unit is less than that of other polysaccharide sulfates which does not contain any carboxyl residue.

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Summary

Anticoagulant activity of synthetic heparinoids was determined and its relationship to the sulfur content and/or to the molecular size (viscosity) was investigated. Carboxyl residue at C-6 in the glucose unit was proved to weaken the toxicity. Heparin, oxidized starch sulfate and sulfate of the reduced product of oxidized starch were compared for their stability against acids and alkalis.

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28. **Masaya Namekata**: Studies on Oxidized Starch Sulfates for Medical Purposes. III.*1 Inhibition of the Proteolytic Action of Pepsin by Sulfates of Oxidized Starch and its Reduced Products.

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It is known that sulfate-containing polysaccharides inhibit the proteolytic action of pepsin. The inhibitory action of natural or semi-synthetic polysaccharide sulfates, such as chondroitin sulfuric acid, heparin, polyhydromannuronic acid sulfate, and amylose sulfate upon proteolysis has been reported,[1~4] but these were limited to comparative studies on the inhibitory action of polysaccharides.

In the previous reports of this series from this Laboratory, the preparation of oxidized starch, its reduced products and their respective sulfates and their blood anticoagulant activities were reported. In the present paper, the inhibitory action of oxidized starch sulfate and sulfate of reduced product of oxidized starch on the proteolytic action of pepsin is reported.

Experimental

Determination of Proteolytic Action of Pepsin——The proteolytic action of pepsin was determined by the method of Bonfil, *et al.*[3,5] 1 cc. of crystalline pepsin solution (1 mg./cc.), adjusted to pH 1.6 by addition of conc. HCl, was placed in a test tube, then either 1 cc. of a solution of polysaccharide sulfates or 1 cc. of distilled water (both were adjusted to pH 1.6 by conc. HCl) was added,

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and the contents were mixed by swirling. The mixture was allowed to equilibrate in a water bath at 37° for 10 min. Into this mixture 1 cc. of a substrate consisting of 3% dried human plasma in a solution adjusted to pH 1.6 with conc. HCl was added. Following the incubation for 30 min. at 37°, 10 cc. of 10% CCl_3COOH was added to stop the reaction. The content was allowed to stand for 15 min. before the precipitated protein was removed by filtration (Toyo Roshi No. 6). To 2 cc. of the filtrate in a test tube, 5 cc. of 0.55*M* Na_2CO_3 and 1 cc. of phenol reagent of Folin and Ciocalteu[5] (diluted to 3 volumes with distilled water before use) were added and mixed. The intensity of the color due to the liberated tyrosine was determined in a Coleman Junior Spectrophotometer at 660 mμ and the quantity of tyrosine was calculated from the calibration curve of the standard tyrosine.

The proteolytic activity of pepsin was estimated by determining tyrosine liberated from the substrate protein due to the hydrolysis and the degree of inhibition by polysaccharides was expressed in percent against the value of reference pepsin.

Results and Discussion

Inhibitory activity of oxidized starch derivatives on pepsin was determined in a concentration of 1~7 mg./cc. and compared with those of heparin (Table I and Fig. 1). It

Table I. Effect of Oxidized Starch Sulfate, Sulfate of Reduced Product of Oxidized Starch, and Heparin on the Proteolytic Action of Pepsin

Compound No.[a]	S (mole)	[η]	Anticoagulant activity U/mg.	Inhibitory action of samples in various concentrations (mg./cc.)[b]				
				0	1	3	5	7
ROS18-S. 1	1.75	0.039	27.7	100	75.1	48.3	32.0	28.0
ROS26-S. 1	0.25	0.015	0.3	100	88.5	71.6	58.0	50.0
ROS26-S. 3	1.18	0.015	9.6	100	81.0	55.6	38.4	31.0
OS27-S. 3	1.19	0.008	2.5	100	73.0	54.2	40.1	32.2
ROS.Y-S. 1	1.89	0.034	42.4	100	70.3	44.8	28.5	25.0
Heparin	(9.41%)	0.037	126	100	91.6	72.3	56.1	48.0

[a] OS-S (oxidized starch sulfate), ROS-S (sulfate of reduced product of oxidized starch).
[b] Expressed in % against the value of reference pepsin.

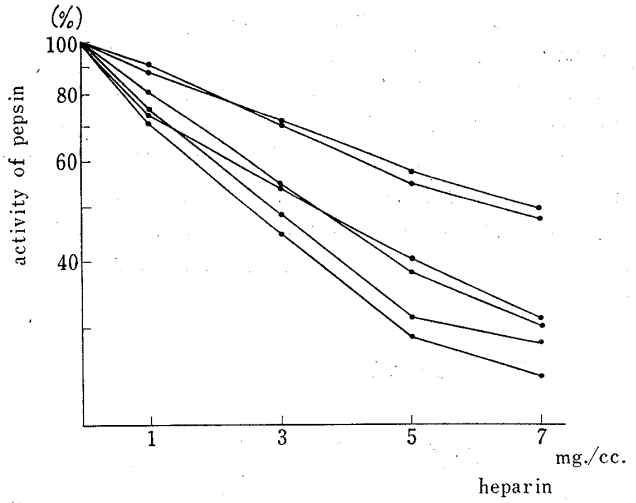

Fig. 1.
Inhibitory Activity of Heparin

was found that oxidized starch and reduced product of oxidized starch, having no sulfate groups, have no inhibitory action on pepsin, but their sulfates have equal or stronger activity than heparin, though their anticoagulant activity is 1/3 to 1/5 that of heparin. It was also noted that the inhibitory activity of oxidized starch sulfate and sulfate of reduced product of oxidized starch were proportional to the sulfate group content and the dose-response curves are almost linear in a concentration range of 1~5 mg./cc. of the same sample. From these results, it could be considered that the inhibitory activity

of oxidized starch sulfate and sulfate of reduced product of oxidized starch is due to their sulfate group and is affected greatly by their sulfur content or their electronegativity, but not so much by their intrinsic viscosity ([η]) or the degree of polymerization ($\overline{DP}$).

To make this clear, further examination was made on oxidized starch sulfate and sulfate of reduced product of oxidized starch of various sulfur contents, having a viscosity of 0.006~0.008, in a concentration of 1, 3, and 5 mg./cc.

From its results, summarized in Table II and Fig. 2, it was confirmed that, among the samples of [η], 0.007~0.008 the inhibitory activity was proportional to the sulfur content, but the activity of the samples having [η] value of 0.006 is weak, though it contained more sulfur than the sample having [η] value of 0.007. It can therefore be concluded that the activity depended not only on sulfur content but also on $\overline{DP}$. In this series of the sample having [η] value of 0.006~0.008, both anticoagulant activity and inhibitory action were proportional to the sulfate group content.

Table II. Effect of Oxidized Starch Sulfate and Sulfate of Reduced Product of Oxidized Starch with Various S Content ([η] 0.006~0.008) on Proteolytic Activity of Pepsin

Compound No.	S (mole)	[η]	Anticoagulant activity U/mg.	Inhibitory action of samples in various concentrations (mg./cc.)			
				0	1	3	5
OS26-S. 1	0. 55	0. 007	0. 3	100	86. 3	69. 1	57. 4
OS26-S. 2	0. 80	0. 007	0. 4	100	76. 3	64. 2	52. 5
OS26-S. 3	0. 94	0. 007	0. 5	100	76. 0	64. 2	52. 2
OS26-S. 5	1. 00	0. 007	0. 6	100	75. 0	63. 2	51. 5
OS26-S. 9	0. 82	0. 006	0. 1	100	86. 1	72. 7	62. 9
OS27-S. 3	1. 19	0. 008	2. 5	100	73. 0	54. 2	40. 1
ROS26. F4-S	1. 84	0. 006	2. 2	100	84. 0	67. 5	55. 2

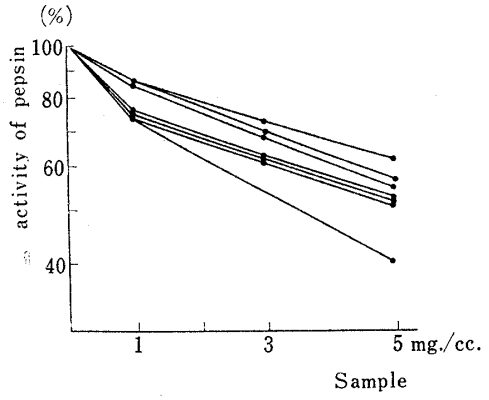

Fig. 2. Inhibitory Activity of Sulfates of Oxidized Starch and its Reduced Product on Pepsin

From the results summarized in Table III and Fig. 3, it is obvious that among the samples having [η] value of 0.010~0.015, the inhibitory activity is proportional to sulfur content and is much higher than those of the samples listed in Table II. With the samples of sulfate of reduced product of oxidized starch with high sulfur content, the inhibitory activity is more correlated with viscosity than with anticoagulant activity, but with the samples with a sulfur content less than 1.0 mole/glucose unit, both anticoagulant activity and inhibitory action are enhanced to the same extent with increasing sulfur content and [η] value.

To find the effect of $\overline{DP}$ on inhibitory activity, samples of various [η] values, having a sulfur content of more than 1.5 moles/glucose unit, were examined for their inhibitory activity. From the results shown in Table IV and Fig. 4, it is noted that among the

Table III. Effect of Oxidized Starch Sulfate and Sulfate of Reduced Product of Oxidized Starch with Various S Content ([η] 0.010~0.015) on Proteolytic Activity of Pepsin

Compound No.	S (mole)	[η]	Anticoagulant activity U/mg.	Inhibitory action of samples in various concentrations (mg./cc.) 0	1	3	5
ROS26-S. 1	0. 25	0. 015	0. 3	100	88. 5	71. 6	58. 0
ROS26-S. 2	0. 99	0. 015	7. 2	100	80. 8	55. 6	38. 4
ROS26-S. 3	1. 18	0. 015	9. 6	100	80. 0	53. 0	35. 4
ROS26-S. 4	1. 59	0. 015	11. 3	100	74. 4	48. 4	31. 0
ROS27-S. 1	1. 23	0. 011	14. 8	100	78. 0	59. 8	44. 6
ROS12-S. 1	0. 80	0. 010	2. 3	100	85. 0	64. 8	50. 4
OS17-S. 1	1. 54	0. 010	5. 5	100	83. 0	58. 5	42. 2
OS.Y-S. 1	1. 04	0. 012	8. 4	100	85. 5	63. 0	45. 7

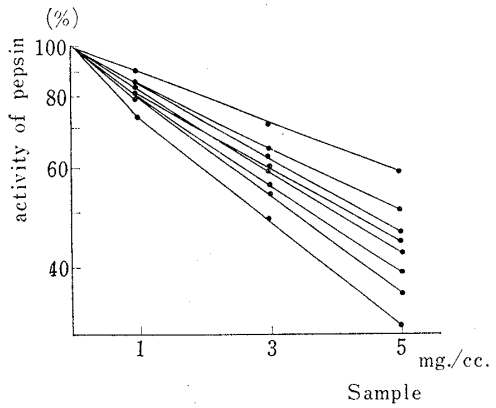

Fig. 3. Inhibitory Activity of Sulfates of Oxidized Starch and its Reduced Product on Pepsin

Table IV. Effect of Oxidized Starch Sulfate and Sulfate of Reduced Product of Oxidized Starch of Various Viscosity (S : 1.5~1.89 mole/glucose unit) on Proteolytic Activity of Pepsin

Compound No.	S (mole)	[η]	Anticoagulant activity U/mg.	Inhibitory action of samples in various concentrations (mg./cc.) 0	1	3	5
ROS26. F1-S	1. 54	0. 001	8. 6	100	81. 5	57. 8	39. 0
ROS26. F2-S	1. 65	0. 009	5. 5	100	76. 0	51. 0	34. 9
ROS26. F3-S	1. 53	0. 008	4. 2	100	82. 0	58. 2	39. 9
ROS26. F4-S	1. 84	0. 006	2. 2	100	84. 0	67. 5	55. 2
ROS26-S. 4	1. 59	0. 015	11. 25	100	74. 4	48. 4	31. 0
ROS17-S. 1	1. 67	0. 038	3. 58	100	71. 0	45. 2	29. 1
OS17-S. 1	1. 54	0. 001	5. 5	100	83. 0	60. 5	42. 2
ROS.Y-S. 1	1. 89	0. 033	42. 4	100	70. 3	44. 8	28. 5
ROS18-S. 1	1. 75	0. 039	27. 7	100	75. 1	48. 3	32. 0
OS12-S. 3	1. 68	0. 001	6. 2	100	78. 2	52. 4	34. 6

samples of 1.5 to 1.9 moles/glucose unit, sulfur content the inhibitory activity was not so affected by viscosity, especially above 0.015 [η].

This fact was supported by the fact that the anticoagulant activity was not parallel to the inhibitory activity. In a preceding paper,*1 it was reported that the anticoagulant activity was proportional to $\overline{DP}$ among the samples with certain sulfur content. On the other hand the inhibitory activity was proportional to $\overline{DP}$ up to 0.015 [η] value and, beyond this critical value, the activity was scarcely affected by $\overline{DP}$ or viscosity.

In Table V, results on other polysaccharide sulfates, heparin, chondroitinsulfuric acid, and dextran sulfate, each composed of different saccharide, are recorded. It seems

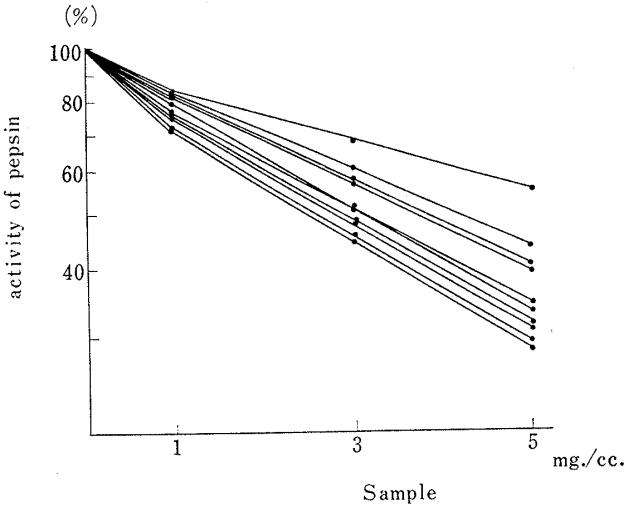

Fig. 4.

TABLE V. Effect of Other Polysaccharide Sulfates on Proteolytic Activity of Pepsin

Sample	S (%)	[η]	Anticoagulant activity U/mg.	Inhibitory action of samples in various concentrations (mg./cc.)			
				0	1	3	5
Dextran sulfate	17.8	0.2761	29.5	100	82.8	48.6	28.4
Chondroitin sulfuric acid	6.6	0.0178	0.23	100	99.6	86.7	74.4
Heparin	9.41	0.0366	126	100	91.6	72.3	56.1

that the inhibitory activity is not affected by the difference in the composing saccharide, but depends on the degree of sulfation.

Statistical analysis of multiple correlation among inhibitory action, sulfate content, and [η] of the 18 samples from the data on oxidized starch sulfate and sulfate of reduced product of oxidized starch shown above, were as follows :

			p	
Multiple coefficient	Z	against	X Y	<0.01
	X		Y Z	>0.01
	Y		X Z	>0.01 <0.05
Partial coefficient	Z Y	at fixed	X	>0.01 <0.05
	X Z		Y	<0.01
Simple coefficient	Z	against	X	<0.01
	Z		Y	>0.05

where X : Sulfate group content (mole/glucose unit)
Y : Intrinsic viscosity
Z : Inhibitory activity on pepsin (expressed in percent of proteolytic activity of un-inhibited pepsin, at 5 mg./cc. of the sample concentration).

All of the correlation of inhibitory action against sulfur content is highly significant at $p=0.01$, but the partial correlation of inhibitory activity against viscosity is significant at $p=0.05$, and simple correlation of inhibitory activity against viscosity is insignificant. These results indicate that inhibitory activity on pepsin is greatly affected by the sulfate content and not so much by $\overline{DP}$.

The fact that pepsin inhibiting activity and anticoagulant activity were not closely correlated was obvious from the experiment on sulfate of reduced product of oxidized starch with sulfur content of more than 1.5 moles/glucose unit having a different viscosity and on heparin and dextran sulfate. This is also evident from the correlation coefficients on anticoagulant activity against viscosity and sulfur content. Correlation

of pepsin inhibiting activity is significant only against sulfate content, while that of anticoagulant activity is significant against not only sulfate content but also against viscosity, as anticoagulant activity is influenced strongly by $\overline{DP}$.

Levey, *et al.*[2] reported that hyaluronic acid, having no sulfate group, had no inhibitory effect on pepsin, while heparin, having the most strong anticoagulant activity, was the most active inhibitor, and that inhibitory activity of chondroitinsulfuric acid, sodium polyhydromannuronic acid sulfate was proportional to their anticoagulant activity. From these results, it was concluded that the inhibitory activity of polysaccharide sulfate was mainly due to their sulfate group. On the other hand, Placer, *et al.*[4] studied both the inhibitory effect on pepsin and anticoagulant activity of heparin, amylose sulfate, cellobiose sulfate, and hyaluronic acid sulfate, and reported that there was no correlation between inhibitory effect and anticoagulant activity, as the former was not influenced by their molecular weight.

In the present work, the result showing that the inhibitory action depends on the sulfate group is coincident with those of Levey, *et al.*[2] and of Placer, *et al.*,[4] and the result on correlation between inhibitory action and anticoagulant activity of the samples agrees with that of Placer, *et al.*[4] From the present experiments on correlation between inhibitory action and viscosity of the sample, using a series of samples of various viscosity and sulfate content, it is concluded that the activity is affected not only by the sulfate content but also ristricted by a certain minimum viscosity.

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Summary

The inhibitory effect of polysaccharide sulfates on the proteolytic action of pepsin is due to their sulfate group and is enhanced with the increasing sulfate group content. The effect of $\overline{DP}$ on the inhibitory activity is weaker than that of the sulfur content, but the minimum critical value is necessary for the appearance of inhibitory action. Inhibitory effect is influenced largely by $\overline{DP}$, especially in samples with a small sulfur content, and slightly by samples with a large sulfur content. The inhibitory activity by polysaccharide sulfates is not correlated to their anticoagulant activity.

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