Mechanism of the Antipeptic Action of Anionic Carbohydrate and Its Clinical Application for the Treatment of Peptic Ulcer

AKIRA ISHIMORI

Department of Internal Medicine,* Tohoku University School of Medicine, Sendai

Since the report of Babkin and Komarov in 1932,1 the ideal antipeptic agents have been searched and recently the synthetic sulfated polysaccharide2 is thought to be promising one. Although our knowledge on antipeptic agents has increased ever since, the exact mechanism of the antipeptic action of these substances has poorly been systematically understood. Accordingly no reasonable and systematic way to develop the antipeptic preparations has yet been known.

In the present study various kinds of anionic carbohydrate preparations were used besides dextran sulfate preparations (DS). DS was modified systematically in various ways in order to study the mechanism of its antipeptic action and to study the relationship between the antipeptic activity and the chemical structure. The clinical investigation was also performed to relate the antipeptic action and the effect on peptic ulcer.

METHODS

A) In vitro experiment
a) The antipeptic action on the crystallized pepsin: The hog pepsin that was recrystallized

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* Director: Prof. S. Yamagata.
Pepsin (3x crystallized) made by Nutritional Biochemical Corp., U.S.A. and hemoglobin purchased from Wako Junyaku Kogyo K.K., Japan were used. Various preparations of dextran esterified were offered by Meito Sangyo Co. Ltd., Nagoya, Japan.
three times, was used at the concentration of 0.01 mg/ml in 0.04 N HCl unless otherwise mentioned. To determine the antipeptic activity of test preparations, the peptic activity of pepsin-HCl solution was measured according to the method of Glass and Wolf with or without a presence of test preparations; First, 5 ml of pepsin solution was placed in a test tube with or without various amounts of test preparations. The incubation was carried out at 35.5°C for 30 minutes. Then the digestion was carried out for period of 10 minutes by adding 5 ml of 2.0% hemoglobin dissolved in the HCl solution (pH 1.5) which was prewarmed to 35.5°C. Exactly at the end of the digestion 5 ml of 10% trichloracetic acid was added and the contents were mixed throughly by the inversion of the rubber stoppered test tube. The digestion was carried out at pH 1.5 unless otherwise mentioned. The tubes were allowed to stand more than 10 minutes in the room temperature before the filtration through Toyo-roshi No. 4 filter paper. To 5 ml of filtrate was added 18.5 ml of 0.45 N NaOH and then 1.5 ml of the phenol reagent of Folin-Ciocalteu was used for the estimation of the liberated tyrosine-like substances at 640 nm using a Shimazu Spectrophotometer Model QV-50.

The control was prepared in the same way except the destruction of pepsin by mixing the pepsin solution with 10% trichloracetic acid at the beginning. The experiment was performed in duplicate in all instances and the grade of digestion (PU) was sought from the difference between the readings of the digestion and the control tubes according to Glass' formula.

In order to express the degree of the antipeptic activity of the test prepration, the inhibition rate was sought according to the following formula.

\[
\text{Inhibition rate(%) = } \frac{\text{PU without test preparation} - \text{PU with test preparation}}{\text{PU without test preparation}} \times 100
\]

In most experiments with DS, DS with 18% of the sulfur (S) content prepared from the source dextran of the mean molecular weight 7,500 was used unless otherwise mentioned.

b) The antipeptic action on proteases of the native specimen: The antipeptic action of DS on proteases contained in the native specimen such as gastric juice and urine, was determined by both the modified method of Glass for gastric juice and the newly developed method for urine. In the latter determination, urine was concentrated 7 times quantitatively to get rid of interfering substance and then diluted 200 times with HCl at various pHs. The incubation of diluted urine was carried out with the same amount of 2.0% hemoglobin solution at 35.5°C for 16 hours.

In addition, proteases were isolated from gastric mucosa of the surgically removed stomach by means of DEAE cellulose column chromatography and the effect of DS on pH activity curve of each protease was studied. The same preparation of DS as used in the experiment a) was used.

B) Paper electrophoresis

In order to prove the probable chemical binding between DS and pepsin or protein substrates, DS was incubated either with pepsin or various protein substrates at various pH levels in the room temperature for 30 minutes, then 0.05 to 0.1 ml of the mixture containing 0.5 to 2.0 mg of DS and 1.0 to 3.0 mg of the protein substrate was applied and run on the paper using a series of Teorell-Stenhagen buffer of corresponding pH. The Toyo Filter Paper No. 51 and the horizontal type of Kayagaki paper electrophoretic apparatus were used. The strength of electric current, the running time, pH and the ionic strength of the buffer solution were adjusted for each run as described later, so as to obtain the best electrophoretic pattern. Immediately after the cessation of electrophoresis, paper strips were dried in the oven at 100 to 110°C for 30 minutes and stained either with Amidoblack 10B or PAS.

Since DS of the mean S-content 19.3% prepared from the source dextran of the mean molecular weight 34,100 was well separated under the electrophoresis used and well stained by PAS, it was used exclusively throughout this experiment unless otherwise mentioned.
C) Immunoelectrophoresis

In order to analyze the protein constituents of the ulcer-covering so-called 'white coating' which is observed under the gastroscopy, the specimen was taken from the surface of the ulcer lesion under the fiberscopy, and placed immediately in the test tubes which contain a small amount of phosphate buffer of pH 8.6 and kept for an overnight at 4°C. The following morning the agar immunoelectrophoresis was performed, unless the Benzidin occult blood test for the specimen was positive. The standard procedures for the analysis of serum protein were applied using the veronal buffer of pH 8.6 and ionic strength 0.1 as the bridge buffer, and 110 volts on each slide glass for 1 hour. Antisera were purchased from Behring Werke, West Germany.

RESULTS

A) In vitro experiment

a) Antipeptic action on the crystallized pepsin: Since the antipeptic action of the anionic carbohydrate preparation is believed to depend on esterification, it may be influenced by changing the type of esterification. Dextran of the mean molecular weight 55,000 was esterified to the same degree (15.5% in terms of the mean S-content) in three ways and their antipeptic activities were compared. As shown in Fig. 1, an increase in the dose of each preparation brought the higher antipeptic activity, but dextran sulfate (DS) shows higher inhibitory effects than dextran phosphate and dextran carboxymethylate.

The source of carbohydrate is also known to influence the antipeptic activity. Fig. 2 shows various sulfated carbohydrates with different antipeptic activities. It is noted that the preparations of large molecular weight, namely DS and amylopectin sulfate show more effective inhibition than others in each corresponding dose, and inhibited the peptic digestion completely at the higher dose. The preparations of small molecular weight, namely sorbitol sulfate and sucrose sulfate show no noticeable antipeptic activity at the lower dose. The finding suggests that the degree of sulfation and molecular weight in addition to the structure of carbohydrate decide the antipeptic activity of each preparation.

![Fig. 1. The art of esterification of dextran and inhibition rate.](image-url)
To confirm these possibilities, dextran of the mean molecular weight 190,000 was sulfated to various degrees and the antipeptic activities were determined. It is seen in Fig. 3 clearly that there is a parallel relationship between the S-content and the antipeptic activity except in the lower S-content range than 6%. Such exception has been observed in the other series of experiment with DS prepared from the source dextran of molecular weight 220,000. It is also seen that DS of the low S-content does not show 100% of inhibition rate even at the highest dose of 40 mg, which corresponds nearly half of hemoglobin in the solution.

To see the effect of molecular weight on the antipeptic activity, dextrans of various molecular weights are sulfated to the same degree, namely the mean S-content of 18%. The comparison of the inhibitory effect and molecular weight was made at various different doses of DS as shown in Fig. 4. It is seen that the
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Fig. 4. The relationship between mean molecular weight of source dextran and inhibition rate of DS with S-content of 18%.

![Graph showing the relationship between mean molecular weight of source dextran and inhibition rate of DS.](image)

Inhibitory effect increases with the increase of molecular weight in the range of molecular weight up to about 16,000, but in the higher molecular weight than that, no significant increase of inhibitory effect is observed and it remains rather relatively steady. It should be pointed out, however, that this effect of molecular weight on the antipeptic activity becomes unclear in the high dose of DS such as 40 or 20 mg.

Concerning the mechanism of the antipeptic action of the anionic carbohydrate preparation, the effect of incubation order was studied. Fig. 5 shows that when DS is incubated with pepsin, then the substrate is added, the inhibitory action is more effective than when DS is incubated with the substrate first, then pepsin is added under each corresponding condition. It seems to suggest that DS acts on both pepsin and the substrate, but differently in the degree.

Fig. 5. The effect of changing of incubation order on inhibition rate of DS.

![Graph showing the effect of changing of incubation order on inhibition rate of DS.](image)
To see the influence of pH on the inhibitory effect, pH activity curve of the crystallized pepsin was sought with or without a presence of DS as shown in Fig. 6. Although the inhibitory effect tends to decline with the elevation of pH, it is difficult to draw the definite conclusion owing to the low proteolytic activity at the pH level far apart from the optimal pH of pepsin.

b) Antipeptic action on proteases of the native specimen: pH activity curve of proteases in the native gastric juice was measured with or without a presence of DS as shown in Fig. 7. It should be mentioned that an increase of the dose of DS can inhibit the proteolytic activity completely in the whole pH range.

The same sort of the experiment was done with urinary proteases. Since most proteases in urine are known to exist as proenzymes, the effect of DS was studied in the following two ways. In the first, urine was acidified first to convert proenzyme to an active form before adding DS, while in the second, DS was added to urine first and acidified thereafter. Fig. 8 shows that except the range around pH 1.5 DS depresses pH activity curve in the same manner in both experiments.

Fig. 9 demonstrates that pH activity curves of all proteases isolated from gastric mucosa by means of DEAE cellulose column chromatography are depressed by DS.
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The inhibitory effect of DS on pH activity curve of pepsin in native gastric juice.

B) Paper electrophoresis

The relationship between the incubation order of DS, pepsin and the substrate, and the antipeptic action demonstrated in the in vitro experiment described above strongly suggests that the antipeptic activity is attributed to the chemical binding of anionic carbohydrate to not only pepsin, but also the substrate. This chemical reaction is believed to occur stronger in the lower pH than at the isoelectric point of the substrate or enzyme.

Pepsin was run on the paper for 4.5 hours at 0.5 mA/cm with or without the pretreatment of incubation with DS for 30 minutes in Teorell-Stenhagen buffer of ionic strength 0.101 and pH 1.8 in the room temperature. As shown in Fig. 10 the incubation changed the degree of migration of pepsin, which is stained as a single band with Amidoblack 10 B and the bound DS is well recognized superimposed on the pepsin band. It apart from the free DS band.

The same sort of the experiment was repeated with hemoglobin instead of pepsin using Teorell-Stenhagen buffer of pH 6.2 and ionic strength 0.054. The results demonstrate clearly that DS binds also to hemoglobin and changes the rate of migration of the latter as seen in Fig. 11.
Using the same method, the chemical binding between DS and various plasma proteins was studied at different pHs.

Fig. 12 shows that all plasma proteins bind to DS firmly at pH 3.6. On the contrary, the small amount of DS binds to plasma proteins leaving most of DS free at pH 9.0 as shown in Fig. 13. The degree of binding is different among proteins.

The effect of pH-elevation upon the protein - DS complex formed at the low pH was studied. Using McIlvaine buffer, 1 mg of DS was incubated with 0.02 ml of serum at pH 3.6 for 30 minutes in the room temperature and the mixture was subjected to a paper electrophoresis at pH 7.0 for 3 hours at 0.5 mA/cm. Fig. 14 demonstrates that the compound of DS and serum formed at pH 3.6 split mostly during the electrophoresis at pH 7.0 leaving only the small amount of bound DS. The same sort of experiment was carried out with the Na-Al salt of dextran sulfate (APD) with the different result. Namely most of APD remained in the bound form after the electrophoresis as seen in Fig. 14. (All dextran sulfate preparations except APD are used in the form of Na salt.)
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Fig. 9. The inhibitory effect of DS on pH activity curves of proteases separated from human gastric mucosa by DEAE cellulose column fractionation.

Fig. 10. The paper-electrophoretic pattern of pepsin-DS complex.

Fig. 11. The paper-electrophoretic pattern of hemoglobin-DS complex.

C) Immunoelectrophoresis

Using antihuman rabbit serum, the ulcer lesion covering ‘white coating’ was analyzed immunoelectrophoretically and compared with serum as shown in Fig. 15. It is seen that ‘white coating’ contains proteins of the serum origin.

‘White coating’ was also analyzed immunoelectrophoretically using antifibrinogen rabbit serum. Fig. 16 demonstrates that ‘white coating’ contains the fibrinogen-like substance which migrates towards the cathode – the opposite direction to plasma fibrinogen. Since plasma incubated with the acid-pepsin solution behaves similarly as shown in Fig. 16, the finding may be interpreted to reflect the structural change of plasma proteins probably by acid-pepsin contained in the native gastric juice at the surface of the ulcer lesion.
DISCUSSION

Using synthetic dextran sulfate (DS) Tomizawa and Kondo\textsuperscript{11} reported that it inhibits strongly ulcerogenesis in rats with pylorus ligation, while Bianchi and Cook\textsuperscript{12} found the same effect with synthetic amylopectin sulfate. The use of these synthetic substances is believed to have opened the way of a new approach to study the mechanism of antipeptic action of the anionic carbohydrate preparations in addition to the old way with the natural substances.\textsuperscript{13-16} In the present investigation various kinds of synthetic anionic carbohydrate preparations were used and the relationship between the inhibitory effect and the chemical structure was sought systematically.

Fig. 13. The effect of incubation with DS at pH 9.0 on the paper-electrophoretic pattern of proteins.
(A) Incubation of serum with DS and subsequent electrophoresis carried out at pH 7.0

![Image of electrophoresis results for DS, Serum, and Serum+DS](image1)

(B) Incubation of serum with DS first carried out at pH 3.6, then subject to electrophoresis at pH 7.0

![Image of electrophoresis results for Serum+DS and Serum+APD](image2)

Fig. 14. The influence of pH elevation to 7.0 on serum protein-DS complex produced at pH 3.6.

![Image of immunoelectrophoretic analysis](image3)

Fig. 15. The immunoelectrophoretic analysis of 'white coating' covering ulcer lesion of the stomach (a)
S: serum WC: white coating abs: antihuman rabbit serum

The results obtained in the present study indicate that both the type of esterification and the source of carbohydrate influence the inhibitory effect as shown in Figs. 1 and 2. Namely the sulfation was most effective. Further analysis revealed that its antipeptic activity depends on the degree of esterification and molecular weight as shown in Figs. 3 and 4. The statement made by Cook et al.⁴ that a certain molecular size is critical for pepsin-inhibition seems likely but with the further addendum that a critical molecular size exists not in single, but
multiple. In general it can be stated safely from the results obtained in the present study that the antipeptic activity increases with the increase of molecular weight within a certain range of lower molecular weight (lower than about 16,000 in case of DS), but there is no such relationship in a range of higher molecular weight. It should be pointed out that the sharper isolation of the source carbohydrate of a certain molecular weight may demonstrate more clearly the multiple existence of critical molecular sizes. It is interesting to note that this molecular weight-antipeptic activity relationship appears to be more evident in the lower dose of DS such as 2.5 or 5.0 mg in the present digestive system, suggesting that the experiment to compare the antipeptic activity should be carried out using various doses of test preparations as in the present investigation.

Fischer and Fischer and Astrup reported already that heparin forms stable salts with many proteins owing to its strongly acid nature and this reaction is reversible one that follows the mass law. Jaques stressed that the reaction between heparin and proteins alters the properties not only of proteins such as isoelectric point, precipitability by various precipitating agents etc., but also of heparin such as anticoagulant activity etc., depending upon the dissociation constant of the reaction. He also stated that heparin possibly combines proteins in the far alkaline side of their isoelectric points, although the elevation of pH tends to increase the dissociation of the compound. This may be applicable for other anionic carbohydrate than heparin owing to their similar chemical structures. In fact, Sasaki and Noguchi found that DS and fibrinogen combine even in the physiological pH range where both of them have negative net charges and
suggested that other intermolecular binding forces than the electrostatic force should be considered, since sodium carboxymethyl cellulose carrying-COO⁻ does not combine fibrinogen under the same condition. Vocac and Alphin²¹ stated the same view basing on the experimental results with lignosulphonate.

It is noted, however, that anionic carbohydrate added to the digestive system combines not only with the substrate protein, but also with the enzyme protein, which was shown by means of paper electrophoresis in the present study. Anderson¹⁵ and Martin et al.²² stated that sulfated polysaccharides diminish the peptic activity in vitro mainly by reacting with and protecting the substrates, rather than by inactivating the enzyme through chemical binding. However, the present study demonstrated that when the incubation order is changed, the inhibitory effect is significantly changed as shown in Fig. 5. It indicates that the chemical reaction between pepsin and DS is also important to decide the degree of inhibition. Namely the free hemoglobin remaining unbound after incubated with DS is digested easily by pepsin added afterwards giving the low inhibition rate, while the active pepsin remaining unbound after incubated with DS could not be so much, giving the high inhibition rate. It should be noted that there would be no appreciable difference between the both experiments in the amount of unbound hemoglobin at the time of digestion since the small amount of pepsin is used in order to maintain the zero kinetics order during the digestion. The view that both reactions between DS and the substrate, and between DS and pepsin are important to decide the degree of the antipeptic activity is supported strongly by the findings in which DS is indicated to bind both pepsin and the substrate protein. Therefore, 'direct antipeptic action' is designated for the action of anionic carbohydrate on pepsin and 'indirect antipeptic action' for the action of anionic carbohydrate on the substrate protein hereafter. Accordingly the opinion of Levey and Sheinfeld²³ that the antipeptic action of sulfated polysaccharide bases on the chemical reaction between pepsin and sulfated polysaccharide is also regarded as seeing only one side of the mechanisms, 'direct antipeptic action.'

As to the influence of pH on the antipeptic activity, the report by Jaques¹⁹ that a shift in pH towards the alkaline side causes an increase of the dissociation of the compound of heparin-proteins, seems to favour the view that an elevation of pH produces a low antipeptic activity. The dissociation increases the amount of the unbound substrate protein and the active pepsin in the digestive system leading to the digestion of the former by the latter. However, the results shown in Fig. 6 show no definite evidence to support the above view and coincide with the report of Anderson.²⁴ This may be partly due to the lack of the accuracy in measuring the peptic activity at the pH level far apart from the optimal pH of pepsin.

Nevertheless, the influence of pH on the compound formation between DS and various plasma proteins was clearly demonstrated by paper electrophoresis as shown in Figs. 12 and 13. Proteins bind DS far less at pH 9.0 than pH 3.6, but the degree of chemical binding is somewhat different from protein to protein. Furthermore the experiment shown in Fig. 14 proves that the chemical binding of
protein and DS is reversible, although the dissociation of the compound occurs very slowly even under the electric charge. This is regarded to be the important feature of anionic carbohydrate from the clinical point of view. Since the ulcer lesion-covering so-called 'white coating' which is visible under the gastroscopy was found to constitute partly from plasma proteins as demonstrated in Figs. 15 and 16, the above findings are thought to show a clinical usefulness of anionic carbohydrate as the antipeptic drug. When anionic carbohydrate is administered orally to the patient with peptic ulcer, it binds chemically not only with pepsin in gastric juice, but also with 'white coating' on the surface of ulcer. The direct antipeptic action in the gastric juice corresponds to the action of antacid which acts only temporally and terminates with the evacuation of gastric content into duodenum within several to several ten minutes. However, anionic carbohydrate which binds to 'white coating' remains there and continues to protect the ulcer lesion from the powerful digestion by the native gastric juice, thus the healing of ulcer will be promoted. Such indirect antipeptic action upon the ulcer lesion should be regarded as the most important effect of the antipeptic drug. It is believed to form the base to evaluate the clinical usefulness of the test preparation.

It should be remembered that the chemical binding between anionic carbohydrate and protein is reversible and the elevation of pH accelerates the dissociation of the compound. Accordingly it is not advisable to administer the antipeptic drug together with powerful antacids such as sodium bicarbonate, although most antacids are not able to bring intragastric pH high enough to prevent the compound formation. The combined use of the antipeptic drug and the anticholinergic drug is highly recommendable not only theoretically, but also from the clinical experience. Furthermore, since it binds to various proteins in food, it is more reasonable to administer it when the stomach is empty.

Now, it is apparent that it would be more beneficial if we can minimize the dissociation of the compounds formed between anionic carbohydrate and proteins. In an attempt to find out such an antipeptic drug, Na of the ordinal DS was substituted with Al to various degrees. Fig. 14 demonstrates that APD, one of the preparations produced in this way, remains in the bound form with serum protein even after electrophoresis. From the precipitation experiment, it was thought that Al-Al bridges might be formed between Al atoms in the compound, thus the compound is united firmly each other and becomes much less dissociable. The clinical usefulness of APD was already proved with the excellent results in the double blind trial and will be reported soon.

Recently the multiplicity of gastric protease has been reported demanding the reevaluation of the antipeptic drug. The present study is designed to see the antipeptic action on pH activity curves of native gastric juice and urine. However, the experimental results show that it acts on all proteases in these biological fluids as shown in Figs. 7 and 8. And it was confirmed further that all proteases isolated from gastric mucosa by means of DEAE cellulose column chromatography were inhibited by DS without exception as shown in Fig. 9.
In the present study the several approaches to develop the ideal antipeptic drug are shown, namely the selection of the material with the proper molecular size, the type and the degree of esterification and the device to make the anionic carbohydrate-protein compound less or un-dissociable. The treatment of peptic ulcer with the antipeptic drug can be regarded as a more physiological approach than the antacid therapy which has been widely used, because the natural occurrence of antipeptic substances as a constituent of gastric mucus is believed to be responsible for the major defence of the gastric wall against peptic digestion and the breakdown of this defence mechanism is regarded to lead to the occurrence of peptic ulcer. Anionic carbohydrate repairs the breakdown of this defence mechanism. One of the characteristic features of peptic ulcer is its frequent recurrence, and the prevention of the recurrence is as important as the treatment of the active ulcer. Accordingly the appearance of the new drugs with the physiologically acting mechanism for the long term administration has been waited. Antipeptic drug is believed to satisfy such demands.

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References


