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Improved Enantioselectivity of Subtilisin Carlsberg Towards Secondary Alcohols by Protein Engineering

Robin Dorau[a],[b], Tamás Görbe[c] and Maria Svedendahl Humble*[a],[d]

Abstract: Generally, the catalytic activity of subtilisin Carlsberg (SC) for transacylation reactions using secondary alcohols in organic solvent is low. Enzyme immobilization and protein engineering was performed to improve the enantioselectivity of SC towards secondary alcohols. Possible amino acid residues for mutagenesis were found by combining available literature data with molecular modeling. SC variants were created by site-directed mutagenesis and evaluated for a model transacylation reaction containing 1-phenylethanol in THF. Variants showing high *E*-values (>100) were found. However, the conversions were still low. A second mutation was made and both *E*-values and conversions were increased. The most successful variant, G165L/M221F, showed increased conversion (up to 36%), enantioselectivity (*E*-values up to 400), substrate scope and stability in THF, compared to wild-type.

Introduction

Nowadays, the industrial demand of stereospecific syntheses to obtain enantiomerically enriched products using sustainable and environmentally compatible methods is increasing. The ability of enzymes to catalyze selective transformations under mild conditions makes them highly attractive for green chemistry as industrial biocatalysts.[1,2]

By enzyme-catalyzed kinetic resolution (KR), enantiomerically enriched alcohols of high purity can be prepared from alcohol racemates on industrial scales. Enantiomerically enriched alcohols are industrially important synthetic building blocks for synthesis of various pharmaceuticals.[3,4] Normally, the KR is obtained by an enantioselective transacylation reaction using an acyl donor in organic solvent. Then, one alcohol enantiomer is acylated by an acyl donor and the other alcohol enantiomer is enriched. The enantioselectivity of the transacylation reaction can be controlled by using either (*R*)- or (*S*)-selective enzymes, such as (*R*)-selective lipases or (*S*)-selective serine proteases.[4,5] For (*R*)-selective KR reactions, *Candida antarctica* lipase B (CalB, Novozyme 435) is commonly applied. For (*S*)-selective KR reactions, subtilisin Carlsberg (SC) and CalB W104A have been used. The later enzyme, CalB W104A, was created by rational design to show reversed enantioselectivity towards secondary alcohols.[6,7] CalB W104A has been applied to resolve various secondary alcohols.[6-9]

SC is a serine protease naturally secreted from *Bacillus licheniformis*.[10] SC is industrially applied as an ingredient in various detergent products.[11] For synthetic organic chemistry applications, SC has been used to enrich the (*S*)-enantiomer of a racemic alcohol mixture by dynamic kinetic resolution (DKR) systems combining SC and a ruthenium complex in organic solvent.[12-16]

SC and CalB are serine hydrolases, which react by the similar reaction mechanism and contain an oxyanion hole as well as a catalytic triad (Asp-His-Ser). Despite that both enzymes have a different overall fold, their active sites are mirror images.[17] Both enzymes have limited space for one enantiomer of the secondary alcohol, but the mirror-imaged orientation of their catalytic sites favors the opposite enantiomer according to Kazlauskas rule.[18-19] Both lipases and SC react faster as the medium (M) sized group of a secondary alcohol is positioned into a small (stereoselectivity or S_1') pocket and the large (L) group is directed towards the solvent (Figure S1). In SC, the methyl (M) group of 1-phenylethanol is positioned into the S_1'pocket and the phenyl (L) group is directed towards the solvent (Figure 1). Generally, SC shows low enantioselectivity in KR of secondary alcohols and low organic solvent tolerance, compared to CalB.

The activity and enantioselectivity of an enzyme in organic solvent has been shown to depend on its structural flexibility and thus on the preparation technique and the reaction conditions (solvent and temperature).[20-22] Numerous preparatory methods have been shown to affect the enantioselectivity and activity of SC to various extents.[13, 23-27] However, the main bottleneck for efficient application of such stabilized SC preparations is the low stability in organic solvent.[28,29] Various methods (additives, CLEA, surfactants and immobilization) have been explored to improve its activity in organic solvent and at elevated

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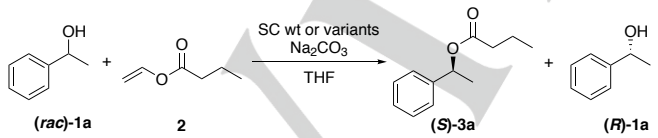
temperatures.[13,16,30-35] Previously, pre-treatments of SC with KCl salt or surfactants (Brij 56 and octyl-β-D-glycopyranoside) prior lyophilization have improved the enzyme performance in terms of activity, operational stability and enantioselectivity in organic solvent.[13,36] It has previously been proposed that the enzyme treatment method applied prior to immobilization is more important for the enzyme activity and enantioselectivity, than the type of immobilization technique.[30]

As an alternative method to the previously mentioned chemical- or reaction condition engineering, protein engineering can be applied to genetically modify the enzyme. SC belongs to the protein family of subtilisins, which has numerous members sharing high sequence identities, structure similarity and catalytic properties.[37,38] The subtilisins served as model proteins for the development of the early protein engineering strategies. As a result, over 50% of the amino acid residues in SC have been subjected to mutagenesis.[37] To date, there are few reports on protein engineering of SC for improved transacylation activity in organic solvent. However, a variant of Subtilisin *Bacillus lentus* (SBL) (M222C) showing both increased stereoselectivity and conversion for the transacylation of 1-phenylethanol using *N*-acetyl-*L*-phenylalanine vinyl ester as acyl donor in acetonitrile, was created by site-directed mutagenesis.[39]

Due to the current lack of efficient (*S*)-selective enzymes for enantioselective transacylation of racemic secondary alcohols in organic solvent, this work aims at improving the enantioselectivity of SC by protein engineering.

Results and Discussion

Initially, the enantioselectivity of SC (AGN35600.1)[40] towards 1-phenylethanol (**(*rac*)-1a**) in THF was explored using a model transacylation reaction (Scheme 1). For this, the enzyme was applied in (a) free form, (b) free and treated with surfactants (Brij 56 and octyl-β-D-glycopyranoside), (c) immobilized on EziG 2 and treated with surfactants and (d) immobilized on Accurel MP1000 and treated with KCl. All enzyme preparations were lyophilized 24 h prior use and all reactions were performed under argon atmosphere.



Scheme 1. The model transacylation reaction used to evaluate the enantioselectivity of SC wild-type (wt) and variants.

The use of (a) free SC in solution for the model reaction resulted in an *E*-value close to 4. Previously, *E*-values of 2-3 have been published.[13] The use of treated enzyme preparations, in free or in immobilized form (b-d), resulted in a tenfold increased *E*-value (40) compared to (a). Importantly, the use of free enzyme in water solution, immobilized enzyme on EziG 2 or Accurel MP1000 without surfactant or KCl treatment, did not result in any transacylation activity for the model reaction. Despite this, all enzyme preparations showed activity in a proteolytic activity assay using a synthetic peptide, *N*-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide.

Since only moderate *E*-values (40) were obtained using the pre-treatment methods (surfactants and KCl) and immobilization techniques (EziG 2 and Accurel MP1000), protein engineering guided by molecular modeling based on literature data was applied.

By molecular modeling, the (*S*)- and (*R*)-configuration of the tetrahedral intermediate of the model transacylation reaction (Scheme 1) were built into a crystal structure of SC (PDB ID: 1YU6[41]. In SC, the methyl group of (*S*)-1-phenylethanol should be directed into the S_1' pocket and the phenyl group should be directed towards the solvent (Figure 1 and S1B).[18-19,42] Theoretically, the large phenyl group should not fit well into the S_1' pocket and therefore the (*R*)-1-phenylethanol should react slower than the (*S*)-1-phenylethanol.

The size of the S_1' pocket is restricted by a conserved methionine (M221). The numbering of the amino acid residues in 1YU6[41] is shifted one residue, starting from amino acid residue 56, compared to the mature protein sequence of SC (AGN35600.1)[40]. Therefore, the amino acid residue M222 in 1YU6[41] corresponds to residue M221 in SC AGN35600.1[40]. The same type of restriction of the alcohol binding pocket is shown in lipases. For example, CalB has a tryptophan (W104) at the similar position. The size of the side chain of the amino acid residue at this position has been shown to affect both the enzymes enantiomeric preference and the enantioselectivity.[6,7,43] Previous mutagenesis studies on subtilisin *Bacillus amyloliquefaciens* (SBA) have shown that the methionine sulfur atom next to the catalytic serine (M222 in SBA) is prone to oxidize. The formation of a sulfoxide at this position results in enzyme inactivation, probably due to destabilization of the transition state intermediate.[44-46] This may be the reason why an inert atmosphere (addition of argon) is required to obtain conversion for the model reaction using SC. Estell *et al.* created 19 variants on position M222 in SBA and evaluated the oxygen resistance. Two variants, M222A and M222S, showed increased resistance towards oxidation.[44] Also, the mutation M222F in subtilisin BPN´ resulted increased peptide ligation activity in organic solvent.[47] Based on these literature data, mutagenesis of SC on the corresponding position (M221) might result in a more stable enzyme. The initial molecular modeling indicated that an exchange of M221 by other amino acid residues might result in a reshaped S_1' pocket.

Initially, five enzyme variants on position M221 (M221A/C/S/F/W) were created by site-directed mutagenesis. All created variants showed proteolytic activity both on skim milk

agar plates and in a proteolytic activity assay. However, the proteolytic activity of M221F and M221W was low. It is important that the enzyme variants retain the natural proteolytic activity. Otherwise, the pro-peptide cannot be cleaved off after secretion and the enzyme will remain inactive.[48,49]

Two variants, M221C and M221F, showed higher *E*-values for the model transacylation reaction (Scheme 1) compared to wild-type (Table 1). Variant M221C showed a synthetically useful *E*-value (>100), followed by M221F (*E* = 67). However, the conversions were still unsatisfying (5%) after 48 h. Variant M221S showed similar *E*-value as the wild-type. No *E*-values could be determined for variants M221A and M221W due to the low conversion (<1%) after 48 h. Possibly, more accurate *E*-values could have been determined if higher conversions were obtained.

With the prospect to improve the conversion of the model reaction, while retaining the high enantioselectivity shown by the M221 variants, further mutation points were searched for. At the bottom of the S_1 pocket, a glycine (G166 in 1YU6[41] and G165 in SC AGN35600.1[40]) is found. Previous mutagenesis studies on the corresponding position in SBA affected both the size of the S_1 pocket as well as the natural peptide amino acid side chain preference.[50] In addition, variants of SC on this position (G165L/I/Y) were previously found in a gene library aiming on improved perhydrolysis activity by increasing the substrate specificity towards methyl esters.[51-52]

Again, the literature data and molecular modeling was used as the base for the design of one variant on position G165. The SC G165L variant was created by site-directed mutagenesis and was after enzyme preparations tested for the model transacylation reaction (Table 1). SC G165L showed a slightly higher *E*-value (46) and conversion (8%) compared to wild-type enzyme.

Then, the G165L mutation was combined with the M221C/F/S variants and further evaluated for the model reaction (Table 1). The double variants showed good *E*-values (>100). Variant G165L/M221F showed the highest conversion (20%) after 48 h.

Table 1. Enantioselectivities[a] (*E*-values) of immobilized[b] SC wild-type and variants for transacylation of **(*rac*)-1a** and vinyl butyrate in THF measured after 48 h.[c]

SC variant	ee_S [%]	ee_P [%]	Conv. [%]	*E* (*S*)
Wild-type	3	95	3	39
M221A	0	87	0.2	n.d.[d]
M221C	6	99	5	>100
M221F	5	97	5	67
M221S	3	98	3	41
M221W	0	86	0.1	n.d.[c]
G165L	8	95	8	46
G165L/M221C	18	98	16	>100
G165L/M221F	25	98	20	>100
G165L/M221S	6	99	6	>100

[a] The enantiomeric ratio (E) was calculated using Equation 2.

[b] The enzymes were immobilized on EziG 2 material and co-lyophilized with surfactants.

[c] Reaction conditions (1 mL): Immobilized SC on EziG 2 (10 mg), Na_2CO_3 (53 mg), vinyl butyrate **2** (75 mM), 1-phenylethanol **(*rac*)-1a** (50 M), dodecane (100 mM) in THF at 25 °C. The reactions were flushed with argon and analyzed by chiral GC.

[d] Not determined due to low conversion (<1%).

The substrate specificity of the M221C and G165L/M221C/F/S variants for (*S*)- and (*R*)-1-phenylethanol was determined and compared to the wild-type enzyme (Table 2). In order to increase the *E*-value towards (*S*)-1-phenylethanol (**(*S*)-1a**), the substrate specificity (k_{cat}/K_M) towards the **(*S*)-1a** should be increased, while the corresponding value for the (*R*)-1-phenylethanol **(*R*)-1a** should be decreased. Both SC M221C and G165L/M221F showed increased substrate specificity towards **(*S*)-1a** and reduced substrate specificity for **(*R*)-1a**. Both SC G165L/M221C and SC G165L/M221S showed higher substrate specificities for both **(*S*)-** and **(*R*)-1a**. The G165L/M221F variant showed the highest *E*-value of 160.

Table 2. Kinetic parameters[a] (k_{cat}/K_M) determined for immobilized[b] SC wild-type and variants (M221C, G165L/M221C, G165L/M221S and G165L/M221F) for transacylation[c] of (*S*)- and (*R*)-1a, separately, in THF at 25 °C.

SC variant	$(k_{cat}/K_M)_R$ [s^{-1}]	$(k_{cat}/K_M)_S$ [s^{-1}]	*E* (*S*)	$(K_M)_R$ [M]	$(K_M)_S$ [M]
Wild-type	0.064	2.5	39	0.41	0.25
M221C	0.042	5.9	140	2.1	0.38
G165L/M221C	0.11	15	140	3.4	0.51
G165L/M221F	0.047	7.7	160	>6.8	0.64
G165L/M221S	0.22	13	57	6.8	1.4

[a] Enzyme kinetics was performed under pseudo-one-substrate conditions. The concentration of vinyl butyrate **2** was kept constant at 200 mM. Separate reactions were performed for (*S*) and (*R*)-1a.

[b] The enzymes were immobilized on EziG 2 material and co-lyophilized with surfactants.

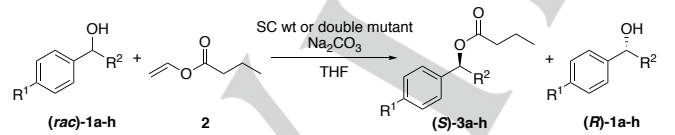
[c] Reaction conditions (1 mL): Immobilized SC on EziG 2 (10 mg), Na_2CO_3 (53 mg), vinyl butyrate **2** (200 mM), 1-phenylethanol **(*rac*)-1a** (0.1-1.0 M), dodecane (100 mM) in THF at 25 °C. The reactions were flushed with argon and monitored by chiral GC.

The enantioselectivity of SC G165L/M221F was explored for different secondary alcohols in comparison to SC wild-type (Table 3). Within the functionalization of the para-position (R^1), the electronic effects of the substituents were explored. The 1-(4-methylphenyl)ethan-1-ol **(*rac*)-1b** worked well with SC, G165L/M221F. However, the enantioselectivity was a bit lower 43 (Table 3, entry b). The electron donating 1-(4-

methoxyphenyl)ethan-1-ol **(*rac*)-1d** gave an *E*-value of 139, which is a huge increase compared to the wild type enzyme, which provides very low activity towards this specific substrate (Table 3, entry d). SC G165L/M221F showed a high *E*-value (110) for 1-(4-chlorophenyl)ethan-1-ol **(*rac*)-1c**. The corresponding value for the SC wild-type for the same substrate was 41 (Table 3, entry c). The aliphatic part of the model substrate **(*rac*)-1a** was also substituted with longer alkyl chains (R^2-groups) (Table 3): 1-phenylpropan-1-ol **(*rac*)-1e** (entry e), 1-phenylbutan-1-ol **(*rac*)-1f** (entry f), 1-phenylpentan-1-ol **(*rac*)-1g** (entry g) and 1-phenylheptan-1-ol **(*rac*)-1h** (entry h). SC G165L/M221F showed high *E*-values (>200) with great enantiomeric excess (99%) for all substrates with longer alkyl chains. However, the conversions were still low.

The alcohols, **(*S*)-1a** and **(*S*)-1e-h**, were modelled into a model of the G165L/M221F variant based on 1YU6[41]. According to the modeling, the new variant has opened up a new cavity in the S_1' pocket that harbors longer alkyl chains of secondary alcohols (Figure 2). In the wild-type enzyme (1YU6[41]), the side chain of M221 is directed towards the catalytic S220. In the model of G165L/M221F, the amino acid side chain of F221 is positioned in the opposite direction compared to the position of the native methionine side chain. Consequently, a new cavity is created in the S1′ pocket of G165L/M221F due to the M221F mutation. All modeled secondary alcohols (Table 3 a, e-h and Figure 2) were able to fit the alkyl group (methyl to heptyl) into the S_1' pocket of the G165L/M221F variant while retaining the required hydrogen bonding network. This was not possible in the wild-type model, which can only fit a methyl group in its S_1' pocket (Figure 1). The amino acid residue G165 (or L165) is located in the bottom of the S_1 pocket (Figure S4 and S5). In the wild-type model, the alkyl chain of vinyl butyrate does not fill up the space in the S_1 pocket. In order to reduce this pocket and to improve the binding of the butyl chain, amino acid residue G165 was substituted by a leucine. According to the model, the G165L mutation restricts the S_1 cavity (Figure S4 and S5).

Table 3. Enantioselectivity (*E*) and conversion [%] of immobilized[a] SC wild-type and G165L/M221F for transacylation of (*rac*)-secondary alcohols **(*rac*)-1a-h** and vinyl butyrate in THF measured after 48 h.[b]



(*rac*)-1a-h 2 (*S*)-3a-h (*R*)-1a-h

Entry[b]	R^1	R^2	Wild-type[a]	G165L/M221F[a]
a	-H	$-CH_3$	44, 3 %	120, 36%
b	$-CH_3$	$-CH_3$	7, <1%	43, 31%
c	-Cl	$-CH_3$	41, 6%	110, 36%
d	-OMe	$-CH_3$	2, <1%	140, 26%
e	-H	$-C_2H_5$	0, 0%	210, 6%
f	-H	$-C_3H_7$	0, 0%	220, 8%
g	-H	$-C_4H_9$	0, 0%	210, 6%
h	-H	$-C_7H_{11}$	0, 0%	210, 7%

[a] The enzymes were immobilized on Accurel MP1000 in phosphate buffer (0.1 M, pH 7.8) containing 1 M KCl.
[b] Reaction conditions (1 mL): Immobilized SC on Accurel MP1000 (10 mg), Na_2CO_3 (53 mg), vinyl butyrate **2** (750 mM), secondary alcohol **(*rac*)-1a-h** (500 M), dodecane (100 mM) in THF at 23 °C.[13] The reactions were flushed with argon and monitored by chiral GC.

Finally, the enantioselectivity of SC wild-type and G165L/M221F for the model reaction was explored at different reaction temperatures (Table 4). In contrast to the wild-type enzyme, the enantioselectivity of G165L/M221F variant was shown to be significantly affected by the temperature. The highest *E*-value (400) was determined for the G165L/M221F variant at 7 °C. The corresponding value for SC wild-type at 7 °C was 40. The *E*-value shown by the G165L/M221F variant at 23 °C was lower than previously measured in the kinetic study using the two alcohol enantiomers in separate reaction vials.

Table 4. Enantioselectivities (*E*-values) and conversion [%] of immobilized[a] SC wild-type and G165L/M221F for transacylation[b] of (*rac*)-1-phenylethanol and vinyl butyrate in THF measured after 120 h.

Temperature [°C]	Wild-type	G165L/M221F
7	40, 3%	400, 11%[c]
50	21, 3%	69, 20%

[a] The enzymes were immobilized on Accurel MP1000 in phosphate buffer (0.1 M, pH 7.8) containing 1 M KCl.
[b] Reaction conditions (1 mL): Immobilized SC on Accurel MP1000 (10 mg), Na_2CO_3 (53 mg), vinyl butyrate (750 mM), 1-phenylethanol (500 M), dodecane (100 mM) in THF.[13] The reactions were flushed with argon and monitored by chiral GC.
[c] Measured after 43 h.

Finally, the stability of the most successful variant SC G165L/M221F in THF was compared to wild-type (Figure 3). Purified enzyme solutions (1 mg/mL) were prepared in sodium phosphate buffer and diluted in THF. The remaining relative activity of SC wild-type and G165L/M221F during storage in THF was measured using a proteolytic activity assay. The wild-type enzyme lost 30% activity after 15 minutes of incubation and after 1 h <1% activity remained. Variant G165L/M221F remained 50% of the initial activity after 1 h of storage in THF. In order to evaluate the stability of each mutation in G165L/M221F, the single variants G165L and M221F were also explored. The three SC variants were found to be more stable than the wild-type enzyme in THF. The stability of the double variant was shown to depend on the M221F mutation.

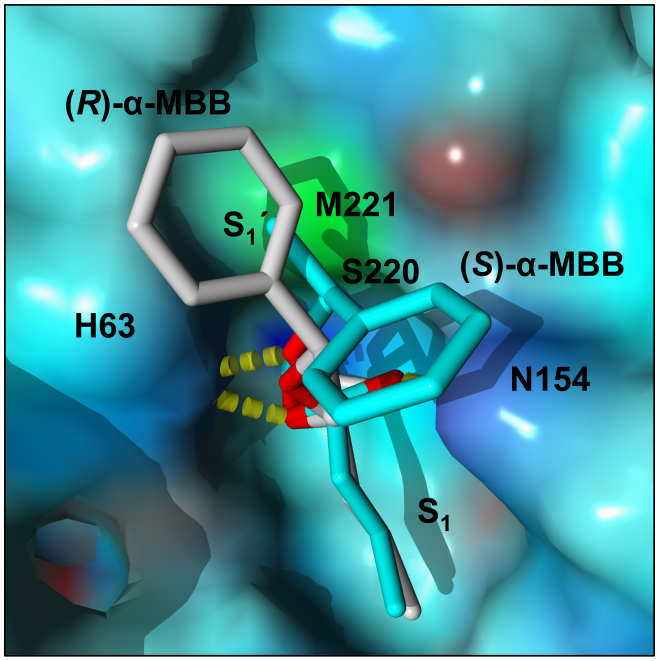


Figure 1. The tetrahedral intermediates of the model reaction (Scheme 1) built into SC wild-type (PDB ID: 1YU6[41]). The enzyme structure is shown in surface mode in element colors. The (*S*)-α-methylbenzyl butyrate ((*S*)-α-MBB) is shown in cyan and the (*R*)-α-methylbenzyl butyrate ((*R*)-α-MBB) is shown grey. The catalytic important amino acid residues (H63 and S220) and the amino acid residues that build up the oxyanion hole (N154 and S220) are labeled. The amino acid residue M221 is labeled as well as the S_1 (acyl) and the S_1´ (alcohol) binding pockets. The alkyl chain of the acyl donor, vinyl butyrate, is coordinated into the S_1 binding pocket. The alkyl chain of the secondary alcohol is directed into the S_1´ binding pocket and the phenyl group is directed towards the solvent. G165 is located in the bottom of the S_1 pocket and is displayed in Figure S4. Molecular graphics created with YASARA[53,54] and PovRay[55].

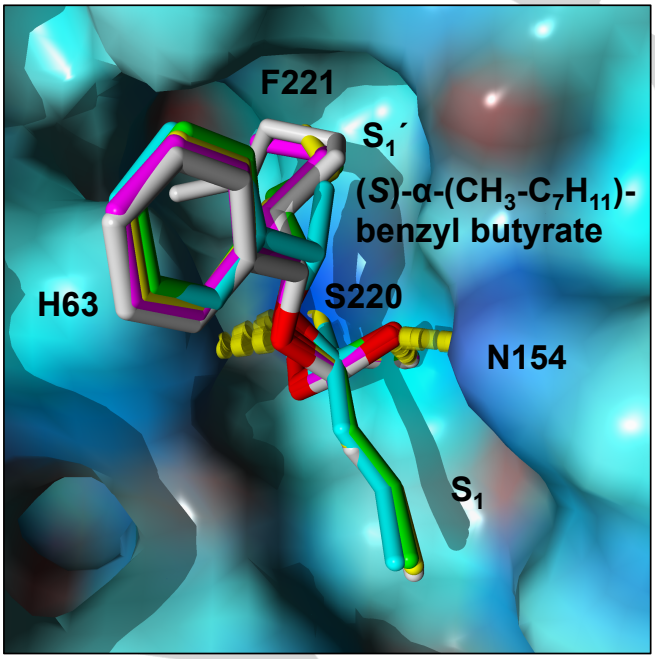


Figure 2. The tetrahedral intermediates of (*S*)-α-MBB (cyan), (*S*)-α-ethylbenzyl butyrate (green), (*S*)-α-propylbenzyl butyrate (yellow), (*S*)-α-butylbenzyl butyrate (magenta) and (*S*)-α-hexylbenzyl butyrate (grey) are coordinated into the active site of SC G165L/M221F. The alkyl chains of the secondary alcohol are coordinated into the S_1´ binding pocket and the phenyl group of the alcohol is directed towards the solvent. The alkyl chain of the acyl donor, vinyl butyrate, is coordinated into the S_1 binding pocket. L165 is located in the bottom of the S_1 pocket and is displayed in Figure S5. The enzyme structure is shown in surface mode in element colors. Molecular graphics created with YASARA[53,54] and PovRay[55].

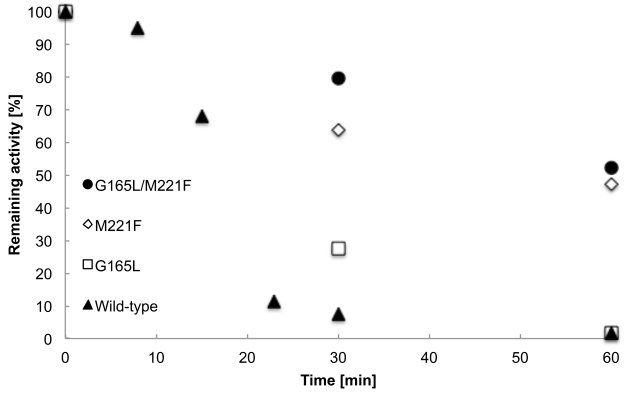


Figure 3. Remaining relative activity of SC wild-type, G165L, M221F and G165L/M221F during storage in THF by a proteolytic activity assay containing *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide in phosphate buffer (0.1 M, pH 7.8). The residual activity is expressed as a percent of an enzyme control.

Conclusions

As previously been concluded by others[30], the enantioselectivity of SC was more dependent on the treatment of the enzyme prior to immobilization, than the type of immobilization technique. However, immobilization of SC to Accurel MP1000 in phosphate buffer (0.1 M, pH 7.8) supplemented with 1 M KCl resulted in efficient immobilization (5% enzyme was remaining in solution after 90 min) (Figure S6). In addition, the lyophilized enzyme preparations were stable for several months when stored at room temperature in a desiccator saturated with LiCl to provide a low water activity. A new SC variant, G165L/M221F, was created by site-directed mutagenesis. This variant showed *E*-values >100 towards (*S*)-1a in the model reaction. By enzyme kinetics, the G165L/M221F mutations were shown to increase the enzyme substrate specificity towards the desired **(*S*)-1a**, while the undesired (*R*)-enantiomer was reduced. In contrast to the wild-type enzyme, the enantioselectivity of G165L/M221F was shown to be significantly temperature dependent. The highest *E*-value was obtained at 7 °C for the model reaction.

The M221F mutation opened up new space in the S_1´pocket as well as improved the enzyme stability in THF. The G165L/M221F variant showed *E*-values >200 for secondary alcohols (**(*S*)-1e-h**) harboring longer alkyl chains (C_2H_5 to C_7H_{11})

than a methyl group. These substrates, with propyl to heptyl groups, were not possible to convert using the wild-type enzyme.

Experimental Section

Materials

All commercially available chemicals were purchased from Sigma-Aldrich and used without further purification. Other chemicals (alcohols and esters) for KR were synthesized as described in section "Synthesis of substrates and products".

The *B. subtilis* WB600 cells and the gene AGN35600.1[40] encoding for subtilisin Carlsberg AprE [*Bacillus paralicheniformis* ATCC 9945a] inserted in a commercial *E. coli-Bacillus* shuttle vector pHY300PLK (Takara Bio, Inc., Shiga, Japan) together with its promoter and pre-pro-sequence[51] were kindly provided by the research group of Prof. Dr. Ulrich Schwaneberg (RWTH Aachen University in Germany).

All primers (for his-tag insertion and site-directed mutagenesis) are found in the list of primers in the Supplementary Information. The primers were purchased from Eurofins Genomics. Also, Eurofins Genomics performed DNA sequencing.
For PCR product purification and DNA plasmid extraction, a GeneJET PCR Purification Kit (K0701) and a GeneJET Plasmid MiniPrep Kit (K0502) from Thermo Fisher Scientific were used.

Molecular modeling

Molecular modeling was performed using the software YASARA (Version 16.4.16)[53,54] and the crystal structure with PDB ID: 1YU6[41] obtained from the RCSB Protein Data Bank. All molecular numbers mentioned in this section is according to the pdb file.
Initially, the molecules A, C and D in 1YU6[41] were deleted. The remaining object (Mol B) was cleaned. The hydrogen atom (HG) on S221 OG was deleted and added on H64 NE2 as HE2. A simulation cell of 5 Å around all atoms was set.
To remove bumps and correct the covalent geometry, the structure was energy-minimized with the Amber99 force field[56], using an 8.0 Å force cutoff and the Particle Mesh Ewald algorithm[57 to treat long range electrostatic interactions. After removal of conformational stress by a short steepest descent minimization, the procedure continued by simulated annealing (timestep 2 fs, atom velocities scaled down by 0.9 every 10th step) until convergence was reached, i.e. the energy improved by less than 0.05 kJ/mol per atom during 200 steps.
The tetrahedral intermediates of (*R*)- and (*S*)-α-MBB were stepwise built into the structure from S221 OG. Energy minimizations (using the protocol above) were performed on the tetrahedral intermediate structures of (*R*)- and (*S*)-α-MBB. During the energy minimizations, the protein backbone remained fixed and the S221 residue remained free. As a final step, energy minimizations were performed on free structures.
Mutations were made to the two wild-type models containing either (*R*)- or (*S*)-α-MBB (Figure S2 and S3). The selected amino acid residues for amino acid exchange were swapped into other amino acid residues. After energy minimization (using the protocol above), the following was measured: (i) Hydrogen bond distances between; S221 OG and H64 HE2 or S221 O6 (from alcohol) and H64 HE2; (ii) Angles between; S221 OG, H64 HE2 and H64 NE2 or S221 O6 (from alcohol), H64 HE2 and H64 NE2. The atom numbering (according to the mature protein) is shown in Figure S2 and the obtained data is shown in Table S1.
In order to explore the acceptance of SC G165L/M221F towards 1-phenyl alcohols with longer alkyl chains than methyl, the previous model containing (*S*)-α-MBB was modified from methyl to propyl, butyl, pentyl and heptyl (C_2H_5 to C_7H_{11}) and saved as separate files. All files were energy minimized (using the protocol above). The obtained models were structurally aligned and compared (Figure 2) using Mustang[58].

H_6-tag insertion

The gene of SC was provided in the pHY300PLK shuttle vector.[51] A Histidine-tag (H_6-tag) was inserted at the C-terminus of the SC AGN35600.1[40] gene. The primers for the H_6-tag insertion were designed to partially overlap in the area of the insert. The PCR product was purified, the pHY300SC template was removed by *DpnI* (FD1704, Thermo Fisher Scientific) treatment (37 °C, over night) and the digested PCR-product was repurified. The obtained PCR construct (pHY300SCH_6) was sequenced after transformation and stored at -20 °C.

Site-directed mutagenesis

Point mutations were introduced in pHY300SCH_6 by site-directed mutagenesis using a two-stage PCR method.[59,60] Starting with two PCR reactions (50 µl), each containing either the forward or the reverse primer (0.5 µM), single stranded mutated fragments of the gene were created in separate tubes.
PCR reaction, first step: 98 °C for 30 s, one cycle; 98 °C for 10 s, 55 °C for 30 s, 72 °C for 3 min, 3 cycles. The reactions were then combined in one tube (100 µL). Second step: (98 °C for 30 sec, one cycle; 98 °C for 10 sec, 55 °C for 30 sec, 72 °C for 3 min, 15 cycles; 72 °C for 5 min, one cycle).
The obtained PCR products were purified, digested with *DpnI* and re-purified. The obtained PCR constructs were stored at -20 °C.

Transformation

The mutated DNA plasmids were transformed into *E. coli* DH5α by electroporation (2.5 kV, 200 Ω, 25 µF). Aliquots of *E. coli* DH5α (40 µl) were thawed on ice (30 min) prior to addition of purified DNA plasmid (1 µl, 50 ng/µl). After the electroporation, SOC-medium (0.5 mL) was immediately added to the cells. The transformation solution was incubated for 2 h at 200 rpm and 37 °C. Then, the transformation solution was spread out on LB-Amp agar plates and incubated at 37 °C overnight.

Then, one colony of *E. coli* DH5α containing pHY300SCH$_6$ (wild-type or mutant) was cultivated in LB-Amp medium overnight at 37 °C and at 200 rpm. The plasmid was isolated and purified. The wild-type and the mutations were confirmed by sequencing.

After sequencing, the pHY300SCH$_6$ (wild-type and mutants) were transformed to *B. subtilis* WB600 for protein expression. The electroporation of *B. subtilis* WB600 was performed according to Lu *et al*.[61] Aliquots of electrocompetent *B. subtilis* WB600 were thawed on ice for 30 min before addition of plasmid DNA (1 µl, 50 ng/µl). Subsequently, the cells were transferred to the electroporation cuvette, incubated on ice for additional 5-10 min and subjected to electroporation (2.4 kV, 200 Ω, 25 µF). LB medium (1 mL) supplemented with D-sorbitol (0.5 M), and D-mannitol (0.38 M) was immediately added to each cuvette. The transformation solution transferred to 15 mL Falcon tubes before incubation for 3-6 h at 37 °C and 220 rpm. Then, the transformation solution was spread on LB-Tet-Milk agar plates containing 15 µg/mL tetracycline and 1.5 % skim-milk in LB agar.

Protein expression

The *E. coli-Bacillus* shuttle vector pHY300PLK allows protein expression in *B. subtilis*.[62] The pHY300SCH$_6$ (wild-type or variants) were produced in *B. subtilis* WB600, which continuously secrets the expressed enzyme into the cell culture supernatant. One colony of *B. subtilis* WB600 containing pHY300SCH$_6$ (wild-type or variants) was grown in LB medium (4 mL) containing 15 ug/mL tetracycline overnight at 37 °C and 180 rpm in 50 mL Falcon tubes. A fraction of the overnight culture (0.5-1 mL) was used to inoculate shake flask cultures (200-250 mL). The protein expression was performed in unbaffled glass shake flasks (2 L) using 1.5x LB medium (tryptone 15 g/L, NaCl 7.5 g/L, yeast extract 7.5 g/L) supplemented with tetracycline (15 µg/mL) during 60-70 h at 37 °C at 180 rpm. Then, the supernatant was separated from the cells by centrifugation (JA-10, 8500 rpm, 30 min, 4 °C). The supernatant, containing the secreted SC, was collected and sterile filtered (0.45 and 0.2 µm). The total protein amount was determined by Bradford[63] using Bio-Rad Protein assay solution (Bio-Rad, #5000001). The pH of the protein solution was set to 7.8 prior to further application or storing at 4 °C.

Protein purification

The supernatant solutions containing his-tagged SC wild-type or variant enzymes were manually purified on a HisTrap™ HP column (17-5247-01, GE Healthcare). The column was pre-washed with a sodium phosphate buffer (20 mM, pH 7.8) containing sodium chloride (500 mM) and the protein was eluted with a sodium phosphate buffer (20 mM, pH 7.8) containing sodium chloride (500 mM) and imidazole (500 mM). The collected protein solutions were concentrated and the buffer was exchanged to sodium phosphate buffer (100 mM, pH 7.8) using Amicon Ultra (15 mL) centrifugal filters with a molecular weight cut-off of 10,000 (Merck). The amount of purified protein was measured on NanoDrop™ 1000 Spectrophotometer at 280 nm. The purified enzyme solutions were stored at 4 ℃ prior further use.

Immobilization on EziG and co-lyophilization with surfactants

Immobilization of SC on hydrophobic EziG 2 material was made using sterile filtered (0.45 and 0.2 µm) cell culture supernatant in order to obtain simultaneous protein purification and immobilization.[64] The EziG 2 material was added in 4-fold excess, compared to the amount of protein in solution. For SC wild-type, about 300 mg EziG 2 per L cell culture supernatant was applied. The amount of protein was measured by Bradford[63]. The immobilization solution was incubated at 30 °C and 200 rpm for 1 h in 0.5 mL media bottle. Then, the carrier material was collected by centrifugation at 750 x g for 2 min, washed twice with sodium phosphate buffer (0.1 M, pH 7.8), washed twice with surfactant solution (Octyl β-D-glucopyranoside (2.5 mg/ml) and Brij™ 58 (2.5 mg/ml) dissolved (37 °C, 220 rpm) in sodium phosphate buffer (0.1 M, pH 7.8) according to Borén *et al*.[13], pelleted by centrifugation at 1000 x g for 1 min, and finally, the supernatant was removed. The immobilized enzyme preparation on EziG 2 was rapidly frozen in liquid nitrogen and lyophilized overnight.

Immobilization on Accurel MP1000

Accurel MP1000 (particle size <1500 m) was pre-wetted in ethanol (95%). Thereafter, the beads were washed 3-fold with sodium phosphate buffer (100 mM, pH 7.8). Purified enzyme solution (~10 mg) was diluted to in sodium phosphate buffer (100 mM, pH 7.8) containing KCl (1 M). The diluted enzyme solution (10 mL, 1 mg/mL) was added to wet Accurel MP1000 (1 g) in a glass vial (20 mL). The immobilization solution was incubated at room temperature on an end-over-end rotation wheel for 1-2 h. The enzyme activity was measured before, during and after the immobilization using a proteolytic activity assay (SI Figure S6). As no proteolytic activity could be detected (1-2 h) in solution, the liquid solution was discarded by pipetting. The immobilized enzyme preparation was lyophilized overnight, without previous freezing.

Storage of immobilized enzyme preparations

The immobilized and lyophilized enzyme preparations were stored at 23 °C (room temperature) in a desiccator containing a saturated water solution of LiCl.

Transacylation reactions using SC immobilized on EziG

The enantioselectivity of the obtained immobilized SC and variants on EziG 2 were explored for the model reaction (Scheme 1) using: 50 mM (*rac*)-1a, 75 mM vinyl butyrate, 25 mM dodecane in THF. The THF was dried over activated molecular sieves (3 Å) for at least 12 h prior use. To the reaction solution (1 mL), both enzyme prepared on EziG 2 (10 mg/mL), dry Na_2CO_3 (53 mg/mL) was added. In order to avoid air exposure, argon gas was added to the reaction tubes. Spontaneous

transacylation reactions without enzyme were not observed. The reaction mixture was incubated at 30 °C and 220 rpm in an incubator shaker. Reaction samples (50 µL) were taken to GC vials containing cyclohexane, after 0 h, 2 h, 4 h, 24 h and 48 h.

The kinetic parameters for SC wild-type and SC variants were measured using the **(*R*)-** and **(*S*)-1a** in separate reactions tubes in a pseudo-one-substrate approach. Therefore, vinyl butyrate was used at a constant concentration of 200 mM[20,28,33,42] and dodecane (100 mM) was added as an internal standard. The concentration of 1-phenylethanol was varied from 10 mM to 1000 mM (10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 75 mM, 100 mM, 200 mM, 500 mM, 1000 mM). The reaction mixtures were incubated at 30 °C and shaking at 220 rpm in an incubator shaker. Reaction samples (50 µl) were taken at 0 h, 2 h, 4 h, 6 h, 24 h and 48 h to cyclohexane prior to GC analysis.

Transacylation reactions using SC immobilized on Accurel MP1000

Due to a low stability of SC on EziG, further reactions were made with enzyme immobilized on Accurel MP1000. In addition, the substrate concentrations were increased (1 mL): 500 mM racemic alcohol, 750 mM vinyl butyrate, 100 mM dodecane in dry THF[13].

GC analysis

All collected reaction samples were analyzed by gas chromatography (GC) using a chiral column (CP-ChiraSil-DEX CB 25 m x 0.32 mm x 0.25 µm) without prior storage. For the model reaction (Scheme 1) the following GC-method was used: 90 °C hold 4 min, 5 °C/min to 150 °C, 70°C/min until 200 °C and 200 °C hold 2 min.

A calibration curve was prepared in THF using *rac*-α-MBB in different concentrations: 1 mM, 5 mM, 10 mM, 25 mM, 50 mM, 75 mM and 100 mM, while keeping the internal standard (dodecane) constant at 100 mM. The ratio between the concentration of one enantiomer and dodecane was used to calculate the concentration (mM) of the obtained products.

Calculation of enantiomeric ratio and reaction yield

The enantiomeric ratio (*E*-value) was calculated using two different methods. As racemic secondary alcohols were applied in the reaction solution, equation (1) was used for calculation of the *E*-values.[65] As the two enantiomers were applied in separate reaction solutions, equation (2) was applied for the calculation of the *E*-values.[66] The conversion of the reaction was calculated according to equation (3).[66]

Equation 1:

$$E = \frac{\ln\frac{1 - ee_s}{(1 + ee_s)/ee_p}}{\ln\frac{1 + ee_s}{(1 + ee_s)/ee_p}}$$

Equation 2:

$$E = \frac{kcat/KM\ (S)}{kcat/KM\ (R)} = \frac{v(S)}{v(R)}$$

Equation 3:

$$\text{Conversion} = \frac{ee_s}{ee_s + ee_p}$$

Synthesis of substrates and products

Substrates and products, that were not commercially available, were synthesized according to literature procedures.

Procedure A: The secondary alcohols, **(*rac*)-1f-h**, were synthesized from the corresponding ketones. The ketone (27 mmol) was solved in MeOH (50 mL) and NaOH was added to get the solution basic. The mixture was cooled down to 0 °C and 1.6 equivalents of $NaBH_4$ was added in small portions. The solution was stirred overnight in room temperature. The reaction was diluted with 50 mL water and acidified with concentrated HCl solution. The MeOH was evaporated under vacuum and the water solution was extracted with EtOAc. The organic phase was washed with brine solution, separated, dried with anhydrous Na_2SO_4. The solvent was evaporated. The product was analyzed by NMR spectroscopy. The yield was quantitative in each case and further purification was not necessary.

1-phenylbutan-1-ol ((*rac*)-1f): The synthesis followed Procedure A. The product appeared as a colorless oil with the mass of 3.77 g (93% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] 7.37-7.26 (m, 5H) 4.70-4.66 (m, 1H), 1.85-1.64 (m, 3H), 1.51-1.25 (m, 2H), 0.94 (t, 3H, *J* = 7.3 Hz), ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] 144.9, 128.4, 127.5, 125.9, 74.4, 41.3, 19.0, 14.0.

1-phenylpentan-1-ol ((*rac*)-1g): The synthesis followed Procedure A. The product appeared as a colorless oil with the mass of 4.26 g (96% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] 7.37-7.27 (m, 5H) 4.69-4.65 (m, 1H), 1.85-1.68 (m, 3H), 1.45-1.23 (m, 4H), 0.90 (t, 3H, *J* = 7.1 Hz), ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] 145.1, 128.6, 127.6, 126.0, 74.9, 39.0, 28.1, 22.8, 14.2.

1-phenylheptan-1-ol ((*rac*)-1h): The synthesis followed Procedure A. The product appeared as a colorless oil with the mass of 4.78 g (92% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] 7.36-7.27 (m, 5H) 4.68-4.65 (m, 1H), 1.85-1.66 (m, 3H), 1.45-1.25 (m, 8H), 0.87 (t, 3H, *J* = 6.8 Hz), ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] 145.1, 128.6, 127.6, 126.0, 74.9, 39.3, 31.9, 29.3, 26.0, 22.8, 14.2.

Procedure B: To be able to identify the enantioselectivity (*ee*) of the products, the (rac)-1-phenylethyl butyrate derivatives **(*rac*)-3a-h** were synthesized. The alcohol substrate **(*rac*)-1a-h** (1 mmol) was solved in CH_2Cl_2 (3 mL). 3.4 equivalents of butyric anhydride and 0.25 equivalents of DMAP were added to the solution and stirred at room temperature overnight. The reaction mixture was diluted with CH_2Cl_2 and washed with water four times. The organic phase was separated and dried with anhydrous Na_2SO_4. The solvent was evaporated. The product was purified by column chromatography with Al_2O_3 to be able to remove the acid residues and it was analyzed by NMR spectroscopy.

1-phenylbutyl butyrate ((*rac*)-3f): The synthesis followed Procedure B. The product appeared as a colorless oil with the mass of 212 mg (96% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] 7.34-7.26 (m, 5H), 5.77-5.74 (m, 1H), 2.35-2.26 (m, 2H), 1.94-1.85 (m, 1H), 1.78-1.61 (m, 3H), 1.41-

1.22 (m, 2H), 0.94-0.90 (m, 6H), ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] 173.1, 141.2, 128.5, 127.9, 126.6, 75.7, 38.7, 36.7, 18.9, 18.6, 14.0, 13.8.

1-phenylpentyl butyrate ((*rac*)-3g): The synthesis followed Procedure B. The product appeared as a colorless oil with the mass of 220 mg (94% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] 7.36-7.27 (m, 5H) 5.76-5.72 (m, 1H), 2.33-2.29 (m, 2H), 1.95-1.86 (m, 1H), 1.81-1.73 (m, 1H), 1.70-1.61 (m, 2H), 1.34-1.20 (m, 4H), 0.92 (t, 3H, *J* = 7.5 Hz), 0.87 (t, 3H, *J* = 7.1 Hz), ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] 173.1, 141.2, 128.5, 127.9, 126.6, 76.0, 36.7, 36.3, 27.8, 22.6, 18.6, 14.1, 13.8.

1-phenylheptyl butyrate ((*rac*)-3h): The synthesis followed Procedure B. The product appeared as a colorless oil with the mass of 234 mg (89% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] 7.35-7.27 (m, 5H) 5.75-5.72 (m, 1H), 2.36-2.26 (m, 2H), 1.94-1.85 (m, 1H), 1.80-1.61 (m, 3H), 1.32-1.22 (m, 8H), 0.92 (t, 3H, *J* = 7.4 Hz), 0.86 (t, 3H, *J* = 6.9 Hz), ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] 173.1, 141.2, 128.5, 127.8, 126.6, 76.0, 36.7, 36.6, 31.8, 29.1, 25.6, 22.7, 18.6, 14.2, 13.8.

Stability measurement

SC wild-type and variants were purified and diluted (1 mg/mL) in sodium phosphate buffer (0.1 M, pH 7.8). A tenfold dilution of the enzyme solution was made in THF in glass vials. Samples were taken during 60 min and immediately analyzed for remaining activity using a proteolytic activity assay.

Proteolytic activity assay

A proteolytic activity assay based on hydrolysis of a synthetic peptide *N*-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (S7388 Sigma) was applied in order measure the proteolytic activity of SC wild-type, different SC variants, batches and immobilization. The proteolytic activity was monitored after all working steps and during the immobilization (on Accurel MP1000). Aliquots of the synthetic peptide was diluted to 20 mM in DMSO and stored at -20 °C. Reactions (1 mL) containing; sodium phosphate buffer (990 µl, 0.1 M, pH 7.8), synthetic peptide (5 µl, 20 mM) and enzyme solution (5 µl), were prepared. The initial hydrolysis reaction rates were measured at 410 nm on a Varian Cary® 50 UV-Vis spectrophotometer. A standard curve of *p*-nitroanilide (31569 Sigma-Aldrich) was prepared (0.02-0.14 mM).

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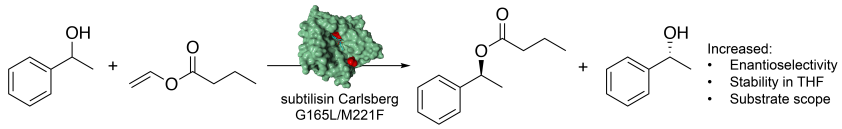

The performance of the serine protease subtilisin Carlsberg (SC) was improved towards transacylation reactions using secondary alcohols in THF. By enzyme immobilization, the enantioselectivity of SC was increased tenfold. By further protein engineering, an enzyme variant (G165L/M221F) showing increased; conversion, enantioselectivity (*E* >100), substrate scope and stability in THF, was created.

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