QUANTITATIVE SEPARATION OF STARCH COMPONENTS ON A CELLULOSE COLUMN[*]

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(Received April 12th, 1973)

SUMMARY

Ethanol-induced adsorption of amylose on a cellulose column equilibrated with ethanol–urea is used for the quantitative separation of amylose and amylopectin from starch. Under these conditions, amylose is completely retained on the column. The column is washed free of amylopectin and the adsorbed amylose is eluted by gradient elution with ethanol–urea. Samples containing 300–400 mg of starch can be successfully fractionated on a column packed with defatted cellulose (20 g) with a recovery of $95 \pm 2\%$. The elution profile reveals the heterogeneity of native amylose.

INTRODUCTION

In plants, the polysaccharides deposited in starch granules consist mainly of amylose (linear) and amylopectin (branched), the isotactic homopolymers of D-glucose. Amylose consists of linear chains of D-glucose linked through α-D(1→4) bonds, and is heterogeneous with respect to molecular weight (1.5×10^5 to 2.4×10^6). Amylopectin contains short linear chains of α(1→4)-linked D-glucopyranose residues, which are interlinked mainly by α(1→6) linkages to form a highly branched structure. Amylopectin is heterogeneous with respect to degree of branching and molecular weight (1×10^6 to 6×10^7).

Chromatographic procedures for the separation of starch components are mostly based on the differences in solubility[1] and the preferential adsorption[2-5] of one of the components or its complexes with a suitable ligand[6]. These procedures are useful in the detection of trace amounts of the starch components in purified preparations of amylose and amylopectin. Paper chromatography may be useful for the micro-scale determination of amylose and amylopectin from different starches. However, the use of strong acids and alkalis in presence of oxygen is bound to cause some degradation of the starch components.

Tanret[7,8] has reported that amylose can be removed by selective adsorption on cotton-wool. Schoch[9] showed that Tanret's method was unable to remove the residual amylose and that the fatty acids extracted from the cotton-wool suppressed the forma-

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[*] A preliminary report of this work was presented at the 5th All-India Symposium in Biophysics, Bombay, December, 1970
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tion of the blue-coloured amylose–iodine–iodide complex. Gilbert et al.[10] investigated the use of defatted cotton-wool for the removal of trace amounts of amylose by adsorption from amylopectin fractions. Their experiments indicated that the amylose can be preferentially adsorbed on defatted cotton-wool, but the process is inefficient.

Our earlier studies[11,12] have shown that trace amounts of amylose from the amylopectin fraction can be removed by batchwise adsorption on defatted cellulose from ethanol–urea. The adsorbed amylose can be eluted with urea (2–4 M). This work has now been extended to the ethanol-induced adsorption of amylose on a cellulose column equilibrated with ethanol–urea for the quantitative separation of amylose and amylopectin from starch. The isolated products are characterized by determining their blue values, iodine binding capacities and β-amylolysis limits.

EXPERIMENTAL

Materials

Water distilled in an all-glass unit was used. All of the reagents used were of AnalaR grade. Commercial ethanol was purified according to the procedure described by Vogel[13]. Urea was recrystallized from ethanol.

Urea solution. Acidification of the urea solution just before use ensured the removal of cyanate by decomposition. A freshly prepared urea solution (10 M) from recrystallized urea gave a negative test for cyanate[14]. Routinely, the urea solutions were acidified to pH 4.0 with citric acid (1 M) before use and the desired pH was obtained by adding NaOH (1 M).

Starch solution. Potato starch was prepared according to the method described by Schoch[15] and the dried starch granules were defatted by repeated extraction with hot methanol (85%, v/v). Defatted starch granules were equilibrated with saline (0.14 M) containing EDTA (10^{-3} M) at room temperature (26–28°) for 16–18 h and further treated with chloroform–n-butanol (15:3) to remove trace amounts of proteins[16].

The defatted wet potato starch (200 mg) was dispersed in 10 ml of buffered urea solution (10 M, pH 6.2) at room temperature (26–28°) with mild stirring for 70–80 h[11,12]. The dispersed starch was precipitated by adding an equal volume of 95% ethanol. This precipitate was dissolved in urea solution (2 M, pH 6.2) and preserved at room temperature.

Defatted cellulose. Chromatographic cellulose powder (Whatman No. 1) was exhaustively defatted by repeated extractions with hot aqueous methanol (85%, v/v) and air-dried.

Iodine solution. A stock solution (1 l) containing iodine (2 g) and KI (20 g) was diluted (1:10) with water and used for the determination of the blue value and iodine binding capacity of the starch components.

Characterization of amylose and amylopectin

This was achieved by determining the blue value according to the method of Bourne et al.[17], the iodine binding capacity on a micro-scale[11,12] based on the procedure of Lansky et al.[18] and the β-amylolysis limit value by a standard procedure[11,12].

Sweet potato β-amylase was purified according to the original procedure of Balls et al.[19], further modified by Nakayama and Anagase[20]. The purified β-amylase

had a specific activity of 200 units/mg of protein. The maltose liberated by the action of β-amylase was determined by the 3,5-dinitrosalicylic acid procedure[21].

The total carbohydrate content was determined by the thymol–$FeCl_3$–HCl procedure[22].

Viscosity determination

The intrinsic viscosities of amylose and amylopectin in NaOH (0.5 *M*) were determined essentially according to the method of Schoch[15] and the experimental details outlined by Greenwood[23].

Methods

The influence of varying the concentration of ethanol on the retention of amylose on defatted cellulose was studied in a batchwise experiment in order to determine the optimum concentration of ethanol for the column chromatographic procedure.

A system containing 1.0 ml of starch (20 mg) in urea (2 *M*), plus 4.0 ml of defatted cellulose slurry (12.5%, w/v) equilibrated in urea (2 *M*) plus 5.0 ml of different concentrations of ethanol (10–100%, v/v) in urea (2 *M*), was placed in different centrifuge tubes (1.5 × 10.0 cm) and incubated at 30 ± 0.1° for 3 h. The tubes were shaken
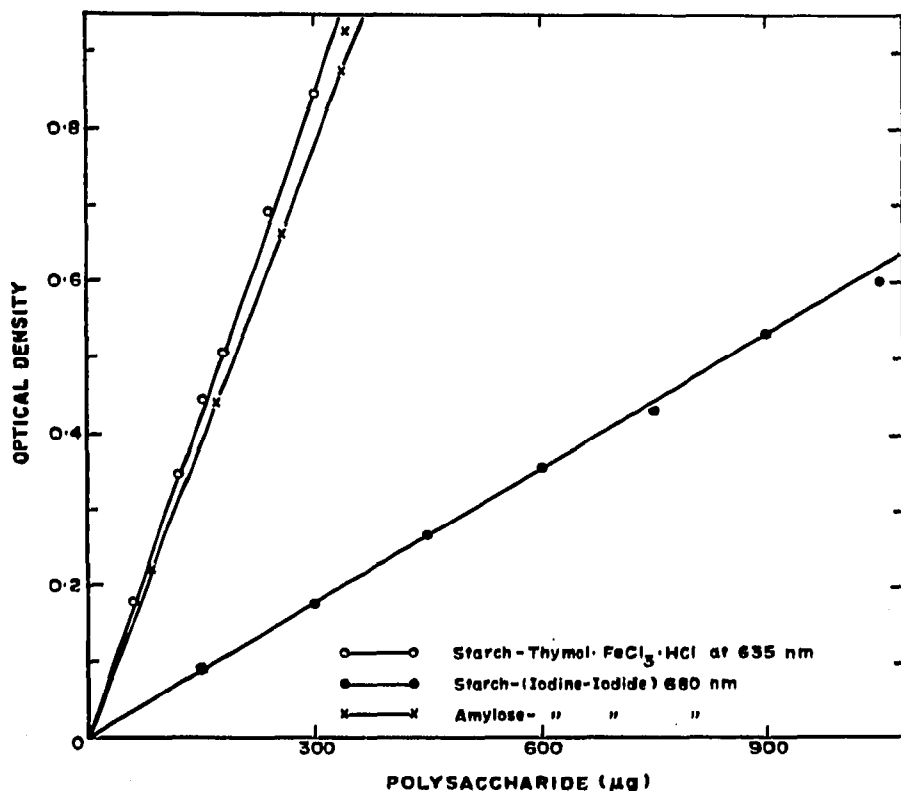

Fig. 1. Standard curves for the determination of total polysaccharide content by the thymol–$FeCl_3$–HCl method and amylose by iodine–iodide blue complex in the chromatographic separation of starch components.

intermittently on a Vortex mixer. After incubation, the cellulose was collected by centrifugation at 2500 g, washed three times with 5.0 ml volumes of the appropriate ethanol–urea mixture, and the adsorbed amylose was eluted with 5.0 ml of urea (2 M). The amylose was determined by its blue coloration with iodine (Fig. 1). The results of a typical experiment are illustrated in Fig. 2.
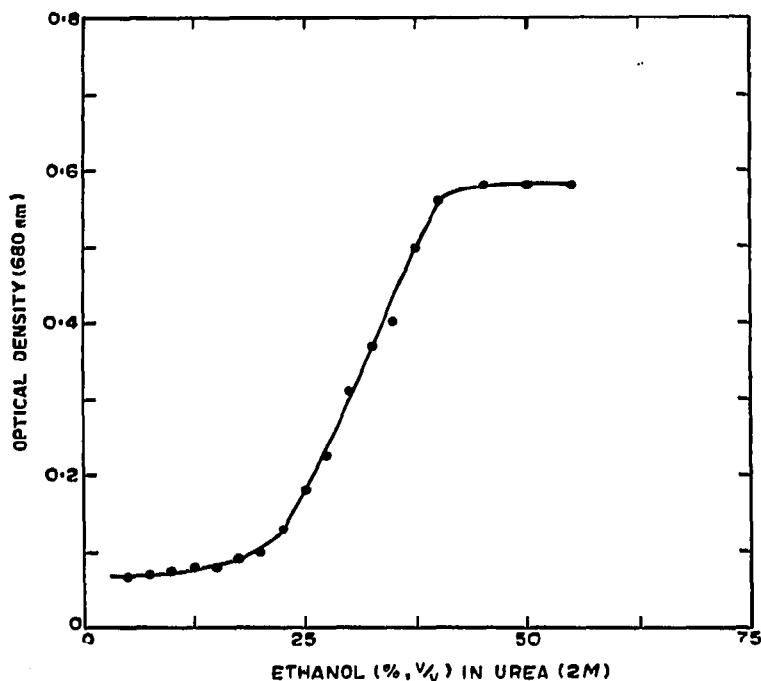

Fig. 2. Influence of the varying concentration of ethanol on the retention of amylose on defatted cellulose.

Starch in urea solution (2 M), on equilibration with cellulose, showed no retention of either component. However, the adsorption of amylose on defatted cellulose in ethanol–urea gradually increased as the concentration of ethanol was increased from 19 to 35% (v/v); above this concentration there was no increase in the adsorption of amylose. Complete precipitation of starch from urea solution (2 M) was observed at an ethanol concentration of about 38–40% (v/v). This precipitate had a gummy consistency and clogged the column. An ethanol concentration of 32.0% (v/v) was found to be suitable for retaining the amylose quantitatively on the cellulose column. The complete elution obtained of the ethanol-induced adsorption of amylose implies that it is a reversible process.

Column chromatographic system. The column consists of a jacketed Pyrex glass chromatographic tube (1.5 × 25 cm), two Pyrex glass capillary (1.0 mm) end-pieces and nylon cloth discs (400 mesh), as shown in Fig. 3. The nylon discs at both the ends are covered with a layer of sand (1.0 cm), which prevents the clogging of the nylon netting. The defatted cellulose powder was equilibrated with ethanol–urea (32%, v/v; 2 M) with mild stirring for 3–4 h at room temperature (26–28°). It was allowed to settle
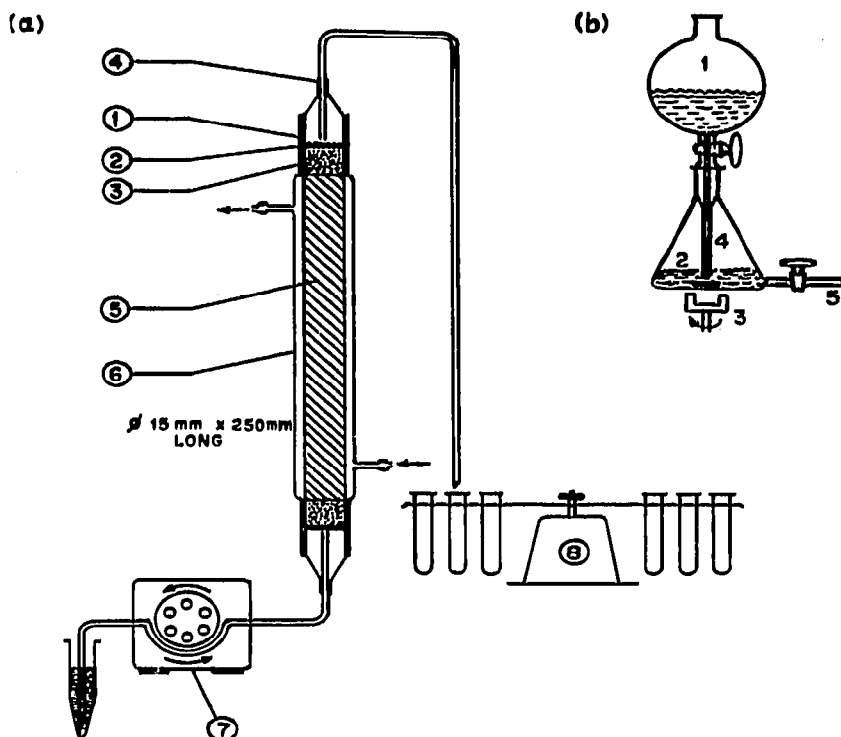
Fig. 3.(a) Chromatographic assembly. 1 = Tygon tube; 2 = nylon disc; 3 = sand; 4 = capillary (I.D. 3 mm); 5 = cellulose; 6 = water-jacket; 7 = peristaltic pump; 8 = fraction collector. (b) Constant-volume gradient mixture. 1 = Reservoir (500 ml); 2 = constant-volume mixing chamber (250 ml); 3 = magnetic stirrer; 4 = capillary (I.D. 0.03 mm, height 90 mm); 5 = capillary (I.D. 1.0 mm).

under gravity and the supernatant solution containing the fines was removed by decantation. Three or four treatments were found to be adequate to ensure the complete removal of the fine particles. The cellulose slurry in ethanol–urea was carefully added to the column in small portions and a uniform packing was achieved by using nitrogen* under pressure. Precautions are necessary in order to prevent the entry of air bubbles into the column. The column packing was carried out under dust-free conditions as far as possible. The column was connected to the fraction collector assembly through capillary polythene tubing and maintained at $30\pm0.5°$. The assembly (Fig. 3) consists of an automatic fraction collector, a drop counter, a peristaltic pump, a constant-volume gradient mixer and a circulating thermostat.

Operation of the column. To 10.0 ml of dispersed starch solution (200 mg) in urea (2 M), an equal volume of ethanol–urea (64%, v/v; 2 M) was added with gentle stirring. The rates of addition of ethanol and stirring were adjusted so that no precipitate was formed and the solution remained clear. The solution was equilibrated at 30° for 10 min and centrifuged at 2500 g for 5 min so as to ensure that a homogeneous solution was obtained. The clear starch solution was introduced on to the cellulose

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* Nitrogen gas under pressure from a nitrogen cylinder was passed through a filter of cotton wool so as to remove the dust.

column from the bottom, through a peristaltic pump operated at a flow-rate of 30 ml/h. It was observed that for the complete retention of amylose, the solution had to be recycled through the column three times. After the third passage, the solution emerging through the column consisted of a pure amylopectin fraction. Complete removal of amylopectin from the cellulose column was achieved by passing 4–5 bed volumes (bed volume 30 ml) of ethanol–urea (32%, v/v; 2 M) through the column. The polysaccharide content of the fractions was determined by the thymol–FeCl$_3$–HCl procedure[22]. The fractions containing the amylopectin were combined and an equal volume of 95% ethanol was added in order to precipitate the amylopectin. The precipitate was washed free of urea with 60% ethanol.

Regeneration of the cellulose column. After the elution of amylose, the cellulose column could be regenerated by passing 3–5 bed volumes of ethanol–urea (32%, v/v; 2 M) and can then be used two or three times. Further use is limited owing to the high resistance of the cellulose column.

Recovery of the cellulose. The defatted cellulose used was regenerated by treating it with an alkaline solution (NaOH, 0.1 M) for 1 h with gentle stirring, washed free of alkali and equilibrated with ethanol–urea (32%, v/v; 2 M) for further experiments.

RESULTS AND DISCUSSION

Earlier attempts to separate starch components by column chromatographic procedures have been only partly successful. Furthermore, the amylose and the amylopectin fractions isolated by these procedures have not been critically characterized. Amylose and amylopectin are isotactic high-molecular-weight homopolymers of D-glucose that differ only in their structures, and their chromatographic separation presents some special problems. Isotactic polymers, owing to their uniform chain nature, display a strong tendency to crystallize, which is more pronounced in amylose (linear) than in amylopectin (branched).

A neutral aqueous solution of dispersed starch is metastable and on standing becomes opalescent owing to the association of amylose molecules, known as retrogradation. As a consequence, the amylose chains associate through hydrogen bonds, become insoluble and are precipitated. On the other hand, the amylopectin forms fairly stable solutions. Retrogradation of amylose is essentially an irreversible process, but the precipitate can be dissolved by heating it at elevated temperatures or treating it with alkali. Hence it is evident that the precipitate of retrograded amylose is likely to clog the column and its insoluble nature demands drastic conditions for elution. It should be emphasized that for a successful column chromatographic separation, the starch components should be molecularly dispersed, and the adsorption should be reversible.

Our earlier studies[11,12] have shown that starch dispersed on heating in buffered urea (2 M, pH 6.2) forms a clear solution that is stable for several months. Furthermore, the amylose and the amylopectin can be isolated by fractional precipitation with ethanol.

Ethanol is known to form a helical complex with amylose with a characteristic "V"-type X-ray diffraction pattern[24]. According to Erlander and Tobin[25], urea helps to stabilize the helical conformation of amylose in solution. The amylose chain is sufficiently flexible to undergo trans-conformational changes in aqueous solution.

Amylose can complex with a variety of ligands, such as 1-butanol, cyclohexanol, iodine, and dimethyl sulphoxide, to form helical complexes that form fairly stable solutions and prevent precipitation of the amylose by retrogradation. The amylose retained on the defatted cellulose from ethanol–urea is probably in a helical form and the ethanol-induced adsorption is shown to be completely reversible.

TABLE I

CONSTANT GRADIENT ELUTION SYSTEM

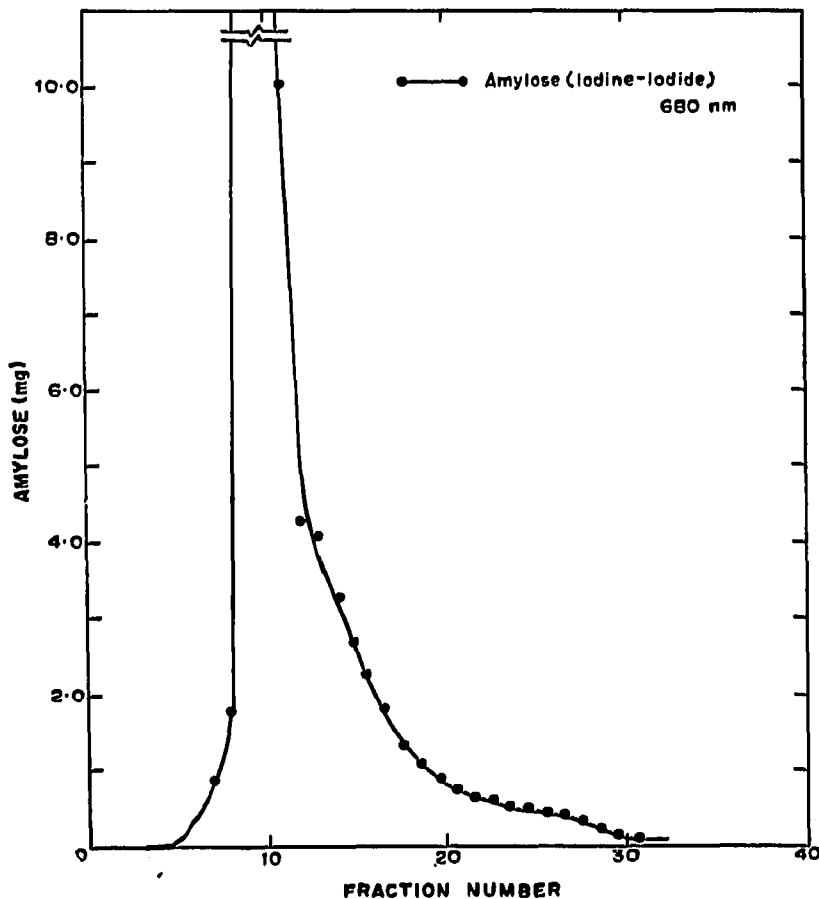| Constant volume mixing chamber	Reservoir	Elution profile (Fig. No.)
(a) Urea (2 M)	Urea (2 M)	4
(b) 25 ml ethanol (32%, v/v) in 2 M urea		5
(c) 50 ml ethanol (32%, v/v) in 2 M urea		6
(d) 75 ml ethanol (32%, v/v) in 2 M urea		7

Fig. 4. Elution profile of amylose (potato) with urea (2 M pH 6.2).

The amylose adsorbed on the cellulose column from the ethanol–urea system was eluted by the systems shown in Table I, and the elution profiles obtained are illustrated in Figs. 4–7. The amylose content in the fractions was determined at 680 nm on treatment with iodine (KI_3) and the total polysaccharide at 635 nm by the thymol–$FeCl_3$–HCl procedure[22]. The two methods give similar results, indicating that the polysaccharide eluted is essentially amylose (Fig. 5). The amylose fractions were combined and amylose was recovered by adding an equal volume of 95% ethanol. The precipitate was washed free of urea with 60% ethanol.

The results of the characterization of the amylose and the amylopectin fractions are given in Table II. It is evident that the amylose is free from amylopectin contamination, as judged from the iodine-binding capacity and the blue value. The β-amylolysis limit of 98.0% suggests that it is essentially a linear polymer. The intrinsic viscosity, $\eta = 3.56$, indicates a high molecular size of the potato amylose. Similarly, the results for amylopectin suggest that it is free from amylose and the properties compare favourably with those of pure potato amylopectin isolated by the conventional fractionation procedure[15].

Amylose on elution with urea (2 M) alone, emerges as a concentrated zone of 5.0 ml. The elution curve (Fig. 4) is not symmetrical (Gaussian distribution curve) but
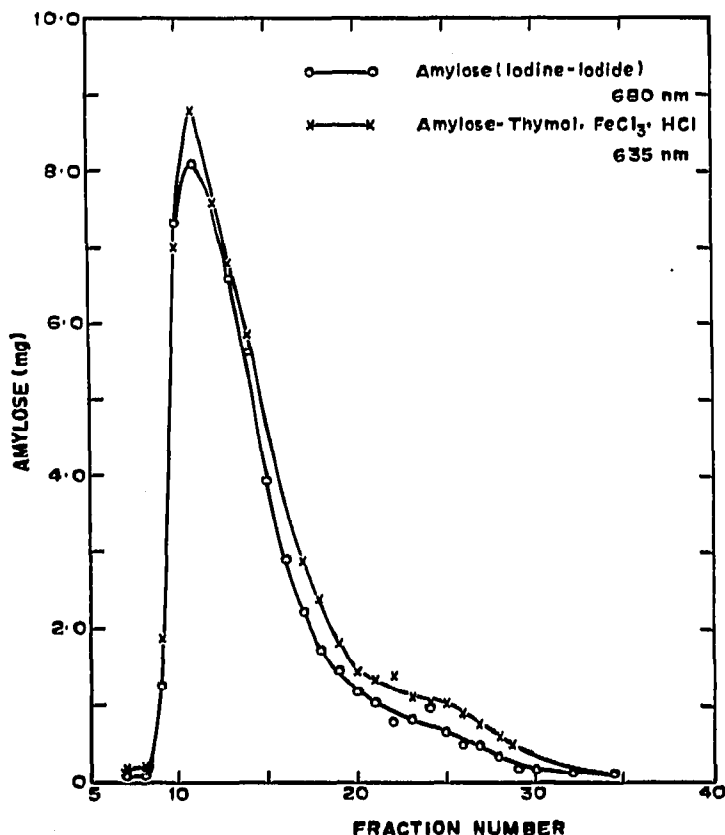

Fig. 5. Elution profile of amylose (potato) with a gradient of ethanol (25 ml; 32%, v/v)–urea (2 M).
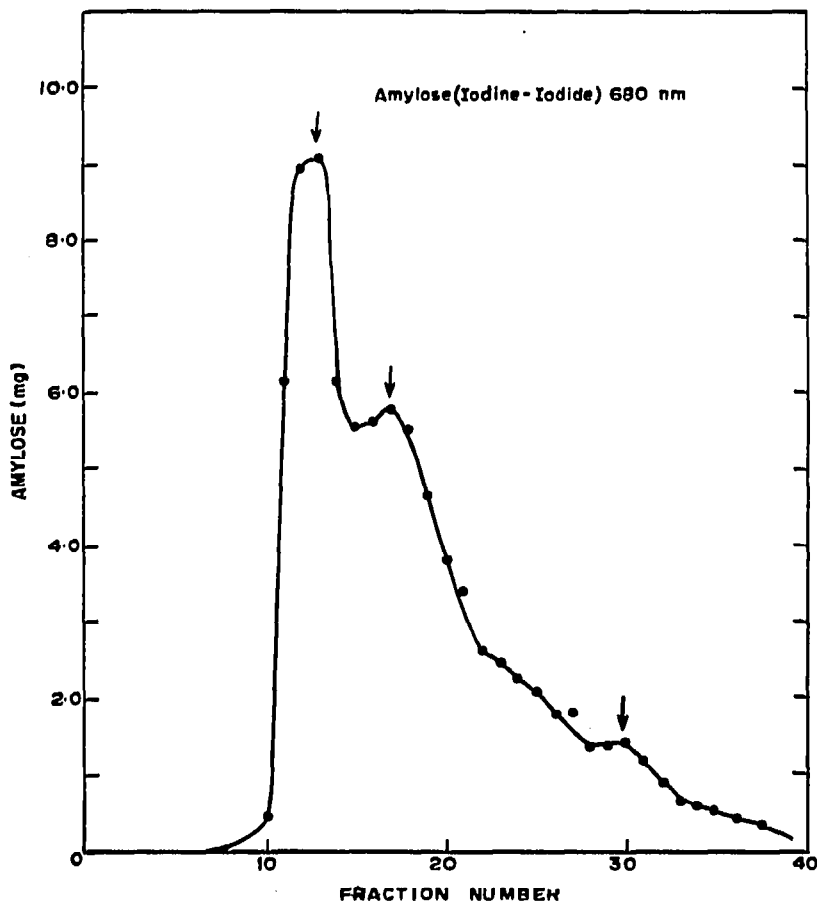
Fig. 6. Elution profile of amylose (potato) with a gradient of ethanol (50 ml; 32%, v/v)–urea (2 M).

TABLE II

CHARACTERIZATION OF THE POTATO STARCH AND ITS COMPONENTS

Component	Iodine binding capacity	Blue value	β-Amylolysis limit**	Intrinsic viscosity (η)
Starch	4.2	0.52	62.0	2.36
Amylopectin*	0.2	0.16	51.0	1.49
Amylose*	19.8	1.42	98.0	3.56

* Pooled fractions.
** Percentage conversion in maltose.

is slightly skewed, indicating a slight heterogeneity. This elution system can be used for the quantitative isolation of the amylose on a micro-scale.

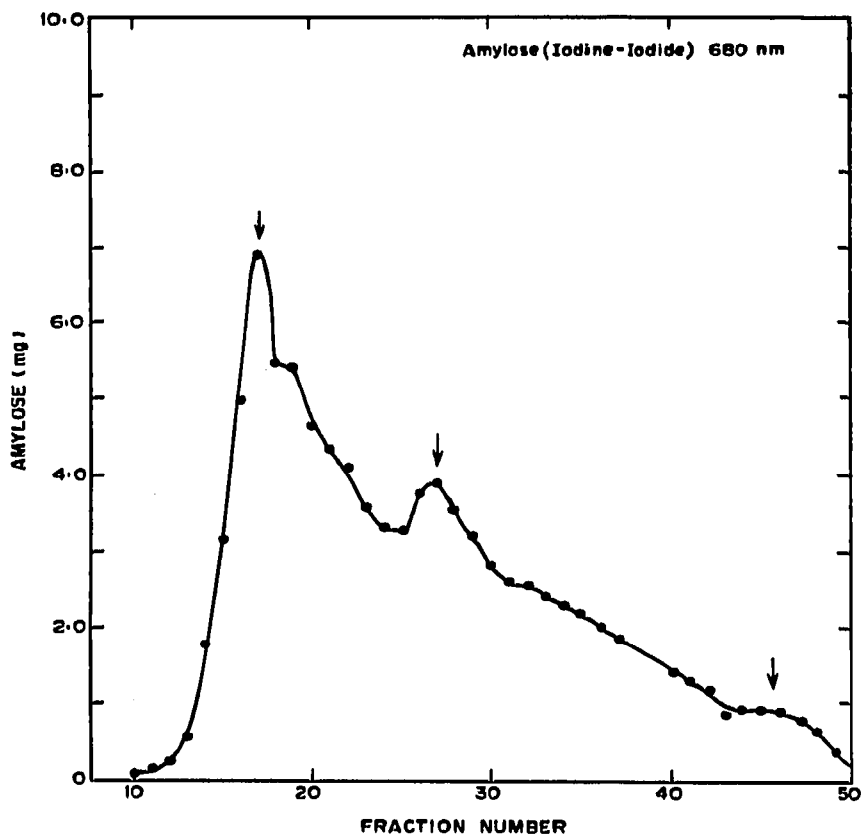The gradient elution profiles of the alcohol-induced amylose on the defatted

Fig. 7. Elution profile of amylose (potato) with a gradient of ethanol (75 ml; 32%, v/v)–urea (2 M).

cellulose show a stepwise resolution as the volume of the ethanol–urea system in the mixing chamber is increased from 25 to 75 ml. The elution profiles (Figs. 5–7) indicate the heterogeneity of the potato amylose, which is in reasonable agreement with the subfractionation data on the potato amylose[26]. The peaks representing the amylose fractions are not well resolved, but it appears that there are three major and two minor peaks. The overlapping of the peaks may be due to the similar sizes of the amylose molecules. Further work is required in order to characterize these fractions by determining their molecular weights.

Attempts to re-chromatograph the eluted amylose or the pure amylose in ethanol–urea were unsuccessful because the column developed a high resistance to the flow of the solvent. Hence the presence of the amylopectin appears to have a beneficial effect in minimizing the resistance to the flow of the solvent through the cellulose column, but it significantly lowers the amylose retention capacity of the cellulose column. The solution must be recycled two or three times for complete removal of the amylose, although the cellulose is far in excess.

The method has also been found to be applicable to the separation of the components of tuber and cereal starches.

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