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A COMBINATORIAL APPROACH TO CHEMICAL MODIFICATION OF SUBTILISIN *Bacillus lentus*

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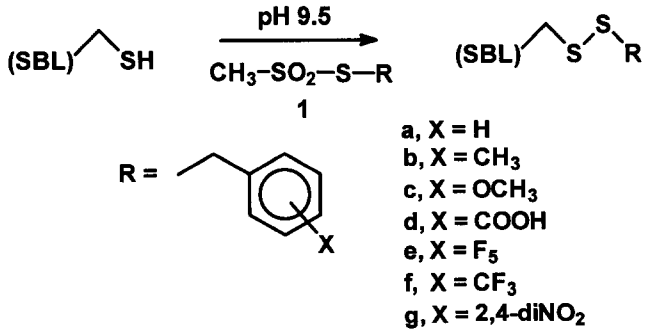
Abstract. The reaction between methanethiosulfonate reagents and cysteine mutants of subtilisin is quantitative and can be used to prepare chemically modified mutant enzymes (CMMs) with novel properties. The virtually unrestricted structural variations possible for CMMs presents a preparative and screening challenge. To address this, a rapid combinatorial method for preparing and screening the activities of CMMs has been developed.

The enzymatic activity of subtilisin *Bacillus lentus* (SBL) has been systematically modified by combined site-directed mutagenesis and chemical modification.[1] Because SBL has no natural cysteine residue, a cysteine introduced by site-directed mutagenesis provides a unique center for highly selective chemical modification with methanethiosulfonate (MTS) reagents[2] (**1**, Scheme 1) to give chemically modified enzyme mutants (CMMs). The virtually unlimited structural variations that can be introduced in this way present a challenge with respect to identifying the best CMMs for a given transformation. We have now addressed this issue by developing a combinatorial-like approach to the preparation and screening of CMMs. Reaction with MTS reagents is quantitative, rapid and nearly non-denaturing in most cases,[1b] and thus provides an excellent basis for a rapid enzyme modification screen on microtiter plates to explore new CMMs. To illustrate the method, modifications at three key active site locations of SBL were studied, namely N62C located in the S_2 pocket, L217C in the S_1' (leaving group) pocket and S166C in the S_1 pocket.[3]



Scheme 1

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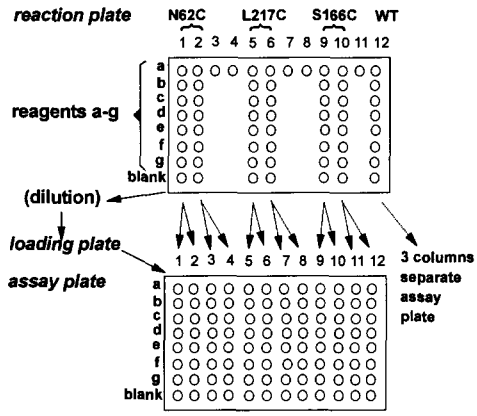
To date, kinetic constants of fully characterized CMM have been evaluated by the method of initial rates with a colorimetric assay. Amidase activity was followed by the release of p-nitroaniline from the tetrapeptide substrate succinylalanylalanylprolylphenylalanyl p-nitroanilide (sucAAPF-pNa). The analogous thiobenzyl ester substrate (sucAAPF-SBn) does not have a chromogenic leaving group, so detection of hydroysis requires reaction of the thiobenzyl leaving group with 5,5′-dithiobis-2,2′-nitrobenzoate (DTNB, Ellman's reagent). We now report the extension of these techniques to a rapid screening method, in which the colorimetric enzyme assay is performed on 96-well microtiter plates. First we evaluated existing, fully characterized,[1] CMMs for amidase and esterase activity, in order to validate the screen. We then generated an array of new CMMs, with **1a–g**, on a 96-well plate and screened the new CMMs for amidase and esterase activity using the 96-well colorimetric assay (Scheme 2). The advantage of this screen is that promising combinations of cysteine mutants and new MTS reagents can be detected without the large CMM quantities required previously.



Scheme 2

In the 96-well enzyme activity assay, k_{cat}/K_M is obtained from the rate of product formation (v), using the limiting case of the Michaelis-Menten equation at low substrate concentration, $v \approx (k_{cat}/K_M)$ [S][E] for [S] << K_M, as an approximation, where [S] and [E] are the substrate and enzyme concentrations, respectively. Enzyme stock solutions were prepared in 5 mM MES with 2 mM $CaCl_2$ pH 6.5 at 5×10^{-7} M for amidase and 5×10^{-8} M for esterase assays, respectively. Substrate solutions were prepared in DMSO. The amidase substrate (sucAAPF-pNa) stock was 1.6 mM, which gave 0.08 mM in the well. The esterase substrate (sucAAPF-SBn) stock solutions were 1.0 mM, which gave 0.05 mM in the well. Assays were carried out in 0.1 M Tris pH 8.6 with 0.005% Tween. Tris buffer for the esterase assay contained 0.375 mM DTNB, and was generally used immediately as the DTNB teneded to decompose at this high pH. A sample of each enzyme solution (~150 μL) was placed in a well in the 1st, 5th or 9th column of an enzyme loading plate. Rows A to G contained enzymes and row H contained MES buffer. On a separate assay plate (Corning, flat bottom, 96-well), 10 μL of substrate

solution and 180 μL of buffer were dispensed into wells along columns to be used in a run. Columns 1–4 on the assay plate contained four replicates of the enzymes in column 1 of the loading plate; columns 5–8 contained four replicates of the enzymes in column 5 of the loading plate, etc. Reactions were initiated by transferring 10 μL of enzyme solution from the loading plate to the assay plate with an 8-channel pipette. For amidase assays, four columns were initiated for one run, for esterase two columns were initiated. The time delay between addition of enzyme to the first column and onset of reading was 22–30 s (amidase) and 10–15 s (esterase). Immediately after initiation, the plate was placed on a Multiskan MCC340 reader (programmed in the kinetic mode, filter 414 nm, lag time 0.0 min., interval 5 s, with automatic background subtraction of blank row H) and was read for 1.0 min (amidase) or 30 s (esterase).[4] The output from the reader represented the average rate of change in absorbance at 414 nm min^{-1}, measured at 5 s intervals over the total time programmed. These data were converted to rates in M s^{-1} using ε_{414} = 8581 $M^{-1}cm^{-1}$ for *p*-nitroaniline ε_{414} = 8708 $M^{-1}cm^{-1}$ for 3-carboxylate-4-nitrothiophenolate. The rates were corrected for active enzyme concentration, and the four replicates for each enzyme were averaged.

Table 1. k_{cat}/K_M obtained for amidase activity at pH 8.6 [a]

Enzyme	Type of assay	k_{cat}/K_M ($s^{-1}mM^{-1}$)
WT	cuvette [b] (standard [c])	76 ± 7 [f]
WT	cuvette (low substrate [d])	75
WT	96-well[e] (low substrate)	75 ± 5 [f]
WT	96-well (standard)	80 ± 17 [c]
L217C-$SCH_2C_6H_5$	96-well (low substrate)	52 ± 6 [f]
L217C-S $CH_2C_6H_5$	96-well (standard)	48 ± 3 [c]
L217C-$S(CH_2)_5CH_3$	96-well (low substrate)	113 ± 18 [f]
L217C-$S(CH_2)_5CH_3$	96-well (standard)	97 ± 12 [c]

[a]All assays were done at room temperature (20 °C) For assays in 1 mL cuvettes, the cell holder was thermostatted to 20 °C. [b]Kinetic assay in 1 mL cuvettes. [c]Complete enzyme kinetics by the method of initial rates, where k_{cat}/K_M was obtained from k_{cat} and K_M values and the errors were obtained from the curve-fitting error in k_{cat} and K_M. [d]Measurement of k_{cat}/K_M using the low substrate approximation (see text). [e]Assay performed on microtiter plates instead of cuvettes. [f] Mean standard error of 4 replicates.

Gratifyingly, the values of k_{cat}/K_M (amidase) obtained using the 96-well assay (Table 1) are the same as those obtained by the initial rates method in 1 mL cuvettes at room temperature.[5] The k_{cat}/K_M value obtained in the cuvette at 20 °C using a complete kinetic evaluation and the value calculated from the rate at the lowest substrate concentration were the same, indicating that the low substrate approximation held for WT (Table 1). To further validate the method, complete kinetic experiments were performed on 96-well plates by running 8 different substrate concentrations along the rows and one enzyme per column. Values of k_{cat}/K_M obtained from these experiments were the same as values obtained with the low substrate approximation for WT and two L217C CMMs, confirming that the low substrate approximation also held for CMMs. Similarly, k_{cat}/K_M values for esterase obtained on the plate did not differ significantly from values obtained with the standard assay (Table 2).

Table 2. k_{cat}/K_M obtained for esterase activity at pH 8.6.[a]

Enzyme	Type of assay	k_{cat}/K_M ($s^{-1}mM^{-1}$)
WT	96-well (low substrate)	3321 ± 256
L217C	cuvette (standard)	5540 ± 798
L217C	96-well (low substrate)	5563 ± 419
N62C	cuvette (standard)	4380 ± 655
N62C	96-well (low substrate)	4151 ± 198
N62C-SCH_3	cuvette (standard)	10100 ± 1287
N62C-SCH_3	96-well (low substrate)	8739 ± 765

[a]Conditions as in Table 1.

Recent modifications at S166C with benzyl-MTS, (**1a**) led to surprising improvements in esterase relative to amidase activity, even though the ester and amide substrates had the same P_1 group.[6] Accordingly, we became interested in screening the effect of introducing benzyl groups substituted with electron-releasing and -withdrawing groups as a further means of modulating amidase and esterase activity. For the micro-scale preparations of these new CMMs, combinations of 7 different aromatic MTS reagents (**1a–g**) and 3 cysteine mutants (N62C, L217C and S166C), as well as a WT control were set up on a 96-well plate as shown in the top part of Scheme 2. Two replicates of each mutant were prepared with each reagent. One reagent, benzyl MTS (**1a**), had already been evaluated in large scale modifications, and was included for comparative purposes. The reaction mixture in each well consisted of 20 μL of enzyme solution (in 5 mM MES, 2 mM $CaCl_2$, pH 6.5, *ca.* 8 × 10^{-5} M), 40 μL of CHES buffer (70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5) and 10 μL of MTS reagent in acetonitrile (*ca.* 1 × 10^{-2} M). The blanks on the reaction plate were 20 μL of MES, 40 μL of CHES and 10 μL of acetonitrile. The reactions were left at room temperature and were tested for residual thiol groups after 2 h in the following way. On a separate plate, 10 μL of reaction mixture was added to 60 μL of DTNB-containing Tris buffer (pH 8.6) and the monitoring plate scanned 15 min later at 414 nm. No difference between the WT and the modified cysteine mutants was detected, indicating that the reaction was complete. To quench the reaction, 10 μL of MES pH 6.5 was added to each well, and 20 μL of the mixture from each well was diluted with 980 μL of MES buffer (in Eppendorf tubes). An amidase loading plate was prepared with the diluted mixtures, and an esterase loading plate with 10 μL of diluted mixture and 90 μL of MES buffer in the appropriate wells. From this point on the assays were the same as described earlier for purified, fully characterized, CMMs. Each reaction replicate was assayed in duplicate (Scheme 1). The k_{cat}/K_M values were calculated from the above limiting case Michaelis-Menten equation, basing [E] on the concentration of active enzyme in the parent mutant and assuming no significant denaturation. Since little difference was observed between the two reaction replicates, the 4 values for each combination were averaged in the final results.

The amidase k_{cat}/K_M value for N62C-**a** and S166C-**a** obtained in the screen is ~2-fold lower than the value obtained for the fully characterized enzymes (Table 3). This is consistent with the initial assay validation

Table 3. Comparison of data from the screen and the corresponding fully characterized enzymes.

Enzyme	Reagent	Amidase k_{cat}/K_M (s^{-1} mM^{-1})[a]	Amidase k_{cat}/K_M (s^{-1} mM^{-1})[b]	Esterase k_{cat}/K_M (s^{-1} mM^{-1})[1]	Esterase k_{cat}/K_M (s^{-1} mM^{-1})[2]
		screen	*fully characterized*	*screen*	*fully characterized*
WT		121 ± 3	178 ± 15 [c]	3890 ± 170	3560 ± 540 [c]
N62C	**1a**	207 ± 13	379 ± 35 [c]	6510 ± 560	6330 ± 1360 [c]
	1c	86 ± 6	93 ± 4	5680 ± 1000	7870 ± 160
	1d	74 ± 11	442 ± 36	7680 ± 1410	50700 ± 2160
	1e	111 ± 3	198 ± 44	7520 ± 710	14260 ± 590
S166C	**1a**	10 ± 1	20 ± 1 [c]	1160 ± 140	4920 ± 1320 [c]
	1c	17 ± 1	68 ± 2	1746 ± 120	5798 ± 106
	1d	16 ± 1	72 ± 3	2370 ± 91	11460 ± 400
	1e	14 ± 0	67 ± 3	784 ± 75	5198 ± 145

[a]Mean standard error from 4 replicates, obtained at room temperature (see text). [b]Values and error calculated from individual k_{cat} and K_M values obtained at 25 °C. [c]Data from ref 6.

results. Excitingly, the factor by which amidase k_{cat}/K_M values for N62C-**a** and S166C-**a** differed from WT was the same as for the fully characterized CMMs, indicating that the rapid screen was giving the correct relative pattern for amidase activity. With respect to esterase rates, the k_{cat}/K_M values for WT and N62C-**a** were the same in the screen and the characterized enzyme (Table 3), again consistent with the initial validation data. However, for S166C-**a** the screening procedure underestimated the esterase k_{cat}/K_M value by ~5-fold, possibly because of unaccounted for denaturation and/or the lower temperature at which the screen was carried out. The seven WT control treatments had the same esterase and amidase activity, and hence the same esterase/amidase ratio (Figure 1), indicating that the reagents themselves did not cause differential denaturation.

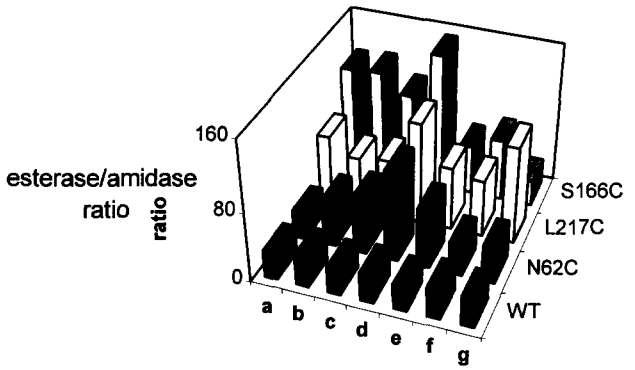


Figure 1. k_{cat}/K_M esterase/amidase ratios for modifications by **1a–g** at positions 62,166, and 217.

The screen results (Figure 1) demonstrated that N62C-**d** and **e** had greatly improved esterase activity compared to WT. In the 166 family, some CMMs also had very favorable esterase/amidase ratios, while in the 217 group, only L217C-**d** was noteworthy. To evaluate the accuracy of these screen results, we prepared and fully characterized N62C- and S166C-**c**, **d**, and **e** (Table 3). Satisfyingly, the k_{cat}/K_M values from the screen and

the characterized enzymes agreed within the error limits discussed previously, except for both 4-carboxybenzyl (**d**) CMMs for which amidase and esterase rates were each ~5-fold underestimated in the screen (Table 3). The suspicion that this was significant denaturation in both CMMs was confirmed by establishing that the concentrations of active CMMs were 4-fold lower than expected from the parent mutant. Nevertheless, the screen clearly showed that both 62- and 166-**d** CMMs had improved esterase activity relative to the benchmark S-benzyl (**a**) variants (Table 3). Furthermore, the screen correctly predicted significantly improved esterase/amidase specificity ratios for N62C- and S166C-**c**, **d**, and **e** (Figure 2).

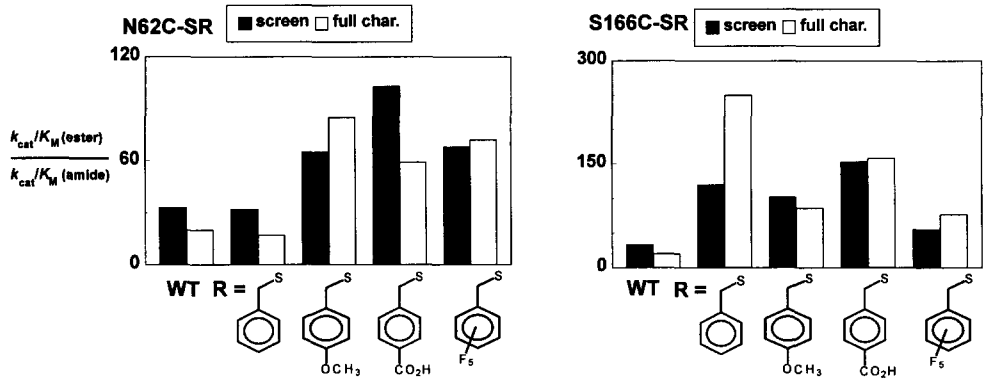


Figure 2. Comparison of the esterase/amidase specificity ratio from the screen and the corresponding fully characterized enzyme.

In summary, we have developed a rapid, microscale, combinatorial assay for the modification of subtilisin cysteine that reliably evaluates amidase and esterase activities of the CMMs generated. To our knowledge, this is the first combinatorial method for chemical modification of enzymes, and surveying their kinetic behavior.

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References and Notes:

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3. Bott, R. R.; Ultsch, M.; Kossiakoff, A.; Graycar, T. P.; Katz, B.; Power, S. *J. Biol. Chem.* **1988**, *263*, 7895.
4. Reading much beyond the nearly linear part of the progress curve (up to ~50% conversion) leads to underestimation of the rate.
5. For normal[1] assays at 25 °C, amidase values are 2-fold higher, but esterase rates are largely unaffected.
6. Plettner, E.; DeSantis, G.; Stabile, M.; Jones, J. B., unpublished observations.