Supplemental Data 1

Supplemental Methods

Purification of bovine β-trypsin − Bovine β-trypsin was purified from a commercial TPCK treated product by chromatography on soybean trypsin inhibitor-immobilized agarose with pH-step gradient elution to separate α- and β-forms of the enzyme (1, 2). Purified β-trypsin was stored in 1 mM HCl containing 10 mM $CaCl_2$. The β-trypsin concentrations was determined from the absorbance at 280 nm using the absorption coefficient of 1.54 $L^{-1} \cdot g^{-1} \cdot cm^{-1}$ and molecular weight of 23,900 (1).

Gel electrophoresis − Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 10% gel as described previously (3). Prior to SDS-PAGE, samples were boiled for 5 min in loading buffer containing 2% SDS, and in the presence or absent of reducing reagent. The gels were visualized by staining with Coomassie Brilliant Blue.

Preparation of immobilized pepsin column − Pepsin-immobilized column was prepared according to the method described previously (4). Pepsin was immobilized on POROS-20AL support through the formation and reducing of Schiff's base with pepsin. The immobilized

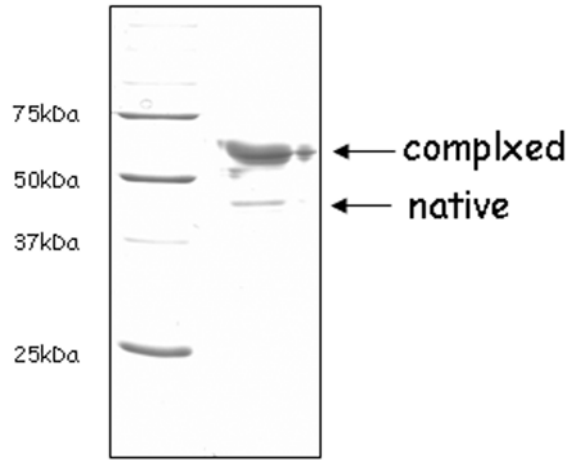
pepsin was packed in clean stainless steel columns (2 mm × 50 mm: Alltech) using AKTA explorer 100 system until back pressure reached 12 MPa. To remove the peptic peptides produced by autolysis among immobilized pepsins, additional extensive pumping was performed with 0.1% formic acid at room temperature until UV absorbance at 215 nm did not decrease further.

LC and ESI-MS spectrometry − The LC/MS (liquid chromatography and mass spectrometry) system for H/D-EX experiment contained two Agilent LC pumps, a sample injector, 10-ports valve, immobilized-pepsin column, C18-Trap column (Zorbax C18, 5×0.3 mm, 5 μm), and analytical C18 column (Magic C18, 3×50 mm, 5 μm). The injection valve, sample loop (100 μl), and all the columns were submerged in a 0~4 ºC ice bath containing 10% of ethanol (Fig. S3). In order to achieve better ionization and sensitivity, peptides were eluted and ionized through post-splitting (1: 19) to a tapered micro electrospray emitter. Finnigan LTQ linear ion-trap mass spectrometer was alternatively used to obtain ultra-zoom scan spectra (MS) or tandem mass spectra (MS2) with acquisition method in which MS survey scan from 350 to 1,500 m/z is followed by ultra-zoom scan spectra (10 m/z of precursor window) or tandem mass spectra (2 m/z of precursor window) for 15 min-data acquisition. Dynamic exclusion was used with 12 sec of duration and 8 m/z of mass window. Several air contaminant ions (371.0, 391.50,

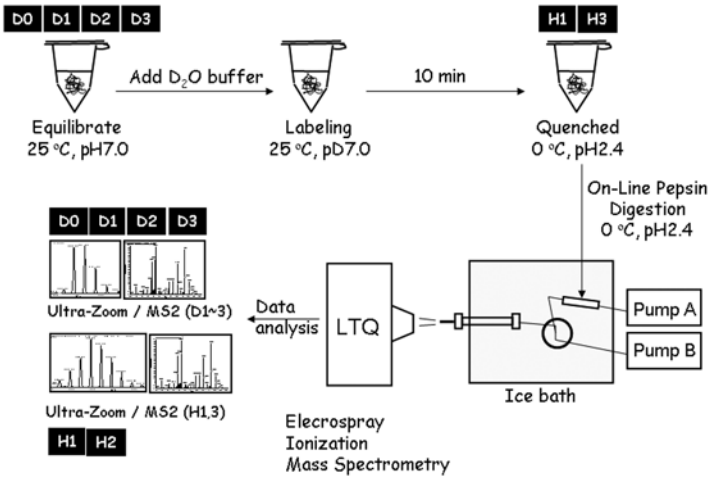
436.10, 445.20, 462.10, 536.10, and 610.10 m/z) were rejected with a window (low = 1.2, high = 3.2) from precursor ion lists. Tandem mass spectra were acquired with 30% of normalized collision energy. Ultra-zoom scans with 15-points smoothed data were further used for the calculation of average mass.

References

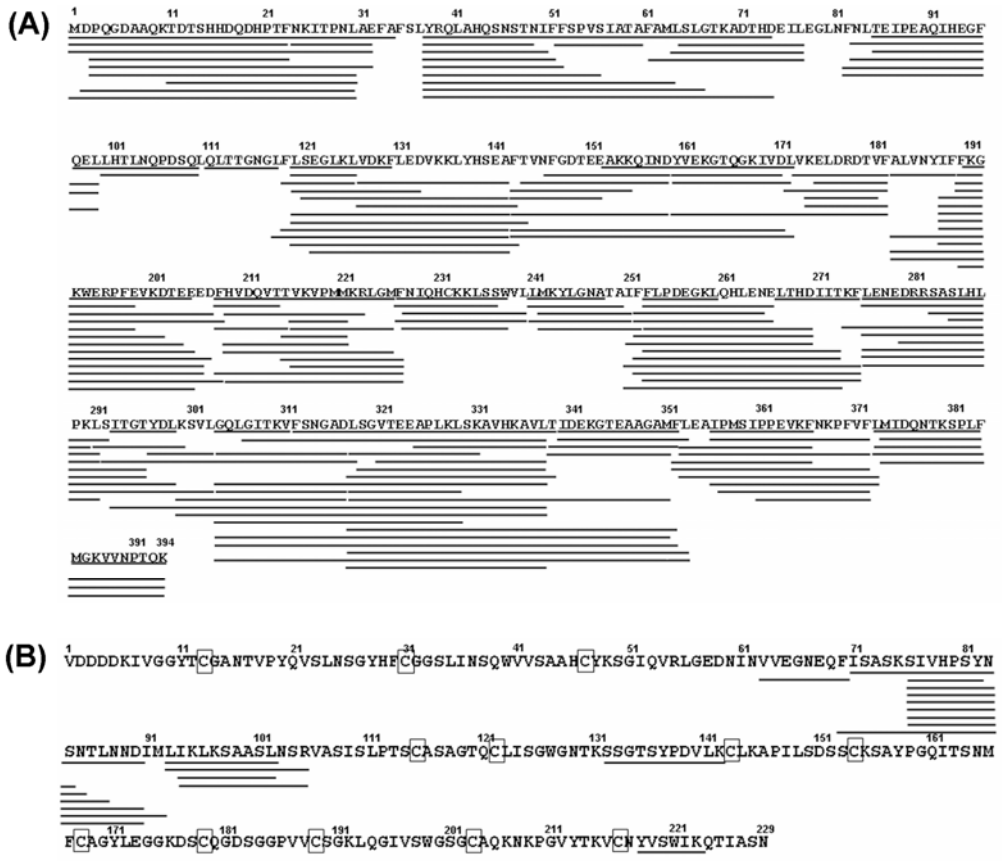
1. Robinson, NC, Tye, RW, Neurath, H, & Walsh, KA (1971) Isolation of trypsins by affinity chromatography. *Biochemistry* **10**: 2743-2747
2. Yung, BY & Trowbridge, CG (1980) A calorimetric comparison of trypsin and its anhydro modification in complex formation with Kunitz soybean inhibitor. *J Biol Chem* **255**: 9724-9730
3. Laemmli, UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685
4. Wang, L, Pan, H, & Smith, DL (2002) Hydrogen exchange-mass spectrometry: optimization of digestion conditions. *Mol Cell Proteomics* **1**: 132-138



Supplemental Fig. 1. SDS-PAGE of α_1AT complexed with β-trypsin. Complex formation of α_1AT was achieved with β-trypsin purified by step-gradient at pH 3.2 from soybean trypsin inhibitor column. Mixture of α_1AT and purified β-trypsin were incubated (α_1AT: active β-trypsin = 1:1) for 10 min in 20mM phosphate buffer (pD 7.0) at 25 ºC. The α_1AT-β-trypsin complex was analyzed on a nonreducing gel: *(left lane)* size markers; *(right lane)* α_1AT-β-trypsin complex.



Supplemental Fig. 2. Overview of HD-EX coupled mass spectrometric analysis with on-line pepsin column. H1 indicates native form of α_1AT and H3 indicates α_1AT-β-trypsin complex in H_2O. D series (D0-D3) indicate deutrated samples in D_2O buffer. D0 is a fully deutrated α_1AT in 7M urea-D4 (D_2NCOND_2) buffer (pD 8.0); D1 is deutrated native form of α_1AT; D2 is α_1AT deutrated during the complex formation with β-trypsin; and D3 is α_1AT-β-trypsin complex deutrated for 10 min at 25 ºC in D_2O buffer. The final deuterium content was 91.6% in all the samples.



Supplemental Fig. 3. Sequence coverage of (A) α_1AT and (B) β-trypsin. The lines indicate peptide fragments generated by using on-line pepsin immobilized column. The boxes in trypsin sequence indicate cysteine residues participating disulfide bond formation. Tandem mass spectrometry of the native (H1) and complexed α_1AT (H3) in H_2O identified 169 overlapping peptic fragments of α_1AT (98.2% coverage) and 16 peptic fragments (26.2% coverage) of β-trypsin. On-line digestion by immobilized-pepsin column was successfully accomplished for all the samples (H1, H2, D1, D2, and D3) but digestion patterns were not exactly identical among different samples.