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ENZYMATIC AND CHEMICAL METHODS FOR THE ANALYSIS OF STARCH

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Optimized experimental set-ups utilized for these studies are a sephacryl gel system for the analysis of native and fractionated starch and, especially combined with enzymatic debranching, low pressure TSK systems. Results will be presented and discussed for potato starch from ripe tubers of Ukomo granules previously fractionated by sedimentation. N-butanol precipitation and separation of re-solved precipitate utilizing the above mentioned SEC-system yield different domains of molecular weight distribution for further investigations. Chemical analysis (methylation analysis) provides average values of degree of branching and information about specific positions of the glucose-residues within the investigated polysaccharide molecule.

INTRODUCTION

Starch is a commonly used raw material as well in food- as in nonfood industries. Therefore the interest in defined raw materials as well as the interest concerning the structure of potato starch increases. Most publications on it assume uniformity in structure of polysaccharides from ripe turbes. But the starch granules show a great variety in size and also differ in the structure of their polysaccharides. In this paper, only the large starch granules of diameters between 50 and 80 μm are dioscussed. Another problem treated as axion is the existance of amylose and amylopect whereas amylose is considered to be a linear molecule, although Banks and Greenwold found branchpoints in amylose already in 1966 [3]. We found in our investigations out that the polysaccharide ran through a continuous shift from a long-chain to a short-chain branched structure. Separation of two fractions is only possible because only beyond a critical chain-length n-butanol precipitation of the polymer successfully works.

MATERIALS AND METHODS

Starch sample: sedimented potato starch granules of Ukomo isolated from ripe tubers in the laboratory.

Fractionation: for better characterization of the components the isolated starch was fractionated by n-butanol [5].

Gelchromatography: SEC: for the investigation of native starch as well as for preparative SEC Sephacryl gels (Pharmacia, Sweden) were used. The gel consists of allyldextrans cross linked with N,N'-methylen-bis-acrylamid. The gels possess eccelent stability and allow high flow rates [8, 9, 10, 11]. HPSEC: TSK gels (Merck, Germany) consisting of hydrophilic vinylpolymers and Superose 6 (Pharmacia) were used to analyze the samples after enzymatic debranching.

Column data: Sephacryl S-200 dimension 135 × 16 mm ID, S-200 940 × 16 mm, S-400 900 × 16 mm, S-1000 1290 × 16 mm; eluent: degassed 0.005 N NaOH with 0.002% NaN_3 (to prevent microbal growth); flowrate 45 ml/h; total volume V_t 650 ml, void volume V_0 230 ml. prep Syst.: S-200 310 × 26 mm, S-1000 850 × 26 mm; V_t 610 ml, V_0 200 ml, flowrate 96 ml/h. HPSEC: TSK HW 40, HW 50, Superose 6 dimension 290 × 10 mm ID each; eluent: degassed 0.005 M KCl + 0.003% NaN_3; flowrate 36 ml/h; V_t 70 ml, V_0 20 ml, pressure 3 bar.

Enzyme: promozyme 200 L (NOVO, Danmark) purified by affinity chromatography [1, 2, 7]. β-cyclodextrin was used as a ligand and for eluation. A HW 40 column was used to restore the acitivity.

Debranching: the sedimented starch granules were dissolved and precipitated with n-butanol [5]. The separeted fractions were put on the preparative system and the resulting 10 ml fractions were pooled in three areas of molecular weight (figure 1). The samples were precipitated by aceton, dissolved in 90% DMSO, diluted with 50 mM acetate buffer containing 5 mM β-mercaptoethanol to stabilize the pullulanase.

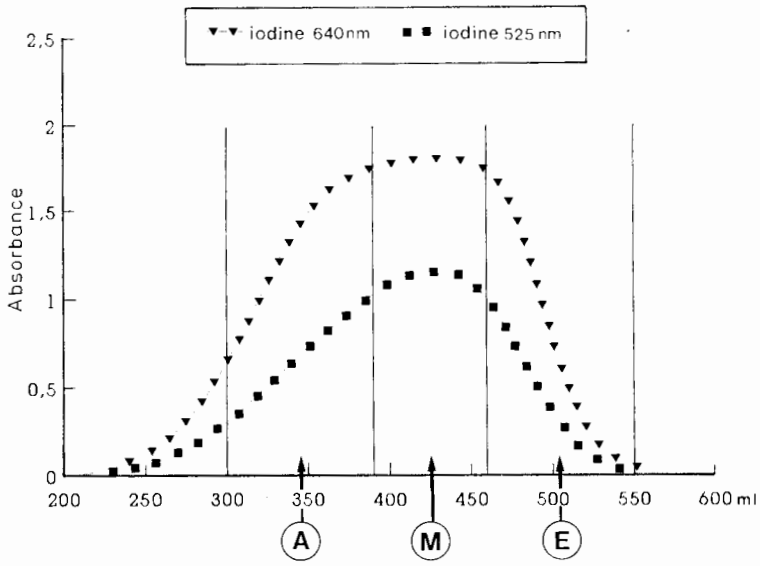

Fig. 1. Pooling of amylose in three fractions with different molecular weight: A (high), M (midrange), E (low) moleculare weight. Absorbance of the iodine complex measured at 640 and 525 nm

METHYLATION ANALYSIS

Methylation was performed by the method of Ciucanu and Kerek [4]. Hydrolysis: To 1-2 mg of methylated material 400 μl of 4 M trifluoroacetic acid (TFA) was added, and the sample was heated for 2 h at 100°C. After drying under nitrogen the sample was treated twice with mathanol in order to remove all the TFA.

REDUCTION

100 μl 1 M NH_3 and appr. 4 mg $NaBH_4$ were added, the vial was closed and kept for 2 h at room temperature. Then the reaction mixture was neutralized with acetic acid (pH 5) and dried. After treatment with 500 μl of acetic acid and 500 μl of methanol the sample was dried under N_2-gas.

ACETYLATION

200 μl acetic acid anhydride was added and the mixture held at 100°C for 60 minutes. After drying the sample with N_2, 1 ml of dichloromethan was added. As a last step the organic layer was washed with 2 portion of water (5 ml), dried and 2 μl was used for GLC. GC: Carlo Erba Instruments HRGC 5300 Mega series; MS: Ion Trap Detektor Finigan MAT; Column: DB1701 capillarycolumn; Pressure: 150 bar; Temperature program: from 140°C rising with 10°C/min to 200°C and futher 4°C/min to 260°C.

RESULTS AND DISCUSSION

Although SEC provides separation of polymers up to 100 million Dalton characterization of starch polysaccharides is insuffcient exclusively utilizing this method. The identical range of hydrodynamic volume for different types of molecular structure of starch polysaccharides has been found and the results are demonstrated in Fig. 2 for native starch, n-butanol precipitate and n-butanol supernatant.

Only a combination of enzymatic hydrolysis of the α-1.6-linkages with following chromatographic separation of the degradation products on an low pressure SEC system allows specific statements. A futher advantage of this method is, that in only needs about one hour for each analysis run. Essential items are also: the total dissolving of the sample because only dissolved molecules are available for enzymatic action; the total debranching (the velocity of the enzyme action depends on the degree of branching, if necessary a further addition of enzyme completes the debranching); no additional activities in the enzyme (a purification step by affinity chromatography is necessary to remove even the slightest traces of side activities, especially amylases).

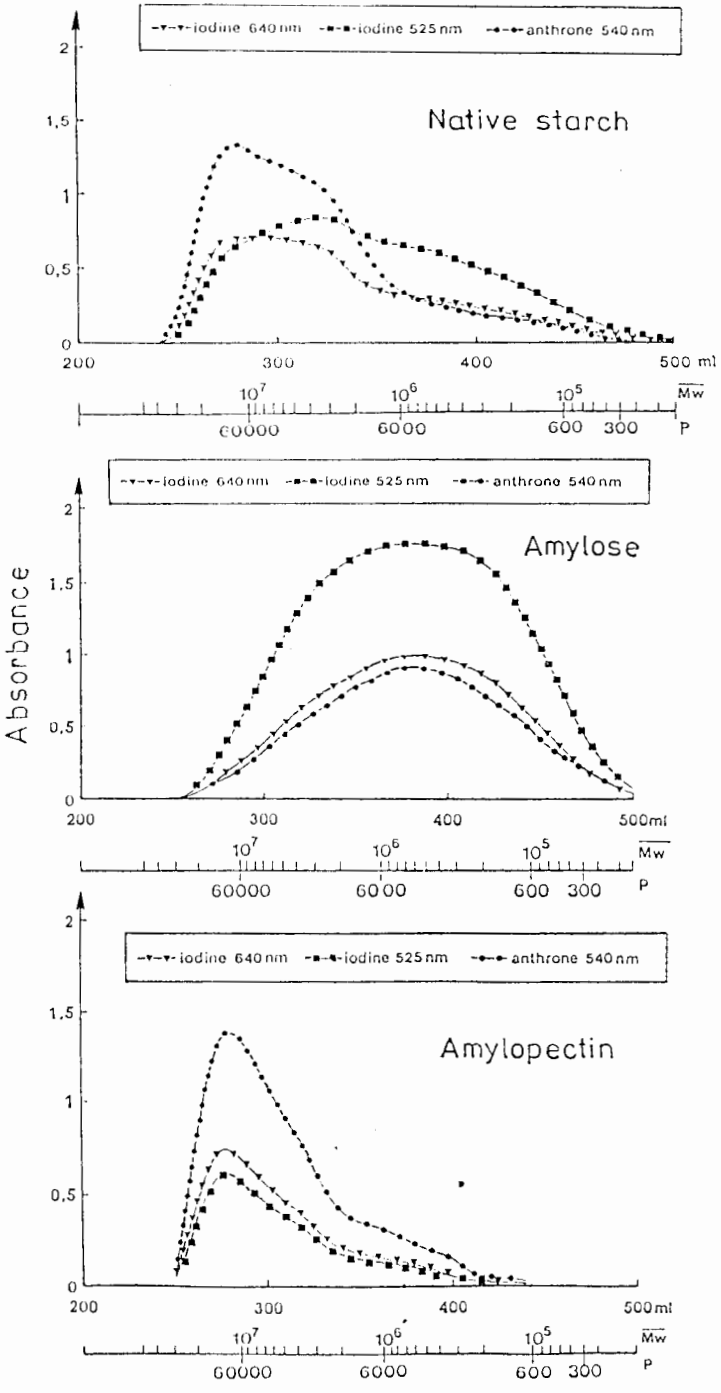

Fig. 2. Molecular weight distribution of starch determined by separation on an allyldextran system before and after fractionation with n-butanol. Calibration was done with pullulan standards

Table 1. Molecular weight average from SEC

	$\overline{Mw}$	$\overline{Mw}$	debranched $\overline{Mn}$	DP
Amylose				
A	1 mill.	110,000	14,000	84
M	450,000	122,000	19,500	117
E	85,000	23,000	12,000	72
Amylopectin				
A		5,100	3,100	19
M	5 mill.	5,700	3,400	20
E		6,300	3,800	23

In Table 1, the results for amylose and amylopectine from specified starch granules (diameter between 50 and 80 μm [5] in the areas of molecular weight A, M and E) are presented. As an essential result amylose has to be considered to be branched. With increasing molecular weight the number of short chains increases forming the branched component amylopectine. The partition of relatively long chains that was found especially in large granules indicated a shift in the concentration ratio between branching and α-1.4 synthesizing enzymes in favour of the synthases attached to the surface of the granules [6]. Because of the large surface of mature granules and the high supply of substrate the forming of longer chains is possible. This fact explains that amylose is located in the periphery of a granule whereas amylopectine is located in the center. Amylopectine of mature granule is homogeneously branched in all molecular weight fractions and shows a typical bimodal distribution with maxima at a DP of 18 and 54 glucose units which are transferred by the branching enzymes.

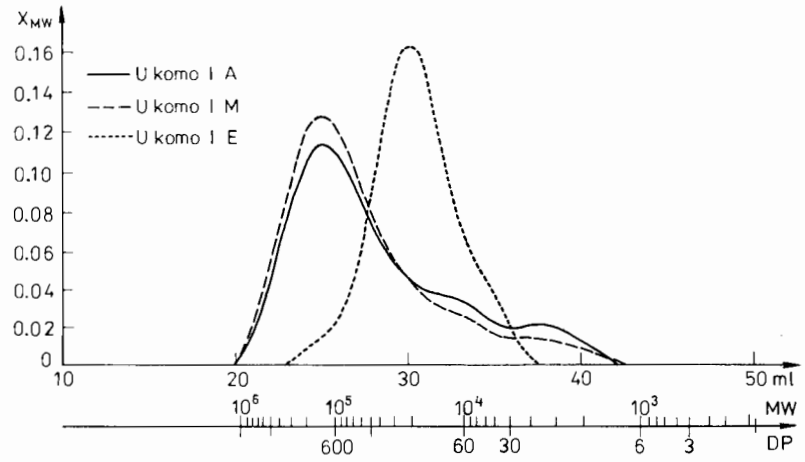

Fig. 3. High-, midrange- and low molecular weight of amylose after debranching with pullulanase. Samples were chromatographed on an TSK system; chromatograms are normalized to the area 1.0

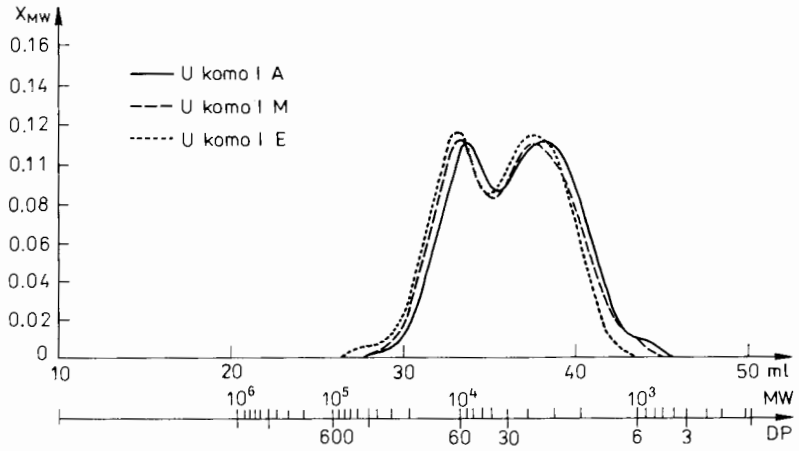

Fig. 4. Amylopectin fractions after debranching with pullulanase

The results of enzymatic debranching and chromatographic distribution of the degradation products can be proved by methylation analysis. Statements are only possible concerning the statistical average and within the reliable working range of the method. Table 2 compares the calculated number average with the results from chemical analysis. It has to be stated that the results of the chemical analysis of amyloses which contain a relatively small amount of α-1.6-linkages have already shown considerable differences whereas the enzymatic analysis have shown good reproducability.

Tabele 2.

	DPn chemical	DPn enzymatic
Amylose		
A	70	87
M	100	121
Amylopectin		
A	24	19
M	26	21

These investigations show that a combination of chromatographic and enzymatic plus chemical methods give essential information about the molecula-re structure of starch which is of great interest for both, fundamental research and industrial application.

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ENZYMATYCZNE I CHEMICZNE METODY ANALIZY SKROBI I JEJ PRODUKTÓW

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Streszczenie

Kontynuując wcześnicjsze badania nad strukturą skrobi, przedstawiono zastosowania chemicznych i enzymatycznych metod służących do określania struktury częsteczkowej skrobi ziemniaczanej. Jedną z ważnych metod dla określenia masy cząsteczkowej jest metoda sączenia żelowego (SEC). Najkorzystniejszymi układami doświadczalnymi wykonywanymi do tych badań są modyfikowane żele akrylowe stosowane w przypadku analizy skrobi naturalnej jak i frakcjonowanej, a szczególnie niskociśnieniowe systemy pracujące na polimerach winylowych (TSK) z uprzednim enzymatycznym usuwaniem rozgałęzień. Omówione wyniki badań dotyczą ziarn skrobi ziemniaczanej, uprzednio rozdziclonych przez sedymentację, otrzymanej z dojrzałych bulw Ukomo. Wytrącenie n-butanolem i rozdział ponownie rozpuszczonych osadów z wykorzystaniem wyżej wymienionych metod sączenia żelowego (SEC) daje różnorodne frakcje o zróżnicowanej masie cząsteczkowej dla naszych badań. Analiza chemiczna (metylowanie) dostarcza ustaleń co do stopnia rozgałęzienia i informacji o położeniu reszt glukozowych w obrębie badanej cząsteczki polisacharydu.