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Supplementary Information

A Protease Inhibition Strategy Based on Acceleration of Autolysis

Yan Lv, Jianbin Zhang, Hao Wu, Shan Zhao, Yizhe Song, Shujun Wang, Bing Wang, Guojun Lv* and Xiaojun Ma*

Laboratory of Biomedical Materials Engineering, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China

Correspondence to: lgj1802@dicp.ac.cn, maxj@dicp.ac.cn

Experimental Methods

1 Materials

Dextran sulfate (DS, $\overline{M}_w$ = 500 kDa), trypsin (from bovine pancreas, potency ≥ 3000 BAEE units mg^{-1}), Ethyl N-benzoyl-L-arginine hydrochloride (BAEE, 342.8 Da, 98%), were all purchased from Aladdin Industrial Inc. (China) and used directly without any further processing. Insulin (from porcine pancreas, 27 units mg^{-1}, Wanbang Biopharmaceuticals Co. Ltd., China). Autolysis-resistant trypsin (Sequencing grade modified trypsin, potency ≥ 17569 BAEE units mg^{-1}, Promega Corporation, USA). All other chemicals were of analytical grade and commercially available.

2 Trypsin autolysis kinetics curves in the presence of different concentrations of DS.

2.1 Autolysis experiments

First, 4 mL DS (0.0002 - 2 mg mL^{-1}) containing tris-HCl (50 mM, pH = 8.0) was preheated at 37 °C. Then, 4 mL of trypsin (0.08064 mg mL^{-1}, 240 BAEE units mL^{-1}) containing $CaCl_2$ (1 mg mL^{-1}, 9 mM) and HCl (1 mM) was added followed by vigorous stirring. After each incubation period at 37 °C, an 800 μL sample was immediately acidified by adding 200 μL of 75 mM HCl and cooled to room temperature. A negative control containing no DS was performed according to the same protocol. A 100% activity control is a negative control maintained at 4 °C rather than 37 °C all the time. All experiments were replicated three times.

2.2 Determination of the residual activity

The residual activity of trypsin in each sample was analyzed within 30 minutes using BAEE as a substrate and an indicator. 500 μL sample at room temperature was mixed with 2.5 mL of BAEE (0.8 mM) containing tris-HCl (25 mM, pH = 8.0), immediately, the increase in absorbance caused by the hydrolysis of BAEE to N-α-benzoyl arginine was recorded with UV spectroscopy (Shimadzu, UV-2550) at 5-second intervals for 1.5 minutes. The residual activity of trypsin at a certain time is proportional to the degradation rate calculated by linear fitting their corresponding UV curves. All the relative residual activity was calculated via normalization according to the 100% activity control and given as a mean ± SD (n=3).

2.3 Curves fitting and half-life values calculation

Autolysis kinetics curves of trypsin incubated with DS was drawn by fitting the tryptic residual activity at different time using a modified first-order equation (**Table S1**). The half-life of trypsin

incubated with certain concentration of DS was calculated by **equation S1**.

$$t_{\frac{1}{2}} = \frac{1}{k}\ln\left(\frac{A_i - 100\%}{A_i - 50\%}\right) \qquad \text{(S1)}$$

3 Inhibition effects of different anionic polymers towards trypsin.

According to the protocol of **section 2.1**, 400 μL trypsin (0.08064 mg mL^{-1}, 240 BAEE units mL^{-1}) containing $CaCl_2$ (0.9 mM or 9 mM) and HCl (1 mM) was mixed with different anionic polymers (400 μL 0.08064 mg mL^{-1}) containing Tris HCl (50 mM, pH = 8.0) followed by incubation at 37 °C for 10 min. After that, 200 μL HCl (75 mM) was added to terminate the autolysis. A negative control containing no polymer was performed according to the same protocol. A 100% activity control is a negative control maintained at 4 °C rather than 37 °C all the time. All experiments were replicated three times. Residual activity in the samples was analyzed within 30 minutes refer to the method in **section 2.2**.

4 Autolysis-resistant trypsin control experiment to prove that the autolysis feature of trypsin is as essential for its inhibition by DS.

According to the protocol of **section 2.1**, 400 μL modified trypsin containing $CaCl_2$ (0.1 mg mL^{-1}, 0.9 mM) and HCl (1mM) was mixed with 400 μL DS (2 μg mL^{-1}) containing Tris HCl (50 mM, pH = 8.0) followed by incubation at 37 °C for 15 min. After that, 200 μL HCl (75 mM) was added to terminate the autolysis. A negative control containing no DS was performed according to the same protocol. A 100% activity control is a negative control maintained at 4 °C rather than 37 °C all the time. All experiments were replicated three times. Residual activity in the samples was analyzed within 30 minutes refer to the method in **section 2.2**.

5 Characterization of the tryptic autolysis products during incubation with DS using SDS-PAGE.

5.1 Samples preparation

4 mL trypsin (2 mg mL^{-1}) containing $CaCl_2$ (1 mg mL^{-1}, 9 mM) was mixed with 4 mL DS (0.496 mg mL^{-1}) containing Tris HCl (50 mM, pH = 8.0). After each incubation period at 37 °C, an 800 μL sample was immediately mixed with 200 μL of 1 M Na_2SO_4 and cooled in an ice bath to terminate the autolysis acceleration. A negative control containing no DS was performed according to the same protocol.

5.2 SDS-PAGE

24 μL sample was mixed with 6 μL loading buffer (×5) followed by 5-minutes incubation at 95 °C. Then, 20 μL sample were enriched by a 5% gel (pH 6.8) and separated by a 15% gel (pH 8.8) at 100 V for 60 minutes. The SDS-PAGE gel was stained overnight using Coomassie brilliant blue and imaged in an optical imaging system (Carestream, Gel Logic 2200 PRO, USA).

6 Characterization of the tryptic autolysis products during incubation with DS using MALDI-TOF.

6.1 Samples preparation

400 μL trypsin (2.5 mg mL^{-1}) containing $CaCl_2$ (1 mg mL^{-1}, 9 mM) was mixed with 400 μL DS (0.62 mg mL^{-1}) containing Tris HCl (50 mM, pH = 8.0) followed by incubation at 37 °C for 2 hours. After that, sample was purified using an ultra-filtration centrifuge tube (Molecular weight cut-off, MWCO = 100 kDa) at 1500×g for 10 minutes to remove DS from sample, since it was proved that the DS hindered the ionization of large autolysis peptides in MALDI-TOF. A negative control containing no DS and maintained at 4 °C all the time was performed according to the same protocol.

6.2 Mass spectrometry characterization

The samples were characterized with a MALDI-TOF/TOF 5800 Mass spectrometry system (AB SCIEX, USA), which was operated in the reflection mode (for mass range 600 - 4,000 m/z) or linear model (for mass range 4,000 - 30,000 m/z) for positive ions. Prior to analysis, the instrument is calibrated with a mixture of peptide standards (Mass Standards Kit for Calibration of AB SCIEX TOF/TOF instruments) with a maximum mass measurement error tolerance of ± 0.5 m/z. Samples aliquots (0.5 μL) were pipetted onto an Opti-TOF 384 MALDI target plate (AB SCIEX) and left to dry at room temperature. overlaid with 0.5 μL of a matrix solution and left to dry: α-cyano-4-hydroxycinnamic acid (CHCA, 10 mg in 0.5 mL of 0.1% TFA and 0.5 mL acetonitrile) for mass range 600 - 4,000 m/z; sinapic acid (SA, 10 mg in 0.66 mL of 0.1% TFA and 0.33 mL acetonitrile) for mass range 4,000 - 30,000 m/z. Mass spectra were accumulated from 1,000 laser shots at laser intensity 4,000 for mass range 600 - 4,000 m/z; 5000 for mass range 4,000 - 10,000 m/z; 6,000 for mass range 10,000 - 30,000 m/z.

7 **Equilibrium-dialysis control experiment** to prove that the direct contact rather than Ca^{2+}-deprivation between DS and trypsin is necessary for the autolysis acceleration.

10 mL DS (4 μg mL^{-1}) was isolated in a dialysis bag (MWCO = 7 kDa), which was completely submerged

in 20 mL solution containing $CaCl_2$ (0.2 mg mL^{-1}, 1.8 mM) and Tris HCl (50 mM, pH = 8.0). The whole setup was maintained for 4 hours at 4°C aiming to reach the mass transfer equilibrium of Ca^{2+}. Afterwards, it was incubated for 10 minutes at 37 °C after the addition of 10 mL trypsin (0.1613 mg mL^{-1}, 480 BAEE units mL^{-1}) into the solution outside the dialysis bag. 800 μL sample was withdrawn followed by mixing with 200 μL ice-cold HCl (75 mM). The residual activity in sample was measured according to the **section 2.2,** The result should be multiplied by 0.75, since the trypsin cannot penetrate the dialysis membrane diluting in the whole system. A negative control containing no DS was performed according to the same protocol. A 100% activity control is a negative control maintained at 4 °C rather than 37 °C all the time. A positive control was performed by directly blending DS with trypsin without a dialysis bag undergoing the same 10-minutes incubation at 37 °C. All experiments were replicated three times.

8 Characterizations of DS-trypsin complexes (DTCs)

8.1 Visual study of the formation and disintegration of DTCs

1 mL DS (1.24 mg mL^{-1}) containing Tris HCl (50 mM, pH = 8.0) was preheated at 37 °C in a glass container. 1 mL trypsin stock solution (10 mg mL^{-1}) containing $CaCl_2$ (1 mg mL^{-1}) and HCl (1 mM) was rapidly added into container with stirring, taking pictures immediately, and then the mixture had been kept at 37°C until it became transparent. Finally, another milliliter of trypsin stock solution was added into the same container, the above operation was repeated three times in total.

8.2 TEM imaging of DTCs

1 mL trypsin (1 mg mL^{-1}) containing $CaCl_2$ (1 mg mL^{-1}) and HCl (1 mM) was added drop-wise into equivolume DS (0.248 mg mL^{-1}) dissolved in Tris HCl (50 mM, pH = 8.0). Vigorous magnetic stirring of the solution was implemented as it sat in an ice bath. 100 μL sample was pipetted onto an 200-mesh carbon-coated copper grids and left to dry in a vacuum, then overlaid with 100 μL of phosphotungstic acid and vacuum dried again. The morphology of the DTCs was studied by TEM (JEOL, JEM-2100, Japan) at 200 kV.

8.3 Size and quantity variation of DTCs characterized by dynamic light scattering

1.5 mL trypsin (0.08064 mg mL^{-1}, 240 BAEE units mL^{-1}) containing $CaCl_2$ (0.1 mg mL^{-1}, 0.9 mM) and HCl (1 mM) was mixed with 1.5 mL DS (2 μg mL^{-1}) containing Tris HCl (50 mM, pH = 8.0) at 25°C. The average hydraulic diameter and count rate of DTCs in the sample were continually monitored by dynamic light scattering every 1 - 5 minutes using a Zetasizer (Malvern, UK., nano-ZS

90, 4mW, He–Ne laser, 633nm). Scattering light was detected at 90° by the automatically adjusted laser attenuation filters and the measurement position within the cell at 25 °C. A viscosity (0.8863 mPa·s) and a refractive index at 633 nm (1.330) of distilled water at 25 °C were used for data analysis. The instrument was routinely checked and calibrated using the standard reference latex dispersion (Malvern, Zeta potential transfer standards, Malvern, UK) and polystyrene particles (Nanosphere size standards, Duke Scientific Corporation, USA). Each experimental point was given as a mean value of 8-times scanning. The trendlines were drawn based on 8-points moving average method.

The average count rate was also monitored during the experiments. This parameter represents the scattering intensity of the sample in the absence of laser attenuation filters (adjusters of the laser power), therefore it can be used for comparison in the scattering intensity (kcps) between samples and indicates the particles concentration in the sample. The average count rate is calculated from the measured count rate divided by the attenuation factor.

9 Molecular weight variation of DS during autolysis acceleration characterized by gel permeation chromatography (GPC).

500 μL DS (5 mg mL^{-1}) dissolved in Tris HCl (50 mM, pH = 8.0) was mixed with 500μL trypsin stock solution at different concentrations (1, 3, 10 mg mL^{-1}). Such mixture was incubated at 37°C for 2 hours and then stored in 4 °C until analysis. A negative control containing no trypsin was performed according to the same protocol.

The characterization was carried out with a GPC system, which consisted of an HPLC pump (Waters 515) and a refractive index detector (Waters 2414, RIU-FS = 20 μRIU, 30 °C). After filtration through 0.22 μm, 20 μL sample was separated on a 7.8 × 300 mm column of TSK-gel G5000PWXL (TOSOH, Japan). The column was equilibrated and eluted with 25 mM Tris HCl buffer (pH 8.0) at 30 °C (flow rate: 0.7 mL min^{-1}).

10 The influence of various physical environments on the inhibitory effect of DS

10.1 The preparation of biological samples

All animal studies were performed in accordance with the institutional guidelines of Animal Care and Use Committee of Dalian Medical University, China. Male Sprague-Dawley rats (180 - 220 g) were executed after anesthesia. The small intestine (from 2 cm below the pylorus to 2 cm above the

caecum) was lavaged using 20 ml deionized water, the lavage fluid was gathered from about 5 mL to 15 mL. Meanwhile, the lung was lavaged using 10 ml deionized water. Then, the brain and lung were isolated and homogenated by repetitive freeze-thawing and ultrasonication (400W, 5s, 20 times) with water (1:9 w/w). All of the lavage fluid recovered and tissue homogenate were centrifuged and kept at -20 °C. The cell lysate of more than 10^6 HepG2 cell in 1 mL deionized water was prepared by ultrasonication (400W, 5s, 20 times) and kept at -20 °C after centrifugation.

10.2 Autolysis experiments

According to the protocol of **section 2.1**, 75 μL trypsin (0.4032 mg mL^{-1}, 1200 BAEE units mL^{-1}) containing $CaCl_2$ (9 mM) and HCl (1 mM) was mixed with different biological samples (150 μL) and incubated at 37 °C for 5 min, then, DS (75 μL 0.1 mg mL^{-1}) containing Tris HCl (50 mM, pH = 8.0) was added and incubated for 10 min. After that, 75 μL ice-cold Na_2SO_4 (1 M) was added to terminate the autolysis. A negative control containing no biological sample was performed according to the same protocol. A 100% activity control is a negative control maintained at 4 °C rather than 37 °C all the time. Residual activity in the samples was analyzed within 30 minutes refer to the method in **section 2.2**.

11 Insulin protection study using DS for preventing degradation by trypsin.

800 μL of artificial intestinal fluid containing Tris-HCl (24 μmol, pH = 8.0), $CaCl_2$ (0.6 mg), and trypsin (144 BAEE units) was prepared. Then, 200 μL of inhibitor DS was added, and the mixture was incubated for 10 minutes. After adding insulin (200 μL, 0.3 mg), the solution was incubated at 37°C for 2 hours. A 200 μL sample was immediately acidified by adding 100 μL of 0.2 M HCl. Each sample was analyzed by HPLC (Waters e2695) with a UV detector (Waters 2998). Undegraded insulin was separated on a C_{18} column (4.6 × 250 mm, Elite, Dalian, China) at 40°C, and the mobile phase consisted of 29 vol% acetonitrile and 71 vol% aqueous phase (0.2 M Na_2SO_4, 45 mM H_3PO_4-ethanolamine buffer, pH = 2.3). All experiments were replicated three times. The inhibitory potency was reflected by IC_{50}, which represents the inhibitor concentration required for 50% inhibition of insulin degradation.

Additional Figures and Tables

Table S1. Fitting data of autolysis kinetics curves

fitting equation: $A(t) = (100\% - A_i)e^{-kt} + A_i$

Concentrations of DS mg mL^{-1}	k ×10^{-3}	A_i %	R*	Half-life min	Multiple
0	1.92	42.76	0.9970	1077	1
10^{-4}	20.0	35.85	0.9984	75.44	14.3
3×10^{-4}	24.2	19.51	0.9993	40.06	26.9
10^{-3}	286	13.25	0.9998	3.006	358
10^{-2}	542	7.513	0.9992	1.463	736
10^{-1}	467	15.28	0.9996	1.912	563
1	470	19.54	0.9995	2.066	521

*: Goodness of fitting represented by the square root of coefficient of determination (R^2).

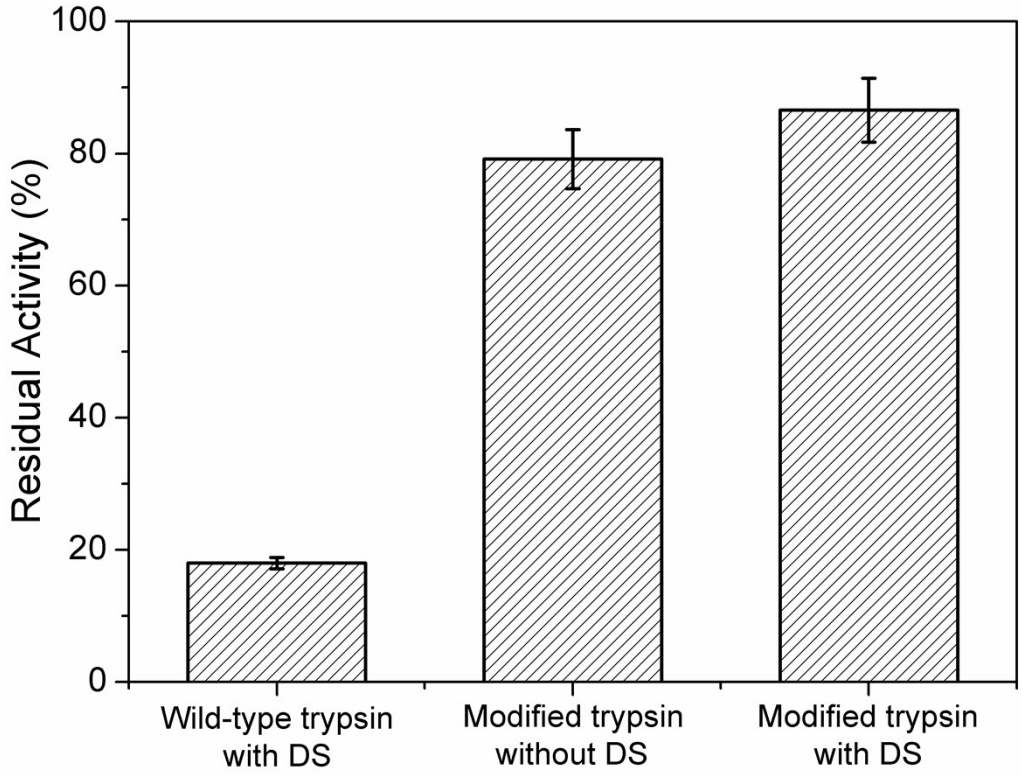

Fig. S1 The inhibition effect of DS on an autolysis-resistant modified trypsin compared with wild-type trypsin (SD, n = 3).

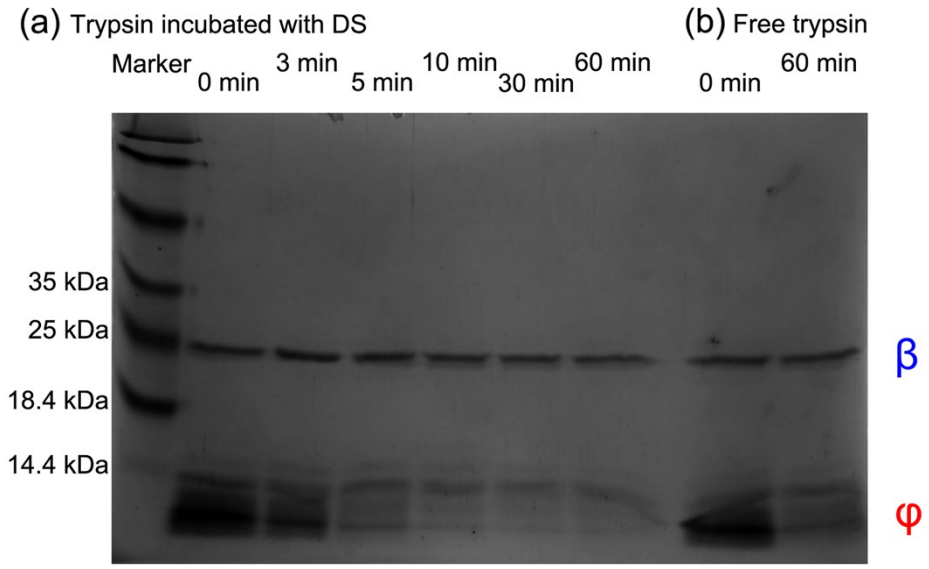

Fig. S2 SDS-PAGE image of the autolytic products (a) incubated with DS at 37 °C for 2 hours compared with free trypsin (b). φ: double-chain φ-trypsin. β: single-chain β-trypsin.

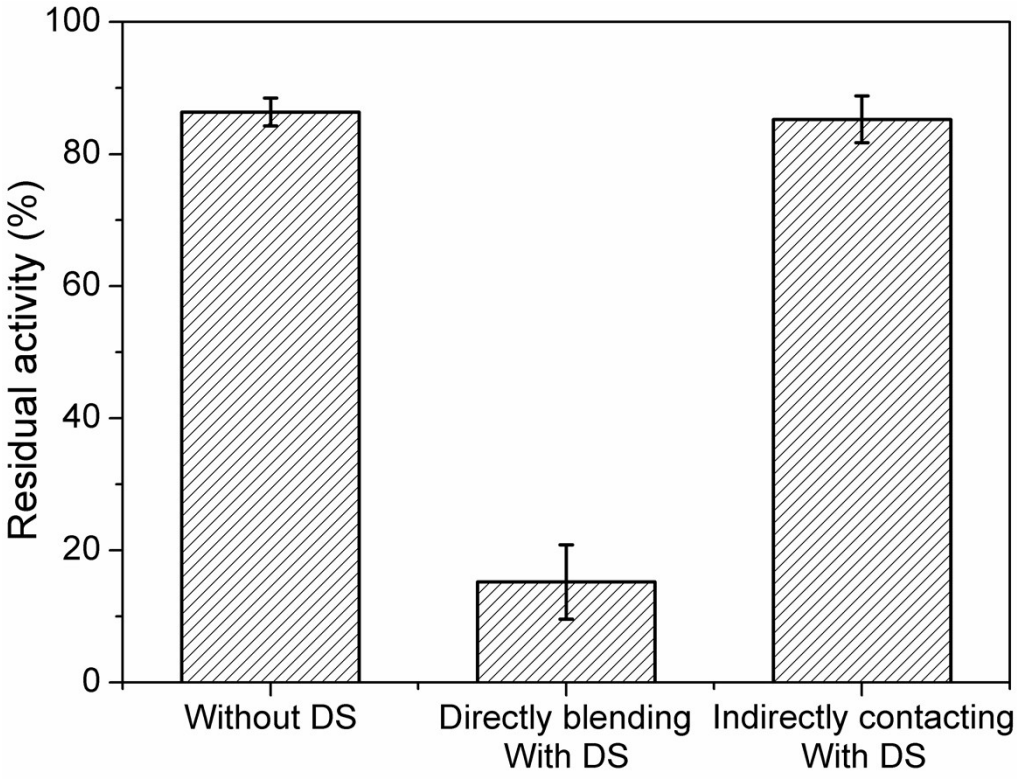

Fig. S3 The residual activity of trypsin after 10-minutes incubation at 37 °C with DS in different contact mode (SD, n = 3).

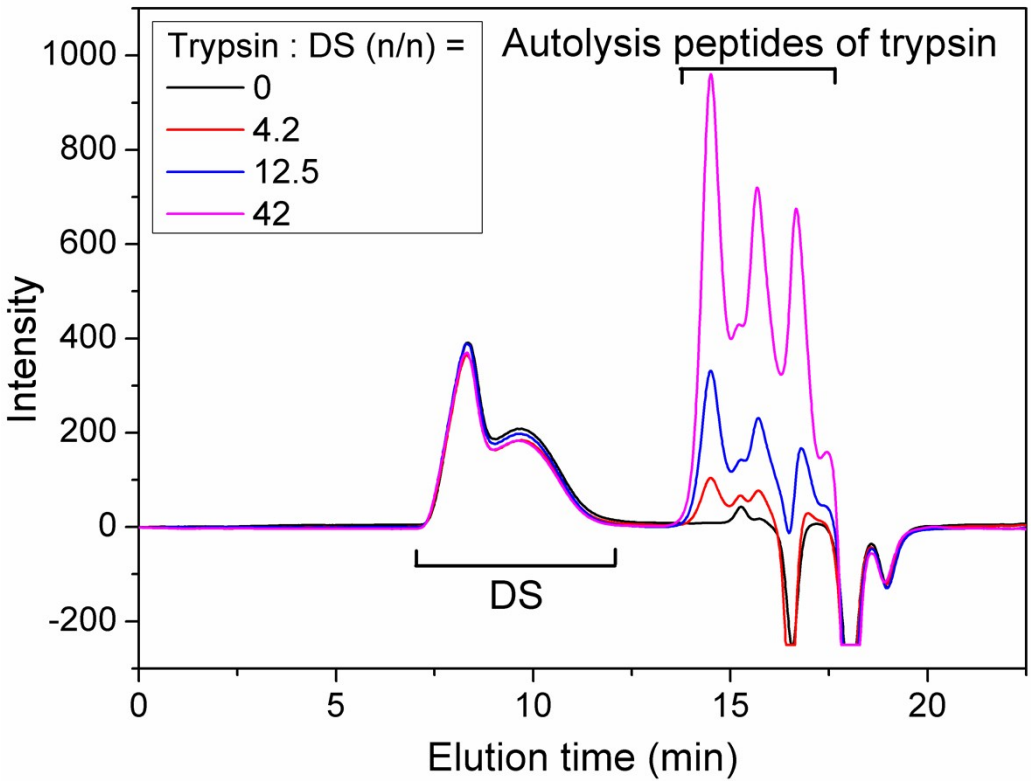

Fig. S4 The molecular weight distribution of DS after incubation with different molar ratios of trypsin for 2 hours at 37 °C characterized by GPC.