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The Influence of Substrate Structure on the Kinetics of the Hydrolysis of Starch by Glucoamylase

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ABSTRACT

In this research the influence of substrate structure on the kinetics of the enzymatic hydrolysis of starch by glucoamylase was evaluated. For this purpose, two substrates of different form and molecular weight were used. In one case, the kinetics of the hydrolysis corresponds to a typical Michaelis-Menten behavior; in the other, a decrease of the hydrolysis rate occurred once a determined substrate concentration was surpassed. The structural differences between the starches, which caused important differences on the rheological properties of their solutions, justify the observed differences in their behavior. Branching of the substrate exerts two opposite effects on the hydrolysis rate because it allows the increase of the number of available points for the enzymatic attack, although the branching increases the steric hindrances and, consequently, the mass transfer resistances. The balance between these two effects is clearly dependent on the substrate concentration.

Index Entries: Glucoamylase; starch; kinetic; substrate structure; weight distribution.

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Nomenclature: M_n, number average molecular weight; M_w, weight average molecular weight; M_o, molecular weight at peak maximum; M_n/M_w, polydispersivity; K, consistency factor (cp); n, flow behavior index; K_m, Michaelis -Menten constant (g/L); K_i, inhibition constant (L/g); V_{max}, maximum velocity (g/L/min).

INTRODUCTION

Glucoamylase is, with α-amylase and glucose isomerase, one of the most widely used enzymes in industry (*1*). This enzyme acts in the recurrent exohydrolysis of the glycosidic linkage α-1,4 of the polysaccharides, releasing successive units of glucose (*2*).

Different authors determined the kinetics of the hydrolysis of starch by glucoamylase. The obtained values of the kinetic parameters (Table 1) differ largely from each other, even in order of magnitude. These important differences are mainly attributed to a) the different characteristics of the employed enzymes, obtained from different culture media and sometimes poorly purified, which can convey the presence of other amylases (mainly α-amylase); and b) the use of free or immobilized enzyme.

However, there are very few studies dealing with the influence of the characteristics of the substrate (physical properties, structure) on the kinetics of the hydrolysis. In a previous work (*12*), the importance of mass transfer resistances on the kinetics was shown. For this purpose, three polysaccharides of quite similar characteristics, starch, amylopectin and glycogen, were considered. The results showed a decreasing reaction rate zone when starch and amylopectine were employed as substrates. Several experiments performed in different conditions were carried out:

1. At lower substrate concentration, but keeping the same substrate/enzyme ratio;
2. Increasing the apparent viscosity of the media by adding an inert thickener agent; and
3. Working at static or shaken conditions.

The obtained results clearly showed that the rate of the process was controlled by mass transfer rate.

In this study, the influence of the structure of one particular substrate (starch in this case) on the kinetics of the enzymatic hydrolysis was evaluated. For this purpose two types of soluble starch, with different molecular structure and physical properties, in aqueous solutions, were used.

MATERIALS AND METHODS

Enzyme

The enzyme used was amyloglucosidase NOVO produced from a submerged fermentation by *Aspergillus niger*. Its activity is of 300 AGU/mL.

Table 1
Kinetic Parameters of the Hydrolysis of Various Starches

Substrate	K_m, g/L	V_{max}, g/L min	References (3–11)
Starch (M_W = 5.000)	1.5	1.94	Pieters and Bardeletti (1992)
Starch (M_W = 19.000)	1.97	8.97	Fujii and Kawamura (1985)
Starch (M_W = 42.000)	4.33	19.82	Fujii and Kawamura (1985)
Starch (M_W = 100.000)	0.21	0.59	Imani et al. (1986)
Starch	0.34	0.02	Steverson et al. (1984)
Starch	0.83	2.22	Tsekova (1984)
Starch	15.94	0.62	Wang et al. (1984)
Starch	0.9	0.03	Saha et al. (1979)
Starch	7.0	0.49	Szajáni et al. (1985)
Starch	20.9	0.21	Przybyt and Sugier (1988)

One unit of amygloglucosidase NOVO (1 AGU) is an amount of enzyme that produces 1 μmol of glucose per min in the following conditions: Maltose 10 g/L at 25°C, pH 4.3, for 30 min. The enzymatic solution used was prepared by diluting (1:1000) the enzymatic preparation with distilled water.

Substrates

Two types of soluble starch supplied from Merch and Panreac from here, A and B, respectively) were used. In each case, the solutions were prepared dissolving the starch in an acetate buffer 0.15-*M*, pH 4.4.

Kinetic Method

A kinetic analysis of data based on the initial rates method was followed. It was found that a linear relationship between the hydrolysis rate and substrate concentration was maintained during the considered period (10 min). Selected pH and temperature were 4.4 and 40°C, respectively. The working method was described in a previous work (*13*).

Sugar Analysis

DUBOIS and DNS methods (*14,15*) were employed for the determination of the total and reducing sugar concentration, respectively. In all kinetic studies total and reducing sugars and the ratio between them were determined.

Rheology

A rotational viscometer, Brookfield Synchro-Lectric, model LVT with a UL adapter, was used to study the rheology of the different solutions

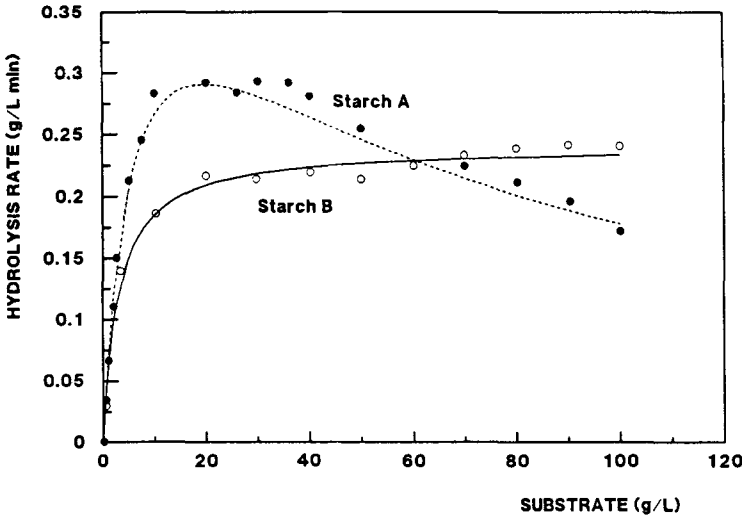

Fig. 1. Hydrolysis rate vs substrate concentration for starch A (●) and B (○).

prepared with the two starches. A series of experiments at velocity gradients between 0.02 and 0.8 s^{-1} for starch concentrations between 10–90 g/L, was carried out.

Molecular Weight Distribution

The molecular weight distribution of both substrates was obtained by gel permeation chromatography, using a column S805-S804 and NaOH (0.05-*M*) as eluent.

RESULTS AND DISCUSSION

Kinetic Results

In Fig. 1, the kinetic profile of the hydrolysis of aqueous solutions of starches A and B are shown The velocity of hydrolysis of starch A is higher than starch B at the lower substrate concentrations (below 60 g/L). For instance, at a substrate concentration of 20 g/L, the hydrolysis of A is approx 50% quicker than of B. However, when operated at higher starch concentrations, starch B is hydrolyzed more easily. Furthermore, while the hydrolysis of starch B follows a characteristic Michaelis-Menten behavior, the hydrolysis rate of starch A increased with substrate concentration up to a maximum, and from then a progressive decrease takes place.

The experimental results of hydrolysis of starch B were adjusted to a Michaelis-Menten equation (Eq. 1). Equation 2, corresponding to a model that postulates an inhibition by substrate behavior, was applied to model the hydrolysis of starch A. In Fig. 1, the experimental data and the curves

Table 2
Kinetic Parameters
Obtained in Hydrolysis by Glucoamylase
of Two Starches with Different Characteristics[a]

	Starch A	Starch B
K_m, g/L	6.22	2.98
V_{max}, g/L/min	0.47	0.24
K_i, L/g	0.016	P

[a] Assuming a conventional model by Michaelis-Menten with (A) and without (B) substrate inhibitions.

corresponding to the two equations are represented; as can be seen, data fitting is very satisfactory. From these data the corresponding kinetic parameters, shown in Table 2, were determined.

$$V = V_{max} \cdot S / (K_m + S) \quad (1)$$

$$V = V_{max} \cdot S / (K_m + S + K_i \cdot S) \quad (2)$$

It seems unlikely that substrate A exerts an inhibitory effect in the concentration range at which starch B shows a typical Michaelian behavior. Therefore, in order to determine the influence of the structure of both substrates on the hydrolysis rate, three kinds of experiments were done:

1. Rheological characterization of A and B starches;
2. Determination of reducing/total sugar ratio; and
3. Molecular weight distribution.

Rheological Characteristics of the Solutions

Determination of the rheological characteristics of substrates A and B can be useful in evaluating the diffusional difficulties of the enzyme in the reaction mixture. Because of the mechanism of the glucoamylase, which releases a unit of glucose from the starch in each step, it could be expected that apparent viscosity decreases in a very gradual way in the course of the hydrolysis. Therefore, mass transfer limitations should play a more relevant factor than in the similar case of α-amylase, whose hydrolysis α-1.4, determined randomly produces a rapid decrease on the apparent viscosity.

The rheological characterization of the substrate solutions was followed by rotational viscometry, operating at different velocity gradients $(-dV/d_r)$ and at substrate concentrations ranging between 10 and 90 g/L. The obtained results fit very well the Ostwald-de Waele equation (Eq 3). The values obtained for the flow behavior index (n) were $0.82 < n < 0.92$ for solutions of starch A and $0.88 < n < 0.92$ for B; these values allowed us to identify their pseudoplastic, non-Newtonian, behavior.

$$\tau = K \cdot (-dV / d_r)^n \quad (3)$$

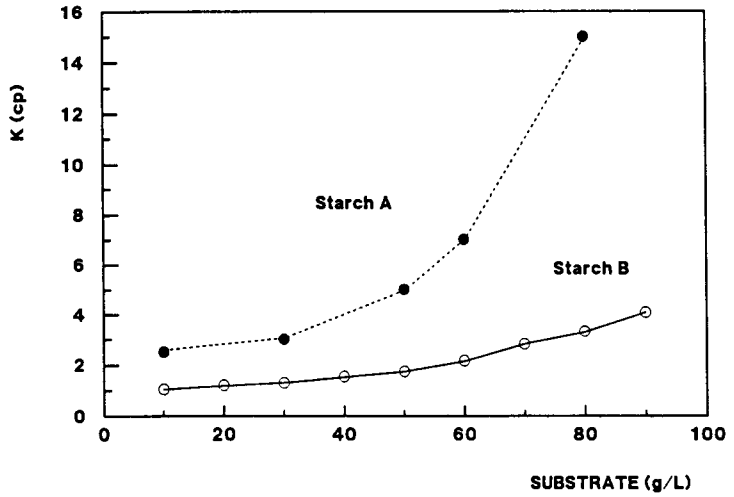

Fig. 2. Consistency index, K, vs substrate concentration for starch A (●) and B (○).

The consistency factor (*K*) gives a good indication of the apparent viscosity of the solutions. In Fig. 2, the variation of the consistency factor against substrate concentration for starches A and B is shown. The apparent viscosity of starch A is always higher than B. Furthermore, the differences between them increase dramatically from a substrate concentration of 40 g/L. As observed in Fig. 1, from this value the hydrolysis rate of starch A decreases very fast. It is interesting to point out that the hydrolysis velocity of substrate A, at 50 g/L, is very similar to that of substrate B at 90 g/L; their apparent viscosities are almost the same. These results may induce one to consider that a mass transfer limitation can be responsible for the decrease of hydrolysis rate of starch A.

Reducing Sugars/Total Sugars Ratio

The information given for this parameter is relevant in order to evaluate the relative branching degree of starch; more branching implies a higher availability of susceptible points to the enzymatic action. The ratio reducing sugars/total sugars of substrates A and B was 0.4 and 0.1, respectively. Because of this, the higher hydrolysis velocity of starch A at moderate concentrations is easily explained: at these conditions the positive effect of branching overcomes the diffusional restrictions to the enzymatic action because of its higher apparent viscosity.

Molecular Weight Distribution

It is also important to determine the possible influence of the molecular weight of the substrates on the hydrolysis rate: a higher molecular weight can result in an increase of the hindrances reaching the attack points by the active centers of the enzyme. Figure 3 shows the chromatograms from which the molecular weight of both substrates, presented in

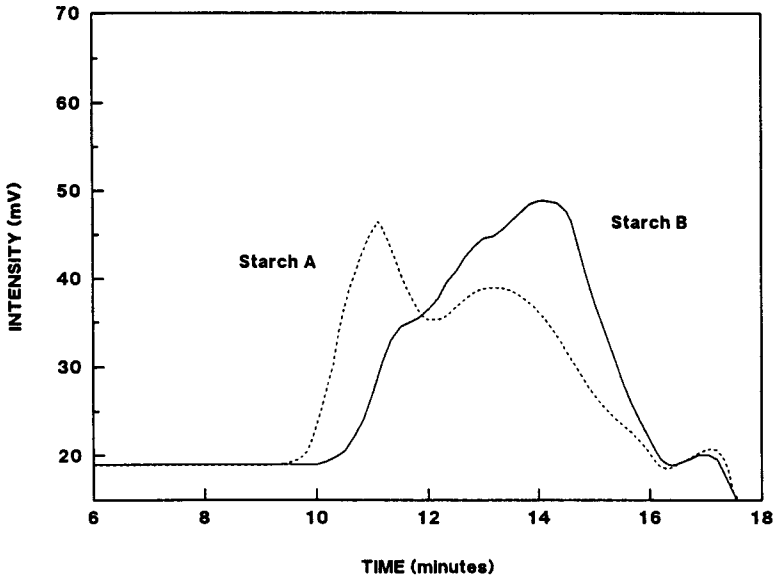

Fig. 3. Chromatograms of gel permeation for determining molecular weight distribution, for starch A (----) and B (——).

Table 3
Structural Parameters of Starches A and B

	Starch A	Starch B
M_n	46630	26240
M_w	316900	103000
M_o	517100	25670
M_n/M_w	6.795	3.924

Table 3, were determined. It is evident that starch A exceeds B in the average molecular weight number (M_n) (46,630 vs 26,240) and also in the average molecular weight (M_w) (316,000 vs 103,000). It is important to point out that the M_w of the glucoamylase is approx 97,000 (*16*), so that it can be said that substrate A reaches more than triple the above value; substrate B reached the same range. Furthermore, it can be observed how the polydispersivity (M_n/M_w) of starch A presents a noticeably wider band than starch B. The conclusions obtained from the overall analysis of these results agrees well with the preceding evaluations based on the considerations of the reducing/total sugars ratio.

CONCLUSIONS

A different kinetic behavior of the hydrolysis of two starches by glucoamylase, Michaelian kinetics and apparently inhibited by substrate, was observed. This apparent discrepancy can be analyzed by considering,

simultaneously, the rheological properties and also size and molecular weight distribution of both starches. The factors related with size and molecular shape provide a simple explanation for the kinetic results and for the rheological characteristics as well. Although at low substrate concentrations the hydrolysis rate mainly depends upon the available points to the enzymatic action, which is a direct function of the branching of the molecule, as the substrate concentration increases, the branching itself increases the steric hindrances. This effect causes the reaction rate to be slower in respect to those determined at lower concentrations by using other substrates of lower molecular weight and lesser ramification.

Our results indicate that when the kinetics of a particular enzymatic process are studied, it is important to consider also the rheological and structural properties of the substrates. It could be dangerous to only consider the numerical analysis of the values of the kinetic parameters, as it can lead to wrong conclusions about the mechanism of the reaction.

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REFERENCES

1. Andres, C. (1983), *Food Processing* **45,** 40–43.
2. Wang, D., Cooney, C., and Demain, A. (1986), *Fermentation and Enzyme Technology.* Wiley, United Kingdom.
3. Pieters, B. R. and Bardeletti, G. (1992), *Enzyme Microb. Technol.* **14,** 361–370.
4. Fujii, M. and Kawamura, Y. (1985), *Biotechnol. Bioeng.* **27,** 260–265.
5. Imani, K., Shiomi, T., Uchida, K., and Miya, M. (1986), *Biotechnol. Bioeng.* **28,** 1721–1726.
6. Steverson, E. M., Korus, R. A., Admassu, W., and Heimsch, R. C. (1984), *Enzyme Microb. Technol.* **6,** 549–554.
7. Tsekova, K. V. (1984), *Comptes Rendues Acad. Bulgare Sci.* **37,** 1681–1683.
8. Wang, H. L., Swain, W., and Hesseltine, C. W. (1984), *J. Food Sci.* **49,** 1210–1211.
9. Saha, B. C., Mitsue, T., and Ueda, S. (1979), *Starch* **31,** 307–314.
10. Szajáni, B., Klamar, G., and Ludvig, L. (1985), *Enzyme Microb. Technol.* **7,** 488–492
11. Przybyt, M. and Sugier, H. (1988), *Starch* **40,** 171–174.
12. Miranda, M., Murado, M. A., Sanromán, A., and Lema, J. M. (1991), *Enzyme Microb. Technol.* **13,** 142–147.
13. Sanromán, A., Chamy, R., Núñez, M. J., and Lema, J. M. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 527–538.
14. Dubois, M., Giles, U. A., Hamilton, I. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem.* **29,** 350–356.
15. Bernfeld, P. D. (1951), *Adv. Enzymol.* **12,** 379–427.
16. Solomon, B. (1979), *Adv. Biochem. Eng.* **10,** 131–177.