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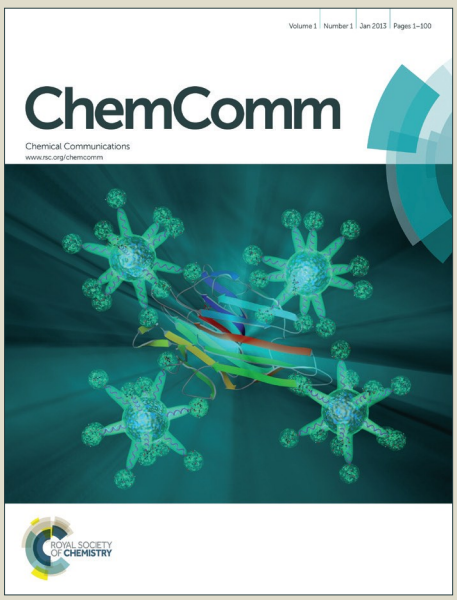

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A Protease Inhibition Strategy Based on Acceleration of Autolysis

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We report on an iconoclastic strategy for inhibiting proteases via autolysis acceleration. We show that proteases can be concentrated and induced to rapidly self-digest by a biocompatible polymer served as an efficient catalyst. This new generation of protease inhibitors may find applications in the treatment of various protease-dependent diseases.

Protease inhibitors (PIs) have attracted increasing attention due to their ability to regulate physiological metabolism and their use as alternative therapies for cancer[1], AIDS[2], Alzheimer's[3] and other diseases.[4] However, the large-scale applications of conventional PIs (usually proteins or peptides) are limited by their low efficiency and high biodegradability, which is due to their conventional inhibition strategy of the "lock and key" theory. Hence, iconoclastic and more efficient strategies are necessary in order to design more efficient and stable PIs for clinical use.

Most exocrine proteases (e.g. trypsin, α-chymotrypsin) undergo autolysis, or self-digestion,[5] and their autolytic rate is concentration-dependent. By creating autolysis-acceleration protease inhibitors, called AA-PIs, it is possible to increase the local concentration of a protease and increase its collision probability thereby increasing its autolytic rate. Finding suitable AA-PIs that would be specific for different kinds of proteases would be a considerably promising but also challenging task.

For example, trypsin[6] is one of the most widespread proteases in the body and is positively charged (pI = 10.5) under physiological conditions. An effective AA-PI for trypsin must "attract" and "trap" trypsin without hindering its autolysis. Therefore, it must have many negative charges and a sufficiently large molecular weight. Although most anionic polymers (APs) are satisfactory, only some of them have an inhibitory effect towards trypsin in a physiological environment (Fig. 1). This is because single-charged APs (each polymer residue carries one net charge) tend to form a gel with Ca^{2+} instead of combining with trypsin. For instance, sodium alginate, polyacrylic acid, and carboxymethyl cellulose form a gel in the calcium ion concentration of human blood (2.5 mM); by contrast, the inhibition efficiency of double-charged APs, such as dextran sulfate (DS) and heparin sodium, are almost unaffected.

DS is the sulfonic acid derivative of dextran. It is easily prepared and biocompatible. We used this lead compound, or model AA-PI of trypsin, to evaluate our novel inhibition strategy and characterized its mechanism.

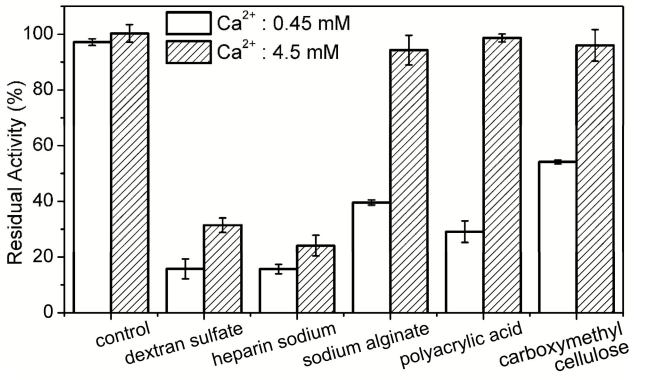

Fig. 1 Inhibitory effects of different anionic polymers toward trypsin. Trypsin was incubated with polymer (1:1 w/w) in the presence of 0.45 mM or 4.5 mM Ca^{2+} at 37°C (SD, n = 3) for 10 minutes.

Bovine pancreatic trypsin was incubated with different concentrations of DS at 37°C in artificial intestinal fluid containing 4.5 mM Ca^{2+}. Trypsin's residual activity was immediately suspended and measured after each incubation period (Fig. 2a). Our data showed that the half-life of trypsin was shortened more than 730 times by the presence of DS compared with the negative control, and this occurred at an extraordinarily low inhibitor-protease molar ratio of 1:84. Based on these data, this AA-PI polymer is likely the most efficient trypsin PI discovered thus far. All of the deactivation

kinetics of trypsin follow first-order kinetics (Eq. 1, fitting data: Table S1).

$$A(t) = (100\% - A_i)e^{-kt} + A_i \quad \text{Eq. 1}$$

The constant k represents the deactivation rate and A_i likely represents the percentage of inert trypsin not involved in the rapid deactivation process. The half-life of trypsin $t_{1/2}$ was calculated according to a formula deduced from Eq. 1 (Fig. 2b), which sharply declined with the increase in DS concentration.

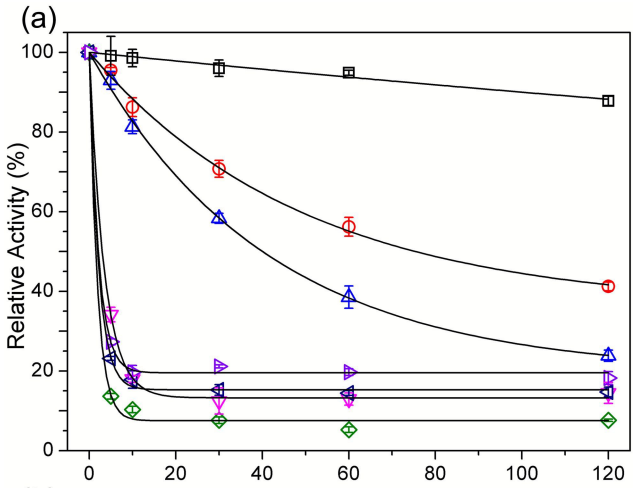

Fig. 2 The effect of DS concentrations on the autolysis rate of trypsin. (a) The autolytic kinetics of 120 BAEE units mL^{-1} trypsin incubated with various concentrations of DS at 37°C (SD, n = 3). (b) The relationship between the activity half-life of trypsin and the trypsin-DS molar ratio.

Unlike traditional PIs, the autolysis induced by AA-PIs is considered to be one of the underlying causes of tryptic deactivation. To test this hypothesis, an autolysis-resistant trypsin (modified by reductive methylation) was used as a control (Fig. S1).[7] There was no significant difference in the autolytic rate of this modified trypsin, even in the presence of DS. The result illustrates that the autolytic property of a protease is necessary for AA-dependent inhibition.

The autolytic products of trypsin were studied by MALDI-TOF (Fig. 3) and SDS-PAGE (Fig. S2).[8] Unlike the negative control, the great majority of trypsin molecules incubated with DS were rapidly degraded into small peptides over time, such as m/z 659.3, 824.5, 1699.5, 2011.1, and 4347.6. It was shown in Fig. 3 that double-chain trypsin (φ-trypsin) was more sensitive to AA than single-chain forms (β-trypsin). The φ-trypsin,[9] which consists of chain A (5.1 kDa) and chain B (17.9 kDa), was from β-trypsin (23 kDa) cleaved at Lys170-Asp171.

At this point, trypsin inhibition had been related to its autolysis acceleration (AA) by DS, but the precise mechanism of this process was unknown. Considering that Ca^{2+} is a cofactor for trypsin and stabilizes its structure, we determined whether AA was associated with Ca^{2+}-deprivation.[10] An equilibrium-dialysis control experiment was carried out by blocking the ability of DS from directly contacting trypsin using a dialysis bag (Molecular weight cut-off = 7 kDa) (Fig. S3). Our data confirmed that, although Ca^{2+}-deprivation can still occur, the autolytic rate of trypsin was no longer affected by DS. Our observations show that direct contact between AA-PIs and proteases is necessary for AA, while Ca^{2+}-deprivation is not a factor in this process.

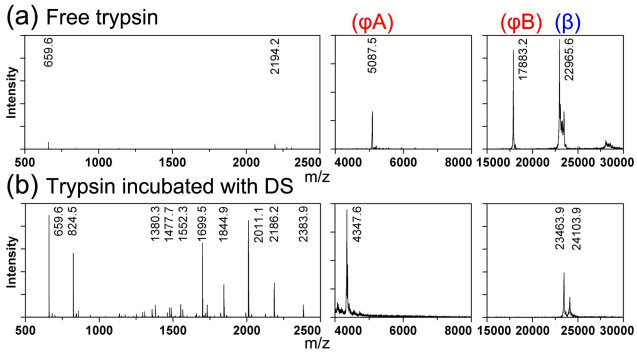

Fig. 3 MALDI-TOF mass spectrum of the autolytic products (b) incubated with DS at 37 °C for 2 hours compared with free trypsin (a). φA, φB: chain A and chain B of double-chain φ-trypsin. β: single-chain β-trypsin.

The data showing that direct contact between the protease and AA-PIs is necessary for AA, supported our initial hypothesis that rapid autolysis of trypsin was induced by the enrichment effect of DS. To determine whether the local concentration of trypsin was enhanced by AA, the DS-trypsin complexes (DTCs) were characterized by transmission electron microscopy (TEM, Fig. 4b). A visible increase in turbidity (Fig. 4a), caused by DTC formation, was observed immediately after mixing the DS with high concentrations of trypsin. If the solution was maintained at 37 °C, this turbidity would disappear and the solution would regain its transparency. This observation may indicate that numerous trypsin molecules were coalesced around the DS molecules via electrostatic interactions[11] and that they were rapidly degraded into fragments. Afterwards, such fragments departed from the DTCs and free DS were released. More importantly, such DS molecules could once again interact with newly-added trypsin and again form DTCs (re-establish turbidity).

In fact, the DTCs play an important role in AA. To test this, extremely high concentrations of ionic strength sodium sulphate (0.2M Na_2SO_4) was added to the solution, to prevent DTCs formation by inhibiting electrostatic interactions (Fig. 4c).[12] As a result, the DTCs and turbidity both disappeared, and the autolytic rate of trypsin was no longer affected by DS. The importance of DTCs in this process led us to focus on the dynamics of DTC formation and disintegration, which were monitored in situ using dynamic light scattering. As shown in Fig. 4d, the count rate represents the scattering intensity which is positively correlated with the quantity of DTCs.[13] After mixing the trypsin with DS, both the count rate and hydraulic diameter of the DTCs increased until they reached a maximum, then the size remained fairly constant, and the quantity of DTCs gradually declined to near zero.

According to the above DTC experiments, the AA process could be divided into three stages (Fig. 5). First, the bigger and more plentiful DTCs are formed as a result of continual adsorption of free trypsin, which is then degraded into small peptides within the DTCs, as was demonstrated by the SDS-PAGE and MALDI-TOF data. The peptides leave the DTCs since their electrostatic interaction with DS are substantially weakened, by the less effective superimposition of electrostatic potentials around the peptide chains.[14] Second, after the aggregation-dispersion dynamic equilibrium is reached, the DTCs begin to reduce as the free trypsin is consumed. Third, the majority of DTCs collapse and turn into free DS until the concentration of

free trypsin is lower than its critical aggregation concentration. The molecular weight of catalyst-like DS remains constant throughout the process (Fig. S4).

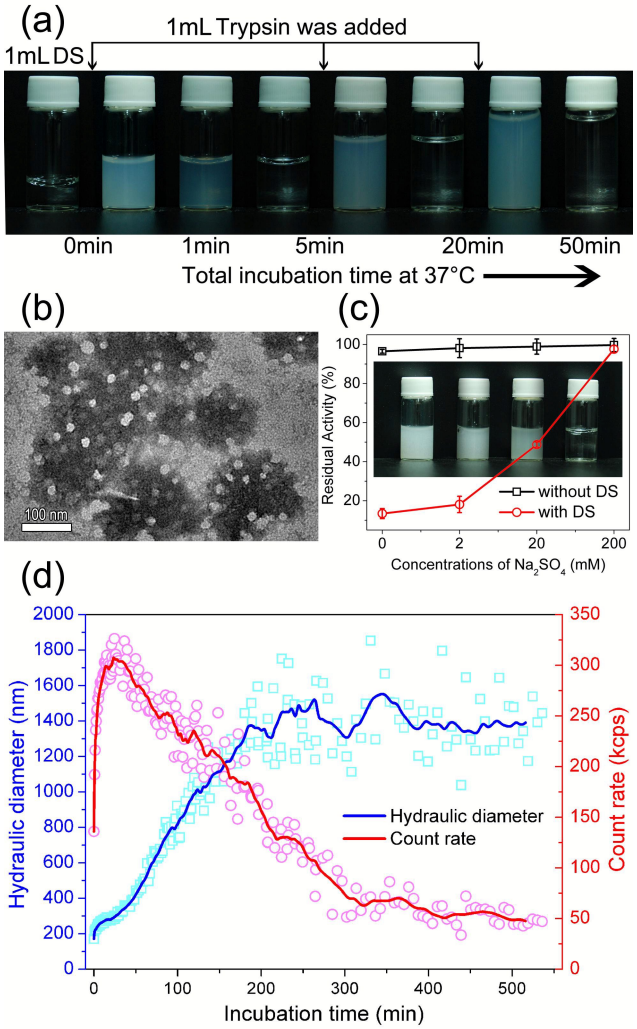

Fig. 4 Characterization of DS-trypsin complexes formed at the molar ratio of 1:84. (a) Alternate formation and disintegration of DTCs after each addition of trypsin (5 mg mL^{-1}) into DS at 37°C were observed. (b) TEM images of DTCs after negative staining. Bar: 100 nm. (c) The turbidity (insert) of trypsin incubated with DS and its residual activity after 10-minutes incubation at 37 °C in the presence of different concentrations of Na_2SO_4, compared with free trypsin without DS. (d) The quantity and size changes of DTCs over time monitored using dynamic light scattering at room temperature.

Finally, to evaluate the feasibility of the AA-PIs as an effective inhibitor, we determined AA-PI's ability to inhibit the trypsin-induced degradation of insulin, a protein highly sensitive to proteolysis (Fig. 6).[15] Insulin was mixed with trypsin (120 BAEE units mL^{-1}) and different concentrations of DS and incubated at 37°C for 2 hours. The rate of degradation of insulin was significantly inhibited by AA-PI. The inhibition efficiency of DS at low concentration is especially impressive, since its half-maximal inhibitory concentration (IC_{50} = 0.1 μg mL^{-1}) was about 200 times lower than that of commercial soybean trypsin inhibitor. However, the insulin remaining undegraded was unable to reach 100% and even fell slightly when excessive DS was used, such unpreventable degradation of insulin may be due to the inert trypsin (represented by A_i in Eq. 1) which was the last several survivors absorbed on a DS

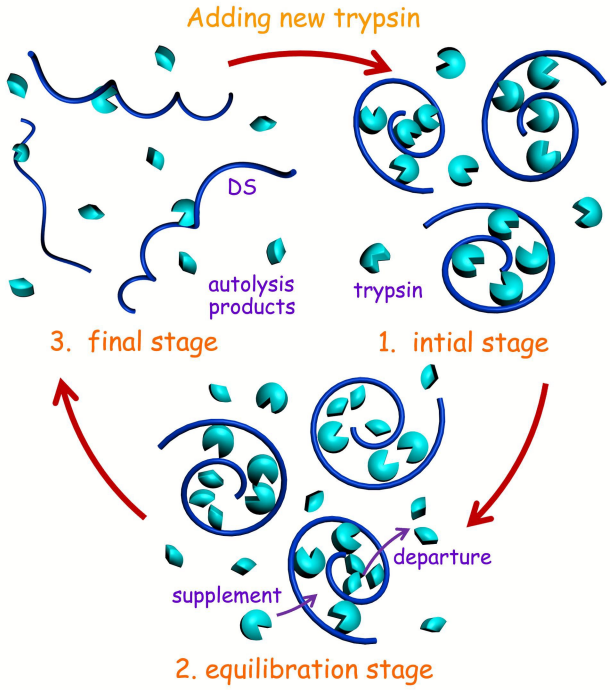

Fig. 5 Possible mechanism of trypsin autolysis acceleration in the presence of DS.

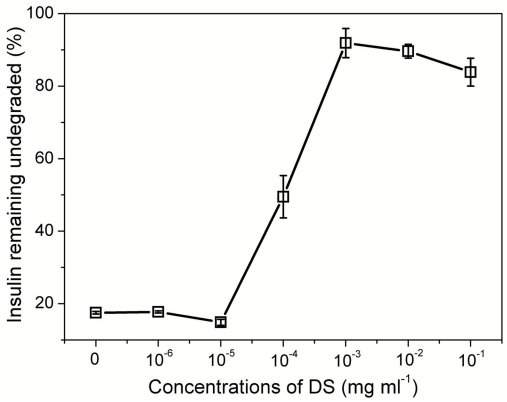

Fig. 6 The rate of degradation of insulin by trypsin (120 BAEE units mL^{-1}) in the presence of different concentrations of DS at 37°C for 2 hours (SD, n = 3).

molecule. These observations confirmed the earlier trypsin autolysis experiments in Fig. 2.

In addition, the effect of the physiological environment was considered. The inhibitory efficiency of DS in the presence of cellular lysate (10^6 mL^{-1} HepG2 cells, ultrasonication), lavage fluid (Sprague-Dawley rat intestine or lung lavaged by 10 mL deionized water), or tissue homogenate (Sprague-Dawley rat lung or brain tissue was homogenated with deionized water (1:9 w/w) by ultrasonic) was evaluated in vitro. All samples (account for 50% of reaction system) were directly mixed with trypsin before the DS was added. As shown in Fig. 7, DS still maintains a significant inhibitory effect even in the presence of various kinds of interfering substances. However, normal saline leads to a loss of this inhibitory efficiency. The latter is probably due to the loss of electrostatic interaction between DS and trypsin, and the ionization of charged groups is weakened by ion shielding. Hence, unmodified DS AA-PI can only be applied under low ionic strength conditions. Improving the

specific interaction between AA-PIs and the tryptic inactive site is the next step in our research.

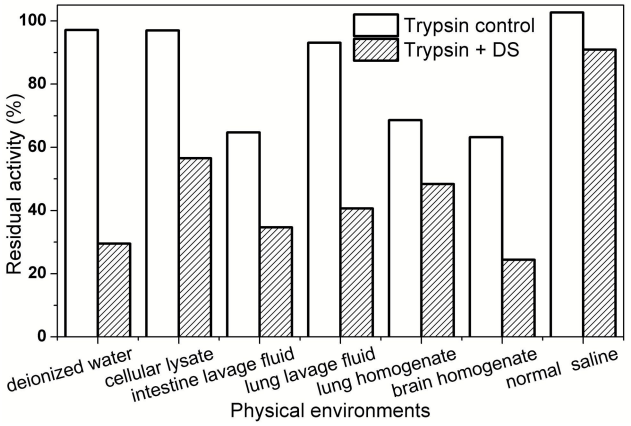

Fig. 7 The influence of various physical environments on the inhibitory effect of DS towards trypsin in 10 min. Physical environments including HepG2 cells' ultrasonic lysate, lavage fluid of rat intestine or lung, homogenate of rat lung or brain tissue (with deionized water 1:9 w/w) and normal saline.

Conclusions

In summary, we demonstrated the mechanism by which cationic proteases are inhibited by APs. We showed that, DS could be used to protect insulin from trypsin degradation in vitro. DS was 200 times more effective than commercial soybean trypsin inhibitor; the half-life of tryptic activity was shortened for more than 730 times in the presence of DS. Such a high efficiency is mainly due to the catalytic property of AA-PIs which gather the proteases and create a high local concentration of proteases to accelerate their autolytic rate. Even more importantly, the autolytic products depart from the inhibitor-protease complexes due to the abatement of electric potentials. Otherwise, the steric hindrance of the autolytic peptides will block access to the new proteases, ultimately inducing the inefficiency of the AA-PIs somewhat akin to the process of catalyst poisoning[16]. Finally, the aggregation of proteases and the dispersion of their autolytic peptides create a rapid autolytic circulation.

AA as a potential strategy for protease inhibition allows us to regulate the physiological metabolism of cells or organ systems using tiny amounts of stable and biocompatible polymers. More specific AA-PIs are necessary in order to inhibit various deleterious pathogenic proteases such as matrix metalloproteases, retroviral protease and β-secretase. If we can design new AA-PIs, specifically identifying and adsorbing the non-active site of these pathogenic proteases, and remove the autolytic products from their catalytic center, we will be able to use these new inhibitors to bring revolutionary new therapies to the treatment of diseases.

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Notes and references

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