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EXPERIMENTAL PROCEDURES

Materials. 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and Boc-amino acids were obtained from Nova Biochem (San Diego, CA), Boc-Arg (*p*-toluenesulfonyl)-OH and Boc-Asn(xanthyl)-OH were obtained from Bachem Bioscience (King of Prussia, PA). S-trityl-mercaptopropionic acid was obtained from Peptides International (Louisville, KY). Boc-Gly-OCH_2Pam-resin and N,N,-diisopropylethylamine (DIEA) were obtained from Applied Biosystems (Foster City, CA). MBHA resin was obtained from Peninsula Laboratories (Belmont, CA). All solvents of high purity were purchased from Fischer. Trifluoroacetic acid (TFA) was obtained from Halocarbon (River Edge, NJ). HF was purchased from Matheson Gas (Cucamonga, CA). Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, Bovine RNA, RNase A and Subtilisin were obtained from Sigma-Aldrich, Inc.

Peptide Synthesis. Peptides were prepared by manual solid phase peptide synthesis (SPPS) typically on a 0.4 mmol scale using the *in situ* neutralization/ HBTU activation procedure for Boc-chemistry as previously described [1]. The peptide coupling was carried with 5-fold excess (2.2 mmol) of activated amino acid for a minimum of 15 minutes. After each coupling, yields were determined by measuring residual free amine with the quantitative ninhydrin assay[2]. Side-chain protected amino acids used in this study were: Boc-Arg (*p*-toluenesulfonyl)-OH, Boc-Asn(xanthyl)-OH, Boc-Asp(*O*-cyclohexyl)-OH, Boc-Cys(4-methylbenzyl)-OH, Boc-Glu(*O*-cyclohexyl)-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Ser(benzyl)-OH, Boc-Thr(benzyl)-OH, and Boc-Tyr(2-Br-Z)-OH. Other amino acids were used without side-chain protection.

After chain assembly was completed, the peptides were deprotected and cleaved from the resin by treatment with anhydrous HF for 1 hr at 0°C with 4% *p*-cresol as a scavenger. After cleavage, peptides were precipitated with ice-cold diethylether, dissolved in aqueous acetonitrile and lyophilized.

TMPAL Resin : 2.2 mmol N^{α}-Boc Leu was activated with 2.0 mmol HBTU in the presence of 3.2 mmol DIEA and coupled for 16 min to 0.4 mmol MBHA resin (99.9% coupling yield). (Note: Boc-Leu Pam resin has also been used as the initial solid support) Next, 0.6 mmol S-trityl mercaptopropionic acid was activated with 0.54 mmol HBTU in the presence of 1.2 mmol DIEA and coupled for 16 min to Leu-MBHA resin (99.94% coupling). The resulting trityl-associated mercaptopropionic acid-leucine (TAMPAL) resin was used as a starting resin for polypeptide chain assembly following removal of the trityl protecting group with 2 × 1 min treatments with 2.5% triisopropylsilane and 2.5% H_2O in TFA. The C-terminal amino acid Boc-Asp(OcHex)-OH/Boc-Thr(Bzl)-OH was coupled using standard coupling protocol, and after the chain elongation the C-terminal thioester peptide was obtained by cleavage with anhydrous HF.

High Pressure Liquid Chromatography (HPLC). Analytical reversed-phase HPLC was performed on a Hewlett Packard HPLC 1050 system using Vydac C-18 columns (5 μm, 0.46 x 15 cm). Semipreparative reversed-phase HPLC was performed on a Rainin HPLC system using a Vydac C-18 column (10 μm, 1.0 x 25 cm). Linear gradients of acetonitrile in water/0.1% TFA were used to elute bound peptides. The flow rates used were 1 ml/min (analytical), and 5 ml/min (semipreparative). Preparative reversed-phase HPLC was performed on WatersDelta Prep-4000 HPLC system using Vydac C-18

column (5.0 x 25 cm). The peptides were purified using linear gradients of acetonitrile in water in water/0.1% TFA with a flow rate of 30 mL/min.

Mass Spectrometry. Electrospray ionization mass spectrometry (ESI-MS) was performed on an API-III triple quadrupole mass spectrometer (PE-Sciex). Peptide masses were calculated from the experimental mass to charge (*m/z*) ratios using MacSpec software (Sciex). Theoretical masses of peptides and proteins were calculated using MacProMass software (Beckman Research Institute, Duarte, CA).

Native Chemical Ligation. The ligation of unprotected synthetic peptide segments was performed as follows: 0.1 M sodium phosphate, pH 6.3, containing 6 M guanidine, and 2% (v/v) thiophenol was added to dry peptides resulting in a final peptide concentration of 1 mM at a pH ≈ 5 (lowered due to addition of thiols and TFA from the lyophilized peptide). The pH of the solution was measured with pH indicator strips from colorpHast pH 4-7. The ligation reaction was performed at room temperature and vortexed periodically to equilibrate the thiol additives and monitored by HPLC and ESI-MS until completion.

Conformationally Assisted Ligation. The self-associating peptides of CI2, CI2(1-39) [C-terminal thioester] and CI2(40-41) [N-terminal Cysteine/Methionine] were ligated under folding conditions using 0.1 M sodium phosphate, pH 6.3, and 2%(v/v) thiophenol. The ligation was performed at room temperature with periodic vortexing and the final pH of the reaction was ≈ 5. The progress and completion of the ligation reaction was monitored by HPLC and ESI-MS. In a typical reaction 2.6 mg (0.57 μmol) of CI2(1-39) and 2.0 mg (0.6 μmol) of CI2(40-64)M40C were taken in 0.5 mL of 100 mM sodium phosphate pH

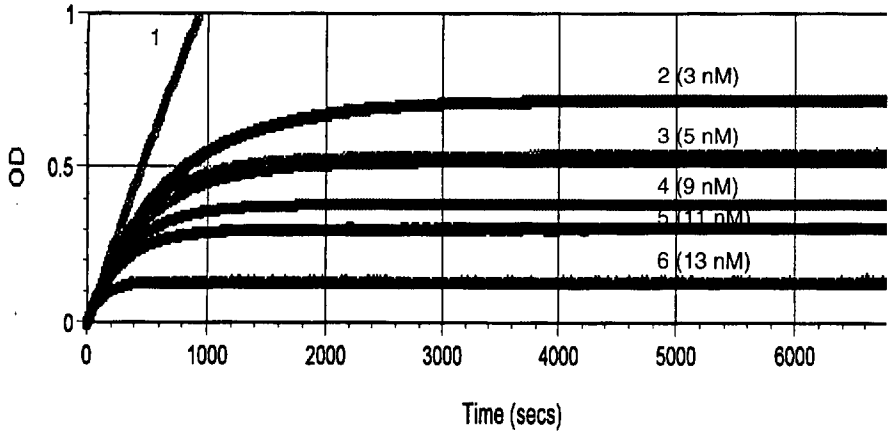
6.3, and 2% (v/v) thiophenol to obtain 3.05 mg of the pure CI2(1-64)M40C in 72% yield after HPLC purification.

Chymotrypsin Inhibition Activity Assay : The assay was performed using Subtilisin carlsberg enzyme using OPTI*max* microtiterplate reader from Molecular Devices. The substrate used was succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, enzyme concentration was calculated from initial rates of substrate hydrolysis. The reactions were carried out in 0.1 M Tris-HCl buffer pH 8.4, with a enzyme concentration of 0.25 nM and substrate concentration of 1 mM in a volume of 0.1 mL with varing inhibitor concentration of 0, 3, 5, 7, 9 and 11 nM. The reactions were followed at 412 nm monitoring the substrate hydrolysis. Ki was determined as described by Fersht et al.,[3] using eq-1. There was no reversal of inhibition even after long time on any of the mutants.

$(v_o-v_s)/v_s = [I]/[Ki(1+[S]/Km)]$ ----(1)

The Table shows the Ki Inhibition of Sutilisin Carlberg by CI2 and Mutants

CI2/mutants	**K_i (pM)**
CI2 reported	12.6
CI2 - T39D/A16G	32.9±4.6
CI2 - T39D	47.1±3.7
CI2 - T39D/M40C	79.2±5.2
CI2 - T39D/M40C/A16G	54.6±2.3



The curves 1-6 on the graph represent the substrate hydrolysis by subtilisin monitored in presence of varying amounts of Inhibitor (0, 3, 5, 9, 11&13 nm).

RNase Activity Assay . The RNase A was assayed by following the change in absorbancy of RNA on RNase A digestion, using Cary1 Bio/UV-Visible spectrophotometer. Bovine RNA (1mg/mL) was taken in 0.1 M Tris-HCl, 2 mM EDTA pH 7.8 the steady UV reading was monitored at 25°C, for a few minutes, quickly RNase A (10 μL of 0.1 μg/mL) was added with stirring and the decrease in the OD at 298 nm was monitored for atleast 5 min. The rate of absorbancy change was estimated and the activity was determined by the decrease in OD at 298 nm over 1 min, due to hydrolysis of RNA as described by Oshima et al.[4] (1 unit of enzyme corresponds to 0.01 absorbancy change/min in the above assay).

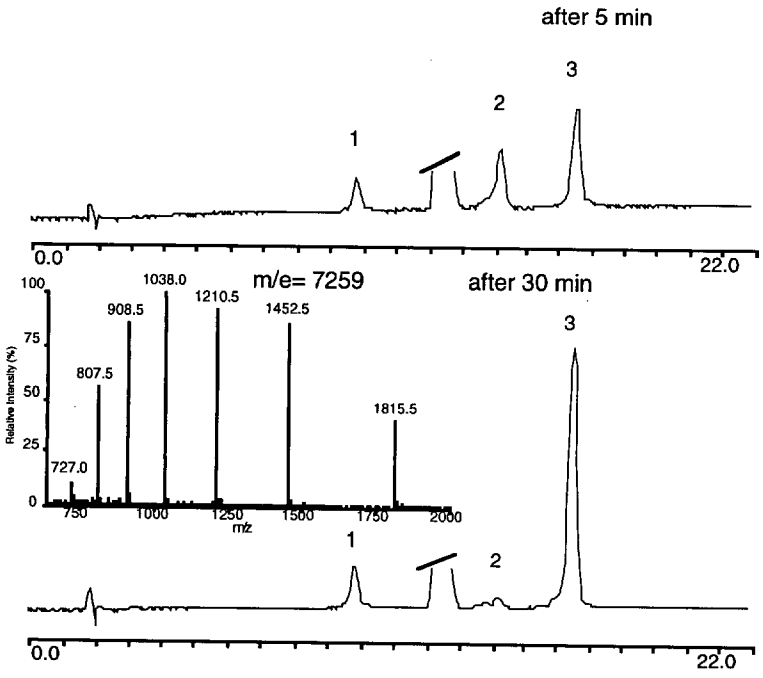
RNase A activity determined by RNA digestion

Source of RNase A	Units of RNase A/mL
RNase A (commercial sample)	1.52 units
RNase A (semisynthetic)	1.46 units

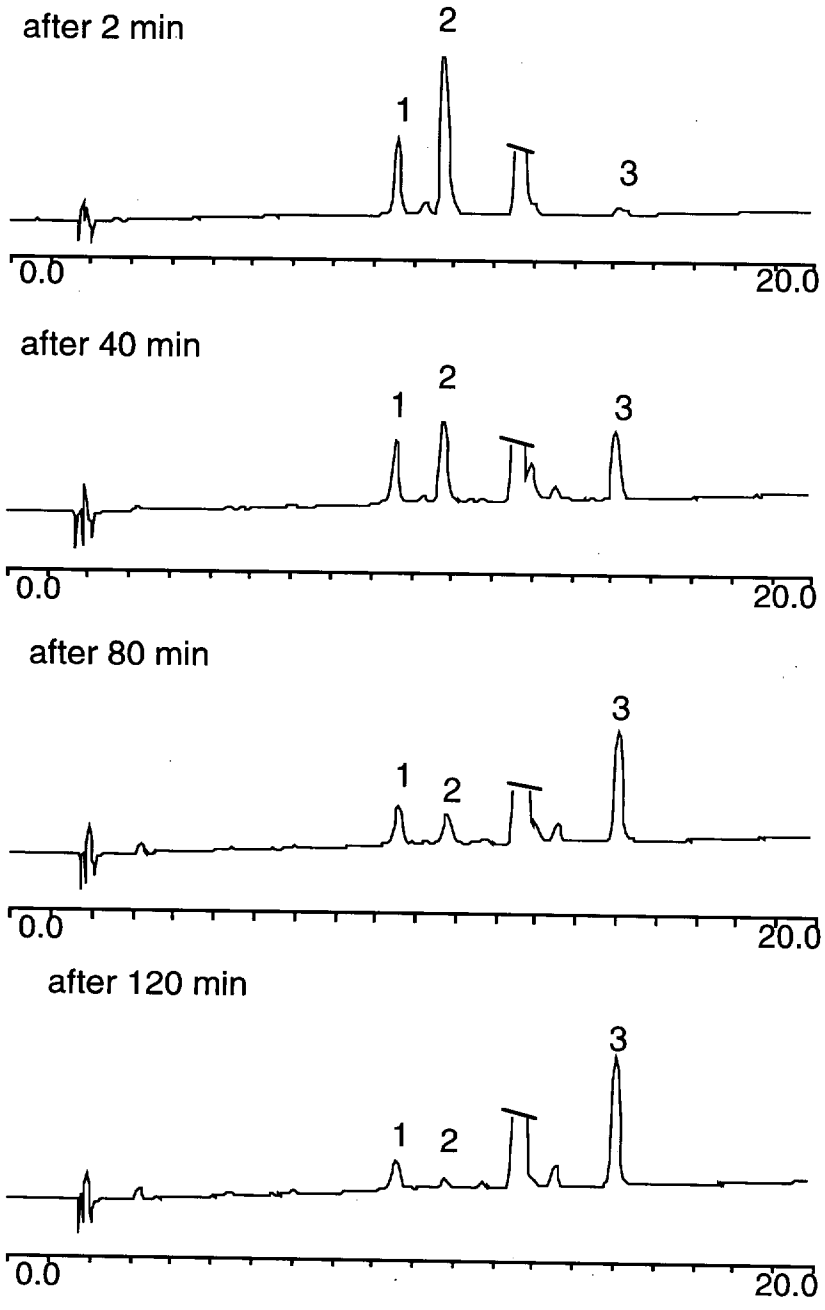
Molecular Masses of Peptides and Proteins Prepared

Peptides/protein	Molecular weights observed	Molecular weights calculated (average)
CI2(41-64)	3003.3 ± 0.5	3003.5
CI2(41-64)M40C	2975.6 ± 0.6	2975.5
(1-39)T39D	4514.5 ± 0.7	4514.2
(1-39)A16G/ T39D	4499.9 ± 1	4500.2
(1-64) T39D	7300.2 ± 0.9	7299.6
(1-64)M40C/ T39D	7272.7 ± 1.2	7271.6
(1-64)A16G/ T39D	7287.2 ± 1.5	7285.6
(1-64)A16G/M40C/ T39D	7258.9 ± 1.5	7257.5
S-pep	2366.5 ± 0.7	2366.5
S-pro	11535.2 ± 1	11534.0
RNase A	13683.4 ± 2	13682.3

The HPLC chromatogram showing Ligation of CI2(1-39)thioester (1) with CI2($_{cys}$40-64) (2) to give ≈70% product (3) in less than 5 min. The ligation was essentially complete after 30 min.



The HPLC chromatogram showing ligation of CI2(1-39)T39D thioester (2) with CI2(40-64)(1) to give the product (3) in 2 hrs.



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