SOYBEAN OLIGOSACCHARIDES

ISOLATI ON BY GEL FILTRATION AND IDENTIFICATION BY ACETYLATION

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In a preceding paper KAWAMURA and KIMURA[1] reported on the identification of soybean oligosaccharides, sucrose, raffinose, and stachyose, by the melting point and specific rotation of acetylated compounds. However, the stachyose tetradecaacetate seemed to be yet impure. Moreover the papar of ONUKI[2] was overlooked, which described acetylation of stachyose. Therefore, the acetylation should have been reexamined.

It was necessary to prepare a considerable amount of stachyose in order to reexamine the acetylation. Carbon column chromatography as recommended by WOLFROM and THOMPSON[3] was applied to defatted soybean meal, but it took much time to collect a considerable amount of stachyose by this method. Recently the dextran gel filtration has been extended to lower molecular compounds, e. g. Sephadex G-15[4] may be applied to molecular weights lower than 1500. By using Sephadex G-15 we can prepare stachyose very easily, because this technique can give us first the compound with the highest molecular weight, contrary to carbon column chromatography which gives us first the compound with the lowest molecular weight.

This paper reports the isolation of oligosaccharides, especially stachyose, from defatted soybeans (raw and heated) by gel filtration and the melting point and specific rotation of free and acetylated oligosaccharides.

Experimental

1. Isolation of Oligosaccharides from Defatted Soybean Meal

1. 1. *Samples* A representative variety of the U. S. southern district, Hampton, was used. The oil was extracted with hexane from the soybean (1962 crop) by the ordinary factory process. Some experiments were made on this defatted flake itself, while some other experiments were made on the defatted flake autoclaved at 120° for 10 min at the moisture content of 20%.

1. 2. *Extraction of sugars* Pulverize 30 g of the raw or autoclaved defatted soybean flake in an electric mixer with 250 ml of 80% ethanol. Wash the pulverized sample into an Erlenmeyer flask with 50 ml of 80% ethanol. Reflux for 1 hr. Centrifuge and wash the residue well with 80% ethanol and then with distilled water. Filter. Remove ethanol from the filtrate and washing. Add saturated lead acetate solution to deproteinize. Centrifuge. Add 2 *N* sodium carbonate to the solution to remove excess lead ions. Adjust the pH to 6.8. Concentrate the sugar extract to about 100 ml below 40° with a rotary evaporator. Pass the concentrated sugar extract through a mixture of ion-exchange resins Amberlite IRC-50 and IR-45 (1 : 1) packed into a tube of 30 mm × 450 mm. Concentrate the deion-

ized extract again with a rotary evaporator to about 15 ml. Keep in a deep freezer at −20°.

1. 3. *Separation of sugars by carbon column chromatography*[1-3] Stir and mix 120 g Darco G-60 and 120 g Celite 545 with 300 ml 50% ethanol. Put into a tube of 36 mm × 450 mm. Wash with 50% ethanol and distilled water. Add 10 ml concentrated sugar extract on the top of the column, connected to a fraction collector. Elute with distilled water, 4, 8, 15, and 30% ethanol subsequently. Take each fraction of 20 ml. Determine sugar on 1 ml of each fraction by the phenol-sulfuric acid colorimetry. Collect the sugar-containing fractions and detect the sugar by paper chromatography with butanol-pyridine-water (6 : 4 : 3) as the solvent system and 3% *p*-anisidine hydrochloride in water-saturated butanol as the spraying reagent. Collect the fractions containing the same sugar, concentrate under reduced pressure, and crystallize from 95% ethanol or subject to acetylation after drying under reduced pressure to anhydrous powder.

1. 4. *Separation of sugars by gel filtration*

1. 4. 1. *Preliminary experiments* Put 35 g (dry weight) Sephadex G-15 into a glass tube of 12.5 mm × 900 mm. Let it swell with 350 ml distilled water for 3 hr. Wash well with distilled water. Connect to a fraction collector. Dissolve 0.2 g each of glucose, sucrose, and raffinose, in 2 ml water. Put 0.5 ml mixed solution on the top of the column. Elute with distilled water. Collect the fractions of 2 ml. Detect the sugar by the phenol-sulfuric acid method. Identify and determine the sugar by spotting 10–50 μl on paper by paper chromatography. The effluent speed was set to 6 or 4 ml/hr. (See Figs. 1 and 2.)

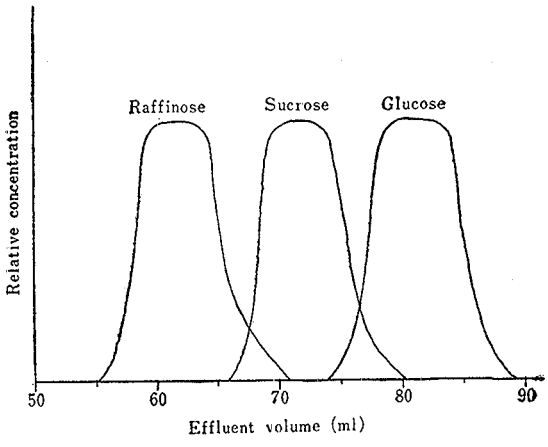

Fig. 1. Separation of standard sugars by gel filtration
(Effluent speed 6 ml/hr)

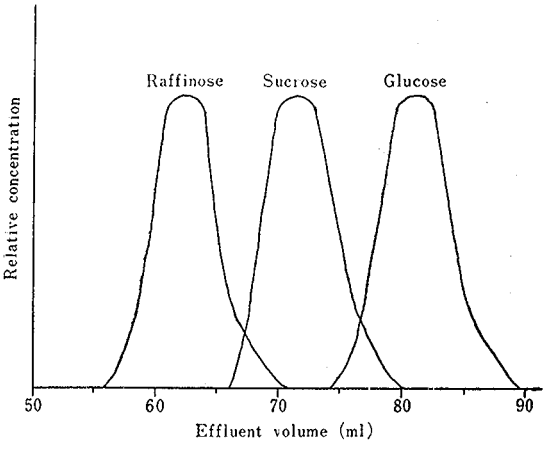

Fig. 2. Separation of standard sugars by gel filtration
(Effluent speed 4 ml/hr)

The amount and fraction numbers of the three sugars were almost the same in these 2 effluent speeds. Only the peaks of the curves of relative concnetration vs. fraction number were sharper with 4 ml/hr than with 6 ml/hr. The results were entirely the same when the experiment was repeated 3 times under the same conditions.

Put 0.5 ml sugar extract from defatted soybeans on the top of the same column. Examine the separation of sugars (verbascose, stachyose, raffinose, sucrose, and glucose)

at the effluent speed of 6 ml/hr. (See Fig. 3.)

In this case pure stachyose was collected in the fractions ranging 53–61 ml. The mixed fractions of 62–72 ml was concentrated and again put to gel filtration and the result was that pure stachyose was again collected at 54–61 ml of effluents. Thus stachyose can easily be isolated purely.

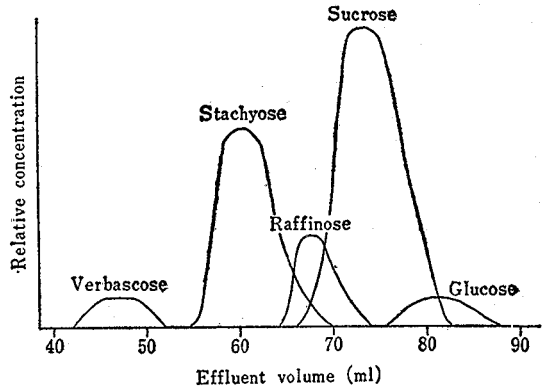

Fig. 3. Separation of oligosaccharides from raw or heated defatted soybean meal

1. 4. 2. *Isolation of stachyose from defatted soybeans by gel filtration*

Sugars were extracted from 500 g defatted soybeans by refluxing with 2 L 80 % ethanol (3 times) and washing with 1 L distilled water. Total filtrate was concentrated to 1 L. The concentrated sugar extract was deproteinized with lead acetate and sodium carbonate as described above. However, it was not deionized and adjusted to pH 6.8. Then it was concentrated under reduced pressure to 100 ml, and the concentrated extract was kept in a freezer.

Gel filtration was made as follows (See Fig. 4). Extend the bottom (1) of the long glass tube (2) of 30 mm × 2000 mm. Narrow the lower end, and attach a glass cock (3) for regulating the effluent speed. Put 350 g (dry weight) Sephadex G-15 into this tube. Connect the tube to a fraction collector (4). Place a 2 L flask (5) containing distilled water for elution, at the level lower than the top of the glass tube. Connect the top of the tube and the 2 L flask (5) with a siphone (6) set with a valve (7). Make two more holes through the rubber stopper at the top of the long glass tube (2) than the hole for that siphone. Pass two small glass tubes through the two holes. Attach a pinch cock (8) to one of the two glass tubes for pouring the sample solution and attach a valve (9) to the other glass tube for air inlet. Wash well the entire glass tube (2) with distilled water. Certify that no sugar is detected by the phenol-sulfuric acid method. Set the fraction collector so as to make 6 ml each of fraction at the effluent speed of 50 ml/hr and to collect 200 test tubes in 24 hr, total amount being 6 ml × 200=1200 ml. Do not stop the effluence from the column, but close the siphone valve (7) and open the air inlet valve (9). Pour 5 ml

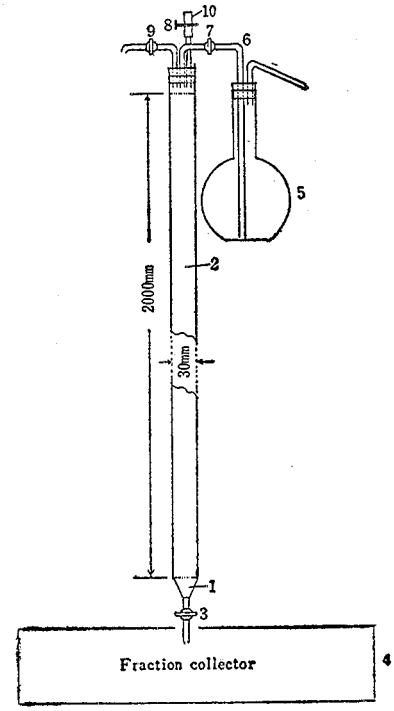

Fig. 4. Apparatus for gel filtration (See the text for explanation)

sample solution through the small glass tube with a pipette which adapts well with the rubber tube (10) of the pinch cock (8) rapidly, when the liquid level within the glass tube lowers down to the top of the Sephadex column. Close the air inlet valve (9), when the sample solution completely spreads over the top of the column. Simultaneously begin the collection. The sample in the pipette will be poured into the glass tube by the force of effluence. Open the air inlet valve (9) and pour about 5 ml distilled water through the sample inlet hole (10) rapidly, when the total amount (5 ml) of the sample solution is poured. Make the thickness of water about 1.5-2 cm over the top of the colum. Close the air inlet (9) and sample inlet (10) holes, and open the siphone valve (7). It is necessary to take care that the liquid should not come down the top of the column to produce air bubbles in the column. A part of the effluent in each test tube is examined if any sugar is present by the phenol-sulfuric acid method and if present the sugar was identified and estimated by paper chromatography. Measure the electrical resistance on each fraction by a kiloohmmeter to examine the amount of salts present.

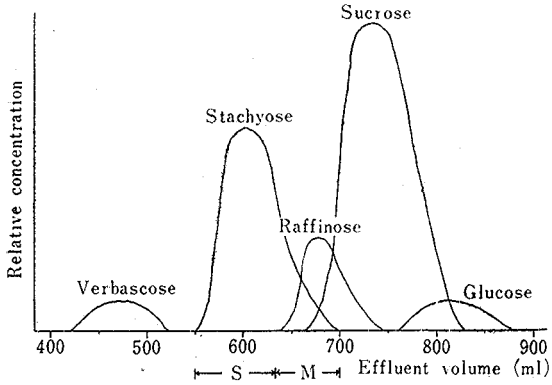

Fig. 5. Separation of oligosaccharides from defatted soybean meal by gel filtration
S: pure stachyose fractions
M: fractions of mixture consisting of stachyose, raffinose, and sucrose

The result is shown in Fig. 5. This is entirely similar to Fig. 3, only the scale is tenfold. Electric resistance was measured to see the separation of salts. The effluents 850-930 ml showed 7-8 kΩ/cm; thus they contain salts. The effluents 6-690 and 1080-1200 ml showed 200 kΩ/cm which is equal to the resistance of deionized water used in this experiment; thus these effluents do not contain additional salts.

Paper chromatography showed entirely the same results on various effluents, when applied at the 2nd, 5th, and 15th times of gel filtration.

The fractions 640-700 ml (M of Fig. 5) were collected, concentrated, and reseparated. The effluents 550-630 ml (S of Fig. 5) was concentrated to 20 ml sirup which contained no other sugar than stachyose as revealed by paper chromatography.

This experiment was made primarily for isolating stachyose and no considerations were paid to isolation of other sugars. Figs. 1-3 and 5 show only relative concentrations according to the coloration of developed spots on paper chromatogram.

This experiment was made at the room temperature, but in January, i. e. at 3-8° and no consideration was thought necessary to prevent the effects of microbial enzymes.

2. Identification of Soybean Oligosaccharides from Melting Point and Specific Rotation of Free and Acetylated Compounds

2. 1. *Materials and methods* Sugars were isolated from extracts from raw and autoclaved flakes of Hampton soybeans by carbon column chromatography and later by gel

filtration.

Acetylation was made with sodium acetate catalyst.[5] Reflux 10 parts pure and anhydrous sugar with 5 parts anhydrous sodium acetate and 70 parts acetic anhydride for 10–14 min in an oil bath (120–30°). Pour into 500 parts ice water and keep in a refrigerator for 4 hr with intermittent stirring. Discard supernatant and add 500 parts ice water. Pulverize the sirupy solids. Keep in a refrigerator for further 2 hr. After 2–3 repetitions of these procedures, filter the solid material. Wash with water. Dissolve in about 50 parts 95% ethanol by warming. Keep at the room temperature to crystallization. Put in a refrigerator to grow crystals. Filter crystals, dissolve in 95% ethanol, and recrystallize. Repeat this recrystallization 3 times. Dry at reduced pressure (at 30°), and measure the melting point and specific rotation.

Optical rotation was measured by a photomagnetic direct reading polarimeter.

2. 2. *Results* Anhydrous sucrose (1.00 g) and 0.89 g raffinose isolated from soybeans gave 1.5 g crystalline sucrose acetate aud 1.2 g crystalline raffinose acetate respectively. But 0.42 g stachyose did not give crystalline acetate even after several months of storage after acetylation (it gave about 0.5 g white powder).

More (6 g) stachyose was used for acetylation, which was obtained from soybeans by gel filtration. It did not give crystalline acetate as Onuki[2] reported. Thus it was dissolved in 95% ethanol, cooled, and the precipitate formed was centrifuged. This procedure was repeated until no increase in melting point was noted (7 times).

The characteristics of free and acetylated sugars were entirely similar for those obtained from raw and autoclaved soybean flakes. They are shown in Tables 1 and 2.

Table 1 The melting point and specific rotation of the free, anhydrous sugars from raw or autoclaved soybean flakes

Sugar	m.p. Found	m.p. Literature	$[\alpha]_D$ Found	$[\alpha]_D$ Literature
Sucrose	176–180°	184–185°[6]	+66.7°/24°(H_2O)	+66.5°(H_2O)[6]
Raffinose	118–120°	118–120°[7]	+123.4°/20°(H_2O)	+123.1°(H_2O)[7]
Stachyose	170–172°	170° [2]	+146.2°/20°(H_2O)	+146.3°(H_2O)[2]

Table 2 The melting point and specific rotation of the acetates of sugars from raw or autoclaved soybean flakes

Acetate	m.p. Found	m.p. Literature	$[\alpha]_D$ Found	$[\alpha]_D$ Literature
Sucrose octaacetate	87°	87° [8]	+59.0°/20°($CHCl_3$)	+59.6°($CHCl_3$)[8]
Raffinose hendecaacetate	98–101°	99–101°[9]	+97.4°/16°(EtOH)	+92°(EtOH) [9]
Stachyose tetradecaacetate	94–95°	95–96°[2]	+120.1°/16°(EtOH)	+120.2°(EtOH) [2]

Discussion and Conclusion

Gel filtration with Sephadex G-15 was successfully applied to separation of oligosaccharides of soybeans, especially stachyose. Verbascose and other sugars may be separated as well, but this paper concerns effective preparation of stachyose. In this connection gel filtration is superior to carbon-Celite column chromatography, since the former technique gives us first the highest-molecular compound in a mixture.

The data for melting point and specific rotation of stachyose tetradecaacetate of the preceding paper[1] should be corrected to the present data. This acetate could not be crystallized as Onuki[2] noted. However, it was purified by 7 "recrystallizations." The data coincide well with those of Onuki and it proves that raw and heated soybean meal contains stachyose.

As to the heat treatment many data have been obtaind in KAWAMURA's laboratory on sugars by paper chromatography. Now it is conclusively stated that raw and heated defatted soybeans contain sucrose, raffinose, and stachyose as the main oligosaccharides.

Summary

The soybean oligosaccharides (sucrose, rsffinose, stachyose, and verbascose) were separated by gel filtration with Sephadex G-15. Especially preparation of stachyose from defatted soybeans by gel filtration was described in detail. Sucrose, raffinose, and stachyose were identified through melting point and specific rotation of free and acetylated compounds.

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大豆少糖類のゲル濾過による分離とアセチル化による確認

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要旨　川村，木村[1]は大豆の少糖類，サッカロース，ラフィノース，スタキオースをアセチル化し，その融点と比旋光度を測定した．しかしそのときのスタキオーステトラデカアセタートは不純のようであったし，小貫[2]の報文を見おとしていた．そのためアセチル化を再検討する必要があり，それにはスタキオースを相当量大豆からとり出す必要がある．炭素カラムクロマトグラフィー[3]では非常に時間がかかる．そこで最近市販されたセファデックスG-15[4]を用いて，スタキオースを容易に分離し得ることをたしかめた．このゲル濾過によると分子量の高い化合物がさきに得られるので都合がよい．ここに分離した純粋のスタキオースと前報でも扱ったサッカロースとラフィノースの無水糖ならびにそれぞれのアセチル化物につき融点と比旋光度を求めた．

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