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ALTERING THE SPECIFICITY OF SUBTILISIN *B. LENTUS* BY COMBINING SITE-DIRECTED MUTAGENESIS AND CHEMICAL MODIFICATION

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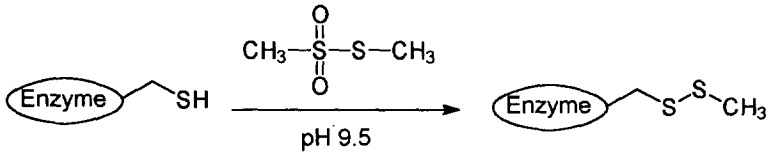
Abstract: The thiol side chain of the M222C mutant of the subtilisin from *Bacillus lentus* (SBL) has been chemically modified by methyl-, aminoethyl-, and sulfonatoethylthiosulfonate reagents. Introduction of charged residues into the active site of the enzyme reduced the catalytic efficiency with Suc-AAPF-pNA as the substrate, but resulted in better binding of sterically demanding boronic acid inhibitors.

Modifying enzyme properties by site-directed mutagenesis is limited to natural amino acid replacements, although molecular biological strategies for overcoming this restriction have been devised recently.[1] However, the latter procedures are not generally easy to apply in most laboratories. In contrast, controlled chemical modification of enzymes offers broad potential for facile and flexible modification of enzyme structure, thereby opening up extensive possibilities for controlled tailoring of enzyme specificity.

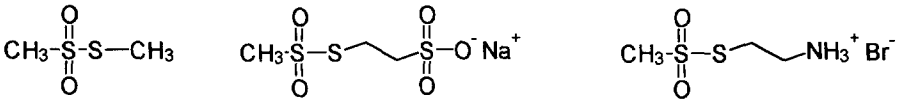
Changing enzyme properties by chemical modification has been explored previously, with the first report being in 1966 by the groups of Bender[2] and of Koshland,[3] who created a thiolsubtilisin by chemical ($CH_2OH \rightarrow CH_2SH$) transformation of the active site serine residue of subtilisin BPN' to cysteine. In 1985, interest in chemically produced artificial enzymes, including some with synthetic potential, was renewed by the Kaiser group,[4] and more recently by Hilvert[5] and by Suckling.[6]

Our interest in using chemical modifications to tailor enzyme specificity was stimulated by the reports that for cysteine-containing enzymes, reaction of a cysteine-thiol side chain with alkyl alkanethiosulfonates provides a rapid, specific, and quantitative method of chemical modification that can be performed under mild conditions and is independent of the nature or structure of the added group.[7] The S_N2-type reaction involved in such modifications is outlined in Scheme 1. This method can be combined with the controlled introduction,



Scheme 1. Representative chemical modification of an enzyme's cysteine residue by methyl methanethiosulfonate.

by site directed mutagenesis, of a cysteine residue specifically targeted for alkanethiosulfonate modification, as has been described for carboxypeptidase Y,[8] staphylococcal nuclease,[9] and for a subtilisin-like enzyme.[10] This unified site-directed mutagenesis–chemical modification approach is ideally suited to prokaryotic enzymes such as subtilisins, which contain no natural cysteine. It is therefore the strategy that we have adopted for exploring the potential of chemical modification to alter the specificity of the subtilisin from *B. lentus* (SBL). In this initial study, the M222C (BPN′ numbering) mutant of SBL has been modified by reaction with methyl methanethiosulfonate (**1**), sodium 2-(sulfonatoethyl)methanethiosulfonate (**2**), and (2-aminoethyl)methanethiosulfonate hydrobromide (**3**).[11]



1 **2** **3**

Results and Discussion

The mutant enzyme SBL-M222C was prepared and purified as described previously,[12] and appeared as a single band on native PAGE after the final chromatographic step. It was then reacted on a 25 mg (9.4×10^{-7} mol) scale in CHES buffer (2.5 mL, 70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5) with a 100-fold molar excess of each of reagents **1**, **2**, or **3** (100 μL of a 0.90 M solution; **1** in EtOH, **2** in CHES buffer, **3** in MeOH). Each reaction was monitored by measuring changes in specific activity (in 0.1 M TRIS pH 8.6, 0.005% Tween 80, 1% DMSO), as reflected by the changes in absorbance (Figure 1) due to the release of *p*-nitroaniline from the Suc-AAPF-pNA substrate (1 mg/mL) purchased from Sigma. The reaction products SBL-M222C-SMe, SBL-M222C-$SEtSO_3^-$, and SBL-M222C-$SEtNH_3^+$ from the reaction of SBL-M222C with **1**, **2**, and **3**, respectively, were purified on disposable PD-10 Sephadex® G-25 M (Pharmacia) desalting columns, followed by dialysis against Millipore-water, and then lyophilization. The purity (>90%) of each modified enzyme was confirmed by native PAGE analysis and by electrospray mass spectroscopy (ES-MS),[13] which showed in each case that only the desired 222C alkylation had taken place.

To ensure that inadvertent reactions at residues other than 222 were not occurring, SBL-WT itself was incubated with reagents **1**, **2**, and **3**. No change in activity over time was detected with any of the reagents,

thereby excluding the possibility of chemical modifications at untargeted residues. To further verify that only the single, introduced, cysteine residue was modified, and was wholly responsible for the observed changes in specific activity, the parent SBL-M222C enzyme was regenerated from SBL-M222C-SEtNH_3^+, by reduction of its chemically created disulfide bond with 100 equiv of β-mercaptoethanol (BME) at pH 9.5 under nondenaturing conditions. After 24 h of this treatment, the specific activity was fully restored to that of unmodified SBL-M222C. Application of dithiothreitol (DTT)[14] under similar conditions failed to effect the desired restoration of WT activity. The requirement of a large excess of BME for the reductive regeneration, and the ineffectiveness of DTT, indicates that access to the modified site is sterically constrained.

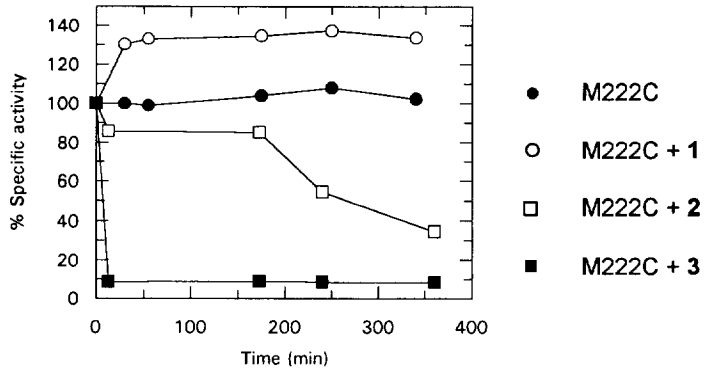
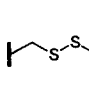


Figure 1. Observed specific activities during chemical modifications of M222C.

Each alkylation by **1-3** proceeded quite quickly, as exemplified by the reaction of SBL-M222C with **1**, which was complete in less than 30 min, and with the specific activity increasing to 130% of SBL-M222C itself. (SBL-WT is 2.8 times as active as the SBL-M222C mutant in this assay). This result parallels that for a similar modification of a mutated "subtilisin-like" enzyme by **1**.[10] Conversely, an immediate drop in enzyme activity was observed when SBL-M222C was aminoethylated with **3**. This reduced activity on the introduction of -SEtNH_3^+ at position 222 parallels the situation observed with subtilisin BPN′, whose activity is reduced by 14-fold on replacing its M222 residue by lysine (K).[15] Thus as would be expected, the -SEtNH_3^+ group and the amino butyl side chain of lysine engender similar activity-reducing effects. In contrast to the situation for the reaction of **3**, whose aminoethyl group is protonated under the pH 7.5 assay conditions, the progress of reaction curve for **2**, with its negatively charged sulfonato group, was very different. After the addition of the first aliquot of **2**, the activity dropped slightly. However, after a second aliquot was added to the mixture, the activity slowly dropped to almost the level of the M222C-SEtNH_3^+. While both SBL-M222C-SEtSO_3^- and SBL-M222C-SEtNH_3^+ still retain enough protease activity to hydrolyze the Suc-AAPF-pNA substrate, their turnover numbers are much lower than that of SBL-M222C itself, or its methyl modified M222C-SMe

derivative. There remains the possibility that some of the low hydrolytic activity observed for the sulfonate and amine variants stems from the residual contamination by unmodified M222C.

Table 1. Kinetic Parameters for Modified Enzymes.[a]

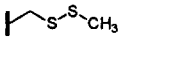
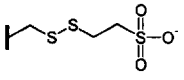
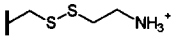
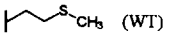
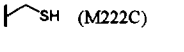
Enzyme (structure at 222)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} M^{-1})
 (WT)[b]	48.0 ± 2	0.55 ± 0.06	8.7×10^4
 (M222C)[b]	17.3 ± 0.4	0.77 ± 0.05	2.2×10^4
	33.3 ± 0.9	0.68 ± 0.04	4.9×10^4
	1.64[c] ± 0.05	0.55 ± 0.05	$3.0^c \times 10^3$
	1.06 ± 0.02	0.61 ± 0.05	1.7×10^3

(a) Michaelis-Menten constants were measured at 25 °C according to the initial rates method in 0.1 M phosphate buffer containing 0.5 M NaCl at pH 7.5 with Suc-AAPF-pNA as the substrate. Active enzyme concentrations were determined via PMSF titration.[16]
(b) From reference 12.
(c) Enzyme concentration determined by weighing lyophilized powder.

The kinetic parameters for all the chemically modified enzymes are recorded in Table 1. The K_M remains relatively constant for all of the modifications. However, with the charged ethylsulfonate or ethylamino group modification, k_{cat} drops drastically. The reasons for the different k_{cat} effects cannot yet be accounted for, although it may be that the close proximity of the charged modifications to histidine 64 will adversely impact the role of proton transfer for this latter residue during catalysis.[15] Furthermore, when the hydrophobic -SMe group is attached to cysteine at 222, the k_{cat} value is virtually restored to that of SBL-WT. The high activity for M222C-SMe is not unexpected since the hydrophobic nature and steric bulk of the modified cysteine residue mimic those of the methionine residue of the SBL-WT enzyme, with the only difference being the replacement of a sulfur atom by a $-CH_2$ group.

Although K_M for Suc-AAPF-pNA was not affected by the chemical modification of SBL-M222C, since the 222 position is adjacent to the entrance of the S_1 pocket it was considered likely that such modifications could cause changes in S_1 pocket binding characteristics. Accordingly, the modified enzymes were subjected to an S_1-specificity evaluation by probing with boronic acids as competitive inhibitors, using a previously described strategy.[12]

Table 2. Inhibition constants for Chemically Modified Enzymes from M222C[a]

Enzyme (structure at 222)	K_I (μM) 1	2	3	4	5	6
	123 ± 8	11.2 ± 0.7	139 ± 9	3.8 ± 0.3	153 ± 10	820 ± 54
	146 ± 15	15.6 ± 1.5	176 ± 17	11 ± 1	25 ± 2	290 ± 28
	108 ± 9	10.5 ± 0.9	127 ± 11	8.9 ± 0.8	40 ± 3	178 ± 16
 (WT)	141 ± 22	23 ± 4	150 ± 23	9.2 ± 1.4	327 ± 50	801 ± 68
 (M222C)	155 ± 36	23 ± 5	204 ± 47	18 ± 4	31 ± 7	241 ± 5

(a) Inhibition constants were determined using Waley's method[17] in 0.1 M phosphate buffer containing 0.5 M NaCl at pH 7.5 with Suc-AAPF-pNA as the substrate.

The K_I data show that each modified enzyme tolerates a wide variety of substituents on the phenyl ring of a boronic acid inhibitor. Interestingly, the biphenyl boronic acid **5** and the phenylalanine analogue **6** show the greatest variations in K_I values with the different enzymes. The highest K_I values are manifest for the more hydrophobic WT and M222C-SMe enzymes, while for the more hydrophilic, M222C, M222C-$SEtSO_3^-$, and M222C-$SEtNH_3^+$ catalysts, the K_I values are reduced by 4- to 5-fold for these same inhibitors. These differences may well be due to the fact that the highly polarizable side chains of cysteine of M222C, and the charged side chains of the modified enzymes alter the environment around the active site nucleophile, Ser221, thus affecting the catalytic triad and its properties.

Abbreviations: CHES: 2-(cyclohexylamino)ethanesulfonic acid, MES: 4-morpholinethanesulfonic acid, TRIS: tris(hydroxymethyl)aminomethane, PAGE: polyacrylamide gel electrophoresis

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