Chapter 9

Summary, General Discussion and Perspectives
Summary

This thesis describes the application of lipolytic enzymes of Bacillus subtilis for the enantiopure production of the chiral intermediate 1,2-O-isopropylidene-sn-glycerol (IPG) out of racemic ester compounds. (+)-IPG is an important chiral synthon in the synthesis of the biologically active form of several well-known pharmaceuticals and endogenous compounds, such as phospholipids, β-adrenoceptor antagonists, and platelet aggregating factors. To improve the yield of enantiopure IPG as compared to production using wild type lipase/carboxylesterase, we assessed two different strategies to specifically evolve these biocatalysts into high enantioselectivity: paralogous gene analysis and directed evolution using phage display selection.

To discover IPG-enantioselective esterase genes in the genome of B. subtilis, we chose to study B. subtilis strain 168 as a model system, since (i) its genome sequence had just been elucidated at the start of this research in 1998 (Kunst et al., 1997) and (ii) it is generally recognised as safe'. The lead compound in our search for homologues was the carboxylesterase NP of B. subtilis strain Thai I-8, as this enzyme was characterised to be an excellent biocatalyst in the kinetic resolution of chiral carboxylic acid esters, such as naproxen and ibuprofen methyl esters. In contrast, it was found to show only modest enantiopreference towards chiral alcohol esters, such as 1,2-O-isopropylidene-sn-glycerol esters.

Chapter 2 presents the identification of the ybfK gene of B. subtilis 168. This gene was identified by the B. subtilis 168 genome project as an unknown gene with high homology (64% on protein level) to the B. subtilis Thai I-8 carboxylesterase NP. We developed a novel method to clone this carboxylesterase NP paralogue into its homologous host, B. subtilis. After overexpression of the corresponding protein in the cytoplasm of B. subtilis, the enzyme was purified through anion exchange and hydrophobic interaction chromatography. The purified YbfK consisted of 296 amino acids and showed an apparent molecular mass of 32 kDa (SDS-PAGE). Comparison of the specific activities and enantioselectivities of YbfK and carboxylesterase NP towards caprylate esters of IPG revealed that YbfK produces (+)-IPG out of its caprylate ester with 99.9% enantioselectivity. Since the enzyme is an efficient carboxylesterase it has been renamed cesB.

In chapter 3 the isolation, cloning, expression and characterisation of the virtually identical homologue of the carboxylesterase NP, carboxylesterase NA of B. subtilis 168 (98% identity, corresponding to 6 different amino acids difference) is described. The gene encoding carboxylesterase NA has been renamed cesA. All three highly homologous esterases were fully characterised with respect to their applicability towards the enantiospecific hydrolysis of a wide range of compounds. Comparison of their specific activities towards several compounds revealed rather different substrate specificities, while
their physico-chemical properties were retained. We concluded that carboxylesterase NP and CesA are particularly suited for the enzymatic conversion of naproxen esters, while CesB offers enantiopure (+)-IPG from its caprylate ester.

Chapter 4 describes the development of a sensitive, specific, reproducible and fast chiral gas chromatography method for the determination and quantification of the enantiomers of IPG. This assay can be used for the determination of the enantioselectivity of lipases/esterases (mutants) in kinetic resolution experiments.

For our alternative approach for the evolution of highly enantioselective enzymes variants, we introduced a novel phage display strategy to be able to quickly and reproducibly select enzyme variants with improved characteristics towards IPG out of a pool of (randomly) constructed enzyme mutants.

Chapter 5 describes the application of this method for the future selection of Bacillus CesA mutants with improved enantioselectivities. For the first time, a method was described for the presentation of an intracellular esterase of B. subtilis on the phage coat protein g3p. We assessed the effect of several signal peptide sequences, both Sec and Tat pathway dependent, on the translocation of the carboxylesterase in order to optimise the phage display of this enzyme. Functional display of the cytosplasmic Bacillus carboxylesterase could be achieved only when Sec-dependent signal peptides were used. Although the use of a Tat-dependent signal peptide can result in carboxylesterase precurosr processing, proper phage display seems to be impossible. This strategy can serve as a model for future cloning of (intracellularly produced) heterologous proteins.

In chapter 6 this phage display method was assessed to improve the enantioselectivity of B. subtilis lipase A by directed evolution. The lipA gene was cloned upstream of the phage g3p encoding sequence and downstream of a modified g3p signal sequence in the phagemids pCANTAB 5E. After functional characterisation of LipA on the phage (demonstrating its proper folding), the interaction with enantiomeric phosphonate suicide inhibitors (see chapter 7), covalently coupled to (+)- and (-)-IPG stereoisomers (Sc and Rc inhibitor, respectively), was studied. These inhibitors can be used for the future selection of LipA variants with improved enantioselectivities by the phage display technology.

The synthesis of the immobilised enantiomeric phosphonate suicide inhibitors is described in detail in chapter 7. We succeeded in attaching chiral phosphonate-based enzyme inhibitors covalently to solid supports.

We developed a dual selection method for the isolation of mutants using immobilised enantiomeric phosphonate suicide inhibitors (chapter 8). Saturated PCR mutagenesis was performed for the site-specific conversion of a stretch of amino acids near the active site of Bacillus LipA. After expression of these mutants on bacteriophages, dual selection on (+)- and (-)-IPG phosphonate suicide inhibitors resulted in the isolation of variants with an
inverted enantioselectivity. The mutants were further characterised by determination of their Michaelis-Menten parameters. The 3D structures of the $Sc$ and $Re$ inhibitor – LipA complexes were determined to provide a structure-relationship for the chiral characteristics of the enzyme. For the first time, we have shown that enantioselectivity can be inverted using dual selection in combination with the phage display technology. This implies that a selection system has become available for the directed evolution of the enantioselective properties of $B. subtilis$ LipA. Moreover, this technique appears to be generally applicable for the selection of any enantioselective hydrolytic enzymes towards any chiral substrate ester.

**General discussion and perspectives**

In the last part of this thesis, the results of the previous chapters will be placed in a broader context. This also allows to describe the perspectives for future research.

It is generally accepted that improving the enantioselectivity of an enzyme is a complex topic. On the one hand it can be argued that screening may provide suitable enzymes/bacterial strains for the desired enantioselectivity. Possible strategies include testing commercially available purified enzymes, screening different wild type bacterial strains or searching potentially interesting enzymes using analysis of elucidated genome sequences. However, it should be noted that identification of highly enantioselective enzymes by classical enzyme screening programmes is frequently hampered by a low expression or excretion level under wild type conditions, which seriously restricts the chance of finding the ‘perfect’ enzyme. In addition, wild type enzymes are often produced by the host organism as a mixture of isoenzymes, differing in amino acid sequence or glycosylation pattern, and are sold as crude mixtures for practical reasons and cost-effectiveness (Bornscheuer, 2002b). As a consequence, proper characterisation of the biocatalyst requires cloning and expression of the gene of interest. Nevertheless, we demonstrated that a detailed analysis of available genome sequences in combination with homology alignments with well-characterised and highly enantioselective enzymes can reveal novel enzymes with interesting characteristics.

Although (classical) screening continues to be an important source for biocatalysts, an efficient alternative is represented by the construction of novel enantioselective enzyme using rational design or directed evolution (Arnold, 2001; Bornscheuer & Pohl, 2001; Bornscheuer, 2002b; Chen, 2001; Henke et al., 2003; Kazlauskas & Weber, 1998; Kazlauskas, 2000). Knowledge of the 3D structure, often provides the rational means to create novel biocatalysts (Kazlauskas, 2000). The success of this approach obviously is dependent on the availability of the 3D structure of the enzyme and, unfortunately, quantitative predictions on enantioselectivity are often not reliable (Henke et al., 2003; Kazlauskas, 2000). When we started this research project in 1998, the 3D structure of the
Bacillus LipA was not yet elucidated. Thus, an attractive alternative was found in a directed evolution approach. In general, directed evolution consists of the generation of large mutant libraries followed by high-throughput screening or selection to identify desired mutants (Arnold, 1998; Arnold & Volkov, 1999; Bornscheuer, 2002b; Farinas et al., 2001; Jaeger & Reetz, 2000; Jaeger et al., 2001; Ness et al., 2001; Powell et al., 2001; Reetz, 1997b; 2000; Tao & Cornish, 2002; Wahler & Reymond, 2001). We have decided to evaluate the use of phage display selection for the creation of enantioselective Bacillus LipA variants towards IPG esters.

First, we succeeded in developing a novel phage display method for the improvement of the enantioselective properties of B. subtilis LipA towards IPG esters. This method made use of a fast and reproducible dual selection strategy applying enantiomeric phosphonate suicide inhibitors. For the first time, we were able to show that the enantioselective properties of an enzyme can be inverted using dual selection in combination with the phage display. Although the success of the phage display method had already been established for the selection of biocatalysts with improved or novel activities and properties (Atwell & Wells, 1999; Avalle et al., 1997; Danielsen et al., 2001; Demartis et al., 1999; Jestin et al., 1999; Soumillion et al., 1994; Vanwetswinkel et al., 1995; 1996; Widersten et al., 2000), its application in enhancing enantioselectivity has never been shown.

In spite of these findings, the basic principle of applying the phage display technology for the selection of the most enantioselective enzyme or group of enzymes out of a large library of enzyme mutants remains rather controversial. Most obviously, one could argue that the selection does not depend on full catalytic activity but only on binding of the enzyme to the substrate – inhibitor complex (Forrer et al., 1999). As a result, enantioselective binding is predestined to select enzymes displaying unacceptably low activity (in the worst case a single turnover only). This was demonstrated recently by Danielsen et al. (2001). These investigators failed to select improved lipase variants on biotinylated phosphonate suicide inhibitors. They speculated that the complex process of folding and secretion of this lipase hampered the expression in a heterologous host while interfacial activation complicated the design of an selection protocol. We hypothesised that these problems could possibly be overcome by using the Bacillus LipA as a model catalyst, since this enzyme does not require chaperones for efficient folding and interfacially activation at a water-lipid interface. In fact, this thesis clearly demonstrates that catalytically fully active Bacillus LipA mutants with improved enantioselectivities can be selected using phage display, implicating that a selection system has become available not only for the directed evolution of the enantioselective properties of B. subtilis LipA, but also for the selection of other enantioselective lipolytic enzymes. Therefore, this technique can result in the construction of enzyme variants specific towards certain biochemical processes and can contribute to the production of more enantiopure pharmaceuticals.
in the identification of the so-called hot-spots involved in IPG substrate binding. These regions can be further explored in a second round of mutagenesis using for example localised PCR mutagenesis in order to allow all amino acid substitutions.

- **DNA shuffling:**
  
  Selected variants can be further optimised by DNA shuffling, which elegantly allows combining of enantioselective properties. A pool of DNA sequences is randomly fragmented (e.g. by treatment with DNase I). The gene fragments are assembled into a library of full-length genes by repeated cycles of denaturation, annealing and DNA polymerase extension (Stemmer, 1994).

Secondly, another potential method for selection of enantioselective lipase variants is based on growth. The growth of a bacterial host can be made dependent of lipase with the use of certain synthetical compounds. For example, the chiral alcohol of interest can be coupled to a lipid or esters as the sole carbon source. Only the cells encoding a variant lipase which can hydrolyse the supplied substrate ester will be able to divide, resulting in survival (growth). After some consecutive rounds of mutagenesis and selection an enantioselective variant can be evolved in a stepwise manner.

Yet, it has to be stated that the use of IPG as model substrate to evaluate phage display in the directed evolution of an enantioselective biocatalyst might result in rather disappointing results. Recently, the 3D structure of LipA was elucidated (Van Pouderoyen et al., 2001). Due to its small size and the absence of a separate lid domain, which is present in the larger lipases, the *B. subtilis* LipA was regarded as a minimal α/β hydrolase fold enzyme. One might argue that the active site cleft could be relatively small as compared to other lipases and that for this reason, the *Bacillus* LipA would be ideally suited for the enantiopure production of IPG. More detailed information about the active site cleft was obtained after the elucidation of the 3D structure of *B. subtilis* LipA covalently bound to the enantiomeric phosphonate inhibitors. These 3D structures illustrate that the active site cleft of LipA is solvent exposed and relatively opened. As a consequence, both stereoisomers of an IPG ester can be converted and only a slight enantiopreference is observed. Although the mutation of one amino acid (LipA N18I) does account for an inverted enantioselectivity, more generations of mutants will have to be constructed to evolve LipA of *B. subtilis* into a highly enantioselective enzyme for the kinetic resolution of IPG esters. Most likely, loops have to be added in regions near the active site of LipA to sterically hinder the binding of one of the enantiomers of IPG. In this respect, it is worthwhile mentioning that these experiments are currently under investigation.

Third, it is tempting to suggest that highly enantioselective LipA mutants can be obtained as well when a more bulky alcohol, such as a secondary alcohol, instead of IPG (a primary alcohol) is used as a ligand at the phosphonate inhibitor. On could for example think of chiral beta-blockers, such as propranolol and sotalol.
Our mutant, selected by phage display as described above (chapter 8), had an inverted enantioselectivity compared to the wild type LipA. Unfortunately, the observed enantioselectivity of our mutant, LipAN18I, is insufficient for an industrialised process. Several suggestions can be made to further improve the enantioselectivity of LipA towards chiral substrates, such as IPG esters.

First of all, consecutive generations of mutants have to be constructed. One could think of several approaches:

- **Site directed mutagenesis:**
  
  During this research, several other mutant libraries were constructed. Unique restriction sites (ApaLI, Eco47III, XhoI) were introduced by silent mutations in the lipA gene allowing the cloning of 4 additional mutant libraries. Spiked oligonucleotides were used to construct these mutant libraries directed towards stretches of 5 amino acids flanking the active site of the LipA. These oligonucleotides resembled the lipA sequence for approximately 80%. Theoretically, this would yield a mutant library which is composed mostly of single and double mutations. We have cloned 4 mutants libraries directed towards amino acids: 11-15; 104-108; 132-136; and 154-158, respectively. After transformation of E. coli TG-1, mutant libraries consisting of approximately 50,000 to 100,000 colonies were obtained. It should be mentioned that this should be more than sufficient to include all possible single and double mutations. Subsequently, E. coli TG-1, containing the mutant libraries, was infected with M13KO7 helperphages to produce phage particles containing the phagemid genome and a fusion of g3p and the mutant enzymes. Bio-panning of these phages on the enantiomeric phosphonate suicide inhibitors (as described in chapter 8) using the dual selection method resulted in the isolation of phages without a lipase insert or with lipase inserts containing multiple deletions and frame shifts. Only a minor percentage of these phages contained an inactive lipase molecule. The most obvious explanation for this phenomenon is found in the localisation of these mutant libraries near the active site of the LipA. Most likely, the applied selection method was too stringent for selection of interesting mutants out of these libraries. In a following attempt, shorter incubation times could be assessed. In addition, these mutant libraries could be cloned in the variant LipAN18I.

- **Saturation mutagenesis:**
  
  Due to the small size of the Bacillus LipA compared to other lipases, mutant libraries can be directed towards each codon of LipA (181 mutant libraries in total). Saturated mutant libraries of each codon in LipA were constructed separately using PCR with NNS (N = A, C, G, or T; S = C or G) oligonucleotides to avoid incorporation of stop codons. Currently, these mutant libraries are cloned in pCANTAB 5E. After transformation of E. coli TG-1, these libraries can be expressed on bacteriophages and, subsequently, dual selection experiments can be performed. This approach might result
Finally, instead of lipases, carboxylesterases may be attractive enzymes for the enantiopure production of chiral pharmaceuticals. A wide range of pharmaceutically interesting chiral substrates and intermediates can be covered, as was shown in chapter 3. The directed evolution of these enzymes may, however, be hampered by the lack of a 3D structure of these enzymes. During this thesis, we initiated experiments for the elucidation of the crystal structure of carboxylesterase NP of *B. subtilis* Thai I-8 and CesA of *B. subtilis* 168 in collaboration with the Department of Biophysical Chemistry of the University of Groningen. Although a wild type carboxylesterase data set was obtained, no heavy atom derivatives could be obtained to solve the phase problem. In another attempt to elucidate the structure, the seleno-methionine labelling of all the methionines present in the carboxylesterase was assessed. The *in vivo* incorporation of the amino acid seleno-methionine during protein biosynthesis at AUG codons has proven to be a successful method to obtain phases for smaller proteins. Although the enzyme was purified from the cytoplasm of *B. subtilis* strain DB430 and the incorporation of seleno-methionine was confirmed by matrix assisted laser desorption/ionisation (MALDI) analysis, no enzyme crystals could be obtained so far. Unfortunately, up till now no 3D structures of homologous esterases are available which excludes structure elucidation by molecular replacement.