ABSTRACT

MAHAMMAD, SHAMSHEER. Enzymatic reactions in water-soluble polymer solutions: rheology and kinetics. (Under the direction of Dr. Saad A. Khan).

Water-soluble polymers and their derivatives are widely used in many applications that include paints and coatings, detergents, drug delivery, and, oil and gas production. The rheological properties of solutions containing these polymers are determined by the various intermolecular interactions between the polymer molecules in the solution. These intermolecular interactions depend on molecular weight, degree of branching and the molecular architecture of the polymers. As such, it is often necessary to modify the structure of these polymers to tailor their rheological properties for a particular end use. Since the structure of naturally occurring water-soluble polysaccharides can be easily modified using enzymes, they are now widely being used in many of the aqueous formulations. In this regard, the main objective of this research is to explore the use of enzymes to control the rheology of aqueous solutions containing polysaccharides and thereby, understand the interrelationship between the enzymatic modification and the resulting rheological consequences.

In the first part of the project, we focus on modifying the structure of guar galactomannan using three glycosidase enzymes, β-mannanase, β-mannosidase and α-galactosidase, at different combinations and proportions. We investigate the effect of synergistic hydrolysis by multiple enzymes in terms of viscosity reduction patterns during the hydrolysis reactions. We develop a mathematical model based on Michaelis-Menten kinetics to predict the changes in molecular weights and molecular weight distribution during the hydrolysis reaction. The model is evaluated using the molecular weight distribution data measured during the depolymerization of guar using β-mannanase enzyme. We also develop a rheokinetic model combining the kinetic model with the viscosity-molecular weight relationship. The rheokinetic model is used to estimate the kinetics parameters by tracking changes in steady shear viscosity during the enzymatic reactions. The effects of the combined

action of enzymes on degradation rates are quantified in terms of variation in rate constants and other model parameters.

In the second part of the project, we focus on modulating the rheology of synthetic polymer that is not biodegradable. Our approach is to modulate intermolecular interaction between the polymer molecules by adding cyclodextrins. The synthetic polymer used in this study is a hydrophobically modified associative polymer. The polymer has a comb-like structure with hydrophobic groups randomly attached to the polymer backbone. The intermolecular interaction between the hydrophobic groups forms a transient network resulting in the thickening of the solution containing them. The cyclodextrins encapsulate the hydrophobes, disrupts the network, and causes a reduction in viscosity and other viscoelastic properties. Subsequent degradation of the cyclodextrin using an amylase enzyme enables complete recovery of the original rheological properties. We develop mathematical models to study the thermodynamics of cyclodextrin-hydrophobe complexation and the kinetics of the enzymatic reactions. We show that the model parameters can be estimated by measuring changes in the rheological properties during the cyclodextrin-hydrophobe complexation and subsequent enzymatic degradation process. The effects of enzyme-concentration, temperature and polymer concentration on the kinetics of enzyme reactions and recovery of the original rheological properties are also investigated.

ENZYMATIC REACTIONS IN WATER SOLUBLE POLYMER SOLUTIONS: RHEOLOGY AND KINETICS

SHAMSHEER MAHAMMAD

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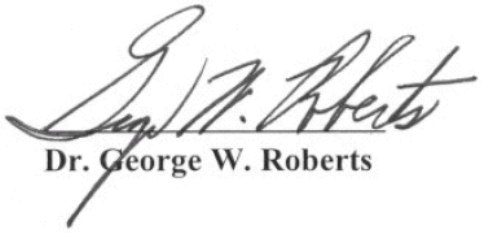

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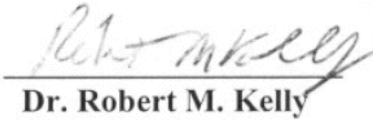

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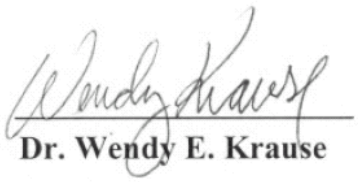

Dr. Wendy E. Krause

Dr. Saad A. Khan
Committee Chair

BIOGRAPHY

Shamsheer Mahammad was born on October 2, 1972, in Mangalore, a beautiful coastal city in southern India. His interest in chemistry and his aptitude for Mathematics prompted him to pursue a career in Chemical Engineering. He received his Bachelor of Engineering degree in Chemical Engineering from National Institute of Technology, Karnataka (formerly known as Karnataka Regional Engineering College, Surathkal), in 1994. He then moved to Chennai, India to attend Indian Institute of Technology, Madras, where he received his Master of Technology degree in Chemical Engineering in 1996. After graduation, he was employed with Galaxy Surfactants Ltd., Bombay (India) as a Project Executive, working on design, trouble-shooting and scale-up of process equipments in surfactant manufacturing plants. He was with Galaxy Surfactants Ltd. till March 1999 and then he joined Bhabha Atomic Research Centre (BARC), Bombay (India) as a Scientific Officer. He was employed with BARC till July 2001, working on modeling and simulation of flow and heat transfer processes in a nuclear steam generation plant. He entered North Carolina State University in the Fall of 2001 and began working on his doctoral dissertation.

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CHAPTER 1

INTRODUCTION AND OVERVIEW

Abstract

In this chapter, we introduce the topic of this dissertation: “Enzymatic reactions in water-soluble polymer solutions: rheology and kinetics”. Most of the naturally occurring polysaccharides are water-soluble and have the potential to replace many synthetic polymers in developing environmentally benign processes. The polysaccharides are being used in a variety of applications because of their biocompatibility, biodegradability and natural abundance. The enzymes, because of their specificity, offer a powerful method to modify the structure of the polysaccharides to get desired physiochemical properties depending on applications. The structural modifications also alter the interaction of the polysaccharides with other synthetic and biopolymers. In this regard, we explore the use of enzymes to modify the rheology of solutions containing polysaccharides.

1.1. Introduction

There is a growing interest in the use of polysaccharides due to their biocompatible, biodegradable and environmentally benign characteristics. Polysaccharides are high molecular weight polymers of monosaccharides such as glucose, fructose and mannose, where these monosaccharide units are linked through glycosidic bonds. Although they are made up of single type of building blocks, their enormous diversity has led to a bewildering variety of species, structures and properties all performing a large variety of functions of great significance[1]. They can also easily undergo chemical[2] and biochemical modification[3] to generate novel products with unique rheological and applicative properties. The interactions of some of the polysaccharides with other synthetic and biopolymers have further increased their range of applicability[4-7]. The versatility in the structure and properties of the polysaccharides and their derivatives, along with their modifications find widespread applications including food[8], cosmetics[9], petroleum[10] and pharmaceutical[11] industries.

The polysaccharides can have linear, branched or cyclic structures containing residues of only one type of monosaccharides or of different types of monosaccharides. The polymers of one type of monosaccharide units are called homopolysaccharides (e.g. cellulose, starch, etc) and those containing different monosaccharide units are known as heteropolysaccharides (e.g., glucomannans: polymers of glucose and mannose, galactomannans: polymers of galactose and mannose sugars, Figure 1.1). Cyclodextrins(CD) are cyclic polysaccharide with six or more glucose units arranged on a doughnut shaped ring as shown in Figure 1.2[12]. The CDs with 6, 7, 8 glucose units are called α, β, γ CD respectively. Depending on their structure, monomer composition and conformations, the polysaccharides show different physical and chemical properties. Although large varieties of polysaccharides have a multitude of industrial uses, in many cases they need to be further modified to improve their applicability across the wide spectrum of end-uses. In the last few decades, wide ranges of chemical derivatives of these polysaccharides were developed[13]. The chemical modifications improved the rheological properties of their solutions and hydrogels, while still maintaining their biodegradability and biocompatibility.

Enzymes, because of their stereo and regio-specific catalysis, have proven useful in modifying the structure of the polysaccharides[14,15]. These enzymes not only work under very mild conditions, but their activity can also be easily modulated depending on the process conditions such as temperature, pH, and ionic strength. Typical enzymatic modifications involve backbone scission[16], debranching[17], decyclization[18] and cross-linking[3]. *In this research, we enzymatically modify the structure of guar-galactomannans and cyclodextrins to illustrate the backbone-scission, debranching and decyclization reactions. The consequences of these structural modifications are investigated in terms of changes in rheological and microstructural properties of solutions containing the polysaccharides.*

The structural modification of guar galactomannan using debranching or backbone scission enzymes or combinations of both directly affects the viscosity of their solutions. Hence the extent of structural modifications and the kinetics of enzymatic reactions can be quantified in terms of changes in viscosity during the enzymatic reactions. However decyclization and subsequent chain-scission of cyclodextrins does not affect the rheology of CD solutions. In this case, we approach indirectly by complexing the CD with hydrophobically modified associative polymers. The complexation of CD with the polymer changes viscoelastic behavior of the associative polymer solution. These changes in viscoelastic properties are used to study the kinetics of the enzymatic reactions. Understanding various issues surrounding the above enzymatic reactions are important in developing more efficient methodologies to modify the rheological and microstructural properties of solutions and gels containing polysaccharides. In the following paragraphs, we briefly introduce the two major thrust areas investigated in this research and outline the project goals for each of them.

1.2. Enzymatic modification of rheology of guar-galactomannan

In this part of the research, we focus on modifying the structure of guar-galactomann, a naturally occurring hetero-polysaccharide of mannose and galactose monosaccharides using glycosidase enzymes. Guar is a graft copolymer, containing a backbone of mannose units linked together by β-1,4 bonds. Galactose units are randomly attached to the backbone

via α-1,6 linkages[19]. Three different types of glycosidase enzymes act on the guar molecule: β-mannanase cleaves interior β-1,4 linkages between mannose units, β-mannosidase enzyme cleaves β-1,4 linkages only from the nonreducing end of a guar molecule, α-galactosidase enzyme debranches the guar molecule[20]. Figure 1.1 shows the structure of a guar molecule with the locations of enzyme actions.

The structural modifications of guar using β-mannanase and α-galactosidase enzymes have been widely studied[21-23]. The backbone cleavage by β-mannanase enzyme significantly reduces the viscosity of guar solution. Hence, guar is used as a fracturing fluid in hydraulic fracturing to suspend propping agents, such as sand. The β-mannanase enzymes are used as breakers that degrades guar and facilitates flow of gas/oil at a controlled rate[24]. The low molecular weight guar produced by enzymatic degradation is also used as a dietary fiber supplement[25].

Guar has the highest M:G (mannose:galactose) ratio among all the naturally available galactomannan. Debranching of the guar using α-galactosidase enzyme produces galactomannans with different M:G ratios some of which can mimic the properties of other natural galactomannans such as locust bean. The debranched guar can associate themselves at the debranched portion of the mannose units via hydrogen bonding, and the molecular association is known as hyperentanglement[23,26,27]. Hyperentanglement increases solution viscosity and can also lead to gel formation. The debranched guar also has a higher tendency to form complexes with other polysaccharides such as xanthan and forming gels[28,29]. Because of these enhanced thickening and gelling properties, enzyme-modified guar is of considerable interest in food and pharmaceutical applications.

However, there are no systematic studies on degradation of guar by more than one enzyme acting simultaneously or sequentially. Hence, in this part of the research, we investigate the action of the three enzymes β-mannanase, β-mannosidase and α-galactosidase in different combinations and proportions. In particular, we explore the synergistic action of these enzymes, if any, on the structural and rheological properties of the guar

galactomannans. Along the same vein, it is also important to understand the kinetics of enzymatic reactions to quantify the changes in rheological and microstructural properties that takes place during the hydrolysis reactions. However, there are no mathematical models reported in the literature that can accurately predict the changes in molecular weight (MW), molecular weight distribution (MWD) and viscosity of the guar galactomannan solutions. Understanding these experimental and modeling issues is important in developing enzymatic processes to control the rheological and microstructural properties of guar solutions and gels, and form the specific goals of this part of the project that is delineated below.

1.2.1. Develop a mathematical model to study the kinetics of enzymatic reactions

Our focus during this study is to develop a mathematical model based on Michaelis Menten kinetics to predict the changes in MWD during the enzymatic degradation of guar. Previous kinetic models on enzymatic depolymerization of guar were simplified models assuming zero order or first order reaction kinetics. These models did not take into account the enzyme-substrate complexation and cannot predict the changes in entire MWD during the enzymatic reaction. The proposed model takes into account the effect of enzyme-substrate complexation on the overall kinetics of the reaction and effects of galactose branches on the enzyme-substrate complexation and subsequent degradation of guar molecule.

1.2.2. Investigate the hydrolysis of guar galactomannan using multiple glycosidase enzymes

In this study, we focus on hydrolyzing the guar using the three enzymes together in different proportions and combinations. We develop a more efficient hydrolysis method for structural modification of the guar galactomannan using hyperthermophilic versions of the enzymes. Enzymes synthesized by hyperthermophiles (microorganism with optimal growth temperatures greater than 80^0C) are called hyperthermophilic enzymes. They show the same catalytic action as their mesophilic counterparts, but are highly thermostable and are optimally active at high temperatures[30]. The activity of the hyperthermophilic enzymes can be further controlled depending on temperature, pH and enzyme source.

The enzymatic reactions are carried out in situ in a rheometer and the progress of the reaction is tracked by measuring the variation in the zero shear viscosity during the reaction. The viscosity of a guar solution decreases continuously during the enzymatic reactions. Since the viscosity is a function of MW, monitoring the changes in viscosity during the enzymatic reaction allows us to estimate the kinetic parameters. A rheology-based kinetic model is developed to estimate the reaction rate constants, and the synergistic effects of multiple enzymes are measured in terms of variation in these rate constants. This model makes use of the kinetic model developed to estimate the changes in MWD during the enzymatic reactions. The rheometric method offers a simple and convenient method to study the kinetics of enzymatic reactions compared to the conventional methods.

1.3. Enzymatic modification of rheology of hydrophobically modified associative polymers

Polysaccharides interact with other synthetic or biopolymers in solution leading to increase or decrease in the viscosity of the solution[31]. The synergistic interaction between some of the polysaccharides and the other biopolymers produce hydrogels. Recently, it has been found that cyclodextrins can be used to remove the hydrophobic interaction in associative polymers[32-34]. Associative polymers are natural or synthetic polymers with hydrophobic groups attached at two ends of the polymer molecule or grafted to the polymer backbone as shown in Figure 1.3. The interactions between the hydrophobic groups result in an increase in viscosity of the solutions, thereby rendering them useful as thickeners in many industrial applications[35]. Although the hydrophobic interactions are important from a rheological standpoint, it is also often necessary to control these interactions for ease of handling prior to end use. Cyclodextrins encapsulate the hydrophobic groups in their hydrophobic annulus and reduce the viscosity of the solution by several orders of magnitude[36]. Although the reduction in viscosity improves the processability of formulations containing associative polymers, it is necessary to get back the original rheological properties for their intended use. One of the novel ways to recover the original rheology of the solution is to enzymatically degrade the CD.

To illustrate this hypothesis we will use hydrophobically modified alkali soluble emulsion (HASE) polymer as a model associative thickener and focus on developing a methodology to control the rheology of HASE polymer solutions using CDs and enzymes. The HASE polymer is a copolymer of methacrylic acid, ethylacrylate and a macromonomer containing hydrophobes as shown in Figure 1.4[37]. The hydrophobic macromonomer is composed of C_{22} alkyl hydrophobic groups. These groups are separated from the polymer backbone by a polyethylene oxide (PEO) spacer of 40 moles of ethoxylation. The macromonomer is attached to the polymer backbone through a urethane linkage.

Many α-amylase enzymes derived from different microbial sources have been proven to degrade CD[38]. We will use a commercially available α-amylase enzyme derived from *Aspergillus oryzae* for this study. The rheological consequences of complexation of CD with hydrophobes and the subsequent degradation of the CD depend on various factors such as polymer concentration, type of CD, CD concentration, pH, temperature, and enzyme concentration. Understanding the overall effects of these factors is important in developing efficient enzymatic processes. Hence, this particular research thrust has the following goals.

1.3.1. Examine the thermodynamics of CD-hydrophobe complexation in associative-polymers

The complexation of CD with polymer hydrophobe is an equilibrium process, where CDs bound to the hydrophobes are in equilibrium with free CDs in the polymer solution. Such equilibrium can be represented in the form of Langmuir type adsorption isotherm[34]. It is essential to estimate the binding constant of the adsorption isotherm to quantify the strength of binding and to understand the thermodynamics of the complex process[39]. The complexation of CD with small organic molecules had been the subject of systematic thermodynamic studies to estimate the binding constant, enthalpy, entropy and free energy change of complexation. These studies employed a wide variety of experimental techniques including spectrophotometry, ultra filtration, and chromatography. These methods are adequate when the concentrations of host compounds do not affect the adsorption isotherm. However, these methods cannot be used for high viscosity polymer solutions, and dilution of

the polymer solution affect the complexation. Taking into account the drawbacks of the conventional methods, we develop a new rheometric approach that uses the changes in viscoelastic properties of associative polymer solution due to CD-hydrophobe complexation. The HASE polymer forms a transient network due to intermolecular hydrophobic interaction. The encapsulation of hydrophobes by CD disrupts networks and reduces network junction density and the dynamic moduli of the polymer solution. The model assumes that the reduction in dynamic moduli is directly proportional to the CD adsorbed on to the hydrophobes. Assuming 1:1 equilibrium between the CD adsorbed on the hydrophobes and the free CD in the solution, binding constants are calculated. The variation in binding constant with temperature is used to estimate the enthalpy, entropy and free energy change of the complexation. Experiments are carried out using α and β CDs and their relative strengths of binding on hydrophobes are estimated.

1.3.2. Investigate enzymatic manipulation of hydrophobic interactions in associative polymers

Although the kinetics of enzymatic degradation of CDs has been extensively studied with different types of enzymes[40], no kinetic studies have been reported so far on the degradation of CD in the presence of polymer molecules that have affinity to form inclusion compounds with the CD. Previous kinetic studies on degradation of CD used chromatographic and spectrophotometric techniques to analyze the degradation products. These methods require dilution of the reaction mixture and test conditions (i.e., temperature, pH, ionic strengths) quite different from that of the reaction mixture. Further, these methods fail while analyzing the reaction mixture containing associative polymers, as the dilution and the test conditions affect the polymer solubility and the complexation of CD with the hydrophobes. In addition, reaction mixtures containing hydrophobic polymers pose greater difficulty in chromatographic analysis as the hydrophobic groups affect the performance of chromatographic columns. Taking into account these drawbacks of the conventional methods, we develop an alternate method based on rheometry to study the kinetics of enzymatic reactions. To the best of our knowledge, no rheology-based kinetic model has

been reported so far on enzymatic degradation of CD in the presence of polymer that can form inclusion complex with CD. The kinetic model takes into account CD-hydrophobe complexation, and allows for estimation of binding constants for the CD-hydrophobe complexation.

1.4. Thesis overview

We provide here for convenience, an overview of the work presented in this thesis. However, each chapter is presented in a stand-alone format.

In Chapter 2, we provide a detailed literature review of guar galactomannans, associative polymers, cyclodextrins and the enzymes that are used in this research. A brief literature survey on rheokinetics of polymerization/depolymerization reactions in general or enzymatic reactions in specific is also presented in this chapter. Chapter 3 summarizes the prominent experimental techniques used in this research. In Chapter 4, we describe the mathematical models developed to study the kinetics of enzymatic depolymerization of guar galactomannan. Rheological investigation of degradation of guar galactomannan by multiple glycosidase enzymes is presented in Chapter 5. Chapters 6 and 7 describe the research on modifying the rheology of hydrophobically modified associative polymers using CD and enzymes. A rheometric study of the thermodynamics of CD-hydrophobe complexations is presented in Chapter 6. Chapter 7 presents the rheological investigation of enzymatic degradation of CD and subsequent recovery of rheological properties. In Chapter 8, we summarize the outcome of this research and provide recommendations for future research in this area.

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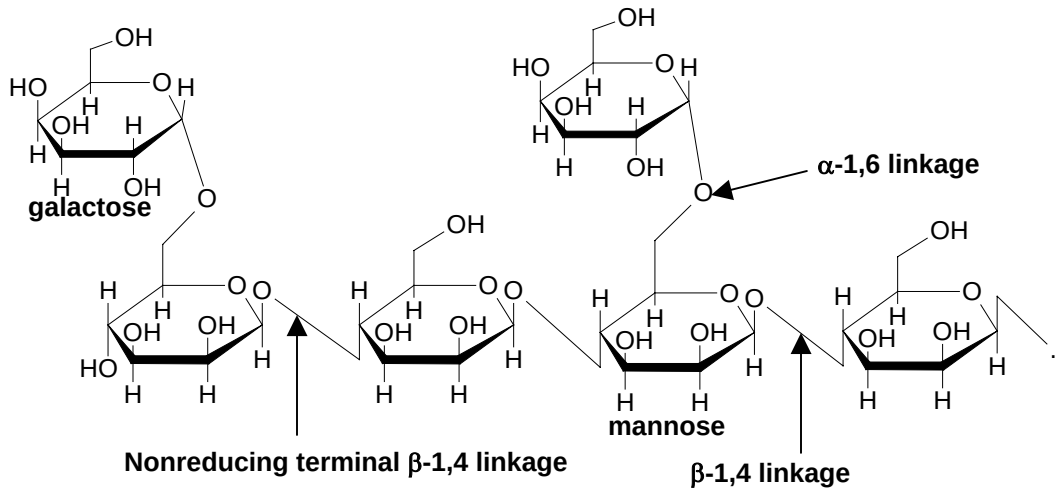


Figure 1.1: Structure of guar galactomannan showing sites for action of three different enzymes. β-mannanase cleaves all β-1,4 linkages; β-mannosidase cleaves only terminal β-1,4 linkages; α-galactosidase cleaves α-1,6 linkage.

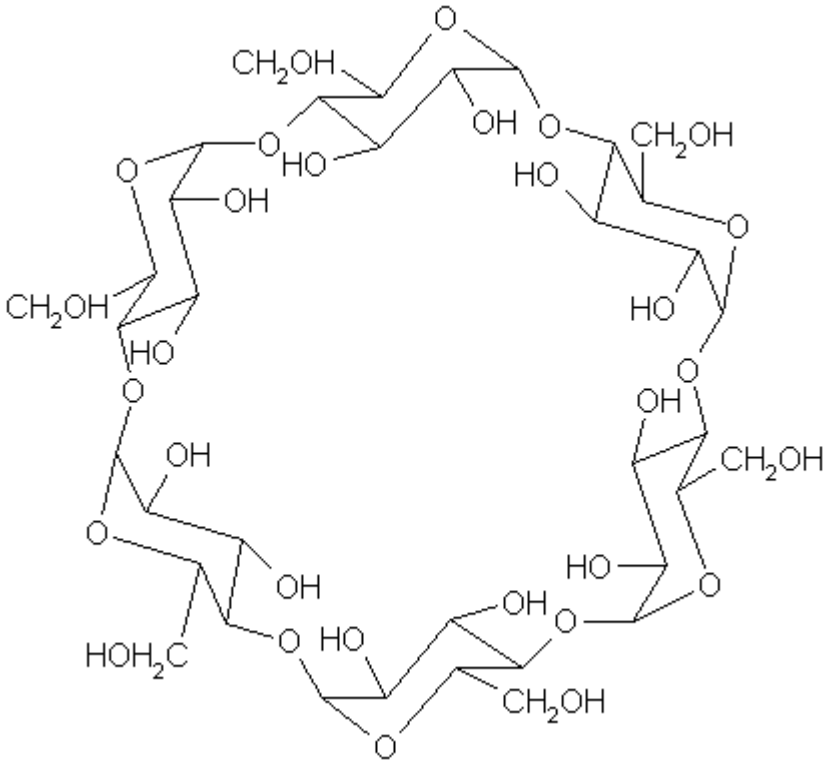
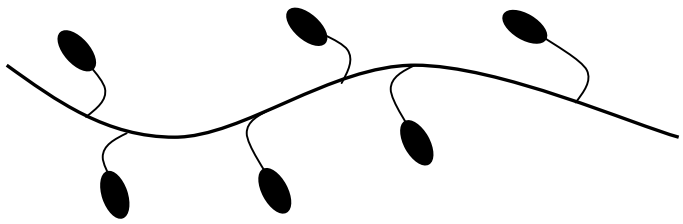
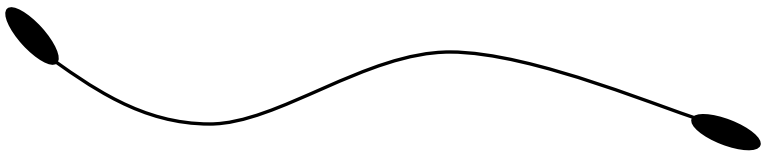


Figure 1.2: Structure of α-cyclodextrin showing six glucose units arranged in the form a ring



(a) Comblike associative polymer



(b) Telechelic associative polymer

Figure 1.3: Schematic of two types of associative polymers. (a) Comblike associative polymers with hydrophobes attached randomly to the polymer backbone. (b) Telechelic associative polymers with hydrophobic groups attached to the two ends of the polymer backbone.

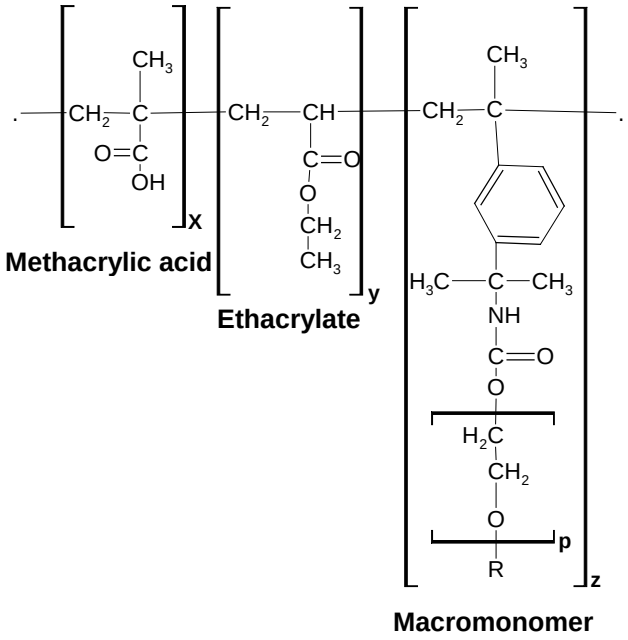


Figure 1.4: Structure of HASE polymer: composition of monomers x/y/z = 43.57%/56.21%/ 0.22% by mole; number of moles of ethoxylation, p=40, hydrophobic group (R) in the macromonomer is $C_{22}H_{45}$.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

Abstract

In this chapter, we focus on the polymers of interest, namely guar gum, associative polymers and cyclodextrins, and briefly explain their structure and physiochemical properties. The readers are introduced to different types of enzymes used in this study. We briefly explain the rheokinetics of polymerization or depolymerization reactions and their applications in studying the enzymatic reactions. We then provide a brief overview of the literature to date that deals with enzymatic modification of guar gum and the modification of rheology of associative polymer solutions.

2.1. Guar galactomannan

Galactomannans are heteropolysaccharides containing residues of galactose and mannose monosaccharide units. The basic structure of all galactomannan contains a linear backbone of β-1,4 linked mannopyranosyl units to which α-1,6 linked galactopyranosyl units are attached as shown in Figure 2.1[1].

Although all naturally occurring galactomannans have the same basic structure, they differ in their mannose to galactose (M:G) ratio depending on the source[2]. Three types of galactomannans are harvested from the seeds of leguminous plants on an industrial scale: guar gum, locust bean gum and tara gum. Guar gum is harvested from the endosperm of C*yamopsis tetragonoloba* and has an M:G ratio of 2.0, tara gum from *Cesalpinia spinosa* has an M:G ratio of 3 and locust bean gum (LBG) from *Ceratonia siliqua* has an M:G ratio of 4. The M:G ratio is a key factor in determining the rheological properties[3]. Without any galactose branches, mannan linear backbone will aggregate due to intermolecular hydrogen bonding of cis-hydroxyls on mannose that will subsequently lead to gelation or precipitation in water[4]. As galactose branches are added, they sterically prevent intermolecular hydrogen bonding and aggregation. Hence guar gum has the highest solubility in water, whereas LBG and tara-gum do not dissolve in cold water; heating causes thermal breakdown of intermolecular hydrogen bonding allowing dissolution[5].

Guar is being used in a wide variety of commercial and industrial applications such as hydraulic fracturing, pharmaceuticals, textiles, cosmetics, detergents, health-care and personal care products[6]. In addition guar is safe for human consumption and is mixed with other biopolymers to thicken or bind food products. Since guar has the lowest M:G ratio among the commercially available natural galactomannans, galactomannans with higher M:G ratio could be easily obtained by debranching guar. The debranched guar mimic the properties of other natural galactomannans depending on the extent of debranching[7].

2.1.1. Modification of guar

The native guar is modified by both chemical and biochemical methods to extend its range of applications. Three major categories of the modified guar are found in literature: (i) derivatized guar (ii) guar-hydrogels and (iii) enzyme modified guar.

Derivatized guar: Guar gum is derivatized by one or more of the reactions such as oxidation, hydroxyalkylation, carboxymethylation, cyanoethylation, quarternization and sulfation[8]. Some examples of derivatized guar are hydroxypropyl guar, carboxymethyl guar and hydroxypropyl-carboxymethyl guar. Although no significant change in the rheological properties occur by derivatization[9], it substantially improves the solubility in cold water as well as other organic solvents such as methanol, ethanol and ethylene glycol.

Recently the hydrophobically modified derivatives have attracted the interest of many industries such as oil drilling[10], cosmetics[11], healthcare and personal care industries[12,13]. Part of this stems from the fact that the modified guars act like surfactants in aqueous media, and can be used as dispersant or emulsifiers[14]. In addition, these polymers behave like associative polymers[15-17]. Like associative polymers, the rheology of their aqueous solution can be modulated using surfactants[18,19] or cyclodextrins which form inclusion compounds with hydrophobic part of the molecule[20,21].

Guar hydrogels: Hydrogels are usually made of hydrophilic polymer molecules, which are cross-linked either by chemical bonds or other cohesion forces such as ionic interaction, hydrogen bonding or hydrophobic interaction. Hydrogels are elastic solids with a remembered reference configuration to which the system returns even after being deformed for a long time[22]. The interesting characteristics of some of the gels is that they can be made to respond to changes in the environmental conditions such as temperature, pH, ionic concentration, electric field, solvent, external stress or combination of these[23]. Because of these unique properties, hydrogels find numerous applications in pharmaceutical, agricultural, biomedical and consumer-oriented fields.

Both physical and cross-linked guar hydrogels are synthesized. In physical gelation, guar is treated with other biopolymers or synthetic polymers, their synergistic interaction leads to a gel[24,25]. Guar hydrogels are also synthesized by reacting it with multifunctional cross-linking agents[26,27]. Gelation of guar produces significant rheological changes by transforming individual guar molecules into a polymer network. The network structure of guar hydrogels can be used to encapsulate drugs for pharmaceutical applications. It can also impart greater thermal stability[28] to guar and provide a way to tailor the texture of food products such as yogurt.

Enzymatically modified guar: Three glycosyl hydrolase enzymes can degrade guar into individual sugars, galactose and mannose. The enzymes are β-mannosidase, β-mannanase and α-galactosidase. The β-mannosidase cleaves a single mannose unit from the non-reducing end of the guar molecule. β-mannanase cleaves the interior glycosidic bonds between adjacent mannose units in the backbone of the guar molecule and α-galactosidase cleaves α-1,6 bond between the mannose and galalctose units.

The hydrolysis by β-mannanase enzyme decreases molecular weight (MW) and viscosity of the guar solution[29]. The β-mannanase enzymes are also found to degrade guar derivatives[30] and hydrogels[31]. The action of α-galactosidase enzymes debranches guar and the debranched guar form intermolecular hydrogen bonds that lead to hyperentanglement and subsequent gelation of guar solution[32]. The α-galactosidase treated guar gum forms a physical hydrogel on complexation with helical polysaccharides like xanthan[4,33]. The enzyme-modified guar gum finds applications in a wide variety of area including food, textiles and pharmaceuticals. These modified guar are also used to synthesize chemical and physical gels that can be used as rheology modifiers[4].

Guar degrading enzymes: The guar degrading enzymes are found in a wide range of plants and animals and are synthesized by a variety of microorganisms. However, only a few highly purified preparation of this enzyme have been reported. This is due in part to the low levels of activity in source materials and in some cases, to instability of the enzymes. Hydrolysis by

these enzymes is affected by degree and pattern of substitution of the main chain by α-D-galactosyl residues. Hence the catalytic action of the enzymes depend on the source of enzymes and also the source from which guar is harvested[34,35]. The enzymes, purified from hyperthermophilic sources have the same catalytic activity, but improved thermal stability and higher temperature optimums compared to their mesophilic cousins[36]. Hyperthermophilic enzymes offer a unique opportunity to develop industrial applications at high temperatures. Enzymes are also used to cross-link the guar chains[37].

2.1.2. Rheological properties of guar galactomannan solutions

The naturally-occurring guar galactomannans are high MW (MW~$2x10^6$), polydisperse (PDI >2) polysaccharides[38]. The rate of dissolution and hydration of the guar depends on the average molecular weight, concentration and the temperature of dissolution[39]. It was also observed that the dissolution temperature affects the viscosity of guar solution. A plot of viscosity vs. dissolution temperature shows a maximum at around 60^0C[40]. This behavior was related to differences in molecular structure of the polymers dissolved at different temperatures.

Aqueous solutions of guar loose viscosity over time. The inherent microorganisms present in guar powder degrade guar to low MW polysaccharide products in a relatively short period of time at room temperature[41]. Guar solutions also undergo degradation under acidic or basic conditions[42].

The intrinsic viscosity of guar gum has a Mark-Houwink-Sakurada (MHS) relationship of $[\eta]=3.04x10^{-4}\ M_w^{0.75}$ dl/g[43] and a characteristic ratio of 11.87 as determined by gel permeation chromatography (GPC) and dilute solution viscometry. The degraded guar gum shows lower Huggins coefficient (approximately 0.4) compared to that of the native guar (approximately 0.79)[42,44,45]. This suggests that intermolecular association via hydrogen bonding is weakened on hydrolysis of the guar.

The steady shear rheology of guar gum solutions show a shear thinning behavior over wide ranges of temperatures and concentration[43]. The zero-shear viscosity of the guar increases with concentration and decreases with temperature. The concentration dependency of steady shear viscosity is found to follow a power law with a power-law exponent of 3.5[46]. Figure 2.2 shows the master curve at 25^0C obtained after time-temperature superposition of flow curves of 1% guar solution measured at different temperatures. The high viscosity of guar solution is attributed to their high molecular weight and their ability to form intermolecular hydrogen bonds. The intermolecular interactions depend on the conformation of guar molecules that are affected by solvent conditions like temperature and pH or the presence of salts[47], surfactants or sugars[48] in the solution.

Figure 2.3 represents a dynamic frequency spectrum of guar solutions[49]. Aqueous solutions of the guar galactomannan show a typical entanglement network response under dynamic oscillatory experiments[49,50]. Two storage modulus plateau zones, very broad relaxation spectra, and strong dependencies on MW, polymer concentration and temperature are observed[32,49,51]. The dynamic modulus increases proportionally as c^3 (c=guar concentration)[46]. The viscoelastic response, at high frequencies, is attributed to conformational relaxations of multi-chain structures and, at low frequencies, to interchain bonding effects[32].

2.2. Cyclodextrins

Naturally occurring cyclodextrins (CD) are cyclic oligosaccharides that are composed of α-1,4 linked D-glucopyranose units, as shown in Figure 2.4[52]. They are produced through the degradation of starch by the enzyme CD glucosyltransferase. The CDs composed of 6, 7, 8 glucopyranose, usually referred to as α-CD, β-CD and γ-CD respectively, are the most abundant in nature and studied extensively. The CDs with higher ring size are synthesized from starch using α-glucanotransferase enzymes[53,54]. The CDs have doughnut like annular structure with hydrophilic outer shell and hydrophobic annular interior. The solubility of CDs vary with their size in an irregular manner and the annular diameters and volumes increase substantially from α-CD to γ-CD [55].

Much of the interest in CDs arises from their ability to include or encapsulate a guest molecule inside their annuli to form complexes usually described as inclusion compounds. These complexes are unusual in that only temporary physical bonding occurs between the CD and the guest. The driving forces leading to the formation of inclusion compounds include electrostatic interaction, van der Waals interaction, hydrophobic interaction, hydrogen bonding, release of conformational strain, exclusion of cavity-bound high-energy water, and charge-transfer interaction[56]. The majority of the CD complexes reported involve a guest with a hydrophobic moiety, which resides inside the hydrophobic annulus of CD[57]. The CDs with suitable hydrophobic or hydrophilic molecules attached to the CD ring are known as modified CDs. The modification of CDs changes the solubility and the complexation behavior from their unmodified counterparts[58].

The complex forming characteristics of CDs have attracted attention in a wide range of applications including food, textiles, cosmetics and pharmaceutical industries[59]. They are being used to produce stereo-specific polymers[60]. The CD inclusion compounds have been used extensively in separation science because they discriminate between positional isomers, functional groups, homologs and enantiomers[61]. Now CDs are also being used to control the rheology and improve the processability of polymeric systems. Their ability to control the rheology stems from the fact that they remove the hydrophobic interaction between the polymer chains[62]. The encapsulation of hydrophobic groups by CD helps in studying the difference between the structure and solution properties of hydrophobically modified associative polymers from that of their unmodified counterparts.

2.2.1. Cyclodextrin degrading enzymes

The enzymes that degrade α-1,4 linkages between glucose units of starch molecules also degrade α-1,4 linkages in CDs. However their activity varies depending on the types of CDs. The variety of enzymes catalyzing the above hydrolysis reactions are α-amylases, β-amylases, amyloglucosidases, α-glucosidases, pullulanases, cyclodextrinases and cyclodextrin glycosyltransferases[63-65]. The hyperthemophilic versions of these enzymes have

same catalytic activity as their mesophilic cousins, but are found to function at extreme conditions (temperature, pH, salt concentrations) prevailing in many industrial applications[66].

2.3. Associative polymers

Associative polymers have recently drawn considerable interest due to their original and specific rheological properties that distinguish them from other polymers in terms of their viscosity enhancement[67-69]. These hydrophobically modified polymers either have a telechelic structure in which the chains are end capped with the hydrophobic groups or more complicated comb-like structure in which the hydrophobic groups are randomly grafted to the polymer backbone Hydrophobically modified Ethoxylated Eurethane (HEUR) polymers are one class of telechelic polymers[70], and Hydrophobically modified Alkali Soluble Emulsion (HASE) polymer is an example for the associative polymers with a comb like structure.

HASE polymers are one class of water-soluble associative polymers having a comb like structure with pendant hydrophobic groups randomly attached to polyelectrolyte backbone[71]. Figure 2.5 represents the chemical structure of a typical HASE polymer considered in our study. The polymer backbone is a polyelectrolyte composed of a copolymer of methacrylic acid (MAA) and ethylacrylate (EA). The polymer is hydrophobically modified by randomly grafting a number of hydrophobic macromonomer to the polymer backbone. The hydrophobic macromonomer is composed of C_8-C_{30} alkyl or aryl hydrophobic group. These groups are separated from the polymer backbone by a PEO spacer, 5-40 moles of ethoxylation. The macromonomer is usually attached to the polymer backbone via a urethane linkage.

HASE polymers have several advantages over other associative polymers in terms of cost and wide formulation latitude[72]. They are currently being used as rheology modifiers in a wide range of applications, including paint formulations, paper coatings, personal and home care products, UV-photo protecting, aerated emulsions, fabric softeners and glycol based aircraft anti-icing fluids[73-76]. HASE polymers enhance the solution properties via

several thickening mechanisms that include intermolecular interaction between ethacrylate groups[77] or hydrophobic groups attached to the backbone[78]. Neutralization of the backbone methacrylic acid elongates the backbone due to electrostatic repulsion between COO^- groups, resulting in entanglement and viscosity enhancement[79]. However, the main contribution comes from the intermolecular hydrophobic interaction between the hydrophobic-groups[78].

Although the associative nature of HASE polymers makes them attractive from a rheological standpoint, the ability to control these interactions is often necessary. It is desirable to deactivate hydrophobic groups for ease of handling during solution preparation and reactivate them prior to end use. Further, it is often necessary to remove the hydrophobic interactions for characterization of the polymers using techniques such as light scattering and gel permeation chromatography[80].

Recently it has been found that the viscosity of HASE solution can be decreased dramatically by adding cyclodextrins[62]. Cyclodextrins encapsulates the hydrophobic groups of the polymer forming an inclusion compound, thus deactivating the hydrophobic interaction. By adding a suitable surfactant that has higher affinity complex with CDs than the hydrophobes, the solution rheology can be recovered[81]. Another novel way to recover back the viscoelasticity of the solution is to enzymatically degrade the cyclodextrins and reactivate the hydrophobic interaction[82].

2.3.1. Rheological properties of hydrophobically modified associative polymers

Figure 2.6 represents a typical dynamic frequency spectrum of HASE solutions. The dynamic frequency spectrum resemble that of an entangled polymer solution with the cross over of G'(elastic nodulu) and G"(viscous modulus) curves occurring at a low frequency. In aqueous alkaline media, the hydrophobic groups on the HASE polymer dynamically associate forming a transient network. The topology of the network so formed depends on the polymer concentration, molecular architecture, temperature, pH and the presence of other molecules like surfactants, ions etc. The dynamic rheological properties of the polymer solutions depend on the topology of the network. In contrast to the simple linear telechelic

associative polymers, a Maxwell model with single characteristic time cannot represent the network dynamics of the HASE polymer. This deviation from the classical Maxwellian response in oscillatory shear arises from the coexistence of both hydrophobic associations and topological entanglements[83]. Also the complexity of the hydrophobic association varies depending on number of hydrophobes in the network junctions.

Subtle variations in molecular architecture of HASE polymer can cause significant changes in the microstructure and rheological properties of the polymer systems[84]. At a particular concentration of hydrophobes on the backbone, the strength of the polymer network or dynamic moduli decreases with increasing ratio of MA/EAA, and the extent of decrease increases with increasing macromonomer concentration. This behavior suggests that electrostatic interactions imparted by methacrylic acid are more dominant when compared to hydrophobic interactions as the macromonomer composition is increased[85]. The steady shear viscosity and storage modulus also increase with the degree of ethoxylation[86]

The rheological behavior of the HASE polymer is strongly affected by type and composition of solvents. Figure 2.7 shows the steady shear behavior of a 3% HASE solution in a mixture of water and propylene glycol (PG) with different PG proportions. In general, the steady shear profile shows a weak shear thinning behavior at low shear rates followed by stronger shear thinning region at higher shear rates. There is also a decrease in the relative viscosity (defined as the steady shear viscosity divided by the solvent viscosity) as the PG content increases. In addition, the shear thinning behavior seems to be more pronounced in "water-rich" solvents. The reduction in the relative viscosity with PG content reflects changes in the polymer microstructure, possibly a weakening of the hydrophobic associations, as the solvent quality changes[87].

The formation of transient network via hydrophobic interactions is greatly affected by type and concentration of surfactants. Addition of surfactants to these polymer systems leads to unusually large rheological changes over relatively small ranges of surfactant concentrations[68]. The selection of a surfactant is dictated by the solubility of the surfactant in the system which is, in turn, controlled by temperature, pH, nature of the surfactant

hydrophobe, and the presence of co-solvents[88]. For example, the interactions between nonionic surfactants and HASE polymers result in either increase or decrease in viscosity and dynamic moduli of HASE solutions depending on the surfactant concentration, hydrophilic-lypophilic balance (HLB) of the surfactant[89].

The HASE polymer is supplied in the form of latex dispersion at a pH of 4 and has a water-like viscosity. The addition of NaOH causes ionization of the methacrylic acid groups leading to dissolution of latex particles[79]. The electrostatic repulsion between the COO^- ions on the backbone results in the expansion of polymer chain and facilitates the intermolecular hydrophobic interaction leading to increase in the viscosity of solution[90-93]. It has been observed that the viscosity of the solution increases with increasing pH till the neutral pH. Further increase in pH does not significantly change the solution viscosity. At this point the electrostatic repulsion is balanced by the intermolecular hydrophobic interactions. However on addition of salts, the salt ions shield the electrostatic interaction between the COO^- ions on the backbone, resulting in coiling of polymer chains due to intramolecular hydrophobic interactions. This changes the viscoelasticity of the polymer solutions from predominantly elastic to viscous behavior. Also at a certain level of added salt, shear-thickening behavior is observed at high shear stresses. The shear thickening is caused by an increase in the network junction densities due to the conversion of intramolecular to inter-molecular associations[93,94].

2.4. Rheokinetics

When studying the kinetics of ordinary chemical reactions involving low molecular weight reactants and products, the viscosity of the reaction mixture is not an important parameter. Therefore, the viscosity of the reaction mixture does not depend on the degree of conversion, but only on the reaction conditions, such as temperature of the mixture. However major changes in viscosity occur during polymerization or depolymerizations because of the formation or disappearance of high molecular weight molecules. The changes in viscosity of the reaction mixture will certainly have an effect on kinetics of reactions involving polymer molecules and vise a versa. Therefore, understanding the changes in rheological properties during polymerization or depolymerization process is very important in view of optimizing

the reaction conditions for maximum conversion[95]. This coupling between the changes in rheological properties and the kinetics of polymerization process is a relatively new field of research and has been called rheokinetics[96].

Kinetics of reactions are generally studied by measuring the concentration of reactants and the heat released using spectrophotometric, calorimetric or chromatographic techniques. Rheokinetics is an alternative technique that uses changes in viscoelastic properties to estimate the kinetic parameters, and is very useful when the conventional methods fail for the reaction mixtures containing polymers.

The starting point for deriving a rheokinetic model is the power law function that relates viscosity (η) to average molecular weight (M_w) and polymer concentration (C) as given by the following equation.

$$\eta = K M_w^a C^b \qquad (2.1)$$

Here, K, a, and b are constants. Both M_w and C are functions of time or conversion and the function depends on the type of polymerization[97-99].

The conventional techniques for kinetic studies do not allow investigation of the influence of flow conditions on the kinetics of polymerization. Rheokinetics is based on the measurement of the rheological properties that involve some kind of flow. Therefore, by simply varying flow conditions in the experiments, it is possible to measure the effect of flow on the kinetics of polymerization reactions.

However, measuring the viscosity of a polymerizing sample is not an easy task, as the reactions are very fast and the viscosity builds up by several orders of magnitude within a few minutes. Fortunately, commercially available present rheometers allow in *situ* tracking of changes in rheological properties during polymerization reactions.

In our proposed research, the modification of rheology involves the hydrolysis of glycosidic bonds in the polysaccharides. Since there are changes in the rheological properties

during the enzyme reaction, it is possible to study the kinetics of enzymatic reactions by monitoring the changes in rheological properties. In the case of degradation of guar, there is a change in molecular weight that can be estimated from the kinetics of degradation. Since the viscosity is correlated to molecular weight, the kinetic parameters can be estimated from the changes in viscosity during the enzymatic process. In case of an associative polymer, the enzyme breaks cyclodextrin-hydrophobe complexes and reactivates the hydrophobic interaction between the associative polymers. These interactions create network junction between the polymers and increase the viscosity and other viscoelastic properties. The transient network theory predicts that the storage modulus at high frequencies is directly proportional to molar concentration of network junctions. Addition of CDs reduces number of network junctions and subsequent degradation of CDs by enzymes restores the network junctions. Hence, by observing the changes in the dynamic frequency spectrum of the associative polymers during the degradation of CD, we can estimate the kinetic parameters.

2.5. Previous research

2.5.1. Enzymatic modification of guar galactomannan

Previous research involving enzymatic modification of guar solutions and hydrogels has been focused on their applications in food, petroleum and pharmaceutical industry and limited to rheological and microstructural characterizations. These studies involved structural modification using individual enzymes either β-mannanase or α-galactosidase.

In food industry, guar is used as thickening and water binding agent, and usually appear along with other polysaccharides and/or proteins[100-103]. Hence considerable efforts have been made in understanding the rheological consequences of interactions between guar and other food ingredients[48,104,105]. In this direction, enzymatic modification allowed greater flexibility in tailoring the guar to meet the requirements in desired end products[106]. For example, debranched guar is used as a low cost substitute for locust bean gum to create synergistic gels with xanthan[107,108]. The interaction between guar and whey proteins has been found to enhance the performance of heat induced whey protein gels[109,110]. Enzymatic

modification of guar offers a unique method to tailor the rheological properties of heat induced guar-whey protein hydrogels while optimizing their composition for low carbohydrate high protein food products[111]. Low molecular weight galactomannans produced by hydrolysis of guar by β-mannanase enzyme have been used as a supplement for dietary fibers[112].

In the petroleum industry, guar and its hydrogels are being used as hydraulic fracturing fluids[113,114]. During hydraulic fracturing process, guar needs to be degraded to lower the viscosity and facilitate the flow of gas and oil from wells. Traditionally, oxidative breakers such as potassium or ammonium persulfate have been used for the degradation[115]. The oxidative breakers are low molecular weight compounds that can leak off into the surrounding rocks before the necessary degradation has occurred. Enzymatic degradation offers a powerful, environmentally benign alternative to the degradation using oxidative breakers.

Tayal *et al*[29] and Cheng *et al*[30,42] studied the degradation of guar using β-mannanase enzyme. The degradation was quantified in terms of changes in zero shear viscosity and molecular weight distribution. The kinetics of degradation was found to follow zero order kinetics and the polydispersity index increased during the hydrolysis. It was believed that the nonrandom enzyme attack increased the PDI during the hydrolysis reactions. And the nonrandomness can arise either due to the presence of galactose side chains or local confinement of enzymes in a high viscosity guar matrix. But no theoretical models were developed that can explain the increase in PDI during the hydrolysis. The above studies used mesophilic enzymes that are limited to low temperature applications, because the mesophilic enzymes are denatured at high temperatures. The thermophilic versions of the above enzymes have optimum activity at elevated temperatures and can be used in high temperature fracturing of deep oil wells.

Burke *et al*[116] developed a method to control the degradation of guar by β-mannanase enzyme using tris(hydroxymethyl)aminomethane, a reversible, pH-dependent inhibitor. In the presence of inhibitor and pH 9.0, the enzyme did not degrade guar and no reduction in

solution viscosity was observed. However, when the pH was reduced to 4.0, significant reduction in viscosity was observed. This reversible, pH-dependent inhibition is possibly a result of the protonation of the inhibitor that avoids the interaction of the inhibitor with active sites on the enzymes.

In hydraulic fracturing, guar is often cross-linked using multifunctional chemicals such as borax and titanium to create hydrogels[117]. Tayal *et al* [31] studied the degradation of guar-borax hydrogels using enzymes. The study reveals that during enzymatic degradation, the complex viscosity of the hydrogel decreases slowly at the beginning followed by a higher rate of decrease at latter times. The viscosity reduction pattern is exactly opposite to that of guar solution, where viscosity reduces at a faster rate at the beginning and the rate reduces at longer times. The slower rate of degradation during the initial stages of the degradation of guar hydrogels is due to limited mobility of the enzyme in the hydrogel. As the hydrogels are degraded, the enzymes can freely move and attack the degradable bonds at a higher rate.

Guar hydrogels are widely used as pharmaceutical excepients in oral drug delivery formulations. Such orally administered drugs have to pass through several formidable barriers in gastrointestinal tract before delivering the drug to target sites for the treatment of local diseases. For example, to achieve successful colonic delivery, a drug needs to be protected from absorption and dissolution in the upper GI tract, and then be abruptly released into the proximal colon, which is considered the optimum site for colon-targeted delivery[118]. Although the hydrogels can undergo swelling in the environment of stomach and small intestine, the cross-linking in the hydrogel avoids the premature release of drugs in the GI tract. Hence, they are widely used in colon targeted drug delivery systems. The drug release profile can be controlled by varying the extent of cross-linking, type of cross-linker and the polymer concentrations in the hydrogel. Wide varieties of guar hydrogels have been synthesized and characterized for their swelling and drug release properties under various release environments[26,27,119-123]. Burke *et al*[124,125] investigated diffusional properties of guar solutions and hydrogels and, showed that the drug mobiliy inside the guar hydrogels can be controlled via enzymatic restructuring of the guar gum.

2.5.2. Modification of rheology of HASE polymer solutions

Since the HASE polymers are used as rheology modifiers, most of the previous research involving HASE polymer focused on rheological characterization of their solutions. Many insightful works have been reported that take into account effects of temperature, salt, surfactants and cosolvents on the rheological properties of HASE solutions. These studies are helpful in understanding the rheological behaviors of the polymer in their formulations for different applications. Abdala *et al*[87] studied the effect of solvent composition (water and polyethylene glycol) on the dynamic and steady rheological properties of HASE polymer solutions. In some of the formulations, both the HASE polymers and surfactants are used, where HASE polymer acts as thickener and the surfactants impart surface-active properties. Understanding the intermolecular interaction between the HASE polymer and surfactants are important to optimize their composition in the formulations. In this regard, extensive studies have been carried out on the effect of surfactants on the rheological properties of HASE polymers[88,126-129]. Since the HASE polymers are polyelectrolytic in nature, their rheological properties are susceptible to changes in pH and salt content in the solution[90,93,94,130-132]. Although aqueous solutions of HASE polymer show regular dependency of temperature on viscosity and other rheological properties, they show abnormal behavior with temperature in the presence of salts and/or surfactants[132,133].

Recently, considerable interest has been generated in modulating the rheology of hydrophobically modified associative polymer solutions using CDs[20,62,82,134,135]. The CDs encapsulate the hydrophobes within their hydrophobic cavity and disrupts the transient network formed through intermolecular hydrophobic interactions. Abdala *et al*[136] investigated the effect of α and β CD on solution rheology of HASE polymers. They showed that binding of CD on the polymer hydrophobes reduces viscosity and other viscoelastic properties of HASE polymer solutions by several order magnitude. On addition of surfactants, that has a higher binding affinity to form complex with CD, the hydrophobic interactions are restored and the rhelogical properties are recovered. However, complete recovery is not possible as the polymer surfactant interactions are inevitable[137].

2.6. Conclusions

Most of studies mentioned above on enzymatic degradation of guar used individual enzymes, either β-mannanase or α-galactosidase. No systematic studies have been carried out so far on degradation of guar using multiple enzymes in different combinations and proportions. In addition, the kinetics of enzymatic degradation of guar is not clearly understood and no mathematical models are available that can predict the changes in entire molecular weight distribution during the degradation process or the concomitant changes in viscosity.

The rheology of HASE polymer solution depends on various factors that include pH, temperature, and the presence of additive such as salts, surfactants and CDs. The hydrophobic interactions in HASE polymers can be modulated using surfactants and CDs. Although enzymes are found to break the inclusion compounds formed between CD and hydrophobes on the polymer, the mechanism and kinetics of CD-hydrophobe complexation and the subsequent recovery of rheological properties are not clearly understood. In addition, there is a lack of understanding of the thermodynamics of CD-hydrophobe complexation in associative polymers. These unresolved issues form the crux of this thesis.

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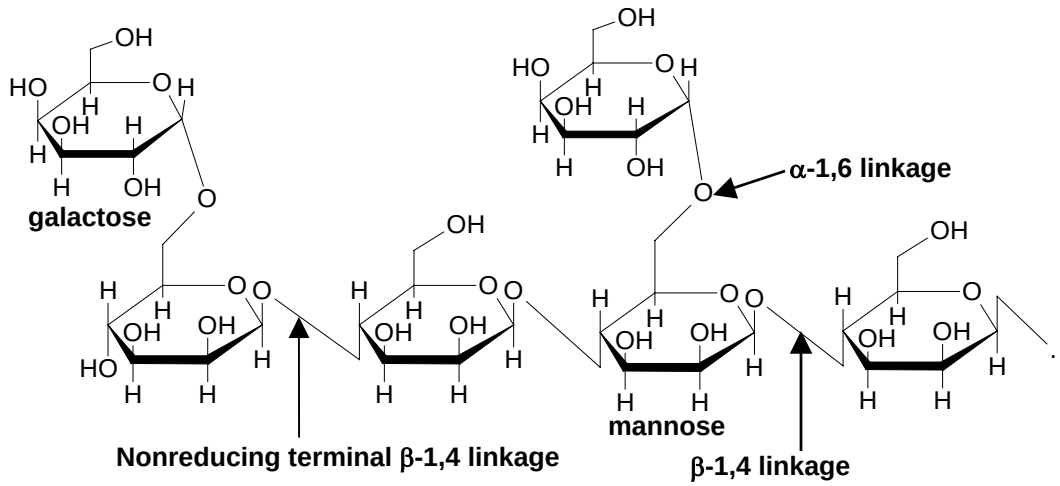

Figure 2.1: Structure of guar galctomannan showing sites for action of three different enzymes. β-mannanase cleaves all β-1,4 linkages; β-mannosidase cleaves only terminal β-1,4 linkages; α-galactosidase cleaves α-1,6 linkage.

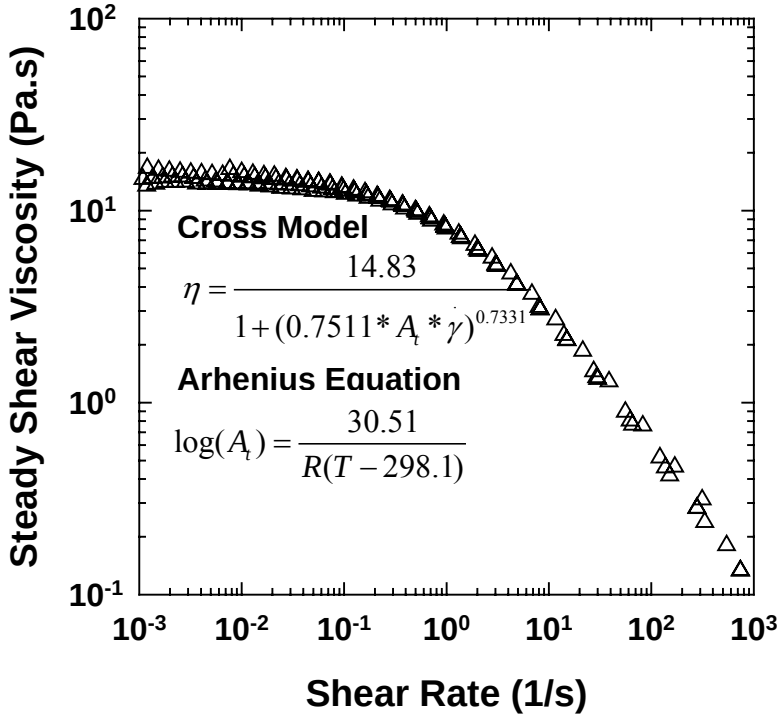

Figure 2.2: Steady shear behavior of 1% guar solution. The master curve is obtained by time-temperature superposition of the steady shear viscosity curve of 1% guar measured at 25^0C, 50^0C and 75^0C. The master curve can be fit to a Cross Model and the shift factors follow Arhenius equation as shown in the figure.

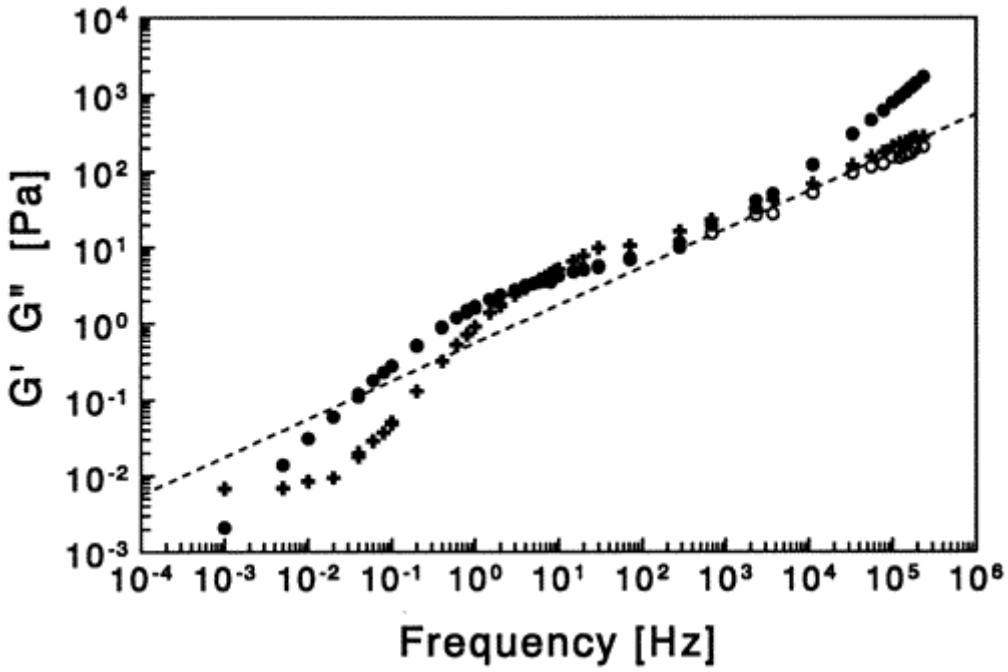

Figure 2.3: Dynamic frequency spectrum of 0.4%(w/w) guar solution measured at 20^0C. The graph is reproduced from the reference [49][49]. The symbols "+" and "●" represent elastic modulus (G') and viscous modulus (G") respectively.

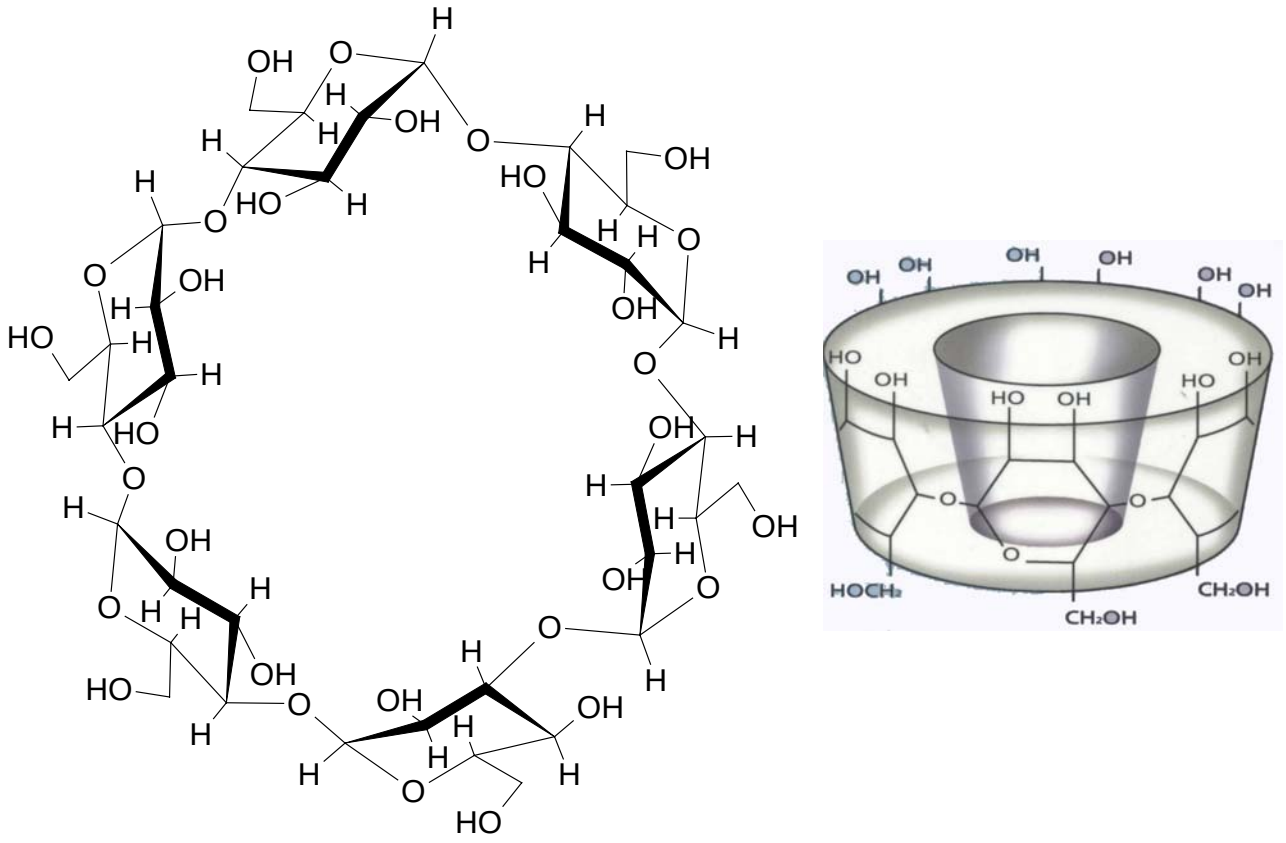

Figure 2.4: Structure of a cyclodextrin. The cyclodextrins are doughnut shaped cyclic oligosaccharides as shown in the figure on the right. The size of the cyclodextrins varies depending on number of glucose units in the ring.

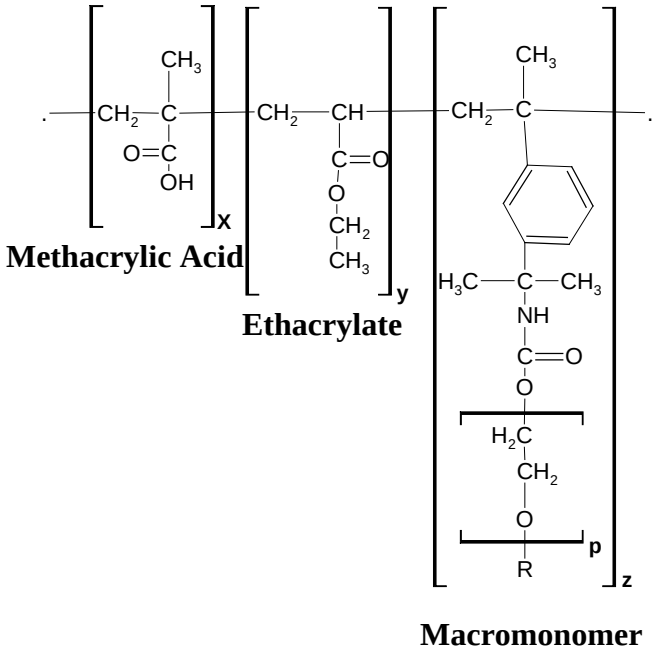

Figure 2.5: Structure of a HASE polymer: composition of monomers x/y/z = 43.57%/56.21%/0.22% by mole; number of moles of ethoxylation (p=40), hydrophobic group (R) in the macromonomer is $C_{22}H_{45}$

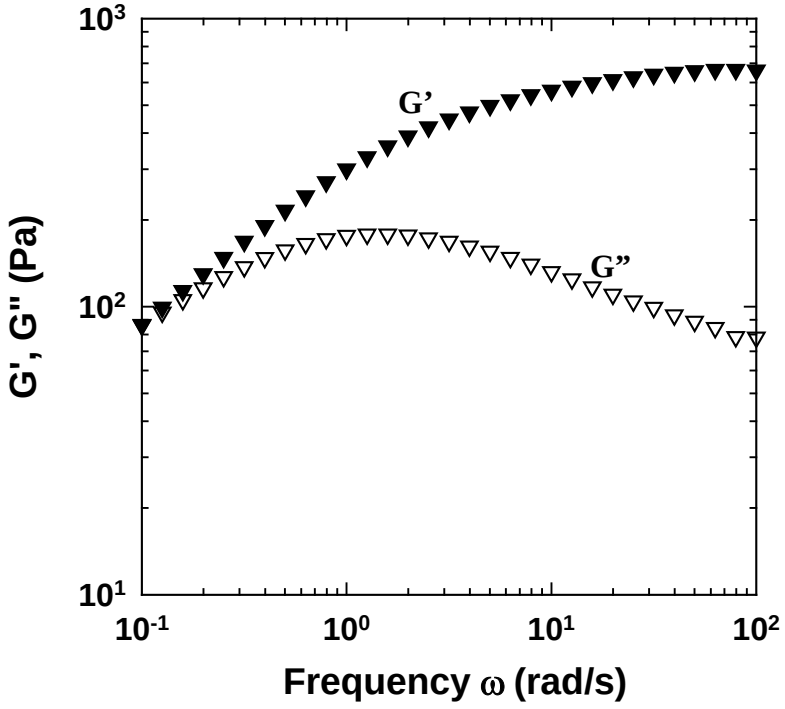

Figure 2.6: Dynamic frequency spectrum of 3% w/w hydrophobically modified associative polymer solution measured at 25^0C. Closed symbols represent G' and the open symbols represent G"

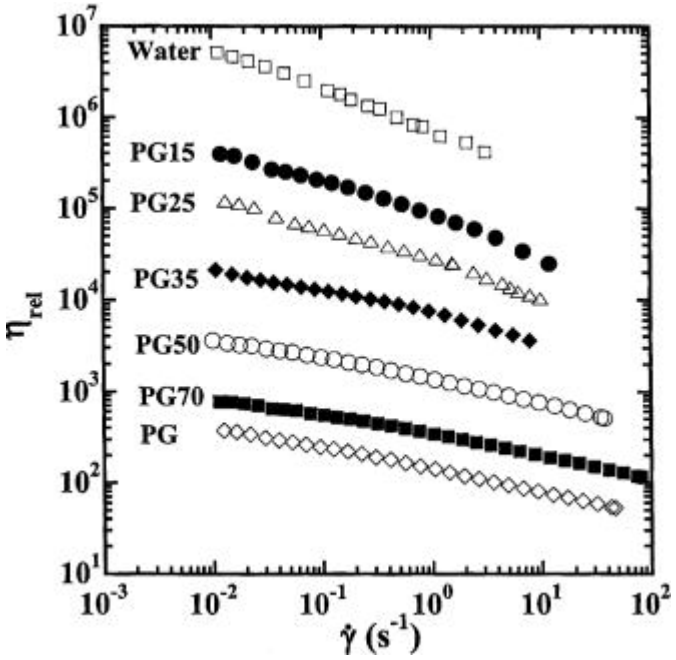

Figure 2.7: Steady shear viscosities of a 3% polymer solution in different proportions of the cosolvents. The graph is reproduced from the reference [87][87]. The numbers on the graph represent the weight percentage of propoylene glycol in the solvent.

CHAPTER 3

EXPERIMENTAL METHODS

Abstract

In this chapter we present a brief overview of the different experimental techniques and methods used in this research. Many experimental techniques can be used to quantify the enzymatic degradation of polymers, and the resulting physiochemical consequences. These include but are not limited to rheology, size exclusion chromatography, and confocal laser scanning microspy. Our research primarily focused on rheological techniques. However, data collected by previous investigators using gel permeation chromatography (GPC) are also used in our study while evaluating theoretical models developed during this research. The GPC technique is therefore discussed briefly in this chapter for the interested reader.

3.1. Rheological characterization

Rheology is defined as the science that deals with the deformation of material as a result of applied stress[1]. Two major rheological properties are of particular importance to polymer science: viscosity and viscoelasticity[2]. In the following paragraphs, we define these two properties and the rheological experiments used for their measurements.

3.1.1. Viscosity

Viscosity is a property of fluids that indicates resistance to flow. When a stress is applied over a volume of material, a deformation occurs. Newton's law states that applied stress is directly proportional to strain rate (or rate of deformation) and the proportionality constant is known as viscosity. During measurement of the viscosity, a sample is subjected to a constant shear stress by applying continuous rotation on a rotational instrument known as rheometer. The response of the sample is measured in terms of strain rate. The experiment is repeated at different shear stresses (τ), to get viscosity (η) as a function of strain rate ($\dot{\gamma}$) as given by the following equation. This type of rheological experiment is known as **steady shear experiment.**

$$\eta\left(\dot{\gamma}\right)=\frac{\tau}{\dot{\gamma}} \tag{3.1}$$

The effect of shear rate on viscosity can be represented by a power law equation given by,

$$\eta = k\left(\dot{\gamma}\right)^{n-1} \tag{3.2}$$

According to the behavior of η as a function of shear rate, the fluid can be classified as Newtonian (constant viscosity or n=1), pseudoplastic or shear thinning (viscosity decreases with shear rate or n<1) and dilatants or shear thickening (viscosity increases with shear rate or n>1). The pseudoplastic fluids with a yield point are called as plastic fluids. The

plastic fluids resist deformation below the yield stress[3,4]. The steady shear behavior of these types of fluids is shown in Figure 3.1.

Figure 3.2 represents a typical variation in viscosity of a polysaccharide hydrocolloid as a function shear rate. At very low shear rates, the hydrocolloid shows a Newtonian behavior with viscosity (η_0) independent of shear rate. The Newtonian viscosity is also known as zero shear viscosity. The zero shear viscosity is a function of molecular weight and concentration of the polymer in solution, usually represented by a power law,

$$\eta_0 = K M_w^a C^b \tag{3.3}$$

where, K, a, and b are constants, M_w and C represent the molecular weight and concentration of the polymer in solution respectively. At moderate shear rates, the viscosity decreases with shear rate following a power law of the form given by Equation (3.2). At higher shear rates, the viscosity again becomes independent of shear rate. At rest, polymer molecules are coiled and entangled to form an irregular shape that offers sizable resistance to flow, i.e., a high viscosity. At very low shear rates, these shapes are not disturbed and the resistance to flow (or viscosity) remains constant. With increasing strain rate the polymer molecules uncoil, disentangle, stretch and orient in the direction of applied force. The molecular alignment allows polymer molecules to slip past each other more easily and hence decreases viscosity. At very high shear rates, the viscosity will approach asymptotically a finite constant level, where the optimum of perfect orientation has reached[4]. Going to even higher shear rates cannot cause further shear thinning.

We have extensively used the steady shear rheological experiments to characterize enzymatic degradation of guar galactomannan. During enzymatic degradation, the zero shear viscosity of guar solutions decreases due to a decrease in MW of the guar molecules. Therefore, the extent of degradation can be measured in terms of changes in viscosity of the guar solution. The enzymatic reactions are carried out in situ in a rheometer, and the zero shear viscosity is measured at different intervals of time. During the reactions, the reaction mixture is held at a constant stress within the Newtonian region. This type of rheological test

is known as **peak hold test**[5] and provides an effective way to characterize and quantify the rate of degradation of a polysaccharide during enzymatic degradation. Figure 3.3 represents the changes in viscosity of 1% guar solution during degradation by β-mannanase enzyme at 75^0C and pH 7.0. The viscosity was measured at a stress of 1 Pa.

3.1.2. Viscoelasticity

Many of the polymeric solutions are viscoelastic in nature, i.e., they show both viscous (liquid like) and elastic (solid like) characteristics. With viscoelastic materials, only part of the deformation produced by applied stress returns to zero on removal of the applied force. The remaining part of the deformation will not return to zero when the force is removed. These two parts of the deformations are called as elastic and viscous deformations respectively. On the other hand, if the applied force varies sinusoidally with time, then the resulting strain will lag behind the shear stress by a phase angle δ. The phase angle δ is zero for an ideally elastic solid and 90^0 for an ideally viscous liquid. Correspondingly, viscoelastic materials exhibit a phase angle between 0 and 90^0. The elastic and viscous nature of polymeric materials can be measured using **dynamic rheological experiments** as explained below.

The dynamic rheological technique is a useful tool in probing microstructure of materials without disturbing the structure. In dynamic oscillatory experiments, both the applied stress and the resulting strain vary sinusoidally as given by the following equations.

$$\tau = \tau_0 \sin\left(\omega t + \delta\right) \tag{3.4}$$

$$\gamma = \gamma_o \sin\left(\omega t\right) \tag{3.5}$$

In the above equations τ_0 and γ_0 represent the amplitudes of stress and strain respectively; δ is the phase angle between the stress and strain waves, and ω is the frequency of oscillation. Using trigonometric identities, the stress wave can be decomposed into an in-phase and out-of-phase component.

$$\tau = \tau_o \cos(\delta)\sin(\omega t) + \tau_o \sin(\delta)\cos(\omega t) \quad (3.6)$$

The in-phase component corresponds to the ability of the material to elastically store energy and the out of phase component corresponds to its ability to dissipate energy. For viscoelastic materials at small strain amplitude, the resulting stress can be represented as a linear combination of in-phase and out-of-phase components as given by the following equation.

$$\tau = G'\gamma_0 \sin(\omega t) + G''\gamma_0 \cos(\omega t) \quad (3.7)$$

This behavior is known as linear viscoelasticity and the range of strain or stress amplitudes, where the linear viscoelasticity is applicable is known as linear viscoelastic (LVE) region. In the above equation, G' is the storage modulus, related to the stress in-phase with the strain and G" is the loss modulus, related to stress out-of-phase with the strain as given by the following equations.

$$G' = \frac{\tau_o}{\gamma_0}\cos(\delta) \quad (3.8)$$

$$G'' = \frac{\tau_o}{\gamma_0}\sin(\delta) \quad (3.9)$$

The loss tangent is the ratio of the viscous modulus to the elastic modulus.

$$\tan\delta = \frac{G''}{G'} \quad (3.10)$$

In the LVE region, the moduli G' and G" are only functions of frequency and give valuable information about the nature of the material. For example, a perfectly elastic material will have all the stress in phase with strain, i.e. G"=0. For a perfectly viscous liquid, all the stress will be out of phase with strain, i.e. G'=0. Figure 3.4 schematically represents the frequency dependence of G' and G" for typical viscoelastic materials. For viscous

liquids, G”>>G’ at all frequencies and the moduli increase with frequency. For elastic solids both G’ and G” are independent of frequency with G’>>G”. Many of polymeric solutions show liquid like property at low frequencies and solid like properties at high frequencies. The two curves of the moduli cross over at a particular value of angular frequency known as the cross over frequency. At the cross over frequency, G’=G” or tanδ =1. The cross over frequency depends on various factors such as concentration and molecular weight of the polymer and, intermolecular interaction between the polymer molecules. For highly entangled polymer solution, the cross over appears at low frequency as shown in the Figure 3.4

Figure 3.5 represents the dynamic frequency spectra of hydrophobically modified associative polymer solutions. The dynamic spectrum shows predominantly elastic behavior where G’ and G” curves cross over at low frequencies. The crossover frequency decreases with increasing concentration of the polymer. Similar types of dynamic behaviors are obtained for highly entangled polymer solutions, where the entanglement depends on molecular weight and concentrations of the polymer in their solutions.

We used dynamic rheology to characterize associative polymer solutions containing cyclodextrins. The changes in dynamic moduli in the presence of cyclodextrin are used to quantify the intermolecular interaction between the polymer and cyclodextrins. A detailed description of the interactions between the associative polymer and cyclodextrins are given in the following chapters. In the presence of CD, the dynamic moduli decrease by several orders of magnitude. Subsequent degradation of CD by enzymes recovers the original rheological properties. The changes in rheological properties during the enzymatic reactions are used to study the kinetics of enzymatic reactions. The enzymatic reactions are carried out *in situ* in a rheometer and the extent of reaction is tracked by monitoring changes in dynamic moduli at different intervals of time. During the reaction, a sinusoidal stress of constant amplitude within the LVE region is applied and the dynamic moduli is measured at a single frequency. This type of test is called as **dynamic time sweep test[5].** Figure 3.6 represents a dynamic time

sweep test carried out to track the enzymatic degradation of cyclodextrins in 1% HASE polymer solution.

3.2. Gel permeation chromatography (GPC)

Gel permeation chromatography is a powerful technique that can be used to determine complete molar mass distribution of a polymer[6,7]. This technique involves injecting a dilute polymer solution into a solvent stream, which then flows through a column or a series of columns packed with beads of a porous gel. GPC is a form of size exclusion chromatography in which the polymer molecules elute from the chromatography column in order of decreasing molecular size in solution. The smaller polymer molecules will pass through most of the pores and thus have a longer flow path and elute out after the larger molecules.

The essentials of a GPC apparatus include a solvent pump, injection valve, column bed and detectors. The molar mass information is obtained in the form of a chromatogram, a plot of detector response as a function of elution time. In conventional GPC, the molecular weight is calculated using a universal calibration curve, which is a plot of hydrodynamic volume of molecules versus elution time[8,9]. Unless the standards used to obtain the calibration curve have exactly the same size and conformation as the unknown polymer, the molecular weights obtained will always be relative and never absolute. This difficulty can be overcome by using different detectors in series after the SEC column[10-12]. Each detector contributes to a different but complementary variable, the light scattering detector give the molecular weight, the viscometer gives intrinsic viscosity and molecular size and the refractometer or UV spectrophotometer measures the concentration of the sample. The combination of these detectors quantify the molecular weight changes during the degradation of biopolymers and allows estimation of a range of structure informations, such as molecular weight, radius of gyration, intrinsic viscosity, in a single experiment.

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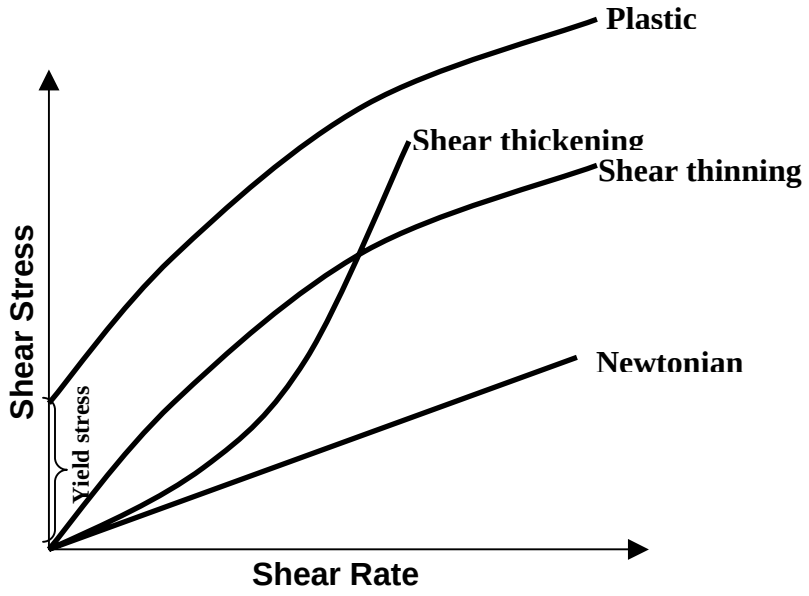

Figure 3.1: Schematic representation of flow behavior of different types of fluids.

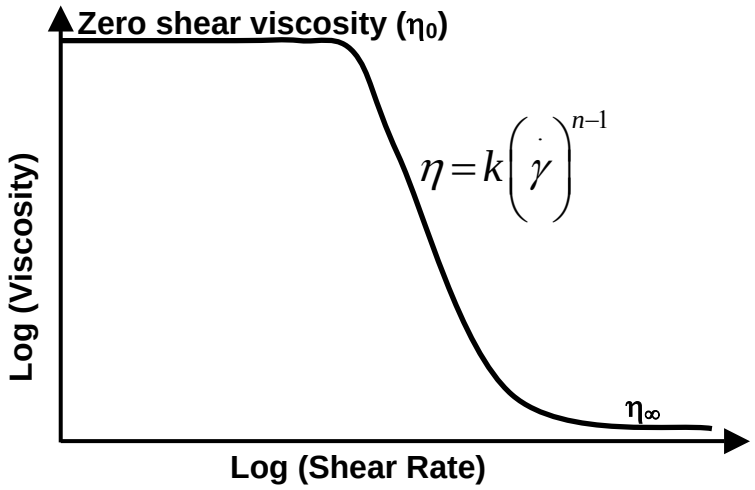

Figure 3.2: Typical flow behavior of polysaccharide solutions.

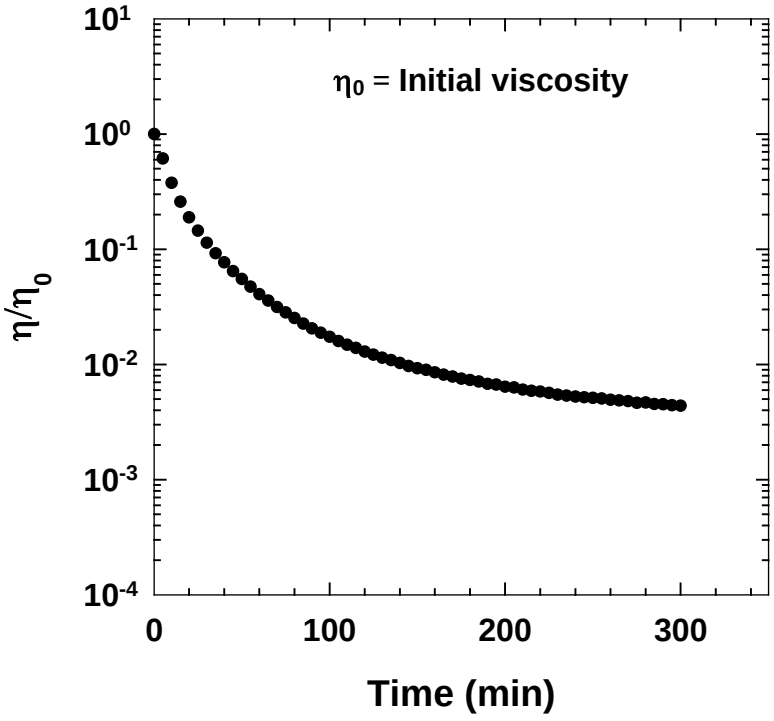

Figure 3.3: Changes in normalized viscosity of 1% guar solution measured at 75^0C during enzymatic degradation of the guar

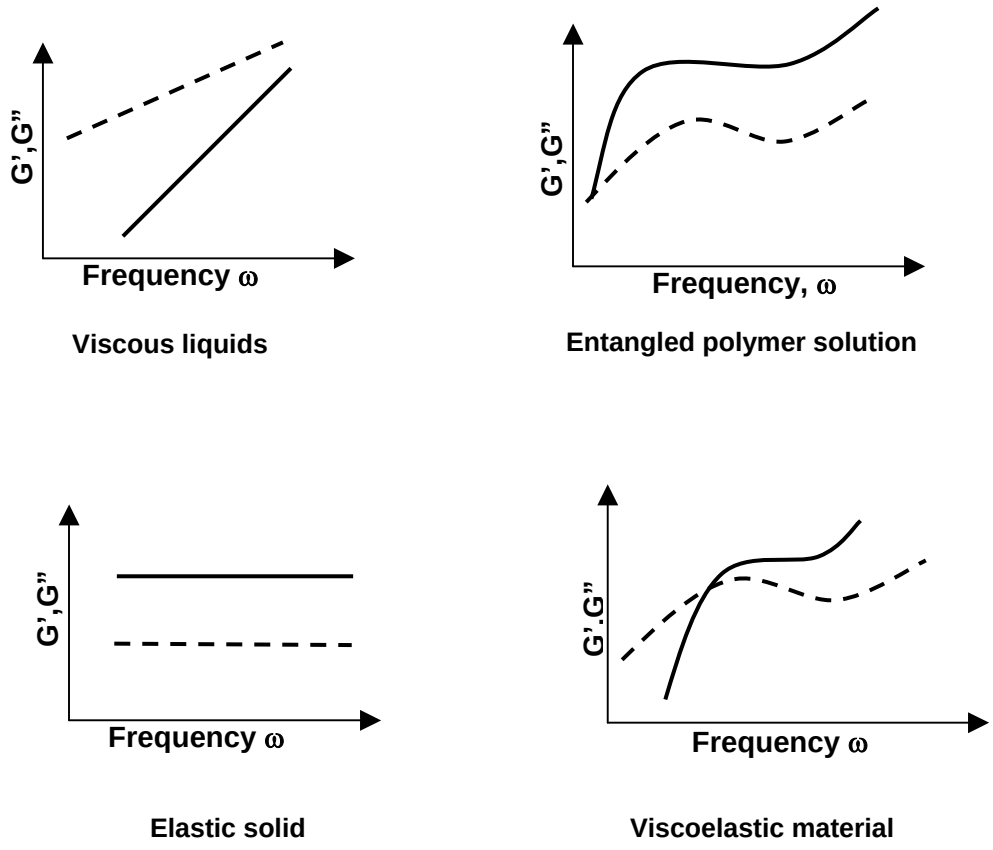

Figure 3.4: Schematic representation of elastic modulus and viscous modulus as a function of frequency for different type of materials. In the above figures, the solid line represents G' and the dashed line represents G".

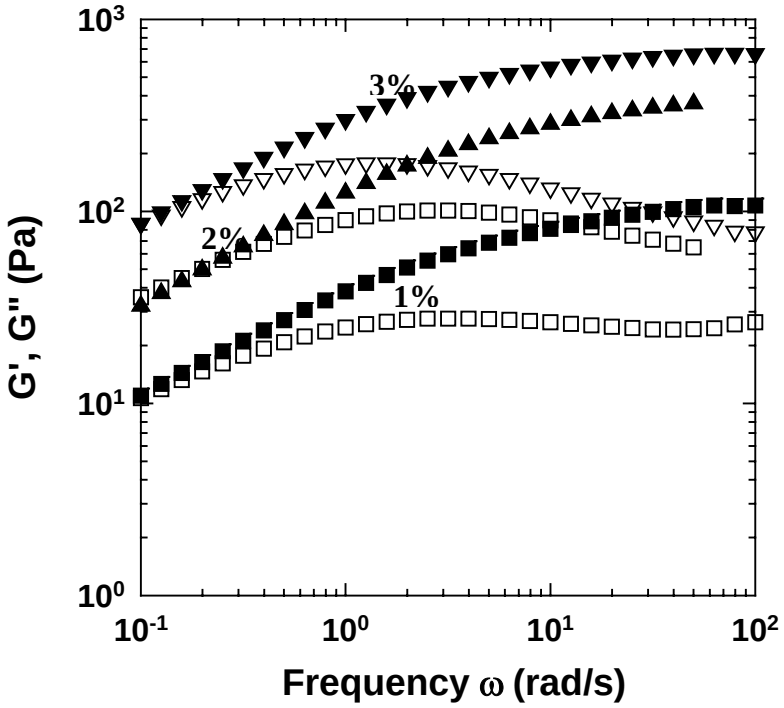

Figure 3.5: Dynamic frequency spectrum of hydrophobically modified associative polymer solutions at different concentrations. In the above figure, the closed symbols represent G' and the open symbols represent G".

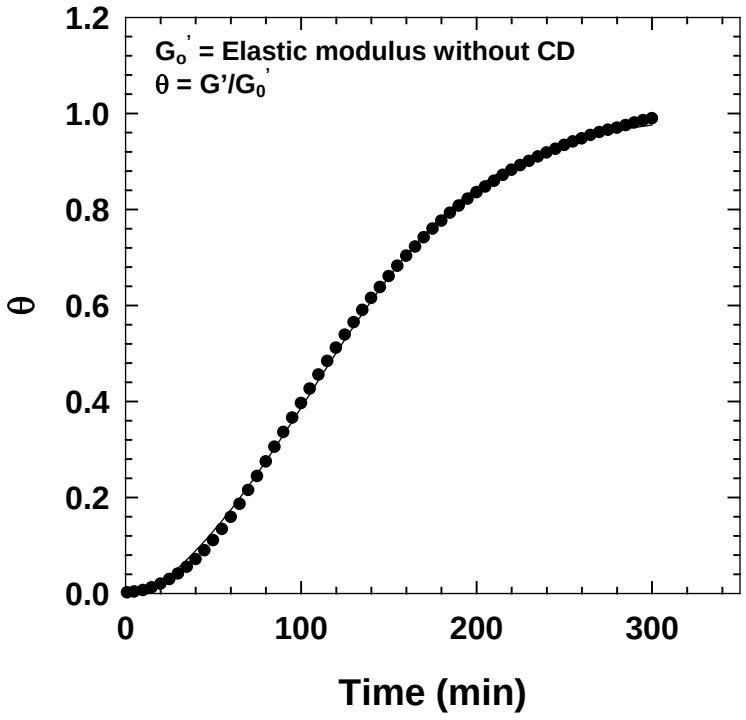

Figure 3.6: Changes in the normalized elastic modulus of 1% solution of HASE polymer with cyclodextrin during enzymatic degradation of the cyclodextrin. The dynamic moduli are measured at a stress amplitude of 1Pa and frequency of 1 rad/s

CHAPTER 4

KINETICS OF ENZYMATIC DEPOLYMERIZATION OF GUAR GALACTOMANNAN

Chapter 4 is essentially a manuscript by Mahammad, S., Prud'homme, R. K., Roberts, G. W., and Khan, S. A., submitted to ***Biomacromolecules***

Kinetics of Enzymatic Depolymerization of Guar Galactomannan

Shamsheer Mahammad[1], Robert K. Prud'homme[2], George W. Roberts[1] and Saad A. Khan[1]

[1]Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC 27695-7905
[2]Department of Chemical Engineering, Princeton University, Princeton, NJ 08540

Abstract

A new mathematical model based on Michaelis Menten (MM) kinetics is developed to predict the changes in molecular weight distribution (MWD) during the enzymatic depolymerization of guar galactomannan. The model accounts for the effect of branching by considering the guar molecule as a substrate having three types of bond with different MM kinetic parameters. The overall kinetics of the enzymatic reactions then can be represented in terms of composite kinetic parameters that are functions of the MM parameters for the individual bonds. The depolymerization is assumed to follow a random scission mechanism, in which an enzyme randomly attacks the substrate molecule at any one of the three types of bonds, and leaves the substrate on cleavage of the bond. Expressions for the variation in molecular weights during depolymerization are developed by applying moment generating techniques to the kinetic model. The model is evaluated against the complete MWD obtained using gel permeation chromatography. During the initial stages of depolymerization, the enzymatic reaction is in the zero-order regime of MM kinetics and the polydispersity index (PDI) increases with time. Subsequently the PDI decreases, as the depolymerization tends to follow first order kinetics. We also show that for a zero-order, random or nonrandom scission, the variation of PDI with time can exhibit a maximum. These analyses confirm that an increase in PDI during the depolymerization is not necessarily due to nonrandom scission, as previously concluded.

4.1. Introduction

Guar galactomannan is a naturally occurring polysaccharide consisting of a linear backbone of β-1,4-linked mannose units with randomly attached α-1,6-linked galactose branches, with the ratio of mannose to galactose units being approximately 2 (Figure 4.1)[1]. Guar and its derivatives are extensively used in many applications including food, oil recovery, drug-delivery, personal and health-care products[2] because of their low cost, natural abundance, and ability to impart thickening and other desirable functionalities. In many of these applications, depolymerization of the guar is required to tailor the rheological and microstructural properties of specific products[3,4]. Consequently, a study of the depolymerization of the native guar to different extents and molecular weights is needed to understand the structure and molecular weight dependence of the physiochemical properties of the polymer[5-7]. While chemical, thermal and mechanical methods are traditionally used for degradation[8,9], enzymes offer a powerful alternative[10] because of their specificity. Guar is susceptible to hydrolysis by three types of enzymes: β-mannanase cleaves the β-1,4 linkages between mannose backbone units; β-mannosidase cleaves the terminal β-1,4 linkages from the nonreducing end of the guar molecule; and α-galactosidase cleaves the α-1,6 linkages between the mannose and galactose units[5,11]. Figure 4.1 shows a schematic of a guar molecule together with the sites for enzyme action.

Although the effects of enzyme modification on rheology and microstructure of guar solutions and gels have been studied[12-16], the kinetics of the enzymatic depolymerization of guar is not clearly understood. In previous investigations, the kinetics of depolymerization by β-mannanase enzyme was measured and compared with the kinetics of degradation by acid hydrolysis[6] and ultrasonication[9]. The enzymatic hydrolysis showed similar viscosity and molecular weight reduction patterns compared to those of ultrasonication and acid hydrolysis. Enzymatic hydrolysis resulted in a broadening of the molecular weight distribution (MWD), i.e., an increase of the polydispersity index (PDI). However, ultrasonication and acid hydrolysis resulted in a narrowing of the MWD, i.e., a decrease of the PDI. Although the enzymatic depolymerization was shown to follow a zero-order

kinetics[6,9], the kinetic model could not explain why PDI increased during the depolymerization. In previous studies of β-mannanase catalyzed depolymerization of guar[6,9], the increase in the PDI was attributed to nonrandom enzyme attack on the β-1,4 linkages in the guar backbone. It was hypothesized that the non-randomness might result from local confinement of the enzymes in the concentrated guar matrix, leading to high levels of hydrolysis in regions around the enzyme and to no degradation in regions inaccessible to the enzyme[6]. This is in contrast to hydrolysis by acids and bases or by mechanical methods, where all degradable bonds in a guar molecule are equally reactive.

The enzymatic hydrolysis of polymers involves the interaction between an enzyme and a polymer molecule, forming an intermediate complex at an active site on the polymer molecule[17]. Some of the factors that affect enzyme-substrate complexation are branching and conformation[18-22]of polymer molecules and the accessibility of bonds to the enzyme attack. Since guar is a branched polymer with galactose side chains randomly attached to the backbone, the presence of the branches can hinder enzyme attack and lead to different reaction rate constants for different bonds in the guar chain. Although nonrandom enzymatic attack was used as one of the possible explanations for the increasing PDI that was observed during the enzymatic hydrolysis of guar[6], no quantitative kinetic model was developed to show how nonrandom scission increases the PDI.

In this study, we first develop a zero order kinetic model to predict changes in the MWD during the depolymerization of guar by β-mannanase enzyme. The model reveals that both random and nonrandom bond scission lead to a PDI that increases initially and then decreases as the depolymerization reactions progress. Consequently, one cannot conclude that enzymatic depolymerization follows a nonrandom scission mechanism, based solely on changes in PDI with time. In a few studies, enzymatic reactions have been treated as first order with respect to the concentration of degradable bonds[23-25]. In particular, Yoon *et al.*[25] showed that the PDI increases during microbial depolymerization of polyesters if the reaction follows first order random scission kinetics. Although some of these models were able to predict changes in MWD during depolymerization, they do not provide a physical

interpretation of enzymatic reactions, where the concentration of enzyme-substrate complex determines the rate of reaction.

In order to gain further insight into the behavior of the enzymatic hydrolysis of guar, we develop a new mathematical model based on Michaelis Menton (MM) kinetics. There are many studies showing that Michaelis Menten kinetics describes the enzymatic depolymerization of polymers[18,19,26,27]. However, their focus has been on estimating the MM kinetic parameters from experimental data. No model has been developed based on MM kinetics to predict the changes in MWD during enzymatic degradation of polymers.

The presence of branches on the backbone of galactomannans and other polysaccharides changes the action pattern and activity of endo-acting glycosidase enzymes on the backbone[5,11,18,19,28]. These effects have been studied via computer simulations[9,29]. In the present model, we treat the effect of galactose branches on the β-mannanase activity by considering the guar molecule as a substrate with three types of active sites (bonds) randomly distributed on the backbone as shown in Figure 4.2. The three types of bonds are: (1) the β-1,4 bond between two unsubstituted mannose units, (2) the β-1,4 bond between an unsubstituted and a substituted mannose unit, and (3) the β-1,4 bond between two substituted mannose units. Each type of bond will have different MM kinetic parameters. However, the β-mannanase enzyme attacks randomly at any of these bonds to form an enzyme-substrate complex, followed by degradation of the bond. We assume that the degradation follows a single scission mechanism[17], where the enzyme leaves the substrate upon cleavage of a bond. We develop a kinetic rate equation for depolymerization of guar molecules with a distribution of chain lengths assuming that the kinetic parameters are independent of chain length. We then apply moment generating techniques[30] to the kinetic rate equation to develop equations for the rate of change of number-average and weight-average molecular weights as a function of the kinetic parameters.

4.2. Results and discussion

4.2.1. Mathematical modeling

4.2.1.1. Nonrandom zero order depolymerization kinetics

According to the zero order model, the rate of degradation of guar to be independent of substrate concentration, but is proportional to enzyme concentration. In this model, nonrandom scission is taken into account by considering the rate constant to be a function of both chain length and the position of the hydrolysable bond on the chain. For zero order scission kinetics, the rate of change of concentration of guar molecules of chain length "i" is given by,

$$\frac{dP_i}{dt} = \left\{ \sum_{j=i+1}^{\infty} (k_{j,i} + k_{j,j-i}) - \sum_{j=1}^{i-1} k_{i,j} \right\} E_T \qquad (4.1)$$

In the above equation, $k_{i,j}$ is the rate constant for the hydrolysis reaction occurring at the j^{th} bond on a polymer chain of i monomer units. E_T is the total concentration of enzyme in the reaction mixture, and P_i is the concentration of a molecule with chain length "i". The first summation on the right hand side of the above equation represents the rate of formation of molecules of chain length "i" due to degradation of molecules of chain length j>i at the "i"th bond or "(j-i)"th bond. The second summation shows the rate of degradation of the molecules of chain length "i" at any of the i-1 bonds. For random scission kinetics, all k_{ij} are equal.

When a zero-order model is used, it is mathematically possible for the concentrations of polymer molecules with a specific chain length to become negative unless the reaction is "turned off" when all of the molecules of that chain length have been consumed. This can be accomplished by setting all of the rate constants that apply to this chain length equal to zero.

$$k_{i,j} = 0; \quad 1 \le j \le i-1; \quad P_i = 0$$

For molecules with high values of "i", $k_{i,j}$ may become zero at relatively short times, especially if the initial concentration of molecules with this chain length is low. On the other hand, the initial concentration of a particular short chain (low i) molecule may be zero such that $k_{i,j}=0$ for this chain length, initially. However as molecules of these chain lengths are formed during the depolymerization reaction, the values of $k_{i,j}$ become finite. We do not attempt to account explicitly for these types of behavior since they depend on the initial chain length and on the final extent of depolymerization.

The following development is based on the assumption that all of the chain lengths that are present in the initial distribution are continuously present until the end of the reaction and that no polymers are formed with a chain length that is not present in the initial distribution. From Equation (4.1), the changes in the moments of chain length distribution can be derived[30], as follows.

For the n^{th} moment,

$$\frac{d\mu_n}{dt} = \sum_{i=1}^{\infty} i^n \frac{dP_i}{dt} = -\sum_{i=1}^{\infty}\sum_{j=1}^{i-1}\left[i^n - j^n - \left(i-j\right)^n \right] k_{i,j} E_T = -k_n E_T \tag{4.2}$$

Equation (4.2) can be used to derive the following equations for μ_0, μ_1 and μ_2

$$\frac{d\mu_0}{dt} = \sum_{i=1}^{\infty} \frac{dP_i}{dt} = \sum_{i=1}^{\infty}\sum_{j=1}^{i-1} k_{i,j} E_T = k_0 E_T \tag{4.3}$$

$$\mu_0 = \mu_{00} + k_0 E_T t \tag{4.4}$$

$$\frac{d\mu_1}{dt} = \sum_{i=1}^{\infty} i \frac{dP_i}{dt} = 0 \tag{4.5}$$

$$\mu_1 = \mu_{10} \tag{4.6}$$

$$\frac{d\mu_2}{dt} = \sum_{i=1}^{\infty} i^2 \frac{dP_i}{dt} = -\sum_{i=1}^{\infty}\sum_{j=1}^{i-1}\left[i^2 - j^2 - (i-j)^2 \right] k_{i,j} E_T = -k_2 E_T \tag{4.7}$$

$$\mu_2 = \mu_{20} - k_2 E_T t \tag{4.8}$$

In equations (4.4), (4.6) and (4.8), μ_{00}, μ_{10}, μ_{20} are the initial moments of the chain length distribution before the hydrolysis reactions begin. The equations for changes in number average (X_n) and weight average (X_w) degrees of polymerization (DP) can be derived using Equations (4.4), (4.6) and (4.8).

$$\frac{1}{X_n} = \frac{\mu_0}{\mu_1} = \frac{1}{X_{no}} + k_0^{'} t \tag{4.9}$$

$$X_w = \frac{\mu_2}{\mu_1} = X_{wo} - k_2^{'} t \tag{4.10}$$

$$\text{where, } k_0^{'} = \frac{k_0 E_T}{\mu_{10}};\ k_2^{'} = \frac{k_2 E_T}{\mu_{10}} \tag{4.11}$$

The change in PDI (Q) with time is given by,

$$Q = \frac{X_w}{X_n} = \left(\frac{1}{X_{no}} + k_0^{'} t \right)\left(X_{wo} - k_2^{'} t \right) \tag{4.12}$$

Here, X_{no} and X_{wo} are the initial number average and weight average degrees of polymerization respectively.

The PDI versus time curve will have an extremum, if

$$\frac{dQ}{dt} = k_0^{'} X_{wo} - \frac{k_2^{'}}{X_{no}} - 2k_0^{'} k_2^{'} t = 0 \tag{4.13}$$

If an extremum exists, it must be a maximum since,

$$\frac{d^2Q}{dt^2} = -2k_0^{'}k_2^{'} < 0 \tag{4.14}$$

The maximum value of Q (Q_{max}) and the time (t_{max}) at which the maximum occurs can be derived using equations (4.12) and (4.13) respectively.

$$t_{\max} = \frac{1}{2}\left[\frac{X_{wo}}{k_2^{'}} - \frac{1}{k_0^{'}X_{no}}\right] \tag{4.15}$$

$$Q_{\max} = \frac{1}{4k_0^{'}k_2^{'}}\left(\frac{k_2^{'}}{X_{no}} + k_0^{'}X_{wo}\right)^2 \tag{4.16}$$

Equation (4.15) shows that if $\frac{k_2^{'}}{k_0^{'}} \geq X_{wo}X_{no}$, a maximum will not occur and the PDI will decline monotonically with time. However, if the initial molecular weight of the polymer is sufficiently high, $X_{no}X_{wo}$ will be greater than $\frac{k_2^{'}}{k_0^{'}}$. In this case, the PDI will increase to the maximum given by Equation (4.16), and then will decrease. The above results show that the PDI can either increase or decreases for both random and nonrandom scission under zero order kinetics depending on relative values of $X_{wo}X_{no}$ and $\frac{k_2^{'}}{k_0^{'}}$.

In a previous investigation, it was shown that the PDI can increase for random scission under first order kinetics[25]. The kinetics of enzymatic depolymerization of guar also were studied for two cases of nonrandom scission, central scission and Gaussian scission.[9] In the central scission case, enzyme preferentially attacks at the center of the polymer chain, whereas in Gaussian scission, the variation in the rate constant along the chain follows a Gaussian distribution with the maximum at the center. However, the experimental data did not fit either of these mechanisms for first order kinetics. Previous analyses have been based on simplified kinetic models of enzyme-substrate complexation, i.e., either first or zero order

kinetics. In addition, previous models allowed more than one enzyme to be attached to a guar molecule. However, in view of the very small ratio of enzyme to hydrolysable bonds, the present model permits no more than one enzyme to be attached to a single polymer molecule. In the following section, we develop a new mathematical model based on the above picture of enzyme-polymer interaction, and on a full MM kinetic model to extend the range of previous kinetic analyses.

4.2.1.2. Michaelis Menten kinetics

The enzymatic reactions of a single substrate with a single type of active site can be represented as [31]

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (4.17)$$

The free enzyme (E) attaches to the substrate (S) and forms an enzyme-substrate complex (ES). The enzyme then acts on the substrate to release the products and free enzyme. The kinetic pathway may involve single scission, where an enzyme molecule leaves the substrate on degrading a bond, or multiple scission, where the enzyme remains attached to a substrate molecule until all the degradable bonds in the molecule have been cut. A pathway intermediate between single scission and multiple scission also may be followed[17]. We assume that the depolymerization follows a single scission mechanism, as the presence of galactose side chains may preclude the multiple scission mechanism.

In enzymatic depolymerization reactions, every degradable bond is often considered to be a substrate[26,32]. However, it is physically not possible for a guar molecule to hold enzymes at all degradable bonds, as the MW of a repeat unit between the degradable bonds (~270) is much smaller than the MW of the enzyme (~50000). Moreover the enzyme concentration is much lower than that of the guar. In a typical guar hydrolysis reaction, the β-mannanase enzyme concentration is of the order 10^{-10} mmol/ml whereas the guar concentration is of the order of 10^{-5} mmol/ml. Consequently, in the following development, the guar molecule is considered to be the substrate, rather than a degradable bond. A guar

molecule can complex with an enzyme at any of the three types of active sites on the guar backbone, as discussed earlier. The reactions at each of these sites are assumed to follow MM kinetics, but with different kinetic parameters at each type of site. We assume these kinetic parameters to be independent of polymer chain length.

Equation (4.17) can be modified to take into account the multiple types of active sites on a guar backbone. For an enzyme acting on a j type bond on a polymer chain containing i monomer units, Equation (4.17) can be written as:

$$E + P_i \underset{k_{2j}}{\overset{k_{1j}}{\rightleftharpoons}} \left[EP_i\right]_j \xrightarrow{k_{3j}} E + P_k + P_{i-k} \tag{4.18}$$

$$\frac{d\left[EP_i\right]_j}{dt} = k_{1j}\left[E\right]P_i - k_{2j}\left[EP_i\right]_j - k_{3j}\left[EP_i\right]_j \tag{4.19}$$

Assuming pseudo steady state for each type of enzyme-substrate complex,

$$\left[EP_i\right]_j = \frac{k_{1j}}{k_{2j} + k_{3j}}\left[E\right]P_i = \frac{\left[E\right]P_i}{K_j} \tag{4.20}$$

where, $K_j = \dfrac{k_{2j} + k_{3j}}{k_{1j}}$ (4.21)

In the above Equation, $\left[EP_i\right]_j$ represents the concentration of enzyme substrate complex formed at j type bonds on a guar molecule of chain length i, and $\left[E\right]$ is the concentration of free enzyme. The mass balance on the total enzyme gives:

$$E_T = \left[E\right] + \sum_{i=2}^{\infty}\sum_{j=1}^{3}\left[EP_i\right]_j = \left[E\right] + \sum_{i=2}^{\infty}\sum_{j=1}^{3}\frac{\left[E\right]P_i}{K_j} \tag{4.22}$$

Hence, $$\left[E\right]=\frac{E_T}{1+\sum_{i=2}^{\infty}P_i\sum_{j=1}^{3}\frac{1}{K_j}} \tag{4.23}$$

Assuming K_j is independent of guar concentration and chain length, Equation (4.23) can be simplified as

$$\left[E\right]=\frac{E_T}{1+\frac{1}{K_M}\sum_{i=2}^{\infty}P_i} \tag{4.24}$$

where, $$\frac{1}{K_M}=\sum_{j=1}^{3}\frac{1}{K_j} \tag{4.25}$$

The overall rate of formation of polymer molecules of chain length "i" is written as:

$$\frac{dP_i}{dt}=-\sum_{j=1}^{3}k_{3j}\left[EP_i\right]_j+\sum_{l=i+1}^{\infty}\frac{2}{l-1}\sum_{j=1}^{3}k_{3j}\left[EP_l\right]_j \tag{4.26}$$

The two terms in the right hand side of Equation (4.26) represent the rate of disappearance and rate of formation of polymer molecule containing i monomer units respectively. The factor $\frac{2}{l-1}$ is the probability of formation of polymer molecules of i monomer units from the polymer molecules containing “l”(l>i) monomer units.

Substituting for $\left[EP_i\right]_j$ using Equations (4.20) and (4.24) we get,

$$\frac{dP_i}{dt}=\frac{k'E_T}{K_M+\sum_{i=2}^{\infty}P_i}\left[-P_i+2\sum_{l=i+1}^{\infty}\frac{P_l}{l-1}\right] \tag{4.27}$$

The above equation shows that the depolymerization kinetics can be represented by a modified MM kinetic equation with composite kinetic parameters given by:

$$\frac{1}{K_M} = \sum_{j=1}^{3} \frac{1}{K_j}; \quad k' = K_M \sum_{j=1}^{3} \frac{k_{3j}}{K_j} \tag{4.28}$$

Equation (4.27) is applicable only for i>2;

For i=1, $$\frac{dP_1}{dt} = \frac{k' E_T}{K_M + \sum_{i=2}^{\infty} P_i} \left[2 \sum_{l=2}^{\infty} \frac{P_l}{l-1} \right] \tag{4.29}$$

In the following sections, we use Equations (4.27) and (4.29) to derive equations for molecular weight distributions using moment generating techniques.

4.2.1.2a Determination of molecular weight distribution (MWD) and polydispersity

The generating function[30] is defined as:

$$G(s,t) = \sum_{i=1}^{\infty} s^i P_i \tag{4.30}$$

From Equations (4.27) and (4.29),

$$\frac{dG}{dt} = \frac{k' E_T}{K_M + \sum_{i=2}^{\infty} P_i} \left[-\sum_{i=2}^{\infty} s^i P_i + 2 \sum_{i=1}^{\infty} s^i \sum_{l=i+1}^{\infty} \frac{P_l}{l-1} \right] \tag{4.31}$$

The above equation can be simplified, after expanding the summations.

$$\frac{dG}{dt} = \frac{k' E_T}{K_M + \sum_{i=2}^{\infty} P_i} \left[-\sum_{i=2}^{\infty} s^i P_i + 2 \sum_{i=2}^{\infty} s \left(\frac{s^{i-1} - 1}{s-1} \right) \left(\frac{P_i}{i-1} \right) \right] \tag{4.32}$$

The moments of the MWD, $\mu_0, \mu_1, \mu_2,$ are given by,

$$\mu_0 = \sum_{i=1}^{\infty} P_i = \lim_{s\to 1}(G) \tag{4.33}$$

$$\mu_1 = \sum_{i=1}^{\infty} iP_i = \lim_{s\to 1}(s\frac{\partial G}{\partial s}) \tag{4.34}$$

$$\mu_2 = \sum_{i=1}^{\infty} i^2 P_i = \lim_{s\to 1}\left\{s\frac{\partial}{\partial s}\left(s\frac{\partial G}{\partial s}\right)\right\} \tag{4.35}$$

The equations for the rates of change of the moments can be obtained from equations (4.32)-(4.35) after applying L'Hopitals rule.

$$\frac{d\mu_0}{dt} = \frac{k'E_T\left(\mu_0 - P_1\right)}{K_M + \left(\mu_0 - P_1\right)} \tag{4.36}$$

Here, P_1 is the concentration of monomer units and (μ_0-P_1) is the concentration of polymers with at least one hydrolysable bond.

$$\frac{d\mu_1}{dt} = 0 \tag{4.37}$$

$$\frac{d\mu_2}{dt} = \frac{-\frac{k'}{3}\left(\mu_2 + \mu_1 - 2P_1\right)}{K_M + \left(\mu_0 - P_1\right)} \tag{4.38}$$

In the following sections, two limiting cases of Equations (4.36) and (4.38) are examined to derive equations for average molecular weights and PDI. In a previous investigation[32], we have shown that the reaction becomes diffusion controlled above 3% (w/w) of guar solution. However, the following analyses will be accurate enough to predict the changes in MWD during enzymatic hydrolysis of guar in the concentration range of <3%(w/w).

Initial stages of depolymerization: In a previous investigation[32], zero-order kinetics were observed in the guar concentration range of 0.1-3% w/w over a reaction time of 20 hours.

This can be true only if $K_M << (\mu_0 - P_1)$ in Equation (4.36). Initially the concentration of monomers P_1 is very low. Moreover, the total concentration of substrate molecules containing degradable bonds (μ_0-P_1) increases during the initial stages of reaction due to cleavage of interior β-1,4 glycosidic bonds. If the molecular weight of the guar is high, the total probability of cleavage at interior bonds is higher than that of terminal bond cleavage. Hence the rate of increase in μ_0 is greater than the rate of increase in P_1. If $K_M << (\mu_0 - P_1)$, Equations (4.36) and (4.38) can be further simplified:

$$\frac{d\mu_0}{dt} = k' E_T \tag{4.39}$$

$$\frac{d\mu_2}{dt} = \frac{-k' E_T (\mu_2 + \mu_1)}{3\mu_0} \tag{4.40}$$

The rate of change of number (X_n) and weight (X_w) average degree of polymerization (DP) is given by,

$$\frac{d}{dt}\left(\frac{1}{Xn}\right) = \left(\frac{k' E_T}{\mu_1}\right) = k'' \tag{4.41}$$

$$\frac{dX_w}{dt} = \frac{-k''(X_w + 1)}{3\left(\frac{1}{X_n}\right)} \tag{4.42}$$

Note that the apparent rate constant k" is proportional to the total enzyme concentration, E_T and inversely proportional to weight percentage of guar in solution (~μ_1).

Integration of equations (4.41) and (4.42) gives the variation of X_n and X_w with time.

$$\frac{X_{no}}{X_n} = 1 + X_{no} k'' t \tag{4.43}$$

$$\frac{(X_{wo}+1)^3}{(X_w+1)^3} = 1 + X_{no}k''t$$
$$or\ \frac{X_{wo}^3}{X_w^3} = 1 + X_{no}k''t, \quad if\ X_w >> 1 \tag{4.44}$$

The polydispersity index Q is given by:

$$Q = \frac{X_w}{X_n} = Q_0\left(1 + X_{no}k''t\right)^{\frac{2}{3}} \tag{4.45}$$

Where, Q_0 represents the initial PDI. Equation (4.45) shows that the polydispersity index increases during the initial stages of depolymerization.

For $X_{no}k''t < 1$, equation (4.44) can be simplified using a Taylor series expansion,

$$\frac{X_w}{X_{wo}} = 1 - \frac{1}{3}X_{no}k''t \tag{4.46}$$

As expected, Equations (4.43) and (4.46) have the same form as the corresponding equations for zero-order kinetics given in equations (4.9) and (4.10).

Late stages of depolymerization: During the late stages of reaction, $K_M >> \mu_0 - P_1$, when the number of substrate molecules with degradable bonds, less the number of monomer units decreases below K_M. The reaction then follows pseudo first order kinetics, where the rate of depolymerization is proportional to the concentration of substrate molecules with degradable bonds, as shown in Equation (4.47). This stage will occur when X_n is so low that the hydrolysis of guar molecules at the terminal ends results in an increase in concentration of monomer units (P_1) accompanied by a decrease in the concentration of substrate (μ_0-P_1) containing hydrolysable bonds. Under these conditions, Equations (4.36) and (4.38) simplify to

$$\frac{d\mu_0}{dt} = \frac{k' E_T \left(\mu_0 - P_1\right)}{K_M} \tag{4.47}$$

$$\frac{d\mu_2}{dt} = \frac{-k' E_T \left(\mu_2 + \mu_1 - 2P_1\right)}{3K_M} \tag{4.48}$$

The variation of polydispersity index is then given by:

$$\frac{dQ}{dt} = \frac{k' E_T}{3K_M}\left[2Q - \frac{1}{X_n} - 3\frac{X_w P_1}{\mu_1} + 2\frac{P_1}{X_n \mu_1}\right] \tag{4.49}$$

As the reaction progresses, $P_1 \rightarrow \mu_0$ and the concentration of monomer units will be higher than the total concentration of oligomers and polymers. Hence, Equation (4.49) simplifies to

$$\frac{dQ}{dt} \approx \frac{k' E_T}{3K_M}\left[-Q - \frac{1}{X_n} + 2\frac{1}{X_n^2}\right] \tag{4.50}$$

$$For\ Q > 1,\ \frac{1}{X_n} >> \frac{1}{X_n^2}$$
$$\therefore \frac{dQ}{dt} \leq 0 \tag{4.51}$$

Hence the polydispersity should decrease during the latter stages of hydrolysis.

4.2.2. Model evaluation

The enzymatic hydrolysis of guar solution has been studied using β-mannanase enzyme[6,32]. The reaction was carried out with 0.5% (w/w) guar in aqueous solution at room temperature and neutral pH. The enzyme concentration in the reaction mixture was 2×10^{-4} U/ml. The changes in MWD during the reactions were measured using gel permeation chromatography (GPC) with pullulan standards. We use the MWDs obtained from these experiments to evaluate the MM kinetic model.

Figure 4.3 shows the MWD at various times during the enzymatic depolymerization of guar using β-mannanase enzyme. The data points shown in the figure are reproduced from the curves in reference 6. The MWD was fit to a Zimm Schultz (Z-S) distribution[33] as given by Equation (4.52) using Sigma Plot 8.0. The solid lines in Figure 4.3 show the resulting Z-S distributions. The parameters "a" and "b" in the Z-S distribution are related to the weight and number average molecular weights by equations (4.53) and (4.54).

$$w(P_i) = \frac{a^{b+1}}{b!} M_i^b \exp(-aM_i) \tag{4.52}$$

$$a = \frac{1}{M_W - M_n} \tag{4.53}$$

$$b = \frac{M_w}{M_W - M_n} \tag{4.54}$$

The number average (M_n) and weight average (M_w) molecular weights were calculated using Equations (4.53) and (4.54) respectively at each of the times in Figure 4.3. The values of X_n and X_w were then calculated from M_n and M_w values as given by Equations (4.55) and (4.56).

$$X_n = \frac{M_n}{M_r} \tag{4.55}$$

$$X_w = \frac{M_w}{M_r} \tag{4.56}$$

In the above equations, M_r is the average molecular weight of a repeat unit. In this analysis, we assumed guar as a graft copolymer of mannose and galctose with a galactose molecule attached as a side group to every alternate mannose molecule. Hence, the average molecular weight of a repeat unit (M_r) was taken as 270, i.e., the average of molecular weights of an unsubstituted mannose unit (~180) and a mannose unit substituted with single molecule of galactose unit (~360).

Figure 4.4 shows the variation in calculated values of M_n, M_w and PDI. As expected, both the molecular weights continuously decrease with time. The reductions in M_n and M_w are rapid initially and slower at longer times. However the PDI increases continuously for the range of reaction time studied.

The kinetic parameter for guar hydrolysis was obtained using Equations (4.43) and (4.44) as shown in Figures 4.5 and 4.6. The apparent rate constant (k") determined from both plots is approximately the same. The values of k" obtained from Figures 4.5 and 4.6 are $14x10^{-6}$ hr^{-1} and $13x10^{-6}$ hr^{-1} respectively. Although the depolymerization was carried out for 20 hrs, the linearity of Figures 4.5 and 4.6, and the agreement of the values of k" from the plots suggests that the reaction lies within the initial regime of MM kinetics over this whole range of time. Analysis of previous results[6,9] also shows that only a very small fraction (<0.5%) of the original bonds were cleaved during the entire time studied.

In another study[34] on the enzymatic depolymerization of guar, a linear relationship was observed between $1/X_n$ and time, and between the apparent zero-order rate constant (k") and the reciprocal of guar concentration. These results are in agreement with our derivations (Equation 4.41). In Figure 4.7, we use the MWDs obtained from reference 9 to estimate the rate constant for the enzymatic degradation of 0.7% guar solution. As discussed previously, the MWDs were fit to a Zimm-Schultz distribution to estimate M_w and M_n. In the Figure 4.7, we plot (X_{no}/X_n) and $(X_{wo}/X_w)^3$ against time to estimate the apparent rate constant k" as given in Equations (4.43) and (4.44). The good linear fit obtained for both sets of data supports the validity of our approach. The difference in the apparent rate constant obtained from the two lines could be attributed to be experimental error.

In Figures (4.5), (4.6) and (4.7), we estimated the kinetic rate constants assuming linear variations in both $1/X_n$ and $(1/X_w)^3$ with time. This is true only if the entire range of reaction time studied falls in the zero-order regime of Michaelis Menten kinetics as described earlier. Equations (4.43) and (4.46) show that the Michaelis Menten kinetics can be reduced to a form of zero order scission kinetics (Equations 4.9 and 4.10) for the initial stages of the reaction, when $X_{no}k"t<<1$. In Figure 4.8, X_w is plotted against time for the MWD data of

Reference 9 and the figure shows that X_w decreases linearly with time as given by Equations (4.10) and (4.46). This also corroborates our assumption that the entire reaction time studied falls within the zero order regime of Michaelis Menten kinetics.

The changes in PDI predicted by MM kinetics (Equation 4.45) and zero-order nonrandom kinetics (Equation 4.11) are shown in Figure 4.9. For predicting the PDI using MM kinetics the average of the slopes of the two lines in Figure 4.7 was used as $X_{no}k$" (=0.2). For predicting the PDI using zero order kinetics, the average of slopes of the lines in Figure 4.7 was used as k_0' (=0.2) and, the slope of the line in Figure 4.8 was used as k_2'(=0.023). The zero-order kinetic model shows a maximum in the PDI versus time curve, whereas the PDI increases monotonously for MM kinetics, during the reaction time studied. The MM kinetic model predicts that PDI should decrease when the concentration of guar molecule containing at least one degradable bond becomes small compared to K_M. Since guar is used as a rheology modifier in many of its applications, previous kinetic studies have been limited to time durations for which the maximum extent of viscosity reduction occurred. Analysis of previous results[6,9] shows that only a very small fraction (<0.5%) of the original bonds were cleaved during the entire time studied. During this time duration, the concentration of polymer molecules containing at least one degradable bond increases and probably does not go below K_M. Hence PDI should increase during the entire reaction time studied. The zero order models do not take into account the enzyme-substrate complexation and are unable to accurately predict the kinetics for the later stages of the degradation process where the concentration of enzyme-substrate complex limits the rate of degradation.

4.3. Conclusions

In this study, we develop two mathematical models to examine the kinetics of enzymatic degradation of guar galactomannans: zero order nonrandom scission kinetics and MM kinetics. These models address many of the unresolved issues in prior investigations such as broadening of molecular weight, random versus nonrandom scission, and effects of branches on degradation kinetics, and provide a detailed analysis of the changes in the entire molecular weight distribution during the enzymatic hydrolysis process.

The enzymatic degradation of guar during the initial stages can be described by zero-order nonrandom or random scission kinetics. The model predicts that for zero order kinetics, the PDI can increase for either random or nonrandom bond scission. Hence the increase in PDI during the enzymatic degradation is not necessarily due to nonrandom scission of bonds, as previously concluded. However, the zero-order model does not take into account the enzyme-substrate complexation behavior and fails to predict accurately the changes in MWD during the entire depolymerization process. As such, a new MM kinetic model has been developed that takes into account enzyme substrate complexation and the effects of galactose branches on the enzyme-polymer complexation. The model is evaluated against two sets of experimental data and fits the data well. The model predicts the PDI to increase and MM rate equation to approximate zero order during the initial stages of reaction, but the PDI to decrease and the rate equation to tend towards first order kinetics as reaction progresses.

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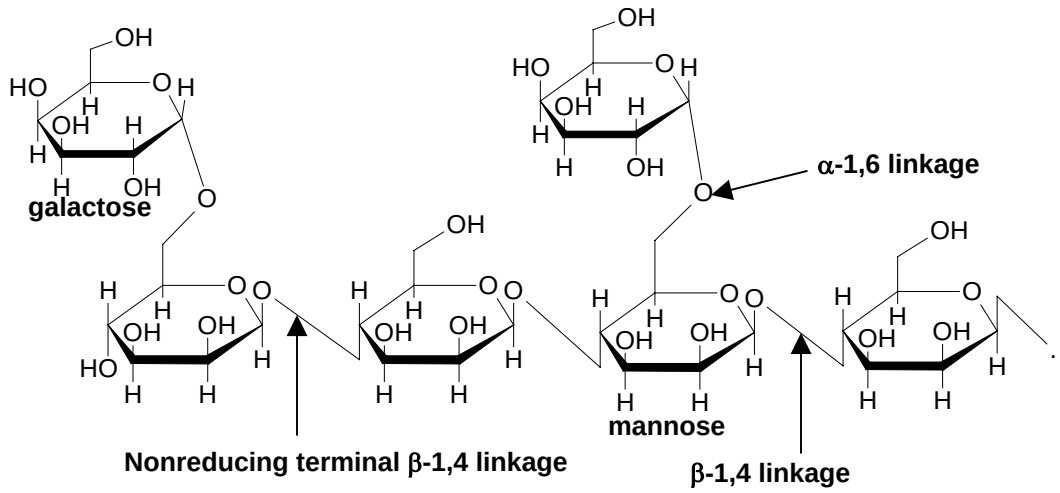
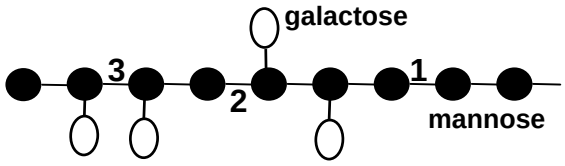


Figure 4.1: Structure of guar galctomannan showing action of three different enzymes. β-mannanase cleaves all β-1, 4 linkages, β-mannosidase cleaves only terminal β-1, 4 linkages, and α-galactosidase cleaves α-1, 6 linkages.



1. **Bond between two unsubstituted mannose units**
2. **Bond between a substituted and an unsubstituted mannose units**
3. **Bond between two substituted mannose units**

Figure 4.2: Schematic of a guar molecule showing three types of β-1, 4 bonds.

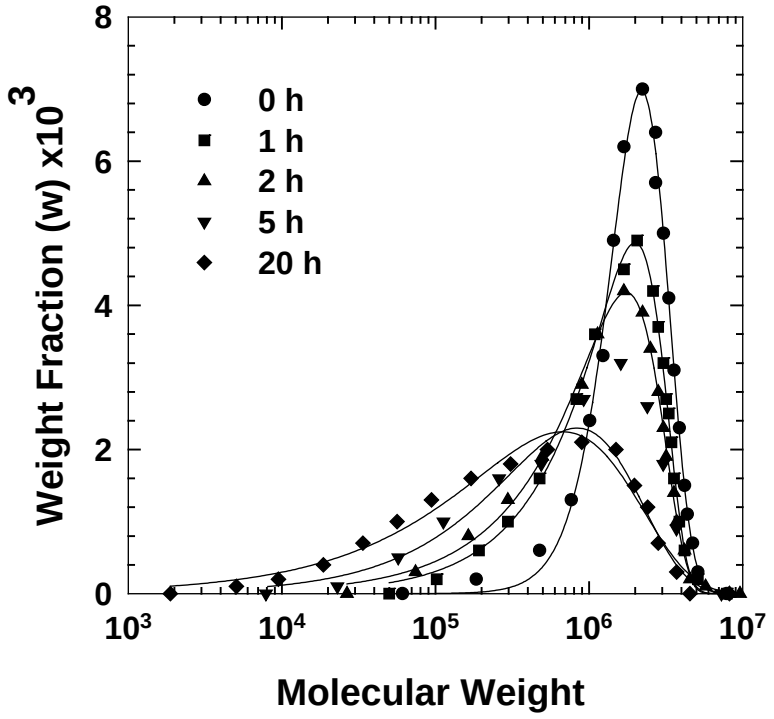


Figure 4.3: Changes in MWD during hydrolysis of guar by β-mannanase at 25^0C. The data points were obtained from the MWD curves in reference [6]. The solid lines are fits of the Zimm-Schultz distribution function to the data points.

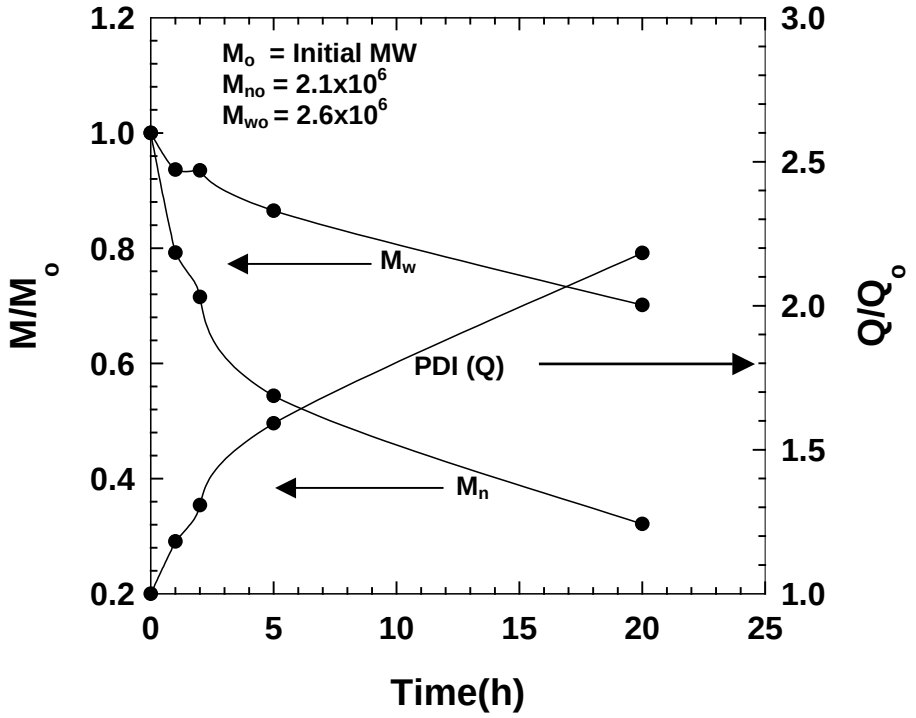


Figure 4.4: Changes in MW and PDI during the depolymerization of guar by β-mannanase enzyme. The solid lines connect the data points, which were obtained from the MWD curves in reference [6].

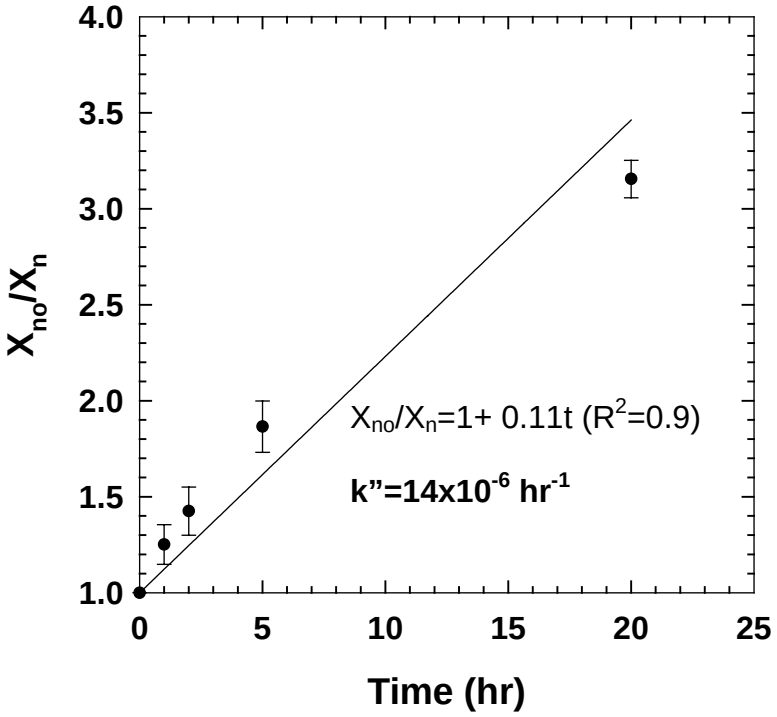


Figure 4.5: Estimation of kinetic rate constant for the data of reference [6] using Equation (4.43). The error bars represent the residuals estimated from the regression analysis.

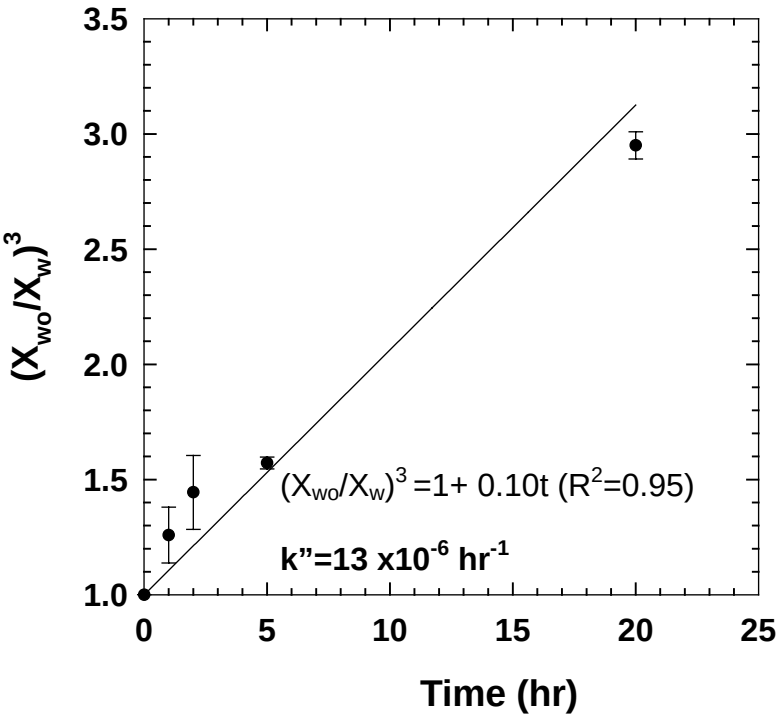


Figure 4.6: Estimation of kinetic rate constant for the data of reference [6] using Equation (4.44). The error bars represent the residuals estimated from the regression analysis.

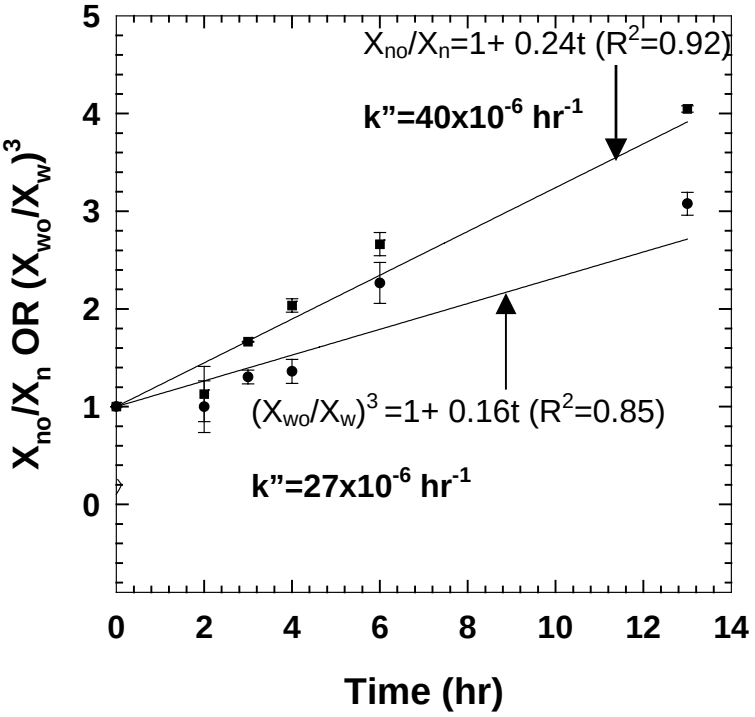


Figure 4.7: Evaluation of MM kinetic model for the data of reference [9]. The error bars represent the residuals estimated from the regression analysis.

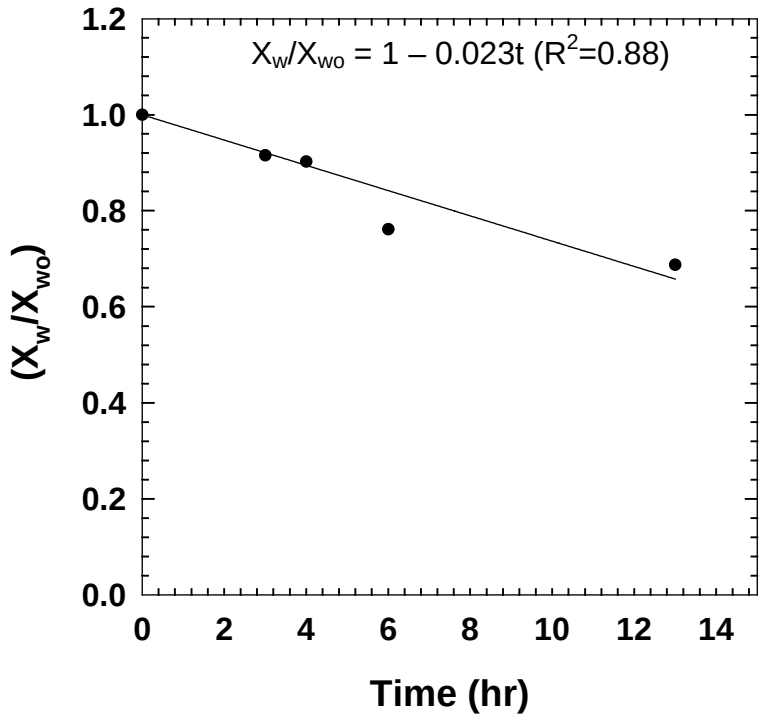


Figure 4.8: Evaluation of zero-order nonrandom scission kinetic model for the data of reference [9].

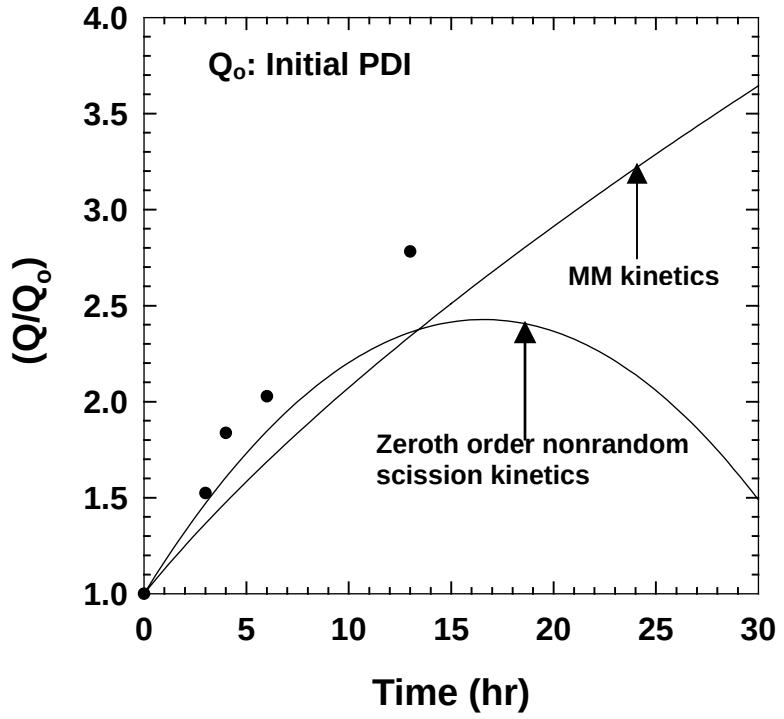


Figure 4.9: Prediction of PDI using MM kinetics and zero-order nonrandom scission kinetics. The data points are calculated from the MWD data of reference [9]. For the MM kinetic model, an average of $X_{no}k$" (0.20 hr^{-1}) estimated from the lines of Figure 4.7 is used. For zero order nonrandom scission kinetics, the rate constants k_0' = 0.20 hr^{-1} estimated from Figure 4.7 and k_2' = 0.023 hr^{-1} estimated from Figure 4.8 are used.

CHAPTER 5

RHEOLOGICAL INVESTIGATION OF HYDROLYSIS OF GUAR GALACTOMANNAN USING MULTIPLE GLYCOSIDASE ENZYMES

Chapter 5 is essentially a manuscript by Mahammad, S., Comfort, D. A., Kelly, R. M., and Khan, S. A. prepared for submission to ***Biomacromolecules***

Rheological Investigation of Hydrolysis of Guar Galactomannan using Multiple Glycosidase Enzymes

Shamsheer Mahammad, Donald A. Comfort, Robert M. Kelly and Saad A. Khan
Department of Chemical and Biomolecular Engineering
North Carolina State University, Raleigh, NC, 27695-7905

Abstract

Guar galactomannan, a naturally occurring polysaccharide, is susceptible to enzymatic hydrolysis by three enzymes:β-mannosidase, β-mannanase and α-galactosidase. The β-mannosidase cleaves a single mannose unit from the non-reducing end of the guar molecule. The β-mannanase cleaves interior glycosidic bonds between adjacent mannose units, while the α-galactosidase cleaves the galactose side branches off the guar. In this study, we examine the hydrolysis of guar using hyperthermopilic versions of these enzymes together in different proportions and combinations. The enzymatic reactions are carried out *in situ* in a rheometer, and the progress of the reaction is monitored through measuring the variation in zero shear viscosity. A rheology-based kinetic model is developed to estimate the reaction rate constants and interpret the synergistic effects of multiple enzymes in terms of these rate constants. We find that the presence of α-galactosidase affects the action of both β-mannanase and β- mannosidase. However, this effect is more pronounced when the α-galactosidase and β-mannanase or β-mannosidase enzymes are added sequentially than simultaneously. This is possibly because debranching of the guar facilitates the attack on β-1,4 linkages by both β-mannanase and the β-mannosidase enzymes and increases the rate of hydrolysis by the individual enzymes.

5.1. Introduction

Galactomannans are naturally occurring heteropolysaccharides of galactose and mannose monosaccharide units. The molecular architecture of all galactomannans consists of a linear backbone of β-1,4 linked mannopyranosyl units with α-1,6 linked galactopyranosyl branches as shown in the Figure 5.1[1]. Although all naturally occurring galactomannans present this structure, they differ in their mannose:galactose (M:G) ratio. Among the naturally occurring galactomannans, guar gum is the one used most often industrially, and has an M:G ratio of ~2[2].

The widespread use of guar galactomannan is based on its structural properties that lead to high viscosity polymer solutions containing them[3]. Native guar can be modified by both chemical and enzymatic methods to extend its range of applications[4,5]. Guar and its modifications have thus spawned a variety of commercial and industrial uses in hydraulic fracturing[6], food[7], pharmaceuticals[8,9], textiles[10], cosmetics[11], detergents[12], health-care and personal care products[13]. In addition, guar is safe for human consumption and is commonly mixed with other biopolymers to thicken or bind food products[14-17]. Since guar has the lowest M:G ratio among commercially available natural galactomannans, galactomannans with higher M:G ratio can also be obtained by debranching guar, these modified guar are expected to mimic the properties of other natural galactomannans[16,18-20].

Many applications of guar require control of its molecular weight (MW), molecular weight distribution (MWD) and the intermolecular interactions to tailor its rheological and microstructural properties. For instance, partially hydrolyzed guar gum can be used as a substitute for dietary fibers because of its low viscosity and high solubility[7,21,22]. The biodegradability and hence the release characteristics of guar gum as a tablet matrix can be modulated by judicially restructuring its architecture[9,23]. In applications of guar solutions as hydraulic fracturing fluids, the outflow of oil/gas is facilitated by controlled hydrolysis of the guar solution. Guar is often used in many food products along with other polysaccharides[24] and proteins[25]. The molecular interaction between guar and other food ingredients can be further controlled by debranching the guar and changing the M:G ratio[26], to optimize

composition of the food ingredients without compromising functionality. The degradation of guar can also be used to obtain different galactose or mannose oligomers[27].

Guar can be depolymerized by several mechanisms including acid hydrolysis[28], ultrasonication[29], irradiation and enzymatic hydrolysis[30,31]. Among all these methods, enzymatic hydrolysis of guar gum offers a powerful approach to restructure the molecular architecture because of their regiospecific and stereospecific catalysis. Three glycosyl hydrolase enzymes can hydrolyse guar into individual sugars, galactose and mannose[19]. These three enzymes are β-mannosidase, β-mannanase and α-galactosidase. The β-mannosidase cleaves a single mannose unit from the non-reducing end of the guar molecule. The β-mannanase cleaves interior glycosidic bonds between adjacent mannose units and the α-galactosidase cleaves the galactose branches from the backbone. Figure 5.1 shows the structure of guar galactomannan with the locations of enzyme action.

The major focus on enzyme modification of guar and other galactomannans has been on hydrolyzing the mannose backbone to reduce solution viscosity[31,32], and to modify the structure of guar molecule by cleaving off the galactose side chains[16,18-20,33]. However no studies have been reported so far on the hydrolysis of guar or any other galactomannans using multiple glycosidase enzymes. In this research, we hydrolyze guar using the glycosidase enzymes in different combinations and proportions and examine if there is any synergy between the enzyme actions. Our notion is based on the premise that studies on other polysaccharides have showed synergism between the glycosidase enzymes[34-39] and, the action of β-mannanase is affected by the M:G ratio and the substitution pattern of galactose side chains on the mannose backbone[40]. A unique feature of this work is the combination of viscometry, rheokinetic model and hyperthermophilic enzymes to examine this issue.

Enzymes synthesized by hyperthermophiles (microorganism with optimal growth temperatures greater than 80^0C) show the same catalytic action as their mesophilic counterparts, but are highly thermostable and are optimally active at high temperatures[41-44]. We carry out the enzymatic reactions *in situ* in a rheometer and track the extent of hydrolysis

by monitoring the changes in zero shear viscosity, which is known to be sensitive to polymer MW. We also develop a rheokinetic model to estimate enzymatic reaction rate constants and interpret synergistic effects of multiple enzymes in terms of variation of these rate constants.

5.2. Rheokinetics of enzymatic degradation

Enzymatic reactions are usually assumed to follow Michaleis Menten kinetics given by the following equation.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \tag{5.1}$$

The enzyme (E) attaches to the substrate (S) (guar molecules) and forms an enzyme-substrate complex (ES). Then the enzyme acts on the substrate to release the product (P) and free enzyme. Based on this mechanism, the rate of degradation of guar molecule of "i" monomer units (P_i) can be given by the following equation[45].

$$\frac{dP_i}{dt} = \frac{k' E_T}{K_M + \sum_{i=2}^{\infty} P_i}\left[-P_i + 2\sum_{l=i+1}^{\infty}\frac{P_l}{l-1}\right] \tag{5.2}$$

In the above equation K_M is the Michelis constant, k' is the catalytic rate constant and E_T is the total enzyme concentration in the solution. It can be shown that during the initial stages of reaction, when total amount of substrates (guar molecules) containing hydrolyzable bonds are higher than K_M, the reaction tends to follow zero order kinetics. For the zero order kinetics, the variation of weight average degree of polymerization (X_W) or weight average degree of polymerization (M_W) is given by[45],

$$\frac{X_{wo}^3}{X_w^3} = \frac{M_{wo}^3}{M_w^3} = 1 + k''t \tag{5.3}$$

where, X_w is the weight average degree of polymerization and the subscript "0" refers to the conditions before the hydrolysis reactions begin. The apparent rate constant k'' is a function

of enzyme concentration (E_T), initial molecular weight (M_{no}) and the polymer concentration (c_p) as given by the following equation.

$$k'' = \left(\frac{k' E_T M_{no}}{c_p} \right) \tag{5.4}$$

The relationship between the viscosity of a polymeric solution and its molecular weight can be represented in the following form of equations,

$$\begin{aligned} \eta &= A\, M_w^{\alpha}, \ for\ M_w \geq M_e \\ \eta &= B\, M_w^{\beta}, \ for\ M_w < M_e \end{aligned} \tag{5.5}$$

where, M_e is the entanglement molecular weight. The entanglement MW and the parameters A, B, are dependent on polymer concentration. For polymeric melts, the exponents α, β are 3.4 and 1.0 respectively. Graessley *et al*[46] showed that the exponent a=3.4 for polystyrene at concentrations between 25 and 100%. Weintjes *et al*[47] have reported α=5.8 for the guar solution in the entangled solution region. The unusually high value of the coefficient compared to that of polymeric melts were attributed to the intermolecular interactions between the guar molecules. However later it was found that these exponents are very sensitive to temperatures[48] and polydispersity of the polymer[49]. Equation (5.5) can therefore be modified to take into account the effect of polydispersity.

$$\eta = \sum_{i=1}^{\infty} K_i w_i M_i^a \tag{5.6}$$

where, the subscript "i" represents number of monomer units in a molecule. The coefficient K_i and the exponent "a" depend on the molecular weight of the molecule.

Guar is a polydisperse polymer with molecular weights in the range above and below the entanglement molecular weight. In the following kinetic model, we assume guar to be a mixture of two fractions, one having weight average MW higher than M_e and the other

having the weight average MW lower than M_e. This assumption will simplify Equation (5.6) to,

$$\eta = w_e K_e M_{we}^{\alpha} + w_u K_u M_{wu}^{\beta} \tag{5.7}$$

where, w_e and w_u represents the weight fraction of entangled and unentangled MW fractions respectively and,

$$w_e + w_u = 1 \tag{5.8}$$

Further, the average MW of the mixture can be given by,

$$M_w = w_e M_{we} + w_u M_{wu} \tag{5.9}$$

During the initial stages of reaction, when w_e tends to unity, the rate of change of viscosity can be obtained combining Equation (5.3) with (5.7):

$$\frac{\eta_0}{\eta} = \left(1 + k"t\right)^{\frac{\alpha}{3}} \tag{5.10}$$

$$\ln\left(\frac{\eta_0}{\eta}\right) = \frac{\alpha}{3}\ln\left(1 + k"t\right) \tag{5.11}$$

$$When \;\; k"t \ll 1$$

$$\ln\left(\frac{\eta_0}{\eta}\right) \approx \frac{\alpha}{3} k"t = k_0 t \tag{5.12}$$

$$where, k_0 = \frac{\alpha}{3} k" = \frac{\alpha}{3}\left(\frac{k' E_T M_{no}}{c_p}\right) \tag{5.13}$$

As the reaction progresses, the MW will decrease in both the entangled and the unentangled fractions. This will result in some of the molecules from the entangled fraction

to go into the unentangled fraction. The weight fraction of entangled fraction decreases continuously and that of unentangled fraction increases. Towards the late stages of reaction when w_u tends to unity, the rate of decrease in viscosity of the guar solution can be given by,

$$\frac{\eta_0}{\eta} = \frac{K_e M_{w0}^{\alpha-\beta}}{K_u}\left(1 + k''t\right)^{\frac{\beta}{3}} \tag{5.14}$$

At the entanglement MW,

$$K_e M_e^{\alpha} = K_u M_e^{\beta} \tag{5.15}$$

Using Equation (5.15) in (5.14)

$$\frac{\eta_0}{\eta} = \left(\frac{M_{wo}}{M_e}\right)^{\alpha-\beta}\left(1 + k''t\right)^{\frac{\beta}{3}} \tag{5.16}$$

$$\ln\left(\frac{\eta_0}{\eta}\right) = \left(\alpha - \beta\right)\ln\left(\frac{M_{w0}}{M_e}\right) + \frac{\beta}{3}\ln\left(1 + k''t\right) \tag{5.17}$$

$$\textit{For guar}, M_{wo} \gg M_e \textit{ and } \quad \alpha > \beta,$$

$$\textit{If } \left(\alpha - \beta\right)\ln\left(\frac{M_{w0}}{M_e}\right) \gg \frac{\beta}{3}\ln\left(1 + k''t\right) \tag{5.18}$$

$$\ln\left(\frac{\eta_0}{\eta}\right) = \frac{\alpha - \beta}{3}\ln\left(\frac{M_{w0}}{M_e}\right) \approx \text{constant} \approx c$$

Equations (5.12) and (5.18) show that during the initial stages of enzymatic degradation $\ln\left(\frac{\eta_0}{\eta}\right)$ increases linearly with time, whereas during the late stages of reactions, when the molecular weight of the polymer goes below M_e, the variation in $\ln\left(\frac{\eta_0}{\eta}\right)$ with time is

negligible. These two types of asymptotic behavior can be represented by a single equation of the following form.

$$\ln\left(\frac{\eta_0}{\eta}\right) = \frac{ct}{d+t} \tag{5.19}$$

Where, $\frac{c}{d} = k_0$ (5.20)

The parameter k_0 is the apparent rate constant that can be estimated from viscosity vs. time curve and is proportional to the rate constant (k') for the enzymatic reaction as given by Equation (5.13).

In the above equation,

$$c = \ln\left(\frac{\eta_0}{\eta_\infty}\right) \tag{5.21}$$

where, η_∞ is the final viscosity that can be obtained on enzymatic degradation and d is the time required for $\ln\left(\frac{\eta_0}{\eta}\right)$ to reach a value $\frac{c}{2}$. The variation of rate constant with temperature can be represented by Arrhenius equation.

$$k_0 = A\exp\left(\frac{-\Delta E}{RT}\right) \tag{5.22}$$

Here, ΔE represents the activation enrgy of reaction and "A" is proportional to the frequency of enzyme substrate interaction. The rate of reduction in viscosity at any time can be derived from equation (5.19) to give the following expressions.

$$\frac{d\eta}{dt} = \eta_0 \frac{-k_0 d^2}{(d+t)^2}\exp\left(-\frac{ct}{d+t}\right) \tag{5.23}$$

$$For \quad t \ll d$$

$$\frac{d\eta}{dt} = -\eta_0 k_0 \exp\left(-k_0 t\right) \tag{5.24}$$

$$At \quad t = d$$

$$\frac{d\eta}{dt} = \eta_0 \frac{-k_0}{4} \exp\left(-\frac{k_0 d}{2}\right) \tag{5.25}$$

$$For \quad t >> d$$

$$\frac{d\eta}{dt} = \eta_0 \frac{-k_0 d^2}{t^2} \exp\left(-k_0 d\right) \tag{5.26}$$

These equations are used in conjunction with experimental results to estimate reaction rate constants and interpret results.

5.3. Materials and methods

Guar gum was purchased from Sigma-Aldrich and purified to remove all the materials that are insoluble in their aqueous solution[50]. Solution of guar gum was obtained by sprinkling guar gum slowly into the vortex of water to the concentration of 10mg/ml. This solution was vigorously mixed for 2 hours followed by low shear mixing for 24 hours. Fisher Scientific Dynamix mixture was used for the mixing. The solution was centrifuged at 10000g for 30 minutes. The supernatant from the centrifuged solution was collected and mixed with two volumes of ethanol. The guar precipitates in the ethanol/water mixture, and the precipitate was separated, and lyophilized at 100 mTorr for 48 hours. The dried guar was crushed to get fine powder using mortar and pestle. A 1.1% solution of the purified guar was prepared in phosphate buffer at pH 7 using 0.2 mg/ml of sodium azide as bactericide and 0.05M sodium thioslulfate to minimize the thermal degradation of guar. For enzymatic reactions, the 1.1% solution was diluted to 1% using the phosphate buffer after adding the required amount of enzymes.

For higher concentration of guar solutions, purified guar was dissolved in desired quantity of pH 7 phosphate buffer containing 0.05M sodium thiosulfate and 0.2 mg/ml of sodium azide. The solution is heated to 80^0C and mixed for 15 minutes followed by digestion at 50^0C to get a clear solution.

All three hyperthermophilic enzymes used in this study, were produced at NCSU hyperthermophile laboratory from *Thermatoga maritima*. The enzymes were supplied in an aqueous solution form containing approximately 3 mg/ml of protein. The activities of β-mannanase, α-galactosidase and β-mannosidase were 0.106U, 0.316U and 0.0341U respectively. One unit of activity (U) is equivalent to micromoles of glycosidic bonds broken per minute per micro liter of enzyme solution at 75^0C and pH 7. The above enzyme stock solutions were stored at 5^0C in a refrigerator and diluted to required activity as and when required. All enzyme reactions were carried out at 75^0C and at a pH of 7, unless otherwise noted.

The enzymatic reactions were carried out *in situ* in a TA instruments AR-2000 stress-controlled rheometer using a couette geometry. The reaction mixture was subjected to a shear stress within the Newtonian region of the guar solution and changes in zero shear viscosity of the guar solution was measured as a function of reaction time to track the extent of reactions. TA-Advantage Data Analysis and Sigma Plot-8.0 softwares were used for curve fitting and data analysis.

5.4. Results and discussion

5.4.1. Steady shear viscosity of guar during enzymatic degradation

Figure 5.2 represents the steady shear viscosity of 1% native guar solution at 25 ^{0}C. The graph is obtained by time temperature superposition of steady-shear viscosity curves measured at different temperatures ranging from 25^0C to 75^0C. The steady shear behavior of 1% guar gum shows a zero shear viscosity region at low shear rates followed by a non-Newtonian region with a slope of ~0.7 at high shear rates. Figure 5.3 represents the changes

in steady shear viscosity of 1% guar solutions at different time intervals during enzymatic degradation of the guar using β-mannanase enzyme. The reaction was carried out in 1.05% guar solution at 75^0C and the samples were collected at different time intervals. The sample solution pH was adjusted to 12 using 2N NaOH to terminate all enzymatic reactions. The solution was then diluted to 1% using DI water. Steady shear viscosity measurements were then carried out for the degraded samples at 25^0C and pH 12. As the depolymerization continues, the critical strain rate increases and the slope of the non-Newtonian region decreases and finally the non-Newtonian region vanishes.

In all the following experiments on enzymatic reactions, the reaction mixture was subjected to a constant stress (τ) within the Newtonian region of the undegraded guar solution and the viscosity of the guar solutions was measured at different time intervals during the reactions. Since the Newtonian region of guar solution broadens during the degradation, the measured viscosity will be equal to "zero shear viscosity" during the entire degradation process.

5.4.2. Degradation of guar by individual enzymes

Figure 5.4 compares the changes in viscosity of the 1% guar solution when reacted with different enzymes at 75^0C. During the reactions, the activities of all the enzymes were maintained at $1.2x10^{-6}$ U/ml. In this and all subsequent figures, we plot the ratio of the viscosity of the native guar to that of the enzyme-hydrolyzed guar so that a larger number corresponds to more degradation of guar. We find that the action of β-mannanase reduces viscosity by more than 90% within 3 hours. This is because β-mannanase reduces molecular weight substantially by cleaving the backbone of guar molecule. The use of α-galactosidase enzyme cleaves of the galactose branches from the backbone. Pai *et al*[51] showed that debranching of guar using α-galactosidase can result in more hydrogen bonding between the galactose depleted mannose backbone and eventual gel formation. However, we do not observe any increase in viscosity during the reaction at 75^0C; instead, the viscosity decreases slightly. We believe that no hydrogen bonds are formed in our case due to the high

temperature. This is in contrast to Pai *et al*'s results, which were monitored at room temperature, Even in this case; the process was slow, taking three weeks to form a gel. The small decrease in viscosity observed in our case can be attributed to the slight change in MW by debranching of the guar molecule. Finally, the β-mannosidase enzyme cleaves of mannose units from the non-reducing end of the guar molecule and does not result in any significant decrease in viscosity either. We conducted an experiment without any enzyme to check if there is any significant reduction in viscosity due to thermal degradation of guar. High temperature processing of guar gum can decrease solution viscosity[52]; however, addition of sodium sulfite has reduced the thermal degradation of guar[53] and the changes in viscosity due to thermal degradation during the reaction is observed to be insignificant.

5.4.3. Rheometric estimation of kinetic rate constants

Figure 5.5 represents variation in viscosity during the degradation of guar using β-mannanase enzyme for two different concentrations of the enzyme. The figure shows that the rate of reduction in viscosity increases with increasing concentration of the enzyme. The plot is fit to an equation of the form given by the Equation (5.19) and the fitting parameters are shown in the graph. We are able fit the data in to the equation with regression coefficient $R^2 \approx 1$. The apparent rate constants (k_0) estimated from the plot are $0.11 min^{-1}$ and $0.092\ min^{-1}$ respectively for the enzyme concentrations, $1.2x10^{-5}$ U/ml and $1.2x10^{-6}$U/ml. These values indicate that the rate increases linearly with enzyme concentration. The figure also shows that the parameter "d" decreases with increasing enzyme concentration and the values of "d" estimated from the graph are 62 min and 382 min for the enzyme concentrations, $1.2x10^{-5}$ U/ml and $1.2x10^{-6}$U/ml respectively. For these values of "d" the rate of reduction in viscosity at time $t>d$ is negligible as given by Equation (5.26). Hence the parameter "d' essentially represents the time required for all the guar molecules in the entangled fraction to go into unenetangled fraction.

Figure 5.6 represents the changes in viscosity during the enzymatic degradation of guar for three different concentrations of guar. For this experiment, a 2% guar stock solution was used to obtain the other two concentrations of 1.5% and 1%. These solutions were

subjected to β-mannanase action at an enzyme concentration of $1.2x10^{-5}$ U/ml. Figure 5.6 shows that the rate of degradation decreases with guar concentration. The apparent rate constant estimated from the graph are shown in the figure and the rate constants decrease with increasing guar concentration as given by Equation (5.13). The rate constant obtained for the degradation of 1% guar, prepared by diluting 2% guar solution (Figure 5.6) is lower compared to that obtained directly from purified guar (Figure 5.5). Since 2% guar solution is prepared by dissolving the purified guar at 80^0C followed by digestion at 50^0C, these thermal processes must have degraded the guar to some extent resulting in a decrease in the initial molecular weight. These observations on variation of apparent rate constant (k_0) with enzyme concentration, polymer concentration and the initial molecular weight, corroborates our derivation in Equation (5.13), where it is shown that k_0 is directly proportional to initial MW and enzyme concentration and it is inversely proportional to guar concentration.

5.4.4. Degradation of guar by the simultaneous action of β-mannanase and α-galactosidase enzyme

Figure 5.7 shows the variation in viscosity of 1% guar solution during the enzymatic degradation of guar with different concentration ratios of β-mannanase and α-galactosidase enzymes. The β-mannanase enzyme concentration in the reaction mixture was kept constant at $3.6x10^{-6}$ U/ml and α-galactosidase concentration was varied from 0 to $3.6x10^{-3}$ U/ml. Figure 5.7 shows that as the α-galactosidase concentration is increased from 0 to 100 times the β-mannosidase concentration, there is no significant change in the viscosity reduction pattern during the initial stages of the reaction; however, the rate of viscosity reduction increases somewhat during the later stages of the reaction. With an increase in α-galactosidase concentration by another ten fold, there is a significant enhancement in viscosity reduction rate not only at long times but also at short times.

The effects of simultaneous action of α-galactosidase and β-mannosidase enzyme are further probed by using different combinations of these two enzymes as shown in Figure 5.8. In this case, the β-mannanase concentration was maintained at $1.2x10^{-5}$ U/ml and α-

galactosidase concentration was varied from 0 to 100 times the concentration of β-mannanase enzyme. No significant change in viscosity reduction pattern is observed when the enzyme concentration ratio is varied from 0 to 10 as observed in Figure 5.7. However, when the ratio is increased to 1:100, there is a significant change in the viscosity reduction pattern during the entire hydrolysis time. In this case, the apparent rate constant (k_0) (=0.179min^{-1}) is higher for the mixture of the two enzymes compared to that with β-mannanase (k_0=0.106 min^{-1}) alone.

The β-mannanase enzyme acts on interior β-1,4 linkages on the mannose backbone, and the presence of galactose side chains may hinder the action of β-mannanase enzyme. With the increase in concentration of α-galactosidase enzyme more α-1,6 linkages are removed, making β-1,4 linkages easily accessible by β-mannanase enzyme. Hence the rate of degradation of guar increases with increasing α-galactosidase concentration.

In contrast to Figure 5.7, where the initial rate constant (k_0) essentially remains constant when the α-galactosidase concentrations are increased from 0 to 1000 times the β-mannanase concentration ($3.6x10^{-6}$ U/ml), Figure 5.8 shows that, when the β-mannanase concentration is increased to $1.2x10^{-5}$ U/ml, there is a significant increase in the rate constant when the α-galctosidase concentration is 100 times the β-mannanase concentration. Hence, Figures 5.7 and 5.8 show that the viscosity reduction pattern depends not only on the concentrations of individual enzymes but also on their composition in the mixture.

In both the experiments represented in Figures 5.7 and 5.8, there is a reduction in the value of "d" with increasing concentration of α-galactosidase. This shows that, the time required for complete unentanglement of guar molecule decreases due to simultaneous action of the two enzymes.

Figures 5.7 and 5.8 also show that; at a fixed β-mannanase concentration, the final viscosity (η_∞) decreases with increasing α-galactosidase concentration. The action of β-mannanase on native guar results in blocks of galactose substituted galactomannan

oligomers, whereas by debranching the guar weight of the galactose substituted units in the final solution are considerably reduced and hence the final viscosity of guar decreases on debranching the guar using α-galactosidase enzyme.

5.4.5 Degradation of guar by the sequential action of α-galactosidase and β-mannanase enzyme

Figure 5.9 shows the viscosity reduction pattern of native and debranched guar. In this case guar solution was first treated with α-galactosidase enzyme at a concentration of $1.2x10^{-3}$U/ml and left overnight in a water bath at 75^0C. Then the solution was treated with $1.2x10^{-5}$U/ml of β-mannanase enzyme and viscosity reduction was monitored in a rheometer. The figure shows that the rate of viscosity reduction increases considerably by prior debranching of the guar molecule. Figure 5.9 shows that the sequential action of the enzymes increases the apparent rate constant (k_0) to 6 times that for the native guar. This is in contrast to Figure 5.8, where the simultaneous action of β-mannanse and α-galactosidase enzyme increases the apparent rate constant to twice that for the β-mannanase action alone. Although in Figures 5.8 and 5.9, guar is treated with same concentration of the enzymes, the increase in rate constant is more prominent in sequential action than that in the case of simultaneous action.

As observed in the simultaneous action of enzymes (Figures 5.7 and 5.8), the prior debranching of guar also decreases the time required for unentanglement of guar molecules. Figure 5.9 also shows that the final viscosity achieved on complete degradation of debranched guar is less compared to that obtained after complete degradation of native guar.

In order to get further insight on how the debranching affects the enzyme action on guar backbone, we conducted enzyme reactions at different temperatures on both the native guar and the debranched guar. As explained in previous experiments, the reactions were carried out *in situ* in a rheometer to develop viscosity vs. time curve at each temperature. The apparent rate constant (k_o) is determined after fitting the graphs to the model Equation (5.19). Figure 5.10 shows the variation in the rate constant with temperature for both the native and

debranched guar. The data is fit to an Arhenius equation as shown in the figure to estimate the activation energy and the frequency factor. The results show that rate constant for debranched guar is higher compared to that of native guar at all temperatures. However, activation of energy of reaction is essentially unaffected by debranching the guar; But, there is an order of magnitude increase in the frequency factor indicating that debranching the guar facilitates guar-enzyme interaction, resulting in enhanced rate of hydrolysis.

5.4.6. Degradation of guar using the mixture of β-mannosidase and β-mannanase enzyme

Figure 5.11 shows the simultaneous action of β mannanase and β-mannosidase on guar hydrolysis. For this study, higher concentration of the β-mannosidase enzyme (2.4×10^{-3} U/ml) was used, as lower concentrations of β-mannosidase enzyme did not bring any significant changes in viscosity reduction patterns. The β-mannanase enzyme concentration was maintained at 1.2×10^{-5}U/ml. We observe no significant change in the rate of reduction in viscosity during the initial stages of reactions. However, the rate of reduction in viscosity increases during the later stages of reaction. The final viscosity obtained by combined actions of β-mannanase and β-mannosidase is lower than that obtained with the individual action of either β-mannanase or β mannosidase enzyme. The backbone cleavage by β-mannanase enzyme creates more terminal β-1,4 linkages that can be degraded by β-mannosidase enzyme. Hence the simultaneous action of these two enzymes result in low molecular weight oligomers of mannose or galactomannans. Hence the final viscosity obtained is lower compared to that obtained by β-mannanse enzyme.

5.4.7. Degradation of guar using the mixture of β-mannosidase and α-galactosidase enzyme

Figure 5.12 shows the effect of debranching on the viscosity reduction pattern by β-mannosidase enzyme. The debranching of guar significantly enhances the rate of the viscosity reduction by β-mannosidase enzyme. The initial rate constant is higher for

debranched guar compared to that of native guar. This indicates that β-mannosidase enzyme may be cleaving only nonreducing terminal mannose units that are not attached with galactose side groups. If a β-mannanase enzyme removes mannose units one by one from the nonreducing terminal of a chain, the presence of galactose side groups obstruct the progress of the degradation further along the chain.

5.5. Conclusions

We hydrolyzed guar using three different hypertherophilic glycosidase enzymes at different concentrations, ratios and combinations. Synergistic hydrolysis is observed between different enzymes acting on guar galactomannan. The synergism depends on enzyme concentrations and ratio, as well as the combination of enzymes used. The presence of α-galactosidase enzyme affects the viscosity reduction pattern of both β-mannanase enzyme and β-mannosidase enzyme. However, the effect is more pronounced when α-galactosidase and β-mannanase or β-mannosidase enzymes are added sequentially than simultaneously. A rheokinetic model is developed and the model agrees well with the experimental data. Both the debranched and the native guar showed same activation energy for β-mannanse action. However the debranching considerably increases the frequency of enzyme-guar interactions. The debranching of guar decreases the time required for complete unentanglement of guar molecules during the degradation of the guar by β-mannanase enzyme. The residual viscosity obtained on complete degradation of debranched guar is lower when compared to that obtained on complete degradation of native guar.

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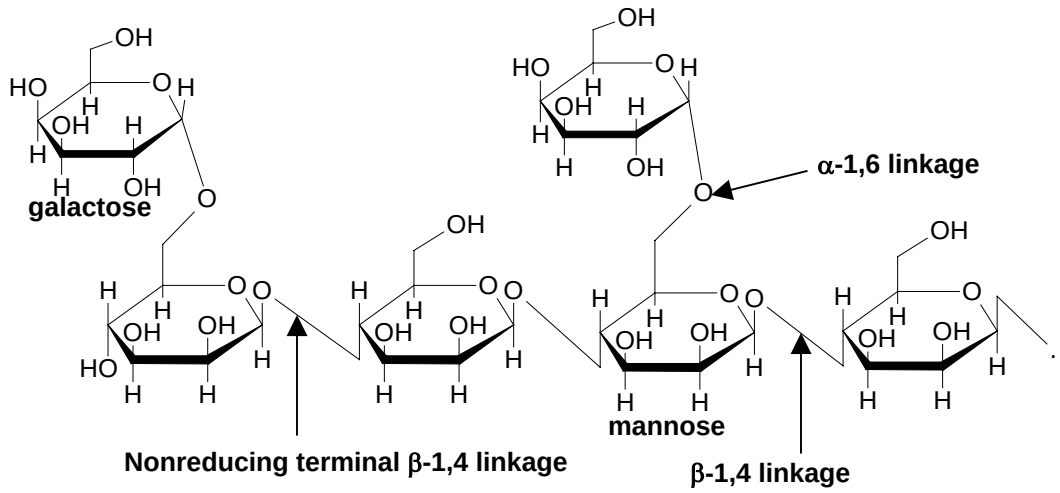

Figure 5.1: Structure of guar galctomannan showing active centers of three different enzymes. β-mannanase cleaves interior β-1, 4 linkages; β-mannosidase cleaves only nonreducing terminal β-1, 4 linkages; α-galactosidase cleaves α-1, 6 linkages.

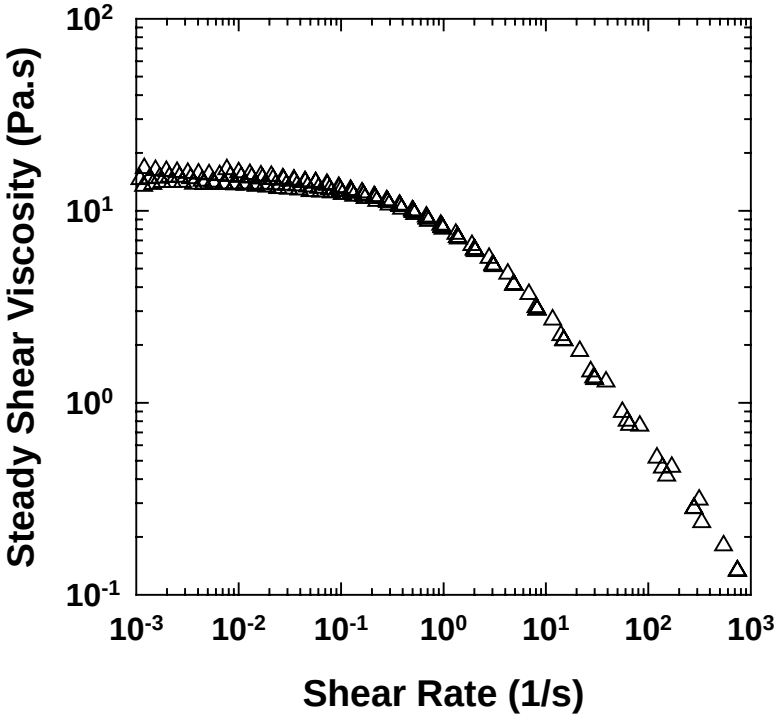

Figure 5.2: Steady shear behavior of 1% guar solution. The figure shows the master curve at 25^0C obtained by time temperature superposition flow curves measured at 25^0C, 50^0C, 60^0C, and 75^0C.

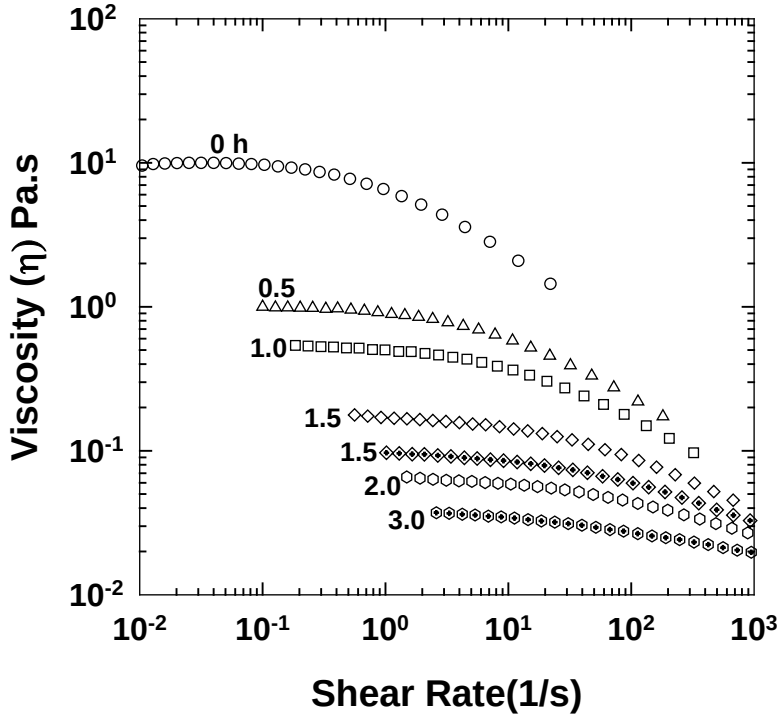

Figure 5.3: Changes in steady shear viscosity of guar gum during the degradation by β-mannanase enzyme. The activity of the enzyme in the solution is 1.2×10^{-6} U/ml. The numbers in the figure indicates degradation time in hours.

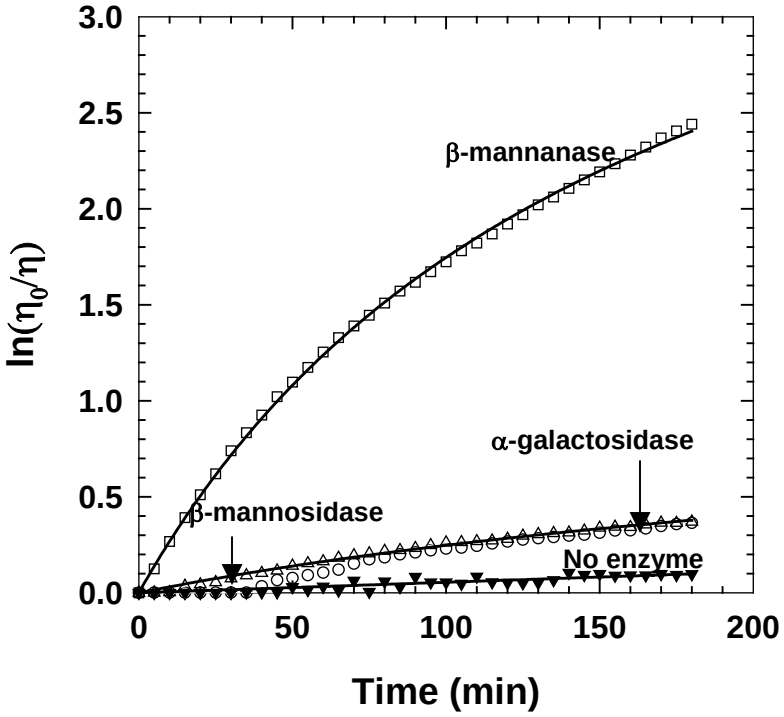

Figure 5.4: Degradation of 1% guar solution by three different enzymes monitored in terms of the viscosity ratio of undegraded guar (η_0) over degraded guar(η). The activities of all the enzymes are $1.2x10^{-6}$U/ml.

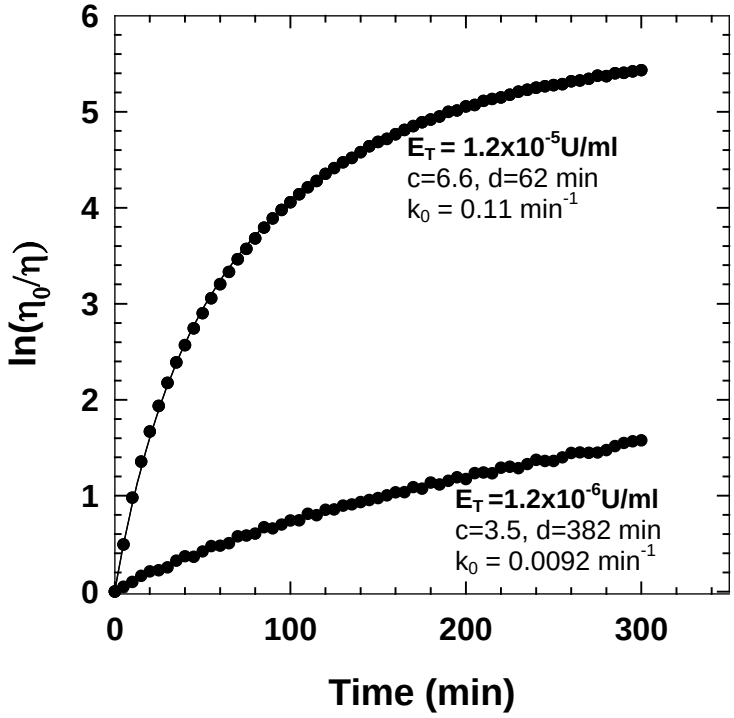

Figure 5.5: Rheometric estimation of kinetic parameters for the degradation of 1% guar solution at 75^0C and pH 7 using two different concentrations of β-mannanase enzyme. The solid lines represent the model fit to the experimental data.

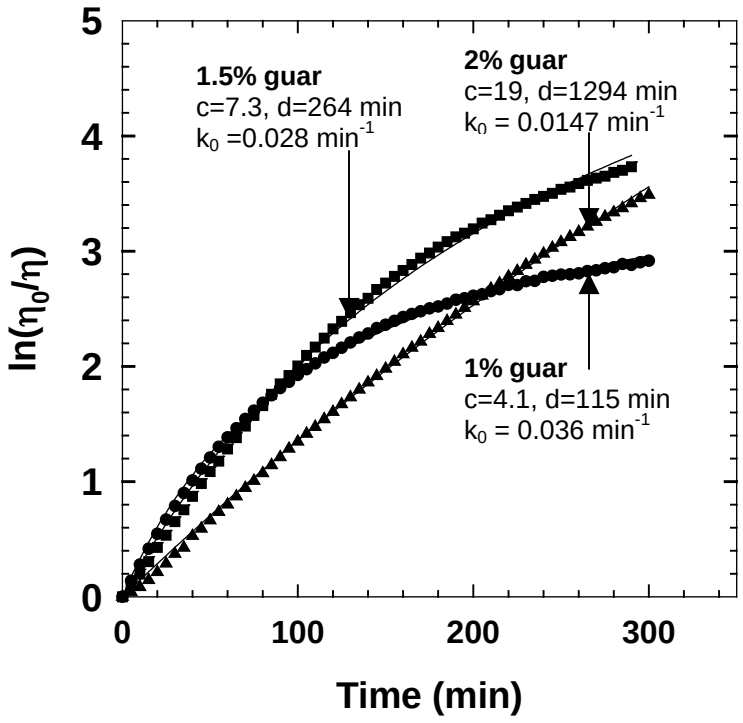

Figure 5.6: Rheometric estimation of kinetic parameters for the degradation of guar solution with different concentrations of guar at 75^0C and pH 7 using β–mannanase enzymes at the concentration of $1.2x10^{-5}$U/ml. The solid lines represent the model fit to the experimental data.

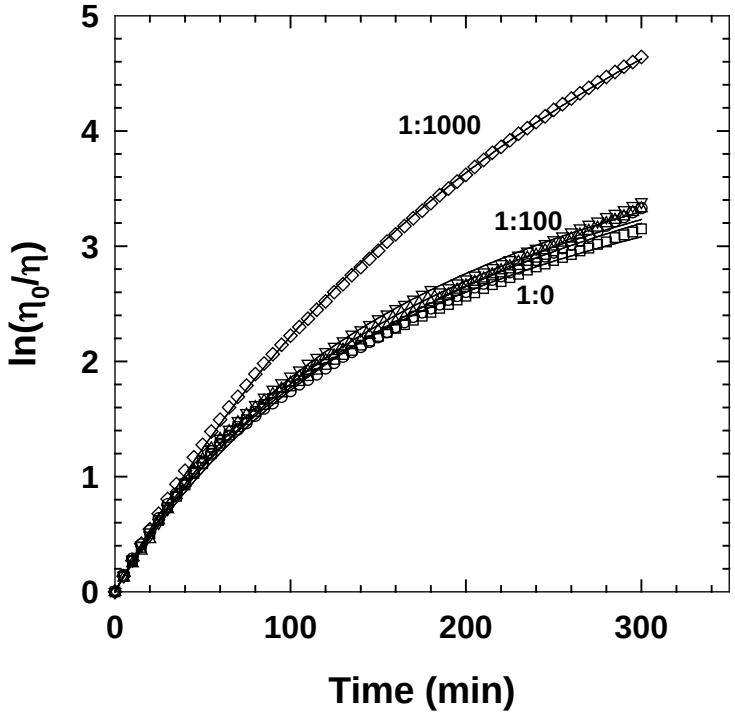

Figure 5.7: Degradation of guar by simultaneous action of β-mannanase and α-galactosidase measured in terms of viscosity ratio of undegraded guar (η_0) over degraded guar (η). The activity of β-mannanase enzyme in the solution is 3.6×10^{-6} U/ml and the ratios shown in the figure are the ratios of β-mannanase to α-galactosidase activity.

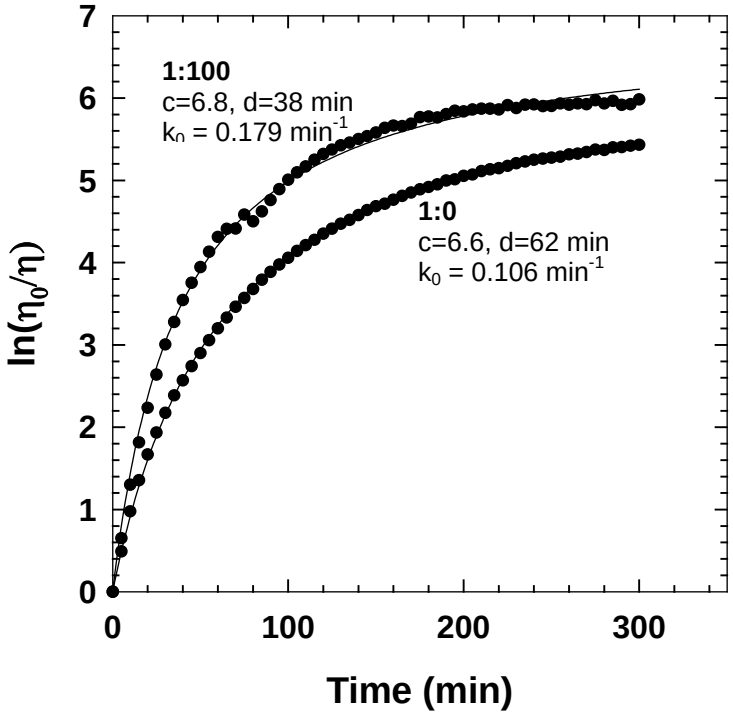

Figure 5.8: Degradation of guar by simultaneous action of β-mannanase and α-galactosidase. The activity of β-mannanase enzyme in the solution is 1.2×10^{-5} U/ml and the ratios shown in the figure are ratios of β-mannanase to α-galactosidase activity.

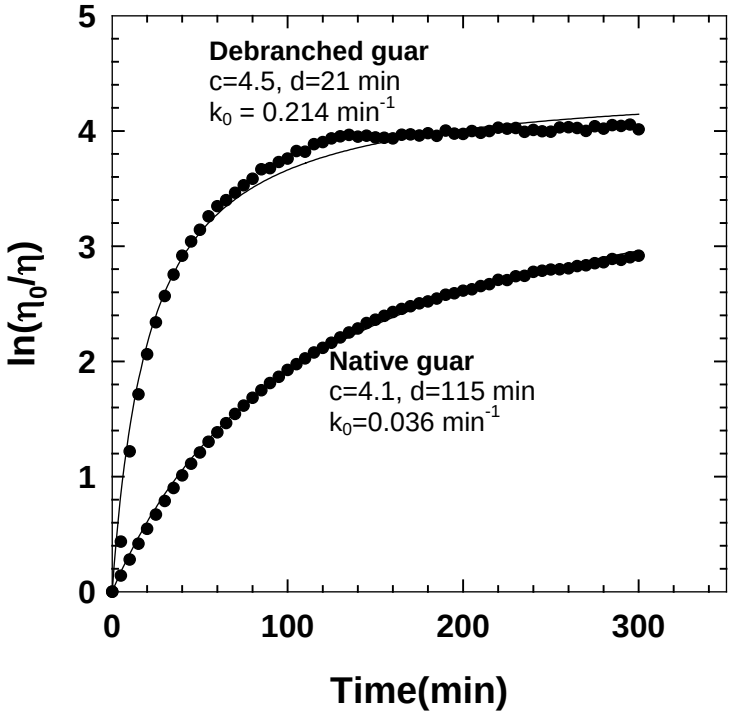

Figure 5.9: Degradation of guar by sequential action of β-mannanase and α-galactosidase enzyme. The native guar is debranched by treating with $1.2x10^{-3}$ U/ml of α-galactosidase for 24 hours. The debranched guar is then treated with $1.2x10^{-5}$U/ml of β-mannanase.

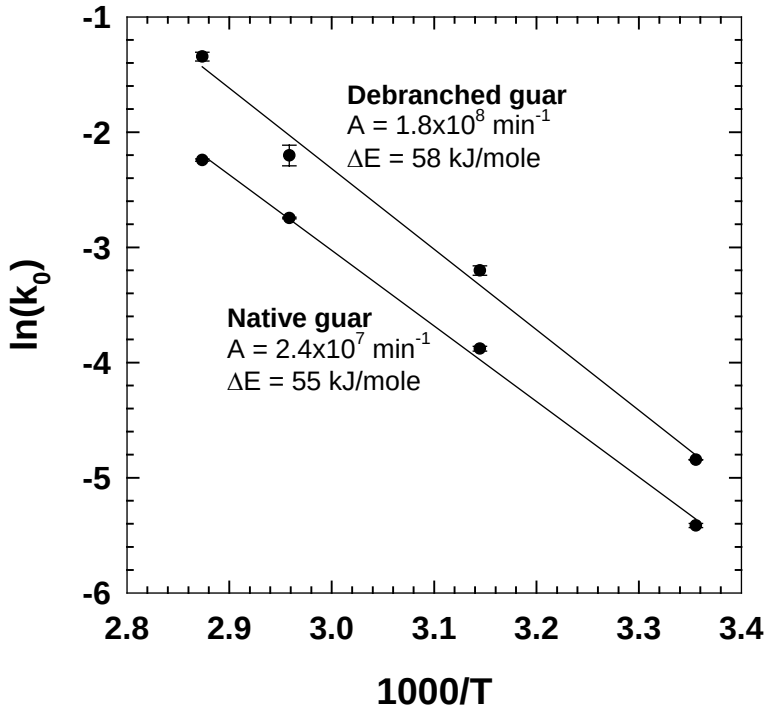

Figure 5.10: Effect of temperature on the hydrolysis of native and debranched guar by β-mannanase. The native guar is debranched by treating with $1.2x10^{-3}$ U/ml of α-galactosidase enzyme for 24 hours. The activity of β-mannanase enzyme in the reaction mixture is $1.2x10^{-5}$ U/ml.

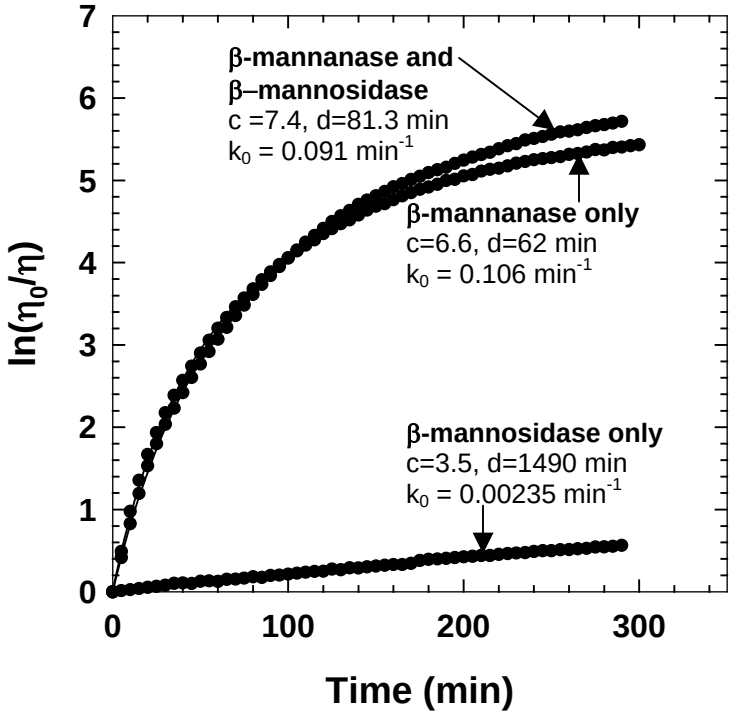

Figure 5.11: Degradation of guar using the mixture of β-mannanase and β-mannosidase. The activities of β-mannanase and β mannosidase enzymes in the solution are $1.2x10^{-5}$ U/ml and $2.4x10^{-3}$U/ml respectively.

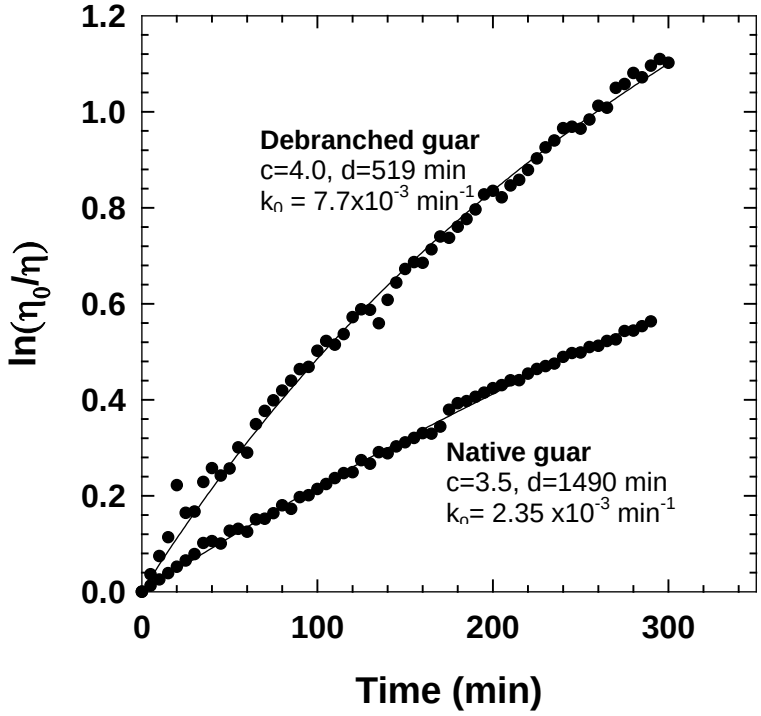

Figure 5.12: Degradation of native and debranched guar by β-mannosidase. The activity of β mannosidase enzyme in the solution is 2.4x10^{-3}U/ml. The native guar is debranched by treating with 1.2x10^{-3} U/ml of α-galactosidase enzyme for 24 hours.

CHAPTER 6

RHEOMETRIC STUDY OF CYCLODEXTRIN-HYDROPHOBE COMPLEXATION IN ASSOCIATIVE POLYMERS

Chapter 6 is essentially a manuscript by Mahammad, S., Roberts, G. W., and Khan, S. A., prepared for submission to ***Macromolecules***

Rheometric Study of Cyclodextrin-Hydrophobe Complexation in Associative Polymers

Shamsheer Mahammad, George W. Roberts and Saad A. Khan
Department of Chemical and Biomolecular Engineering,
North Carolina State University, Raleigh, NC, 27695-7905

Abstract

We develop a new rheology based method to study the CD-hydrophobe complexation in hydrophobically modified associative polymer solutions. The associative polymers have comblike structure with hydrophobic groups randomly attached to the polymer backbone. Intermolecular interactions between the hydrophobic groups form a transient network resulting in thickening of the polymer solutions. On addition of cyclodextrins to the solution, the hydrophobes are encapsulated within the hydrophobic cavity of the cyclodextrins (CD). This reduces viscoelastic properties of the polymer solution by several orders of magnitude. We exploit the existence of a dynamic equilibrium between the CD adsorbed to the hydrophobes and free CD in the solution, to develop a rheology based Langmuir type adsorption isotherm for estimating the binding constant for molecular complexation. The model is based on the assumption that amount of CD adsorbed is proportional to reduction in elastic modulus of the polymer solution due to the encapsulation of the network junctions by CD. The effects of temperature on binding constant are studied to estimate the enthalpy, entropy and free energy change of complexation. Experiments are conducted with both α and β CD at different polymer concentrations and temperatures to estimate the relative strength of binding of the CDs. The complexation with α-CD has a higher equilibrium constant and enthalpy change indicating stronger adsorption of α-CD to hydrophobes compared to that of β-CD.

6.1. Introduction

Hydrophobically modified alkali soluble emulsion (HASE) polymers are water-soluble associative polymers having a comb-like structure with pendant hydrophobic groups randomly attached to the polymer backbone[1]. In aqueous solutions, the hydrophobic groups associate to form a transient network resulting in thickening of the polymer solutions[2,3]; thereby making associative polymers highly desirable as rheology modifiers in variety of applications including paints and coatings[4], aircraft anti-icing fluid[5], personal and homecare products etc[6]. In many of these applications, controlling the hydrophobic interaction often desirabl. The hydrophobic interactions may pose problems in the characterization of the polymer using GPC and light scattering[7]; thereby, necessitating a need to prevent them. It is also sometimes necessary to isolate the hydrophobic interactions to study the factors responsible for their unique rheological properties. The hydrophobic interactions in solutions are affected by pH[8], ionic strength[9], temperature[10] and surfactants[11,12]. However, complete reversibility of the associative phenomena cannot be achieved by these parameters. Further, addition of salts, and/or surfactants into the solution may cause complications in the final use of the polymer solutions.

Considerable interest has recently been generated in modulating the intermolecular hydrophobic interactions by encapsulating the hydrophobic groups via formation of inclusion compounds with cyclodextrins[7,13-16]. Cyclodextrins, doughnut shaped cyclic polysaccharides, can encapsulate the hydrophobes within their annular cavity and deactivate the hydrophobic interactions. This reduces the rheological properties (e.g. viscosity and viscoelasticity) by several orders of magnitude[14-16]. An important feature of the CD-hydrophobe complexation is their reversibility that can be achieved by using suitable surfactants which has higher affinity for CD than the hydrophobes[16] or by addition of enzymes that can degrade cyclodextrins[17].

The complexation of hydrophobes with CD is an equilibrium process and the measurement of their equilibrium constant is important in describing and understanding the thermodynamics of the complexation process. Several classes of compounds which can be included in natural and modified cyclodextrins have been subjected to systematic

thermodynamic studies[18]. These studies focused on finding equilibrium binding constants for the complexation of CD with small organic and inorganic guest molecules. The variation in the binding constant with temperature was used to estimate the free energy, enthalpy and entropy of the complexation. However, only a limited number of studies for the complexation thermodynamics of CD and associative polymers have been reported[15,19,20]. These work mainly focused on determination of binding constant for telechelic associative polymers, which cannot be carried over for our system. In addition, no attempt has been made to estimate other thermodynamic quantities such as enthalpy, entropy, and free-energy change of complexation.

A wide variety of experimental methods including spectrophotometry, ultrafiltration, chromatography, refractometry, conductometry, and NMR have been employed in the determination of thermodynamic quantities for the complexation of CD with small molecules[21]. A few attempts have also been made to extend the above techniques to study the complexation thermodynamics of surfactants and polymers with CD[19,22-30]. Tutaj *et al*[23] used spectroscopic displacement method, in which phenolphthalein was used as a competitive chromophore, to determine the binding constant of β-cyclodextrin with surfactants. The method is based on decolorization of the solution containing phenolphthalein dye in the presence of β-CD. β-CD encapsulates the phenolphthalein dye and decolorizes the solution. The extent of decolorization depends on the amount of CD present in the solution. On addition of surfactants, the phenolphthalein from the CD cavity is displaced by the hydrophobes of surfactant or the polymer. Based on the recovery of color, the adsorption equilibrium constant can be determined. Zeying Ma *et al*[20] used this method for the complexation of hydrophobically modified ethoxylated urethanes with CD. Karkasayan *et al*[19] studied the inclusion complexes between methoxypoly(ethylene glycol)s (MPEG)s bearing one hydrophobic group (phenyladamantyl) per chain and β-CD by capillary electrophoresis.

In all of these studies, measurements were carried out at very dilute concentrations, and change in physical properties of solutions due to the complexation was used to determine

the binding constant. This approach is adequate when the concentrations of host compounds do not affect the adsorption isotherm. However, these methods fail for high viscosity polymer solutions, where dilution of the solution affects the complexation. Taking into account the drawbacks of the conventional methods, we develop a new rheometric method in this study; that uses the changes in viscoelastic properties of associative polymer solution due to CD-hydrophobe complexation.

The HASE polymer forms a transient network due to intermolecular hydrophobic interactions. The encapsulation of hydrophobes by CD disrupts the network and reduces network junction density and the dynamic moduli of the polymer solution. The model assumes that the CDs adsorbed on the hydrophobe are in equilibrium with free CDs in solution, following a Langmuir adsorption isotherm. The binding constant for the isotherm is estimated from rheological measurements, assuming the reduction in dynamic moduli due to CD-hydrophobe complexation. The variation in binding constant with temperature is used to estimate the enthalpy and entropy of the complexation.

6.2. Mathematical modeling

6.2.1. Adsorption isotherm

The adsorption of CD to the hydrophobic binding sites on the polymer is given by the following equation,

$$H + CD \underset{k_d}{\overset{k_a}{\rightleftharpoons}} H\text{-}CD \tag{6.1}$$

At equilibrium,

$$K_d = \frac{k_d}{k_a} = \frac{[H][CD]}{[H\text{-}CD]} \tag{6.2}$$

In the above equations, $[CD], [H], [H\text{-}CD]$ represents concentrations of free CD, free and occupied binding sites on the polymer respectively; k_a, k_d are the rate constants for the adsorption and the desorption processes respectively. K_d is known as the binding constant and is equivalent to the inverse of the equilibrium constant for Reaction (6.1).

In the following analysis, we neglect the effect of interactions between hydrophobes on the CD-hydrophobe complexation equilibrium. Hence, the total concentration of the binding sites on the polymer (H_0) is given by,

$$H_0 = [H] + [H\text{-}CD] \tag{6.3}$$

Using Equation (6.3) in (6.2), we get,

$$\frac{[H\text{-}CD]}{H_0} = \frac{[CD]}{K_d + [CD]} \tag{6.4}$$

Equation (6.4) is in the form of the Langmuir adsorption isotherm[21], where K_d measures binding affinity of the complexation. Please note that the higher the affinity of binding, the lower the value of K_d will be.

6.2.2. Rheometric estimation of the binding constant

In this work, we estimate the binding constant based on rheological measurements. From Green Tobolosky theory[31] on rubber elasticity, the high frequency storage modulus or plateau modulus (G_∞) is proportional to junction density in a transient network system.

$$G_\infty = gnkT \tag{6.5}$$

where, g is the proportionality constant, n is the network junction density, k is the Boltzmann constant and T is the absolute temperature. The CDs encapsulate the network junctions and reduces the plateau modulus. We assume that the fractional reduction in the plateau modulus is directly proportional to the fraction of binding sites occupied by CDs.

Hence,

$$\frac{G_{\infty 0} - G_{\infty}}{G_{\infty 0}} = a\frac{[H\text{-}CD]}{H_0} \tag{6.6}$$

In the above equation, G_{∞} and $G_{\infty 0}$ represent the plateau modulus with and without CD respectively. The proportionality constant "a" couples the reduction in the plateau modulus to the amount of the CD adsorbed on the hydrophobes.

It has been shown that the dynamic viscoelastic behavior of associative polymers can be fit into a generalized Maxwell model given by the following equations[2,3,32].

$$G' = \sum_{i=1}^{m} \frac{G_i (\omega\tau_i)^2}{1+(\omega\tau_i)^2} \tag{6.7}$$

$$G'' = \sum_{i=1}^{m} \frac{G_i \omega\tau_i}{1+(\omega\tau_i)^2} \tag{6.8}$$

Here, G_i is the modulus of a relaxation mode with relaxation time τ_i. The multiple relaxation modes arise due to the complexity and nature of the network junctions[33]. In comb-like associative polymers, the nature of network junctions may vary as each polymer molecule may be connected to the network at multiple points.

The limitations of the available rheometers do not allow measurement of the dynamic moduli at high frequencies. One of the easiest and most convenient methods for estimation of plateau moduli is to extrapolate the Maxwell model to high frequencies.

$$G_{\infty} = \sum_{i=1}^{N} G_i \tag{6.9}$$

However, we show in the following experimental sections that, at any CD concentration, the ratio of dynamic moduli with and without CD (G'/G'_0 or G''/G''_0) remains the same at all frequencies. Hence,

$$\frac{G_\infty}{G_{\infty 0}} \approx \frac{G'}{G'_0} \approx \frac{G''}{G''_0} = \theta \qquad (6.10)$$

Using Equations (6.10) and (6.4) in (6.6), we have,

$$1-\theta = \frac{a\left[CD\right]}{K_d + \left[CD\right]} \qquad (6.11)$$

The Equation (6.11) indicates that "θ" will become negative at very high CD concentrations, if a>1.0. Since the negative elastic modulus does not have a physical meaning, the above equation is valid in the CD concentration range; where, value of θ lies between 0 and 1.

Karlson *et al*[14,15] developed a similar method to estimate the binding constant for associative polymer assuming that the Newtonian viscosity $\eta_0 \alpha G_\infty \tau \alpha nkT\tau$. This assumption is true only when the dynamic behavior of polymer follows a single element Maxwell model, but cannot be applied to the HASE polymer, as the dynamic behavior of HASE polymer solution shows multiple relaxation modes.

As shown later in Figure 6.4, 30 moles of CDs are required to completely saturate one mole of the hydrophobes, i.e., only 1 mole of CDs out of 30 moles of CD added to the solution binds to the hydrophobes. Hence we substitute total CD concentration (C) for free CD concentration in Equation (6.11).

$$1-\theta = \frac{a\,C}{K_d + C} \qquad (6.12)$$

We conduct dynamic frequency sweep tests on HASE polymer solutions with different CD concentrations to develop a "θ" vs. CD concentration curve. The curve is fit to Equation (6.12) to estimate the binding constant for CD-hydrophobe complexation.

6.2.3. Thermodynamics of CD-hydrophobe complexation

The relation between the free energy change (ΔG) and the binding constant of the complexation process can be written as,

$$\Delta G = \Delta H - T\Delta S = RT \ln\left(K_a\right) \tag{6.13}$$

Here, ΔH, ΔS represents the enthalpy and entropy change of the complexation process respectively. K_a is the binding constant given in terms of activities ("a") of chemical species participating in the complexation process and the activity of each species can be given by the product of their activity coefficient (γ) and the concentrations. Hence,

$$K_a = \frac{a_H a_{CD}}{a_{H-CD}} = \left(\frac{\gamma_{CD}\gamma_H}{\gamma_{H-CD}}\right)\left(\frac{[H][CD]}{[H\text{-}CD]}\right) = K_\gamma K_d \tag{6.14}$$

K_γ represents the binding constant in terms of activity coefficients.

Using Equation (6.14) in (6.13)

$$RT \ln\left(K_d\right) = \Delta H - T\Delta S - RT \ln\left(K_\gamma\right) \tag{6.15}$$

For dilute solutions (low molar concentration of CD and hydrophobes), $K_\gamma \approx 1.0$,[34]

Hence, $RT \ln\left(K_d\right) = \Delta H - T\Delta S$ (6.16)

The enthalpy (ΔH) and entropy (ΔS) of complexation can be estimated from the temperature dependence of binding constant using the following van't Hoff relationships[35]

$$\left(\frac{\partial T \ln K_d}{\partial T}\right)_P = -\frac{\Delta S}{R} \tag{6.17}$$

$$\left(\frac{\partial \ln K_d}{\partial T^{-1}}\right)_P = \frac{\Delta H}{R} \tag{6.18}$$

In Equations (6.17), and (6.18), ΔH and ΔS are assumed to be constant within a narrow range of temperatures.

The enthalpy and entropy of complexation of many natural and modified CD have been determined[36]. In the overwhelming majority of cases, both ΔH and ΔS are negative and as a consequence CD complexation is said to be enthalpy driven. However, CDs are quite polar molecules and it is only the annular interior, which is hydrophobic. The hydrophobic interactions between semipolar molecules may have either positive or negative ΔH and ΔS. Consequently one should be cautious in deducing CD complex stabilizing interactions in too great a detail from the magnitude and sign of ΔH or ΔS. However, these values will be helpful in determining the relative roles of different interactions[37] that drive the complexation process.

6.3. Materials and methods

The model associative polymer used in this study is a hydrophobically modified alkali-soluble emulsion (HASE) polymer synthesized by UCAR emulsion systems (Dow Chemicals, USA) through emulsion polymerization. It is a copolymer of methacrylic acid, ethacrylate and a macromonomer (MM) containing hydrophobes as shown in Figure 6.1. The composition of these components in the polymer is 43.57%, 56.21%, and 0.22% by mole, respectively. The hydrophobic macromonomer is composed of C_{22} alkyl hydrophobic groups. These groups are separated from the polymer backbone by a polyethylene oxide (PEO) spacer of 40 moles of ethoxylation. The macromonomer is attached to the polymer backbone through a urethane linkage.

The HASE polymer was supplied in the form of aqueous latex of approximately 26% solids. The latex was dialyzed using a spectraphore cellulosic membrane of 10000 MW against DI water for three weeks. The dialysis removes the low MW polymer molecules and the additives added to stabilize the latex. The dialyzed latex was freeze-dried for two days. The dried powder was stored in an airtight bottle at room temperature. HASE solutions were then prepared in DI water using 1M NaOH required exactly to neutralize the methacrylic acid in the polymer molecules. The required quantities of polymer, NaOH and water were mixed together in a beaker and kept in water bath at 50^0C for a week to ensure the complete dissolution and viscosity development. The final solution pH was approximately 7.0±0.2.

The CDs were purchased from Cerestar, Inc, USA. The moisture content in CD was measured using TA Instruments thermogravimetric analyzer TGA-2950 and found to be approximately 13%.

The polymer solution with and without CDs were prepared by diluting a 5% polymer solution with required quantities of water and CD and kept it in a water bath at 50^0C to get a clear solution without any entrapped air bubbles. All samples were used for the experiments within two weeks of their preparation in order to avoid any possible degradation of the polymer in the presence of NaOH[38].

Dynamic rheological experiments were conducted in a TA Instruments AR-2000 stress controlled rheometer. Either a couette or 40 mm, cone and plate geometry was used for our measurements depending on the viscosity of the solution. A dynamic stress sweep test was carried out before the dynamic frequency sweep test to ensure the applied stress was within the linear viscoelastic region. While measuring the viscoelastic properties at different temperatures, the sample was equilibrated at a particular temperature for at least 15 minutes, and high viscosity PDMS oil was used to prevent evaporation loss.

6.4. Results and discussions

6.4.1. Dynamic behavior of HASE polymer solution

Figure 6.2 shows the dynamic frequency spectrum of aqueous solutions of HASE polymer at 1%, 2% and 3% (w/w) concentrations. The dynamic frequency spectra resemble that of typical entangled polymer solutions with cross over of G' and G" occurring at low frequencies and the cross over frequency decreasing with increasing polymer concentration. The figure shows that the dynamic moduli increase with polymer concentration at any frequency. The dynamic behavior of HASE polymer solutions depends on the topology of the transient network of the polymer and the number of active junctions in the transient network increases with polymer concentration[32]. Since the dynamic moduli are directly proportional to the junction density, the dynamic moduli increase with polymer concentrations. However, the net number of active junctions in the network is directly proportional to the net rate of association and dissociation of hydrophobes[39]. Since this rate depends on applied frequency, the number of effective junctions or dynamic moduli depend on frequency as shown in the figure.

6.4.2. Effect of CD-hydrophobe complexation on rheology

Figure 6.3 shows the effect of α and β-CD on dynamic frequency spectrum of 1% HASE solution. The dynamic moduli decrease with increasing CD concentrations up to the CD concentration of 30 moles/mole MM (Figure 6.4). No further reduction in dynamic moduli is observed when more CD is added to the polymer solution (not shown in the figure). However, the dynamic moduli obtained on complete encapsulation of hydrophobe are lower for α-CD compared to that for β-CD. The CDs encapsulate hydrophobes and dissociate the hydrophobes from the polymer network. This reduces the number of junctions in the transient network resulting in decrease in dynamic moduli.

In Figure 6.4, the ratio ($\theta = G'(\omega)/G'_0(\omega)$) is plotted as a function of CD concentration for both α and β-CD. At the same CD concentration, the reduction in G' values is more for α-

CD compared to that for β-CD indicating the higher affinity of α-CD for adsorption compared to that of β-CD. In Figure 6.4, the symbols show the θ values averaged over the frequency range of 0.1-10 rad/s and the error bars represent the deviation from the average value. It is interesting to note that the ratio remains approximately the same at all frequencies as the height of error bars are very small at all CD concentrations. This behavior can be explained as follows. The network junctions formed due to hydrophobic interactions can have different moduli and relaxation time depending on the number of polymer chains participating in the junction[32]. The dynamic behavior of such networks can be represented by generalized Maxwell model with multiple relaxation modes as given by the Equations (6.7) and (6.8). Each relaxation mode can have different number of network junctions depending on the complexity of the network. The CDs encapsulate equal fraction of junctions at each relaxation modes and hence the reductions in modulus of each relaxation mode are equal. However the encapsulations of junctions by CD do not significantly affect the relaxation times. Hence the extent of reduction in moduli remains the same at all frequencies.

6.4.3. Estimation of binding constants

We have shown that the extent of reduction in elastic modulus on the addition of CD is independent of frequency and this ratio at any frequency can be used to estimate fraction of binding sites on the polymer occupied by CD. We measured dynamic frequency spectrum of HASE solution with different amount of CD in the solution. The ratio of elastic modulus with and without CD measured at a frequency of 1 rad/s was used to calculate "θ" values. The variation of 1-θ as a function of CD concentration was fit to the Equation 6.12 to estimate the parameters "a" and K_d. Experiments were conducted using both α and β-CD; varying polymer concentration and temperatures. The estimated values of "a" and "K_d" are tabulated in Table 6.1

Figure 6.5 represents the 1-θ values for 1% HASE solution measured at a frequency of 1 rad/s and a temperature of 25^0C with different CD concentrations for both α and β-CD. The curves are fit to the adsorption isotherm given by Equation (6.12) to estimate the

parameters "a" and K_d. The parameter "a" represents the proportionality constant relating fraction of binding sites on the polymer hydrophobes occupied by cyclodextrins to the reduction in elastic modulus due to the CD. The value of the proportionality constant is approximately 1.05 for α-CD and 1.2 for β-CD. The binding constant K_d is approximately equal to the amount of CD required to saturate 50% of the binding sites. The values of K_d estimated from the plot are 0.13 mM and 0.76 mM for α and β-CD respectively.

In order to compare the values estimated using rheometric techniques, we looked into literature for binding constant values estimated for HASE or similar associative polymers measured using other measurement techniques. Unfortunately no work have been reported on the estimation of binding constants for HASE polymer. However, extensive data are available in literature for binding constants and other thermodynamic parameters for the complexation of small organic molecules with CD. Rekharsky *et al*[36] list binding constants for complexation of different kinds of small organic guest molecules with different types of CD. For the same host-guest complex, the solution conditions and measurement techniques varied among the researchers. There is no consistency in the reported binding constant values for the same host-guest complex. A few studies have been reported on the estimation of binding constants for some CD-polymer complexation[14,15,40]. Since the binding constant changes considerably depending on the type of polymer, CD and solution conditions, it is not advisable to compare our experimental values to the other similar systems and derive any conclusions. However this study on estimation of binding constant is important in understanding the relative strength and mechanism of association.

6.4.4. Effect of temperature on CD-hydrophobe complexation

Dynamic frequency sweep tests were carried out on HASE solutions containing different amount of CD at different temperatures (25^0C, 30^0C, 35^0C, 40^0C). Adsorption isotherms or 1-θ vs. CD concentration curve as shown in Figure 6.5 were developed using the dynamic frequency spectra measured at different temperatures. The parameters "a" and "K_d" were estimated as described above. The proportionality constant "a" did not change significantly with temperature. The variations in the binding constant with temperatures are

represented in the form of van't Hoff plots given in Figure 6.6. The binding constant K_d increases with temperature for both α and β-CD. The increase in temperature decreases the extent of CD adsorption. Hence at any CD concentration, the amount of CD adsorbed decreases with increasing temperatures.

The Figure 6.6 shows the van't Hoff plots for estimation of enthalpy and entropy of complexation in 1% HASE solution containing either α or β CD. The van't Hoff plots are linear indicating that both the enthalpy and entropy of complexation are constant within the temperature range studied. The enthalpy and entropy change of complexation are found to be negative for the complexation. Hence, we conclude that the CD-hydrophobe complexation is enthalpy driven. Negative enthalpy values represent the expulsion of high-energy water surrounding the hydrophobes on complexation. The negative entropy change represents the involvement of adsorbed CD in hydrogen bonding with bulk water or with CD adsorbed to other chains[18].

The enthalpy and entropy change of complexation for α CD is higher compared to those for β-CD. Since α CD has a smaller cavity volume compared to that of β-CD, it can accommodate very few high-energy water molecules surrounding the hydrophobe within the cavity. Also, α-CD has a smaller annular ring and can fit more snuggly on to the hydrophobes. Hence, the complexation with α-CD is stronger leading to higher enthalpy change compared to that of β-CD.

6.4.5. Effect of polymer concentration on CD-hydrophobe complexation

Figure 6.7 shows the effect of polymer concentration on binding constants for both α and β CD at different temperatures. In the figure, $\ln(K_d)$ is plotted against 1/T as in a van't Hoff plot for estimation of the enthalpy of complexation. The enthalpy and entropy of complexation estimated from van't Hoff plots are shown in the figure. The figure shows that the binding constant for α CD is smaller compared to that for β CD at all temperatures and polymer concentrations.

At any temperature, the binding constant increases with increasing polymer concentration for both α and β CD. However, the enthalpy (ΔH) and entropy (ΔS) change due to the complexation essentially remains the same at both the polymer concentrations for both α and β-CD. This shows that the binding constant K_a (Equation 6.14) is independent of polymer concentration. However, the values of K_γ estimated using Equation (6.14) decreases with increasing polymer concentrations, indicating that intermolecular interactions between polymer hydrophobes affect the CD-hydrophobe complexation. This type of behavior is observed when, two molecules are competing for the same adsorption site[41]. In HASE solution with CD, the interactions between hydrophobes and the interaction between CD and the hydrophobe determine the overall dynamic behavior of the transient network. Since the polymer concentration affects the intermolecular hydrophobic interaction that will also affect the CD-hydrphobe complexation. At higher polymer concentrations, the interactions between polymer molecules obstruct the CDs approaching the binding sites on the polymer. Hence the binding constant, i.e., CD required to saturate 50% of the binding sites increases with increasing polymer concentration.

The complexation of CD with polymer hydrophobes is in a dynamic equilibrium, where the net rate of adsorption and desorption determines the amount of CD binding to hydrophobes. Similarly, amount of free hydrophobes available for binding CDs depend on net rate of association and dissociation of hydrophobic groups. Hence, a detailed analyses taking into account equilibria due to both CD-hydrophobe and hydrophobe-hydrophobe interactions are required to give a better picture of CD-hydrophobe complexation processes in HASE polymer solutions.

6.5. Conclusions

In this study we have developed a rheological technique to study the CD-hydrophobe complexation in associative polymer solutions and a mathematical model that permits quantitative interpretation of the data. The model represents the equilibrium between the bound CD and the free CD in the form of a Langmuir type adsorption isotherm. The model parameters are estimated from the rheological measurements assuming that the reduction in

dynamic moduli due CD-hydrophobe complexation is directly proportional to amount of CD adsorbed on to hydrophobes. The adsorption isotherm is a function of polymer concentration, temperature and the type of CD used. The variations in binding constant with temperature is used to estimate enthalpy and entropy of complexation of CD.

The model fits the experimental data very well and the binding constant estimated using the model increases with increasing temperature and polymer concentration. Since both the enthalpy and entropy change of complexation is found to be negative, the complexation is assumed to be enthalpy driven. The values of binding constant, enthalpy, and entropy change indicate that α CD has higher binding affinity compared to that of β-CD. At higher polymer concentrations, the intermolecular interaction between the polymer molecules affects the CD-hydrophobe complexation and increases the binding constant values.

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Table 6.1: Rheometrically estimated binding constants for the complexation between hydrophobes and (a) α-CD (b) β-CD in HASE polymer solutions. The enthalpy and entropy of complexations estimated from van't Hoff plots are also given in the table.

(a) α-CD

HASE concentration	**1%**		**2%**	
Temperature (K)	**a**	**K_d (mM)**	**a**	**K_d (mM)**
298	1.05	0.13	1.20	1.51
303	1.04	0.18	1.23	1.88
308	1.04	0.16	1.28	2.42
313	1.08	0.35	1.35	3.24
ΔH (kJ/mole)		-46		-40
ΔS (J/mole K)		-136		-135

(b) β-CD

HASE concentration	**1%**		**2%**	
Temperature (K)	**a**	**K_d (mM)**	**a**	**K_d (mM)**
298	1.14	0.76	1.19	1.98
303	1.14	0.85	1.18	2.11
308	1.22	1.25	1.14	2.50
313	1.15	1.13	1.26	3.00
ΔH (kJ/mole)		-25		-22
ΔS (J/mole K)		-80		-78

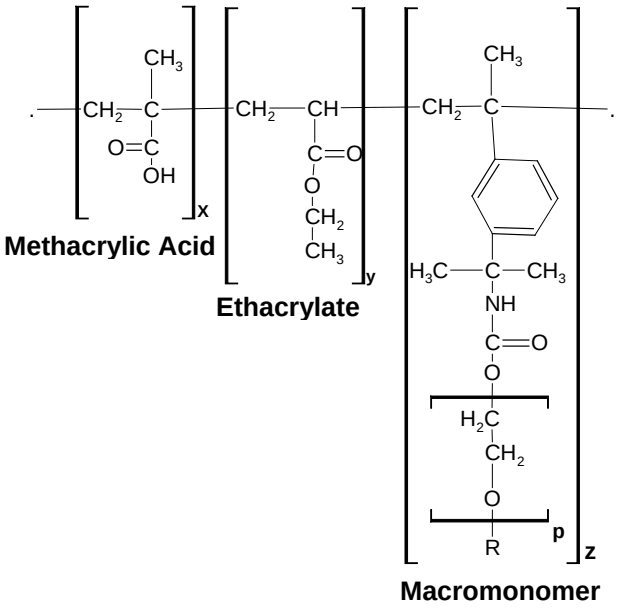

Figure 6.1: Structure of HASE polymer: composition of monomers x/y/z = 43.57%/ 56.21%/ 0.22% by mole; number of moles of ethoxylation (p=40), hydrophobic group (R) in the macromonomer is $C_{22}H_{45}$

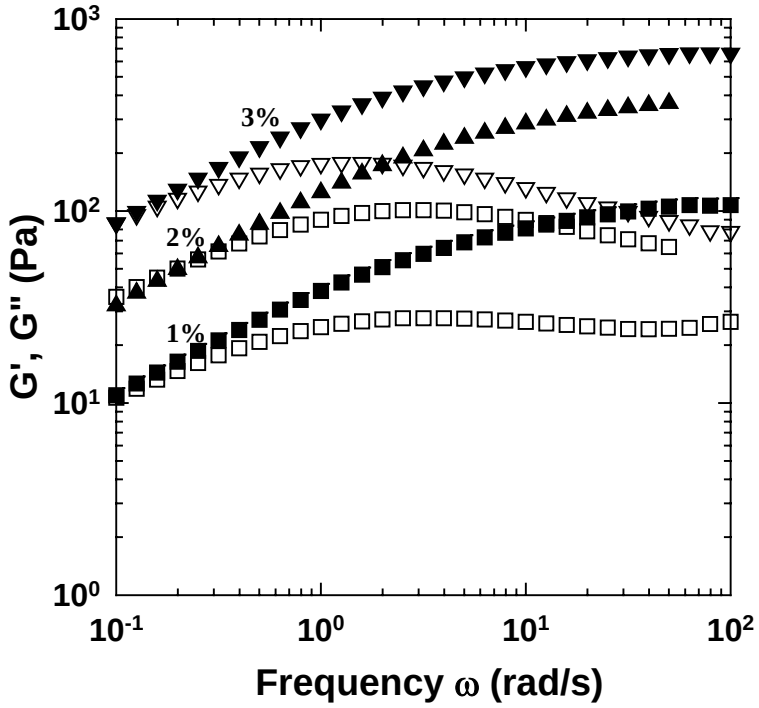

Figure 6.2: Dynamic frequency spectrum of HASE solutions at different concentrations measured at 25^0C. The filled symbols represent the elastic modulus (G') and the open symbols represent the viscous modulus (G").

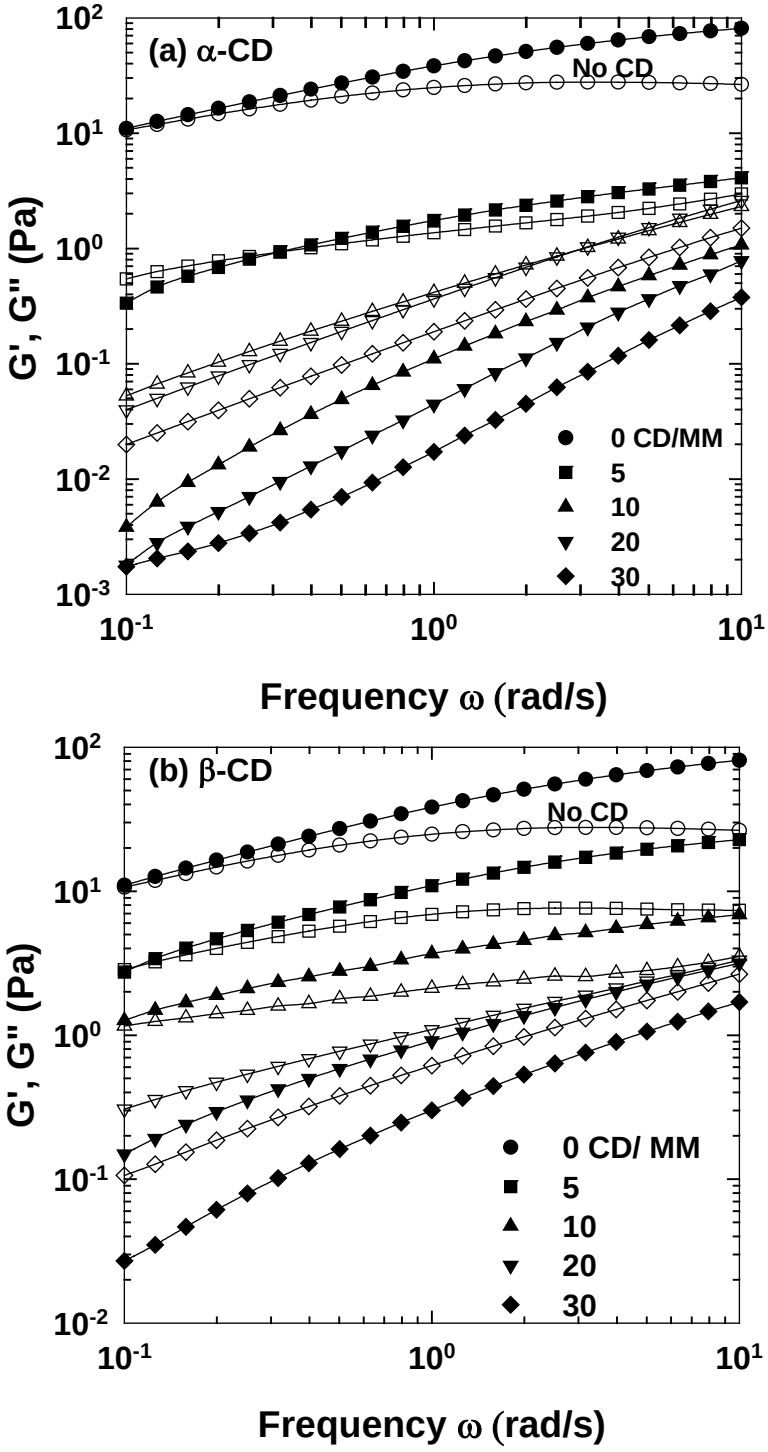

Figure 6.3: Effect of (a) α and (b)β-CD on the dynamic frequency spectrum of 1% HASE solution, measured at 25 ^{0}C. The filled symbols and the open symbols represent G' and G" values respectively. The CD concentrations are in the units of moles/mole MM.

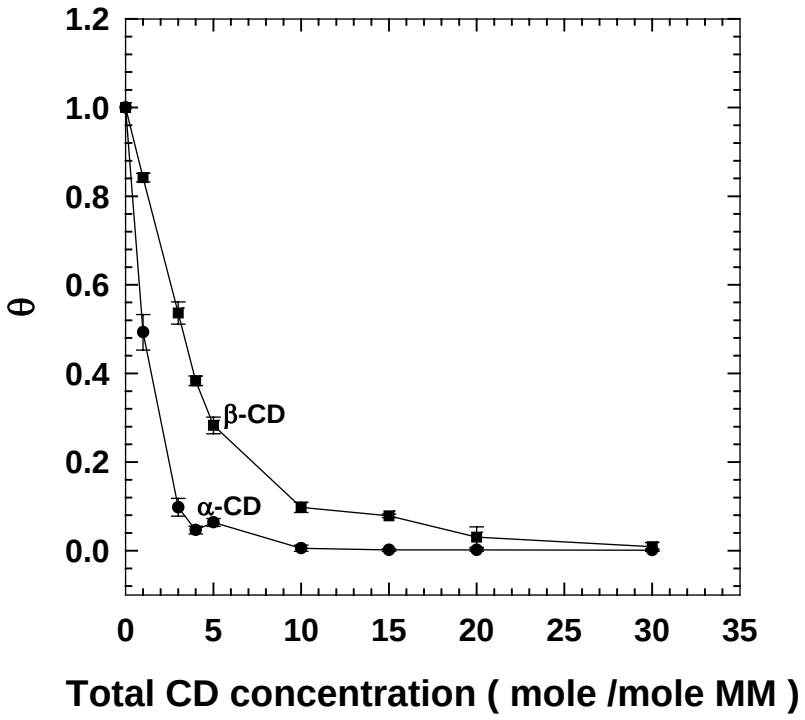

Figure 6.4: Reduction in normalized dynamic moduli (θ) of 1% HASE polymer solution with different amount of CD measured at different frequencies. The symbols show the θ values averaged over different frequencies, and the error bars shows the deviation from the average value.

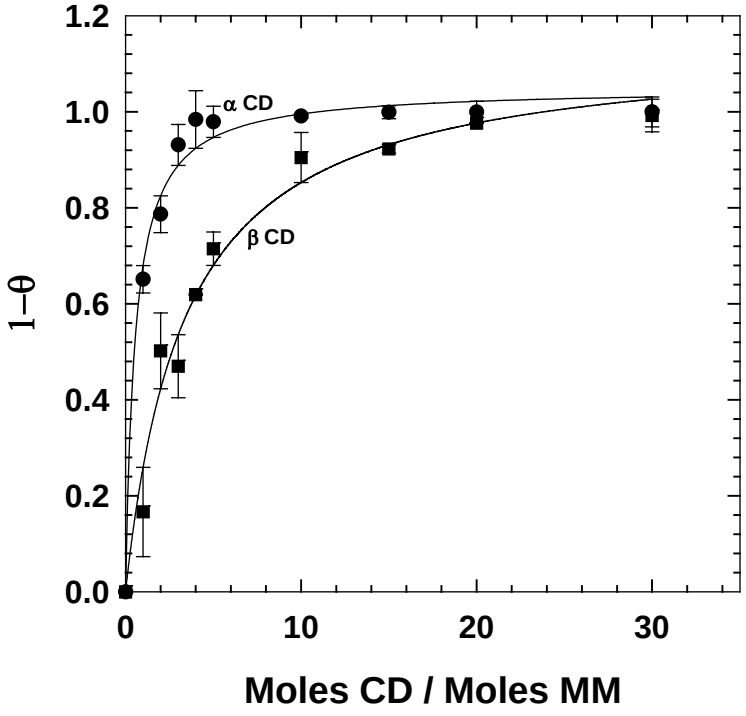

Figure 6.5: Adsorption isotherms for the CD-hydrophobe complexation at 25^0C in 1% w/w HASE solution.

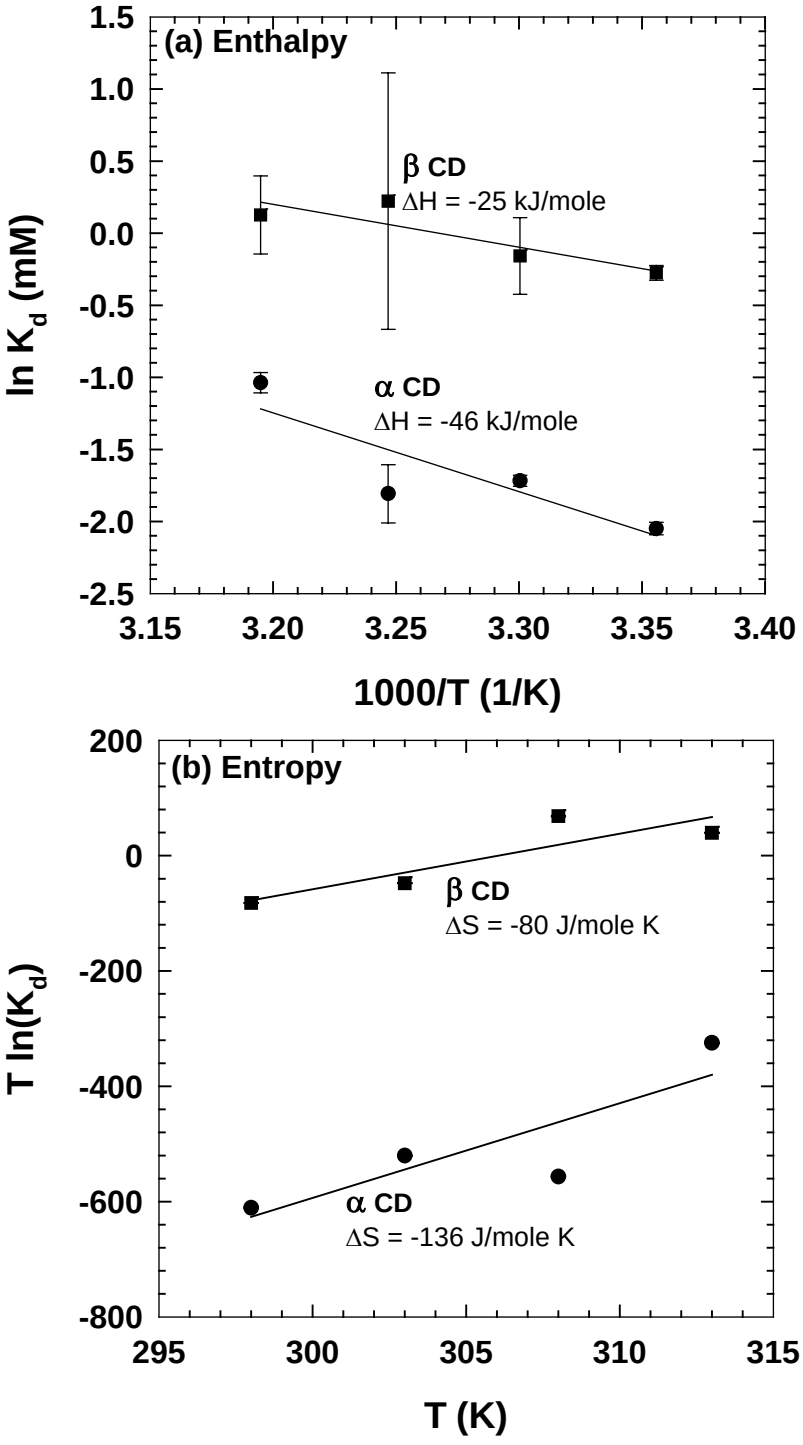

Figure 6.6: van't Hoff plot for estimation of (a) Enthalpy (b) Entropy of CD-hydrophobe complexation in 1% (w/w) HASE solution.

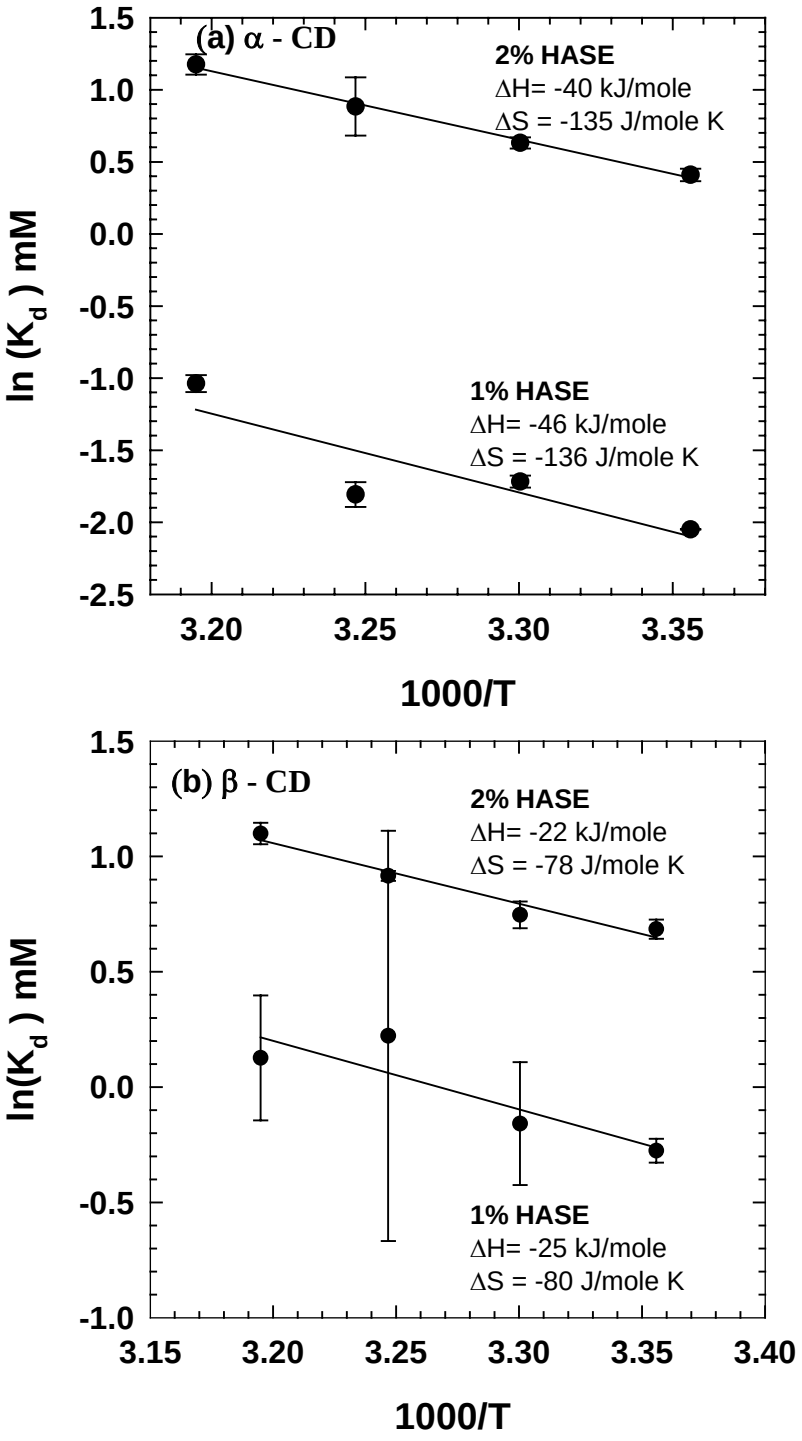

Figure 6.7: Effect of HASE concentration on binding constant (K_d) of (a) α-CD (b) β-CD hydrophobe complexation at different temperatures.

CHAPTER 7

ENZYMATIC MANIPULATION OF HYDROPHOBIC INTERACTIONS IN ASSOCIATIVE POLYMERS

Chapter 7 is essentially a manuscript by Mahammad, S., Roberts, G. W. and Khan, S. A. prepared for submission to ***Macromolecules***

Enzymatic Manipulation of Hydrophobic Interactions in Associative Polymers

Shamsheer Mahammad, George W. Roberts and Saad A. Khan

Department of Chemical and Biomolecular Engineering,
North Carolina State University, Raleigh, NC, 27695-7905

Abstract

A new method is developed to reversibly modulate hydrophobic interactions in associative polymers using cyclodextrins (CD) and enzymes that cause scission of the α-1,4 linkages in cyclodextrins. The associative polymers have a comb-like structure with pendant hydrophobic groups randomly attached to the polymer backbone. The intermolecular interaction between hydrophobic groups forms a transient network resulting in thickening of solutions containing the polymer. The CDs, doughnut-shaped cyclic polysaccharides, encapsulate the hydrophobes within their hydrophobic cavity and eliminate hydrophobic interactions. This results in several orders of magnitude reduction in solution viscosity and other viscoelastic properties. Subsequent degradation of the CDs by enzymes restores the hydrophobic interactions and the original rheological properties. A rheokinetic model is developed to describe the kinetics of the enzymatic reactions. The model accounts for equilibrium between the CD bound to the hydrophobes and free CD in solution. According to the model, the enzyme hydrolyzes only the free CD in the solution, which causes the release of the bound CDs in order to maintain equilibrium. The reaction is assumed to follow Michaelis Menten kinetics and the kinetic parameters are determined by tracking the changes in the viscoelastic properties of the polymer solution during the enzymatic scission of CD. The effects of enzyme concentration, polymer concentration, and temperature on the rate of recovery of the original rheological properties are determined, and are used to validate the model of CD degradation kinetics.

7.1. Introduction

Hydrophobically-modified alkali-soluble emulsion (HASE) polymers are water-soluble associative polymers having a comb-like structure with pendant hydrophobic groups randomly attached to the polymer backbone[1,2] as shown in Figure 7.1. In aqueous solution, the intermolecular interaction between the hydrophobic groups forms a transient network resulting in thickening of the solutions[3]. HASE polymers are widely used as rheology modifiers in a variety of applications including paints and coatings, aircraft anti-icing fluids[4,5].

Although intermolecular hydrophobic interactions between the polymer molecules are attractive from a rheological standpoint, controlling these interactions is often desirable. The hydrophobic interactions in the solution are affected by pH[6], ionic strength[7], temperature[8] and surfactants[9] in the solution. Nevertheless, complete reversibility of the associative phenomena cannot be achieved solely by manipulating these parameters. However, the interaction between hydrophobic groups can be eliminated by encapsulating these groups within the hydrophobic cavity of cyclodextrins (CD) to form inclusion compounds[10-14].

The cyclodextrins are doughnut-shaped cyclic oligosaccharides of glucose units linked together by α-1,4 bonds[15]. The most common CDs, composed of 6,7 or 8 glucose units are denoted as α, β, γ CDs respectively. Various CDs are widely used to encapsulate small drug, fragrance, or flavor molecules in pharmaceuticals, consumer products, or food items[16]. Recently, CDs have been found to encapsulate the hydrophobes attached to polymer chains[13,17,18]. The encapsulation of hydrophobic groups by CD reduces the viscosity and other rheological properties of the solution by several orders of magnitude[10-12].

Although the reduction in the viscosity improves the processability of the polymer solution, it may be necessary to restore the original rheological properties for the intended use of the polymer solution. One of the methods to recover the rheological properties is to add a surfactant that has a higher affinity for CD than the hydrophobes[12,19]. On addition of surfactants, the CDs attached to hydrophobes are desorbed and they form inclusion compounds with hydrophobic groups of the surfactants. However, polymer-surfactant interaction is inevitable in this process[20-22], and complete recovery of the original rheological

properties cannot be achieved[12]. A viable alternative in this regard is to use enzymes that can degrade the CDs.

Cyclodextrins are prone to degradation by microbial and enzymatic attack. Different types of enzymes are found to act on cyclodextrins[23-26]. The amylolytic enzymes that cleave α-1,4 linkages between glucose units of a starch molecule also degrade CD. Another enzyme, cylodextrinase specifically degrades α-1,4 linkages in CDs[23,27]. These enzymes are found to be capable of releasing the encapsulated molecules from the CD cavity[28-31].

The mechanism and kinetics of the enzymatic degradation of CD in the presence of inclusion compounds are not clearly understood. The complexation of CD with host molecules is believed to be an equilibrium process, where the CD adsorbed to the host molecule is in equilibrium with free CD in solution[32]. It is not known whether the enzymes degrade adsorbed CDs or free CDs in solution or both. While degradation of the adsorbed CD can break the inclusion compound, the degradation of free CDs can also release the CD from the inclusion compounds to maintain the equilibrium. In fact, if CD equilibrium is established very rapidly relative to the rate of CD degradation, the exact mechanism is not kinetically relevant.

With CD in aqueous solutions, the activity of α-amylase enzyme increases with increasing CD ring size[25,33], with almost no activity for α-CD. Studies of the active centers on α-amylase enzyme show that the CDs must bind to hydrophobic groups on the enzyme molecule before ring scission can occur[34-36]. Because of the smaller ring size of α-CDs, it is difficult for them to form a complex with hydrophobic binding sites on α-amylase. Similarly, in the presence of polymers or other molecules containing hydrophobic groups, the rate of CD ring scission will depend on the ability of the CD to complex with enzyme active centers, relative to its ability to complex with the other hydrophobic groups in the reaction mixture[37,38].

The kinetics of enzymatic degradation of free natural and modified CDs has been extensively studied with different types of enzymes[25,26,33]. The main focus of these studies has been on estimating kinetic parameters for the enzymatic reaction and studying the effects

of pH, temperature and ionic strength on the kinetic parameters. However, no kinetic studies have been reported on the degradation of CD in the presence of molecules that form inclusion compounds with the CD.

In previous kinetic studies on degradation of CD, chromatographic techniques were used to analyze the degradation products[33,39,40]. These techniques require dilution of the reaction mixture and the conditions (i.e., temperature, pH, ionic strength) that are quite different from that of the reaction mixture. These methods are not appropriate for reaction mixtures containing HASE polymers, as the dilution and the test conditions may affect the polymer solubility and the complexation of CD with the hydrophobes. Also, reaction mixtures containing hydrophobic polymers pose greater difficulty in chromatographic analysis[14], since the hydrophobic groups may affect the performance of chromatographic columns. Taking these drawbacks into account, we have developed an alternate method, based on rheometry, to study the kinetics of the enzymatic degradation of CD in the presence of HASE polymers. Specifically, enzymatic reactions are tracked by monitoring changes in the dynamic moduli of the solution as the reaction proceeds.

The recovery of rheological properties during enzymatic degrdation involves three steps: (1) enzymatic reactions on free CD; (2) dissociation of CD from the hydrophobes to maintain the equilibrium; (3) association of free hydrophobic groups to recover the rheological properties. The degradation of free CD in solution releases the CD adsorbed on the hydrophobes as shown in Figure 7.2. On removal of CD from the hydrophobes, hydrophobic interactions are restored that increases the dynamic moduli. In the following section, we develop a detailed kinetic model for the enzymatic degradation of CD and show how the kinetic parameters can be estimated from rheological measurements.

7.2. Mathematical modeling

7.2.1. Enzymatic degradation of cyclodextrin

The enzymatic reactions involve two steps, ring opening followed by chain scission, as shown in Figure 7.3. In the first step, the CD is cleaved randomly at one of the α-1,4 linkages to form a linear oligosaccharide. In the second step, the enzyme continues to cleave the α-1,4 linkages between glucose units on the linear oligosaccharide, ultimately leading to

the formation of glucose. The ring-opening step and the chain-scission steps will have different kinetic parameters. Moreover, for the chain-scission step, the kinetic rate constant will vary, depending on the position of α-1,4 bond on the oligosaccharide[39]. The enzymatic reactions in both the steps are assumed to follow the Michaelis Menten (MM) kinetics.

$$E + CD \underset{k_{02}}{\overset{k_{01}}{\rightleftharpoons}} (E\text{-}CD) \xrightarrow{k_{03}} E + P_n \tag{7.1}$$

$$E + P_i \underset{k_{ij2}}{\overset{k_{ij1}}{\rightleftharpoons}} (E\text{-}P_i)_j \xrightarrow{k_{ij3}} E + P_j + P_{i-j} \text{ , } for\ i = 2, n\ \&\ j = 1, i-1 \tag{7.2}$$

Equation (7.1) represents the ring-opening step and the Equation (7.2) represents the chain-scission step. In the above equations, P_i represents an oligosaccharide containing "i" glucose units and "n" is the total number of glucose units in a CD molecule. The enzyme-substrate complexes formed with CD and at the j^{th} bond of an oligomer molecule with "i" glucose units are represented by (E-CD) and $(E\text{-}P_i)_j$ respectively.

Cyclodextrin binds to the hydrophobic sites (H) on the HASE polymer forming an inclusion compound, represented as H-CD.

$$H + CD \underset{k_d}{\overset{k_a}{\rightleftharpoons}} H\text{-}CD \tag{7.3}$$

Since enzymes are proteins that may contain hydrophobic/hydrophilic amino-acids[41-44], the enzymes may interact with the HASE polymer to form enzyme-polymer complexes, as represented as E-Π.

$$E + \Pi \underset{k_{de}}{\overset{k_{ae}}{\rightleftharpoons}} E\text{-}\Pi \tag{7.4}$$

In Equations (7.3) and (7.4), H and Π represent the binding sites on the polymer for the adsorption of CD and enzyme molecules respectively. Applying pseudo steady state approximations for all the complexes in Equations (7.1), (7.2), (7.3) and (7.4), we get,

$$\frac{d[E\text{-}CD]}{dt} = k_{01}[E][CD] - (k_{02} + k_{03})[E\text{-}CD] \simeq 0 \tag{7.5}$$

$$\frac{d[E\text{-}P_i]}{dt} = k_{ij1}[E][P_i] - (k_{ij2} + k_{ij3})[E\text{-}P_i] \simeq 0 \tag{7.6}$$

$$\frac{d[H\text{-}CD]}{dt} = k_a[H][CD] - k_d[H\text{-}CD] \simeq 0 \tag{7.7}$$

$$\frac{d[E\text{-}\Pi]}{dt} = k_{ae}[E][\Pi] - k_{de}[E\text{-}\Pi] \simeq 0 \tag{7.8}$$

The last two equations are equivalent to assuming that Reactions (7.3) and (7.4) are in equilibrium.

From Equations (7.5), (7.6), (7.7) and (7.8),

$$[E\text{-}CD] = \frac{k_{01}}{k_{02} + k_{03}}[CD][E] = \frac{[CD][E]}{K_0} \tag{7.9}$$

$$[E\text{-}P_i]_j = \frac{k_{ij1}}{k_{ij2} + k_{ij3}}[P_i][E] = \frac{[P_i][E]}{K_{ij}} \tag{7.10}$$

$$[H\text{-}CD] = \frac{k_a}{k_d}[H][CD] = \frac{[H][CD]}{K_d} \tag{7.11}$$

$$[E\text{-}\Pi] = \frac{k_{ae}}{k_{de}}[E][\Pi] = \frac{[\Pi][E]}{K_\Pi} \tag{7.12}$$

In the above equations, K_0 is the Michaelis constant for degradation of CD and K_{ij} is the Michaelis constant for the degradation of the j^{th} bond on an oligomer with "i" glucose units. The parameters K_d, K_Π are the binding constants for CD-hydrophobe and enzyme-polymer complexation respectively.

From the enzyme mass balance, the total concentration of enzymes (E_T) is given by,

$$E_T = [E] + [E\text{-}CD] + [E\text{-}\Pi] + \sum_{i=2}^{n}\sum_{j=1}^{i-1}[E\text{-}P_i]_j \tag{7.13}$$

Using Equations (7.9) - (7.12) in (7.13),

$$[E] = \frac{K_0 E_T}{K_0 + CD + \frac{K_0[\Pi]}{K_\Pi} + \sum_{i=2}^{n} \frac{K_0}{K_i} P_i} \tag{7.14}$$

where,

$$\frac{1}{K_i} = \sum_{j=1}^{i-1} \frac{1}{K_{ij}} \tag{7.15}$$

For simplicity, we assume that the enzymatic degradation of CD occurs only in solution, i.e., that CD complexed with the HASE polymer does not react directly. Hence, the overall rate of disappearance of free CD from the solution is given by,

$$\frac{d[C]}{dt} = -k_{03}[E - CD] + r_d \tag{7.16}$$

The first term on right hand side of the above equation is the net rate of degradation CDs by enzyme and the second term (r_d) is the rate at which CD desorbs from the HASE polymer in order to maintain equilibrium between free and complexed CD. The rate "r_d" can be derived as follows.

From the balance on hydrophobic binding sites, the total concentration of the binding sites (H_0) can be given by,

$$H_0 = [H] + [H.CD] \tag{7.17}$$

Using Equation (7.17) in (7.11),

$$\frac{[H.CD]}{H_0} = \frac{[CD]}{K_d + [CD]} \tag{7.18}$$

$$\text{Now, } r_d = \left(\frac{d[H.CD]}{d[CD]} \right) \frac{d[CD]}{dt} = -\frac{K_d H_0}{\left(K_d + [CD]\right)^2} \frac{d[CD]}{dt} \tag{7.19}$$

Using Equations (7.9), (7.14) and (7.19) in (7.16),

$$\frac{d[C]}{dt} = \frac{-k_{03} E_T [CD]}{\left\{ K_0 + [CD] + \frac{K_0 [\Pi]}{K_\Pi} + \sum_{i=2}^{n} \frac{K_0}{K_i} [P_i] \right\} \left\{ 1 + \frac{H_0 K_d}{\left(K_d + [CD] \right)^2} \right\}} \tag{7.20}$$

From the mass balance on CD, the total concentration of CD (C) is given by,

$$C = [C] + [H\text{-}CD] = \left[1 + \frac{H_0}{K_d + [CD]} \right] [CD] \tag{7.21}$$

Using Equation (7.21) in (7.20),

$$\frac{dC}{dt} = \frac{-k_{03} E_T [CD]}{\left\{ K_0 + [CD] + \frac{K_0 [\Pi]}{K_\Pi} + \sum_{i=2}^{n} \frac{K_0}{K_i} [P_i] \right\}} \tag{7.22}$$

It has been shown that for degradation of CD by amylase or cyclodextrinase enzymes, the substrate binding affinity parameters (or MM constants) K_0, and K_i do not differ significantly from each other[33,39]. Also the ring-opening step is found to be very slow compared to the chain-scission step[39]. Hence, the concentration of oligomers with at least one degradable bond, $\sum_{i=2}^{n} P_i \approx 0$. For CD degrading enzymes, K_0 is of the order of 80mM[39] and the initial concentration of CD in the reaction mixture is 7 mM for 1% HASE solution. Under these conditions, $K_0 + \frac{K_0 [\Pi]}{K_\Pi} >>> [CD]$. Therefore,

$$\frac{dC}{dt} = -k_c' E_T [CD] = -k_c [CD] \tag{7.23}$$

$$\text{Where } k_c' = \frac{k_{03}}{K_0 + \frac{K_0 [\Pi]}{K_\Pi}} \tag{7.24}$$

Our experiments show that 30 moles of β-CDs are required to completely saturate one mole of the hydrophobes, i.e., only 1 mole of CDs out of 30 moles of CD added to the solution

binds to the hydrophobes. Hence we substitute total CD concentration (C) for free CD concentration ([CD]) in Equation (7.21).

Integrating Equation (7.23),

$$C \approx [CD] \approx C_0 e^{-k_c^{'} E_T t} \tag{7.25}$$

In the above equation C_0 represents the initial concentration of total CD in the solution.

7.2.2. Rheokinetics of enzymatic reactions

According to transient network theory[45], the high frequency modulus or plateau modulus (G_∞) is directly proportional to number of network junctions in a network,

$$G_\infty = gnkT \tag{7.26}$$

where, g is a constant usually taken as unity; n is the junction density, k is the Boltzman constant and T is the absolute temperature. We have observed that the dynamic moduli of HASE polymer solutions decrease on addition of CD to the solution, due to encapsulation of network junctions by CD. We assume that the reduction in the plateau modulus is directly proportional to amount of CD adsorbed to the hydrophobes as given by the following equation.

$$\frac{G_{\infty 0} - G_\infty}{G_{\infty 0}} = a \frac{[H - CD]}{H_0} \tag{7.27}$$

The parameters G_∞, $G_{\infty o}$ represents the plateau modulus with and without CD, respectively.

The proportionality constant "a" couples the reduction in plateau modulus to the fraction of hydrophobic sites on the HASE polymer occupied by CD ([H-CD]/H_0). Our experiments show that the reduction in dynamic moduli with CD is independent of frequency. Hence,

$$\frac{G_{CD}^{'}}{G_0^{'}} = \frac{G_\infty}{G_{\infty 0}} = \theta \tag{7.28}$$

where, G'_{CD}, G'_0 represent the elastic modulus (G') values at any frequency with and without CD respectively.

Using Equations (7.27) and (7.28) in (7.18) and assuming $C \approx [CD]$,

$$1-\theta = \frac{aC}{K_d + C} \tag{7.29}$$

The Equation (7.29) indicates that "θ" will become negative at very high CD concentrations, if a>1.0. Since the negative elastic modulus does not have a physical meaning, the above equation is valid in the CD concentration range; where, value of θ lies between 0 and 1.

The equations for rate of change in θ during enzymatic reactions can be derived using Equation (7.25) in (7.29)

$$1-\theta = \frac{a.C_0 e^{-k_c' E_T t}}{K_d + C_0 e^{-k_c' E_T t}} \tag{7.30}$$

Values of θ are obtained by measuring elastic modulus (G') of the polymer solution during the enzymatic reactions. The variation in θ with time can be fit to equation (7.30) to estimate the parameters a, K_d and k_c'.

The kinetic rate constant k_c, varies with temperature according to Arhenius equation[46].

$$k_{cat} = A \exp\left(\frac{-\Delta E}{RT}\right) \tag{7.31}$$

Here ΔE is the activation energy, A is the frequency factor, T is the absolute temperature and R is the gas constant.

7.3. Materials and methods

The model associative polymer used in this study was a hydrophobically-modified alkali-soluble emulsion (HASE) polymer synthesized by UCAR Emulsion Systems (Dow Chemicals, USA). It is a copolymer of methacrylic acid, ethacrylate and a macromonomer containing hydrophobic groups as shown in Figure 7.1. The compositions of these components in the polymer are 43.57%, 56.21%, and 0.22% by mole respectively. The

hydrophobic macro monomer is composed of C_{22} alkyl hydrophobic groups. These groups are separated from the polymer backbone by a polyethylene oxide spacer containing 40 moles of ethoxylation. The macro monomer is attached to the polymer backbone through a urethane linkage. The polymer was supplied as an aqueous latex of approximately 26% solids. This latex was dialyzed using a spectraphore cellulosic membrane of 10000 MW against DI water for three weeks, changing water once every two days. The dialysis removes low MW polymer molecules and the additives used to stabilize the latex. The dialyzed latex was freeze-dried for 2 days under a vacuum of 100 mTorr. The dried powder was stored in an airtight bottle at room temperature.

The β-CD used in this study was purchased from Cerestar, Inc., USA. The moisture content in CD was measured using a TGA-2950 thermo gravimetric analyzer (TA Instruments, USA). The moisture content in the CD was approximately 13%.

HASE solutions were prepared in DI water using sufficient 1M NaOH to neutralize the methacrylic acid in the polymer. The required quantities of polymer, NaOH, β-CD and water were mixed together in a beaker and kept in a water bath at 50^0C to ensure complete dissolution and viscosity development. The samples were removed from the water bath after a clear solution without entrapped air bubbles was obtained. The final solution pH was 7.0±0.2. The samples were used for the experiments within two weeks of their preparation in order to avoid degradation of the polymer in the presence of NaOH[47].

A commercial α-amylase enzyme (Clarase) was used for this study. The enzyme was a gift from Genencor Inc. USA. The Clarase enzyme is a fungal α-amylase enzyme derived from a selected strain of *Aspergillus Oryzae*. It was supplied in the form of a liquid solution with a specific gravity of 1.15 g/ml. The activity of the enzyme in the solution was 4000 SKBU/g. One SKBU is the concentration of enzyme required to dextrinize 1.0 g of limit dextrin per hour under assay condition[48]. The optimum pH and temperature for the enzyme activity are 5.5-6.5 and 40-50^0C respectively.

The enzymatic reactions were carried out *in situ* in a TA Instruments AR-2000 stress controlled rheometer using couette geometry. Polymer solution with a known amount of β-CD was equilibrated at the desired temperature in a water bath. The preheated HASE

solution with β-CD was then mixed with the desired amount of enzyme and immediately loaded into the rheometer, which had previously been heated to the test temperature. Dynamic time sweep test was carried out to track the enzymatic reactions. The test was carried out at an oscillatory stress of 1 Pa and the dynamic moduli were measured at a frequency of 1 rad/s at five minutes interval. The reaction was stopped when the dynamic moduli values reached a plateau. High viscosity PDMS oil was used to prevent evaporative loss during the reactions at temperatures above room temperature.

7.4. Results and discussions

7.4.1. Effect of CD-hydrophobe complexation on the rheology of HASE polymer

Figure 7.4 shows the effect of β-CD on the dynamic moduli, G' and G", of 1% HASE solution measured at 25^0C and pH 7. In the figure, the ratios of elastic moduli with and without CD are plotted against the CD concentration. The symbols show the average values of θ measured over a frequency range of 0.1-10 rad/s; the error bars represent the deviations in θ from the average value. The figure shows that the dynamic moduli decrease on addition of CD and the most reduction is achieved at around 25 moles of CD/mole of macromonomer. The figure also shows that at any CD concentration, the extent of reduction is same at all the frequencies, thus confirming Equation (7.28), i.e., the ratios G'_{CD}/G'_0 and G''_{CD}/G''_0 are approximately equal at all CD concentrations and frequencies. This behavior can be explained as follows.

The hydrophobic interactions between HASE molecules form a transient network with the network junctions that have different moduli and relaxation times depending on the number of polymer chains participating in the junction. The dynamic behavior of such networks can be represented by the generalized Maxwell model with multiple relaxation modes as given by the following equations[49].

$$G' = \sum_{i=1}^{n} G_i \frac{(\omega\tau_i)^2}{1+(\omega\tau_i)^2}$$

$$G'' = \sum_{i=1}^{n} G_i \frac{\omega\tau_i}{1+(\omega\tau_i)^2} \qquad (7.32)$$

$$where,\ G_i = gn_ikT$$

In the above equations G_i, is the modulus of a relaxation mode with relaxation time τ_i. The G_i are proportional to the junction density (n_i). On addition of CD, some of the junctions in each relaxation mode are broken, resulting in a decrease in the junction densities. However, the extent of reduction in each junction density is same for all the modes and the relaxation times do not change significantly. Hence the extent of reduction in moduli remains the same at all frequencies. This shows that it is sufficient to monitor changes in dynamic moduli at any one frequency to track the enzymatic degradation of CD.

7.4.2. Recovery of rheological properties during enzymatic degradation of CD

In Figure 7.5, we compare the dynamic spectra of a 1% HASE solution with that of an identical solution that had been treated with CD concentration of 30 moles/mole of macromonomer and then with 37 SKBU/g of α-amylase. The figure shows the dynamic frequency spectra measured five hours after the addition of enzyme. We find that that the degradation of β-CD restores the hydrophobic interactions and recovers the dynamic spectrum so that G' and G" match those of the original HASE solution.

Figure 7.6 shows the changes in dynamic moduli of 1% HASE solution with 30 moles α-CD/mole macromonomer, when treated with 37 SKBU/g of α-amylase enzyme. Although α-CD is able to reduce the viscoelastic properties of HASE solution by several orders of magnitude, no change in solution rheology is observed on addition of α-amylase. Since α CD has a smaller ring than β-CD, α-amylase may not be able to access its active sites inside the CD ring. This result is consistent with our assumption that the α-amylase does not degrade CDs adsorbed to the hydrophobes.

7.4.3. Estimation of kinetic parameters from rheological experiments

We conducted dynamic time sweep tests, where the dynamic moduli were measured

as a function of time. The experiments were conducted at a stress level ensuring that it lied within the linear viscoelastic region during the entire reaction process. The enzymatic reactions were tracked by monitoring the changes in G' at a frequency of 1.0 rad/s and the results are represented in a plot of θ vs. time. The θ values increased during the degradation of CD, and reached eventually unity indicating complete degradation of CD. The θ vs. time curve was fit to Equation (7.30) using Sigma Plot 8.0 software to estimate the kinetic parameters a, k_c' and K_d. We conducted the enzymatic reactions at different enzyme concentrations, temperatures and polymer concentrations to study the effects of these parameters on reaction kinetics. The initial concentration of the β-CD was maintained at 30 moles/mole macromonomer in all the experiments. Also pH in the reaction mixture was maintained at 7.0, to obtain maximum enzyme activity while ensuring complete solubility of the polymer.

Figure 7.7 shows the changes in θ of 1% HASE solution at 25^0C during the enzymatic degradation of CD in the solution. Reactions were carried out at different enzyme concentrations and the θ values were plotted against E_T.t. E_T is the total enzyme concentration in SKBU/g, and t is the reaction time in minutes. The model Equation 7.30 fits experimental data very well and also indicates that rate constants ($k_c = k_c'E_T$) increase linearly with enzyme concentration, as expected. The k_c' estimated from the plot is $1x10^{-3}$ $(SKBU/g.min)^{-1}$. The estimated value of the binding constant (K_d) from this experiment is approximately same at all enzyme concentrations with an average of 0.93 mM. This suggests that the presence of enzymes in the solution does not significantly affect the CD-hydrophobe complexation. The proportionality constant "a" does not change significantly with enzyme concentration; the average value of "a" is 1.2.

An alternative approach to verify the validity of our model is to estimate K_d using Equation (7.29) from an independent set of experiments. We measured the dynamic frequency spectrum of 1% HASE solution with different β-CD concentrations at 25^0C. Figure 7.8 represents the change in elastic modulus of the HASE solution measured at a frequency of 1 rad/s as a function of β-CD concentration. The experimental data were fit to Equation (7.27) to estimate the parameters "a" and K_d. The values of "a" and K_d were found

to be 1.2 and 0.8mM respectively. The values of K_d estimated from this experiment and the kinetic experiments are approximately same, and the errors are within the experimental uncertainty limits.

7.4.4. Effect of temperature on kinetics of enzymatic degradation of β-CD

Figure 7.9 shows the changes in θ values of a 1% HASE solution during the enzymatic degradation of β-CD at three different temperatures with an enzyme concentration of 26 SKBU/g. The figure shows that the dynamic moduli recover faster with increasing temperature, indicating the rate of degradation of β-CD increases with temperatures. At a fixed enzyme concentration, the kinetic rate constant is a function of k_{03} and K_0 as given by Equation (7.20). Both k_{03} and K_0 can change with temperature[50]. As a result the kinetic rate constant can either increase or decrease depending on the effects of temperature on k_{03} and K_0 values.

Figure 7.10 shows that effect of temperature on the rate constant (k_c). We find that the rate constant increases with temperature following the Arhenius law, as given by Equation (7.31). At higher temperatures, the rate of degradation of the enzyme-substrate complex is more than offset by the decrease in affinity of enzyme to complex with substrate. Hence the rate constant increases with temperature. Figure 7.10 also shows that the binding constant (K_d) also increases with temperature, suggesting that the affinity of CD to bind to hydrophobes decreases with increasing temperatures.

7.4.5. Effect of polymer concentration on the kinetics of enzymatic degradation of β-CD

Figure 7.11 shows the changes in θ of 1% and 2% HASE solution during the enzymatic degradation of β-CD. The enzyme concentrations used in this study and the kinetic parameters estimated are given in the figure. In fact, Equation (7.24) predicts that k_c should decrease with increasing polymer concentratin (Π) as a result of hydrophobic interactions between the polymer and the enzymes. In addition, the affinity of polymer hydrophobes to bind CDs changes with polymer concentration and the K_d values will be different for different polymer concentration. The figure shows that K_d increases with increasing polymer concentration.

7.5. Conclusions

The encapsulation of hydrophobes by CD reduces the viscoelastic properties by several orders of magnitude. Subsequent degradation of CD by enzymes enables complete recovery of the original rheological properties. The rate of recovery is dependent on polymer concentration, enzyme concentration and temperature. A rheology-based kinetic model is developed. The model fits the experimental data well and is able to predict the effects of various process parameters on reaction kinetics and on the recovery of the original rheological properties. The kinetic model can also be used to estimate the binding constant for CD-hydrophobe complexation. The binding constants estimated using the kinetic model is approximately equal to that obtained using the adsorption equilibrium model.

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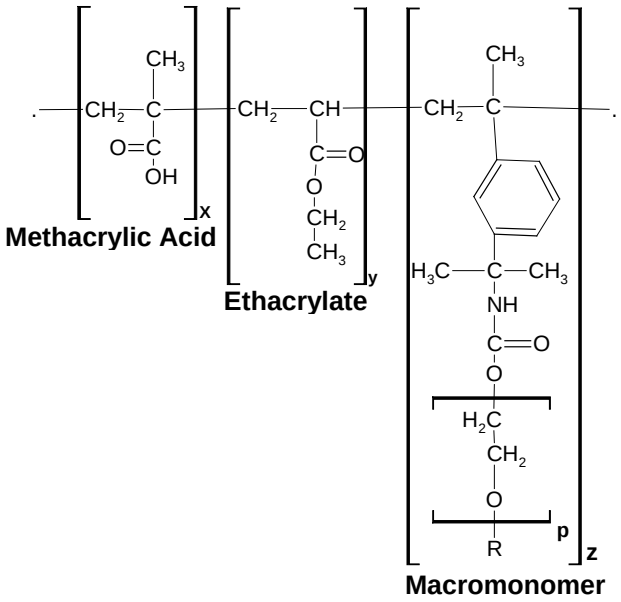

Figure 7.1: Structure of HASE polymer used in this study: composition of monomers x/y/z= 43.57%/56.21%/ 0.22% by mole; number of moles of ethoxylation (p=40), hydrophobic group (R) in the macromonomer is $C_{22}H_{45}$

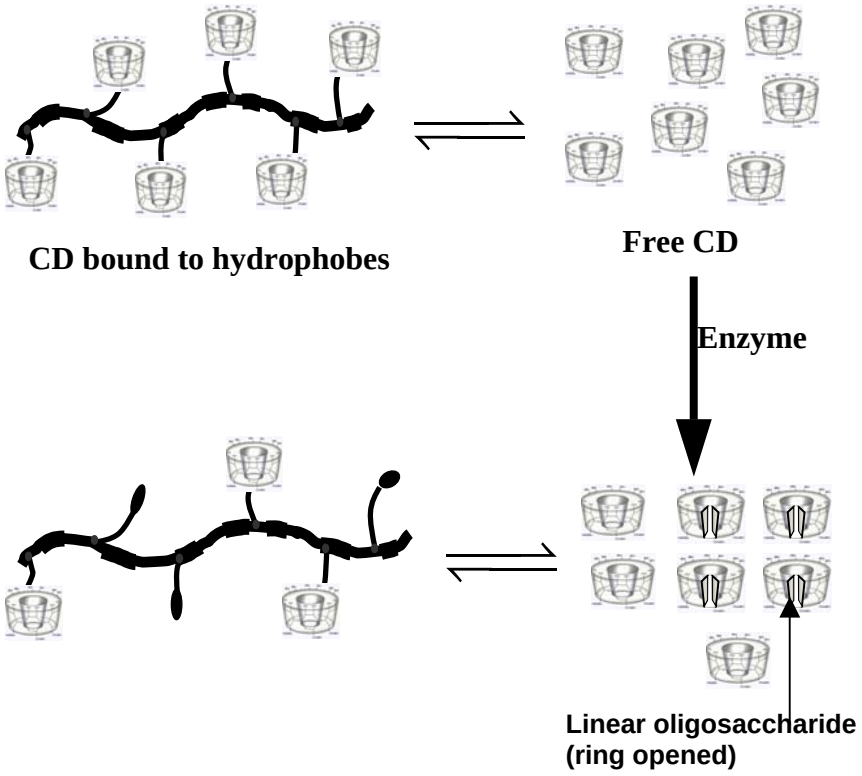

Figure 7.2: Schematic of enzymatic degradation of CD in the presence of CD-hydrophobe inclusion compounds. Enzymes degrade free CD in solution, and the CDs bound to hydrophobes are released in order to maintain the equilibrium.

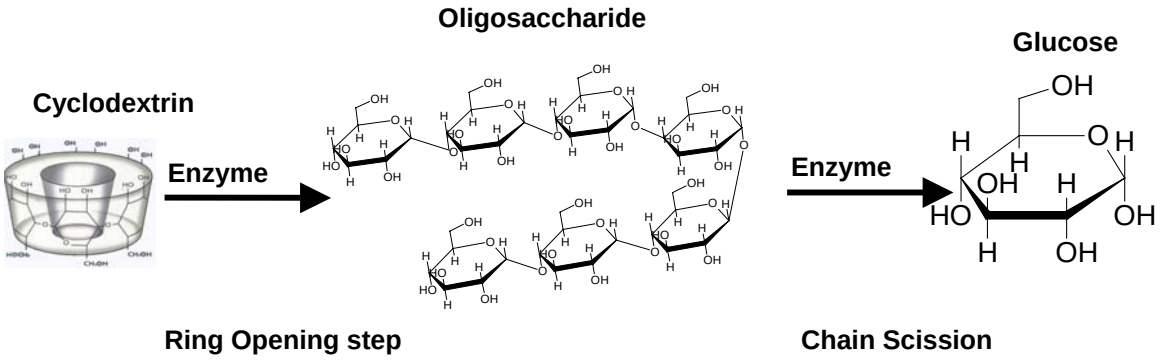

Figure 7.3: Schematic of enzymatic reactions showing ring opening and chain scission steps.

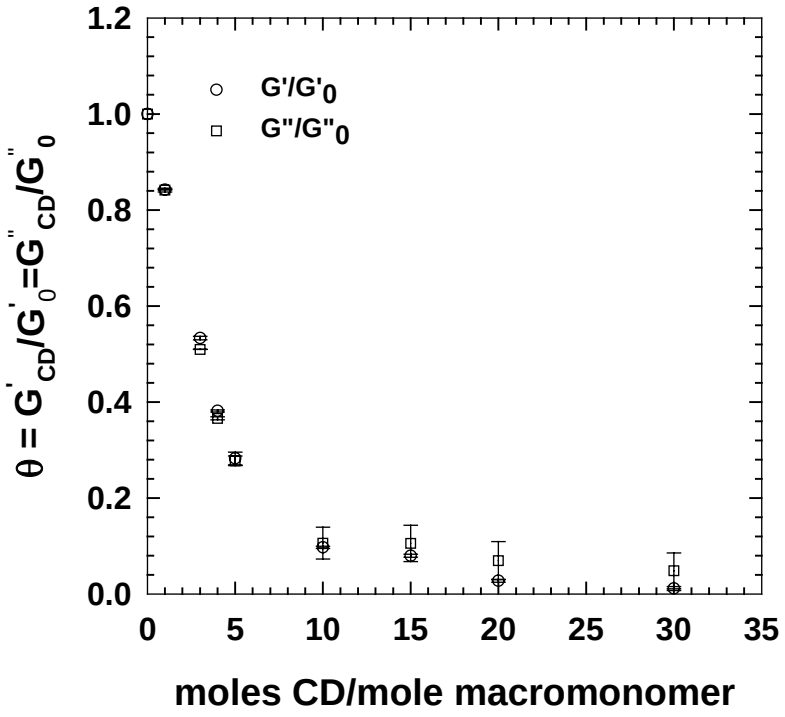

Figure 7.4: Effect of CD on the elastic (G') and viscous (G") moduli of 1% (w/w) HASE solution measured at 25^0C and pH 7. The symbols represent the average values of dynamic modulus over a range of frequency of 0.1-10 rad/s and the error bars represent the deviation from the average value.

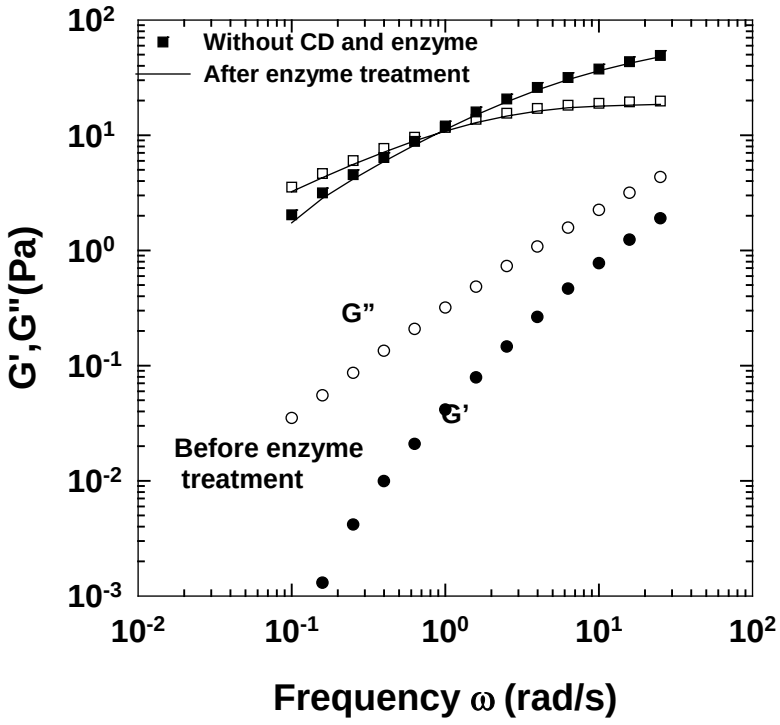

Figure 7.5: Recovery of original dynamic properties of 1% HASE solution at 25^0C and pH 7 after enzymatic degradation of β-CD in the solution for 5 hours. Initial concentration of CD is 30 moles/mole of macromonomer. 37 SKBU/g of enzyme is used for this reaction. Closed symbols show G’ values and open symbols show G” values.

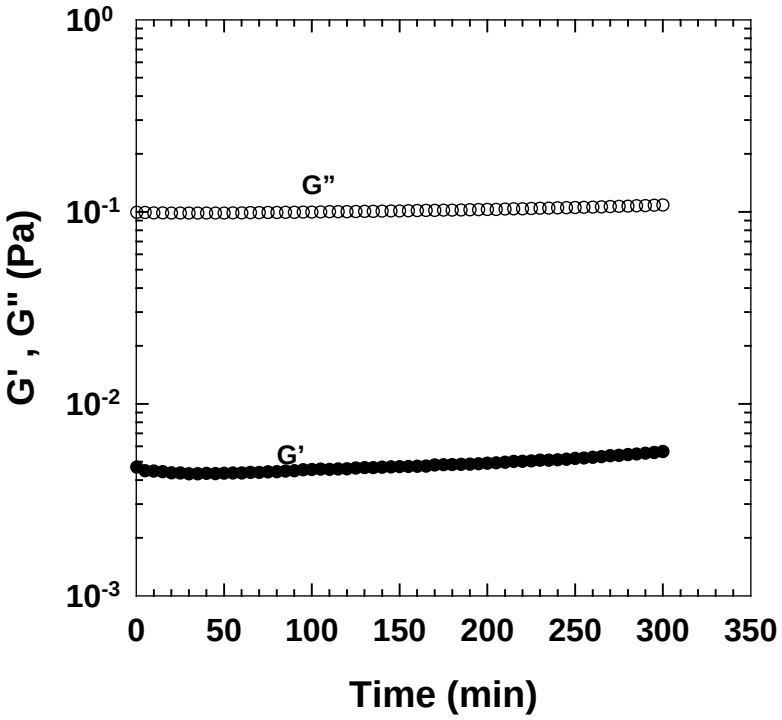

Figure 7.6: Variation in dynamic moduli of 1% HASE solution with 30 moles of α-CD/mole macromonomer during the enzymatic degradation of α-CD at 25^0C and pH 7. The dynamic moduli are measured at a stress of 1Pa and a frequency of 1 rad/s. The enzyme does not degrade α-CD and no changes in dynamic moduli are observed.

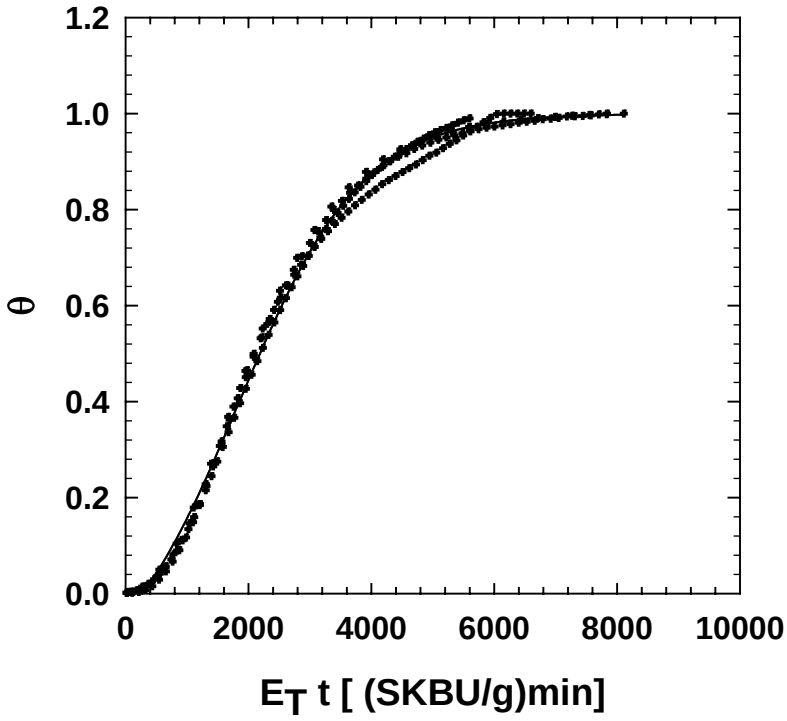

Figure 7.7: Model evaluation: Enzymatic reactions are carried out in 1% HASE solution at 25^0C and pH 7 with enzyme concentrations (E_T) of 19, 26, 37 and 56 SKBU/g. The initial CD concentration in the reaction mixture is 30 moles CD/mole macromonomer. The θ represents the ratio of elastic modulus with and without CD (G'/G'_0) measured at a frequency of 1 rad/s. The solid line shows the model fit.

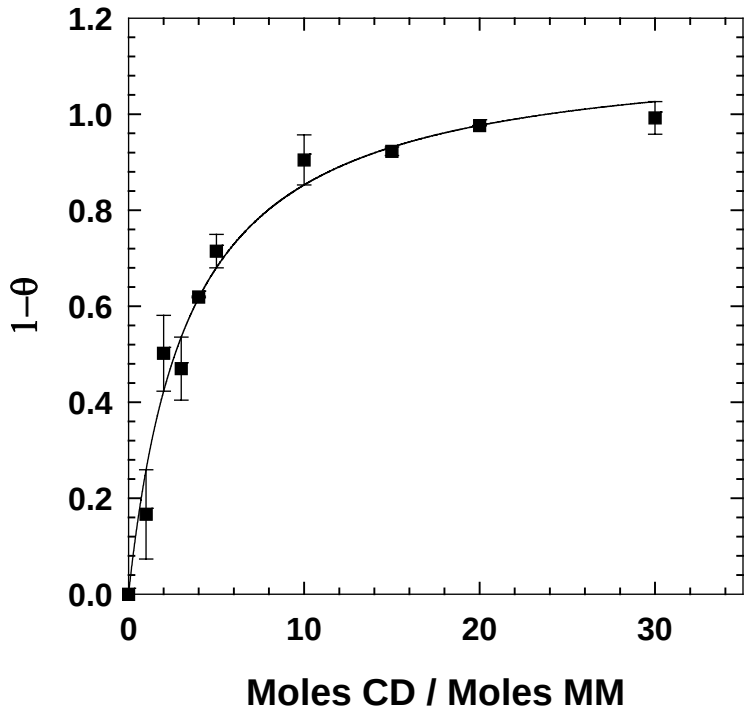

Figure 7.8: Effect of β-CD concentrations on the elastic modulus of 1% HASE at 25 ^{0}C. The elastic modulus is measured at an oscillatory stress of 0.1Pa and frequency of 1rad/s.

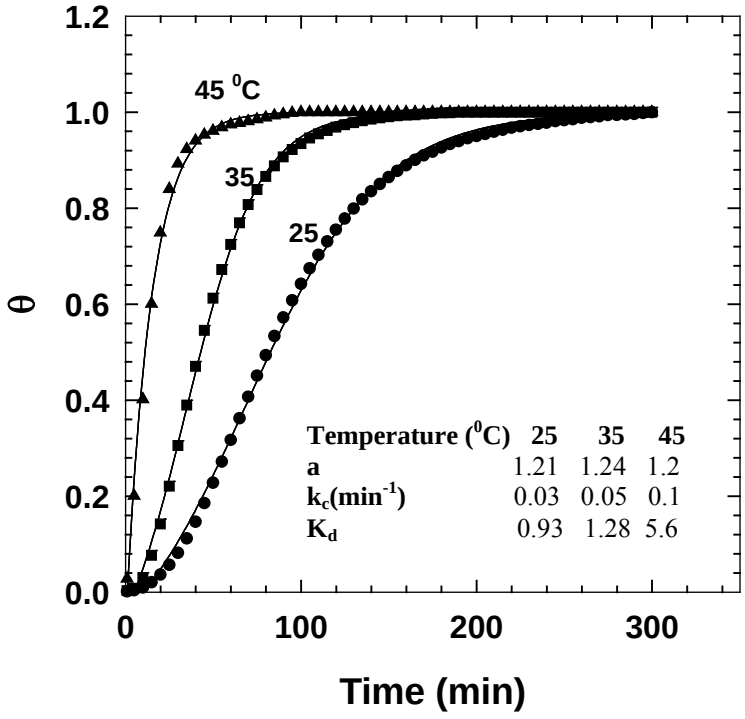

Figure 7.9: Effect of temperature on reaction kinetics: The enzymatic reaction is carried in 1% HASE solution at pH 7.0 with initial CD concentration of 30 moles CD/mole macromonomer. Enzyme concentration = 26 SKBU/g. The solid lines show the model fit.

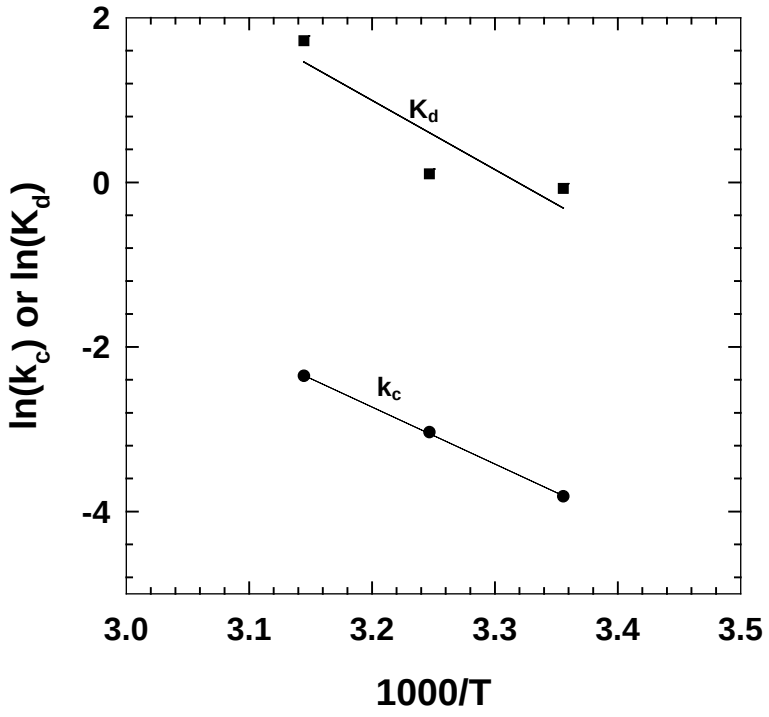

Figure 7.10: Effect of temperature on kinetic rate constant and binding constant: The enzymatic reaction is carried out in 1% HASE solution at pH 7 with initial CD concentration of 30 moles CD/mole macromonomer. Enzyme concentration = 26 SKBU/g.

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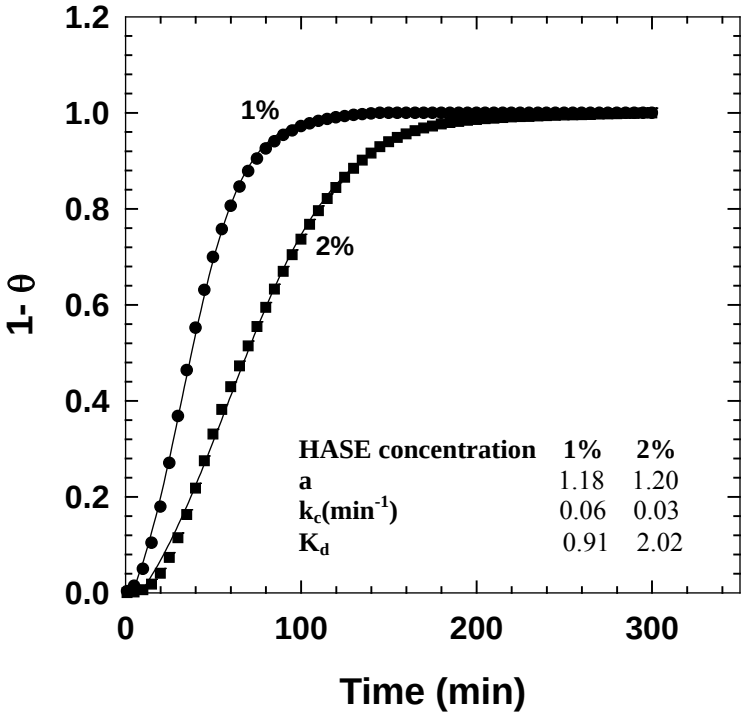

Figure 7.11: Effect of polymer concentration on reaction kinetics: The enzymatic reaction is carried out at 25^0C and pH 7 with initial CD concentration of 30 moles CD/mole macromonomer. Enzyme concentration is 56 SKBU/g for 1% solution and 74SKBU/g for 2% solution. The solid lines show the model fit.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1. Conclusions

In the preceding chapters, we have explored the use of enzymes to control the solution rheology of water-soluble polymers namely, guar galactomannan and hydrophobically modified associative polymers. Our research has mainly focused on understanding the various factors affecting the enzymatic reactions in the polymer solutions. The changes in viscoelastic properties that take place during enzymatic reactions are exploited to study the kinetics of enzymatic reactions. Some of our major findings are summarized below.

- We have developed two mathematical models based on zero order nonrandom scission kinetics and Michaelis Menten (MM) kinetics to predict the changes in molecular weight and molecular weight distribution during the enzymatic depolymerization of guar. These models address many of the unresolved issues in prior investigations such as broadening of molecular weight, random versus nonrandom scission, and effects of galactose branches on the degradation kinetics. The model is evaluated against two sets of experimental data and fits the data well. Both models show that the polydisperisty index increases during the initial stages of the depolymerization. The zero-order model predicts that the PDI can increase for either random or nonrandom bond scission. Hence the increase in PDI during the enzymatic degradation is not necessarily due to nonrandom scission of bonds, as concluded by previous investigators. However, the zero-order model does not take into account the enzyme-substrate complexation behavior and fails to predict accurately the changes in MWD during the entire depolymerization process. The MM kinetic model takes into account enzyme substrate complexation and the effects of galactose branches on the enzyme-polymer complexation. The MM model predicts the degradation to follow first order kinetics and PDI to decrease during the latter stages of the degradation.

- We hydrolysed guar using β-mannanase, α-galactosidase and β-mannosidase enzymes at different concentrations and compositions. The enzymatic reactions are tracked by monitoring the changes in zero shear viscosity of guar solutions. The model developed for the kinetics of enzymatic degradation of guar is used along with viscosity molecular weight relations to develop a rheokinetic model. The rheokinetic model is used to estimate the kinetic parameters from the viscosity vs. time curve. Synergistic hydrolysis is observed between different enzymes acting on the guar molecule. The synergism depends on enzyme concentrations and ratios of enzymes in the reaction mixture. The debranching of the guar by α-galactosiade enzyme enhances the rate of degradation of guar by both β-mannanase and β-mannosidase enzyme. The rheokinetic model agrees well with the experimental data. Both the debranched and the native guar show the same activation energy for β-mannanase action. However, debranching considerably increases the frequency of enzyme-guar interactions.

- We have developed a rheometric model to study the thermodynamics of cyclodextrin (CD) hydrophobe complexation in hydrophobically modified associative polymer (HASE) solutions. The model assumes that the CD-hydrophobe complexation is an equilibrium process following Langmuir type adsorption isotherm. The binding constant for the isotherm is estimated from the changes in viscoelastic properties of HASE solution due to CD-hydrophobe complexation. The adsorption isotherm is a function of polymer concentration, temperature and the type of CD used. Effects of temperature on binding constant are studied to estimate the enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) change of complexation. The study is carried out using both α and β CD at different polymer concentration to estimate the relative strength of binding of the CDs. The complexation with α-CD has higher equilibrium constant and free energy change indicating stronger adsorption of α-CD to hydrophobes compared to that of β-CD. Since both ΔH and ΔS are negative, the complexation is

enthalpy driven. The negative enthalpy indicates that the expulsion of high-energy water surrounding the hydrophobes play a major role in the complexation process; whereas, the negative entropy indicates the formation of hydrogen bonding between CD and bulk water or that between CD molecules attached to different polymer chains. At higher polymer concentrations, the stronger intermolecular interactions between the polymer chains affect the net rate of adsorption and disorption of CDs. This results in increase in binding constant (K_d) values. However, the enthalpy (ΔH) and entropy (ΔS) of the complexation are not affected by polymer concentration.

- We have shown that amylase enzymes can be used to break the inclusion compounds formed between CDs and hydrophobes in HASE polymer solutions. The degradation of CDs restores the hydrophobic interactions between the polymer molecules and recovers the original rheological properties. We have developed a rheokinetic model to study the kinetics of the enzymatic reactions. The model accounts for equilibrium between the CD adsorbed and the free CD in solution. The enzyme hydrolyzes only free CD in the solution and the CDs adsorbed on the hydrophobes are released to maintain the equilibrium. The reaction follows the Michaelis Menten kinetics and the kinetic parameters are estimated by tracking the changes in viscoelastic properties of the polymer solution during the enzymatic reactions. The effects of enzyme concentration, polymer concentration, and temperature are studied on the degradation rate and the subsequent recovery of the original rheological properties. The model fits well with the experimental data and is able to predict the effects of various process parameters on reaction kinetics and the recovery of the rheological properties. The rate constant increases with increasing temperature and enzyme concentrations. The temperature dependency of the rate constant follows Arhenius law. The rate constant decreases with polymer concentrations indicating that hydrophobic interactions between enzyme and polymer inhibit the enzymatic degradation. The kinetic model can also be used to estimate the binding constant for CD-hydrophobe complexation.

8.2. Recommendations

In this dissertation research, we have applied rheological and other scientific principles to characterize and understand various issues concerning the enzymatic reactions in water-soluble polymer solutions. However the scope of the research can be extended to other type of enzymatic reactions and polymer systems. Some of our recommendations for future research are presented below.

8.2.1. Enzymatic cross-linking of guar galactomannans

Chemically cross-linked guar hydrogels are being used in controlled or targeted drug delivery applications[1,2]. The cross-linking reactions are carried out at temperature and pH conditions that may cause degradation of the polymer[3]. Also the unreacted cross-linkers need to be removed before the end use. The enzymatic method offers an environmentally benign alternative to the chemical cross-linking. Moreover the enzymatic reactions occur at mild conditions and the activity of enzymatic reactions can be controlled by changing process conditions such as temperature and pH. Guar can be cross-linked using the enzymes galactose oxidase and catalase[4]. The galactose oxidase enzyme oxidizes the CH_2OH groups on the guar molecule to COOH. The hydrogen peroxide (H_2O_2) produced from the oxidation reaction is detrimental to the enzyme and the catalase enzyme converts H_2O_2 to water. The reactions between OH and COOH groups on the guar molecules will create inter and intramolecular cross-linking, that leads to viscosity enhancement and gelation of the guar solution. Future experiments should investigate the rheological and microstrutural properties of these hydrogels.

8.2.2. Controlled drug release from guar hydrogels via enzymatic degradation of the hydrogels

The diffusion coefficient in guar hydrogels can be changed via enzymatically restructuring the guar molecules[5]. The drug release profile from guar hydrogels are investigated in water with and without enzymes[6,7]. However no mathematical models are

available that can predict the changes in diffusion coefficients of drugs in guar hydrogels during the enzymatic degradation of the hydrogel. The kinetic model developed for the enzymatic degradation of guar can be combined with a relationship between the diffusion coefficient and physical properties of the hydrogel[8] to estimate the changes in diffusion coefficient of drugs in guar hydrogels. The equation for variations in diffusion coefficient with time can be used to estimate the rate of drug release from the hydrogels.

8.2.3. Limiting the β-mannanase activity on guar backbone via hyperentanglement and complexation with xanthan.

Debranching of guar leads to hyperentanglement and subsequent gel formation[9]. The debranched guar also forms complexes with helical polysaccharides[9,10]. We have shown that debranching of guar enhances the rate of degradation by β-mannanase enzyme. The rate of hydrolysis increases because the β-mannanase enzyme can easily access the β-1,4 bonds on the galactose-depleted portion of the guar backbone. Alternatively, we can decrease the rate of degradation of guar by allowing the debranched guar molecules to form hyperentanglement or forming a complex with helical polysaccharides like xanthan. The hyperentanglement or the complexation can obstruct enzyme approaching β-1,4 bonds on the guar backbone, reducing the rate of hydrolysis of guar.

8.2.4. Modulation of rheology of hydrophobically modified polysaccharides

Hydrophobically modified guar (HMG) and its derivatives have attracted many industries because they have been found to be environmentally benign substitute for synthetic associative polymers[11,12]. These polymers are biodegradable and their molecular structure can be further modified using enzymes. The combination of enzymes and CDs can be used to modulate the rheology of solution and gels of HMG. The HMG can be used for drug delivery formulations. The CDs can be used reduce the viscosity of the HMG solutions for uniform loading of drugs into the polymer matrix. Subsequent degradation of CDs will restore the hydrophobic interactions entrapping the drug molecules within the gel network.

8.2.5. Modulation of Rheology of HASE polymer in the presence of surfactants

In many of the applications, HASE polymers are used along with surfactants, where HASE and surfactants impart thickening and surface-active properties respectively[13]. Surfactants can either increase or decrease the viscosity of HASE solutions[14,15]. Controlling these interactions is often necessary to get the required rheological properties without compromising on other applicative properties of the solutions. Cyclodextrins can complex with surfactant hydrophobes[16]. Hence by judicially selecting the type of CDs and their concentration in the solution, the interaction between various molecules in the mixture of HASE, surfactant and CD can be modulated to get required physiochemical properties and optimize their compositions in the mixture.

8.2.6. Functional protein-polysaccharide mixed systems through enzymatic modification:

Both whey protein and guar galactomannans are highly desirable and widely used in food products because of their superior functional properties. Understanding the interactions between whey protein and guar galactomannans on the phase-behavior, rheological and microstructural properties of the mixed system are necessary to optimize their composition in the final food products[17]. The MW and degree of branching of polysaccharide affects the rheological and microstructural properties of protein-polysaccharide systems[18]. Further conformations and molecular architecture of whey protein can be changed by enzymatic cross-linking of the protein molecules[19,20]. Hydrophobic groups on the protein amino acids can be encapsulated by CDs that will in turn modulate hydrophobic interactions in protein molecules. Hence molecular restructuring of guar and whey proteins using enzymes offer a unique method to control the intermolecular interactions between whey protein and guar molecules. This research should utilize rheology, differential scanning calorimetry, confocal microscopy and infrared spectroscopy to study phase behavior, gelation and microstructural properties of the mixed systems, while exploring the effects of temperature, pH, ionic concentration and composition of the protein and the polysaccharide on solution-gel transition and subsequent microstructure development.

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