Enzyme Hydrolysis of Grafted Amylopectin

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ABSTRACT: Previous methods of proof of grafting are based on separation of homopolymers from crude reaction products and further characterization of extracted component. This article reports the proof of grafting by a combined use of viscometry and enzyme hydrolysis that, to our knowledge, has not been reported so far. Two series of graft copolymers of amylopectin with polyacrylamide were synthesized using ceric ion-induced redox initiation technique. In the first series, a variation of ceric ion concentration at fixed acrylamide concentration and in the second series, a variation of acrylamide concentration at fixed ceric ion concentration were undertaken to effect a variation in the number and length of polyacrylamide chains. Qualitatively, it has been observed that there may be some homopolymers formed at a very high acrylamide concentration. The products may at best be a mixture of graft copolymer and homopolymer, but it contradicts the view that the products are purely physical mixtures of polysaccharide and polyacrylamide. © 1998 John Wiley & Sons, Inc. J Appl Polym Sci 70: 2627–2633, 1998

Key words: enzyme hydrolysis; amylopectin-*g*-polyacrylamide; amylopectin; α-amylase; grafting

INTRODUCTION

The phenomenal finding by Mino and Kaizerman,[1] about the possibility of synthesizing graft copolymers on the free radicals produced by the action of ceric ion on structures containing 1,2-diol groups, generated extensive research in graft copolymerization. This has stimulated modification of the properties of a large number of polysaccharides[2] (e.g., starch, cellulose, dextran, amylose, amylopectin etc.) by grafting acrylic polymers onto them. In the author's laboratory, a large number of graft copolymers of polysaccharides (guar gum/xanthan gum/carboxymethyl cellulose/starch/amylose/amylopectin, etc.) have been synthesized by grafting polyacrylamide onto them to develop efficient, shear-resistant,[3] and controlled biodegradable drag reducing[4–7] and flocculating agents.[8–10]

Many attempts have been made to prove the occurrence of true graft copolymers by the use of both chemical and analytical techniques.[11,12] Attempts have been made to separate[13] the homopolymer formed, if any, from the polymerization products and then further analyze the purified product. However, the difficulty encountered in most of the cases is the nonavailability of a solvent that would solubilize only the graft copolymer or the homopolymer, leaving the other component intact. This makes the separation process inefficient. For example, in the present case, polyacrylamide was grafted onto amylopectin. It was difficult to find a solvent for the graft copolymer without dissolving polyacrylamide. So, the idea of separating the two components simply does not work well, and some other approach has to be made.

A twofold approach was made. *First,* a series of graft copolymers were synthesized with varying

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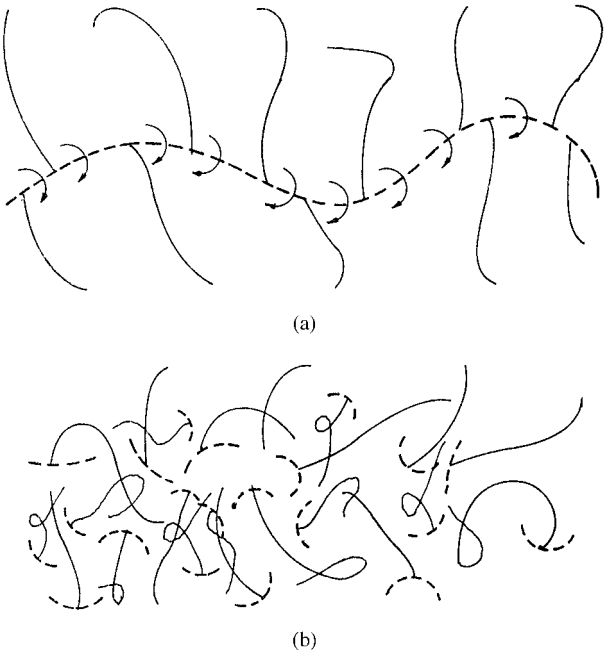

Figure 1 (A) Simplistic view of a graft copolymer of amylopectin and polyacrylamide. Intrinsic viscosity of such a polymer will be a function of both molecular weight and molecular structure. (B) Approximate view of the mixture of polyacrylamide chains after near complete hydrolysis of the backbone polymer (i.e., amylopectin). Intrinsic viscosity will not be the same as in (A). – – – = amylopectin backbone; — = grafted polyacrylamide branches; → = cleavage action. (Adapted from Refs. 18, 19.)

catalyst and monomer concentrations to obtain a variation in the number and length of polyacrylamide chains in the series. The intrinsic viscosity of a polymer, under similar experimental conditions, is a measure of its hydrodynamic volume in solution, which depends on two parameters: molecular structure and molecular weight. For two polymers of approximately the same molecular weight, a linear polymer has a higher hydrodynamic volume than a branched one and consequently a higher intrinsic viscosity.[14] Furthermore, in a series of branched polymers, the one with fewer but longer branches should have a higher hydrodynamic volume—hence a higher intrinsic viscosity than the one with a large number of small branches. Thus, in our series of graft copolymers, there should be a significant variation in the intrinsic viscosity with variation in the monomer and catalyst concentrations. *Second,* a suitable method was chosen to hydrolyze the polysaccharide backbone so that most of the polyacrylamide chains are released. Measurement of intrinsic viscosity both before and after the cleavage of polysaccharide backbone should reflect a great change (Fig. 1), because of the drastic change in molecular weight as well as molecular structure of the original graft copolymer. The change should again be in accordance with the trend in original viscosity of graft copolymers in the series. This change will be observed only if the products are graft copolymers and not physical mixtures of the polysaccharide and polyacrylamide. *Third,* a physical mixture of amylopectin and polyacrylamide was prepared in such proportions that the ratio of two components is approximately the same as should occur in case of graft copolymer (Ap-g-PAM 1), assuming no homopolymer formation. This mixture was subjected to hydrolysis as above, and flow time was measured before and after hydrolysis to observe any possible difference.

The backbone polysaccharide in the present case is amylopectin, a highly branched[15] natural polymer of anhydroglucose units. Amylopectin can be cleaved[16] by two methods: acid hydrolysis and enzyme hydrolysis. The method of acid hydrolysis will not be suitable in this case, because of the presence of polyacrylamide chains that undergo imidization[17] in presence of acid leaving a crosslinked product insoluble in water. As for enzyme hydrolysis, only two are commonly used: amylase (both α and β) and glucoamylase. Glucoamylase has the advantage[16] over amylases in that it can hydrolyze both $1 \rightarrow 4$, as well as $1 \rightarrow 6$ (branch points) links in amylopectin. α- and β-amylases[18,19] do not hydrolyze amylopectin completely because both of them are incapable of hydrolysing action at the branch points. But, the actions of α- and β-amylases differ in that the former acts randomly with an *endo*-mechanism so that all the $1 \rightarrow 4$ links are completely hydrolyzed, producing a mixture of D-glucose, maltose, and low molecular weight α-limit-dextrin ($1 \rightarrow 6$ branch points left intact). On the other hand, β-amylase acts with an *exo*-mechanism from the nonreducing end of the amylopectin. The action stops the moment a branch point is encountered. The result is a mixture of products containing a very high molecular weight product (β-limit-dextrin) along with maltose. This difference in the mechanism of action of α- and β-amylases determines the molecular weight of the endproduct in each case (α-limit-dextrin is of much lower molecular weight compared with β-limit-dextrin). α-Amylase thus degrades the amylopectin to a greater extent, compared with β-amylase. The

aim in the present case was to degrade the amylopectin portion in the graft copolymer as completely as possible to be able to release all of the grafted polyacrylamide chains. The choice was thus reduced to either α-amylase or glucoamylase. Both the enzymes were initially tested with a 1% amylopectin solution to study the degradation characteristics. Ultimately, it was planned to use α-amylase for reasons stated in the Results and Discussion section.

EXPERIMENTAL

Materials

Amylopectin (from corn) and glucoamylase (amyloglucosidase, from *Aspergillus niger*) were purchased from Sigma Chemical Company (St. Louis, MO). α-Amylase (activity: 1300 IU g^{-1}) and ceric ammonium nitrate (CAN) were obtained from Loba Chemie (Bombay, India). Acetone and hydroquinone were supplied by s. d. Fine Chemicals (India). Sodium nitrate and acrylamide (G R Grade) were supplied by E. Merck (Bombay, India). All of the chemicals received were used as such without further purification.

Synthesis

The graft copolymers of amylopectin and polyacrylamide were synthesized by ceric ion-induced redox initiation method. The typical experimental details have been discussed elsewhere.[9] Polyacrylamide was prepared with potassium persulfate initiation of acrylamide as previously described.[20] Its number-average molecular weight (M_n) was evaluated from the intrinsic viscosity [η] using the relation[21]:

$$[\eta] = 6.8 \times 10^{-4} (M_n)^{0.66}$$

Enzyme Hydrolysis and Measurement of Intrinsic Viscosity

The entire study of the hydrolysis characteristics of amylopectin and its graft copolymers with enzymes was conducted at neutral pH (that of deionized distilled water) at room temperature.

The hydrolysis behavior of amylopectin with α-amylase and glucoamylase was studied with a 1% solution of amylopectin. To a solution of amylopectin (5 g/400 mL), 40 mL of glucoamylase solution (2 mg/100 mL) were added and volume made up to 500 mL. The time of mixing with enzyme solution was noted. About 25 mL of this solution was transferred to the Ubbelohde viscometer (CS/S: 0.00527), and the flow time was measured every half hour at a temperature of 27 $\pm$ 0.2°C until it was reduced to $\sim$ 200 s. In a separate experiment, the flow time of 1% amylopectin solution was measured before addition of enzyme solution at 27 $\pm$ 0.2°C. In case of α-amylase, the variation of flow time with a time of 1% amylopectin solution was studied at three different enzyme concentrations, in a similar manner as in the case of glucoamylase. Relative viscosity ($\eta_{rel} = t/t_0$) *versus* time plots were made in each case.

The initial hydrolysis characteristics of the graft copolymers was studied by measuring the flow time of a 0.06 g dL^{-1} solution of each of the graft copolymers both before and after treatment with the α-amylase solution. The flow time of the polymer solution was measured at different time intervals (5 min, 10 min, 15 min, etc.) after treatment with the enzyme solution. It has been found that there was a sharp fall in flow time at the first measurement itself; afterward, the difference, if at all, was quite small. Therefore, it was planned to note only one reading after a prolonged treatment of the polymer solution with the enzyme. Thus, in all cases, the polymer solution was allowed to react with the enzyme solution for 24 h, after which the solution was boiled to destroy the enzyme, cooled to room temperature, and the flow time measured. Results are presented in Table I as t_1 and t_2, respectively. Furthermore, flow times of the physical mixture of amylopectin and polyacrylamide was also noted before and after treatment with α-amylase solution. It may be noted that there was no hydrolysis of the graft copolymers in the absence of enzymes under similar conditions over a time period of 60 days.

The hydrolysis of graft copolymers with α-amylase was conducted in the following manner. A 0.5 g of sample was dissolved with a minimum of stirring (to avoid settling of polymer granules at the bottom of the flask) at $\sim$ 80–90°C in freshly prepared distilled water. The solution was cooled to room temperature. A 10 mL solution of α-amylase (0.1 g dL^{-1}) was added to the polymer solution. It was slowly stirred for $\sim$ 24 h, after which the solution was heated to 100°C to destroy the enzyme. It was cooled and the volume made up to 250 mL.

Viscosity measurements before and after treatment with α-amylase of all the polymers in 1*M* $NaNO_3$ were conducted with the help of a Ubbelo-

Table I Synthesis Details of Graft Copolymers

Solution No.	Polymer	AGU[a] (mol)	Amylase (mol)	CAN (mol 10^{-4})	Conversion[b] (%)	$[\eta_1]$[c] (dL g^{-1})	$[\eta_2]$[d] (dL g^{-1})	t_1[e] (s)	t_2[f] (s)	$t_1 - t_2$ (s)
I	Ap-g-PAM 5	0.0154	0.21	0.5016	70.8	11.38	8.68	347.8	306.1	41.7
II	Ap-g-PAM 1	0.0154	0.21	1.003	87.6	10.61	8.22	310.3	272.5	37.8
III	Ap-g-PAM 6	0.0154	0.21	1.5048	88.13	9.76	7.41	287.55	252.85	34.70
IV	Ap-g-PAM 2	0.0154	0.21	2.006	90.95	6.95	5.45	250.7	230.1	20.6
V	Ap-g-PAM 3	0.0154	0.28	1.003	77.8	9.93	8.37	332.35	293.05	39.30
VI	Ap-g-PAM 4	0.0154	0.14	1.003	84.1	7.46	6.26	240.9	227.7	13.2
VII	Ap-g-PAM 7	0.006	0.28	1.003	78.4	11.68	10.77	367.6	348.1	19.5
VIII	Ap-g-PAM 8	0.006	0.35	1.003	81.6	11.67	10.97	371.2	354.0	17.2
IX	Ap + PAM	—	—	—	—	—	—	307.7	307.2	0.5

[a] Calculated on the basis of anhydroglucose units (AGU; 1 g mol^{-1} of AGU is equal to 162 g).
[b] % conversion is calculated from the relation: Conversion = [(Weight of graft copolymer − Weight of polysaccharide)/Amount of acrylamide] × 100.
[c] Intrinsic viscosity of the graft copolymer.
[d] Intrinsic viscosity of the graft copolymer after hydrolysis.
[e] Time of flow of a 0.06% solution of graft copolymer before hydrolysis.
[f] Time of flow of a 0.06% solution of the graft copolymer after hydrolysis with α-amylase.

hde capillary viscometer (CS/S: 0.00527) at 27 ± 0.2°C. The time of flow was measured for solutions at 5–7 dilutions. The intrinsic viscosity was obtained (from the point of intersection) after extrapolation of two plots [i.e., η_{sp}/C *vs.* C and $\ln(\eta_{rel})/C$ *vs.* C to zero concentration]. Herein, C is the polymer concentration in g dL^{-1}, and η_{sp}/C is reduced viscosity, calculated from the relation $[\eta_{sp}/C = (\eta_{rel} - 1)/C]$ where $\eta_{rel} = \eta/\eta_0 \approx t/t_0$, with t being the time of flow of the polymer solution and t_0 being the time of flow of the solvent at temperature measurement.

RESULTS AND DISCUSSION

It is known that amylopectin has a branched structure.[16] This makes it attain a compact shape in solution, resulting in a lower intrinsic viscosity due to the smaller hydrodynamic volume of the solvated molecules. As we go on grafting polyacrylamide onto it, two changes are possible. One can either obtain a few number of long polyacrylamide chains or a large number of short polyacrylamide chains in the graft copolymer. In the former case, the compact shape of the original amylopectin molecules would be considerably changed due to the presence of long polyacrylamide chains. This would result in a larger hydrodynamic volume leading to a higher intrinsic viscosity. On the other hand, although a large number of short polyacrylamide chains alters the original compact shape of amylopectin, it should nevertheless result in a near spherical shape that in turn helps in a lower hydrodynamic volume and consequent lower intrinsic viscosity.

Table I summarizes the synthesis details and the intrinsic viscosity of all the graft copolymers of amylopectin and polyacrylamide. The results are along expected lines. For example, in the series of graft copolymers I–IV, the concentration of both amylopectin and acrylamide was kept fixed in the feed with varying concentration of catalyst (CAN). Following a simplistic approach, a low concentration of catalyst should initiate a few grafting sites, resulting in longer polyacrylamide chains compared with a high concentration of catalyst that will initiate a larger number of grafting sites that make the average polyacrylamide chains shorter for the same acrylamide concentration. This is reflected in the intrinsic viscosity of the graft copolymers in the series. Comparison of the intrinsic viscosity values between II and IV shows a sharp decrease. On the other hand, a comparison of the same between I and III does not reflect a great change, which could either be due to a comparatively lower monomer conversion or a partial conversion of the acrylamide monomer to homopolymer. In copolymers V and VI, the concentration of amylopectin and CAN was kept fixed, thus reducing the concentration of acrylamide to half. This results in the lowering of intrinsic viscosity in VI that could be due to the shorter polyacrylamide chains. In graft copolymers VII and VIII, a deliberate attempt was made to maintain a very high molar ratio of acrylamide

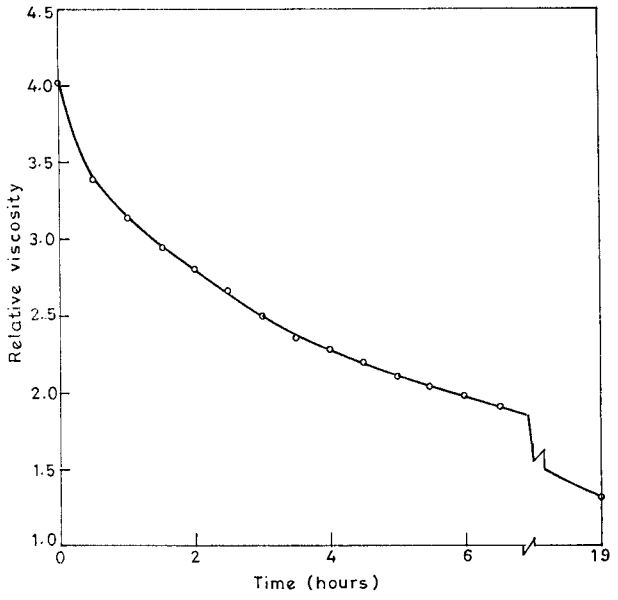

Figure 2 Study of degradation characteristics of a 1% amylopectin solution with glucoamylase (0.1 mg L^{-1}).

to amylopectin (compared with other graft copolymers in the series) at the same catalyst concentration to see the effect of length of grafted polyacrylamide chains on the intrinsic viscosity of graft copolymers. As seen, the intrinsic viscosity values exhibit some difference, although it is not as sharp as it should be when compared with the two graft copolymers II and IV. This indicates that, at a high monomer concentration, the possibility of homopolymer formation might play an important role. The intrinsic viscosity obtained could, therefore, be a mixture of graft copolymer and homopolymer. It may be emphasized that there could be some amount of homopolymers formed at low acrylamide concentration as well, but the percentage conversion is probably higher at a higher monomer concentration in the reaction feed. This observation qualitatively explains the experimental findings of Owen and Shen,[22] who have observed that a monomer concentration of $> 2.0M$ encourages homopolymer formation.

Results of the treatment of 1% amylopectin solution with glucoamylase and α-amylase is shown in Figures 2 and 3. As observed in Figure 2, glucoamylase works excellently, in that it has almost completely degraded the amylopectin in 19 h (at an enzyme concentration of 1.6 mg L^{-1}). Figure 3 shows a similar result of amylopectin with α-amylase at different enzyme concentrations. Comparison of Figures 2 and 3 indicate that glucoamylase at a concentration of 1.6 mg L^{-1} acts almost as good as α-amylase at a concentration of 4 mg L^{-1}. This shows that glucoamylase not only degrades amylopectin completely to glucose, but also does so more efficiently. However, we were guided by two factors while making a selection from among the two enzymes. Glucoamylase is costly and was not readily available. On the other hand, α-amylase was comparatively cheap with ready availability. The former advantage ensured that we could afford the same effect as glucoamylase with a high enzyme concentration of α-amylase. Thus, hydrolysis of all of the graft copolymers were conducted with α-amylase.

A similar trend was expected from the hydrolysis of graft copolymers with α-amylase. However, repeated experiments failed to establish the kind of trend as in Figures 2 or 3. Instead, treatment of a 0.06 g dL^{-1} solution of graft copolymers with α-amylase solution resulted in an initial sharp fall in the time of flow that afterward remained constant with time. This is probably because of the lower amount of amylopectin in the graft copolymer that immediately gets hydrolyzed, leaving only polyacrylamide chains in solution (Fig. 1) that are inactive to α-amylase and with further change in the time of flow. Results of the flow time of a 0.06 g dL^{-1} solution of all of the graft copolymers before and after hydrolysis with α-amylase established the trend observed in the intrinsic viscosity of graft copolymers. Table I shows the time of flow of a 0.06 g dL^{-1} solution of

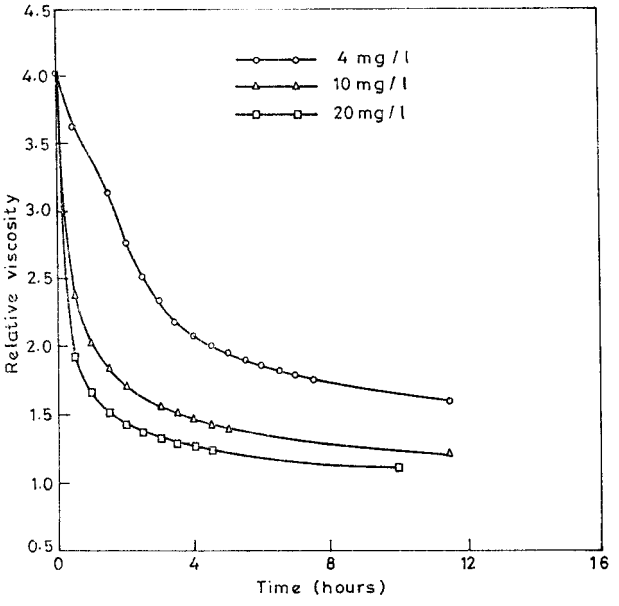

Figure 3 Study of degradation characteristics of a 1% amylopectin solution with α-amylase.

all of the graft copolymers both before and after hydrolysis with α-amylase. It is evident that not only is there a reduction in the time of flow in each case, but also the time of flow varies in different graft copolymers, indicating an occurrence of varying number and length of polyacrylamide chains with varied CAN concentration. The comparison of the difference in flow time between II and IV shows that the polyacrylamide chains are shorter in the case of Ap-g-PAM 2, compared with those in Ap-g-PAM 1. But, as in the case of the intrinsic viscosities of original graft copolymers, the difference in flow times do not differ sharply in the cases of Ap-g-PAM 5 and Ap-g-PAM 6. One observation is very clear from Table I. The difference in the flow time decreases gradually from I to IV, with increasing CAN concentration in the series. This trend qualitatively explains that the variation in CAN concentration in the series actually effects a variation in the number and length of polyacrylamide chains on the amylopectin backbone. Coming to a comparison in the second series of graft copolymers (V–VIII), it is observed that the difference in time of flow is highest in the case of Ap-g-PAM 3. A careful examination reveals that it is close to the value obtained in the case of Ap-g-PAM 5. Ap-g-PAM 5 has a low molar concentration of acrylamide at low CAN concentration (the latter should be responsible for high molecular polyacrylamide branches), in comparison with Ap-g-PAM 3, which has a high molar concentration of acrylamide at a double molar concentration of CAN (increasing acrylamide concentration should result in long polyacrylamide chains, but the simultaneous increase in CAN concentration counters the effect). It seems that a simultaneous variation of monomer and catalyst concentration produces an almost same difference in flow time between the two graft copolymers. Interestingly, the difference in flow time in the cases of Ap-g-PAM 1 and Ap-g-PAM 8 seems unnatural when a larger value can be expected in the case of the latter, especially in view of the increased ratio in molar concentration of acrylamide and amylopectin. But, these values are supported by the intrinsic viscosity values of the original graft copolymers that hardly show any difference. This may be because of a considerable amount of acrylamide getting converted to a homopolymer at such a high monomer concentration. The resulting intrinsic viscosity is probably the effect of a mixture of graft copolymer and homopolymer. This further supports the experimental findings of Owen and Shen.[22]

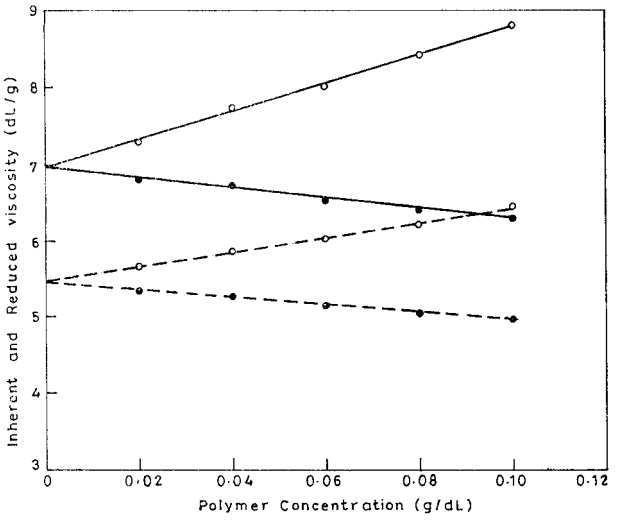

Figure 4 Determination of the intrinsic viscosity of Ap-g-PAM 2. ○ = reduced viscosity (η_{sp}/C); ● = inherent viscosity ($\ln\eta_{rel}/C$); — = before hydrolysis; —— = after hydrolysis with α-amylase.

The last row in Table I shows the flow time of a physical mixture of amylopectin and polyacrylamide ($[\eta] = 6.1$; $M_n \approx 9.75 \times 10^5$) before and after treatment with an α-amylase solution. The difference in flow time is negligible, which may well be within the experimental error. This further supports that the products cannot be physical mixtures of amylopectin and polyacrylamide.

Figure 4 shows the intrinsic viscosity for Ap-g-PAM 2 before and after hydrolysis with α-amylase, with the same for other graft copolymers tabulated in Table I. It is observed that, in all graft copolymers from I to VI, there is a substantial decrease in the intrinsic viscosity after hydrolysis with α-amylase. But, in the cases of Ap-g-PAM 7 and Ap-g-PAM 8, the difference is not much and this further supports the formation of homopolymers.

CONCLUSIONS

The variation of catalyst concentration in the first series of graft copolymers produces a variation in the number and length of polyacrylamide chains. This is evident from their intrinsic viscosity. The variation in monomer concentration keeping the CAN concentration fixed produces another series of graft copolymers. Comparison between the flow

times of a 0.06 g dL^{-1} solution of each of the graft copolymers both before and after hydrolysis with α-amylase indicates that the products are true graft copolymers. But, there seems to be a considerable amount of homopolymer formation at a higher acrylamide concentration. The difference in flow time of a physical mixture of amylopectin and polyacrylamide both before and after hydrolysis shows that the products are true graft copolymers and not physical mixtures.

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