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TETRAHEDRON: *ASYMMETRY*

Chemically modified mutants of subtilisin *Bacillus lentus* catalyze transesterification reactions better than wild type

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Abstract

A combined site-directed mutagenesis and chemical modification strategy has been used to create superior enzyme catalysts for the resolution of racemic primary and secondary alcohols using a transesterification reaction. The chemically modified mutant, N62C–S–CH_3, of subtilisin *Bacillus lentus* catalyzes the transesterification of *N*-acetyl-L-phenylalanine vinyl ester with β-branched primary alcohols faster than wild type. The cysteine mutant, M222C, of subtilisin *Bacillus lentus* gives higher yields (98% and 92% yields with 1-phenylethanol and 2-octanol, respectively, versus 19% and 10% for wild type) and better enantioselectivity than wild type when secondary alcohols are used.

Hydrolase-catalyzed transesterifications are widely employed to resolve racemic alcohols and to stereoselectively acylate prochiral and meso diols.[1] In this regard, serine proteases have found limited application in comparison to lipases and esterases.[1] One reason for this is the high substrate-specificity of many serine proteases compared with other hydrolases.[1,2] Recently, in an effort to extend the synthetic potential of the serine protease, subtilisin *Bacillus lentus* (SBL), we reported the use of *N*-Ac-L-Phe vinyl ester, **2**, as an acyl donor in SBL-catalyzed transesterification reactions with racemic alcohols.[3] This paper illustrates the potential for improving the overall chemical yield and degree of stereoselectivity for these resolutions, using a combined site-directed mutagenesis and chemical modification strategy to alter the substrate specificity of SBL.

The development of enzymes with novel properties can be accomplished by screening wild microbial populations,[4,5] or by site-directed,[2,6–8] or random[9–11] mutagenesis. Previously, we have shown that the combined approach of site-directed mutagenesis and chemical modification of SBL cysteine mutants with methanethiosulfonate reagents permits the facile and controlled introduction of unnatural amino acid side chains, and the creation of novel activities.[12–14] SBL is an ideal candidate for this technique, since it is a well defined enzyme[15] and contains no natural cysteine.

Cysteine mutants of SBL and chemically modified mutants (CMMs) were prepared and characterized as previously described[12] and the best esterases amongst them were selected for preparative evaluation

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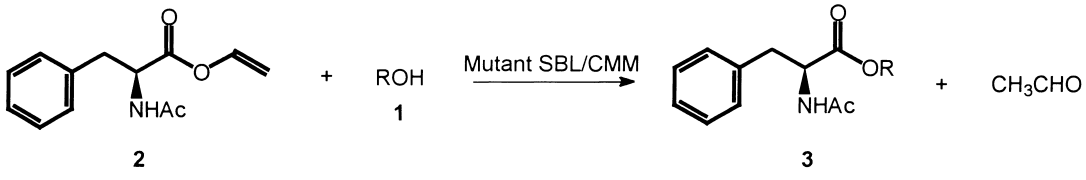
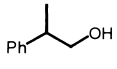
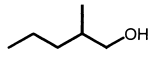
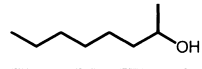
(Scheme 1).[16] Three CMMs (L217C–S–$(CH_2)_2$–SO_3^-, N62C–S–$(CH_2)_2$–SO_3^-, N62C–S–CH_3) and two mutant enzymes (L217C and M222C) were each embedded in a KCl matrix[17] and used to catalyze transesterification reactions in *tert*-BuOH between the acyl donor, **2**, and the racemic primary and secondary alcohols, **1**, as previously described.[3] Two primary alcohols (2-phenyl-1-propanol and 2-methyl-1-pentanol) and one secondary alcohol (2-octanol) were used as representative nucleophiles for the study. The results are given in Table 1. L217C and the L217C–S–$(CH_2)_2$–SO_3^- CMM catalyzed the reaction with the two primary alcohols in similar yields and des to wild type (WT), but only L217C gave as good a yield as WT using 2-octanol as nucleophile. M222C gave lower yields for all three alcohols. N62C–S–$(CH_2)_2$–SO_3^- gave a higher yield of product than WT when 2-phenyl-1-propanol was the nucleophile. The reaction with 2-methyl-1-pentanol, using N62C–S–$(CH_2)_2$–SO_3^- as catalyst gave a significant improvement in the de of the product ester (41%) over WT (26% de). Only one CMM catalyst, N62C–S–CH_3, gave marked increases in product yield for the two primary alcohols (97% for 2-phenyl-1-propanol and 79% for 2-methyl-1-pentanol). No changes in stereochemical preference from WT were observed for any of the CMMs.



Scheme 1.

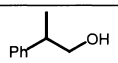
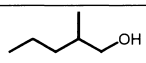
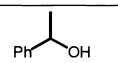
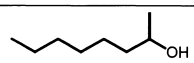
The nature of the solvent and temperature[18,19] are known to influence enantioselectivity, and the effects of these parameters on the N62C–S–CH_3 catalyzed transesterifications were considered next. In this study, CH_3CN was selected as the illustrative solvent since the relatively slow rates in *tert*-BuOH, even at 50°C, precluded the probing of low temperature effects. We included M222C in this part of our study, because it has been found that the M222A mutant of subtilisin BPN′ allowed a faster initial reaction of sterically hindered amine nucleophiles with ester acyl donors.[20] The results are shown in Table 2. In CH_3CN at 4°C, M222C and N62C–S–CH_3 perform better than WT. Both enzymes catalyze the transesterification of primary and secondary alcohols faster than WT and with des that are comparable with WT. Remarkably, they give much higher yields of the product ester than WT when the sterically

Table 1
Yields and de values of **3** from mutant and CMM-catalyzed reactions in *t*-BuOH at 50°C

									
Enzyme	% yield	% de	Abs. Conf.	% yield	% de	Abs. Conf.	% yield	% de	Abs. Conf.
WT[3]	53	30	*R*	58	26	*R*	20	>99	*S*
M222C	20	29	*R*	18	21	*R*	9	>99	*S*
L217C	59	22	*R*	50	12	*R*	19	>99	*S*
L217C-S-$(CH_2)_2$-SO_3^-	49	30	*R*	29	17	*R*	<5	-	*S*
N62C-S-$(CH_2)_2$-SO_3^-	65	32	*R*	59	41	*R*	8	>99	*S*
N62C-S-CH_3	97	24	*R*	79	34	*R*	16	>99	*S*

Conditions: All reactions used 10 equiv. of alcohol **1**, and the acyl donor, **2**, in *t*-BuOH at 50°C for 24 hr (primary alcohols) or for 72 hr (secondary alcohols) as previously described.[3] All yields and de's (HPLC on Chiralcel OD using a hexane:isopropanol eluent) are of purified product, **3**, which was identified by ^1H NMR.[3]

Table 2
Yields and de values of **3** from reactions carried out in CH_3CN at 4°C

								
Enzyme	% yield % de	Abs. Conf.	% yield % de	Abs. Conf.	% yield % de	Abs. Conf.	% yield % de	Abs. Conf.
WT	99, 37 (48 hr)[3]	*R*	91, 4 (24 hr)[3]	*R*	19, 84 (50 hr)	*S*	10, 88 (50 hr)	*S*
M222C	71, 24 (24 hr)	*R*	94, 9 (16 hr)	*R*	98, 93 (44 hr)	*S*	92, 94 (44 hr)	*S*
N62C-S-CH_3	94, 45 (16 hr)	*R*	95, 12 (7 hr)	*R*	40, 80 (50 hr)	*S*	50, 97 (72 hr)	*S*

Conditions: All reactions used 10 equiv. of alcohol, **1**, and the acyl donor, **2**, in CH_3CN at 4°C as previously described.[3] All yields and de's (HPLC on Chiralcel OD using a hexane:isopropanol eluent) are of purified product, **3**, which was identified by ^1H NMR.[3]

hindered secondary alcohols are used as nucleophiles. M222C gives an almost quantitative yield of product ester with 1-phenylethanol and an excellent yield (92%) of ester with 2-octanol. M222C improves the de of the product ester to above 90% for both secondary alcohols, and N62C–S–CH_3 gives the product ester in 97% de for 2-octanol.

From these results, both N62C–S–CH_3 and M222C are seen to be better transesterification catalysts than WT. The reasons for this appear to be different. N62C–S–CH_3 catalyzes the transesterification of primary alcohols with **2** in higher yield and in a shorter time than M222C, but the reverse is true for secondary alcohols, where M222C efficiently couples 1-phenylethanol and 2-octanol with **2** in 98% and 92% yield, respectively. We have proposed[3] that WT gives lower yields with secondary alcohols because branching at the α-carbon of the alcohol is poorly tolerated by the S_1'-pocket[21] of SBL. Residue 222 of SBL is at the boundary between the S_1- and S_1'-pockets, in a region that is in close proximity to where the nucleophile would approach the acyl–enzyme intermediate, in order to deacylate the enzyme and complete the catalytic cycle. Therefore, it is reasonable that if methionine is replaced by the smaller cysteine at position 222, a larger space in this critical region would permit more sterically hindered nucleophiles to react with the acyl–enzyme intermediate. This is exactly what is observed for M222C catalyzed reactions of secondary alcohols. In contrast, residue 62 of SBL is in the S_2 pocket, and therefore it is unlikely that any mutation or modification at this residue would significantly influence the S_1' pocket. Nevertheless, N62C–S–CH_3 does give considerably higher yields than WT with secondary alcohols. Furthermore, this CMM catalyzes the transesterification of primary alcohols much faster than either WT or M222C. It is probable that N62C–S–CH_3 catalyzes transesterification faster than M222C or WT because of a higher turnover rate,[16] but that in the case of secondary alcohols, the improved catalytic efficiency cannot entirely overcome the negative steric hindrance factors.

In conclusion, we note that while the subtilisin CMMs described here clearly do not yet challenge lipase-catalyzed transesterifications, the future potential of the CMM approach is evident from the fact that both N62C–S–CH_3 and M222C are superior transesterification catalysts to WT, with N62C–S–CH_3 giving higher yields in a shorter reaction time in transesterification reactions than WT when primary alcohols are used with **2** as acyl donors. Furthermore, M222C itself is already an excellent catalyst for the transesterification of secondary alcohols.

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