Study on the Kinetics for Enzymatic Degradation of a Natural Polysaccharide, *Konjac Glucomannan*

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Summary: The enzymatic degradation of konjac glucomannan (KGM) was conducted using β-mannanase from an alkalophilic *Bacillus sp.* in the aqueous medium (pH 9.0) at 30°C. The intrinsic viscosity ($[\eta]$), molecular weight (M_w) and molecular weight distribution (MWD) of the degraded KGM were measured. The mathematical relation between $[\eta]$ and M_w, $[\eta] = 5.06 \times 10^{-4} M_w^{0.754}$, was established. The kinetic analysis reveals a dependence of the rate constant (k) on the period of reaction and the initial substrate concentration (c_0) over the range of substrate concentration (1.0~2.0%) used in this work. The results indicate that the enzymatic degradation of KGM is a complex reaction combining two reaction processes with different orders. In the initial phase of degradation k is inversely proportional to c_0, which is characteristic of a zeroth-order reaction; while in the following phase k is independent of c_0, implying the degradation follows a first-order reaction. The reactivity difference in breakable linkages of KGM, the action mechanism of an enzyme on KGM macromolecules, and the theory concerning the formation of an enzyme-substrate complex and 'substrate saturation' can be used to explain such a kinetic behavior. In addition, the enzymatic degradation of KGM was also carried out using the other enzymes like β-mannanase from a *Norcardioform actinomycetes*, β-glucanase Finizym and a compound enzyme Hemicell as a biocatalyst. By comparing and analyzing the degradation processes of KGM catalyzed by four different enzymes, it can be observed that there is a two-stage reaction with two distinct kinetic regimes over a certain range of degradation time for each of the degradation processes. These results are useful to realize controllable degradation of polysaccharides via an environmental benign process

Keywords: degradation; enzymes; kinetics; konjac glucomannan; polysaccharides

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Introduction

In recent years green chemicals and materials, as well as safe and reliable medicines, have drawn much attention due to the present bad environmental situation caused by overburdensome pollutants and due to the increasing anxiousness about public health caused by the production and use of a large number of synthetic chemicals and medicines. On the other hand, developing and utilizing renewable resources have become a very important policy in many developed and developing countries to decrease the demand for and the dependence on limited or depleting resources like petroleum and to realize the goal of sustainable development. As renewable resources, naturally occurring polysaccharides have been revalued because they are environmentally friendly and they possess great potentials for industrial, agricultural, and medical applications as substitutes for some petrochemical products.[1] Konjac glucomannan (KGM) is a kind of natural polysaccharide and renewable resource from the *Amorphophallus konjac* plant. KGM and its derivatives have aroused a great interest of some reasearchers because of their biological activity and many unique physiological and pharmacological functions. It is composed of D-mannose and D-glucose chains linked by β-1, 4-pyranoside bonds; and the molar ratio of mannose and glucose is 1.6 to 1 or 1.69 to 1, depending on the origin of KGM.[2, 3] There are certain short side branches at the C-3 position of the mannoses and acetyl groups randomly present at the C-6 position of a sugar unit.[4, 5] The acetyl groups frequently range from one per six sugar units to one per twenty sugar units.[5] KGM has been widely used in the food, chemical, textile, oil and cosmetics industry because of its unique rheological properties.[6, 7] It is worth noting that KGM or its derivatives may be attractive for biomedical applications since they possess some interesting biological or immunological activities.[8-10]

However, the molecular weight of original KGM is in the range of 10^6 and its solution exhibits a very high viscosity. When it is degraded to a certain extent, KGM can be modified by means of environmentally benign biocatalysis or chemo-biocatalysis, thus leading to valuable applications. For this reason we focused our study on regularities happening in the enzyme-catalyzed degradation of KGM.[11] Although the enzymatic degradation or hydrolysis of KGM have already been studied,[3, 12-15] these researches mainly involved the nearly complete degradation or hydrolysis of KGM, which aimed to prepare monosaccharides,

disaccharides or oligosaccharides, to analyze the structure of KGM and to study the mechanism of this reaction. To our knowledge, the studies on l controllable or imited degradation of KGM conducted by means of an environmentally benign biocatalysis reaction performed under mild conditions, are seldom reported.

In the present work, we investigated the degradation kinetics of KGM catalyzed by β -mannanase from an alkalophilic *Bacillus sp.* (Mannanase I) and compared the characteristics of the degradation kinetics of KGM catalyzed by four different enzymes, thus laying a theoretical and experimental foundation for a controllable degradation of KGM and other polysaccharides via an enviroenmentally benign process. Some results are reported here.

Experimental

Materials and chemicals. Original Konjac glucomannan (KGM), purified powder (3A-PF-120), was a gift from Multi-Ring Health Products, Ltd., China. The β -mannanase (EC.3.2.1.78) from an alkalophilic *Bacillus sp.* (Mannanase I) and another β -mannanase from a *Norcardioform actinomycetes* (Mannanase II) were provided by the Institute of Microbiology, Chinese Academy of Science, β -glucanase Finizym was given by Novo Nordisk Corp., Denmark and a compound enzyme Hemicell was furnished by Chemgen Corp., USA. These enzymes were used as a biocatalyst in the degradation of KGM, respectively. The other chemicals were analytical grade reagent and used as purchased.

Enzymatic degradation. The enzymatic degradation of KGM as a substrate was run in a glass batch reactor containing a certain volume of reaction medium with the optimum pH for the used catalyst, stirred in a thermostat water bath at 30°C. The reaction system was appropriately varied according to the characteristics and activity of the enzyme used as a biocatalyst in the reaction, as shown in Table 1.

At a prescribed time, ethanol was added to inactivate the enzyme, leading to the end of a reaction. The degraded KGM product was repeatedly washed and reprecipitated with water and ethanol, then followed by lyophilization.

Table 1. Reaction system for the enzymatic degradation of KGM catalyzed by four different enzymes, respectively.

Biocatalyst	Reaction Medium		Enzyme Contcentration		Substrate Contcentration
	Buffer Solution	pH			(%)
Mannanase I	glycine-NaOH	9.0	8	mg/dL	1.0 ~ 2.0
Mannanase II	Na_2HPO_4-citric acid	8.0	2	mg/dL	1.0
Finizym	Na_2HPO_4-citric acid	5.0	0.025	mL/dL	1.0
Hemicell	Na_2HPO_4-citric acid	7.0	0.0005	mL/dL	1.0

Viscosity measurement. The degraded KGM sample was dissolved in distilled water at 0.05 g/dL. The intrinsic viscosity of the solution was measured using Ubbelohde-type viscometer at 30 ± 0.5°C.

GPC Measurement. The molecular weight (M_w) and molecular weight distribution (MWD, or M_w/M_n) of the degraded KGM samples were measured at 40°C by gel permeation chromatography (GPC) using a commercial GPC system equipped with a column (Ultrahydrogel 500, Waters Corp., Milford, MA, USA), a pump (Waters, Model 510) and a differential refractometer (Waters, Model 410). The system was calibrated with a monodisperse polyethylene glycol (PEG) standard. The mobile phase was NaCl aqueous solution. Flow rate was 0.6 mL/min.

Results and Discussion

Change in Intrinsic Viscosity of Enzymatically Degraded KGM Products. Figure 1 illustrates the changes in the intrinsic viscosity [η] of the enzymatically degraded KGM samples by Mannanase I with the reaction degradation time t for different substrate concentrations. The [η] indirectly reflects the molecular weight of the measured polymer sample. It is obvious that [η] decreases rapidly in the initial period of the reaction and then slows down as degradation takes place. The viscosity reduction is greater for the reaction system with a lower substrate concentration.

When the degradation of KGM was catalyzed at the same substrate concentration (1.00 %) by differenrt enzymes, the intrinsic viscosity [η] of the enzymatically degraded products was

found to change with the degradation time t following the same trend as shown in Figure 2. Withou a doubt, a decrease in the intrinsic viscosity $[\eta]$ is caused by the degradation of KGM. A comprehensive analysis of the structural features of KGM, the characteristics of a biocatalyst and its catalytic mechanism in this reaction is helpful to explain the above-described behavior.

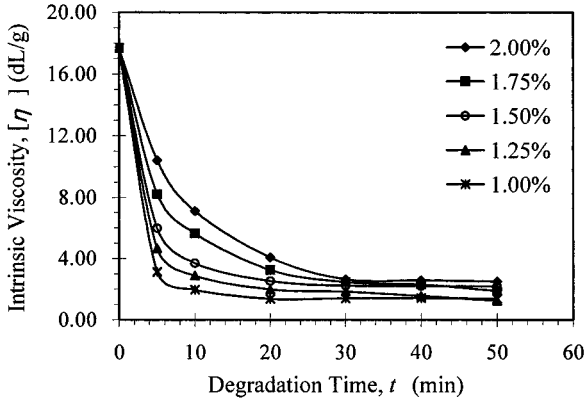

Figure 1. The curves of intrinsic viscosity $[\eta]$ *versus* degradation time t for the enzymatically degraded KGM samples by Mannanase I at different substrate concentrations.

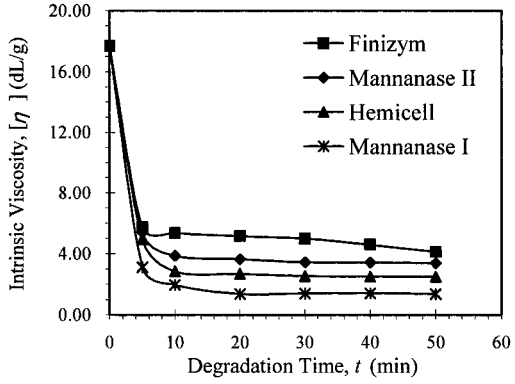

Figure 2. The curves of intrinsic viscosity $[\eta]$ *versus* degradation time t for the enzymatically degraded KGM samples by deferent enzymes at the same substrate concentration, 1.00 %.

Since the macromolecular backbone of KGM is built up of D-mannopyranosyl residue (M) and D-glucopyranosyl residue (G) that are randomly bonded by β-1, 4-glycosidic linkage (→),[12-14, 16] there may exist four different β-1, 4-glycosidic linkages bonding different glycosyl units together: β-mannosidic linkage (M→M), β-glucosidic linkage (G→G), β-mannosyl-glucosidic linkage (M→G) and β-glucosyl-mannosidic linkage (G→M). The studies on the enzymatic and acidic hydrolysis of KGM[12-14, 16] have indicated that theses bonds possess different "strength", which increases following the order M→G, M→M, G→M and G→G. Thus, in the initial period of the enzymatic degradation of KGM the relatively weak bonds, M→G and M→M, can be rapidly broken while the relatively strong links like G→M and G→G remain. As the reaction goes on, the chain length of substrate molecules decreases, leading to a reduction in the susceptible linkages and the active sites of substrate for an enzyme to bind so that the $[\eta]$ or molecular weight of degraded products does not change dramatically.

Establishment of the Relationship between the Intrinsic Viscosity and the Molecular Weight of KGM Samples. The GPC measurements for an original KGM and a series of the degraded KGM samples show that the M_w and MWD of an original KGM are about 9.8×10^5 and 1.46, respectively; and the degraded KGM samples exhibits a wider range of MWD from 1.68 to 1.95. A good linear relationship is observed between LogM_w and Log$[\eta]$ for the degraded KGM solutions as shown in Figure 3. Thus, the mathematical equation, LogM_w = 1.327Log$[\eta]$ + 4.371, is obtained by a linear regression. It means that M_w *versus* $[\eta]$ relationship is in good conformity with the following equation:

$$[\eta] = KM_w^{\alpha} \qquad (1)$$

Based on the calculation, the equation representing M_w *versus* $[\eta]$ relationship for KGM can be established as follows:

$$[\eta] = 5.06\times10^{-4} M_w^{0.754} \qquad (2)$$

N. Kishida et al.[17] studied M_w *versus* $[\eta]$ relationship for partial methylated KGM and obtained the equation

$$[\eta] = 6.37\times10^{-4} M_w^{0.74} \qquad (3)$$

In Eqs. (2) and (3) there are a close α but different K, which is related to the methylation modification of KGM. Eq. (2) can be conveniently used to estimate the molecular weight of KGM by a simple viscometric method.

By using Eq. (2), the [η] *versus t* relationship in Figure 1 and Figure 2 can be transformed into the M_w *versus t* relationship as shown in Figure 4 and Figure 5, respectively.

The above-described change in M_w and MWD suggests that the enzymatic degradation of KGM by β-mannanase proceeds in a random or an endowise mechanism, which is compatible with the catalytic feature of β-mannanase as an endo-acting enzyme.

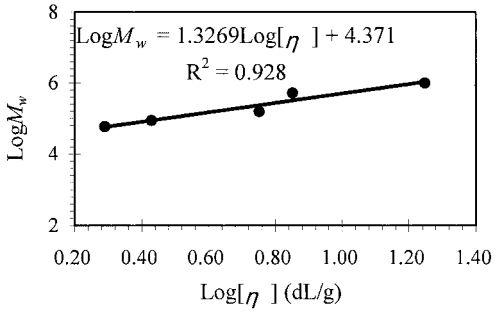

Figure 3. The plot of LogM_w *versus* Log[η] for the aqueous solution of KGM samples prepared by enzyme-catalyzed degradation.

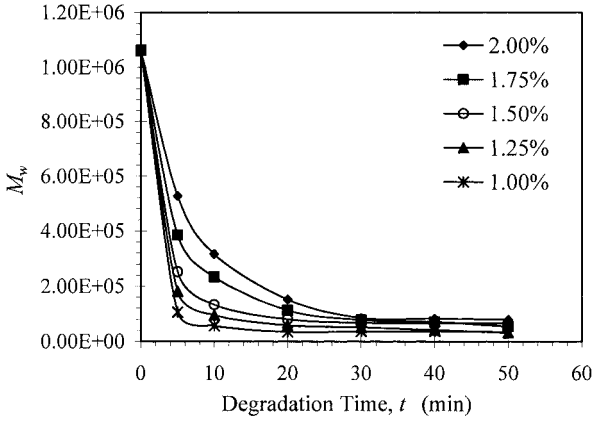

Figure 4. The curves of M_w *versus* degradation time *t* for the enzymatically degraded KGM samples by Mannanase I at different substrate concentrations.

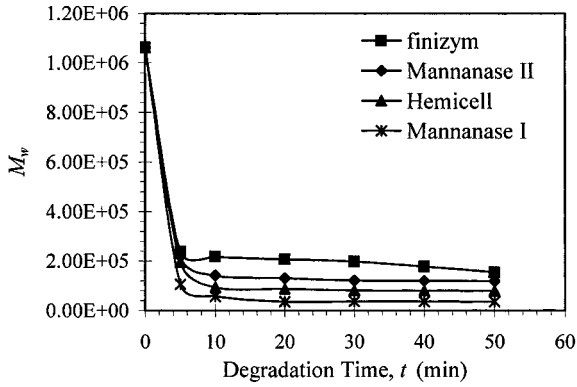

Figure 5. The curves of M_w *versus* degradation time t for the enzymatically degraded KGM samples by deferent enzymes at the same substrate concentration, 1.00 %.

Kinetics of Enzymatic Degradation. A first-order kinetics is generally used to describe the process of polymer degradation. It is expressed by

$$\frac{dL}{dt} = -kL \tag{4}$$

where L represents the total number of breakadable or hydrolyzable linkages in the system, k the apparent rate constant and dL/dt the reduction rate of L. Since there are $(M/m-1)$ linkages in each polymer molecule, where M is the average molecular weight of the macromolecular chain and m is the molecular weight of a monomeric unit. Let N_0 stand for the total number of polymer molecules, then L can be expressed as

$$L = N_0(M/m - 1) \tag{5}$$

If a polymer chain is long enough so as to $m/M << 1$, the following relationship between M and t can be derived

$$\frac{1}{M_t} = \frac{1}{M_0} + \frac{kt}{m} \tag{6}$$

in which M_0 and M_t are the molecular weight of the polymer at time $t = 0$ and t, respectively. Eq. (6) reveals the linear relation between the inverse M_t and degradation time t.

Therefore, we transformed the plots of the M_w *versus* t relationship in Figure 4 and Figure 5 into the plots of the inverse M_w *versus* t relationship as shown in Figure 6 and Figure 7,

respectively. Obviously, regardless of the fact that in each degradation reaction the biocatalyst used or the substrate concentration is different, the plots in Figure 6 and Figure 7 all reveal a unique and two-stage linear relationship between the inverse M_w and degradation time t for the enzymatic degradation systems of KGM, which is consistent with Eq. (6) but exhibits two stages with different slopes. In the initial period of the degradation the straight lines of $1/M_w$ *versus* t exhibit greater slope than those obtained after the degradation has taken place to a certain extent.

Additionally, the slope of $1/M_w$ *versus* t, which is related to the apparent rate constant k, decreases with substrate concentration in the initial stage and tends to the same in the later/next stage. This kinetic behavior differs from the traditional kinetic model of polymer degradation in which no dependence of the rate constant on polymer concentration is considered. That is to say, the theory of a first-order reaction cannot explain the results shown in Figure 6.

The results obtained in our experiments is partly similar to those obtained by A. Tayal et al.[18] and Y. Cheng et al.[19] for the enzymatic degradation of water-soluble polysaccharide such as guar and its derivatives.

In order to make this problem clearer, assuming the enzymatic degradation reaction of KGM is following an nth-order kinetic process, then Eq. (4) becomes

$$\frac{dL}{dt} = -kL^n \quad (n \neq 1) \tag{7}$$

where n is the order of reaction. By integrating Eq. (7) between the limits $t = 0$ and t, and by doing appropriate substitutions, the following equation is obtained:

$$\left[\left(1-\frac{m}{M_t}\right)^{1-n} - \left(1-\frac{m}{M_0}\right)^{1-n}\right] = \frac{k(n-1)}{N_0^{1-n}}t \quad (n \neq 1) \tag{8}$$

For a zeroth-order reaction, $n = 0$, then Eq. (8) becomes

$$\frac{1}{M_t} = \frac{1}{M_0} + \frac{k}{mN_0}t \tag{9}$$

And for a second-order reaction, $n = 2$, then Eq. (8) becomes

$$\frac{1}{M_t} \cong \frac{1}{M_0} + \left(\frac{kN_0}{m}\right)t \tag{10}$$

Consequently the dependence of molecular weight on degradation time can be predicted by Eqs. (9), (6) and (10) for zero-, first- and second-order degradation reaction, respectively. Depending on the order of a reaction, the plot of $1/M_w$ *versus* t can be shown in the form of straight line whose slope is S. From Eqs. (9), (6) and (10) the slope of $1/M_w$ *versus* t plot for an nth-order reaction process can be generalized and expressed as follows:

$$S = \frac{kN_0^{n-1}}{m} \tag{11}$$

Let c_0 stand for the initial polymer or substrate concentration, V for the sample volume and N_{av} for the Avogadro number, then A. Tayal et al.[18] deduced the following equation:

$$c_0 = \frac{N_0 V}{N_{av} m} \tag{12}$$

By substitution we can get

$$S \propto c_0^{n-1} \tag{13}$$

It means that for a zero-order reaction (n=0), S or k is inversely proportional to c_0; and for a first-order reaction (n=1), S or k should be independent of c_0. This can be observed in the enzymatic degradation of KGM by analyzing the change in the slope of $1/M_w$ *versus* t plots. The result is shown in Figure 8. It suggests that the enzymatic degradation of KGM follows a zeroth-order reaction kinetics in the initial phase of the reaction and then a first-order reaction kinetics in the later phase over the whole range of substrate concentration studied.

We have noted the experiments on the enzymatic degradation of guar made by Y. Cheng etal.[19], which show that the substrate concentration range has a great influence on the kinetic behaviors of the enzymatic degradation of guar. At very low guar concentrations, the reaction rate increases with substrate concentration; at intermediate concentrations, the reaction rate becomes independent of substrate concentration; and at very high concentrations, the reaction rate decreases with substrate concentration. In view of the differences in the experiments done by us and by Y. Cheng et al., as well as the structural defferences between guar and KGM, it can be thought that our experimental results are compatible with those obtained by Y. Cheng et al. In fact, the 'real' substrate concentration, or breakable linkages, has been decreased in the later phase of the reaction. The theory concerning the formation of an enzyme-substrate complex and 'substrate saturation" can be used to explain these kinetic

behaviors. In the enzymatic degradation reactions with a given enzyme concentration, increasing the substrate concentration will certainly increase the ratio of substrate to enzyme. When the substrate concentration is high enough for the given enzyme concentration, each enzyme molecule can bind a molecule of substrate to form an enzyme-substrate complex. In this case, increasing the substrate concentration cannot increase the number of complexes formed, and on the contrary increase the viscosity of a reaction system, thus leading to a diffusional resistance to the enzyme mobility in the concentrated polymer solution. The reaction rate then becomes zero-order.

As for the fact that the plots of the inverse M_w *versus* t shown in Figure 7 also exhibit two-stage linear relationship with two distinct kinetic regimes, this indicates that the enzymatic degradation of KGM is a complex reaction where the kinetic mechanism is a combination of at least two reactions with different orders. This may be a universal phenomenon for the degradation of polysaccharides. The acid hydrolysis of cellulose[20] and carageenan [21, 22] also reflected the same kinetic behavior.

The above analysis indicates that the enzymatic degradation of KGM exhibits different kinetic behaviors under different conditions, and it is a complex reaction combining two or more reaction regimes with different orders. The initial short-time reaction regime is of importance because a significant reduction in molecular weight of a substrate takes place in this reaction regime. Therefore, the true order of reaction can be determined only by examining the effect of initial substrate concentration, c_0, on the apparent rate constant, k, or the slope of $1/M_w$ *versus* t plot, S.[18]

For the enzymatic degradation of a polysaccharide such as KGM, guar, cellulose etc, it is reasonable to be characterized using similar equations to Eqs. (9) and (6). If it is assumed that an initial zeroth-order proceeds for time t' and is followed by a first-order reaction up to time t then, the following equations can be derived from Eqs. (9) and (6):

$$\frac{1}{M_{t'}} - \frac{1}{M_0} = \frac{k}{mN_0} t' \quad (0 < t < t') \tag{14}$$

$$\frac{1}{M_t} - \frac{1}{M'} = \frac{k_1}{m}(t - t') \quad (t' < t < t) \tag{15}$$

in which k and k_1 are the rate constants for the zeroth-order and the first-order reaction, respectively.

Eqs. (14) and (15) fit most of experimental data on the enzymatic degradation of polymers. Thus, this approach is of significance for objectively analyzing the kinetic behavior of polymer degradation previously ignored such as the degradation kinetics in the initial short-time phase.

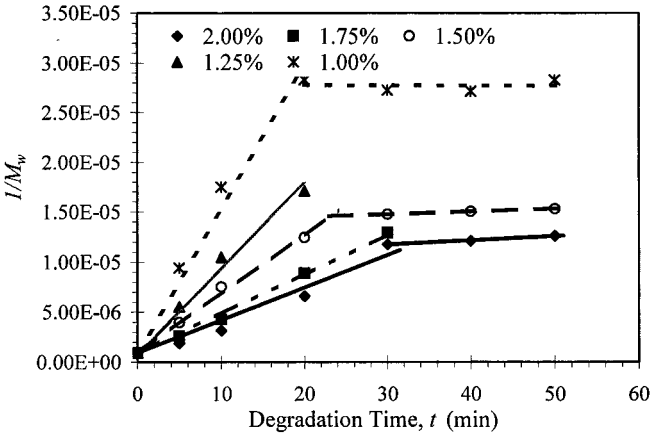

Figure 6. Reciprocal of molecular weight, $1/M_w$, as a function of degradation time t for the enzymatically degraded KGM samples by Mannanase I at various substrate concentrations.

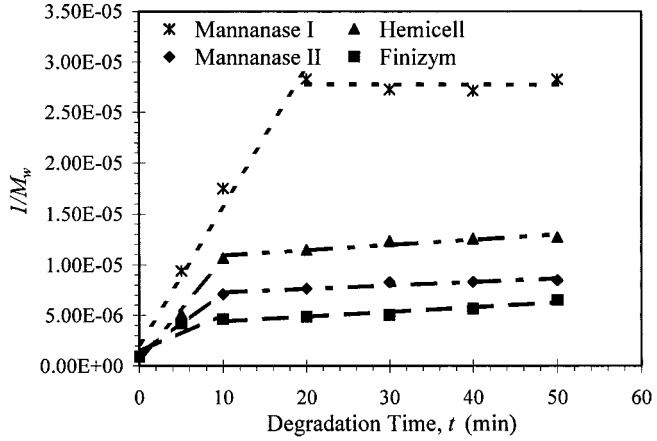

Figure 7. Reciprocal of molecular weight, $1/M_w$, as a function of degradation time t for the enzymatically degraded KGM samples by deferent enzymes at the same substrate concentration, 1.00 %.

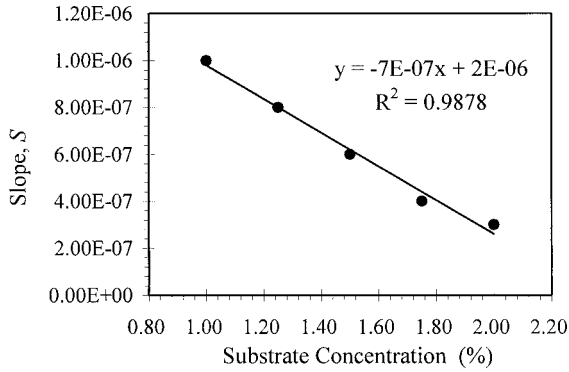

Figure 8. The slope of the straight line of $1/M_w$ *versus* c_0, S, as a function of KGM concentration in the initial period of enzymatic degradation.

Conclusion

1. The relation between $[\eta]$ and M_w of the KGM can be expressed by the equation $[\eta] = 5.06 \times 10^{-4} M_w^{0.754}$ or $\mathrm{Log}M_w = 1.327\mathrm{Log}[\eta] + 4.371$.
2. The enzymatic degradation of KGM catalyzed by Mannanase I is a complex reaction combining at least two reaction regimes with different orders over the range of substrate concentration (1.0 ~ 2.0 %) used in this work. In the initial phase (< 20 min) the degradation follows a zeroth-order reaction kinetics, i.e. its rate constant is inversely proportional to the initial substrate concentration; while in the next phase it follows a first-order reaction kinetics, i.e. the rate constant is independent of the initial substrate concentration. The theory concerning the formation of an enzyme-substrate complex and 'substrate saturation' have been used to explain this kinetic behavior.
3. The enzymatic degradation process of KGM catalyzed by other enzymes such as Mannanase II, Finizym and Hemicell is also a two-stage complex reaction with two distinct kinetic regimes.

These results are helpful to understand the kinetic behaviors of degradation of polysaccharides, thus achieving controllable or limited of KGM and other polysaccharides via an environmental benign enzyme-catalyzed reaction. However, more detailed investigation should be made.

Acknowledgements

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