[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY][1]

Fractionation of Starches from Smooth and Wrinkled Seeded Peas. Molecular Weights, End-group Assays and Iodine Affinities of the Fractions[2]

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RECEIVED APRIL 21, 1952

Smooth-seeded Alaska and wrinkled-seeded Perfection pea starches have been isolated and fractionated with amyl alcohol and *n*-butyl alcohol into amylose and amylopectin. These starches have 35 and 66% amylose, respectively. The number average molecular weight of Alaska pea amylose is 125,000 and Perfection pea amylose is 100,000. Molecular weights of the corresponding amylopectins are 2,000,000 and 140,000. From periodate oxidation and molecular weight data, the degree of branching of these pea-starch amyloses is low and is similar to those of other amyloses prepared by similar methods. Alaska pea amylopectin has an average of 25 glucose residues per terminal non-reducing glucose unit, while Perfection pea amylopectin has 36. Besides the differences in molecular weight and end-group assay of these amylopectins, the iodine potentiometric titration curve of Perfection pea amylopectin is different from that of other amylopectins. These studies show that starch from Alaska peas is similar to cereal and root starches, while starch from Perfection peas is different. A spot test for detecting amylose in starch solution was developed and applied to the amylopectin fractions.

Until 1945, starches from various sources had been divided into two groups: (a) root and cereal starches which have approximately 20 to 30% amylose,[3] and (b) waxy or glutinous cereal starches which are practically pure amylopectin. Nielsen and Gleason,[4] however, showed that wrinkled-seeded peas contain a starch which has an unusually high percentage of the linear component amylose. From the intensity of blue color developed with iodine they concluded that its amylose content is about 75%. Hilbert and MacMasters[5] in a more complete study of the starch from wrinkled-seeded peas concluded that it contains from 60 to 70% amylose. From alkali-lability studies they assumed that the molecular size of wrinkled-pea amylose approximates that of corn amylose and that it is appreciably less than that of potato amylose. Peat, Bourne and Nicholls[6] found that smooth-seeded pea starches contain 29 to 30% amylose, and that starch from Steadfast peas, a wrinkled-seeded type, is 98% amylose. Work recently reported from this Laboratory[7] shows that the amylose content of smooth pea starch is 35 to 37% and that of the wrinkled-pea starch is 65 to 72%.

Analyses show that starch from smooth-seeded peas is similar to that from roots and cereals, while that from wrinkled-seeded peas is different. Further information on their physical properties is desirable; therefore, starches from smooth-seeded Alaska and wrinkled-seeded Perfection peas were isolated by sedimentations from their aqueous suspensions and fractionated with amyl alcohol and *n*-butyl alcohol.[8,9] The properties examined include: (a) molecular weights by osmotic pressure measurements, (b) end-group assays by periodate oxidation, (c) iodine affinities and blue values, and (d) limiting viscosity numbers.[10]

Experimental

Preparation of Starch from Dried Peas.—Dried Alaska and Perfection peas were coarsely milled and the skins were separated from the cotyledons by a cyclone air separator and discarded. The milled peas were soaked overnight in water and ground wet in a hammer mill. Mashing and pressing the mixture on a fine Nylon cloth removed the fiber which was again mixed with water and pressed on the Nylon cloth. The starch settled from the filtrates on standing at room temperature for 2.5 hours. The supernatant liquid was decanted and the starch purified by 4 additional settlings from water. The starch was also treated 2 successive times by standing overnight in 80% ethyl alcohol, filtering and washing with 95% ethyl alcohol and then was dried *in vacuo* at 50°. Analyses of the starches are given in Table I. Yields in percentage of the theoretical from analyses of the peas minus their seed coats are 80% for Alaska and 58% for Perfection starches. The recovery of the wrinkled-pea starch is low and is primarily due to mechanical fracturing of the compound granules into smaller fragments which settle slowly and are difficult to separate from other particles.

TABLE I

ANALYSES OF STARCHES FROM ALASKA AND PERFECTION PEAS

	Alaska pea starch	Perfection pea starch
Starch,[a] %	100	100
Starch–iodine blue value[b]	0.134	0.230
Iodine affinity,[b] %	7.1	13.3
Amylose,[a] %	34.5	66.0
Ash, %	0.05	0.11
Nitrogen, %	.06	.25
Fiber, %	.12	.16
Phosphorus, %	.01	.03

[a] Starch was determined by the sugar–anthrone–sulfuric acid reaction and amylose by the iodine–starch blue value.[7] [b] Blue value per 5 mg. of starch in 500 ml. of solution, reported as the optical density from the Beckman model B spectrophotometer using 1.0-cm. cell and wave length of 6600 Å. Starch–iodine blue values and iodine affinities were determined on samples of the starches which were extracted for 48 hours in a Soxhlet with 95% ethyl alcohol.

Fractionation of Pea Starch.—Pea starches were fractionated with amyl alcohol[11] and *n*-butyl alcohol similar to the method described by Schoch.[8,9] They could not be completely dispersed by autoclaving or refluxing as are starches from other plant sources, so they were treated with

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) Report of a study made under the Research and Marketing Act of 1946.

(3) Recently a new corn hybrid, whose starch is 66% amylose, has been reported by J. W. Cameron in *Genetics*, **32**, 459 (1947), and W. Dvonch, H. H. Kramer and R. L. Whistler in *Cereal Chem.*, **28**, 270 (1951).

(4) J. P. Nielsen and Peggy C. Gleason, *Ind. Eng. Chem., Anal. Ed.*, **17**, 131 (1945).

(5) G. E. Hilbert and M. M. MacMasters, *J. Biol. Chem.*, **162**, 229 (1946).

(6) S. Peat, E. J. Bourne and Mary J. Nicholls, *Nature*, **161**, 206 (1948).

(7) R. M. McCready, Jack Guggolz, Vernon Silveira and H. S. Owens, *Anal. Chem.*, **22**, 1156 (1950).

(8) T. J. Schoch in "Advances in Carbohydrate Chemistry," edited by Pigman and Wolfrom, Vol. I, Academic Press, Inc., New York, N. Y., 1945, pp. 247–277.

(9) Sylvia Lansky, Mary Kooi and T. J. Schoch, THIS JOURNAL, **71**, 4066 (1949).

(10) Nomenclature prepared by the International Union of Pure and Applied Chemistry, *J. Polymer Sci.*, **8**, 257 (1952). Limiting viscosity number is expressed as ml./g. instead of dl./g. previously used for intrinsic viscosity.[9]

(11) This is a mixture of various amyl alcohols.

alkali as follows. One hundred grams of starch was suspended in 4.5 l. of water cooled to 3°, 500 ml. of 10 *N* potassium hydroxide was added and the mixture stirred mechanically for 10 minutes at 3°. Five liters of cold water was added and the solution stirred 5 additional minutes. The solution was acidified with glacial acetic acid to *p*H 5.8 to 6.1, heated on a steam-bath for 3 hours (temperature during the heating was 91 to 93°), and centrifuged in a continuous supercentrifuge to remove a small amount of undissolved material (about 1% which was approximately 40% protein). Eighty ml. of amyl alcohol per liter of the hot solution was added. The mixture was stirred and cooled slowly to room temperature and then stored at 3° for 24 hours. The amyl alcohol–amylose complex was separated by centrifugation and further purified by solution and precipitation with *n*-butyl alcohol[8] repeated three times.

The supernatant liquid, which contained the amylopectin, was concentrated *in vacuo* to one-half its volume. Amyl alcohol was added and the mixture stored at 3° for 3 days. The amyl alcohol–amylose precipitate was removed by centrifugation and discarded. The amylopectin was precipitated from the solution with an equal volume of methyl alcohol. Because of the high concentration of sodium acetate present during the fractionation, it was necessary to redissolve and reprecipitate the amylopectin twice with methyl alcohol in order to decrease the salt content. Percentage yields of the starch fractions are: Alaska pea amylose, 28%; Alaska pea amylopectin, 63%; Perfection pea amylose, 62%; and Perfection pea amylopectin, 23%.

The iodine affinities were determined by the potentiometric titration method of Bates, French and Rundle[12] as modified by Lansky, Kooi and Schoch.[9] The plot of iodine bound *vs.* free iodine of the Perfection pea amylopectin yielded a hyperbolic curve which did not permit an accurate linear extrapolation to the zero axis. For this reason no iodine affinity is given for this fraction. Iodine affinities of the other pea-starch fractions and starch–iodine blue values are included in Table II. In Fig. 1, the potential is plotted against ml. of iodine added for Perfection pea amylose. Alaska pea amylopectin and Perfection pea amylopectin.

TABLE II

IODINE AFFINITY AND STARCH–IODINE BLUE VALUES

	Iodine affinity, % (g. iodine absorbed per 100 g. starch)	Starch–iodine blue value[a]
Amyloses		
Alaska pea	18.7	0.327
Perfection pea	19.1	.333
Amylopectins		
Alaska pea	1.6	0.053
Perfection pea	..	.118

[a] See note *b* under Table I.

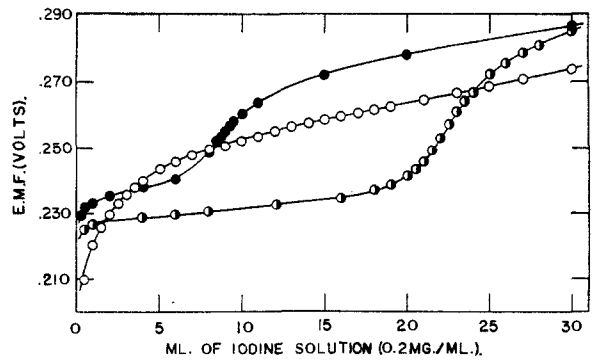

Fig. 1.—Iodine potentiometric titration curves of pea-starch fractions: ●, 90.4 mg. of Alaska pea amylopectin; ◑, 23.0 mg. of Perfection pea amylose; and ○, 90.4 mg. of Perfection pea amylopectin.

(12) L. Bates, D. French and R. E. Rundle, THIS JOURNAL, **65**, 142 (1943).

Viscosities of the pea starch fractions were determined with 1.00 *N* potassium hydroxide as described by Lansky, Kooi and Schoch[9] with an Ostwald-Cannon-Fenske No. 50 pipet, maintained in a thermostated water-bath at 25.00°. "Kinetic Energy" corrections calculated from Wagner's formula[13] were added to the logarithmic viscosity number.[10] Limiting viscosity numbers,[10] which are the infinite dilution values of the logarithmic viscosity numbers, are listed in Table III.

TABLE III

NUMBER AVERAGE MOLECULAR WEIGHTS OF FRACTIONS FROM PEA STARCHES

	Limiting viscosity number,[10] ml./g.	π/C from ordinate, ml./sq. cm.	Molecular weight of acetylated derivative	Calcd. mol. wt. of deacetylated fraction	Degree of polymerization in glucose units
Amyloses					
Alaska pea	176	113	220,000	125,000	770
Perfection pea	134	139	180,000	100,000	630
Amylopectins					
Alaska pea	157	13	2,000,000	1,000,000	6700
Perfection pea	122	101	250,000	140,000	870

Determination of Molecular Weight.—Molecular weights of the acetylated pea-starch fractions were determined by osmotic pressure measurements, with chloroform as solvent. Methods of acetylating and determining the osmotic pressures of the solutions have been described.[14] Static measurements were carried out at all concentrations until a constant value was reached which required 2 to 6 days. The higher concentrations required the least time. The osmotic pressure did not change if it was followed as many as 8 days after the equilibrium was reached. Between runs the zero point with solvent alone which did not exceed ±0.005 cm. was determined for each osmometer. In addition the difference between the capillary rise of chloroform and that of solutions of acetylated starch fractions was found to be less than 0.005 cm., indicating that differences in surface tension were negligible. Intercepts of the ordinate were determined by plotting π/C against C^n and the molecular weights were calculated by the van't Hoff equation. Values of $n = 1.39$ and $n = 2.25$ for acetylated amyloses and amylopectins, respectively, were also found to be applicable with the acetylated pea-starch fractions. Data are shown in Fig. 2 and molecular weights of the pea-starch fractions are given in Table III.

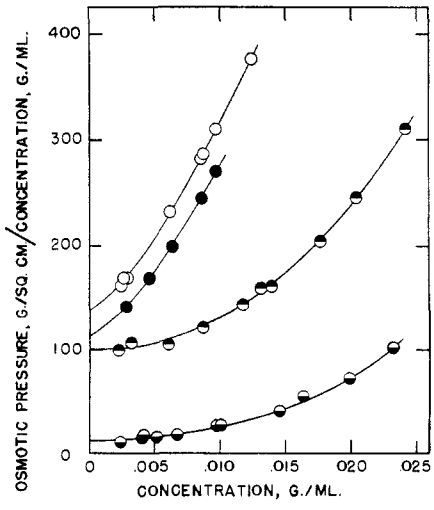

Fig. 2.—Osmotic pressure–concentration relationship of acetylated starch fractions in chloroform solution: ●, Alaska pea amylose; ◒, Alaska pea amylopectin; ○, Perfection pea amylose; and ◓, Perfection pea amylopectin.

(13) R. H. Wagner, *Anal. Chem.*, **20**, 155 (1948).

(14) A. L. Potter and W. Z. Hassid, THIS JOURNAL, **70**, 3774 (1948).

Osmotic pressure measurements of solutions containing less than 0.00243 g. of acetylated Alaska pea amylopectin per ml. were not measured. Osmotic pressure for the 0.243% solution was 0.015 cm. of chloroform and the cathetometer used would measure only to 0.005 cm. The π/C *vs.* C curve shown in Fig. 2 for pea amylopectin acetates in chloroform do not show minima such as was found for corn and tapioca amylopectin acetates by Kerr, Cleveland and Katzbeck.[15]

Oxidation with Sodium Metaperiodate.—The method reported earlier[16] for oxidation of starch fractions with periodate at 3° was applied to the pea-starch fractions, with the exception that the amyloses were dissolved in alkali, neutralized and then subjected to oxidation. These solutions were more dilute than those used previously; therefore, it was necessary to restandardize the method against maltose. Aqueous solutions of maltose and maltose treated with sodium hydroxide and neutralized were oxidized with periodate. The non-reducing terminal glucose units in amylopectin are theoretically responsible for the formic acid produced by oxidation of amylopectin with periodate. Glucose in methyl α-D-glucoside has the same configuration as the non-reducing terminal glucose units in starch. Aqueous solutions of amylopectin and methyl α-D-glucoside were subjected to oxidation with periodate to show that the rates of formic acid production were similar. The concentration and amount of periodate was the same in each experiment and the amount of substrate oxidized was calculated so that there was always the same excess of periodate. It was not necessary to use a mechanical shaker during the oxidation. Results of the oxidation of maltose, methyl α-D-glucoside, amylose and amylopectin are shown in Fig. 3.

One hundred ml. aliquots of the carbohydrate solutions (0.500 g. of amylose, 0.475 g. of amylopectin, 0.222 g. of maltose hydrate or 0.290 g. of methyl α-D-glucoside per 100 ml.) were each oxidized at 3° with 10 ml. of sodium metaperiodate (8.0 g. of $NaIO_4$ per 100 ml.). The amyloses and maltose were dissolved in 1 *N* sodium hydroxide. The maltose solution was allowed to stand 30 minutes. After the addition of 5 volumes of water the solutions were acidified with 0.5 *N* HCl to *p*H 5.0 and diluted to volume. The final concentration of the sodium chloride was 0.1 *M*. Additional samples of maltose dissolved in water were also oxidized. At the end of the oxidation with the periodate the unused periodate was reduced by the addition of 20 drops of ethylene glycol. After standing for an hour the solution was titrated to a green end-point with 0.01 *N* sodium hydroxide (0.1 *N* NaOH was used with the oxidation of the maltose) using a mixed indicator of methyl red and methylene blue (0.04% of methyl red, 10 vol. and 0.04% methylene blue, 1 vol.). Blank determinations were obtained by titrating aliquots of the carbohydrate solutions containing the sodium metaperiodate reduced by ethylene glycol. Ten-ml. aliquots from the methyl α-D-glucoside and the aqueous maltose oxidations were treated with 5 drops of ethylene glycol and then titrated with 0.01 *N* NaOH as described above.

Figure 3 shows that the theoretical amount of 3 moles of formic acid per mole of maltose is produced in about 9 days.

TABLE IV

PERIODATE OXIDATION OF THE STARCH FRACTIONS

	Milliliters of 0.01 *N* NaOH	Degree of polymerization[a]	Glucose residues per terminal glucose unit	Average no. of non-reducing terminal glucose units per molecule
Pea amyloses				
Alaska	2.34	400	..	3.9
Perfection	2.07	450	..	2.1
Pea amylopectins				
Alaska	11.00	...	26.6	...
Perfection	8.13	...	36	...

[a] Calculated from the periodate oxidation data assuming that the amylose molecules are unbranched chains.

(15) R. W. Kerr, F. C. Cleveland and W. J. Katzbeck, THIS JOURNAL, **73**, 111 (1951).

(16) A. L. Potter and W. Z. Hassid, *ibid.*, **70**, 3488 (1948).

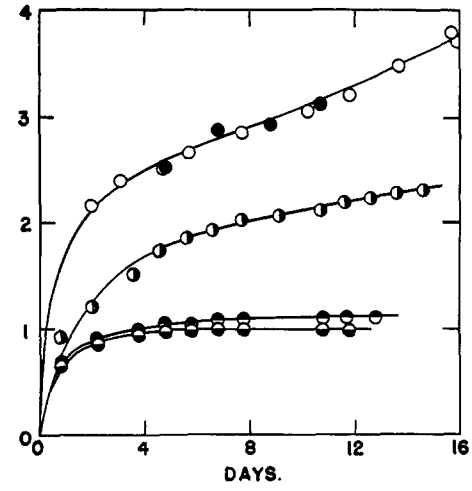

Fig. 3.—Acid produced by periodate oxidation of various carbohydrates for increasing periods of time. Ordinates are indicated in parentheses in the following legend: ○, maltose (moles of formic acid per mole of maltose); ●, maltose treated with 1 *N* NaOH (moles of formic acid per mole of maltose); ◑, Perfection pea amylose (ml. 0.01 *N* NaOH per 0.500 g. amylose); ◒, Alaska pea amylopectin (ml. 0.10 *N* NaOH per 0.475 g. amylopectin); and ◒, methyl α-D-glucoside (moles formic acid per mole of sugar).

The data obtained from the oxidation of pea amyloses and amylopectins are given in Table IV.

Qualitative Spot Test for Amylose.—Examination of Nussenbaum's[17] spot test indicated that amylose could be detected in mixtures of amylose and amylopectin if the amylose was complexed with a fatty acid. However, this failed if the amylose was present to an extent of about 10% or less in the mixture. Amylose in lower proportions may be detected by the following procedure: Whatman No. 1 filter paper is dipped in a 0.15% stearic acid solution in 95% ethyl alcohol and air-dried. Thirty microliters of 0.5% starch solution to be tested is placed on the treated paper and air-dried. The paper sheet is then dipped into 0.1% iodine solution and again air-dried.

Tapioca amylopectin (iodine affinity 0.2%) and mixtures containing 1, 2, 5 and 10% Alaska pea amylose, Alaska pea amylose and amylopectin, Perfection pea amylose and amylopectin, and potato starch were treated as described. Tapioca amylopectin and Alaska pea amylose, each diluted to 0.05% polysaccharide were also spot-tested. Perfection pea amylose, amylopectin and potato starch, each mixed with 8 ml. of amyl alcohol per 100 ml. of 0.5% polysaccharide solution were tested. No change in the results of the starch–amyl alcohol mixtures was observed after standing from 15 minutes to 3 days. The results of these tests are shown in Fig. 4.

Discussion

Molecular weights of amylose[14,18] from various plant sources range from approximately 90,000 to 200,000 while amylopectins from the same plants have been found to be much larger and their sizes range from one to six million.[14,18] The number average molecular weight of Alaska pea amylose is 125,000 and of Perfection pea amylose 100,000, which are similar to those of apple, sago, Easter lily and corn amyloses. Alaska pea amylopectin has a number of average molecular weight of 1,000,000, which is of the same order as that of other amylopectins. Perfection pea amylopectin has a molecular weight of 140,000 one order less than that for other amylopectins.

(17) Siegfried Nussenbaum, *Anal. Chem.*, **23**, 1478 (1951).

(18) A. L. Potter, W. Z. Hassid and M. A. Joslyn, THIS JOURNAL, **71**, 4075 (1949).

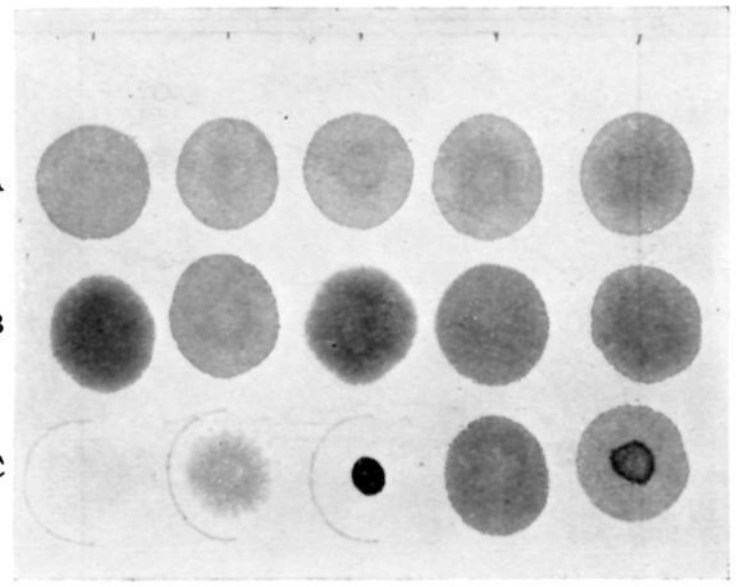

Fig. 4.—Spot test for amylose in amylopectin: A1, tapioca amylopectin; A2, tapioca amylopectin, 1% Alaska pea amylose; A3, tapioca amylopectin, 2% Alaska pea amylose; A4, tapioca amylopectin, 5% Alaska pea amylose; A5, tapioca amylopectin, 10% Alaska pea amylose; B1, Alaska pea amylose; B2, Alaska pea amylopectin; B3, Perfection pea amylose; B4, Perfection pea amylopectin; B5, potato starch; C1, tapioca amylopectin, 0.05%; C2, Alaska pea amylose, 0.05%; C3, Perfection pea amylose treated with amyl alcohol; C4, Perfection pea amylopectin treated with amyl alcohol; C5, Potato starch treated with amyl alcohol.

The degree of branching of pea starch fractions was obtained by oxidation of the polysaccharides with sodium metaperiodate. These data used in conjunction with the molecular weights of the pea amyloses as described earlier[14] indicate that the average number of chains per molecule is 3.9 for Alaska pea amylose and 2.1 for Perfection pea amylose. The degree of branching of amyloses from pea starch is small and in agreement with the values obtained from amyloses prepared by similar methods from other plant sources. Average number of glucose residues per terminal glucose unit was 27 for Alaska pea amylopectin and 36 for Perfection pea amylopectin. End-group assays of amylopectins from other plant sources have been found to range from 22 to 27. The value of 36 for Perfection pea amylopectin is significantly higher than those of other amylopectins.

The iodine potentiometric titration curve in Fig. 1 of Alaska pea amylopectin is similar to a titration curve of a mixture of amylose and amylopectin. The spot test for amylose in amylopectin fractions in Fig. 4 suggests that this fraction has approximately 3 to 5% amylose. Neglecting the portion of the titration curve that indicates amylose, the remainder of the curve is similar to titration curves of amylopectins from other plant sources. These results indicate that portions of the iodine affinity and the starch–iodine blue value of the Alaska pea amylopectin are due to amylose. Assuming that the amylose content of the Alaska pea amylopectin is 5%, the end-group assay for this fraction would be 25 glucose residues per terminal non-reducing glucose unit, the molecular weight would be increased to about 2,000,000, and the starch–iodine blue value would be decreased to 0.036.

The shape of the potentiometric titration curve of the Perfection pea amylopectin (Fig. 1) does not indicate the presence of amylose in this fraction. However, the potential is lower than that obtained with other amylopectins by the addition of the same amount of iodine. This indicates that the iodine is adsorbed by the material. The high starch–iodine blue value of the Perfection pea amylopectin indicates that this fraction might contain amylose, but the spot test in Fig. 4 shows that it does not. The high starch–iodine blue value and the adsorption of iodine as shown by the potentiometric titration curve of Perfection pea amylopectin might be expected because the end-group assay of 36 glucose residues per terminal glucose unit is significantly higher than those obtained from other amylopectins. Some of the branches may be so long that they begin to combine with iodine like amylose. This is not reflected in the shape of the titration curve, possibly because of lack of uniformity in the lengths of the side chains which would give a non-specific titration curve.

The results of this investigation show that starch from smooth-seeded Alaska peas is similar to cereal and root starches, while that from wrinkled-seeded Perfection peas is different. Besides having a high amylose content the amylopectin fraction of the wrinkled-pea starch has a much smaller molecular weight and a smaller degree of branching than amylopectins from other plant sources. The difference in degree of branching is also illustrated in the iodine potentiometric titration curve.

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