Saccharide Constituents of Horse Chestnut (*Aesculus hippocastanum* L.) Seeds
II.* Isolation and Characterization of the Starch

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Dedicated to Corresponding Member of the Slovak Academy of Sciences M. Zikmund, in honour of his 70th birthday

Starch isolated from the seeds of horse chestnut was distributed into fine-grain and coarse-grain portions. Amylopectin was separated and the β-limit dextrin prepared was characterized by periodate oxidation. The α- and β-amylase susceptibilities were measured; it is deduced that the horse chestnut starch possesses a lower amylose content, short outer and longer inner chains than those of the reference potato starch.

Horse chestnut seeds represent — after extraction of triterpenoids with alcohol — an untraditional source of saccharides: in addition to L-arabinose and D-glucuronic acid ethanol can be obtained from the fermentable saccharides [1]. This paper describes the isolation and characterization of starch constituting a dominant portion of the above-mentioned horse chestnut debris.

The debris was filtered through a miller's cloth, floated and alternatively also extracted with dimethyl sulfoxide to afford the starch. Isolation of starch by extraction from the pasty starting material with perchloric acid followed by precipitation with iodine proved unsuccessful on the preparative scale. Washing of the ground material through a fine miller's cloth led to a high yield of starch (*ca.* 70 % of the starting material), which was partly contaminated by a fibrous material. Successive sedimentation of the starch was associated with a concurrent separation of grains according to their size; thus, fine-grain and coarse-grain starches were obtained. On the other hand, extraction with dimethyl sulfoxide afforded a more pure starch, but in a substantially lower yield, so that this material cannot be considered a representative sample. Isolation of starch from the seeds was more recently described [2] as difficult, because common procedures do not sufficiently attack the plasmatic substance needed for liberating starch from the cells; also the starch grains were then supposed as ones of the finest.

Fractions of starch obtained from the debris of horse chestnut were checked for purity and yield by sorption of iodine. This method employed for amylose determination in starches of various plant material revealed relative values of the starch content in the samples and fractions. Further information considering the starch fractions purity was provided by the results of hydrolysis obtained by monitoring the amount of arabinose stemming from the arabinan impurities (*cf.* Ref. [1]).

The crude starch samples containing arabinans according to sorption of iodine, results of hydrolysis and chromatographic determination of products, were fractionated by a partial dissolution in 2 M-NaOH. Starch was dissolved under this condition, whilst a portion of the starting material remaining undissolved was shown to be prevalently an arabinan according to products of hydrolysis. Precipitation of the solution with methanol afforded purified α-glucan fractions of the original fine-grain and coarse-grain starches, existing no more in the native granule form.

The content of amylose in the coarse-grain and fine-grain starches was found to be 14.2 % and 13.8 %, respectively; *i.e.* only a little difference exists between these two fractions, but the total amylose content was lower than that of the raw material commonly used in the starch industry. Nevertheless, the content of amylopectin determined in [2] by the chloral hydrate method was reported to be 73.6 %.

The isolated fine-grain horse chestnut starch was characterized from the viewpoint of its retrogradation; as shown, no retrogradation took place under conditions convenient for this process and no gel separated from the solution.

The horse chestnut starch was the starting material for isolation of amylopectin and preparation of β-limit dextrin. Amylopectin is obtained

* For Part I see Ref. [1].

from the starch solution after amylose was removed (in form of the insoluble butanol complex) by precipitation with methanol. The portion of isolated purified amylopectin, virtually amylose-free (w(amylose) = 0.6 %) amounted to approximately 50 % of the original starch. Amylose freed from the butanol complex was also virtually pure after precipitation with methanol. The β-limit dextrin was prepared by β-amylolysis of amylopectin, removal of maltose and salts by dialysis and freeze drying in 20 % yield with regard to the starting starch.

Composition of the starch and the structure of polysaccharide constituents influenced specifically the course of biochemical degradation. Determination of the enzyme susceptibility was decisive for characterization of the starch isolated. Fine-grain and coarse-grain starches were employed as substrates for α- and β-amylases (Fig. 1). Enzymes characterized by their specific activities

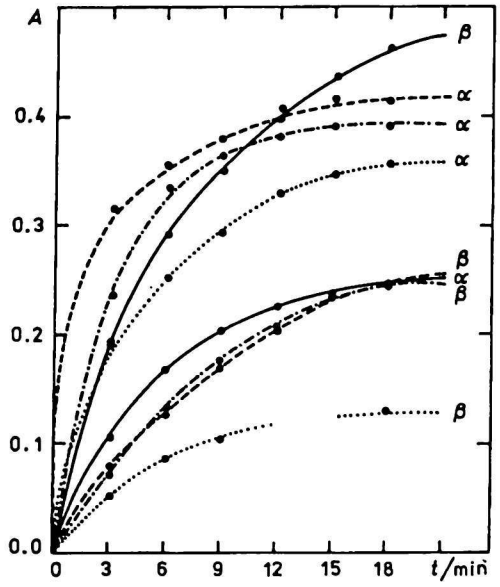

Fig. 1. Amylolysis of potato starch (—), large-grain starch from horse chestnut (-·-·-), fine-grain starch (····), and amylopectin obtained by fractionation of fine-grain starch (----) with α- and β-amylase.

measured with soluble starch were also applied for potato starch serving as a reference.

As found, various results were obtained on α-amylolysis of starches isolated from the ground seeds of horse chestnut. The coarse-grain starch displayed approximately by one half higher initial degradation rate using α-amylase than the fine-grain one; both are better substrates than the potato starch. The respective fine-grain and coarse-grain fractions were by one half and 2.5 times faster degraded than the potato starch. β-Amylolytic degradation of coarse-grain fraction is faster too, but the difference between fractions is not as remarkable as on α-amylolysis. The β-amylolytic degradation rate of horse chestnut starches makes approximately one third of that of the potato starch.

The periodate oxidation offered the most suitable chemical characterization of structural properties of the branched starch components. We found that formic acid was liberated in a lesser extent per the mass of sample and so the horse chestnut starch disclosed lower branching degree than the potato one. (Consumption of less than one equivalent of the periodate oxidation reagent per one D-glucose unit can indicate a residual contamination in products isolated.)

The horse chestnut starch is, according to our findings, characterized by a low amylose content and consequently, by the spontaneous property of its solutions not to retrograde. Due to a low β-amylolysis it could be deduced that the outer amylopectin chains are short and accordingly, the high α-amylolysis is facilitated by the presence of long inner chains. The above-mentioned property of the detailed structure and the over-all lower branching degree is more pronounced with the fine-grain starch.

EXPERIMENTAL

The starch was isolated from the peeled, sprout-free ground seeds of horse chestnut by mixing in methanol (*cf.* Ref. [1] - sample *B*). Water-soluble starch (anal. grade, Lachema, Brno; giving blue colour with iodine, M_r = 5400, *i.e.* DP = 33.3) was used for characterization of the enzyme preparations. The potato starch was a reference starch (Slovenské škrobárne, Spišská Nová Ves). α-Amylase from *Bacillus subtilis* (pH optimum 6.1, specific activity 9.7 nkat mg^{-1} at 20 °C) and β-amylase of barley (pH optimum 5.2, specific activity 22.7 nkat mg^{-1} at 20 °C) (Koch-Light, Colnbrook) were used. All the chemicals were of anal. grade (Lachema, Brno).

The isolated starches and polysaccharides obtained by fractionation were washed with methanol and dried under reduced pressure; preparations with equilibrium moisture were dried at 60 °C over P_2O_5. Solutions of polysaccharides were prepared by dissolving the moistured samples in 2 M-NaOH; the pastes were neutralized with 2 M-HCl after standing in a refrigerator for 0.5 and 20 h, respectively and diluted with water or buffer solution to the required concentration.

The amylose content was determined biamperometrically by sorption of iodine [3]. The sam-

ples were fractionated *via* insoluble butanol—amylose complex [4] and chromatographed by the over-flow descendence technique in acetone—water—butanol (φ_r = 7 : 1 : 2) on a Whatman No. 1 paper; detection with anilinium hydrogen phthalate [5].

The solution for retrogradation was prepared by dissolving starch in 2 M-NaOH, adjusting the pH value to 5.2 and dilution to 25 g dm^{-3} concentration; aliquots withdrawn from the solution without stirring [6] were centrifuged and the supernatant was subjected to iodine sorption.

Purity of the separate isolates was determined by iodine sorption and hydrolysis with 50 % formic acid at 90 °C for 8 h. D-Glucose and maltooligosaccharides were identified in starch hydrolyzates by paper chromatography; residues after isolation of starch contained L-arabinose in addition to D-glucose.

Isolation of Starch

Sieving and floating. The ground material (50 g) suspended in distilled water (1 dm^3) was filtered through a miller's cloth. The filtered material was washed in a high cylinder and allowed to spontaneous sedimentation. The successive sedimentation afforded the fine-grain and coarse-grain fractions of starch; both were centrifuged, several times washed with methanol to remove water. Yields of the fine-grain, coarse-grain fractions, and the residue were 23, 11, and 13 g, respectively. The crude starch fractions were dissolved in 2 M-NaOH, diluted, neutralized, centrifuged and precipitated from the solution by addition of an equal amount of methanol.

Extraction. A suspension of the ground material (5 g) in dimethyl sulfoxide (100 cm^3) was shaken for 48 h, centrifuged (1500 *g*) and the clear supernatant was precipitated slowly by adding methanol (300 cm^3). The precipitated starch was centrifuged, several times successively washed with methanol and ether and dried over P_2O_5. The yield was 0.36 g.

Enzyme Hydrolysis

Solutions of α- or β-amylase were added to the substrates according to their activity at such a rate that the increase of reduction power might remain under 10 % of the maximum value during the first 15 min at 37 °C in phosphate buffer of pH 6.2. The course of hydrolysis was monitored photometrically by the increase of the amount of reducing saccharides as determined by the *Somogyi* [7] and *Nelson* [8] methods on the basis of analytical lines relative to D-glucose at the given pH value.

β-Amylolysis of the saccharide to produce the β-limit dextrin lasting 72 h at room temperature was stopped by heating to 80 °C; the products were obtained after centrifugation, dialysis, and freeze drying.

Periodate Oxidation

The samples of polysaccharides (fine-grain and coarse-grain starches, β-limit dextrin; 150 mg each) were suspended in water (80 cm^3), sodium periodate (10 cm^3, ρ = 30 mg cm^{-3}) and water were added to the 100 cm^3 volume, the mixture was kept in a refrigerator for 1—2 d and the consumption of the oxidation reagent and the content of formic acid formed were determined according to [9] by iodometric titration with biamperometric indication.

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