UDC 615.7[612.115.35]-011

30. Masaya Namekata : Studies on Oxidized Starch Sulfates for Medical Purposes. V.*1 Investigation on the Complex of Pepsin with Sulfates of Oxidized Starch and its Reduced Products.

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It was reported in the previous papers[1,2] that the oxidized starch sulfates had inhibitory action on proteolytic action of pepsin and that the inhibitory action was due to the sulfate group in the oxidized starch sulfates. It has been postulated that both the proteolytic activity on pepsin and protective effect on peptic ulceration of polysaccharide sulfates were caused by the combination of the sulfate with pepsin or mucoprotein,[3~5] but the fact has not yet been confirmed.

In the present series of work, the complex of pepsin with heparin and with sulfates of oxidized starch and its reduced product have been isolated, and confirmed as a complex by electrophoresis. The results are herein reported.

Materials and Method

Sulfates of oxidized starch and its reduced product, the same materials as used in the previous work,[1] heparin and crystalline pepsin were used in this experiment.

Proteolytic action was determined according to the procedure used in the previous work[1] and pH was determined with a Hitachi pH meter. Electrophoretic separation was carried out on 12×26 cm. Toyo Roshi No. 51 filter paper with a Kobayashi Electrophoresis apparatus. Staining was carried out according to the procedure of Hermans, *et al.*,[6] in which polysaccharide sulfates were stained by itself, apart from protein.

After electrophoresis, the filter paper, dried with a current of air at the room temperature, was steeped in neutral HCHO-EtOH solution (mixture of 1 volume of HCHO and 4 volumes of EtOH) for 10 min. to fix the polysaccharide and protein on the paper. Then the filter paper was dried again, soaked in 1% Mucicarmin solution (50% EtOH) for 15 min., and polysaccharide sulfate bands were stained in reddish purple. After rinsing in 10% AcOH, the paper was dried, and soaked in 0.1% MeOH solution of Bromophenol Blue saturated with $HgCl_2$, and the protein bands were stained in greenish blue. The paper was rinsed in 10% AcOH and dried. As the background of the paper remained white, heparin of less than 1 γ could be detected.

Experimental Results

Formation and Confirmation of the Complex of Pepsin with Sulfates*3 of Reduced Product of Oxidized Starch and Heparin——An earlier work showed that the proteolytic activity of crystalline pepsin was decreased to 40~70% of that at pH 1.6 by addition of oxidized starch sulfate, sulfate of reduced product of oxidized starch, or heparin. Since this would indicate that a complex was formed in the solution and that the pepsin activity was decreased by such complex formation, further examination was made to investigate the pepsin-ROS-S complex. A mixture of ROS-S and pepsin solution was separated by electrophoresis, applying a potential of 220 V (3 mA) for 3 hr., on a paper impregnated with a Veronal buffer (pH 8.6) or acetate buffer solution (pH 3.6). The formation of pepsin-ROS-S complex was traced by a color reaction with Mucicarmine (staining agent for ROS-S) and with Bromophenol Blue (staining agent for pepsin). Two bands were detected on the paper impregnated with Veronal buffer, corresponding to ROS-S and pepsin which were applied also for the same paper. On the paper impregnated with acetate buffer, ROS-S band stained with Muci-

*1 Part Ⅳ : This Bulletin, **10**, 177 (1962).
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*3 Abbreviated as ROS-S.

1) Part Ⅲ. M. Namekata : This Bulletin, **10**, 171 (1962).
2) Part Ⅳ. *Idem* : *Ibid.*, **10**, 177 (1962).
3) S. Levey, S. Sheinfeld : Gastroenterology, **27**, 625 (1954).
4) W. Anderson, J. Watt : J. Pharm. and Pharmacol., **11**, 173T (1959).
5) *Idem* : J. Physiol. (London), **147**, 52p (1959).
6) J. Heremans, J. P. Vaerman : Clin. Chim. Acta, **3**, 430 (1958).

carmine and a new band detected with Bromophenol Blue appeared. The new band, moved slightly to the anode side, was different from that of ROS-S or pepsin, and was considered as the pepsin-ROS-S complex. In the experiment on heparin, carried out by the same procedure as on ROS-S, a new band of the complex was also detected with Bromophenol Blue on the paper impregnated with acetate bufler (pH 3.6).

It could be concluded that pepsin would form a complex with ROS-S or heparin in acid solution but not in alkaline solution. To make this clear, further investigation was carried out to isolate the complex of pepsin with ROS-S and heparin. Crystalline pepsin was dissolved in 2% ROS-S solution (pH 5.2) to the concentration of 20.0 (I), 10.0 (II), 5.0 (III), 2.5 (IV), or 1.25 mg./cc. (V), 5 cc. of each solution was transfered to a graduated centrifuge tube, and adjusted to pH 1.6 with 10% HCl. They were stood for 30 min. in a refrigerator and centrifuged at 2000 r.p.m. for 10 min. The precipitate in (I) would be of pepsin, and a flocculent precipitate, which would be the complex, was seen in (II) and (V), the amount of the precipitate in (II) being twice that of (V), and that in (III) and (IV) were less than that in (V). To 10 cc. of ROS-S solution (20 mg./cc.), 100 mg. of pepsin was dissolved and the solution, adjusted to pH of 1.6 immediately, was allowed to stand overnight in a refrigerator. The precipitate, collected by centrifugation, was washed with 0.2*N* HCl until free of ROS-S in the supernatant, checking with Toluidine Blue, as ROS-S is soluble and the complex is almost insoluble in 0.2*N* HCl solution. Majority of the wet precipitate was washed with Me_2CO and Et_2O, and dried over P_2O_5 *in vacuo*. Yield, 58 mg. Both wet and dried precipitates were dissolved in water, adjusted to pH 4.0~4.6 with NaOH, and electrophoresis of the solution was carried out with acetate buffer (pH 3.6). The precipitates were confirmed as pepsin-ROS-S complex (Fig. 1a). In the experiment with heparin by the same procedure, heparin-pepsin complex was also isolated (Fig. 1b).

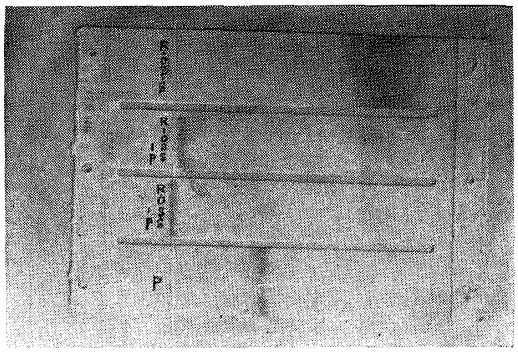

Fig. 1a. Paper Electrophoresis of Pepsin-ROS-S Complex in pH 3.6 Acetate Buffer

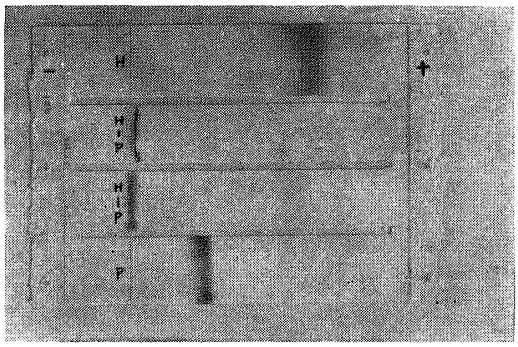

Fig. 1b. Paper Electrophoresis of Pepsin-Heparin Complex in pH 3.6 Acetate Buffer

These two complexes were electrophoretically examined on their stability in the acetate buffer solution of pH 3.6, 4.4, 5.2, and 5.6. It was found that the complex was dissociated into the two compounds in the solution of pH 5.2 and 5.6 (Fig. 2a and 2b).

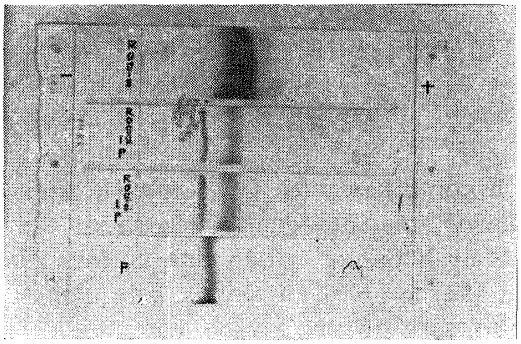

Fig. 2a. Paper Electrophoresis of Pepsin-ROS-S Complex in pH 5.2 Acetate Buffer

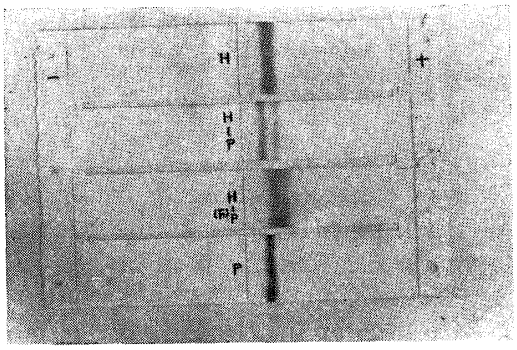

Fig. 2b. Paper Electrophoresis of Pepsin-Heparin Complex in pH 5.2 Acetate Buffer

Proteolytic Activity of the Complex——Proteolytic activity of both complexes (pepsin-ROS-S and pepsin-heparin) was determined with a solution (5 mg./cc.) adjusted to pH 1.6 by the same procedure as in the previous report.[1] They exhibited no proteolytic activity and it was obvious that the pepsin activity was decreased by the formation of an inactive complex with polysaccharide sulfates.

Separation of Pepsin from the Complex and Recovery of its Activity——Since it was clarified that the complex was dissociated in a solution of pH 5.2, further investigations were carried out to bring the complex solutions to pH 5.2 in order to confirm the recovery of the activity of pepsin after the removal of polysaccharide sulfates.

1) Removal of Polysaccharide sulfates as Ba Salt: To 5 cc. of the solution containing pepsin (0.5 mg./cc.) and ROS-S or heparin (2.5 mg./cc.), a small amount of $(AcO)_2Ba$ (powder) was added. The solution (pH 5.2) was allowed to stand until free of ROS-S or heparin in the supernatant, checking with 1% Toluidine Blue solution, and filtered. The filtrate was treated with cation exchange resin (Amberlite IR-120, H-type) to remove the excess of barium.

2) Removal of Polysaccharide Sulfates as the Protamine Complex: To 5 cc. of the solution, 0.2 cc. of aqueous solution of protamine (20 mg./cc.) was added, insoluble protamine-ROS-S complex was filtered off, and the filtrate (pH 2.0) was adjusted to pH 1.6.

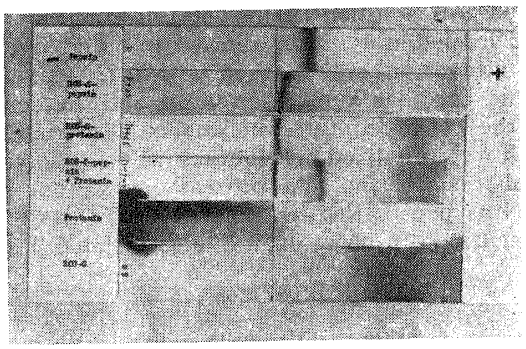

Fig. 3. Paper Electrophoresis of Pepsin-ROS-S Complex and Protamine Complex in pH 3.6 Acetate Buffer

Table I. Recovery of Pepsin Activity, after Removal of ROS-S from ROSS-Pepsin Complex

Expt. No.	Sample	Pepsin activity (%)
(I)	Pepsin alone	100
(II)	Pepsin-ROS-S	33
(III)	Pepsin-ROS-S (Ba treatment)	85.9
(IV)	Pepsin-ROS-S (protamine treatment)	95.5
(V)	Pepsin-ROS-S + protamine	92.3

The proteolytic activity of such a filtrate was determined by the same procedure as previously described. In Table I, the results are shown with that of control pepsin. It was found that the pepsin activity of the solution (II) was only 33% of the control pepsin (I),*4 while those of the filtrates (III)*4 (treated with $(AcO)_2Ba$ and protamine) were 85.7% and 95.5%, respectively. It could be considered that the proteolytic action of pepsin in the pepsin-ROS-S complex was almost completely recovered by removing ROS-S considering the loss of pepsin during the operation. It could also be concluded that pepsin forms a dissociable complex with ROS-S without losing its proteolytic activity. Considering the fact that the proteolytic action of pepsin in the pepsin-ROS-S complex was recovered 92.3% by only addition of protamine, pepsin is in free active form in the solution, while ROS-S was combined with protamine.

Discussion

It was electrophoretically confirmed that pepsin combines with ROS-S or heparin in the solution of pH 1.6~4.4, and such a complex dissociates into each component at the pH above 5.2. The complex of pepsin with ROS-S or heparin, which has no proteolytic activity, was isolated. The activity of pepsin in the complex is almost completely recovered by removing ROS-S or heparin as barium salt or protamine complex. The complex is stained with Bromophenol Blue, not with Mucicarmine. It is postulated from the results described above and the works done by Fischer and others[7~10] that the com-

*4 These solutions were obtained from the complex formation experiment described above.

7) A. Fischer: Biochem. Z., **278**, 133 (1935).
8) K. Meyer, *et al.*: J. Biol. Chem., **119**, 501 (1937).
9) L. B. Jacques: Biochem. J., **37**, 189 (1943).
10) S. M. Partridge: *Ibid.*, **42**, 387 (1948).

plex is formed by the combination of free amino group in pepsin and sulfate groups in ROS-S or heparin.

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Summary

It was confirmed that the complex of pepsin with ROS-S and heparin is formed in an acid solution (pH 1.5~4.4) and that the complex is dissociated at the pH above 5.2. The isolated complex has no proteolytic activity, but it was almost completely recovered by removing ROS-S or heparin as the barium salt or protamine complex. It was confirmed that the inhibitory action of polysaccharide sulfates on proteolytic action of pepsin is caused by the formation of inactive complex of pepsin with the sulfates.

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31. Masazumi Kawanishi : Synthesis of 1-Alkyl-1,2,3,4,6,7-hexahydro-11b*H*-benzo[*a*]quinolizin-2-one Derivatives.

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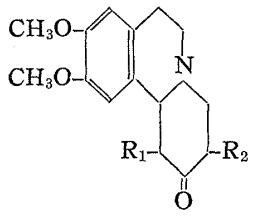
Though it is more than 40 years ago since Pyman[1] had correctly proposed benzoquinolizine as the fundamental skeleton for the Ipecachuanha group of alkaloids, it has never been met in synthetic medicinals until quite recently Schnider, *et al.*[2] ascribed a tranquilizing property to their synthetical products, 3-alkyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b*H*-benzo[*a*]quinolizin-2-ones (B), whose 3-isobutyl derivative was introduced into the clinical field by the Hoffmann La-Roche Inc. as Nitoman.



(A) R_1=alkyl, R_2=H
(B) R_1=H, R_2=alkyl

The present writer, who for some time has been engaged in the study related to rotundine, was interested with the above report and decided to synthesize a series of 1-alkyl-1,2,3,4,6,7-hexahydro-11b*H*-benzo[*a*]quinolizin-2-one derivatives (A) for pharmacological evaluation.

For this objective, it seemed pertinent to develop the method of Ban,[3] which he

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*1 Toda-machi, Kita-adachi-gun, Saitama-ken (川西正純).
1) W. H. Brindley, F. L. Pyman : J. Chem. Soc., **130**, 1067 (1927).
2) A. Brossi, H. Lindlar, M. Walter, O. Schnider : Helv. Chim. Acta, **41**, 119 (1958).
3) Y. Ban : This Bulletin, **6**, 312 (1958).