bond lengths involving non-hydrogen atoms, and bond angles involving non-hydrogen atoms and complete details of the analysis (16 pages); table of observed and calculated structure factors (7 pages). Ordering information is given on any current masthead page.

Conversion of a Protease into an Acyl Transferase: Selenolsubtilisin†

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Well-characterized proteins represent valuable starting points for the construction of new biocatalysts. For example, chemical methodology can be used to introduce non-natural amino acids or catalytic prosthetic groups into preexisting binding pockets to yield hybrid molecules that combine the intrinsic chemistry of the prosthetic group with the binding specificity of the protein template.[1] We are interested in the biological properties of selenium and have developed a chemical approach for converting reactive serine residues in proteins into selenocysteines. We report here the preparation and preliminary characterization of the first artificial selenoenzyme, selenolsubtilisin. Since the organic chemistry of selenium is extensive and of synthetic importance,[2] semisynthetic selenoenzymes with template-imposed selectivities might have considerable practical utility.

Subtilisin is a bacterial serine protease [EC 3.4.21.14] that is chemically[3] and structurally[4] well-studied. Conversion of the active site serine (Ser221) into selenocysteine was accomplished by a two-step protocol, analogous to Polgār's method for making thiolsubtilisin.[5] The side-chain alcohol of Ser221 of subtilisin Carlsberg (Sigma, Protease VIII) was selectively activated by reaction with phenylmethanesulfonyl fluoride (PMSF), and the sulfonylated enzyme (1 mM) was treated with a large excess of hydrogenselenide (ca. 0.5 M) in aqueous buffer (50 mM PIPES, 20 mM $CaCl_2$, pH 6.8) for 36 h at 40 °C. After gel filtration of the crude reaction mixture on Sephadex G-25, the protein fraction was reduced anaerobically with sodium borohydride, and selenolsubtilisin was purified by affinity chromatography on thiopropyl-Sepharose 6B. The yield of selenoenzyme was typically 40–50% based on phenylmethanesulfonylsubtilisin. Greater than 0.95 equivalents of selenium were incorporated per mol of subtilisin (ϵ^{280} 23 500 M^{-1} cm^{-1}) as judged by anaerobic titration of the reduced enzyme with 5,5'-dithiobis(2-nitrobenzoic acid)[6] and by neutron activation analysis.[7]

Serine (and cysteine) proteases cleave amides and esters by a two-step mechanism in which the active site nucleophile is transiently acylated by substrate. We wondered whether a selenol could assume the mechanistic role played by Ser221 in subtilisin. Like thiolsubtilisin,[8] selenolsubtilisin is a poor catalyst of amide hydrolysis, but it does promote the cleavage of activated acyl derivatives. For example, reduced selenolsubtilisin hydrolyzes cinnamoylimidazole under anaerobic conditions via an acyl-enzyme adduct. The covalent intermediate (λ_{max} = 308 nm) was isolated by gel filtration at pH 5 and 4 °C. Deacylation of the enzyme was monitored spectroscopically at 25 °C as a function of pH. For comparison, Carlsberg *O*-cinnamoylsubtilisin (λ_{max} = 289 nm) and *S*-cinnamoylthiolsubtilisin (λ_{max} = 310 nm) were also prepared and studied.[9]

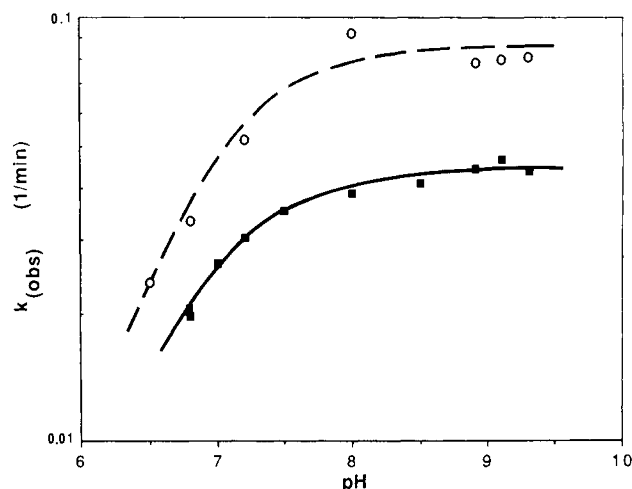

Figure 1. pH-rate profile for the hydrolysis of *S*-cinnamoylthiolsubtilisin (– – –) and *Se*-cinnamoylselenolsubtilisin (—) at 25.0 °C. The data were fit to the equation $k_{obsd} = (k_{lim})/(1 + [H^+]/K_a)$. The k_{lim} values for thiol- and selenolsubtilisin are 0.0837 and 0.0426 min^{-1}, respectively, and the relevant pK_a's are 6.9 and 6.8. Decinnamoylation of native subtilisin Carlsberg is described by a k_{lim} value of 22.8 min^{-1} and a pK_a of 7.7 (data not shown).

Table I. Ratio of Second-Order Rate Constants for Aminolysis and Hydrolysis of Cinnamoylated Subtilisin (O), Thiolsubtilisin, (S), and Selenolsubtilisin (Se)[a]

	$(k_{NH_2}/k_{OH})_O$	$(k_{NH_2}/k_{OH})_S$	$(k_{NH_2}/k_{OH})_{Se}$
n-$BuNH_2$	6	3600	81000
Gly-NH_2	19	7400	27000
NH_2OH	88	1400	7700

[a] Reactions were carried out in 0.1 M borate buffer at pH 9.3 and 25.0 °C, and decinnamoylation was monitored spectroscopically at 300 (O) and 320 nm (S and Se). Products were characterized by reversed-phase HPLC.

As shown in Figure 1, the pH-rate profile for hydrolysis of *Se*-cinnamoylselenolsubtilisin is very similar to that of *S*-cinnamoylthiolsubtilisin. Both curves can be fitted with a kinetic scheme in which optimal activity requires ionization of a group with pK_a of about 7. The titrating species is presumably His64 which is essential for activity in the native enzyme.[3] While the limiting rate constant is within a factor of 2 for thiol- and selenolsubtilisin, deacylation of *O*-cinnamoylsubtilisin is more than two orders of magnitude faster (k_{lim} = 23 min^{-1}). Since the alkaline hydrolysis of structurally analogous esters, thiol esters, and selenol esters is usually comparable, steric effects within the active site, rather than electronic factors, presumably account for this effect. Indeed, the covalent radius of oxygen (0.65 Å) is considerably smaller than that of sulfur (1.03 Å) or selenium (1.16 Å).[10] The properties of the selenium heteroatom thus make it a novel mechanistic probe of steric and electronic effects in enzymatic catalysis.

Selenol esters reportedly undergo aminolysis considerably faster than esters and thiol esters,[11] making them potentially useful for the *synthesis* of amide bonds.[12] We measured the partitioning

† Dedicated to the memory of E. T. Kaiser.

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of the cinnamoyl enzymes between water and a number of amines at pH 9.3 and 25 °C. The aminolysis-to-hydrolysis ratios obtained are summarized in Table I. For each amine studied, the ratio of second-order rate constants increases substantially as one proceeds from subtilisin to selenolsubtilisin. The transfer of the cinnamoyl group to butylamine rather than water, for instance, is 14 000 times more efficient for selenolsubtilisin than for native subtilisin and more than 20 times more efficient than for thiolsubtilisin. Thus, by converting the active site serine into a selenocysteine it is possible to influence the *selectivity* for aminolysis over hydrolysis of the acyl-enzyme intermediate in a dramatic fashion. Kaiser and co-workers[13] have exploited the increased deacylation selectivity of thiolsubtilisin to catalyze peptide bond formation. Selenolsubtilisin, with its substantially higher aminolysis to hydrolysis ratios, might also find application as a specific peptide ligase[14] useful for convergent chemical syntheses of large proteins.

In summary, we have reengineered the active site of subtilisin by chemical conversion of the catalytically essential serine into a selenocysteine. Not only is the selenol group a valuable mechanistic probe but also through this transformation we have turned a proteolytic enzyme into an acyl transferase with selectivity properties suitable for a peptide ligase. In light of current interest[15] in naturally occurring selenoenzymes, we are now extending these studies to examine the redox and metal binding properties of selenolsubtilisin and are also applying our methodology to other protein active sites. We anticipate that the ability to tailor existing enzymes to perform specific functions through chemical modification will make entirely new enzymatic activities generally accessible for use in research, medicine, and industry.

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Investigations of the Biosynthesis of *trans*-(+)-*S*-1-Propenyl-L-cysteine Sulfoxide in Onions (*Allium cepa*)

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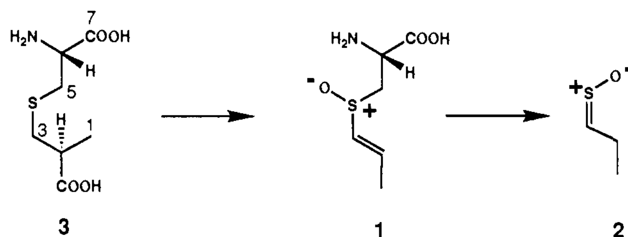
Both onions (*Allium cepa*) and garlic (*A. sativum*) contain a variety of novel sulfur compounds, some of which possess significant pharmacological activity.[1] The unusual amino acid *trans*-(+)-*S*-1-propenyl-L-cysteine sulfoxide (**1**) has been isolated from onions,[2] and it has been shown[2] to be the precursor of the lachrymatory principle characteristic of this plant, (*Z*)-propanethial *S*-oxide (**2**) (eq 1).[3] Investigations by Granroth[4]



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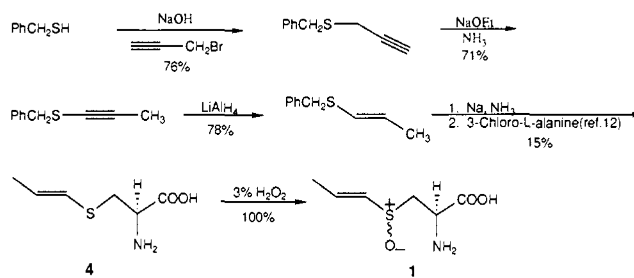
demonstrated that radioactivity from labeled (−)-*S*-(2-carboxy-*n*-propyl)-L-cysteine (CPC) (**3**) is incorporated into **1** by *A. cepa*, but rigorous evidence for the specific incorporation of **3** into **1** was not obtained. We now summarize the results of investigations that (a) provide unequivocal evidence for the specific incorporation of CPC into **1** and (b) place limitations on the number of mechanisms that can be envisioned for this interesting transformation.

In order to investigate the biosynthesis of the amino acid **1**, it was necessary to have access to adequate quantities of the compound for isotope dilution purposes. Attempts to isolate significant quantities of **1** from dehydrated onion powder[5] were unsuccessful, and a total synthesis of **1** reported[6] in 1975 proved to be unsatisfactory. We therefore devised the new synthesis of *trans*-*S*-1-propenyl-L-cysteine sulfoxide that is shown in Scheme I. The synthesis yields an approximately 1:1 mixture of diastereomeric sulfoxides. This mixture is adequate for dilution purposes, since it can be cleanly reduced[7] (70%) back to *trans*-*S*-1-propenyl-L-cysteine (**4**) using $K_3W_2Cl_9$. The sulfide **4** is considerably more stable than the corresponding sulfoxides, and it is therefore more suitable for isolation and purification after isotopic dilution with the sulfoxides.

Biosynthetic investigations began with experiments to prove that CPC is a specific precursor of **1**. 2(*RS*),6(*R*)-[1-^{3}H]CPC was synthesized in the manner outlined in Scheme II and mixed with 2(*RS*),6(*R*)-[^{35}S]CPC prepared by addition of [^{35}S]-L-cysteine to methacrylic acid.[8] The resulting doubly labeled precursor was administered to 3-week-old onion plants by the cotton wick method, and amino acid **1** was isolated by isotope dilution after 3 days. The crude amino acid was reduced to the

Scheme I



Scheme II

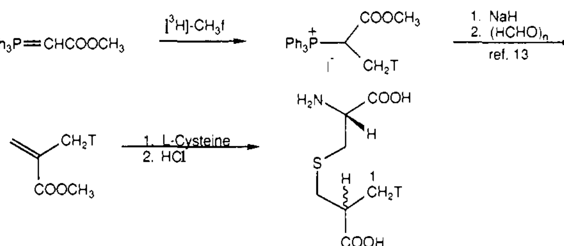


Table I. Administration of Labeled 2(*RS*)-*S*-(2-Carboxy-*n*-propyl)-L-cysteine to *A. cepa*

expt no.	labeling pattern in precursor 3	isotope ratio in precursor 3	isotope ratio in product 1[a]	% ^{3}H retention
1	[^{35}S,1-^{3}H]	^{3}H/^{35}S=5.03	^{3}H/^{35}S=4.68	93
2	[1-^{3}H,5,6,7-^{14}C]	^{3}H/^{14}C = 4.21	^{3}H/^{14}C = 4.09	97
3	[^{35}S,2-^{3}H]	^{3}H/^{35}S = 7.85	^{3}H/^{35}S = 6.50	83
4	3(*RS*)-[^{35}S,3-^{3}H]	^{3}H/^{35}S = 7.35	^{3}H/^{35}S = 3.43	47

[a] In experiments with ^{35}S, the ratio is corrected for decay.

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