On the Carbohydrate Group in Yam Mucin.

By

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Two kinds of yams, *Dioscorea Batatas* Dcne. and *Dioscorea japonica* Thunb., are in common use in Japan as an article of food. They yield a mucilaginous substance when crushed or grated and extracted with water. The chemical nature of this slime was, for the first time, studied by J. Ishii[1]. According to him, the slimy matter which is precipitated from its solution by dilute acetic acid, shows characteristic reactions for protein including that of Molisch and yields a reducing substance on boiling for some time with 5% sulphuric acid. Basing chiefly upon these reactions and its ultimate composition[2], Ishii concluded that the slime belongs to the class of mucins. As he, however, made no detailed study[3] concerning the presence of a carbohydrate group in his preparation, its relation to the true mucins remains yet to be solved[4].

1) Bull. Coll. Agric., Imp. Univ. Tokyo, 2, pp. 97—100.

2) Ishii, l. c. The purified preparation had the following composition: C 52.82, H 7.53, N 14.20, O & S 25.05 and ash 0.41.

3) Too much importance can not be laid upon the Molisch's reaction, as it is known to be too delicate a reaction to be accepted as conclusive evidence of a carbohydrate group in the protein molecule. Cf. T. B. Osborne and J. H. Harris—*The carbohydrate group in the protein molecule*—Jour. Amer. Chem. Soc., 25 (1903), pp. 476—478. That the copper reducing property of the hydrolysis product does not give any clue as to the nature of the reducing substance needs no comment.

4) Cf. T. B. Osborne—*Die Pflanzen Proteine* (Sonderabdruck aus: Asher u. Spiro-Ergebnisse der Physiol., 10 (1910), p. 214.

Mucins[1] are characterized as compounds of the protein molecule with a substance or substances containing a carbohydrate group. The carbohydrate has been identified with glucosamin (chitosamin)[2].

As the occurrence of mucin in the vegetable kingdom has not yet been fully demonstrated, it seemed to us of great interest, to investigate whether or not the glucosamin group is present in the yam mucin and thereby elucidate its true nature.

As the material of our study, the tubers of *Dioscorea Batatas* Dene. were taken and prepared in a manner essentially the same as that followed by Ishii (l. c.), with but slight modifications as our experience suggested. The tubers were grated as thoroughly as possible and then allowed to stand for several hours when starch granules and other substances settled at the bottom of the vessel.

The thick liquid thus obtained was strongly acid in reaction. It was filtered first through linen cloth, then through filter paper, without suction. The last precaution is neccessary, as otherwise some of the starch granules may pass through the filter. The starch was always tested with iodin solution under microscope. The starch-free filtrate was carefully acidified with dilute acetic acid until the concentration of the acid reached to 0.5–1% of the liquid, when a flocculent precipitate was formed in abundance. After standing over night, the clear supernatant liquid was decanted, the precipitate transferred to a filter, and washed well first with 1% acetic acid, then with dilute hydrochloric acid to remove any adhering protein which might be soluble in it, then with a mixture of alcohol and ether and finally with ether alone. When dried in vacuum over sulphuric acid, a yellowish amorphous mass was obtained. About 4.5 grams of the preparation were thus procured

1) Under mucins we include both true mucins and paramucins, the latter as distinguished from the former, chiefly in the property of not being precipitated form its solution by aeetic acid. Cf. Röhmann Biochemie, Berlin, 1908, p. 702.

2) Fr. Müller—Zs. f. Biol., 42 (1901), pp. 468—564.
J. Seemann—Inaug. Diss. Marburg, 1898; Chem. Centr., 1898 II, p. 1271.
C. Neuberg u. F. Heymann–Beiträge z. chem. Physiol. u. Pathol., 2 (1902), p. 201.
H. Steudel—Hoppe-Seylers Zs. physiol. Chem. Strassburg, 34 (1902), pp. 353—384.
A. Oswald—Hoppe-Seylers Zs. physiol. Chem. Strassburg, 68 (1910), pp. 173—180.

from 1 kilogram of the fresh tuber.

Qualitative Reactions.

The preparation obtained in the above described manner showed the following qualitative reactions.

1) It dissolves easily in caustic alkali of about 5% ; in weaker solutions it dissolves with difficulty.

2) It dissolves in strong mineral acids as well as in strong acetic acid.

3) It gives xanthoprotein, biuret, Adamkiewicz's and Millon's reactions but not Liebermann's.

4) Tannin precipitates its solution, while double iodide of potassium and mercury produces a turbidity.

5) The distillate of the substance with hydrochloric acid, 1.06 sp. gr., gives no furfurol reaction with either anilin acetate or phloroglucin solution.

6) On boiling for some time with 5% sulphuric acid, it yields not only the substance which gives biuret reaction but also such as reduce Fehling's solution. The reducing power is still observed even after the hydrolysis product is treated with phosphotungstic acid in the usual manner.

7) It possesses a weak diastatic power, evidently due to the enzyme admixed with it. Reactions for other enzymes were also tested, but with negative results[1].

Identification of Glucosamin.

For the identification of glucosamin in the cleavage products of protein bodies, four methods have been proposed, namely: benzoate method of Fr.

1) As the preparation had diastatic power, our interest was naturally aroused to the study of enzymes in yam tubers. Water extract was made in the manner already described and in it the following enzymes were identified:

A) **Diastase**; B) **Oxidase**; C) **Catalase.**

The detailed report is expected to be published in the near future by one of us.

Müller[1], phenylisocyanate method of H. Steudel[2], oxidation method (norisosaccharic acid) of Neuberg-Wolff[3] and the direct method of A. Oswald.[4] Of these, the last method by Oswald is simplest and allows, at the same time, the direct identification of glucosamin as such. We have therefore attempted to detect glucosamin in the hydrolysis product of the yam mucin according to Oswald's method.

6 grams of the dry preparation were mixed with 240 c.c. of 3% hydrochloric acid and heated in a water bath for 10 hours, with a reflux condenser. The dark brown liquid was filtered, concentrated slowly on a water bath to a syrup and then kept over sulphuric acid. No characteristic crystals of glucosamin hydrochloride appeared even after standing for several weeks. The syrup, however, gave Molisch's reaction as well as biuret reaction very strongly. Our failure to obtain glucosamin hydrochloride was evidently due to the lack of favorable conditions for its separation.

We have therefore tried to isolate it as osazone from the syrup. The syrup was diluted with water and precipitated with phosphotungstic acid in the usual manner. The neutralized syrup was heated with 2 parts of phenylhydrazin hydrochloride and 3 parts of sodium acetate in a water bath for $1\frac{1}{2}$ hours. Characteristic crystals of phenylglucosazone appeared, which when cold was filtered and recrystallized from 60% alcohol. The melting point of the osazone was determined and found to be 203°. Consequently it is probable, if not conclusive, that the syrup contained glucosamin.

Having failed with Oswald's method, we had attempted to identify glucosamin with Neuberg-Wolff's method in the following manner.

30 grams of the preparation were mixed with 40 c.c. of fuming hydrobromic acid, sp. gr. 1.49, and allowed to stand for 2 hours in cold with frequent shaking. At the end of this time, when the whole mass appeared to have been gelatinized, it was diluted with 200 c.c. of water and heated gently in a water bath for $1\frac{3}{4}$ hours with a reflux condenser. The dark

1) Zs. f. Biol., 42 (1901), pp. 468—564.

2) Hoppe-Seylers Zs. physiol. Chem. Strassburg, 34 (1902), pp. 353—384.

3) Ber. D. chem. Ges., Berlin, 34 (1901), pp. 3840—3846.

4) Hoppe-Seylers Zs. physiol. Chem. Strassburg, 68 (1910), pp. 173—180.

brown liquid was filtered and the filtrate was decolorized with animal charcoal. The clear yellowish liquid thus obtained reduced Fehling's solution very strongly. The amount of free hydrobromic acid in the solution was then determined by titration against decinormal alkali solution and about 4/5 of the calculated amount of lead carbonate necessary to neutralize it were added, under strong agitation. After 2 hours standing, the lead bromide was sucked out and the filtrate was concentrated in vacuum under 40°, until nearly all of the remaining hydrobromic acid was driven out. The brownish syrup obtained was boiled with 100 c.c. of 96% alcohol and filtered; the residue was moistened with a little warm water and again extracted with alcohol. This process was repeated several times, until the reducing substance could no longer be detected in the alcoholic extract. The extracts were united, the precipitates formed on standing (chiefly lead bromide) filtered off and then concentrated to a syrup. The syrup was dissolved in 30 c.c. of nitric acid, sp. gr. 1.2, and heated on a water bath until red fumes came off. It was then allowed to cool, 10 c.c. of nitric acid were added and concentrated to a small volume, with constant stirring. About 50 c.c. of water were then added and again evaporated to drive out the nitric acid. Finally the syrup was dissolved in 100 c.c. of water, decolorized with animal charcoal and freed from the remaining hydrobromic acid by the careful use of silver nitrate. The filtrate from silver bromide was exactly neutralized with ammonia and then acidified with a few drops of very dilute acetic acid. Calcium acetate was then added drop by drop to precipitate oxalic acid, neutralized again with ammonia and boiled with concentrated lead acetate solution. When cold the lead precipitates were separated by filtration with suction and washed well with cold water. The precipitates were suspended in 120 c.c. of water and decomposed thoroughly with hydrogen sulphide. The excess of hydrogen sulphide was driven off by boiling the solution, and the filtrate was concentrated on a water bath. The solution thus obtained should contain norisosaccharic acid produced by oxidation of glucosamin, if it were present. To the hot aqueous solution of norisosaccharic acid, an excess of cinchonin was added until its reaction was distinctly alkaline. When cold it was filtered and the filtrate treated in a separating funnel with acetic ether to extract the free

cinchonin remaining in solution. The watery solution was then concentrated to a syrup. On cooling, the crystals of cinchonin salt began to show themselves in the syrup. After 24 hours standing, the crystalline mass was mixed with about 3 c.c. of water, filtered and washed with a little cold water. Colorless needle shaped crystals were obtained, upon recrystallization from hot water, using animal charcoal. Its yield was 0.55 gram. The melting point was found to be 208–209°. The specific rotatory power as determined by Schmidt and Haensch half shadow apparatus was as follows:–

$$[\alpha]_D = \frac{2.1 \times 0.346 \times 25}{0.1017 \times 1} = +177.1°.$$

0.1934 gm. substance dried at 105° gave 1.321 mg. N.

Calculated for $C_6H_{10}O_8(C_{19}H_{22}N_2O)_2$ = N 7.02%

Found..... = N 6.89%

The observed physical constants and analysis clearly indicate that the substance under examination is cinchonin norisosaccharate. The presence of glucosamin group in the yam mucin is hereby fully demonstrated.

Hydrolysis of the Residue.

The hydrolysis products of the protein group of true mucins have received but little attention, owing no doubt chiefly to the difficulty of obtaining sufficient material for the investigation. As a larger portion of the yam mucin used for the separation of glucosamin by hydrobromic acid, in the previous experiment, remained apparently not much affected, we have undertaken to utilize this residue and study the nature of its cleavage products.

The residue, after being dried at a low temperature to drive off the remaining hydrobromic acid, was hydrolyzed by heating with 50 c.c. of 25% sulphuric acid, first in a water bath for 2 hours and then on a sand bath for 12 hours. On cooling, the hydrolysis solution was diluted with 100 c.c. of water, filtered and the filtrate neutralized with baryta. The barium sulphate which separated was sucked out and washed thoroughly by repeatedly boiling

with water. The filtrate was decolorized with animal charcoal and concentrated to crystallization. When the syrup was kept over sulphuric acid for some time, tyrosin began to crystallize in characteristic delicate silky needles. It gave a beautiful red color with diazobenzensulphonic acid in the presence of sodium hydroxide. The tyrosin crystals were separated by filtration and the filtrate kept again over sulphuric acid. Three crops of tyrosin mixed with leucin or of leucin alone were obtained in succession. For the separation of leucin from the mixture, boiling acetic acid was used.

Glutaminic acid hydrochloride was separated by saturating the mother-liquor from leucin, with hydrochloric acid gas and allowing it to stand in an ice box for several days. The yields of the amino-acids follow:

Tyrosin 0.41 gram; leucin 0.25 gram; and glutaminic acid hydrochloride 0.12 gram.

Further purification was not attempted because of their small amounts. They gave following results on analysis.

0.1504 gm. tyrosin gave 0.3311 gm. CO_2 and 0.0895 gm. H_2O.

Calculated for $C_9H_{11}O_3N$ = C 59.66; H 6.07 %

Found ..... = C 60.04; H 6.61 %

0.1450 gm. leucin gave 0.2875 gm. CO_2 and 0.1305 gm. H_2O.

Calculated for $C_6H_{13}O_2N$ = C 54.96; H 9.92 %

Found ..... = C 54.06; H 9.80 %

0.1 gm. glutaminic acid hydrochloride gave 0.1201 gm. CO_2 and 0.0481 gm. H_2O.

Calculated for $C_5H_9O_4N$. HCl = C 32.69; H 5.45 %

Found ..... = C 32.75; H 5.35 %

The presence of other amino-acids is not excluded but the mother-liquor from the glutaminic acid hydrochloride was too little to be examined further.