity residues

We randomised the amino acids Tyr178, Tyr231, Arg255 and Asn266 by building this part of the gene synthetically by hybridising 10 degenerate forward and 10 degenerate backward primers separately to form double stranded pairs of synthetic oligonucleotides between 30 and 100 bps in length. Recombining them in one tube did not result in a library large enough to comprise all possible mutants, although the overlap between each double stranded pair was at least 10 bases. To conquer this problem, we attempted to ligate the double stranded pairs one at a time. The first ligations of single and double pairs resulted in the expected bands, but the combination of these pieces to obtain larger fragments was more difficult to achieve. The presence of non-ligated fragments in the latter ligation mixtures may inhibit the hybridisation of the compatible overhangs, as in the one-tube experiment. Moreover, the amount of correctly ligated DNA of the right size becomes less with every step, resulting in even less of the proper product and a more difficult monitoring of the process. Despite these drawbacks, we were able to produce a mutant library which comprises in theory $3*10^6$ different mutants. This amount covers statistically 94% of the total possible library. Sequencing of randomly picked clones should be performed to confirm this number.

Unfortunately, cloning of the mutagenised gene fragments into the randomised pool of vectors was not very efficient. Transformation resulted only in a total of $1.3*10^5$

different colonies, which represent 0.13% of the possible library of the five totally randomised residues. Plating of this small portion of the total library on minimal medium containing amino adipyl-leucine resulted in no active mutants. Although this can be explained by the small sized library, one may expect a few mutants as shown in a synthetic recombination approach, in which only 0.01% of the total library was screened [95]. Recloning of the ligation product will most probably give some multiple mutants, which display an even better hydrolytic activity towards CPC than SY-77^{Y178H}.
In this research, it has become clear that the combination of rational and random procedures leads to improved enzymatic activity towards the industrially valuable substrates adipyl-7-ADCA and CPC. It is, however, important that both the computational design and the experimental methods are properly evaluated beforehand and afterwards to gain more knowledge about each step in the process, resulting in a more general improvement scheme of biocatalysts.

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