Journal of Chromatography, 319 (1985) 205–214
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,288

RAPID ANALYSIS OF STARCH, AMYLOSE AND AMYLOPECTIN BY HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY*

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(First received August 15th, 1984; revised manuscript received October 10th, 1984)

SUMMARY

Starch components, amylose and amylopectin, were analyzed by high-performance size-exclusion chromatography. These two-components were separated using a two-column system (E-Linear and E-1000) and dimethyl sulphoxide as the mobile phase. The void volume (V_0 = 2.22 ml) was measured using tobacco mosaic virus. Column calibration was accomplished with dextrans of known average molecular weight ($\bar{M}_w$ range = 10,100–2,000,000). The elution volume of amylopectin (V_e = 2.5 ml) indicated that this starch component was fractionated on the column system despite its very large molecular size. Standard curves were prepared from various mixtures of purified corn and wheat amylose and amylopectin. From the linear relationships obtained, the percentages of both components in corn and wheat starches were determined. The method developed proved useful to monitor the purity of amylose and amylopectin preparations, and to estimate rapidly the amylose:amylopectin ratio of starch samples.

INTRODUCTION

Starch contains two major polysaccharide components, amylose and amylopectin. Amylose is generally assumed to be a linear molecule, although recent evidence indicates some non-linear components[1], and amylopectin a branched polysaccharide. The molecular weights of amylose and amylopectin have been estimated as $0.2 \cdot 10^6$–$1.3 \cdot 10^6$ and $4 \cdot 10^8$–$5 \cdot 10^8$, respectively[2,3].

These two components have been separated by traditional column chromatography[4]. Amylopectin is so large that it normally elutes in the void volume of gel columns. Karve *et al.*[5] reported on the separation of starch components by affinity chromatography. In one report[6], the ratios of amylose to amylopectin were measured

* Paper No. 9463 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC, U.S.A. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service, nor criticism of similar ones not mentioned.

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after treatment with isoamylase followed by chromatography of the smaller components formed on a column of Sepharose CL-6B. Adsorption chromatography on cellulose columns has been used by Patil and Kale[7] to fractionate starch components. These methods are useful, but are very time consuming.

It has been tedious to measure directly the amylose and amylopectin composition of starch rapidly owing to the lack of appropriate methodology capable of resolving the two components within a reasonable time. Physically, amylose can be purified by preferential precipitation from amylose–amylopectin mixtures by complexation with *n*-butanol[8]. Methods for the measurement of amylose in starch include an iodine colorimetric method or blue value[9] for approximate determinations and a potentiometric iodine titration[10,11] when greater accuracy is required. These two techniques are based on the formation of an iodine complex with amylose. Indirectly, the amount of amylopectin can be calculated by subtraction of amylose from total starch.

High-performance liquid chromatography (HPLC) has been used for separations and determination of carbohydrate polymers such as maltosaccharides[12], isomaltodextrins[13] and cyclodextrins[14]. Recently, high-performance size-exclusion chromatography (HPSEC) has been used to separate pectin polysaccharides[15], to follow enzymic hydrolysis of beta-limit dextrin[16], and to analyze for starch degradation polymers[17] and other oligomers[18]. The large differences in the apparent molecular weights and structure of amylose and amylopectin should permit a fractionation by molecular size. However, there are no previous reports utilizing HPSEC for the fractionation and determination of these components. The main advantage of a HPSEC method is the short time needed for analysis, usually less than 20 min, and the potential for separations with high resolution. Developments in the area of starch composition analysis are needed to further define structural differences in starch polysaccharides and their relationship to functionality in foods.

The purpose of this investigation was to (1) develop a rapid HPSEC method for the fractionation and determination of starch components and (2) provide useful methodology to check composition and purity of amylose and amylopectin samples.

MATERIALS AND METHODS

Corn amylose and amylopectin are purified research samples from USDA Northern Regional Research Laboratory (Peoria, IL, U.S.A.). Wheat amylose and amylopectin were prepared from wheat starch by Schoch's procedure[19], and wheat starch was prepared from wheat flour (Hard red winter, Eagle variety) following the method described by Wolf[20]. Dextran T10 ($\bar{M}_w$ = 10,100, $[\eta]$ = 0.10), T40 ($\bar{M}_w$ = 42,100, $[\eta]$ = 0.22), T70 ($\bar{M}_w$ = 70,400, $[\eta]$ = 0.28), T500 ($\bar{M}_w$ = 472,000, $[\eta]$ = 0.52) and Blue Dextran ($\bar{M}_w$ *ca.* 2,000,000) were purchased from Pharmacia (Piscataway, NJ, U.S.A.). Tobacco mosaic virus (M_w = 40,000,000, 10 mg/ml solution) was donated by W. G. Dougherty, Department of Plant Pathology. Dimethyl sulfoxide (DMSO), 0.2% water content, (Fisher certified ACS, D-128) was purchased from Fisher (Raleigh, NC, U.S.A.).

Isoamylase (59,000 units/mg protein, 1 mg/ml, Lot. No. 30600) was purchased from Hayashibara Biochemical Laboratories, (Okayama, Japan). Before use, the enzyme solution was replaced with acetate buffer using a collodion bag (Fisher) by

adding 10 ml (5 ml twice) of 0.1 *M* acetate buffer, pH 3.80, to 50 μl of the enzyme solution at 4°C. The diluted buffer–enzyme solution (5 ml) was concentrated to 0.5 ml and diluted to 5 ml with acetate buffer. This enzyme solution can be stored for three months without significant loss of activity.

Distilled and deionized water was used. The water and DMSO were filtered through a fritted disk funnel (pore size, 10–15 μm) and degassed at room temperature. All other chemicals used were chemically pure grade.

HPLC apparatus

A Model 510 pump equipped with a Model U6K universal liquid chromatograph injector and differential refractometer R401 (Waters Assoc., Milford, MA, U.S.A.) were used. The data were collected on an Apple II+ computer equipped with an ADALAB data acquisition/control card and CHROMATOCHART chromatography software for peak area integration (Interactive Microware, State College, PA, U.S.A.). An E-Linear and an E-1000 μ-Bondagel gel permeation chromatography columns (Waters Assoc.) were connected in series. The columns were protected with a guard column (Waters Assoc., 2.5 cm × 3.9 mm I.D.) packed with CO: PELL ODS (Whatman, Clifton, NJ, U.S.A.). The columns were immersed in a 40°C water bath after covering each column with a polyurethane film to prevent rust. The refractometer was also maintained at 40°C with a circulating water bath.

Chromatographic conditions

Starch samples (40 mg of amylose–amylopectin mixtures or dextrans) were dissolved in 2 ml of DMSO in a boiling bath for 5 min, and the solution was centrifuged at 3000 *g* for 5 min. A 20-μl volume of the supernatant solution, removed carefully from the top of the solution, was injected. All samples, including data collected for standard curves, were measured in triplicate. The columns were eluted with DMSO at a flow-rate of 0.2 ml/min. The refractive index detector attenuation was set at 8.

Enzymatic debranching of amylopectin

A 100-μl volume of a 2% solution of amylopectin in DMSO, 100 μl of 0.1 *M* acetate buffer (pH 3.80), 150 μl of water and 50 μl of isoamylase solution were thoroughly mixed and allowed to stand for 17 h at 40°C. A control of the same mixture, with 0.1 *M* acetate buffer replacing the isoamylase solution, was used with each debranching experiment. After completion of the reaction, 80 μl of each mixture was analyzed by HPSEC as described.

RESULTS AND DISCUSSION

Fig. 1 shows a typical profile of an Eagle variety wheat starch sample. Peak Ap (V_e = 2.5 ml) and peak Am (V_e = 3.2 ml) correspond to amylopectin and amylose, respectively. As expected, the larger amylopectin eluted prior to the smaller amylose component. The relative peak areas for each component identified corresponds closely to the known ratio of amylopectin to amylose (*ca.* 75:25) in wheat starches[21]. Treatment of this mixture with isoamylase (17 h at 40°C was found to be sufficient for complete debranching) produced the chromatogram illustrated in Fig.

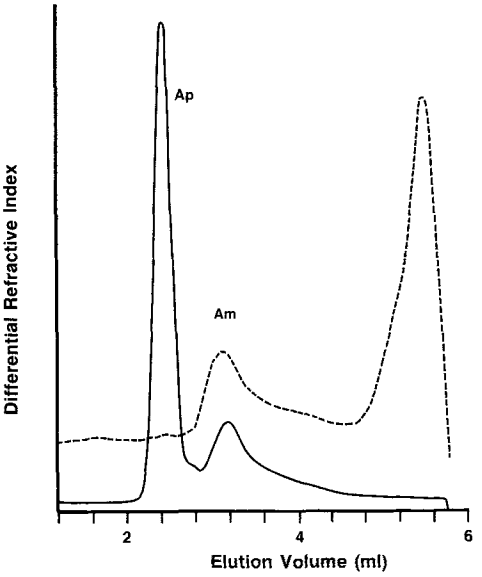

Fig. 1. Elution profiles of wheat starch. (———) Whole wheat starch (variety: Eagle); (--------) after treatment with isoamylase. Peaks: Ap = amylopectin, Am = amylose.

1. As shown, amylopectin is completely degraded to smaller polysaccharide fragments (V_e = 5.8 ml) and amylose appears unchanged. Isoamylase hydrolyzes α-1,6 linkages[22]. The results obtained from treatment with isoamylase serve as additional evidence for identifying peaks Ap as amylopectin and Am as amylose.

Column calibration

Solutions of dextrans of different molecular weights (2,000,000–10,100) were

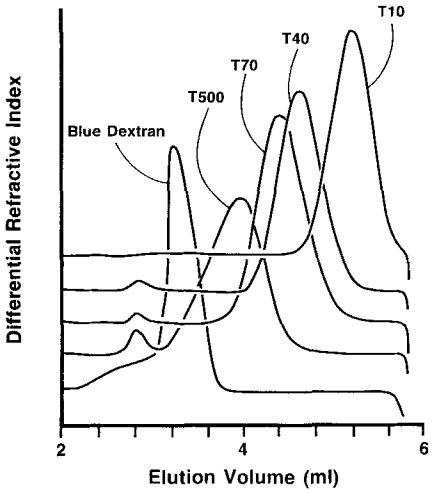

Fig. 2. Elution profiles of dextran standards (10 μl, conc. 20 mg/ml). T10, $\bar{M}_w$ = 10,100; T40, $\bar{M}_w$ = 42,100; T70, $\bar{M}_w$ = 70,400; T500, $\bar{M}_w$ = 472,000; Blue dextran, $\bar{M}_w$ = 2,000,000).

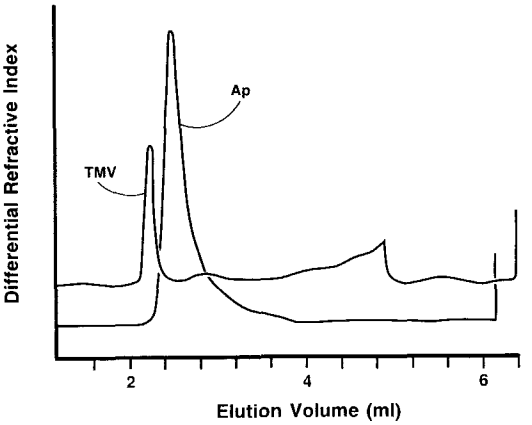

Fig. 3. Chromatograms of tobacco mosaic virus (TMV, 10 μl of 1% aqueous solution) eluting in the void volume and corn amylopectin (Ap, 10 μl of 1% DMSO solution). Conditions: 40°C; mobile phase, water; flow rate, 0.2 ml/min.

used to calibrate the column system. Fig. 2 illustrates the profiles obtained for this series. If water is used as the eluent, rather than DMSO, negligible differences in elution volumes and peak shapes for the dextran solutions were observed.

The determination of the void volume (V_0) was accomplished with tobacco mosaic virus (TMV) using water as the eluent. TMV is a rod-shaped virus, 300 nm × 18 nm, with a molecular weight of *ca.* 40,000,000[23]. As shown in Fig. 3, TMV elutes in the void volume (2.22 ml) and corn amylopectin ($M_w = 4 \cdot 10^8$–$5 \cdot 10^8$) elutes at a larger elution volume. This result indicates that amylopectin is retained on the column system despite its very large molecular size. Presumably, amylopectin may have some affinity or interaction with the column bed or conformational orientation affects its retention. If the rod-shaped structure of TMV, with a Stokes radius of 150 nm, is rotated freely in solution, it would appear globular. Therefore, its shorter retention time relative to amylopectin is not unusual.

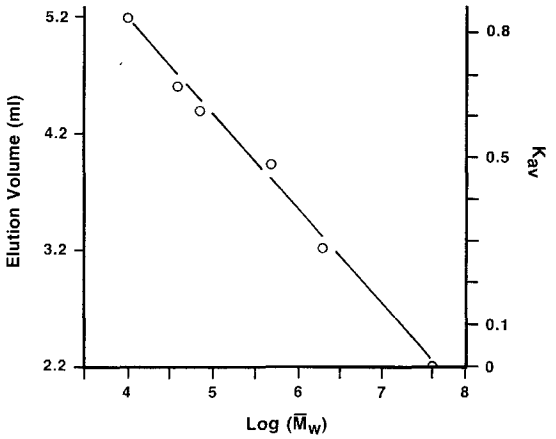

Fig. 4. Relationship between partition coefficient (K_{av}) and log weight average molecular weight ($\bar{M}_w$) of dextran standards.

Partition coefficient (K_{av}) for a series of dextran standards were calculated and plotted against the log of the weight average molecular weight ($\bar{M}_w$) as shown in Fig. 4. A linear relationship for the series of dextran standards (α-1,6) was found. This graph could be used to determine the molecular weight distribution of amylose, assuming that the α-1,4 glucan behaves similarly to the α-1,6 glucan in DMSO. At present, however, it is not possible to make such a comparison since high-molecular-weight standards of α-1,4 glucans are not available.

Standard curves for the determination of corn amylose and amylopectin

Solutions (2%) of corn amylose and amylopectin in DMSO were prepared in various ratios and analyzed to obtain the profiles in Fig. 5. The chromatogram of amylopectin (0% amylose) showed one peak with a small shoulder. After treatment with isoamylase, only the debranched components appeared at V_e of 5.8 ml and no other peaks were detected. Therefore, the amylopectin preparation used for the standard curve was believed to be very pure.

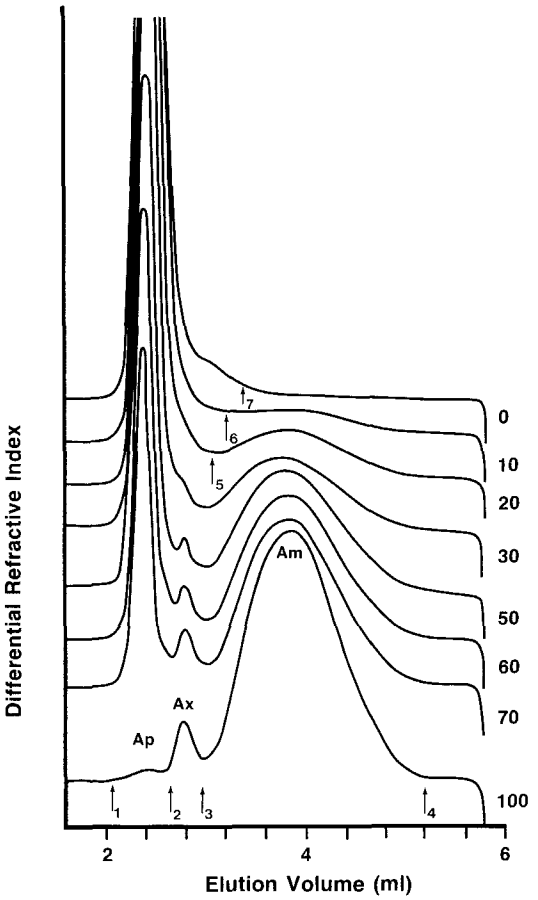

Fig. 5. Elution profiles of samples containing various mixtures of purified corn amylose and amylopectin: 0–100 = percentage of amylose in the sample, Ap = amylopectin, Am = amylose, Ax = unknown component. Arrows 1–7 indicate where areas of each peak were measured.

TABLE I

MEASUREMENTS OF AMYLOSE:AMYLOPECTIN RATIOS IN STANDARD PREPARATIONS

Amylose:amylopectin ratios in standard preparations	*Measured ratio**	*Standard deviation (%)*
75.0:25.0	76.0:24.0	1.89
50.0:50.0	50.8:49.2	0.59
25.0:75.0	28.2:71.8	1.2

* Average of triplicate injections.

The profile of amylose (Fig. 5, 100% amylose) showed two peaks (V_e = 2.8 and 3.8 ml), designated Ax and Am, and a very small broad peak (V_e = 2.4 ml) labeled Ap.. The percentage of these peak areas were Ax = 6.0, Am = 93.0 and Ap < 1. After treatment of this amylose preparation with isoamylase, the area of peaks Ap and Ax decreased and simultaneously new small peaks appeared at a V_e of 5.8 ml. This result indicates that the amylose preparation contains a small amount of branched components. It was noted that degradation of Ap and Ax was not complete, especially peak Ax. Presumably, peak Ax contains some branched components which are not accessible to isoamylase, while Ap is readily hydrolyzed to smaller molecular weight fractions.

Peak area determinations were calculated from the chromatograms depending upon the shape of the elution profile. For example, as shown by the arrows in Fig.

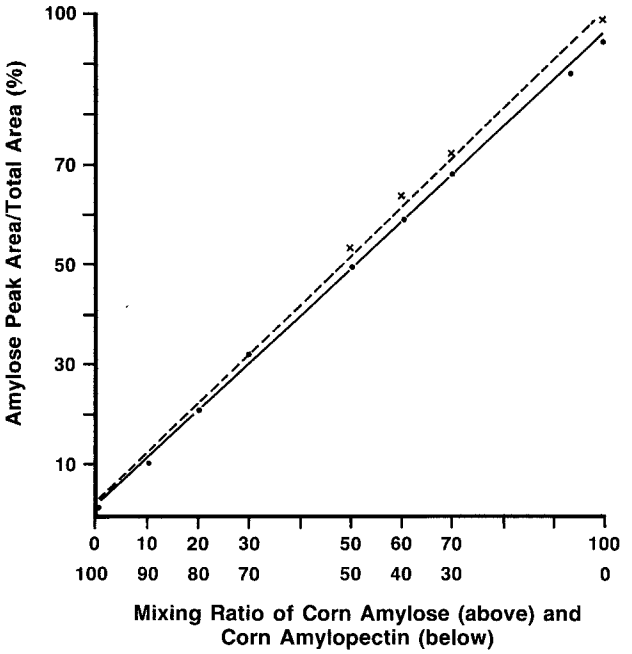

Fig. 6. Standard curves representing the relationship between the mixing ratio of purified amylose and amylopectin preparations *versus* the measured peak area percentages. (———) ratio of Am peak area to total area; (---------) ratio of (Am + Ax) peak areas to total area.

5, the chromatograms for amylose–amylopectin mixtures (100:0, 70:30, 60:40 and 50:50) were measured at the arrows labeled 1–4. In the cases of mixtures containing 30:70, 20:80, 10:90 and 0:100 amylose–amylopectin, the areas were measured at the point of peak minima or at the end of the peak tail, represented as arrows 5, 6 and 7.

Table I lists the data obtained for triplicate injections of three preparations of amylose–amylopectin (75:25, 50:50 and 25:75). The data agree closely with the expected values, and the low standard deviations, measured from triplicate injections, indicate that the analysis is precise and reproducible.

From the area data obtained, two ratios were calculated; Am:total peak area and (Am + Ax):total peak area. Plotting the percentage of each *versus* the mixing ratio of purified amylose and amylopectin produced the linear relationship shown in Fig. 6. Using the mean value found from these two standard curves, the amylose content of two high amylose corn starches (Hylon V, 50% amylose; and Hylon VII, 70% amylose; National Starch and Chemical Co.) and corn starch (Sigma, Lot 42F-0755) were determined as 52.5, 66.0 and 28.5%, respectively. These values were

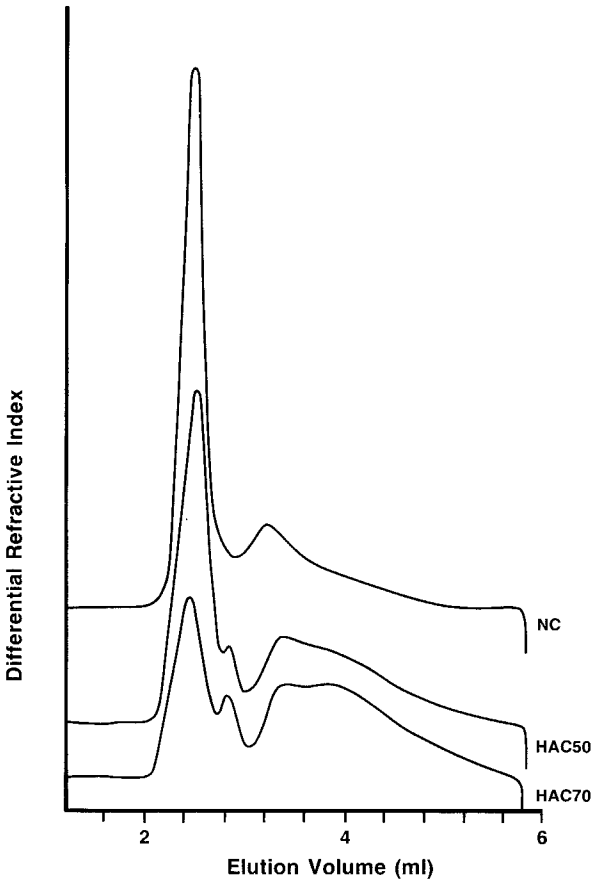

Fig. 7. Elution profiles of corn starches. NC = normal corn starch, HAC 50 = high amylose corn starch (Hylon V), HAC 70 = high amylose corn starch (Hylon VII).

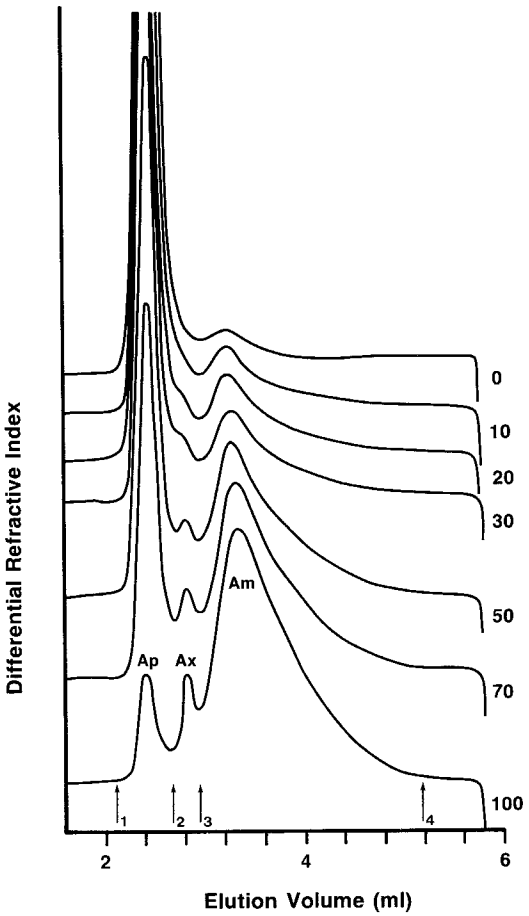

Fig. 8. Elution profiles of samples containing mixtures of purified wheat amylose and amylopectin preparations. Symbols are as those in Fig. 5.

measured from the average of the two standard curves and based on the assumption that the amylose preparation used for the calibration was pure. As shown in Fig. 5, a small amount (less than 1%) of contaminating amylopectin was present in the purified amylose preparation despite efforts to purify the sample. The values of 52.5 and 66.0% are considered very reasonable while the value of 28.5% is slightly higher than the 27% previously reported[24] for normal corn starch. The limiting factor in these determinations is that baseline resolution of amylopectin and amylose is not obtained. Therefore, a very accurate measurement of the amylose content, particularly below 30%, is difficult to achieve due to peak overlap. The profiles of the above three samples are shown in Fig. 7. The chromatograms illustrate that the corn starch samples contain differences in the molecular weight distribution of starch polysaccharides. This appears more prominent within the amylose fraction.

Standard curves for the determination of wheat amylose and amylopectin

As with corn, purified wheat (Eagle variety) amylose and amylopectin were prepared and mixed in various proportions. Fig. 8 shows the profiles obtained from

these preparations. Fractions Ap, Ax and Am were treated with isoamylase. Peak Ap was completely degraded by the enzyme, but peaks Ax and Am remained after the reaction. The peak areas were determined and standard curves plotted as described for determination of the corn starch components. Since the profiles for corn and wheat starch differ, it is recommended that a standard curve be prepared for each sample. The wheat starch sample (Eagle prime starch) shown in Fig. 1 contained 28.5% amylose. Literature values for wheat starch, measured by iodine affinity methods, range from 23.4 to 27.6[21].

CONCLUSION

HPSEC has been shown to be a useful method to monitor the purity of amylose and amylopectin preparations. In addition, the method can be rapidly (less than 20 min) used to estimate the amylose:amylopection ratio of starch samples. Further application of the method, such as separations of debranched and smaller-molecular-weight fragments, show promise of revealing important information concerning the detailed structure of starch components.

ACKNOWLEDGEMENT

We thank Professor W. D. Doughtery for providing the tobacco mosaic virus. The financial assistance of Proctor and Gamble Co. is appreciated.

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