[Chem. Pharm. Bull. 36(9)3675—3677(1988)]

Stimulation by Sulfated Glycans of Proteolysis of Lysozyme by Chymotrypsin

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(Received February 8, 1988)

Proteolysis of native egg white lysozyme by bovine pancreatic α-chymotrypsin was increased about 3-fold in the presence of dextran sulfate (DS). Other naturally occurring polysulfates, heparin and chondroitin sulfate A, also stimulated the proteolysis. Since the unfolded form of lysozyme was hydrolyzed specifically (T. Imoto, H. Yamada, and T. Ueda, *J. Mol. Biol.* **190**, 647, 1986), the enhancement of the proteolysis seemed to be due to unfolding of the protein by sulfated glucan.

Keywords——lysozyme; chymotrypsin; heparin; dextran sulfate; proteolysis

Polyelectrolytes have pronounced effects on isolated proteins as well as cells and tissues. Their effects include activation or inhibition of enzyme activities and stimulation or prevention of certain cellular activities. Sulfated glycans, which are highly anionic, were reported to bind to positively charged macromolecules, especially proteins, by electrostatic interaction.[1] For example, they interacted with basic lysozyme and trypsin in various ways.[2] On the other hand, various polyanions including heparin are also known to activate and inhibit several acidic enzymes.[2a] There is accumulating evidence that some conformational change occurs in antithrombin III (AT III), an acidic protein inhibitor, on its interaction with heparin.[3] Kress and Catanese reported the increased susceptibility of AT III to a protease in the presence of heparin.[4] However, there is no report on enhancement by sulfated glycans of proteolysis of basic proteins. Therefore, in this work, I measured the rate of proteolysis of egg white lysozyme by bovine pancreatic proteases in the presence of sulfated glycans.

Materials and Methods

Materials——Bovine pancreatic α-chymotrypsin and trypsin, crystalline egg white lysozyme, dextran sulfate (DS, molecular weight = 8 kilodaltons (kDa)) and pig intestinal heparin (168 USP units/mg) were obtained from Sigma Chemical Co., St. Louis, MO. Another preparation of DS (molecular weight = 500 kDa) was obtained from Pharmacia Fine Chemicals AB, Uppsala. Sodium hyaluronate and *Micrococcus lysodeikticus* cells were from Seikagaku Kogyo Co., Tokyo, and sodium chondroitin sulfate A was from Nakarai Chemical Co., Kyoto.

Proteolysis of Lysozyme——The assay for proteolysis of lysozyme by proteases was carried out essentially as described previously.[5] The reaction mixture contained 0.5 mg of lysozyme, chymotrypsin (14 μg) or trypsin (20 μg) and sulfated glycan (0.2 mg) in 1.0 ml of 0.1 M NaCl in 50 mM Tris–HCl (pH 8.0). The reaction was terminated by adding 1 ml of 0.44 M trichloroacetic acid in 5% $HClO_4$. The mixture was allowed to stand overnight at room temperature and then centrifuged at $1500 \times g$ for 10 min, and the absorbance of the supernatant at 280 nm was recorded. Mixtures at 0 time served as blanks.

The amidolytic activity of chymotrypsin was measured as described previously[6] in 50 mM Tris–HCl (pH 8.0) with or without 0.1 M NaCl. The assay of bacteriolytic activity of lysozyme was carried out by the method of Smolelis and Hartsell.[7] The first order rate constant was determined by least squares analysis of the initial reaction rates.

Results and Discussion

Since egg white lysozyme is a strongly basic protein, it forms electrostatic salts with

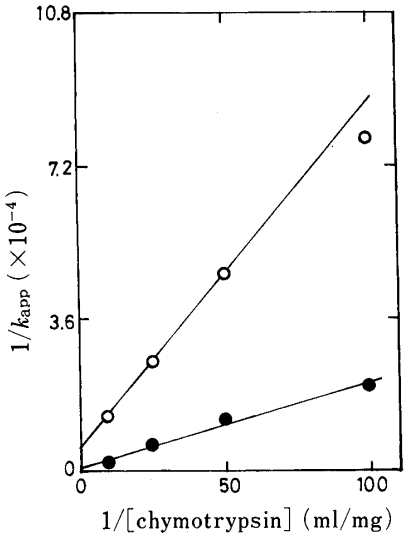

Fig. 1. Stimulation by DS of Proteolysis of Lysozyme by Chymotrypsin

Reaction mixtures containing the indicated concentration of chymotrypsin with (●) or without (○) DS (0.2 mg) and lysozyme (0.5 mg) were incubated at 37 °C for various periods to determine the first order rate constants. The double reciprocal plot of the k'_{app} *vs.* the enzyme concentration is shown.

TABLE I. Stimulatory Effects of Various Glycans on the Proteolysis of Lysozyme by Chymotrypsin

Glycan	Relative proteolysis (%)
DS (M.W. = 8 kDa)	281
DS (M.W. = 500 kDa)	251
Heparin	339
Chondroitin sulfate A	143
Hyaluronate	106

The reaction mixture containing lysozyme (0.5 mg), chymotrypsin (14 μg) and the indicated glycan (0.2 mg) in 50 mM Tris–HCl (pH 8.0) was incubated at 37 °C for 2 h. The extent of proteolysis without glycan was taken as the control and those with glycans were expressed as relative value to the control.

strongly acidic sulfated glycans.[2b] Since their charge-neutralized complexes are insoluble, the reaction mixture was turbid in the presence of the sulfated glycans under the assay conditions used. As reported by others,[8] lysozyme was hydrolyzed slowly by chymotrypsin. DS enhanced this hydrolysis. The first order rate constants of proteolysis in the presence and absence of DS increased with increase in the initial concentration of the protease (Fig. 1). The maximum first order rate constants ($k(s^{-1})$) at enzyme saturation were calculated from the intercepts on the ordinate of extrapolated double reciprocal plots of k'_{app} *vs.* concentration of the protease, and found to be 6.93×10^{-4} and 1.98×10^{-4} in the presence and absence of DS, respectively.

In the absence of NaCl, DS-enhanced proteolysis of lysozyme by chymotrypsin decreased to $65 \pm 4\%$ of that in 0.1 M NaCl. Since the stimulation by DS was significantly higher in the presence of 0.1 M NaCl than in its absence, it was not proportional to the apparent concentration of the ionic complex of lysozyme and DS.

The amidolytic activity of chymotrypsin was not inhibited by DS in the presence of 0.1 M NaCl.[9] However, the activity of chymotrypsin was inhibited by DS in the absence of NaCl. Kinetic analysis of this reaction showed that k_{cat} (s^{-1}) was not affected by DS and was 9.4 in both the presence and absence of DS in 10 mM Tris–HCl (pH 7.4). The k_m values of chymotrypsin for the fluorogenic substrate were 51 and 200 μM in the absence and presence of DS, respectively, indicating that DS caused competitive inhibition of the amidolytic activity of the protease (data not shown). Thus, it seems clear that the increased proteolysis of lysozyme by chymotrypsin is not due to activation of the protease by DS.

The effects of other glycans on the proteolysis of lysozyme by chymotrypsin are shown in Table I. The DS of high molecular weight (500 kDa) was slightly less effective than that of low molecular weight (8 kDa). Of the acidic glycans tested, heparin was the mostly effective on proteolysis of lysozyme by chymotrypsin. Hyaluronate, the least acidic glycan tested, enhanced the proteolysis only slightly. Unlike the stimulating effect of DS, those of naturally occurring acidic glycans appeared to parallel their acidities, probably reflecting their degree of affinity for lysozyme.

Next, I examined whether a lysozyme preparation hydrolyzed by chymotrypsin was active or not in bacteriolysis. In the presence of DS after incubation for 2 h, 43.8% of the lysozyme was hydrolyzed and 43.8% was inactivated. In the absence of DS after incubation for 16 h, 34.0% of lysozyme was hydrolyzed and 32.5% was inactivated. These results indicate that the lysozyme modified by chymotrypsin is enzymatically inactive.

Native lysozyme was also hydrolyzed by trypsin, though to a lesser extent than by chymotrypsin. DS also stimulated proteolysis of lysozyme by trypsin (data not shown). While DS had little instantaneous effect on the intrinsic fluorescence of chymotrypsin, it did have an effect on that of trypsin.[10] Thus, it is unlikely that activation of the proteases through a conformational change is responsible for the apparent stimulation by sulfated glycans of proteolysis of lysozyme.

The reversible denaturation of some globular proteins can be described in terms of an equilibrium between two main conformations (folded state, N, and unfolded state, D) of the protein. Hydrolysis of a protein with a protease appears to proceed via the D form, rather than the N form.[11] When the unfolding rate constant for the $N \rightleftarrows D$ transition of the protein is slower than the rate of proteolysis, it can be determined by measuring the extent of digestion of the protein.[8] They reported a value of 2.2×10^{-4} for the maximum first order rate constant at chymotrypsin saturation at 40 °C in the absence of DS. The value is very close to that in the absence of DS at 37 °C presented here. Thus, all of the results obtained here suggest that DS increases the concentration of the unfolded form of lysozyme by affecting k_1 or k_2 in the $N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D$ transition.

Acknowledgement I thank Professor Kinzo Nagasawa of Kitasato University for encouragement during this work and Miss M. Nakashima for her help.

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