Enzymatic Degradation of Methacrylated Dextrans

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ABSTRACT: Dextran and methacrylated dextrans (dex-MA) were degraded with dextranase, and the formed degradation products were characterized by electrospray mass spectrometry. It was shown that the main degradation product was isomaltose for both dextran and dex-MA. In degraded dex-MA, the main methacrylated product was isomaltotriose. The relative contribution of oligosaccharides with a higher molecular weight (up to isomaltohexaose) and of multiply methacrylated oligosaccharides increased with the degree of substitution of dex-MA. Enzyme kinetics with a three-substrate model showed that the Michaelis−Menten constant for the monomethacrylated substrate was smaller than for the unsubstituted substrate, whereas the Michaelis−Menten constant for multiply methacrylated susbtrates was higher. This indicates a favorable interaction of one methacrylate group with a hydrophobic binding subsite in the enzyme. The maximum degradation rate, however, was substantially lower for the substituted substrates than for the native substrate. From these results, it is concluded that the enzyme hydrolyzes a glycosidic bond between a methacrylated glucopyranose residue and an unsubstituted one in the dex-MA chain. This hypothesis is further supported with electrospray mass spectrometry because of both the presence of an ion formed by fragmentation at the nonreducing end of an oligosaccharide and the absence of oligosaccharides in which the number of methacrylates equals or exceeds the number of glucopyranose residues.

Introduction

The enzymatic degradation of modified polysaccharides (e.g., cellulose, starch and dextran) has been the subject of many studies. Firstly, modification can elucidate more about the mechanism of enzymatic degradation of polysaccharides.[1−6] Secondly, modified polysaccharides are attracting attention in a wide variety of applications in which degradability is often necessary.[1,7−10] Modification of polysaccharides is accomplished by derivatization (of part) of the monomeric units. However, this often results in a retarded enzymatic degradation.[5,8,9,11−13]

Recently, we investigated the release of a model protein (IgG) from enzymatically degrading dextran hydrogels which were obtained by polymerization of methacrylated dextrans (dex-MA) in aqueous solutions.[13] The degradation rate of the gels was not only dependent on the amount of encapsulated dextranase but also on the degree of substitution (DS; the amount of methacrylates per 100 glucopyranose residues). Dextran hydrogels with a low DS (DS 4) could be completely degraded by dextranase. In contrast, no degradation was observed when a gel was prepared using dex-MA with a high DS (>13), although dex-MA itself was susceptible to enzymatic degradation.[13]

This study reports on the degradation of dex-MA by dextranase. These dextran derivatives are synthesized by a coupling reaction of glycidyl methacrylate to dextran. The methacrylic ester is coupled to position 3 and position 2 of the glucopyranose residues in a ratio of 1:1, as determined by ^{13}C-NMR analysis.[14,15] The aim of this study is to obtain insight into the nature of the degradation products and the mechanism of the dextranase-catalyzed hydrolysis of dex-MA.

Experimental Section

Materials. Dextran (T40; M_n = 15.000, M_w = 39.000 as determined by gel permeation chromatography[14]) from *Leuconostoc ssp.*, dextran standards from *Leuconostoc mesenteroides*, maltose, and maltotriose, -tetraose, -pentaose, and -hexaose (further referred to as malto oligomers) were obtained from Fluka AG, Buchs, Switzerland. Glucose was purchased from Genfarma, Maarssen, The Netherlands. Dextranase D-1508 from *Penicillium funiculosum* (EC 3.2.1.11, specific activity 250−500 U/mg protein) was ordered at Sigma NV, Bornem, Belgium. Dex-MA differing in DS were synthesized and characterized according to Van Dijk-Wolthuis et al.[14,15]

Electrospray Mass Spectrometry. Dextran or dex-MA solutions (10 mg/mL) in ammonium acetate (5 mM, pH 5.5) were degraded by dextranase (0.1 U/mL) at 37 °C over 3 days. The solutions (10−20 μL) were injected on a VG Platform II mass spectrometer (Fisons Instruments, Cheshire, U.K.; cone voltage 25−50 V, capillary voltage 4 kV, scan range m/z 150−1200, flow 50 μL/min, source temperature 100 °C). Positively charged ions were produced using an electrospray probe (Fisons Instruments, Cheshire, U.K.). Calibration was done with malto oligomers (ranging from glucose to maltohexaose). The mass spectra were analyzed using Masslynx V.2.1 software (Fisons Instruments, Cheshire, U.K.). For the calculation of the relative amount of a degradation product, the sum of intensities of the peaks assigned to this product was compared with the total sum of intensities of all assigned peaks.

Enzyme Kinetics. Dextran or dex-MA was degraded at different concentrations (0.2−20 mg/mL) in ammonium acetate buffer (5 mM, pH 5.5) at 37 °C. The amount of dextranase (0.01−0.5 U/mL) increased with increasing DS of the dex-MA substrate. Samples were taken every 10 min and heated for 15 min at 95 °C to inactivate the enzyme. The concentration of reducing oligosaccharides in the samples was determined spectrophotometrically with Sumner reagent as described by

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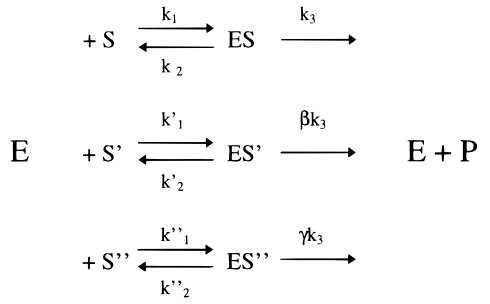

Figure 1. Reaction scheme of an enzyme (E) acting on three different substrates (*S*, *S′*, and *S″*) resulting in a product (P).[16]

Franssen et al.[13] One unit of enzymatic activity (U) is defined as the amount of enzyme that produces 1 μmol/min reducing oligosaccharides, with dextran (10 mg/mL) as substrate at pH 5.5 and 37 °C.

The degradation rate (in μmol/U·min) was calculated as the initial slope of the amount of reducing oligosaccharides in time for several substrates (dextran and dex-MA with DS 4, 8, 13, 27, and 37). The obtained degradation rates (v) were interpreted with a three-substrate model in which substrates S, S′, and S″ have different maximum degradation rates, V_{max}, βV_{max}, and γV_{max} as stated in Figure 1. All substrates have their specific Michaelis−Menten constants, K, K', and K'' respectively. The following equation can be derived from this scheme:[16]

$$v = k_3[\mathrm{ES}] + \beta k_3[\mathrm{ES}'] + \gamma k_3[\mathrm{ES}''] \quad (1)$$

Now, both sides of eq 1 are divided by the total enzyme concentration ($[E]_{total}$; which can also be written as [E] + [ES] + [ES′] + [ES″]):

$$v/[\mathrm{E}]_{total} = \frac{k_3[\mathrm{ES}] + \beta k_3[\mathrm{ES}'] + \gamma k_3[\mathrm{ES}'']}{[\mathrm{E}] + [\mathrm{ES}] + [\mathrm{ES}'] + [\mathrm{ES}'']} \quad (2)$$

The concentration of each species can be expressed in terms of [E] (see Figure 1):

$$v/[\mathrm{E}]_{total} = \frac{k_3[\mathrm{S}]/K + \beta k_3[\mathrm{S}']/K' + \gamma k_3[\mathrm{S}'']/K''}{1 + [\mathrm{S}]/K + [\mathrm{S}']/K' + [\mathrm{S}'']/K''} \quad (3)$$

in which $K = [E][S]/[ES]$ for each substrate. Since

$$V_{max} = k_3[\mathrm{E}]_{total} \quad (4)$$

the equation in the usual Michaelis−Menten form can be obtained:

$$v = \frac{V_{max}[\mathrm{S}]/K + \beta V_{max}[\mathrm{S}']/K' + \gamma V_{max}[\mathrm{S}'']/K''}{1 + [\mathrm{S}]/K + [\mathrm{S}']/K' + [\mathrm{S}'']/K''} \quad (5)$$

In this model, S is defined as a native substrate, S′ as a monosubstituted oligosaccharide, and S″ as a multiply substituted oligosaccharide. All substrates can fill the binding site of the enzyme completely. Assuming a number of binding subsites (n), the fractional substrates S, S', and S'' can be calculated from the total substrate concentration, S_{total}, as follows:

$$S = S_{total}(1 - \mathrm{DS}/100)^n \quad (6a)$$

$$S' = S_{total} n(\mathrm{DS}/100)(1 - \mathrm{DS}/100)^{n-1} \quad (6b)$$

$$S'' = S_{total} - (S + S') \quad (6c)$$

Now eqs 6a−c can be substituted into eq 5. Subsequently, global nonlinear regression analysis (SPSS 6.1.2 for Microsoft Windows 3.11) with the obtained equation was applied on the acquired data in which V_{max}, β, γ, K, K', K'', and n were calculated as unconstrained constants with S_{total} and DS as independent and v as dependent variable, resulting in $n = 14 \pm 6$. The global analysis was repeated with the same equation in which n was substituted by 14, whereas the other constants were kept unconstrained.

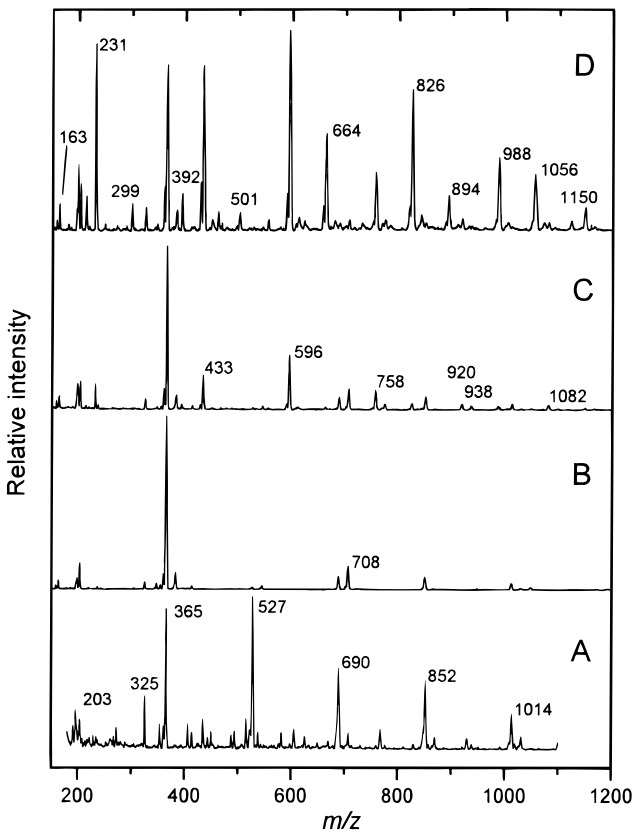

Figure 2. Mass spectra of positively charged ions: (A) a mixture of malto oligomers with a degree of polymerization of 1 to 6; (B) degraded dextran; (C) degraded dex-MA DS 4; (D) degraded dex-MA DS 37. The relative intensities are scaled as a percentage of the highest peak in each mass spectrum. See also Table 1.

Results

Electrospray Mass Spectrometry. In order to characterize underivatized oligosaccharides, electrospray mass spectrometry (ES-MS) has been used for several years[17] and especially ES-MS of metal-coordinated oligosaccharides has proven to be useful.[18,19] A typical mass spectrum obtained in our study by flow analysis of a mixture of malto oligomers up to a degree of polymerization of 6 and in the presence of sodium and ammonium ions is shown in Figure 2A. The relatively high intensity of $[M + Na]^+$ is not a surprise, as it has been reported that this type of adduct ion is very stable.[17] Ammonium adduct ions were present with less intensity. Fragmentation was a minor process and only $[M + H - H_2O]^+$ were observed. Interesting to note was that ions with a low intensity were observed, which represent $[nM + Na]^+$. These ions are multiple molecules coordinated to one sodium ion.[18] Complete assignment of all ions found is given in Table 1.

The presence of abundant ions such as $[M + Na]^+$ in the spectra is very convenient, because this makes it possible to determine the content of an unknown mixture of oligosaccharides by flow analysis, i.e., without separation prior to ES-MS. This is demonstrated for the flow analysis of enzymatically degraded dextran by dextranase (Figure 2B). The mixture contains mainly isomaltose and small amounts of isomaltotetraose and -pentaose (see also Table 1).

Table 1. Peak Assignment in Mass Spectra of a Mixture of Malto Oligomers, Enzymatically Degraded Dextran, and dex-MA (See also Figure 2)

m/z	degree of polymerization	no. of methacrylates	ion description
163	1	0	$M + H^+ - H_2O$ and/or fragmentation[a]
198	1	0	$M + NH_4^+$
203	1	0	$M + Na^+$
231	1	1	fragmentation[a]
299	1	2	fragmentation[a]
325	2	0	$M + H^+ - H_2O$
360	2	0	$M + NH_4^+$
365	2	0	$M + Na^+$
392	2	1	$M + H^+ - H_2O$ and/or fragmentation[a]
428	2	1	$M + NH_4^+$
433	2	1	$M + Na^+$
501	2	2	$M + Na^+$
522	3	0	$M + NH_4^+$
527	3	0	$M + Na^+$
590	3	1	$M + NH_4^+$
595	3	1	$M + Na^+$
663	3	2	$M + Na^+$
689	4	0	$M + Na^+$
707	2 + 2	0 + 0	$2M + Na^+$
757	4	1	$M + Na^+$
825	4	2	$M + Na^+$
851	5	0	$M + Na^+$
893	4	3	$M + Na^+$
919	5	1	$M + Na^+$
937	2 + 3	0 + 1	$M + M' + Na^+$
987	5	2	$M + Na^+$
1013	6	0	$M + Na^+$
1055	5	3	$M + Na^+$
1081	6	1	$M + Na^+$
1149	6	2	$M + Na^+$

[a] Fragmentation according to Figure 3.

In enzymatically degraded dex-MA with low DS (DS 4), monomethacrylated oligosaccharides were detected next to the same products as found in degraded dextran (Figure 2 C and Table 1, e.g., *m/z* 595, isomaltotriose-MA). Ions of monomethacrylated oligosaccharides show a characteristic increase of 68 amu, which corresponds to the methacryl moiety. At high DS (DS 37), also multiply methacrylated oligosaccharides were detected (Figure 2D, e.g., *m/z* 825, isomaltetraose-2MA or *m/z* 1055, isomaltopentaose-3MA), besides unsubstituted and monomethacrylated oligosaccharides.

The mass spectra of degraded dex-MA show a characteristic fragment at *m/z* 231, which was not observed for degraded dextran (Figure 2B) and of which the intensity increases with the cone voltage. This ion could represent the loss of water from a protonated glucose-MA molecule (i.e., $[M + H - H_2O]^+$), which would imply that glucose-MA was formed during the enzymatic degradation. However, this mechanism does not explain why the intensity of the ion at *m/z* 231 increases with the cone voltage. Moreover, sodium adduct ions of glucose-MA could not be detected (e.g., *m/z* 271, $[M + Na]^+$). Alternatively, this ion might be formed by fragmentation at the 1,6 linkage on the nonreducing, methacrylated end of an oligosaccharide, as indicated in Figure 3 (route A), resulting in an oxonium ion, which is a common type of fragmentation.[20] The methacrylic moiety appears to stabilize this ion, since the corresponding unsubstituted ion (*m/z* 163) is only found at a relatively low intensity. An explanation could be that the positive charge can be transferred to a methacryl moiety by a hydride shift, as shown in Figure 3, route B. Despite the mechanism by which the ion at *m/z* 231 is formed, it indicates the presence of a methacrylic group at the nonreducing end of a methacrylated oligosaccharide.

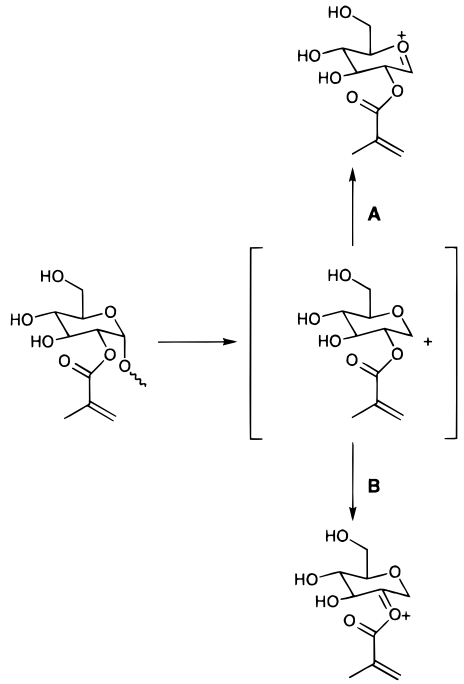

Figure 3. Possible mechanism for the occurrence of the ion at *m/z* 231.

In Figure 4, the relative amount of each degradation product is shown for several DS. The products are organized in degree of polymerization (the number of glucopyranose residues) and the number of methacrylate groups. The main degradation product of dextran is isomaltose. Also glucose and traces of isomaltotetraose and -pentaose were found. Complete degradation of the linear parts of dextran results in the formation of glucose and isomaltose. However, α-1,3 and α-1,4 linkages in the dextran chains result in the formation of isomaltotetraose and isomaltopentaose.[21−23] This characteristic pattern formed by the unsubstituted degradation products is not only found for dextran but also for the methacrylated dextrans (Figure 4B−D). Furthermore, these Figures show that the main substituted degradation product for all dex-MA is monomethacrylated isomaltotriose. For DS 4, the main substituted degradation products have only one methacrylate. As expected with increasing DS, the relative intensity of the monomethacrylated degradation products increases. In addition, di- and trimethacrylated oligosaccharides are detected in degraded dex-MA with a higher DS. The degree of polymerization of these products with multiple substituents is also increased. However, longer oligosaccharides than isomaltohexaose were not detected, which was confirmed by gel permeation chromatography analysis[14] in which M_w of the degradation products did not exceed 1100. Interestingly, Figure 4 demonstrates that the number of methacrylates was lower than the number of glucopyranose residues for all degradation products.

Enzyme Kinetics. Results published by Allen et al.,[24,25] Sigiura et al.,[23] and Walker et al.[21,22] suggest that the number of binding subsites (the maximum number of glucopyranose residues in a polysaccharide that can bind to an enzyme, *n*) is 9−12 for dextranase from *Penicillium funiculosum*. Both gel permeation chromatography and ES-MS analysis show that even at high DS the degradation products of dex-MA contain

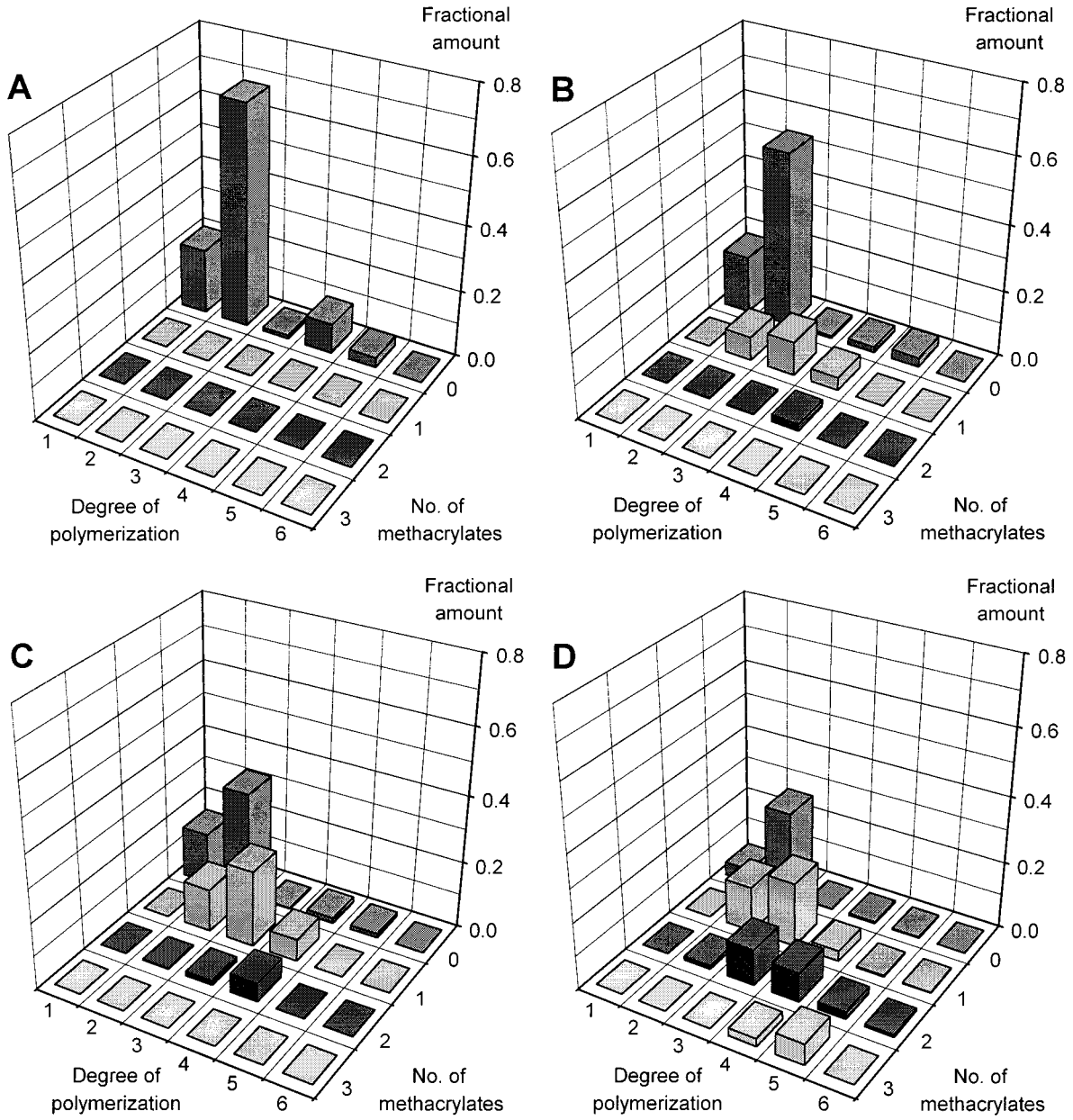

Figure 4. Relative amounts of the degradation products as determined by ES-MS, organized in degree of polymerization (the amount of glucopyranose residues) and the number of methacrylates: (A) dextran; (B) dex-MA DS 4; (C) dex-MA DS 8; (D) dex-MA DS 37. The fractional amount is the sum of intensities of peaks assigned to the particular compound relative to the sum of intensities of all assigned peaks.

six or less glucopyranose residues and up to three methacrylate groups. These products can only be formed when the enzyme is able to hydrolyze glycosidic bonds in methacrylated oligosaccharides. In other words, methacrylated oligosaccharides can also act as a substrate for dextranase. However, it can be expected that the kinetic constants (the Michaelis−Menten constant, K_m and the maximum degradation rate, V_{max}) have different values for the native and the methacrylated substrates.

In Figure 5, the initial degradation rate is plotted against the total substrate concentration for dextran and dex-MA with different DS. With eqs 5 and 6, V_{max}, β, γ, K, K', K'', and n can be calculated from the data shown in Figure 5 using nonlinear regression analysis. The number of binding subsites calculated by the three-substrate model was 14 ± 6. The results of this regression analysis for 14 binding subsites is given in Table 2. Factors β and γ indicate that V_{max} of the substituted substrates is 22% of the unsubstituted substrate. Surprisingly, no difference is found between β and γ, indicating that monosubstituted and multiply substituted substrates are degraded with equal rates. The Michaelis−Menten constant found for an unsubstituted substrate, K, is equal to K_m (0.5−0.6 mg/mL) found for dextran when fitted to the Michaelis−Menten equation, but is slightly greater than values reported in the literature (0.2−0.3 mg/mL[26,27]). Interestingly, K', the Michaelis−Menten constant for monosubstituted substrates, is lower than K, suggesting favorable interactions of a methacrylated glucopyranose residue with a certain binding subsite in the enzyme. However, multiply methacrylated substrates have a reduced affinity for the enzyme ($K'' > K$).

The R^2 of the global regression was determined for each substrate and reaches its maximum at moderate

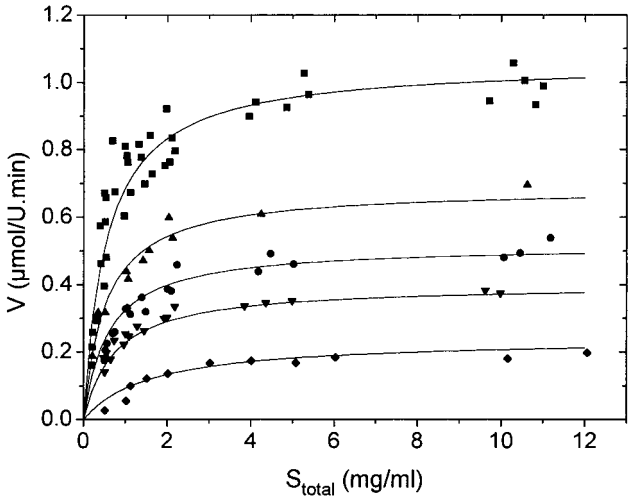

Figure 5. Initial degradation rates for dextran and several dex-MA differing in DS catalyzed by dextranase as a function of the concentration: (■) dextran; (▲) dex-MA DS 4; (●) dex-MA DS 8; (▼) dex-MA DS 13; (◆) dex-MA DS 37. The substrates were degraded 2−4 times in a range of concentrations (0.2−20 mg/mL). The curves represent for each substrate the results of the global nonlinear regression analysis (see also Table 2). One unit of enzymatic activity (U) is defined as the amount of enzyme that produces 1 μmol/min reducing oligosaccharides, with dextran (10 mg/mL) as substrate at pH 5.5 and 37 °C.

DS (DS 8 and 13, $R^2 = 0.994-0.995$). At these DS, all three substrates are present in about equal amounts. On the other hand, S and S' predominate at low DS, whereas at high DS mainly S′ and S″ are present. This can result in the slight decrease of R^2 toward both lower and higher DS ($R^2 \approx 0.98$).

Discussion

In Figure 4, the products of the enzymatic degradation by dextranase are presented as a function of the degree of polymerization and the amount of methacrylates. Interestingly, only degradation products are formed with at least one glucopyranose residue more than the amount of methacrylate substituents. This means that these degradation products must be formed by the hydrolysis of a chain having a degree of polymerization of at least two glucopyranose residues more than the amount of methacrylate substituents. This indicates that dextranase is capable of hydrolyzing a bond between a substituted and an unsubstituted glucopyranose residue, but bonds between two substituted glucopyranose residues can probably not be cleaved by the enzyme. This can be explained by the binding energies of the binding subsites near the catalytic center (Figure 6). It has been reported that several polysaccharide hydrolyzing enzymes have one subsite with antibinding character (a positive binding free energy) next to the catalytic center.[21,24,25] This antibinding subsite might contain apolar amino acids, which give unfavorable interactions with the hydrophilic glucopyranose residues (Figure 6 A, $\Delta G > 0$). However, these apolar amino acids allow hydrophobic interactions with methacrylate groups, which decreases ΔG (Figure 6 B, $\Delta\Delta G < 0$). The binding subsite at the other side of the catalytic center, has often a strong binding character and will preferably bind an unsubstituted glucopyranose residue. If the antibinding subsite is located at the reducing side of the catalytic center, an oligosaccharide with a methacrylate group at the nonreducing end is formed after hydrolysis of the glycosidic bond between a methacrylated and an unsubstituted glucose. This oligosaccharide can be responsible for the fragmentation product found in ES-MS at m/z 231 (Figure 3). This hypothesis is further supported by the results of the enzyme kinetics in which a three-substrate model shows

Table 2. Constants of the Three-Substrate Model Calculated with Nonlinear Regression of Eqs 5 and 6, Assuming That Dextranase from *Penicillium funiculosum* Has 14 Binding Subsites[a]

V_{max} (μmol/U·min)	1.06 ± 0.02
β	0.22 ± 0.04
γ	0.22 ± 0.03
K (mg/mL)	0.54 ± 0.04
K' (mg/mL)	0.41 ± 0.09
K'' (mg/mL)	1.7 ± 0.8
R^2	
global	0.987
dextran	0.987
dex-MA DS 4	0.974
dex-MA DS 8	0.994
dex-MA DS 13	0.995
dex-MA DS 27	0.975
dex-MA DS 37	0.982

[a] The uncorrected coefficient of determination (R^2) at each DS was calculated for the data of the corresponding substrate using full constraint towards the values of the constants.

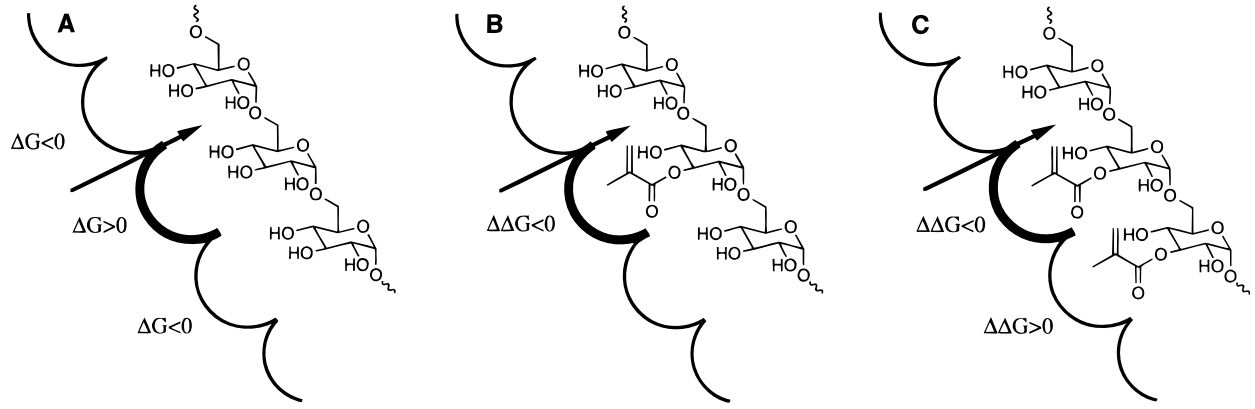

Figure 6. Binding of (A) an unsubstituted, (B) a monosubstituted, and (C) a disubstituted oligosaccharide at binding subsites near the catalytic center of dextranase. The binding subsites of the enzyme are indicated as half circles. Cleavage of the oligosaccharide occurs at the catalytic center, indicated as an arrow. The hydrophobic binding subsite next to the catalytic center is shown in bold. At each binding subsite, the probable sign of the binding free energy (ΔG) for unsubstituted glucopyranose residues is given in Figure A. The change in ΔG ($\Delta\Delta G$) due to the presence of methacrylated glucopyranose residues is given in Figures B and C. Note that the binding location of the second methacrylated glucopyranose residue is probably not restricted to one binding subsite (Figure C).

that the Michaelis−Menten constant for a monosubstituted substrate is lower than the one for the native substrate ($K' < K$), representing the beneficial binding of a methacrylated glucopyranose residue to a hydrophobic binding subsite (see also Table 2). Additionally, multiply substituted substrates show a relatively high Michaelis−Menten constant ($K'' > K$), as a result of unfavorable interactions between a methacrylated glucopyranose residue and another, more hydrophilic binding subsite, of which ΔG will increase (Figure 6 C, $\Delta\Delta G > 0$). Furthermore, the binding of a monomethacrylated substrate decreases the V_{max} compared to the unsubstituted substrate ($\beta < 1$) but multiply methacrylated substrates have a V_{max} similar to that of the monosubstituted substrate ($\gamma \approx \beta$). This large effect of the monomethacrylated substrate compared to multiply methacrylated substrates indicates that one methacrylated glucopyranose residue indeed binds closely to the catalytic center of the enzyme, whereas the other binds at subsites which are located further away.

References and Notes

(1) Aspinall, G. O., Ed. *The polysaccharides*; Academic Press: New York, 1983; Vols. I−III.
(2) Armandt, S.; Drouillard, S.; Schülein, M.; Henrissat, B.; Driguez, H. *J. Biol. Chem.* **1997**, *272*, 2709−2713.
(3) Nojiri, M.; Kondo, T. *Macromolecules* **1996**, *29*, 2392−2395.
(4) Roeser, D. S.; McCarthy, S. P.; Gross, R. A.; Kaplan, D. L. *Macromolecules* **1996**, *29*, 1−9.
(5) Brown, R. G.; Lindberg, B. *Carbohydr. Res.* **1974**, *38*, 369−373.
(6) Suga, K.; van Dedem, G.; Moo-Young, M. *Biotechnol. Bioeng.* **1975**, *17*, 185−201.
(7) Heller, J.; Pangburn, S. H.; Roskos, K. V. *Biomaterials* **1990**, *11*, 345−350.
(8) Crepon, B.; Jozefonvicz, J.; Chytry V.; Rihová B.; Kopecek J. *Biomaterials* **1991**, *12*, 550−554.
(9) Vercauteren, R.; Schacht, E.; Duncan, R. *J. Bioact. Compat. Polym.* **1992**, *7*, 346−357.
(10) Park, K.; Shalaby, W. S. W.; Park, H. In *Biodegradable hydrogels for drug delivery*; Technomic: Basel, 1993.
(11) Brønsted, H.; Hovgaard, L.; Simonsen, L. *STP Pharma Sci.* **1995**, *5*, 60−64.
(12) Vercauteren, R.; Bruneel, D.; Schacht, E.; Duncan, R. *J. Bioact. Compat. Polym.* **1990**, *5*, 4−14.
(13) Franssen, O.; Vos, O. P.; Hennink, W. E. *J. Controlled Release* **1997**, *44*, 237−245.
(14) Van Dijk-Wolthuis, W. N. E.; Franssen, O.; Talsma, H.; Van Steenbergen, M. J.; Kettenes-van den Bosch J. J.; Hennink, W. E. *Macromolecules* **1995**, *28*, 6317−6322.
(15) Van Dijk-Wolthuis, W. N. E.; Kettenes-van den Bosch, J. J.; van der Kerk-van Hoof, A.; Hennink, W. E. *Macromolecules* **1997**, *30*, 3411−3413.
(16) Segel, I. H. In *Enzyme kinetics, behavior and analysis of rapid equilibrium and steady state enzyme systems*; Wiley & Sons: New York, 1975.
(17) Duffin, K. L.; Huang, E.; Henion, J. D. *Anal. Chem.* **1992**, *64*, 1440−1448.
(18) Fura, A.; Leary, J. A. *Anal. Chem.* **1993**, *65*, 2805−2811.
(19) Kohler, M.; Leary, J. A. *Anal. Chem.* **1995**, *67*, 3501−3508.
(20) Blok-Tip, L.; van der Kerk-van Hoof, A.; Heerma, W.; Haverkamp, J.; Kovacik, V.; Hirch, J. *Biol. Mass Spectrom.* **1993**, *22*, 474−480.
(21) Walker, G. J.; Pulkownik, A. *Carbohydr. Res.* **1974**, *36*, 53−66.
(22) Walker, G. J.; Dewar, M. D. *Carbohydr. Res.* **1975**, *39*, 303−315.
(23) Sugiura, M.; Ito, A. *Chem. Pharm. Bull.* **1974**, *22*, 1593−1599.
(24) Allen, J. D.; Thoma, J. A. *Biochem. J.* **1976**, *159*, 105−120.
(25) Allen, J. D.; Thoma, J. A. *Biochem. J.* **1976**, *159*, 121−132.
(26) Sugiura, M.; Ito, A. *Chem. Pharm. Bull.* **1975**, *23*, 1532−1536.
(27) Ludwig, H.; Greulich, K. O. *J. Chem. Res. (S)* **1979**, 30−31.

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