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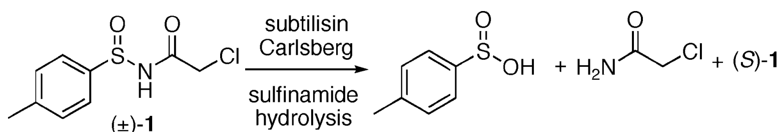

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Unexpected Subtilisin-Catalyzed Hydrolysis of a Sulfinamide Bond in Preference to a Carboxamide Bond in *N*-Acyl Sulfinamides

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Catalytic promiscuity, the ability of an active site to catalyze more than one chemical transformation,[1] contributes to the natural evolution of new enzymes[2] and can generate new catalysts for synthesis.[3] We report a subtilisin-catalyzed hydrolysis of a sulfinamide S−N bond in an *N*-acyl sulfinamide. This reaction is the first enzymatic sulfinamide hydrolysis and also the first case where the enzyme favors the unnatural functional group (sulfinamide) even though the substrate also contains the normal functional group (carboxamide).

Proteases normally catalyze hydrolysis of the amide C−N bond, but can also catalyze hydrolysis of sulfite[4] or sulfonate esters,[5] phosphodiesters,[6] or organophosphate triesters[7] or the condensation of siloxanes.[8] Peptides react via a tetrahedral intermediate, while these analogues must involve alternate geometries. Catalytic promiscuity does not include irreversible inactivation of enzymes by phosphorus compounds,[9] sulfonyl fluorides,[10] β-sultams,[11] or similar inhibitors because inactivation does not regenerate the starting enzyme.

Chiral sulfinamides are versatile auxiliaries for the preparation of chiral amines.[12] Previously, we reported the subtilisin E-catalyzed enantioselective hydrolysis of *N*-chloroacetyl *p*-toluenesulfinamide, **1**, by cleavage of the carboxamide bond (path A in Figure 1a).[13] This highly *R*-enantioselective cleavage is a good synthetic route to enantiopure *p*-toluenesulfinamide. During this work, we discovered a related subtilisin, subtilisin Carlsberg, which favored hydrolysis of the sulfinamide bond (path B, Figure 1a). Mass spectrometry combined with ^{1}H NMR unambiguously identified that hydrolysis of the sulfinamide bond in *N*-chloroacetyl *p*-toluenesulfinamide, **1**, was the major reaction catalyzed by subtilisin Carlsberg. Mass spectrometry identified the sulfinic acid product, while ^{1}H NMR revealed formation of chloroacetamide from S−N hydrolysis, with a small amount of chloroacetic acid from C−N hydrolysis (1:0.37 after 6 h) (Figure 1b), indicating approximately 3 times more substrate reacts via the S−N bond hydrolysis than by C−N bond hydrolysis.

HPLC analysis confirmed that subtilisin Carlsberg favored the S−N bond hydrolysis by approximately 4-fold over C−N bond hydrolysis, and that both reactions were moderately enantioselective (Table 1). The disappearance of the starting compound *N*-chloroacetyl *p*-toluenesulfinamide, **1**, revealed the sum of both reactions. The appearance of the product *p*-toluenesulfinamide revealed the amount of C−N hydrolysis, and the difference revealed the amount of S−N hydrolysis. Control reactions without enzyme revealed ~7% spontaneous hydrolysis, mainly via S−N bond cleavage. For subtilisin Carlsberg (entry 2 in Table 1), approximately 4 times more substrate reacted via S−N bond hydrolysis than by C−N bond hydrolysis: 39 and 9% conversion, respectively, after correcting for chemical hydrolysis. This agrees with the chloroacetamide and small amount of chloroacetic acid in the NMR and a 3−4-fold preference for the S−N reaction by Carlsberg. The S−N cleavage was moderately *R*-enantioselective ($E = 17$), yielding the remaining starting material enriched in the *S*-enantiomer. The products (sulfinic acid and chloroacetamide) are achiral. The C−N cleavage was moderately enantioselective ($E = 6$) and also favored the *R*-enantiomer.

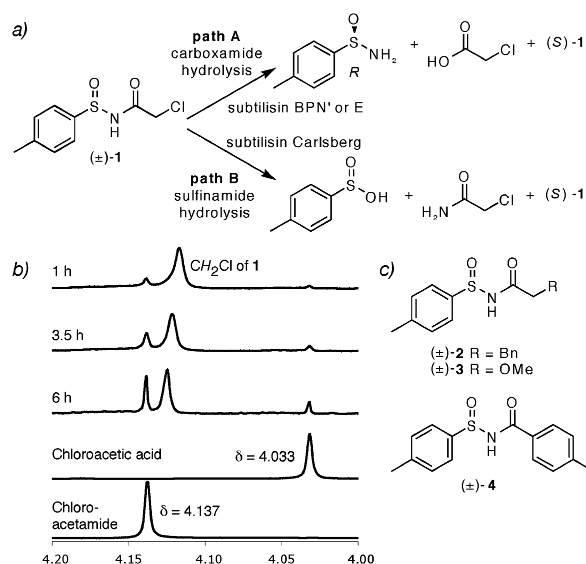

Figure 1. (a) Different subtilisins favor hydrolysis of different bonds in *N*-chloroacetyl *p*-toluenesulfinamide, **1**. (b) Chemical shift of the methylene protons during subtilisin Carlsberg hydrolysis of **1** showing chloroacetamide as the major product. (c) Substrates **2**−**4**.

Two related subtilisins favored C−N hydrolysis in **1**. Subtilisin E (from *Bacillus subtilis* with 70% sequence identity to subtilisin Carlsberg) showed only a highly enantioselective hydrolysis via the C−N pathway (51% conversion, $E > 75$, entry 3), as reported previously.[13] Subtilisin BPN′ (from *Bacillus amyloliquefaciens* with 70% sequence identity to subtilisin Carlsberg and 85% sequence identity to subtilisin E[14]) also favored the C−N pathway (37% conversion, $E > 75$, entry 6) with a small amount of S−N cleavage (5%, entry 6).

Two experiments showed that both the C−N and S−N hydrolyses involved the active site (details in the Supporting Information). First, inhibition of subtilisin with an active-site-targeted inhibitor (phenylmethyl sulfonyl fluoride) eliminated both reactions. Second, **1** competitively inhibited the hydrolysis of the *N*-suc-AAPF-*p*NA peptide substrate[17] with an inhibition constant (K_i) of 7 ± 2 mM, showing that **1** bound to the active site of subtilisin Carlsberg. The affinity of **1** for other subtilisins was similar (subtilisin BPN′: $K_i = 8 \pm 2$ mM; subtilisin E: 12 ± 3 mM).

Subtilisin Carlsberg also catalyzed sulfinamide hydrolysis in three other *N*-acyl sulfinamides, **2**−**4** (Figure 1c, Table 1). The *N*-dihydrocinnamoyl acyl group in **2** mimics phenylalanine's side chain. Subtilisin Carlsberg catalyzed approximately equal amounts

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Table 1. Chemoselectivity and Enantioselectivity of Subtilisin-Catalyzed Hydrolysis of *N*-Acyl *p*-Toluenesulfinamides **1**−**4**

			Total Hydrolysis[a,b]			Sulfinamide Hydrolysis				Carboxamide Hydrolysis			
entry	substrate	subtilisin	conv. %[b]	ee_S, %[c]	amount[d]	% S–N[e]	ee_P calcd[f]	E'[g]	E[h]	% C–N[i]	ee_P, %[j]	E'[g,k]	E[h,k]
1	**1**	none	7	0	0	5	0	N/A[m]		2	0	N/A	
2	**1**	Carlsberg[l]	55	85 (*S*)	6	44 (39)	72 (*R*)	11	17	11 (9)	59 (*R*)	4	6
3	**1**	E	53	98 (*S*)	5	<3	N/A			53 (51)	91 (*R*)	>75	>75
4	**1**	BPN′	49	85 (*S*)	5	10 (5)				39 (37)	93 (*R*)	54	>75
5	**2**	Carlsberg	57	83 (*S*)	6	30	69 (*R*)	7	7	27	57 (*R*)	N/A	4
6	**2**	BPN′	30	44 (*S*)	5	<3				28	94 (*R*)	N/A	46
7	**3**	none	21	0	0	20	0	N/A		1	0	N/A	
8	**3**	Carlsberg	49	27 (*S*)	6	39 (19)	16 (*R*)	1.5	2.2	10	80 (*R*)	10	18
9	**3**	BPN′	27	13 (*S*)	5	17 (0)	<3	N/A		10	90 (*R*)	20	>75
10	**4**	Carlsberg	55	21 (*S*)	20	55	N/A	2	2	<3			

[a] Reactions performed at 25 °C in BES buffer (50 mM, pH 7.2). Reaction times: **1** = 6 h, **2** = 24 h, **3** = 24 h, **4** = 72 h. [b] Total conversion from the disappearance of **1**−**4**. Estimated error ±2% conversion. [c] The ee_S = enantiomeric excess of remaining starting material measured by HPLC. [d] Amount of enzyme (nmol). [e] Calculated amount of the total conversion attributed to S−N bond hydrolysis; the value with the chemical reaction subtracted is in parentheses. [f] Calculated enantiomeric excess for the portion of **1**−**4** that disappeared via the S−N cleavage path. [g] E' = observed enantioselectivity (uncorrected for spontaneous chemical hydrolysis) calculated from conversion, c, and ee_P or ee_S.[15] [h] E = enantioselectivity, corrected for spontaneous chemical hydrolysis using a computer program.[16] [i] Percent C−N conversion; C−N cleavage based on formation of *p*-toluenesulfinamide. [j] Enantiomeric excess of the C−N hydrolysis product, *p*-toluenesulfinamide. [k] Small errors in conversion create large errors in E when E is high. For this reason, the E values are given as lower limits; the E-values calculated from the data are higher in some cases. [l] Three commercial samples of subtilisin Carlsberg gave similar values. [m] Not applicable.

of sulfinamide and carboxamide hydrolysis (30 and 27%, respectively). The *N*-methoxyacetyl compound **3** reacted slowly with subtilisin Carlsberg and gave approximately twice as much sulfinamide hydrolysis as carboxamide hydrolysis (39% S−N hydrolysis (including 20% chemical) and 10% C−N hydrolysis, entry 8). In contrast, subtilisin BPN′ showed only carboxamide hydrolysis with compounds **2** and **3** (entries 6 and 9). The *N*-*p*-toluoyl compound **4** is pseudo-symmetric since both sulfinyl and carbonyl substituents are *p*-tolyl. This compound tests the preference for sulfinamide versus carboxamide hydrolysis in similar steric and electronic environments. Subtilisin Carlsberg catalyzed exclusively a slow sulfinamide cleavage (>10:1, no detected carboxamide hydrolysis) with low enantioselectivity (E = 2, entry 10), while subtilisin BPN′ showed no reaction with compound **4**.

Sulf*on*yl compounds are irreversible inhibitors of serine proteases that form stable sulf*on*yl enzyme complexes, which have been characterized by X-ray crystallography.[18] Peptides containing sulf*in*amide functionality were proposed as protease inhibitors, but synthesis was unsuccessful.[19] Sulfinamide hydrolysis by subtilisin Carlsberg may involve a sulf*in*yl serine intermediate, but we currently have no direct evidence for or against such an intermediate. Sulfatases catalyze the hydrolysis of sulfate esters via a different mechanism. They contain an α-formylglycine residue, not a serine, at the active site and react via a sulfate hemiacetal.[20] Similarly, sulfamidase catalyzes the cleavage of the S−N bond in *N*-sulfoglucosamine to give sulfate.[21]

These results demonstrate that subtilisin Carlsberg catalyzes the unnatural sulfinamide cleavage even when a carboxamide group is available. A related example involved inactivation of the serine protease elastase with a β-sultam (β-lactam analogue), where cleavage favored the strained sulfonamide over the unstrained carboxamide bond.[11] The structurally similar subtilisins BPN′ and E do not cleave these sulfinamide groups, suggesting that a few amino acid substitutions may introduce this catalytic promiscuity.

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Supporting Information Available: Experimental details for synthesis, preparation of enzymes, and analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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