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Characterization of hydroxypropylated potato starch

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Abstract

Unmodified and modified (hydroxypropylated) potato starch were fractionated on a size-exclusion column to obtain amylose and amylopectin fractions. The molar substitution (MS) of modified whole starch was determined to be 0.099, of amylopectin, 0.096, and of amylose, 0.113. The location and distribution of modifying groups on amylopectin and amylose were determined by enzyme-catalyzed hydrolysis, using various combinations of isoamylase, β-amylase, α-amylase and amyloglucosidase, and comparing elution profiles of unmodified and modified amylopectin and amylose digests by size-exclusion chromatography. It was confirmed that amylose was modified to a greater extent than amylopectin, and that further modification of amylopectin occurred close to branch points, probably because amorphous regions are more accessible to the modifying reagent. It was also evident that amylose, likewise, was not uniformly modified.

Keywords: Amylopectin; Amylose; Potato starch

1. Introduction

Starches are composed of unbranched and slightly branched amylose molecules (BeMiller, 1997b), and highly branched amylopectin (Manners, 1989). Derivatization of starches with monofunctional reagents, such as alkylene oxides, restricts intermolecular associations and the ability of starch chains to form junction zones, thereby stabilizing starch pastes and extending the applications of starch. Hydroxypropylstarches are widely used in the food industry. They are prepared by reacting a starch with propylene oxide to low levels of etherification, a molar substitution (MS) of 0.1 being common. Pastes of hydroxypropylated starches have increased clarity and improved freeze-thaw and cold-storage stabilities.

Two of the possibilities for new, chemically modified starch products are the control of reaction sites within starch granules and the control of reaction sites on molecules (amylopectin and amylose) (BeMiller, 1997a). To control any process, it is a prerequisite to understand thoroughly all aspects of the process. Hydrolytic enzymes and size-exclusion chromatography (SEC) have been widely used to examine the molecular architecture of complex carbohydrate polymers (Hood and Mercier, 1978; Hizukuri et al., 1981; Reddy et al., 1993; Steeneken and Woortman, 1994; Bello-Perez et al., 1996). Few investigations of the location of substituents on starch polymers have been reported previously (Hood and Mercier, 1978; Steeneken and Smith, 1991; Steeneken and Woortman, 1994). Based on results of specific enzyme treatments (Hood and Mercier, 1978) and on limited acid-catalyzed hydrolysis (Steeneken and Smith, 1991), it was concluded that substitution was preferentially, if not exclusively, located in amorphous regions. Steeneken and Woortman (1994) reported that the polymers of granular *O*-methyl starches were substituted in a more blockwise manner, while starch polymers methylated in solution were substituted in a more random fashion. The work reported here was undertaken to determine the location and distribution of substituent groups on amylopectin and amylose molecules of granular hydroxypropyl potato starch.

2. Materials and methods

2.1. Fractionation of potato starches

Unmodified and modified (hydroxypropylated) potato starches were fractionated using size-exclusion chromatography (SEC) on a Sepharose CL-2B (Pharmacia Biotech, Piscataway, New Jersey, USA) column (2.6 × 90 cm) to obtain amylose and amylopectin fractions. The void and

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total volumes of the column were determined using Blue dextran 2000 (molecular weight ≈2 000 000, Pharmacia Biotech) and glucose, respectively.

Starch (1%) was stirred with 90% (v/v) dimethyl sulfoxide (DMSO) in a 96°C water bath for 1 h, and then for 24 h at 25°C. An aliquot (15 mg/1.5 ml, dry starch basis) was taken and 6 ml of absolute alcohol was added. The precipitated starch was recovered by centrifugation and washed twice with 70% ethanol to remove DMSO. The precipitated starch was redissolved in water (5 ml) by placing the tube in a boiling water bath and stirring for 20 min. The mixture was centrifuged (3000***g***, 15 min) to remove insoluble residue. The supernatant was filtered using a 5 μm filter.

The filtered sample was placed on the Sepharose CL-2B column and eluted with water containing 0.02% sodium chloride and 0.02% sodium azide, at a flow rate of 20 ml/h. The carbohydrate contents of each fraction (5 ml) were estimated by the phenol-sulfuric acid method (Dubois et al., 1956). The iodine-complex absorbance was determined as follows. To a 1 ml aliquot was added 0.2 ml of 0.2% iodine solution (2 g iodine and 20 g potassium iodide per 1000 ml), and the blue color was measured in a spectrophotometer at 620 nm. Appropriate fractions were pooled to obtain amylopectin and amylose fractions, and the solutions were dialyzed and freeze-dried.

2.2. Molar substitution of modified starch and its amylopectin and amylose fractions

Samples (20 mg) were treated with 20 ml of 1.5 M trifluoroacetic acid at 80°C for 4 h. TFA was removed by evaporation under reduced pressure. The hydrolyzate was dissolved in distilled water, freeze-dried, and then twice exchanged with deuterium oxide. The deuterated samples were analyzed by ^{1}H NMR at 300 MHz (QE 300 Spectrometer, General Electric Co., Fairfield, CT, USA) (pulse delay = 1 s), and MS was calculated (Villwock, 1996).

2.3. Enzyme action on amylopectins

2.3.1. Debranching of amylopectins

Amylopectin from both modified and unmodified potato starches was debranched using isoamylase (Sigma, St. Louis, MO, USA, I 2758). Amylopectin (unmodified and modified, ≈ 5 mg) was dispersed in 2 ml of 0.2 M sodium acetate buffer, pH 3.5; 2 units of isoamylase were added, and the mixture was incubated for 24 h at 20°C. The enzyme was then inactivated by placing the tube in a boiling water bath for 10 min. The digest was centrifuged to remove the precipitated enzyme. The supernatant was fractionated on an SEC column (1.6 × 90 cm) of Biogel P-10 (Bio-Rad Laboratories, Richmond, CA, USA) using 0.02% sodium azide solution as the eluent; 1.5 ml fractions were collected. The void and total volumes of the column were determined using Blue dextran 2000 (molecular weight ≈2 000 000, Pharmacia Biotech) and D-glucose, respectively. Carbohydrate content and iodine-complex absorbance (A_{620}) were determined for each fraction as described above. Appropriate fractions were pooled. The iodine-polysaccharide complex was scanned to determine the absorption maximum (λ_{max}) for each pool.

2.3.2. Action of β-amylase

Amylopectin (unmodified and modified, ≈5 mg) was dispersed in 2 ml of 50 mM sodium acetate buffer, pH 4.8; 2 units of crystalline β-amylase (Sigma, A 7005) were added, and the mixture was incubated for 24 h at 20°C. The enzyme was then inactivated by placing the tube in a boiling water bath for 10 min. The digest was centrifuged to remove the precipitated enzyme. The supernatant was fractionated on Biogel P-10, and the carbohydrate content of each fraction was determined.

2.3.3. Action of isoamylase and β-amylase in combination

Amylopectin (unmodified and modified, ≈5 mg) was dispersed in 2 ml of 0.2 M sodium acetate buffer, pH 3.5; 2 units of isoamylase (Sigma, I 2758) were added, and the mixture was incubated for 24 h at 20°C. The enzyme was inactivated by placing the tube in a boiling water bath for 10 min. The pH was raised to 4.5; 4 units of crystalline β-amylase (Sigma, A 7005) were added, and the mixture was incubated for 24 h at 20°C. The enzyme was then inactivated and removed as described above. The supernatant was fractionated on Biogel P-10, and the carbohydrate content of each fraction was determined.

2.4. Enzyme action on amylose

2.4.1. Action of α-amylase

Amylose (unmodified and modified, ≈ 5 mg) was dispersed in 2 ml of 50 mM sodium acetate buffer, pH 6.8; 2 units of crystalline α-amylase (Sigma, A 6380) were added, and the mixture was incubated for 24 h at 20°C. The enzyme was then inactivated by placing the tube in a boiling water bath for 10 min. The digest was then centrifuged to remove the precipitated enzyme. The supernatant was fractionated on an SEC column (1.6 × 90 cm) of Biogel P-10 (Bio-Rad Laboratories) using 0.02% sodium azide solution as the eluent; 1.5 ml fractions were collected. Carbohydrate content and iodine-complex absorbance (A_{620}) were determined for each fraction.

2.4.2. Action of α-amylase and amyloglucosidase in combination

Amylose (unmodified and modified, ≈5 mg) was dispersed in 2 ml of 0.2 M sodium acetate buffer, pH 6.8; 2 units of crystalline α-amylase (Sigma, A 6380) were added, and the mixture was incubated for 24 h at 20°C. The enzyme was inactivated by placing the tube in a boiling water bath for 10 min. The pH was reduced to 4.5; 4 units of amyloglucosidase (glucoamylase) (Sigma, A 7420) were added, and the mixture was incubated for 24 h at 55°C. The enzyme

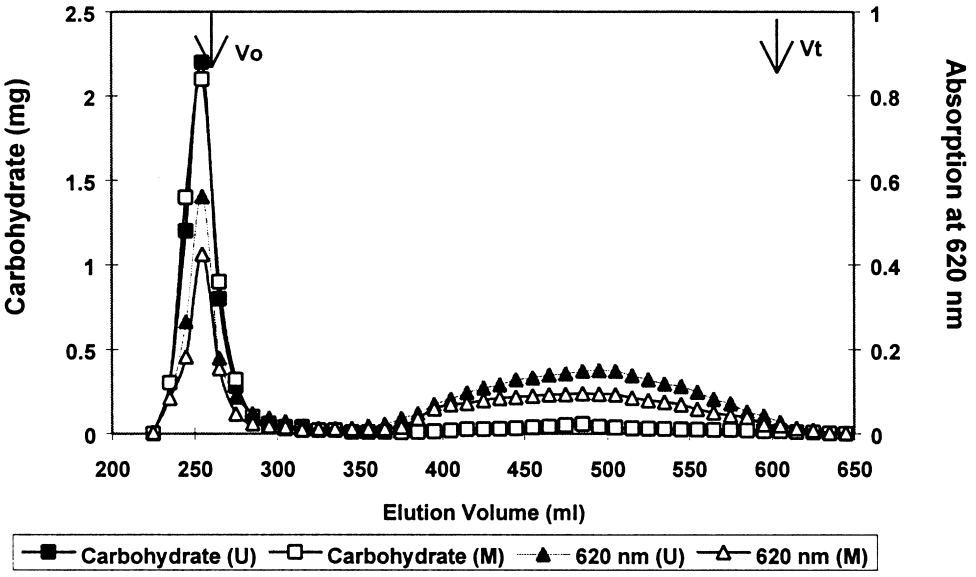

Fig. 1. Elution pattern of unmodified (U) and modified (M) potato starches (Sepharose CL-2B; V_0, void volume; V_t, total volume).

was then inactivated and removed as described above. The supernatant was then added to the Biogel P-10 column and fractionated as described above. The carbohydrate content of each fraction was determined.

3. Results and discussion

3.1. Fractionation of potato starches

Modified and unmodified potato starches each separated into two fractions on Sepharose CL-2B, one larger gel-excluded amylopectin fraction and a smaller gel-included amylose fraction (Fig. 1). Carbohydrate recovery was 85-88%. The A_{620} of modified amylopectin–iodine and modified amylose–iodine complexes was less than that of unmodified amylopectin-iodine and amylose-iodine complexes (Fig. 1), an observation also made by Hood and Mercier (1978). Reduced absorbance undoubtedly results from a hindering of complex formation by the substituent groups.

3.2. Molar substitution

The methyl moieties of hydroxypropyl groups give a distinct ^{1}H NMR peak (1.2 ppm), the area of which was compared with the total area of the peaks of the six glucosyl unit protons (3.1–4.1 ppm). The hydroxypropyl group also contributes three protons in the 3.1–4.1 range, for which a correction was made. Using this technique, the molar substitution (MS) of the whole modified starch was determined to be 0.099, of the amylopectin fraction, 0.096, and of the amylose fraction, 0.113, confirming the finding of Steeneken and Smith (1991) who reported that the degree of substitution (DS) of amylose was 0.21, and of amylopectin, 0.14, when the DS of whole *O*-methylstarch was 0.16. Thus, the amylose fraction was substituted to a greater extent than the amylopectin fraction. Hood and Mercier (1978) concluded that substitution takes place preferentially in amorphous domains of amylopectin molecules. Steeneken and Smith (1991) concluded that substitution takes place preferentially in amorphous regions of granules.

Banks et al. (1973) reported that multiple substitution of amylopectin and amylose molecules of *O*-hydroxyethylstarch occurred only when the MS was greater than 0.45. Therefore, it is likely that the modified amylopectin and amylose fractions in this study were monosubstituted, with most of the substitution at the O(2) position. For DS 0.12 *O*-methylstarch, the percentage of methyl groups located on O(2), O(3) and O(6) was determined to be 69:18:12; the ratios remained constant up to a degree of substitution of 0.8 (Steeneken and Woortman, 1994). Xu and Seib (1997) determined the distribution of hydroxypropyl groups in eight hydroxypropylated wheat and corn starches with DS values ranging from 0.05 to 0.23, and found that the frequency of the hydroxypropyl substitution was: O(2), 67%–78%; O(3), 15%–29%; and O(6), 2%–17%.

3.3. Enzyme action on amylopectin

3.3.1. Debranching of amylopectin

Carbohydrate recovery of debranched amylopectins from the Biogel P-10 column was 93%–98%. Both unmodified and modified amylopectin eluted at the void volume. Elution profiles of the hydrolysis products of the two isoamylase-debranched amylopectins were different. Debranched unmodified amylopectin eluted as three fractions (Fig. 2). The first fraction, which eluted at the void volume (45–65 ml, DP $>$ 50), constituted 19% of the total carbohydrate; the second fraction which eluted at 66–92 ml

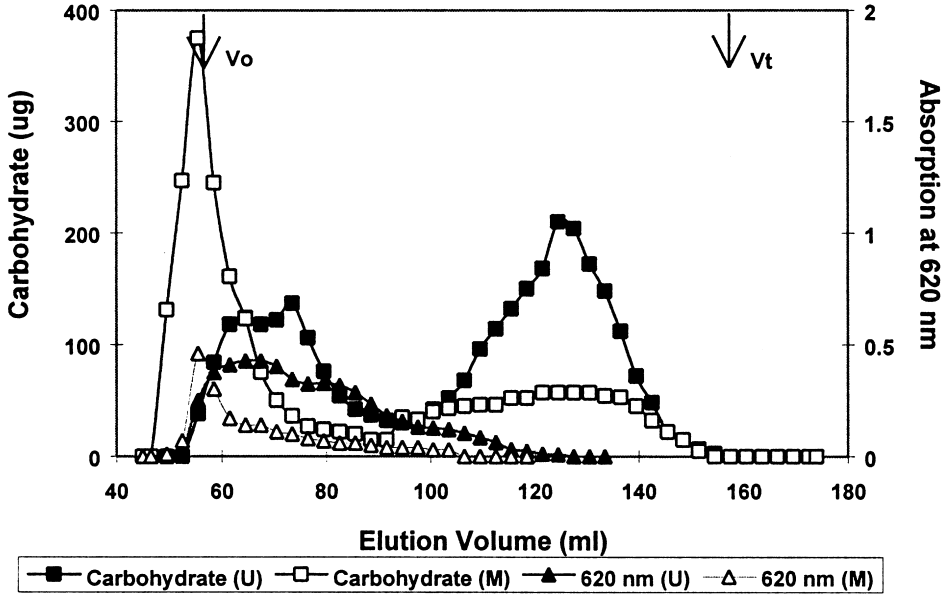

Fig. 2. Elution pattern of debranched unmodified (U) and modified (M) potato amylopectins (Biogel P-10; V_0, void volume; V_t, total volume).

(DP 40–50, 20%), and the third fraction, which eluted at 93–158 ml (DP 10–15, 61%), entered the gel. The first and second fractions were not well resolved. The λ_{max} of the first fraction–iodine complex was 560–600 nm; for the second fraction–iodine complex, it was 540–580 nm. There was little 620 nm absorbance by the third fraction–iodine complex. Reddy et al. (1993) and Bello-Perez et al. (1996) observed that λ_{max} decreased for amylopectin fraction–iodine complexes with a decrease in chain length.

Approximately 58% of the debranched modified amylopectin eluted as a void-volume peak, and 42% entered the gel (Fig. 2). The tail fraction of the void volume constituted 7% of the total carbohydrate loaded onto the column. Approximately 35% of the debranched modified amylopectin eluted as a broad peak (100–158 ml, DP 10–15).

It can be concluded that 58% of the amylopectin was modified close enough to branch points to inhibit isoamylase action. Hood and Mercier (1978) observed that ≈60% of manioc amylopectin was modified sufficiently to inhibit hydrolysis by pullulanase. The λ_{max} of the void volume fraction–iodine complex was between 540 and 580 nm; there was no A_{620} for the iodine complex of the material eluting as a tail (Fig. 2). The tail fraction of the void volume consisted of DP 40–50 material, which could be a population of B2 chains (DP 42–48), as reported by Hizukuri (1986) for potato amylopectin. This fraction was not observed by Hood and Mercier (1978). From the amount

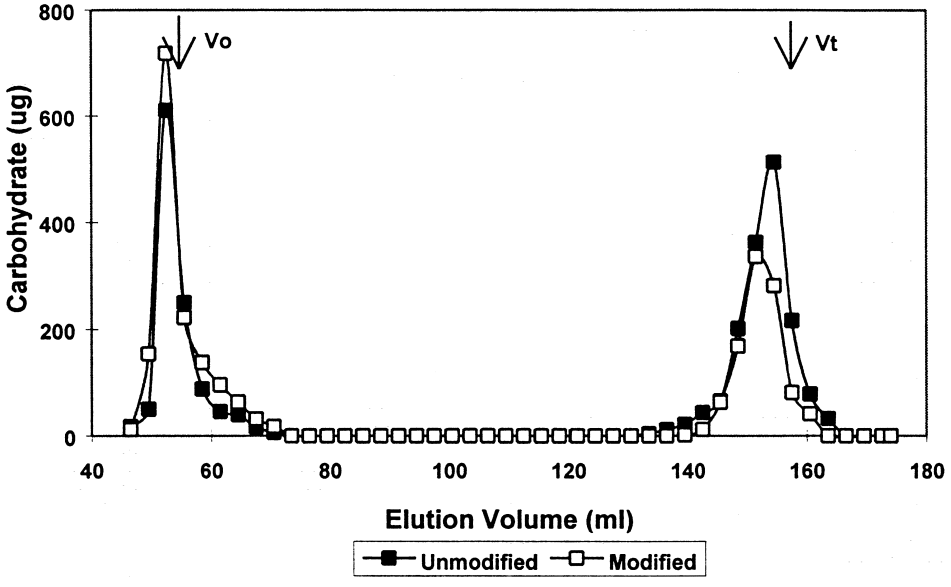

Fig. 3. Elution pattern of unmodified (U) and modified (M) potato amylopectins treated with β-amylase (Biogel P-10; V_0, void volume; V_t, total volume).

of DP 10–15 material eluted, it was evident that ≈43% of the short chains were modified close to the branch points and, therefore, were not released by isoamylase. Each cluster in amylopectin contains a highly branched region that is amorphous (Robin et al., 1974; Manners, 1989). These regions could be the most accessible to the modifying reagents.

3.3.2. Action of β-amylase

Thirty-nine percent of unmodified amylopectin treated with β-amylase, eluted as a void-volume fraction (beta-limit dextrin), while 61% (maltose) eluted as a total-volume fraction (Fig. 3). After modified amylopectin was treated with β-amylase, 53% eluted in the void-volume peak and 47% in the total-volume peak (Fig. 3). The 14% more which was not hydrolyzed in this case must result from cluster chains being modified sufficiently to inhibit β-amylase action. Thus, it appears that there is also some modification towards the nonreducing ends of A and B chains. The 53% in the void-volume peak should contain all the (1,6)-linkages, and probably also all the substituent groups. Beta-amylolysis of unmodified and modified manioc starch occurred to extents of 61% and 34%, respectively (Hood and Mercier, 1978).

Since the MS was 0.096 for modified amylopectin, hydrolysis by β-amylase should have been only ≈10% if the substitution was random. The reason for 47% hydrolysis was either that (1) substitution was non-random, and/or (2) the enzyme used can bypass certain modifying groups. Examination of the maltose fraction by ^{1}H NMR revealed a trace of hydroxypropyl groups, indicating some slight ability of β-amylase to bypass certain minor hydroxypropyl substitution. However, regioselective substitution very close to or below branch points is the main reason for the greater than theoretical hydrolysis.

3.3.3. Action of isoamylase and β-amylase in combination

Both amylopectins were hydrolyzed with a combination of isoamylase and β-amylase. Unmodified amylopectin was completely hydrolyzed to maltose, while 42% of the modified amylopectin was still not hydrolyzed by the combination of enzymes (Fig. 4). The modified amylopectin resisting isoamylase + β-amylase action must have contained most of the branch points and modifying groups, and must have resulted from the action of isoamylase being restricted by the presence of hydroxypropyl ether groups, as previously found for pullulanase (Hood and Mercier, 1978).

The short chains (DP 10–15) and the tail fraction of the void volume peak (DP 40–50) of debranched modified amylopectin (Fig. 2), which combined, totaled 42% of the digest, were hydrolyzed to completion by β-amylase (Fig. 4). This seems to indicate that these chains were linear and devoid of substituent groups. Approximately 50% of the DP ≈15 chains of modified manioc amylopectin were unmodified (Hood and Mercier, 1978). It is likely that the crystalline areas of amylopectin, which are comprised of the short outer chains of DP ≈15 (French, 1972; Robin et al., 1974), were not penetrated by the reagents used in modification. The remaining 72% of the void-volume material (16% of the total carbohydrate) was probably a beta-limit dextrin, resulting from β-amylase degrading the short chains of the void-volume material until the activity was stopped by a branch point or modifying group. Hydrolysis of chains of DP 40–50 by isoamylase + β-amylase, shows that there were regions in amylopectin where even the long chains were not modified. Since no significant amount of oligomers was observed in the isoamylase + β-amylase-treated modified amylopectin, we conclude that there were no short chains that were modified close to their non-reducing ends.

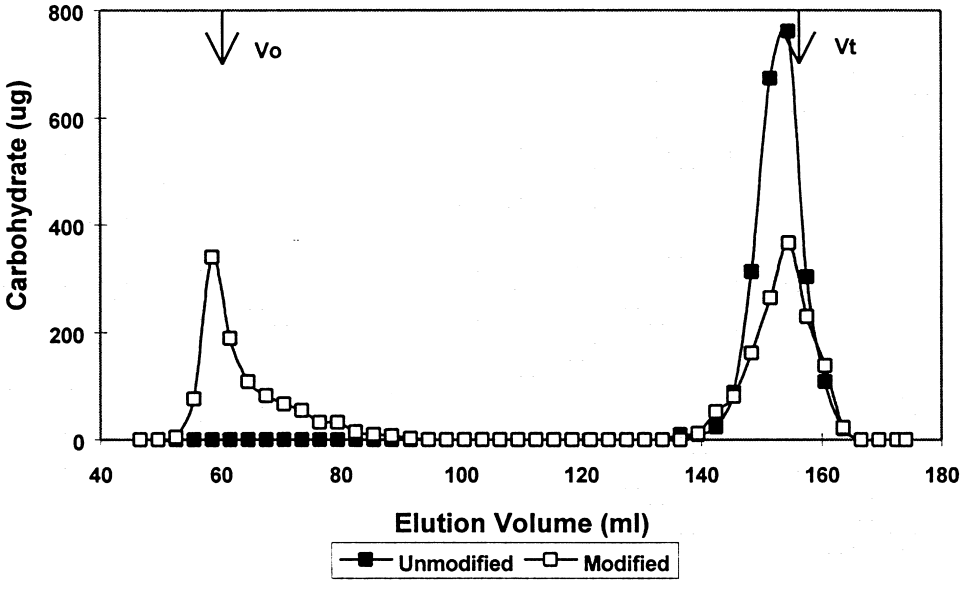

Fig. 4. Elution pattern of unmodified (U) and modified (M) potato amylopectins treated with isoamylase and β-amylase (Biogel P-10; V_0, void volume; V_t, total volume).

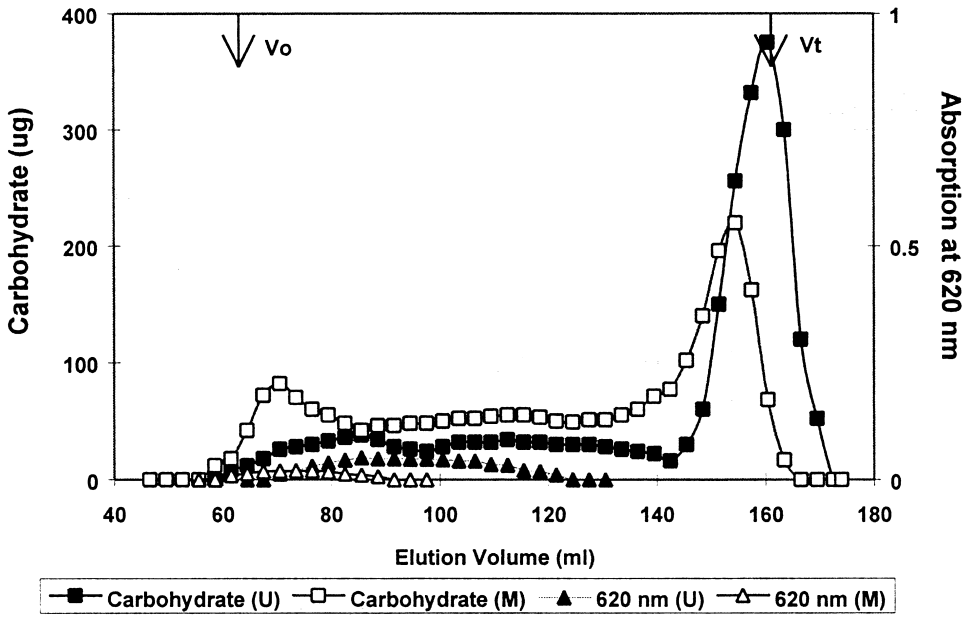

Fig. 5. Elution pattern of unmodified (U) and modified (M) potato amyloses treated with α-amylase (Biogel P-10; V_0, void volume; V_t, total volume).

From these results, it appears that the void-volume peak (42%) contains all the modifying groups, which theoretically means $\approx$ 2.3 groups per 10 glucosyl units of amylopectin (MS 0.23). After pullulanase and β-amylase hydrolysis of modified starch (MS 0.045), Hood and Mercier (1978) also observed highly substituted regions of MS 0.1. It was also determined that substituted monomers in granular *O*-methyl starch tended to occur in clusters (Steeneken and Woortman, 1994).

3.4. Enzyme action on amylose

3.4.1. Action of α- amylase and amyloglucosidase

α-Amylase hydrolyzed $\approx$ 70% of unmodified amylose to low molecular weight material eluting at total volume (Fig. 5). The rest of the amylose eluted as a broad distribution of molecular weight fractions. The hydrolysis products of the modified amylose treated with α-amylase eluted throughout the separation range, and the low molecular weight peak eluted slightly before the total volume peak, indicating the presence of slightly higher molecular weight material than contained in the peak from unmodified amylose after α-amylolysis (Fig. 5). Also, the peak represented only 48% of the total carbohydrate loaded onto the column. The iodine complex of hydrolysis products released from unmodified amylose showed very little 620 nm absorbance; even less absorbance was given by iodine complexes of products released from modified amylose, in keeping with the finding that methylation, at even very low degrees of substitution, diminishes the ability of amylose to form inclusion complexes (Steeneken and Smith, 1991).

A large number of degradation products were released by α-amylase from unmodified starch (Bertoft, 1991). Therefore, the α-amylase-treated amylose digests were treated with amyloglucosidase to distinguish more clearly between hydrolysis products of unmodified and modified amylose. Unmodified amylose was completely hydrolyzed to glucose, while only 60% of modified amylose was converted to glucose (Fig. 6). An amyloglucosidase + α-amylase combination hydrolyzed 98% of unmodified and 70% of modified manioc starch (hydroxypropyl distarch phosphate) to glucose (Hood and Mercier, 1978). The modified amylose which eluted as a trail after hydrolysis by α-amylase, was further hydrolyzed by amyloglucosidase (Fig. 6). The absence of an oligomer peak of any significant amount, and the presence of a wide range of products in the digest, indicated that amylose was not uniformly modified.

From this work, it appears that there were regions in both amylopectin and amylose which were substituted to a greater extent, i.e. that substituent groups occurred in clusters. Steeneken and Woortman (1994) observed that granular *O*-methyl starches were substituted in a more blockwise manner compared with starches methylated in solution.

Enzyme specificity depends on the type of substitution and the source of enzyme. Yoshida et al. (1973) reported that the rate of hydrolysis catalyzed by α-amylase decreased with increased MS of *O*-hydroxyethylstarches, and also that 2-*O*-substitution blocked α-amylase action to a greater extent than 6-*O*-substitution. Although the action of some of the enzymes used may not be completely inhibited by the modifying groups, they can still be used to obtain an understanding of their location. We did obtain NMR evidence that the action of β-amylase was not blocked by all hydroxypropyl groups, but rather that β-amylase did, to a very low degree (data not shown), release small amounts of *O*-hydroxypropylmaltose. The very small amount produced may indicate that this occurred only when the hydroxypropyl

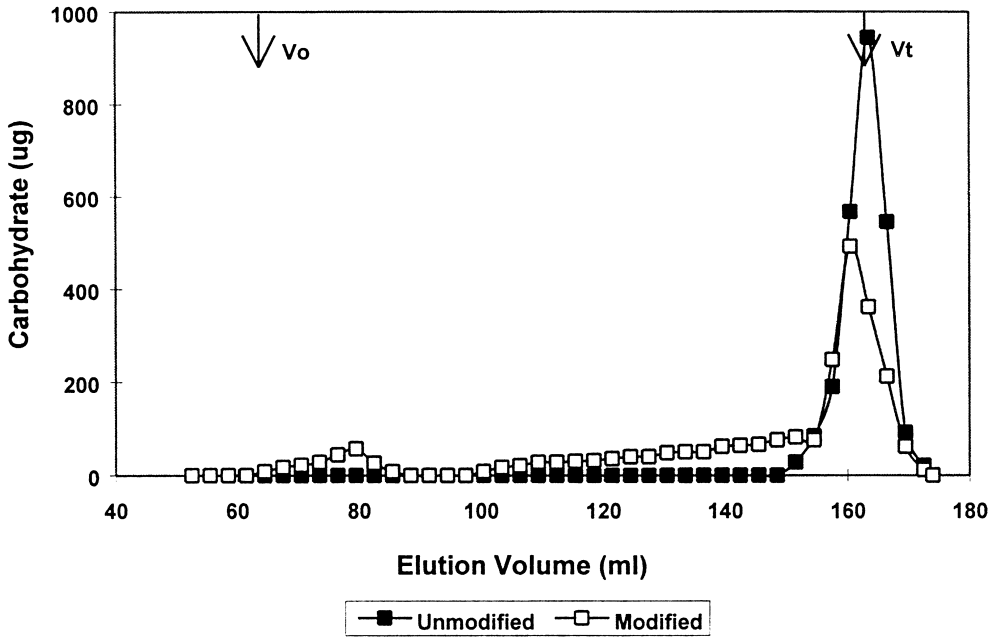

Fig. 6. Elution pattern of unmodified (U) and modified (M) potato amyloses treated with α-amylase and amyloglucosidase (Biogel P-10; V_0, void volume; V_t, total volume).

group was only on one, or a few, of the six hydroxyl groups of a maltosyl unit where substitution could occur, and perhaps at a position where substitution is minor.

From NMR analysis it was clear that amylose was modified to a greater extent than amylopectin, and from enzymic analysis that modification of amylopectin occurred close to branch points, probably because of the accessibility of the amorphous regions to the modifying reagent. It was also evident that there were regions in both amylopectin and amylose that were highly substituted, i.e. that the molecules were non-uniformly modified and the substituent groups somewhat clustered.

Acknowledgements

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