



Kinetic of the enzymatic degradation of chitosan using bromelain: a viscosimetric study.

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Resumen:

Cinética de la degradación enzimática de quitosano usando bromelina: un estudio viscosimétrico. Se realizó la degradación enzimática de quitosano (grado de acetilación (ga) y peso molecular promedio viscosimétrico (M_V) 0,85 y 310 kDa, respectivamente) usando la enzima comercial bromelina. El decrecimiento del grado de polimerización viscosimétrico promedio (X_V) fue estudiado en una solución reguladora de ácido acético (0,3 M)/acetato de sodio (0,2 M) a 25 °C. Bajo estas condiciones experimentales es posible correlacionar la viscosidad intrínseca instantánea $[\eta]$ con M_V a través de la ecuación de Mark-Houwink-Sakurada. Las ecuaciones de Sano, Solomon-Ciuta, Deb-Chatterjee y Ram-Yaseen, las cuales permiten calcular valores de $[\eta]$ con una simple medida de viscosidad relativa (η_{rel}) y/o específica (η_{sp}), fueron empleadas para obtener valores instantáneos de $[\eta]$. También fueron obtenidos valores instantáneos de $[\eta]$ a través de un proceso iterativo usando la ecuación de Huggins. La velocidad inicial de depolimerización (v_o) fue obtenida de soluciones con diferentes concentraciones de quitosano y una concentración fija de bromelina. La constante de Michaelis-Menten (k_M) y la velocidad máxima (V_m) fueron obtenidas usando la representación de Lineweaver-Burk de la ecuación de Michaelis-Menten para cada juego de valores de $[\eta]$, los cuales fueron calculados empleando todos los procedimientos mencionados. Los mejores parámetros cinéticos para la depolimerización del quitosano ($k_M = 3.92 \times 10^{-7} \text{ mol.dL}^{-1}$; $V_m = 9.09 \times 10^{-8} \text{ mol.dL}^{-1} \cdot \text{s}^{-1}$) se obtuvieron cuando se empleó la ecuación de Sano para calcular los valores de $[\eta]$. **Palabras clave:** bromelina, estudios de degradación, depolimerización de quitosano, depolimerización enzimática.

Abstract

Enzymatic degradation of chitosan (degree of acetylation (da) and average viscosimetric molecular weight (M_V) of 0.85 and 310 kDa, respectively) was carried out using commercial bromelain. Decrease of the average viscosimetric polymerization degree (X_V) was studied in a buffer solution of acetic acid (0.3 M)/sodium acetate (0.2 M) at 25 °C. Under these experimental conditions it is possible to relate the instantaneous intrinsic viscosity $[\eta]$ with M_V through the Mark-Houwink-Sakurada equation. Equations of Sano, Solomon-Ciuta, Deb-Chatterjee and Ram-Yaseen, which allow to compute values of $[\eta]$ with a single measurement of relative (η_{rel}) and/or specific viscosity (η_{sp}), were employed to obtain instantaneous values of $[\eta]$. Instantaneous values of $[\eta]$ were also obtained through an iterative process using Huggins equation. Initial velocity of depolymerization (v_o) was obtained from different concentration of chitosan solutions and a fixed bromelain concentration. Michaelis-Menten constant (k_M) and maximum velocity (V_m) were obtained using the Lineweaver-Burk representation of the Michaelis-Menten equation for each set of $[\eta]$ which were calculated employing all procedures mentioned. Best kinetic parameters for chitosan depolymerization ($k_M = 3.92 \times 10^{-7} \text{ mol.dL}^{-1}$; $V_m = 9.09 \times 10^{-8} \text{ mol.dL}^{-1} \cdot \text{s}^{-1}$) were obtained when Sano's equation was employed to calculate $[\eta]$ values. **Keywords:** bromelain, degradation studies, chitosan depolymerization, enzymatic depolymerization.

Introduction

Production of oligopolisaccharides from chitin and chitosan depolymerization is a growing research area due to the wide range of applications that have been derived from these materials. There is *in vitro* evidence, including some of medical significance, that chitosan oligomers enhance the antimicrobial activity against phytopathogenic

fungi [1-3] and bacteria [4-6]. Similarly, chitosan oligomers seem to improve the activity of the native chitosan in the preservation of foods, odontologic treatments, wound protection, etc.

Chitosan depolymerization has been carried out by chemical (i.e. HNO_2 [7]), physical (i.e. sonication [8]) and biological (i.e. enzymes [9-11]) methods. However, when

the proteolytic enzyme bromelain has been used as depolymerization agent, literature is scarce and not conclusive. Thus, while some authors have found that this enzyme is able to produce depolymerization in a good extension [12,13], others have reported a low activity on chitosan degradation [14].

Susceptibility of chitosan to various nonspecific enzymes (i.e. wheat germ lipase [15], lysozyme [16], papain [11], cellulase [17], hemicellulase [18], b-glucosidase [19], etc.) has been reported. However, some authors have attributed this behavior to enzyme purity, which could be contaminated with chitosanases [13,19]. On the other hand, based on results obtained from a genetically engineered lipase [20] others authors assume that these enzymes can catalyze this degradation reaction by themselves. Presumably, hydrolysis of chitosan by this enzymatic preparation follows an acid-base mechanism [21]

Chitosan depolymerization by a nonspecific enzyme such as bromelain may possess much practical importance because this is easily obtained from stem of pineapple, which implies low production costs and use of biocompatible materials. Specific enzymes with high activity toward chitosan depolymerization (i.e. chitosanase) are usually very expensive and most of them unavailable in bulk for commercial exploitation [22]. Usually, most of chitosanases are obtained from microbial sources although it has been reported the isolation and purification from some plants (sweet orange callous tissue [23] and cucumber [24]).

In order to obtain quantitative data on the chitosan depolymerization by bromelain we have carried out kinetic studies by mean of a viscosimetric method. Viscosimetric data were evaluated using several of the viscosimetric equations more reported in the literature to compute $[\eta]$ by mean of a single measure (Table 1). Additionally, an iterative process based on Huggin's equation was employed in order to compare with results coming from of the single point methods. Depolymerization was carried out in acetic acid (0.3 M)/sodium acetate (0.2 M) buffer solution at 25 °C. This medium allows to directly relates changes in the viscosimetric measurements to molecular weight variation of the biopolymer.

Experimental

Chemicals: chitosan (Fluka BioChemika, HMW) was purified dissolving the biopolymer in acetic acid and precipitating it by neutralization (pH 7) with NaOH, then the polymer is filtrated and exhaustively rinsed with deionized water (Millipore) and dried at 50 °C; 1.01×10^{-5} g/dL bromelain (Sigma Chemical Co.) solution in 0.0128

EDTA disodium salt dihydrate (J.T. Baker) was prepared previous to kinetics studies.

Chitosan characterization: M_v was determined from viscosimetric measurements in acetic acid (0.3 M)/sodium acetate (0.2 M) buffer solution. An Ubbelohde type viscometer (Canon) was used. Temperature was maintained at 25.0 ± 0.1 °C using a thermostated water bath (Gallenkamp). $^1\text{H-nmr}$ spectroscopy in D_2O + trifluoroacetic acid (Bruker, DRX 400 MHz) analysis shows an acetylation degree of 0.162.

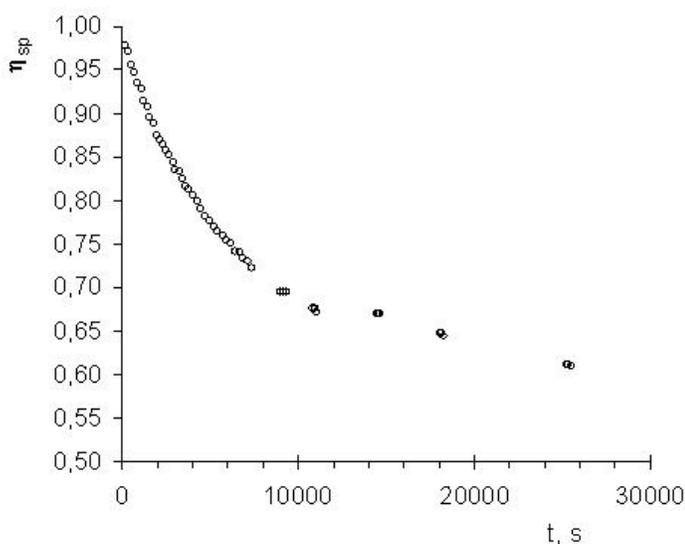
Viscosimetric kinetics studies: a variable amount of standard buffered chitosan solution and fresh buffer solution, both filtrated through a funnel of Hirsch with fritted disc (Sovirel No. 1), were loaded into the viscosimeter (under controlled temperature) in the adequate proportion (see table 2). After thermal stabilization five viscosimetric measurements of the unload enzyme solution were ran to ensure chitosan solutions stability under time experimental time. Then a determined volume of enzyme solution (20 μl) was introduced in the system, stirred and, immediately, consecutive viscosimetric measurements were carried out along the time. Each set of solution concentrations was ran the same day and this procedure was repeated three times with very similar results. Data used to construct table 3 was selected from the better set of results because it is difficult to carry out the viscosimetric measures at the same times in each run.

Viscosimetric data have been treated according to following procedure:

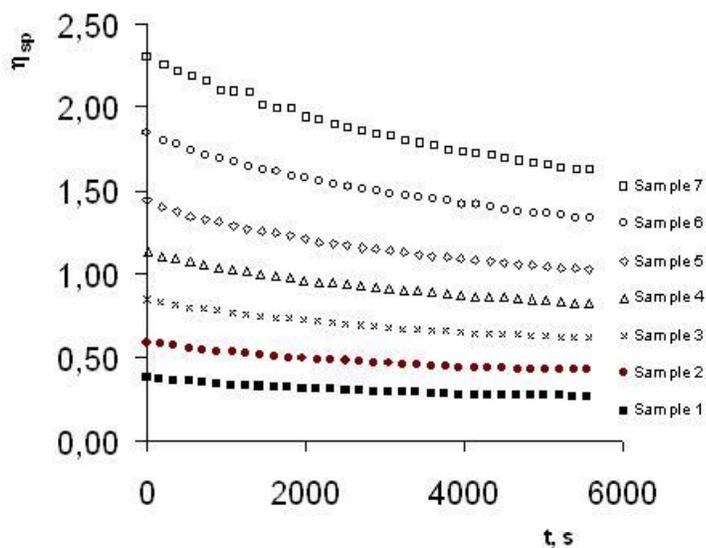
- Relative viscosity (η_r) and/or specific viscosity (η_{sp}) are related to intrinsic viscosity ($[\eta]$) using any of the empirical equations that allow to calculate $[\eta]$ from a single measurement. Table 1 shows some of these equations, although Chee [25] has concluded that some of them (i.e. equations 2, 3 and 4) produce unacceptable results even when diluted polymer solutions are used.
- Average viscosimetric molecular weight (M_v) can be obtained directly from $[\eta]$ using Mark-Houwink-Sakurada (MHS) equation ($[\eta] = KM_v^\alpha$). Chitosan K and α values have been reported by Rinaudo *et al* ($K = 7.4 \times 10^{-4}$ and $\alpha = 0.76$ for a chitosan with $da = 0.15$) in acetic acid/sodium acetate buffer solution at 25 °C [26].
- Initial velocity (v_0) for depolymerization process can be obtained by plotting M_v (or X_v) vs time
- Kinetics parameters (Michaelis-Menten constant (K_M) and maximum velocity (V_m)) are obtained by processing v_0 values according to Michaelis-Menten equation, plotting $1/v_0$ vs $1/c_{p0}$ (Lineweaver-Burk representation).

Table 1. Empirical equations used to intrinsic viscosity determination through the single-point method.

Equation	Number	Reference
$[\eta] = 8[(\eta_{rel})^{1/8} - 1]/c_p$	(1)	[27]
$[\eta] = [2(\eta_{sp} - \ln\eta_{rel})/(c_p)^2]^{1/2}$	(2)	[28]
$[\eta] = [3(\ln\eta_{rel} - \eta_{sp} + 1/2(\eta_{sp})^2)]^{1/3}/c_p$	(3)	[29]
$[\eta] = (\eta_{sp} + \ln\eta_{rel})/2c_p$	(4)	[30]



(a)



(b)

Fig. 1.- (a) η_{sp} with time response for a chitosan/bromelain solution (0.0777 g/dL and 1.02×10^{-5} g/dL, respectively) in acetic acid (0.30 M)/sodium acetate (0.20 M) buffer solution at 25 °C for long time reaction. (b) η_{sp} with time response for diverse chitosan concentrations at fixed bromelain concentrations (9.48×10^{-6} g/dL) in acetic acid (0.30 M)/sodium acetate (0.20 M) buffer solution at 25 °C for initial stages of depolymerization process.

Results and discussion

Experimental M_v value obtained from the starting chitosan sample (309 KDa), perfectly agree with a previous study on similar commercial chitosan sample [31]. Data were plotted according Huggins equation ($\eta_{sp}/c_p = [\eta] + k_H[\eta]^2 c_p$) and a value for $[\eta] = 11.011$ dL/g was obtained. On the other hand, a Huggins constant (k_H) value of 0.536 was also obtained, indicating non appreciable aggregation in the system.

Figure 1(a) shows long time η_{sp} changes vs. reaction time for a chitosan solution (0.0777 g/dL) and bromelain (1.02×10^{-5} g/dL) in acetic acid (0.30 M)/sodium acetate (0.20 M) buffer solution at 25 °C. Similar studies with different enzyme/substrate ratios were conducted

previously. As it can be appreciated, a typical macromolecular enzymatic depolymerization behavior, with an appreciable η_{sp} decreasing along the time, is observed. This result demonstrates that bromelain, in this medium, has a good activity in spite of the low concentration of enzyme employed in this assay. Figure 1(b) shows η_{sp} changes vs. reaction time for the initial stages of the chitosan enzymatic degradation under the experimental conditions showed in table 2.

Average viscosimetric polymerization degree (X_v) values ($M_v = M_0 X_v$ with M_0 = average molar mass of the repetitive units = 167.1 g/mol) and $[\eta]$ values were obtained from figure 1b data, for each time, employing MHS equation and equations 1-4 (table 1), respectively. Similar results

were obtained for all samples, showing good correlation (table 2) when these data were used to obtain values of the Table 2.- Results obtained during degradation studies of different chitosan solutions using bromelain. Correlation coefficient (R^2) are referred to plots of X_v vs. t

Sample	$V_{\text{standar chitosan}}$ (ml)	V_{buffer} (ml)	$c_{\text{po.}}$ ($\text{mol}_{\text{ch.}}\text{dL}^{-1}$)	Eq.	X_v initial	v_{om} ($\text{mol}_{\text{ru.}}\text{mol}_{\text{ch.}}^{-1}\text{s}^{-1}$)	v_o ($\text{mol}_{\text{ru.}}\text{dL}^{-1}\text{s}^{-1}$)	R^2
1	3.0	9.0	8.230x10 ⁻⁸	4	2088	0.2199	1.810x10 ⁻⁸	0.9976
			8.265x10 ⁻⁸	3	2079	0.2180	1.802x10 ⁻⁸	0.9976
			8.566x10 ⁻⁸	2	2006	0.2056	1.761x10 ⁻⁸	0.9976
			8.968x10⁻⁸	1	1916	0.1909	1.712x10⁻⁸	0.9976
			9.180x10 ⁻⁸	H	1875	0.1863	1.710x10 ⁻⁸	0.9976
2	4.5	7.5	1.219x10 ⁻⁷	4	2115	0.2083	2.539x10 ⁻⁸	0.9917
			1.230x10 ⁻⁷	3	2096	0.2046	2.516x10 ⁻⁸	0.9918
			1.296x10 ⁻⁷	2	1989	0.1880	2.436x10 ⁻⁸	0.9921
			1.387x10⁻⁷	1	1859	0.1684	2.335x10⁻⁸	0.9924
			1.417x10 ⁻⁷	H	1822	0.1655	2.345x10 ⁻⁸	0.9889
3	6.0	6.0	1.536x10 ⁻⁷	4	2238	0.2062	3.166x10 ⁻⁸	0.9983
			1.560x10 ⁻⁷	3	2203	0.1995	3.112x10 ⁻⁸	0.9983
			1.676x10 ⁻⁷	2	2050	0.1776	2.9771x10 ⁻⁸	0.9983
			1.836x10⁻⁷	1	1872	0.1526	2.801x10⁻⁸	0.9983
			1.863x10 ⁻⁷	H	1848	0.1528	2.846x10 ⁻⁸	0.9987
4	7.5	4.5	1.844x10 ⁻⁷	4	2330	0.2094	3.861x10 ⁻⁸	0.9946
			1.888x10 ⁻⁷	3	2275	0.1992	3.761x10 ⁻⁸	0.9946
			2.065x10 ⁻⁷	2	2080	0.1721	3.554x10 ⁻⁸	0.9947
			2.316x10⁻⁷	1	1855	0.1421	3.291x10⁻⁸	0.9947
			2.325x10 ⁻⁷	H	1851	0.1388	3.217x10 ⁻⁸	0.9935
5	9.0	3.0	2.127x10 ⁻⁷	4	2424	0.2302	4.896x10 ⁻⁸	0.9912
			2.200x10 ⁻⁷	3	2343	0.2148	4.968x10 ⁻⁸	0.9914
			2.450x10 ⁻⁷	2	2104	0.1809	4.432x10 ⁻⁸	0.9916
			2.8081x10⁻⁷	1	1836	0.1442	4.049x10⁻⁸	0.9920
			2.782x10 ⁻⁷	H	1856	0.1480	4.118x10 ⁻⁸	0.9906
6	10.5	1.5	2.287x10 ⁻⁷	4	2630	0.2279	5.212x10 ⁻⁸	0.9924
			2.399x10 ⁻⁷	3	2507	0.2071	4.968x10 ⁻⁸	0.9926
			2.726x10 ⁻⁷	2	2206	0.1688	4.602x10 ⁻⁸	0.9928
			3.211x10⁻⁷	1	1873	0.1285	4.126x10⁻⁸	0.9931
			3.123x10 ⁻⁷	H	1929	0.1396	4.360x10 ⁻⁸	0.9943
7	12.0	0.0	2.423x10 ⁻⁷	4	2837	0.2337	5.662x10 ⁻⁸	0.9923
			2.581x10 ⁻⁷	3	2663	0.2072	5.348x10 ⁻⁸	0.9926
			2.995x10 ⁻⁷	2	2295	0.1645	4.927x10 ⁻⁸	0.9929
			3.621x10⁻⁷	1	1898	0.1206	4.367x10⁻⁸	0.9933
			3.451x10 ⁻⁷	H	1995	0.1415	4.883x10 ⁻⁸	0.9921

$C_{\text{bromelain init}} = 9.48 \times 10^{-6}$ g/dL; $V_{\text{bromelain init}} = 20$ μL ; $C_{\text{standar chitosan}} = 0.1149$ g/dL.

average depolymerization initial velocity (v_{om}). It is important to clarify that v_{om} , such as it has been determined by plotting X_v vs. reaction time (figure 2), represents an average depolymerization initial velocity value (i.e. average of the moles of repetitive units (mol_{ru}) broken by mol of chitosan by time, expressed in

$\text{mol}_{\text{ru.}}\text{mol}_{\text{ch.}}^{-1}\text{s}^{-1}$) but it does not measure the variation of the concentration of repetitive units being depolymerized. Then, total depolymerization initial velocity (v_o) is obtained, in each sample, by the product $v_{\text{om}}c_p$, and expressed as $\text{mol}_{\text{ru.}}\text{dL}^{-1}\text{s}^{-1}$.

Table 2 shows several interesting results, as for example:

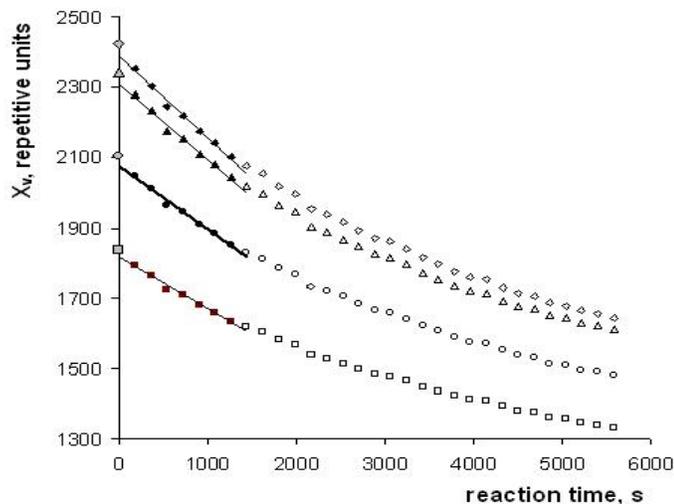


Fig. 2.- X_v changes at the initial stages of depolymerization of a chitosan (0.08614 g/dL) solution using bromelain (9.48×10^{-6} g/dL) in acetic acid (0.30 M)/sodium acetate (0.20 M) buffer at 25 °C. Values for X_v were obtained according to equations 1 (\square), 2 (\circ), 3 (Δ) and 4 (\diamond). Grey symbol correspond to $X_{v \text{ initial}}$ values obtained using the respective equation; bold symbols represent data used to compute v_o .

a) Values of v_{om} have a better linear correlation coefficient (R^2) for most of samples when equation 1 was used to compute $[\eta]$ values.

b) $X_{v \text{ initial}}$ values obtained for each sample tend to increase with chitosan concentration when equations 2-4 were used to calculate $[\eta]$ values.

c) $X_{v \text{ initial}}$ values for each sample (values obtained before adding enzyme to each chitosan solution) are less disperse when equation 1 was used to calculate $[\eta]$ (see table 3), including values obtained through an iterative data treatment using the Huggins equation.

$[\eta]$ values obtained employing this iterative process were computed reordering Huggins equation as:

$$[\eta] = \eta_{sp}/c_p - k_H[\eta]^2 c_p$$

then, $[\eta]$ values are placed into the right side of the

Table 3.- Standard deviation obtained for the average $X_{v \text{ initial}}$ when equations 1-4 and Huggins equation are used to calculate $[\eta]$. $X_{v \text{ initial}}$ values were employed in these calculations for all chitosan solutions.

Equation	Average $X_{v \text{ initial}}$	Standard deviation	% deviation
1	1872	25	1.35
2	2094	114	5.46
3	2315	199	8.58
4	2385	250	10.49
Huggins	1886	55	2.93

equation until an equal value for $[\eta]$ is produced. This intrinsic viscosity value obtained from the iterative treatment, correspond to $[\eta]$ for the polymer in the experimental conditions studied (c_p and η_{sp}). k_H value (0.536) obtained from the initial viscosimetric study is used and it is supposed that remain unvaried during the depolymerization initial stages studied.

On the other hand, we have supposed that initial changes in viscosity are only due to chitosan molecular weight decrease and there are not contributions from conformational changes in the polymeric chain due to a molecular weight transition. According to Tsaih [32], for a chitosan of similar acetylation degree, this transition is expected to appear when X_v is around 1330 ($M_v \sim 223$ KDa), which is far below than experimental X_v values used for our kinetic studies.

Higher $X_{v \text{ initial}}$ values dispersion observed when equations 2-4 are employed to calculate $[\eta]$, which come from the increase of X_v values when concentration is increased, may possibly be attributed to the logarithmic contribution in these equations. These logarithmic contributions are more remarkable for $\eta_{sp} > 1$ or $\eta_{rel} > 1$, as it is observed for more concentrated solutions. On the other hand, due to equation 1 has not any logarithmic contribution produces less dispersed results, which do not show an increasing trend when concentration is increased.

Figure 3 shows the Lineweaver-Burk or double reciprocal representation ($1/v_o$ vs $1/c_{p0}$) using v_o values obtained with all the procedures used to compute instantaneous $[\eta]$ values. v_o values were obtained from similar plots as in figure 2, using either equations 1-4 or the iterative process of Huggins equation. Acceptables linear correlation coefficients are observed for most curves, particularly for curve obtained when Sano's equation was used, although K_M and V_{max} values obtained from each curves are very different. These values (Table 4) were calculate from the slope ($m = K_M/V_{max}$) and intercept ($i = 1/V_{max}$) obtained from each plot in figure 3.

Table 4. K_M and V_{max} values obtained for the initial enzymatic degradation of chitosan with bromelain using the different procedures to calculate $[\eta]$.

Equation used to calculate $[\eta]$	K_M (mol.dL ⁻¹)	V_{max} (mol.dL ⁻¹ .s ⁻¹)	R^2
1	3.92×10^{-7}	9.09×10^{-8}	0.9942
2	7.64×10^{-7}	1.72×10^{-7}	0.9941
3	2.57×10^{-6}	5.67×10^{-7}	0.9934
4	-6.71×10^{-6}	-1.42×10^{-6}	0.9908
Huggins	5.50×10^{-7}	1.17×10^{-7}	0.9872

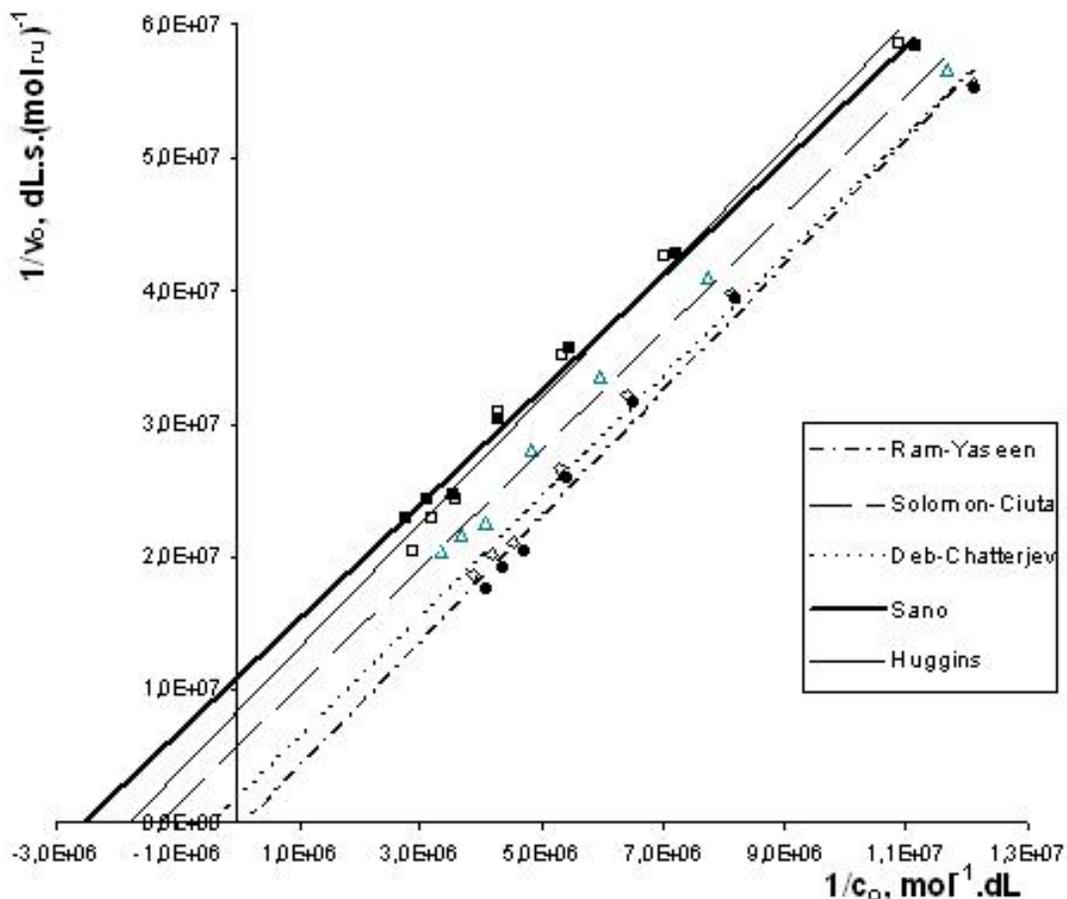


Fig. 3.- Lineweaver-Burk representation (double reciprocal) for depolymerization of chitosan solutions using bromelain (9.48×10^{-6} g/dL) in acetic acid (0.30 M)/sodium acetate (0.20 M) buffer to 25 °C.

Table 5.- K_M and V_{max} values reported in some enzymatic systems using chitosan as substrate.

Enzyme	Sustrate	pH	T (°C)	K_m^*	V_{max}^*
Hydrolase purified from commercial stem bromelain [23]		3.0	50	0.088 % 0.088 g.dl⁻¹	3.50 $\mu\text{mol GlcN}/\text{min}/\text{mg}$ $5.83 \times 10^{-8} \text{ mol.s}^{-1}.\text{mg}^{-1}$
Papain purified from commercial sample [24]	Chitosan ($M_w \sim 71$ kDa, DD $\sim 0,74$) prepared from shrimp chitin	3.5	37	7.14 $\text{mg}.\text{ml}^{-1}$ 0.714 g.dl⁻¹	288.48 $\text{nmoles}.\text{min}^{-1}.\text{mg}^{-1}$ $4.81 \times 10^{-9} \text{ mol.s}^{-1}.\text{mg}^{-1}$
Pronase from <i>Streptomyces griseus</i> [25]	Commercial chitosan	3.5	37	5.21 $\text{mg}.\text{ml}^{-1}$ 0.521 g.dl⁻¹	1.38 $\text{nmoles}.\text{min}^{-1}.\text{mg}^{-1}$ $2.31 \times 10^{-9} \text{ mol.s}^{-1}.\text{mg}^{-1}$
Hydrolase purified from commercial cellulase [26]	Commercial chitosan ($M \sim 560$ kDa, DD $\sim 0,90$)	5.2	60	0.10 mg/ml 0.010 g.dl⁻¹	0.402 $\mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$ $6.70 \times 10^{-9} \text{ mol.s}^{-1}.\text{mg}^{-1}$
Chitosanase purified from <i>Bacillus</i> sp. [27]	Chitosan (DD ~ 0.798)	5.5	37	0.52 $\text{mg}.\text{ml}^{-1}$ 0.052 g.dl⁻¹	$7.71 \times 10^{-6} \text{ mol.s}^{-1}.\text{mg}^{-1}$
Commercial bromelain without purification (this work)	Commercial chitosan ($M \sim 310$ kDa, DD $\sim 0,85$)	4.25	25	0.123 g.dl⁻¹	$9.09 \times 10^{-8} \text{ mol.s}^{-1}.\text{mg}^{-1}$

* Bold values are reported by the authors, after conversion to unique type of units for a better comparison.

By considering that Sano's equation produces (a) an average $X_{V \text{ initial}}$ value more similar to that obtained through the graphic method using Huggins equation, (b) a less dispersed $X_{V \text{ initial}}$ values and (c) a better correlation of the Lineweaver-Burk representation, k_M and V_{max} values obtained with this equation have been selected as the more reliable results. A final consideration to support this selection is based on the fact that Sano's equation is the only relationship that include $c_{po} = K_M$ values in the concentration range evaluated to calculate $[\eta]$.

Finally, we have found that k_M and V_{max} values obtained employing this equation are in an order of magnitude similar to those reported for chitosan enzymatic depolymerization systems, as it can be appreciated in the Table 5.

Conclusions

Kinetic studies of initial depolymerization of the biopolymer chitosan using the non specific enzyme bromelain has been carried out through viscosimetric analysis. Instantaneous intrinsic viscosities during degradation were calculated by either an iterative process using Huggins equation or through four empirical equations employing the single point method under experimental conditions where chitosan MHS viscosimetric constants are well known. Sano's equation produced the more reliable results ($K_M = 0.123 \text{ g.dl}^{-1}$ and $V_{\text{max}} = 4.218 \times 10^{-7} \text{ mol.s}^{-1}.\text{mg}^{-1}$), which are comparable to results obtained from chitosan degradation with specific and nonspecific enzyme.

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