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KINETICS AND SUBSITE MAPPING OF A D-XYLOBIOSE- AND D-XYLOSE-PRODUCING *Aspergillus niger* ENDO-(1→4)-β-D-XYLANASE

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ABSTRACT

A previously described endo-(1→4)-β-D-xylanase produced by *Aspergillus niger* was allowed to react with linear unlabeled and labeled D-xylo-oligosaccharides ranging from D-xylotriose to D-xylo-octaose. No evidence of multiple attack or of condensation and trans-D-xylosylation reactions was found. Maximum rates and Michaelis constants were measured at 40° and pH 4.85. The former increased with increasing chain-length from D-xylotriose through D-xylohexaose to ~70% of that on soluble larchwood D-xylan, and then decreased slightly for D-xyloheptaose and D-xylo-octaose. Michaelis constants decreased monotonically with increasing chain-length. Bond-cleavage frequencies were highest near the reducing end of short substrates, with the locus of highest frequencies moving towards the middle of larger substrates. These data indicated that the endo-D-xylanase has five main subsites, with the catalytic site located between the third and fourth subsites, counting from the nonreducing end of the bound substrate. The subsite to the nonreducing side of the catalytic site strongly repels its corresponding D-xylosyl residue, while the two subsites farther towards the nonreducing end of the substrate strongly attract their corresponding residues. The subsite to the reducing side of the catalytic site moderately attracts D-xylosyl residues, while the next one towards the reducing end has a high affinity for them. The residual error of the numerical estimation was allocated largely to the Michaelis constants of the different D-xylo-oligosaccharides, whose calculated values were appreciably smaller than measured values, especially for shorter substrates. This suggests that the subsite model cannot fully account for the experimental data. Estimated and measured values of maximum rates, bond-cleavage frequencies, and dissociation constant when the active site is fully occupied by substrate agreed more closely with each other.

INTRODUCTION

During the past several years, we have purified to homogeneity five endo-

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(1→4)-β-D-xylanases [(1→4)-β-D-xylan xylanohydrolase, E.C. 3.2.1.8], from a crude *Aspergillus niger* preparation, that have markedly different physical and kinetic properties[1-4]. These enzymes, grouped into three families, differ in (*1*) the products they form, (*2*) whether they preferentially attack soluble or insoluble xylan, (*3*) their degree of activity near branch points, and (*4*) their molecular weights and isoelectric points. It is clear that, in nature, they act in concert to hydrolyze D-xylan and D-xylo-oligosaccharides.

The first of the endo-D-xylanases to be purified differs from the other four in that it produces mainly D-xylobiose and D-xylose from D-xylan and D-xylo-oligosaccharides, whereas the other four yield mainly longer products. This action pattern suggested that its active site is composed of relatively few subsites, indicating that it would be possible to construct its subsite map.

The technique of subsite mapping, pioneered by Robyt and French[5] in their study of the hydrolysis of malto-oligosaccharides with alpha amylase, was extended to more-quantitative applications mainly by the groups of Thoma[6-11] and Hiromi[12-17], using experimental data from varous amylases. With both exo- or endo-hydrolases, a full application of the technique requires the use of a homologous series of oligomeric substrates ranging in chain length up to the one that possesses more monomeric residues than the number of subsites in the active site. From these, the maximum rate (V_m) and Michaelis constant (K_M) of each substrate are determined, along with inhibition constants or their equivalents for smaller nonhydrolyzed materials. For exo-hydrolases, this leads directly to calculation of the free energy between each subsite and the residue of the substrate that it binds. However, with endo-hydrolases, where the location of the catalytic site in the subsite array is not known *a priori* from the product distribution, a series of labeled oligomers must be used in order to determine the frequency of cleavage of each bond in each substrate[8]. These, together with substrate kinetic parameters, lead to binding energies for each subsite. It must be recognized that either multiple attack, where more than one bond of the substrate is cleaved before the substrate is released by the enzyme, leading to a rapid increase of small products, or two-substrate reactions such as condensation and transglycosylation, producing larger products than expected from simple hydrolysis, will distort binding-energy determinations[17-20]. Either, conditions must be chosen where they do not occur, or, their effects must be corrected before binding energies are calculated.

To determine the subsite map of the *A. niger* endo-D-xylanase whose chief products are D-xylobiose and D-xylose, we have conducted a threefold program: measurement of (*1*) product distributions from the hydrolysis of D-xylo-oligosaccharides, to determine whether multiple attack or condensation and trans-D-xylosylation reactions occur; (*2*) V_m values and K_M values for the hydrolysis of D-xylotriose (X_3) through D-xylo-octaose (X_8), along with determination of K_{int}, the dissociation constant of the enzyme–substrate complex when all subsites are occupied by D-xylosyl residues[7], and (*3*) bond-cleavage frequencies (b.c.f.'s) for the hydrolysis of tritium-labeled X_3 through X_8.

MATERIALS AND METHODS

Purification of endo-D-xylanase. — The endo-D-xylanase used in this work has been described[1]; however, it was purified here by two methods that were more sure to achieve homogeneity. The first method, until the last step, was essentially identical to that used earlier[1], using a preparation of crude pentosanase from *Aspergillus niger* (Rohm and Haas Rhozyme HP-150 Concentrate, Lot 3-0007) passed through an Ultrogel AcA 54 gel-permeation column chromatograph, an SP-Sephadex cation-exchange column chromatograph at pH 4.5, and a Sephadex G-50 gel-permeation column chromatograph. The final step, passage through an SP-Sephadex column, differed, in that the elution buffer was 0.01M citrate, pH 5.1, instead of 25mM acetate, pH 4.6, and the linear NaCl gradient was from 0 to 75 mM, not from 0 to 0.3M. This preparation is denoted[2] xylanase 4.

In the second method of preparation, Fraction C, not Fraction B[2], from Ultrogel AcA 34 gel-permeation chromatography was collected, subjected to SP-Sephadex column chromatography at pH 4.5 and Sephadex G-50 column chromatography as previously described[1], and then passed through two SP-Sephadex columns. In the first, a 0.01M citrate eluant buffer was employed with a pH gradient from 4.9 to 5.7, using 0.1-pH unit steps. The desired endo-D-xylanase left the column at pH 5.4. In the final step, the same eluant buffer was used, but at pH 5.1, with a linear NaCl gradient from 0 to 75mM. The material obtained is identified[2] as xylanase 6III.

Isoelectric focusing, with 5:8 ampholyte–poly(acrylamide) gel, of the products of both separation methods routinely yielded one band dyed by Coomassie Brilliant Blue R and one endo-D-xylanase activity peak. Extensive characterization demonstrated that each was identical to the endo-D-xylanase described earlier[1], with an isoelectric point at pH 6.7 and production of D-xylobiose and D-xylose from D-xylan and linear D-xylo-oligosaccharides.

Purification of D-xylo-oligosaccharides. — Samples of X_3 through X_7, of 98% purity or higher, and of X_8 of 91% purity, were generally prepared by hydrolysis of 2% larchwood D-xylan with pH 1 aqueous trifluoroacetic acid (~0.3M, depending on the xylan used) for 15–25 min at 100°. The hydrolyzate, after KOH neutralization of the acid to pH 5, centrifugation, and concentration by evaporation, was passed through a column of 1:1 charcoal powder (MCB Darco G-60)–diatomaceous earth (Manville Celite 560)[21] that had been treated with stearic acid[22], using an aqueous *tert*-butanol gradient of decreasing slope that reached 6–7%. Pooled fractions of the individual D-xylo-oligosaccharides were evaporated to dryness, the residues dissolved in water, and the solutions submitted to a Bio-Rad AG1-X4 strong-base, ion-exchange column in the acetate form, with water as the eluant to remove uronic acids. Preparative h.p.l.c. was conducted on X_5 and X_7 from the ion-exchange column with a Whatman Partisil 10 PAC Magnum 9/50 cyano-amino column and a 7:3 (v/v) acetonitrile–water eluant to eliminate all but minor amounts of other D-xylo-oligosaccharides. This was followed by centrifugation and passage

through the Bio-Rad AG1-X4 column to remove silica.

Purity was measured by four methods: (*1*) h.p.l.c. with a Bio-Rad HPX-42A column at 85° with water as the eluant; (*2*) h.p.l.c. with a Supelcosil LC-NH2 column and 69 or 72% aqueous acetonitrile eluant at room temperature; (*3*) complete hydrolysis with pH 1 aqueous trifluoroacetic acid for 2.5 h at 100°, followed by h.p.l.c. in an HPX-42A column to determine non-D-xylose contaminants; (*4*) complete hydrolysis followed by reducing end-group analysis by the Somogyi-Nelson method[23] standardized with D-xylose, to determine components that are nonreducing and do not chromatograph as oligosaccharides. This group is presumably composed mainly of water. By the first two methods, X_3 had ~1% of oligosaccharides of other chainlengths, while X_4 had 2% of an unknown, X_5 no other oligosaccharide, X_6 2% of X_4 and X_5, X_7 2% of X_5 and X_6, and X_8 9% of all the other smaller D-xylo-oligosaccharides. The third method showed that X_4 and X_5 had ~1% of L-arabinose, while the other products were solely D-xylose. The fourth method led to estimates of 4.7% of inert material in X_3, 3.3% in X_4, 2.6% in X_5, 7.2% in X_6, and essentially none in X_7 and X_8.

Production of tritium-labeled D-*xylo-oligosaccharides.* — D-Xylo-oligosaccharides were partially labeled with tritium at the reducing-end C-1 atom (by Research Products International) by bubbling tritium gas through a pH 7 aqueous solution of 2.8% of carbohydrate at 37° over a catalyst of palladium oxide on barium sulfate[24]. Because this procedure yielded a mixture of labeled products, the samples were repurified by h.p.l.c. with either a Whatman Partisil 10 PXS 10/25 PAC cyano-amino column eluted with 4:1 (v/v) acetonitrile–water, or a Supelcosil LC-NH2 amino column eluted with 7:3 acetonitrile–water.

Determination of action pattern. To determine if multiple attack or trans-D-xylosylation and condensation reactions occurred during D-xylo-oligosaccharide hydrolysis, X_6, X_7, and X_8 were subjected to attack with the endo-D-xylanase in 0.01M sodium citrate buffer at pH 4.85 and 40°. Product distributions of samples taken at 15% conversion, or less, were measured by h.p.l.c. with a Supelcosil LC-NH2 column operated at room temperature with 7:3 (v/v) acetonitrile–water as the eluant.

Kinetic determinations. — Hydrolysis rates at 40° were measured with various concentrations of X_3 through X_8 dissolved in 35mM sodium citrate buffer, pH 4.85, and mixed with different amounts of the endo-D-xylanase. The reactions were conducted in 0.3–3-mL Pierce Reacti-Vials stirred with triangular magnetic stirrers and holding 0.025 to 2 mL of solution. Generally, seven 1–250-μL samples, depending on the carbohydrate concentration, were taken at 2-min intervals, diluted to 1 mL with 2% Na_2CO_3, and ferricyanide reagent[25,26] (4 mL) was added. The latter consisted of 5 g of KCN, 0.34 g of $K_3Fe(CN)_6$, and 20 g of Na_2CO_3 dissolved in 1 L of water. The resulting mixtures were heated for 10 min at 100°, cooled, and their absorbances measured in a 10-mm pathlength cuvet at 420 nm. Reducing end-group production was determined from these absorbances, using as standard the D-xylo-oligosaccharide reactant being tested. Activities were calculated from initial rates obtained by

linear regression of these data, and were normalized to the activity on larchwood D-xylan. Values of V_m and K_M for each substrate were determined from normalized activities at different concentrations of each by use of Wilkinson's method[27]. The value of K_{int} was determined by plotting $(n - m + 1)$, where n is the substrate chainlength and m is the assumed number of subsites, *vs.* $1/K_M$ for X_6, X_7, and X_8. The slope of the resulting straight line is[7] $1/K_{int}$.

Endo-D-xylanase activity on xylan. — Endo-D-xylanase activity was determined with D-xylan made from 2 g of Koch-Light 5702-00 larchwood D-xylan, Lot 90581, which was suspended in 100 mL of 35mM sodium citrate buffer, pH 4.85, stirred overnight at room temperature, and centrifuged to remove undissolved material. To the supernatant liquor was added 1 mL of 0.03% sodium azide.

The assay was conducted at 40° with 0.9 mL of this solution, 1 mL of the same sodium citrate buffer, and 0.1 mL of endo-D-xylanase dissolved in the buffer in a 2-mL Pierce Reacti-Vial stirred with a triangular magnetic stirrer. Samples (100 μL) were taken at 5, 10, 15, and 20 min, diluted to 1 mL with 2% Na_2CO_3, and treated with ferricyanide reagent as before, using X_4 as the standard. The sodium azide in the substrate solution did not affect the assay.

Measurement of bond-cleavage frequencies. — Hydrolyses of purified reducing-end-labeled D-xylo-oligosaccharides with homogeneous endo-D-xylanase were carried out at 40° in 0.01M or 0.05M sodium citrate buffer at pH 4.85. Agitated 300-μL Pierce Reacti-Vials held 200 μL of solution, and 10-μL samples were taken at specified intervals and added to 0.1 μL of M NaOH or 20 μL of 10% Na_2CO_3 solution.

Samples (1 μL) from the NaOH-quenched reaction mixture, or 10 μL from the Na_2CO_3-quenched material, were spotted on E. Merck plastic-backed t.l.c. plates precoated with 0.2-mm thick silica gel 60. After air-drying, the plates were developed at room temperature with a single ascent of 7:2:1 (v/v/v) acetonitrile–water–1-propanol. Dried plates were sprayed with tritium surface autoradiograph enhancer (En^{3}Hance, New England Nuclear) and contact-exposed to X-ray film (Eastman Kodak Type S) for 4 days, to locate radioactive spots corresponding to individual labeled D-xylo-oligosaccharides. These were cut out and added to 10 mL of toluene cocktail (4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazoyl)]benzene in 1 L of toluene). Each sample was counted for 4 min with a Packard TriCarb C2425 scintillation counter. Counts for each D-xylo-oligosaccharide at each sampling time were normalized to the total count at that time, and yielded b.c.f. values of each bond in each D-xylo-oligosaccharide.

Calculation of subsite map. — The number of subsites, location of the catalytic site, values of all binding energies, and kinetic parameters were calculated with the computer program of Allen and Thoma[9]. This program requires the input of V_m and K_M values and their standard deviations for each substrate, b.c.f. values with standard deviations for each bond of each substrate, and the overall K_{int} with its standard deviation. Values of 1 ± 0.2M and 0.5 ± 0.1M were assumed for the K_M values of D-xylose and X_2, respectively, but much different values for these para-

TABLE I

KINETIC PARAMETERS FROM ENDO-D-XYLANASE HYDROLYSIS OF D-XYLO-OLIGOSACCHARIDES OF DIFFERENT LENGTHS AT 40° AND pH 4.85

	Experimental			*Calculated*	
Substrate	*No. of points*	V_m *(relative to* D-*xylan)*	K_M *(mM)*	V_m *(relative to* D-*xylan)*	K_M *(mM)*
X_1	—	—	—	—	1.96
X_2	—	—	—	0.0037	0.855
X_3	8	0.220 ±0.028[a]	102 ±18[a]	0.080	0.845
X_4	10	0.321 ±0.024	4.27 ± 0.77	0.319	0.603
X_5	9	0.645 ±0.046	3.56 ± 0.60	0.488	0.363
X_6	13	0.686 ±0.044	2.29 ± 0.27	0.548	0.278
X_7	6	0.611 ±0.047	1.14 ± 0.20	0.568	0.250
X_8	9	0.589 ±0.048	0.544 ± 0.194	0.589	0.219

[a]Standard deviation.

meters hardly changed the final results. The estimation routine places weights on the experimental estimates of V_m's, K_M's, b.c.f's, and K_{int} in proportion to the reciprocals of the squares of their standard deviations. An acceleration factor (ΔG_a) can be made part of the optimization routine; this factor allows for the increase in the average intrinsic hydrolysis rate with increasing substrate chainlength caused by conformational strain between the active site and the substrate.

RESULTS

Action pattern. — When high concentrations (0.12 to 0.15M) of unlabeled X_6 through X_8 were hydrolyzed with the endo-D-xylanase to low conversions, a symmetrical product-distribution occurred (essentially equal amounts of X_3 and X_5 from X_8 hydrolysis, for instance), indicating the absence of multiple attack. The absence of trans-D-xylosylation and condensation reactions was confirmed in three different ways: (*1*) Neither the hydrolyses of X_6 through X_8 already described nor hydrolyses of labeled X_3 through X_8 at much lower concentrations yielded evidence of production of D-xylo-oligosaccharides larger than the substrate. As the hydrolytic rates of X_6 and larger are essentially constant, measurable amounts of these substances should have accumulated had they been produced. (*2*) No lags at the beginning of hydrolytic reactions involving X_3 through X_8 at concentrations up to several times their K_M values were observed[18]. (*3*) Hydrolyses of X_3 through X_8 followed Michaelis–Menten kinetics, rather than having a sigmoidal dependence of rate on substrate concentration[18].

Kinetic parameters. — Values of V_m and K_M at 40° and pH 4.85 were determined for X_3 through X_8, and are shown in Table I. As expected, K_M decreased monotonically with increasing substrate chainlength. Values of V_m, on the other hand, increased through X_6 and then decreased slightly for X_7 and X_8. The value of

TABLE II

BOND-CLEAVAGE FREQUENCIES FROM ENDO-D-XYLANASE HYDROLYSIS OF LABELED D-XYLO-OLIGOSACCHARIDES AT 40° AND pH 4.85

		No. of bond (from nonreducing end)						
Substrate		*7*	*6*	*5*	*4*	*3*	*2*	*1*
X_3	Exp.	—	—	—	—	—	0.117 ±0.032[a]	0.883 ±0.057[a]
X_3	Calc.	—	—	—	—	—	0.179	0.821
X_4	Exp.	—	—	—	—	0.032 ±0.012	0.541 ±0.035	0.428 ±0.039
X_4	Calc.	—	—	—	—	0.040	0.575	0.386
X_5	Exp.	—	—	—	0.021 ±0.030	0.249 ±0.104	0.570 ±0.047	0.159 ±0.021
X_5	Calc.	—	—	—	0.008	0.279	0.595	0.117
X_6	Exp.	—	—	0.012 ±0.010	0.051 ±0.016	0.480 ±0.041	0.393 ±0.029	0.064 ±0.009
X_6	Calc.	—	—	0.006	0.102	0.500	0.312	0.080
X_7	Exp.	—	0.015 ±0.022	0.092 ±0.023	0.228 ±0.101	0.374 ±0.102	0.252 ±0.035	0.039 ±0.009
X_7	Calc.	—	0.005	0.089	0.233	0.334	0.271	0.069
X_8	Exp.	0.049 ±0.036	0.128 ±0.020	0.112 ±0.041	0.319 ±0.084	0.277 ±0.018	0.080 ±0.070	0.034 ±0.016
X_8	Calc.	0.004	0.075	0.197	0.152	0.283	0.229	0.059

[a]Standard deviation.

K_{int}, calculated from the K_M values for X_6, X_7, and X_8, was 1.46 ±0.31mM, the second value being the standard deviation.

Bond-cleavage frequencies. — Values were obtained at low conversions with low concentrations (15μM–0.15mM) of labeled X_3 through X_8. Two runs were conducted with X_3 and three with X_4, and results were averaged. One run was conducted for each of the other substrates. At shorter chainlengths, bonds closer to the reducing end were preferentially attacked (see Table II). As the chain length increased, the highest incidence of attack moved towards the middle of the chain.

Calculation of the subsite map. — Calculated binding-energies for eight subsites, four to each side of the catalytic site, are shown in Table III for the case where ΔG_a was set to zero. Only subsites 2 through 6, counting from the nonreducing end

TABLE III

BINDING ENERGIES FROM ENDO-D-XYLANASE HYDROLYSIS OF D-XYLO-OLIGOSACCHARIDES AT 40° AND pH 4.85

Subsite no. (from nonreducing end)	*Binding energy (kJ/mol)*
1	0.65
2	−2.40
3	−7.15
4	4.35
Catalytic site	
5	−1.63
6	−3.35
7	−0.52
8	1.54

of the substrate, have major significance. Subsites 4 and 5, which bind D-xylosyl residues on either side of the bond being attacked, strongly repel and moderately attract those residues, respectively. Subsites 2, 3, and 6 are strongly attracting, while subsite 8 is moderately repelling. When ΔG_a was allowed to float, its value was only −237 J/mol, not significantly different from zero. As there were only small variations in subsite maps and calculated V_m, K_M, and b.c.f. values between accelerated and non-accelerated cases, only the latter will be discussed further.

Differences between experimentally obtained values of K_M and those calculated after binding energies were determined were the largest of any of the dependent variables (see Table I). In all cases, calculated values were lower than experimental values, with the differences being greatest with the shortest substrates. Differences between calculated and experimental values of V_m's and b.c.f.'s (see Tables I and II) were smaller. All but one calculated V_m value was smaller than its corresponding experimental value; the calculated value that was higher was for X_2, for which no rate could be experimentally determined. No clear pattern in differences of b.c.f. values was apparent. The calculated value of K_{int} was 1.82mM, compared to an experimental value of 1.46 ±0.31mM. Approximately 68% of the total residual error between experimental and calculated data was found in the K_M values, with 23% associated with the estimate of b.c.f.'s, and 9% with that of V_m's. Less than 1% of the residual error was associated with the K_{int} estimate.

The observation that the experimental value of K_M for X_3 was over a hundredfold larger than its calculated value, as well as being much larger than experimental values of K_M for larger D-xylo-oligosaccharides, led us to test the sensitivity of the calculated results to changes in this parameter, and to data for X_3 in general. Changing the assumed experimental K_M value for X_3 from 102mM to 5mM, barely above the value for X_4, had virtually no effect on calculated values of the binding energies, b.c.f.'s, K_M's, and V_m's. Eliminating all the X_3 data led to somewhat greater, although hardly significant, changes in these four parameters. The residual

error on the estimation decreased less than 25%, and the same skewed relationship between experimental and calculated kinetic parameters still appeared, suggesting that the major portion of the residual error was not associated with the X_3 data. The possibility that two-substrate reactions occurred at high concentrations of X_3, X_4, and X_5 cannot be rigorously excluded, as direct tests to observe them were not performed with these substrates. However, even if they occurred at high concentrations, which is unlikely, given the uniformly negative results when this possibility was tested by presence of products larger than the substrate, presence of an initial lag phase, or lack of adherence to Michaelis–Menten kinetics, they did not occur at the concentrations at which b.c.f. values, which affect the subsite map more than any of the other experimental parameters, were measured.

The fact that estimates of K_M and V_m were nearly always smaller than experimental values indicates that the subsite model does not completely fit the experimental data, as a proper fit should have led to calculated values of K_M and V_m being both higher and lower than experimental values[9]. This deviation suggests that the assumption that there is either no acceleration factor or a constant one is incorrect in this case. Instead, it suggests that the intrinsic bond-hydrolysis rate neither remains constant nor increases exponentially with increasing substrate chainlength, but, instead, varies in a more individual fashion with substrate of different lengths.

Despite the disagreement between calculated and experimental K_M and V_m values, even with a non-zero ΔG_a, the subsite map is capable of explaining why substrates of different lengths have such different V_m's and K_M's, and why individual b.c.f.'s in different substrate vary as they do. There is no significant endo-D-xylanase activity on X_2, because the only productive complex between enzyme and substrate is highly improbable, the result of the highly positive sum of the binding energies of the two subsites adjacent to the catalytic site. With X_3, hydrolysis is possible, because formation of enzyme–substrate complexes across subsites 3, 4, and 5, and across subsites 4, 5, and 6, both yield negative free energies. The difference in free energies of binding between the two productive enzyme–X_3 complexes, the first being much more negative than the second, explains the large difference in the b.c.f. values of the two D-xylosyl bonds of X_3. Values of K_M decrease with increasing chainlength, because the free energies of binding of the complexes formed become progressively more negative as more subsites are involved in substrate binding, as there is only one subsite (the fourth) with a significantly positive binding-energy, and two others (the first and eighth), with fairly small positive energies. Values of V_m increase with increasing substrate chainlength through X_6, because the ratio of productive complexes to total complexes increases. The locus of highest b.c.f. values moves toward the center of the substrate, and becomes more diffuse with increasing substrate chainlength, because the overall free energies of binding of different productive complexes of enzyme and substrate become more similar. Only those complexes with the catalytic site acting on a bond near the end of long substrate chains have free energies of binding much less negative than those involving more subsites. The former are less probable, and therefore yield lower b.c.f. values for bonds near

the end of the substrate.

The structure of the subsite map found with this endo-D-xylanase, with a subsite having a positive binding-energy located adjacent to the catalytic site, is characteristic of endo-hydrolases that produce mainly oligosaccharides but do not catalyze bi-substrate reactions. If the two subsites adjacent to the catalytic site both had negative binding-energies, disaccharides would be hydrolyzed to monosaccharides. The location of the subsite with positive binding-energy to the nonreducing side of the catalytic site suggests that the D-xylosyl residue bound there, whose anomeric bond is the one being cleaved, is strongly distorted, probably from a chair to a half-chair form[7,28]. Similar subsite patterns are found with lysozyme[29] and *Bacillus amyloliquefaciens* alpha amylase[7,10]. Prodanov *et al.*[30], however, found the opposite order of positive and negative binding-energies about the catalytic site of porcine-pancreatic alpha amylase, probably because of the extremely restrictive assumptions they used in order to determine their subsite map. Several other groups have calculated a positive sum of binding energies for the two subsites on either side of the catalytic site, but have not determined how this total is allocated between the sites. This pattern is exhibited by Taka-amylase A from *Aspergillus oryzae*[13] and endo-D-xylanases from both *Cryptococcus albidus*[31] and *A. niger*[32], and, presumably, by an endo-dextranase from *Streptococcus mutans*[33]. it should be noted that the *A. niger* endo-D-xylanase is very different from the one studied in this project, in that it has strong trans-D-xylosylation activity[34] and only four major subsites, two to each side of the catalytic site[32]. Three additional subsites that are situated more to the reducing end of the substrate being bound all have positive binding-energies[32].

Therefore, the endo-D-xylanase described herein is similar in the structure of its active center to others previously subsite-mapped. Parallel work with amino acid sequencing and site-directed mutagenesis of endo-hydrolases, now becoming much easier, has the capacity to extend the knowledge of active-center structure provided by subsite mapping.

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