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Characterization, kinetics, and subsite mapping of Aspergillus niger glucoamylases I and II, and partial purification and characterization of a Chainia endo-xylanase

Michael Martin Meagher

Iowa State University

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Characterization, kinetics, and subsite mapping of *Aspergillus niger* glucoamylases I and II, and partial purification and characterization of a *Chainia* endo-xylanase

Meagher, Michael Martin, Ph.D.

Iowa State University, 1987
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Characterization, kinetics, and subsite mapping of 
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and characterization of a Chainia endo-xylanase

by

Michael Martin Meagher

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Ames, Iowa
1987
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CHAPTER 1.

INTRODUCTION

This dissertation deals with the characterization, kinetics, and subsite mapping of two types of glycoside hydrolases. The objectives of the project are three-fold: 1) To subsite-map glucoamylase I (GAI) and glucoamylase II (GAII) with the malto- and isomaltooligosaccharides to determine if any differences exist between the two forms. This information will give valuable insight into the binding affinity of α-1,4- and α-1,6-linked substrates and may explain why maltose is hydrolyzed thirty times faster than isomaltose. It is the first subsite mapping of an enzyme with a non-preferred homologous substrate group, in this case the isomaltooligosaccharides. 2) To gain a better understanding of the action of GAI and GAII from Aspergillus niger on differently linked substrates. Hydrolysis rate studies were performed with five α-linked disaccharides and two α-linked trisaccharides at different temperatures and pHs to determine their effect on Michaelis-Menten constants. 3) To complete the characterization of an endo-xylanase from the actinomycete Chainia that was conducted in this laboratory by K. B. Bastawde, who brought the enzyme in a semi-purified state from the National Chemical Laboratory in Pune, India.

Chapter 2 consists of a brief introduction to the corn wet-milling industry followed by a discussion of starch conversion technology and the industrial importance of glucoamylase. Next, a literature review is presented which includes characterization, purification, subsite mapping,
and kinetics of glucoamylase. Following that are the results and discussion and finally the conclusions of the work on glucoamylase.

Chapter 3 is composed of five parts. The first section is an introduction to xylanases and some of their characteristics. Next, a literature review of endo-xylanases is presented with an emphasis on bacterial xylanases, followed by experimental procedures, results and discussion, and conclusions and recommendations.

Next, there are three appendices. The first is a derivation of the Hiromi subsite mapping model. The second appendix discusses the production and purification of substrates necessary for the study of glucoamylase kinetics, and the third appendix is a restatement of a model to explain the variation of Michaelis-Menten kinetic constants with changing pH, assuming the enzyme behaves as a dibasic acid.

From this dissertation will eventually come four papers. The first paper will consist of the subsite mapping of GAI and GAII with the malto- and isooligosaccharides. The second will present the GAI- and GAII-catalyzed hydrolysis kinetics of five α-linked disaccharides and three α-linked trisaccharides at different temperatures and pHs. The third paper, which has already been accepted by Carbohydrate Research, deals with the production, purification, and determination of physical constants of α,β-trehalose. This work was a joint venture between Dr. Frederick W. Parrish from the Southern Regional Research Center, Department of Agriculture, New Orleans, and myself. The fourth paper, which is a collaboration with Dr. Bastavde and others at the National Chemical Laboratory and Dr. Louisa Tabatabai at the National Animal Disease Center in Ames, will present the purification and characterization of the Chainia endo-xylanase.
CHAPTER 2.

KINETICS AND SUBSITE MAPPING OF GLUCOAMYLASES I AND II
FROM ASPERGILLUS NIGER

Introduction

The production of glucose from starch originated with Kirchoff in 1811 when he subjected potato starch to hydrolysis with sulfuric acid. Three products were obtained: syrup, solidified syrup, and D-glucose crystals, which were separated from the syrup. The use of this technique to produce D-glucose received little attention until 1922 when Newkirk developed a process that used seed crystals under a controlled rate to produce glucose monohydrate crystals. Since this time the production of D-glucose has grown into a billion pound per year business.

Starch is found in many different plants such as corn, wheat, potato, rice, and manioc. In the United States the principal source of starch is corn. Of the corn produced, 8.72 billion bushels in 1985 (Farm Futures, Dec. 1985, p. 12.), over 74% is used as livestock feed, 20% is used for seed corn and other corn-related products, while only 6% is processed by the corn wet milling industry. Besides D-glucose, there are other products generated from corn starch such as dextrins, conventional corn syrup, high fructose corn syrup (HFCS), and ethanol, which can be used as a gasoline extender. Of these products, corn sweeteners comprise 60% of the starch-based products sold in the United States, with HFCS being the largest item. Since the beginning of isomerization technology in the early 1970s, fructose has increasingly replaced sucrose as a sweetener.
Corn Wet Milling

The starch used for these products, as mentioned above, is obtained from corn by a continuous wet milling process. The process separates the corn kernel into its four major components: the skin or hull; the germ, which contains most of the oil; the gluten, which contains most of the protein; and the starch, the primary product. The typical yields per bushel of corn (25.4 kg at 15.5% moisture) are shown in Table 1.

<table>
<thead>
<tr>
<th>Component</th>
<th>Yield (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>14.4</td>
</tr>
<tr>
<td>Germ (50% corn oil)</td>
<td>1.6</td>
</tr>
<tr>
<td>Corn gluten feed (21% protein)</td>
<td>4.2</td>
</tr>
<tr>
<td>Gluten meal (60% protein)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*Ref. 3, p. 336.

A more detailed discussion of corn wet milling can be found elsewhere³⁴.

Starch Conversion

There are three types of conversion processes for the production of corn sweeteners: 1) acid, 2) acid-enzyme, and 3) enzyme-enzyme. The first method, acid conversion, breaks the starch molecules by high temperature and pressure with acid (usually hydrochloric acid) as the catalyst. Though the process is still used for the production of syrups primarily in the DE* 20-45 range, it is no longer used for the conversion to high DE

*DE is the reducing power of a solution as compared to D-glucose.
syrups those of DE 90-96. The long hydrolysis time needed to attain such high conversion results in the formation of hydroxymethylfurfurals\(^5\) and other colored compounds, along with reversion and condensation products, such as gentiobiose\(^6\).

The second method, acid-enzyme, uses acid to liquefy the starch, usually to a DE level of 10-20, making the starch more accessible to enzymatic degradation. The type of end-product desired determines the enzyme system. If a high DE syrup is desired then glucoamylase is used, while β-amylase or fungal α-amylase will produce a syrup high in maltose.

The final method, enzyme-enzyme, uses enzymes for both steps. The liquefying step, as before, is performed at 80-105°C, except a heat-stable α-amylase is used to thin the starch. Bacterial α-amylase has become the industrial standard. A comprehensive review of liquefaction is given by Reilly\(^7\). The saccharifying step is the same as outlined for the acid-enzyme method.

**Glucoamylase**

One of the most important contributions to starch conversion technology has been the use of glucoamylase. Enzymatic conversion of starch to D-glucose was known as early as 1881\(^8\), but it was not until 1940 when Langlois\(^9\) realized the advantages of the enzymatic route and disclosed a process for producing a solid enzymatic hydrolyzate containing up to 90% (dry basis) D-glucose. Wallerstein\(^10\) in 1951 improved the process by patenting a procedure for the manufacturing of crystalline D-glucose from the hydrolyzate. Kooi et al.\(^11\) further improved yields by the removal of
transglycosylase from glucoamylase preparations.

Glucoamylase is unable to convert starch to 100% D-glucose because of thermodynamic constraints\textsuperscript{12}. Industrially, the yield is 94% D-glucose by weight, with the remaining 6% being oligosaccharides, reportedly maltose, isomaltose, and maltotriose\textsuperscript{7}. This incomplete hydrolysis is caused, in part, by the presence in starch of α-1,6-linked branch points. Glucoamylase is able to cleave α-1,6 bonds, but only at a rate 3.6% that of α-1,4 bonds\textsuperscript{13}. Because of the reduced rate, reversion reactions are already underway before all of the α-1,6-initiated branch points from amylopectin have been removed. The formation of these reversion products along with the unhydrolyzed oligosaccharides affects the downstream production of D-glucose to D-fructose by reducing the yield.

The purpose of this work is to gain a better understanding of the action of glucoamylase on differently linked substrates. This will be accomplished by studying the active site of two glucoamylases, GAI and GAII, from \textit{A. niger}. Both enzymes will be subsite-mapped with malto- and isomaltooligosaccharides to determine if any differences appear between the two forms. To date no one has subsite-mapped both forms from the same species with the maltooligosaccharides. Also, no one has reported subsite-mapping glucoamylase with the isomaltooligosaccharides. This information will give valuable insight into the binding affinity of α-1,4- and α-1,6-linked substrates and enable us to determine if there are any kinetic differences between the two forms. Second, a series of hydrolysis reactions will be run at different pHs and temperatures with α-linked di- and trisaccharides to determine the effect on Michaelis-Menten kinetic
constants. This information will also help in distinguishing any differences between the two forms and will supply a solid foundation of kinetic data for modeling.14

The following sections will review work that has already been performed by others in the field, succeeded by work that has been completed to date in this laboratory.

Previous Work

Characterization

Glucoamylase (α-1,4 glucan glucohydrolase, EC 3.2.1.3) is a typical exo-glucanase in its production of D-glucose from the nonreducing ends of amylose, amylopectin, glycogen, and maltooligosaccharide molecules with inversion of configuration of the anomeric carbon15. Glucoamylase is a typical glycoprotein and is found in a number of fungal species16, but those of industrial importance are Rhizopus and Aspergillus, which are used in Japan and the United States, respectively. Glucoamylase is known to hydrolyze, by a multichain action, other linkages besides α-1,4 and α-1,613,17,18 such as α-1,3, α,α-1,1, α,β-1,1, and α-1,213,18-20. It has also been determined that all cleavage occurs at the same active site21-24.

Glucoamylase is known to exist in multiple forms. Pazur and Ando25, working with an A. niger preparation, Diazyme (Miles Laboratories Inc., Clifton, NJ), found two amyloglucosidases, each possessing different mobility on paper electrophoresis. Lineback et al.26 also isolated and characterized two forms from A. niger and found differences in isoelectric
point, electrophoretic mobility, and, to a small degree, in stability at room temperature for prolonged periods. Since then many others 27-34 have isolated two forms, and even three 35-40, from Aspergillus preparations. Rhizopus species have also been shown to produce multiple forms 41-43, with Tanaka et al. 44 reporting as many as five separate enzymes. It is interesting that Takahashi et al. 43 reported three active and two smaller inactive fragments while Tanaka et al. 44 found five active forms. Multiple forms have also been obtained from Hypocrea peltata 45 and Saccharomyces diastaticus 46.

The presence of multiple forms has been well documented, as mentioned above, but their formation is not completely understood. Yoshino and Hayashida 38,39, working with Aspergillus awamori var. kawachi, suggested that multiple forms can arise from the degradation of the native glucoamylase by acid protease and glycosidase activity. Takahashi et al. 42,43, studying a Rhizopus strain, isolated three active forms, Gluc 1, Gluc 2, and Gluc 3, with molecular weights of 74,000, 58,600, and 61,400, and two inactive forms, H and L, with molecular weights of 16,000 and 14,400, respectively. They found that the two inactive fragments, both glycoproteins, showed a common antigenicity towards Gluc 1 and Gluc 3 but not Gluc 2. Takahashi et al. 43 also found that the N-terminal amino acid of fragment H and Gluc 1 was alanine, while those of Gluc 2 and Gluc 3 were glutamic acid and lysine, respectively. Fragment L did not have a single N-terminal amino acid, and it was suggested that it may have arisen by further proteolytic action on fragment H. The C-terminal amino acid sequence was the same for all three active forms. From the above evidence Takahashi et
al. suggested that Gluc₂ was derived from proteolytic action on Gluc₁. Gluc₃ was thought to be produced by proteolytic modification of Gluc₁ with the release of peptide, but not glycopeptide, from its N-terminal side, which may be further degraded into smaller, undetectable fragments.

In work with A. niger, Boel et al., using molecular cloning techniques, isolated a cDNA copy of the GAI mRNA and found only one glucoamylase gene present in the genome, suggesting that both forms were generated from the same gene. Boel et al. also determined that the smaller fragment, GAII, was transcribed from an mRNA fragment which was identical to the GAI mRNA except for a 169-bp-long intervening sequence which was differentially spliced out. Nunberg et al. were also able to isolate a cDNA copy of the GAI mRNA and found that only one glucoamylase gene existed in the A. awamori genome, suggesting that both forms were derived from the same gene. Nunberg et al. proposed that GAII is a product of either post-translational processing or differential mRNA splicing. Though Nunberg et al. saw a minor immunoprecipitable product, which may correspond to GAII, during in vitro translational studies, they were not able to isolate a cDNA copy of the GAII mRNA.

Because the following work will be concerned only with glucoamylases from A. niger, the discussion from this point on will only pertain to glucoamylases produced by Aspergillus species.

All fungal glucoamylases are glycoproteins containing 5-20% carbohydrate. Lineback and Aria determined that GAI from A. niger contained 13% carbohydrate while GAII was 18% carbohydrate. The predominant sugar was D-mannose, followed by D-glucose and D-galactose, all of which were
linked O-glycosidically to serine and threonine residues. Pazur et al. investigated GAI from *A. niger* and found carbohydrate linked by O-glycosidic bonds to approximately 45 serine and threonine residues with D-mannose as the predominant sugar followed by D-glucose and D-galactose. Pazur et al. suggested that the carbohydrate moieties stabilized the tridimensional structure of the protein. Takahashi et al. purified the major glucoamylase, GAI, from *Aspergillus saitoi*, and estimated that it was 18% neutral sugars and 0.77% glucosamine. Inokuchi et al. purified the minor fraction, GAII, from *A. saitoi* and found that it was 12% neutral sugars and 0.55% glucosamine. In both cases D-mannose was the major carbohydrate with small amounts of D-glucose and D-galactose. Svensson et al. sequenced glucoamylase GAI from *A. niger* and found five N-glycosylated residues and 47 O-glycosylated serine and threonine residues, similar to Pazur et al. Svensson et al. determined there were 99 neutral carbohydrate residues (D-mannose equivalent) plus five residues of D-glucosamine per enzyme molecule. Takahashi et al. determined there were 97 neutral carbohydrates and four glucosamine residues per molecule of enzyme. Using the value of 47 for the number of glycosylated amino acid residues, there are an average of two carbohydrate molecules per amino acid residue.

Attempts to determine the molecular weights of the two forms from *Aspergillus* species have yielded values ranging from 60,000 to 110,000 for GAI and 53,000 to 112,000 for GAII. By chemical analysis Takahashi et al. and Inokuchi et al. determined the molecular weights of GAI and GAII from *A. saitoi* to be 89,400 and 70,200, respectively.
Svensson et al.\(^{51}\), upon sequencing the *A. niger* GAI protein by chemical methods, calculated a molecular weight of 65,424. The corresponding carbohydrate moiety was 17,053, giving a total molecular weight of 82,477. After sequencing GAI and GAII from *A. niger*, Boel et al.\(^{47}\) calculated the molecular weights of the unglycosylated proteins to be 68,234 and 56,668, respectively. Nunberg et al.\(^{48}\) sequenced the GAI gene from *A. awamori* and calculated an unglycosylated molecular weight of 69,000. It is interesting to note that Sierks\(^{53}\) compared the GAI sequences from Boel et al.\(^{47}\) and Nunberg et al.\(^{48}\) and found them to be identical, supporting the molecular weight values. If the molecular weight of the carbohydrate, 17,053\(^{51}\), is added to the Boel and Nunberg values, the molecular weight of GAI is approximately 85,300 to 86,000, respectively. These values are close to that found by Takahashi et al.\(^{31}\), 89,400, for *A. saitoi* GAI, implying similarity between the *A. niger* and *A. saitoi* enzymes. Also, the number of carbohydrate residues determined by Svensson et al.\(^{51}\) and Takahashi et al.\(^{31}\) for GAI, 104 and 101, respectively, are approximately the same. The number of carbohydrate residues for *A. saitoi* GAII was 57 or 12.55\% by weight, as determined by Inokuchi et al.\(^{32}\), which corresponds to an added molecular weight of approximately 8,800. Svensson et al.\(^{52}\) estimated that GAI and GAII were both approximately 20\% carbohydrate. The GAI value was confirmed in a later paper\(^{51}\), but the value for GAII was never addressed. Using the value of 56,668\(^{47}\) for the unglycosylated GAII, and 12.5-20\% carbohydrate content, the molecular weight can vary from 65,000 to 70,800.
The molecular mechanism of glucoamylase has not been determined at this time. It is known that glucoamylase is composed of seven subsites and attacks the non-reducing end of starch, glycogen and related oligosaccharides, releasing D-glucose. Tanaka et al.\textsuperscript{54,55} determined that the non-reducing terminal D-glucosyl residue of a substrate molecule first binds in the second subsite of glucoamylase and then relocates to the first subsite and undergoes hydrolysis. Jolley and Gray\textsuperscript{56,57} found that carboxyl and tryptophanyl residues were necessary for catalytic activity in \textit{A. niger} GAI. Inokuchi \textit{et al.}\textsuperscript{58,59} also found by chemical modification techniques that carboxyl and tryptophanyl groups were necessary for catalytic activity in glucoamylase from \textit{A. saitoi}. Inokuchi \textit{et al.}\textsuperscript{59} found that soluble starch did temporarily protect two tryptophanyl residues from N-bromosuccinimide (NBS) oxidation. Clarke and Svensson\textsuperscript{60} oxidized tryptophanyl residues with NBS in the presence and the absence of both inhibitors and substrates, and found two tryptophanyl residues in both GAI and GAI\textit{I} were protected when oxidation was performed in the presence of acarbose, a known inhibitor. When the acarbose was dissociated, 80% of the enzyme activity remained. Clarke and Svensson\textsuperscript{60} also oxidized glucoamylase in the presence of maltose, maltotriose, and soluble starch and inhibitors such as gluconolactone, maltitol, and deoxynojirimycin. In this case one tryptophanyl residue was protected but the enzyme was deactivated. Their results suggest that one tryptophanyl residue is involved in binding while the other is involved in catalysis. Clarke and Svensson\textsuperscript{61} later identified Trp-120 as the tryptophanyl residue that was involved in binding. Svensson \textit{et al.}\textsuperscript{62}, working with the same system,
found that there are probably three tryptophanyl residues, Trp-212, Trp-417, and Trp-437, that are jointly associated with ligand binding in subsite 1, while Trp-120 is located in subsite 4. Svensson et al. found that when GA1 was oxidized with NBS in the presence of acarbose, GA1 was no longer able to bind to raw starch, but its catalytic activity towards soluble substrates was maintained. Svensson et al. also modified carboxyl residues in the presence of acarbose and found that three carboxyl groups, Asp-173, Glu-179, and Glu-180, were protected, indicating their presence within the active site region.

Purification

The two major forms can be separated by ion-exchange chromatography on a DEAE-cellulose column with elution by either a decreasing linear pH gradient or by an increasing linear gradient of NaCl. Further purification can include ammonium sulfate precipitation, rechromatography on DEAE-cellulose, gel filtration, or preparative isoelectric focusing. The two forms have similar pH optima and cross-react immunologically. GAI has the ability to adsorb onto raw starch and degrade it easily, while GAII is unable to adsorb onto raw starch and hydrolyzes it very slowly. The two forms appear to have similar activity on small maltooligosaccharides and soluble potato starch.

Subsite Mapping

The rate of enzymatic hydrolysis of homopolymers is largely dependent on the degree of polymerization (DP) of linear substrate molec-
The rate parameters, the Michaelis constant, $K_m$, and the maximal rate, $V_m$, have been observed to be related to the DP of the substrate. The $V_m$ increases with DP based on the increased probability of forming productive instead of nonproductive complexes with the enzymatic active site, while the $K_m$ decreases, suggesting a strong interaction between substrate molecules and the active site of the enzyme as the chain length increases. Hiromi and Nitta et al. developed a model which relates the subsite affinities with the measurable rate parameters and the mode of cleavage of the substrates.

The following discussion of the subsite model for depolymerases will concentrate on the model outlined by Hiromi and will pertain only to exo-enzymes.

The first depolymerase enzyme that was studied in great detail was lysozyme. By crystallizing lysozyme in the presence of inhibitors, Blake et al. were able to locate a cleft or active site within the three-dimensional structure of the enzyme using X-ray analysis. By model building they determined that only six residues could make contact with the enzyme and that each individual NAG or NAM residue interacted with specific amino acids. These specific amino acids constitute individual subsites and their sum the active site. In the case of glucoamylase, each D-glucosyl residue will be bound to a corresponding subsite with a certain affinity, $A$. The sum of these individual subsite affinities, $B$, is the unitary part of the standard free energy change, $\Delta G_{n,j}$, or standard affinity, $-\Delta G_{n,j}$, and will be termed the "molecular binding affinity." It represents the net specific interaction between the substrate molecule and the
enzyme. There is also a cratic part of the standard affinity, $-\Delta G_{\text{mix}}$, which arises from the nonspecific random mixing of solute species with solvent, and is common for solution equilibria accompanied by a change in the number of species. This corresponds to the difference between the free energy of mixing of two species, enzyme and substrate, with water and that of a single species, enzyme-substrate, and amounts to $-10.28 \text{kJ/mol}$ at $35^\circ\text{C}$. Thus we have

$$-\Delta G_{n,j} = B_{n,j} - \Delta G_{\text{mix}} = B_{n,j} - 10.28 \text{kJ/mol} \quad (1)$$

where

$$\Delta G_{n,j} = -RT\ln K_{n,j} \quad (2)$$

where $K_{n,j}$ is the association constant for the enzyme-substrate complex ES, $n$ is the number of sites filled, and $j$ is the position of the non-reducing end (Fig. 1). Eq. 2 assumes binding equilibrium between enzyme and substrate, which is the steady-state assumption used in deriving the Michaelis-Menten equation.

As mentioned above, $B$ is the net binding affinity between enzyme and substrate and is expressed by the sum of the subsite affinities of the subsites occupied by the substrate molecule in that binding mode:

$$B_{n,j} = \text{cov.} \left( \sum_{i=1}^{\text{cov.}} L_{A_i} \right)_{n,j} \quad (3)$$
where \((\Sigma)\) indicates that the sum is taken to be for all the subsites covered by the binding. This is one of the fundamental assumptions on which the theory is based.

The second fundamental assumption is that the intrinsic rate constant, \(k_{\text{int}}\), for the hydrolytic cleavage of the substrate linkage in a productive complex is constant, irrespective of the DP of the substrate. Thus the discrepancies in the rate between molecules of different DPs is strictly a function of the probability of occurrence of a productive complex. Both \(K_m\) and \(V_m\) are dependent on the occurrence of productive and nonproductive complexes. The reaction scheme for the hydrolysis of an \(n\)-mer substrate \(S_n\), involving multiple binding modes for productive, \(p\), and nonproductive, \(q\), complexes is written as follows:

\[
\text{NONPRODUCTIVE} \\
\text{PRODUCTIVE}
\]

Figure 1. Schematic representation of the different binding modes of a substrate molecule to the active site of a glucoamylase.
The third assumption is that a substrate molecule can be bound in a variety of modes to form either one productive or several nonproductive complexes (Fig. 1).

The only information needed to generate a complete subsite map for an exo-enzyme is $K_m$ and $V_m$ data for a linear series of substrate molecules. In the case of glucoamylase, those $K_m$ and $V_m$ data are for the maltooligosaccharides from DP2 through DP7. The mathematics are presented in Appendix I.

The subsite map of glucoamylase from four different sources are presented in Table 2 and the corresponding $K_m$ values are listed in Table 3, except for *Rhizopus delemar* [66]. The subsite maps from all four are virtually identical, with the $K_m$ values following similar trends. This is interesting because the properties of the major glucoamylase from *Rhizopus*, $pI = 8.7$ and $MW = 74,000$, are significantly different than those of the major glucoamylase of *Aspergillus*, $pI = 3.86$ and $MW = 90,000$ [73]. These data would suggest that the amino acids which comprise the active sites of these glucoamylases are similar.
Table 2. Subsite affinities of glucoamylase

<table>
<thead>
<tr>
<th>Source</th>
<th>Subsite affinity, kJ/mol</th>
<th>1^a</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>k_{int} s^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh. delemar</td>
<td></td>
<td>0.00</td>
<td>20.31</td>
<td>6.66</td>
<td>1.80</td>
<td>0.92</td>
<td>0.46</td>
<td>0.42</td>
<td>77.0</td>
</tr>
<tr>
<td>Rh. niveus</td>
<td></td>
<td>-2.01</td>
<td>20.76</td>
<td>5.69</td>
<td>2.26</td>
<td>1.34</td>
<td>0.96</td>
<td>0.29</td>
<td>70.1</td>
</tr>
<tr>
<td>Rh. niveus</td>
<td></td>
<td>-0.75</td>
<td>18.38</td>
<td>7.37</td>
<td>3.73</td>
<td>0.75</td>
<td>0.59</td>
<td>0.33</td>
<td>96.0</td>
</tr>
<tr>
<td>Rh. niveus</td>
<td></td>
<td>-0.50</td>
<td>19.76</td>
<td>5.48</td>
<td>1.09</td>
<td>0.92</td>
<td>0.59</td>
<td>0.54</td>
<td>9.8^b</td>
</tr>
<tr>
<td>A. awamori</td>
<td></td>
<td>2.51</td>
<td>18.84</td>
<td>7.03</td>
<td>2.76</td>
<td>1.05</td>
<td>-0.25</td>
<td>---</td>
<td>47.9</td>
</tr>
<tr>
<td>A. saitoi</td>
<td></td>
<td>0.00</td>
<td>19.38</td>
<td>7.24</td>
<td>2.93</td>
<td>0.50</td>
<td>0.33</td>
<td>0.16</td>
<td>43</td>
</tr>
</tbody>
</table>

^aNumber of the subsite beginning at the nonreducing end of a productively bound maltooligosaccharide molecule.

Performed at 0.5°C.

Table 3. K_m values of maltooligosaccharides for glucoamylase

<table>
<thead>
<tr>
<th>Source</th>
<th>K_m, mM</th>
<th>G_2</th>
<th>G_3</th>
<th>G_4</th>
<th>G_5</th>
<th>G_6</th>
<th>G_7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh. niveus</td>
<td></td>
<td>1.20</td>
<td>0.402</td>
<td>0.273</td>
<td>0.204</td>
<td>0.100</td>
<td>0.121</td>
</tr>
<tr>
<td>Rh. niveus</td>
<td></td>
<td>1.54</td>
<td>0.35</td>
<td>0.188</td>
<td>0.141</td>
<td>0.107</td>
<td>0.098</td>
</tr>
<tr>
<td>Rh. niveus</td>
<td></td>
<td>0.82</td>
<td>0.35</td>
<td>0.25</td>
<td>0.19</td>
<td>0.14</td>
<td>0.11</td>
</tr>
<tr>
<td>A. awamori</td>
<td></td>
<td>1.9</td>
<td>0.39</td>
<td>0.25</td>
<td>0.14</td>
<td>0.16</td>
<td>---</td>
</tr>
<tr>
<td>A. saitoi</td>
<td></td>
<td>1.15</td>
<td>0.264</td>
<td>0.17</td>
<td>0.156</td>
<td>0.139</td>
<td>0.129</td>
</tr>
</tbody>
</table>
Kinetics

There have been many reported values for $K_m$ and $V_m$ for the hydrolysis of maltose. Phillips and Caldwell\(^\text{75}\) published the first estimation of $K_m$ for a glucoamylase of *Rh. delemar*. Their value of 6.6 mM is the highest estimate reported. Hiromi et al.\(^\text{23}\) reported a $K_m$ of 1.1 mM at 25°C and pH 5.1 for a *Rh. delemar* glucoamylase while Ono et al.\(^\text{76}\), also working with *Rh. delemar*, estimated a $K_m$ of 1.16 mM at 15°C and pH 5.15. Inokuchi et al.\(^\text{32}\) reported values of 2.7 and 3.0 mM for GAI and GA2 from *A. saitoi* at pH 6.0 and 37°C. Swanson et al.\(^\text{77}\) measured the only $K_m$ value for the *A. niger* glucoamylase. Using a Hanes plot for the modified Michaelis-Menten equation, they obtained a $K_m$ value of 0.6 mM at 40°C and pH 4.5.

For the hydrolysis of isomaltose, Inokuchi et al.\(^\text{32}\) determined values of 36.4 mM for both GAI and GAII at pH 6.0 and 37°C. Hiromi et al.\(^\text{23}\), investigating a *Rh. delemar* enzyme, reported a $K_m$ of 25 mM at 25°C and pH 5.1. Also, Hiromi et al.\(^\text{23}\) reported a value of 6.9 mM for panose at the same conditions.

As the $V_m$'s found in the literature are expressed in many different units, it is more interesting to consider the relative rate of hydrolysis of other substrates compared to maltose. For *A. niger*, Pazur and Kleppe\(^\text{13}\) found isomaltose to have a relative rate of 3.6%, while nigerose and $\alpha,\alpha$-trehalose were 6.6% and 0.074%, respectively. Abdullah et al.\(^\text{78}\), investigating *A. niger*, estimated isomaltose to be 1.3% that of maltose, while isopanose, panose and nigerose were 260%, 73% and 1.1%, respectively. Abdullah et al.\(^\text{78}\) also investigated isomaltotriose, isomaltotetraose, and isomaltopentaose and found values of 8%, 15% and
Inokuchi et al.\(^\text{32}\), working with \textit{A. saitoi}, measured relative rates for isomaltose of 1% for both GAI and GAII. With a glucoamylase of \textit{Rh. delemar}, Hiromi et al.\(^\text{23}\) found values of 3% for isomaltose and 40% for panose.

### Separation and Purification of Glucoamylases I and II

#### Analytical Procedures

**Protein determination**

Protein elution from chromatographic columns was monitored continuously with an ISCO (Instrumentation Specialties Co., Lincoln, NE) Model UA-5 absorbance monitor at 280 nm. Concentrations of GAI and GAIII were estimated from the UV absorbance using \(E_{280}\) values given by Svensson et al.\(^\text{52}\).

**Glucoamylase activity**

Maltose-hydrolyzing activity of the eluent fractions was determined at pH 4.5 and 35°C. Samples (0.5 mL) of enzyme solution were mixed with 1.5 mL of 4% (w/v) maltose solution in 0.05 M acetate buffer. Glucose released in the reaction mixture by the enzyme action on maltose was measured using a Beckman (Fullerton, CA) enzymatic glucose analyzer. The same procedure was used for measuring the activities of GAI and GAIII pools with previous dilutions of the enzyme samples. One unit (1 U) of enzyme activity was defined as the amount enzyme required to release 1 \(\mu\)mol of glucose per minute under the conditions of the assay.

**Transglucosylase activity**

Two different transglucosylase (TG) activity assays were performed by Z. Nikolov with purified glucoamylase pools (GAI and GAIII) using maltose and \(\alpha\)-methylglucoside, respectively, as
substrates. The first assay is based on the differential inhibitory action of acarbose on glucoamylase and transglucosylase as reported by Shetty and Marshall. They found that 100% inhibition of glucoamylase activity in 10% maltose solution occurred at an acarbose concentration of 10 μg/U of glucoamylase activity over a 20-min incubation period at pH 4.5 and 37°C, with no inhibition of transglucosylase activity. Their study showed that transglucosylase activity was affected only at acarbose concentrations higher than 100 μg/U glucoamylase activity. The procedure used in our laboratory to estimate the effect of acarbose on GA and TG activity is described below.

One mL of purified GA (2 DU/mL)* in 0.025 M acetate buffer, pH 4.5, was mixed with an equal volume of the same buffer containing different amounts of acarbose (100-900 μg). After incubating this mixture for 30 min at 35°C, 2 mL of 20% maltose in 0.025 M acetate buffer were added and the incubation was continued for another 24 h. The effect of acarbose on glucoamylase activity was determined by measuring the amount of glucose produced within the first hour of incubation. Glucose in the reaction mixture was determined by HPLC using a Bio-Rad (Richmond, CA) HPX-42A column.

To study the effect of acarbose on TG activity the following procedure was employed. To a 0.01-mL solution of TG (500 units/mL) donated by Dr. John Pazur, where a unit of TG is defined as the amount of

*One Diazyme unit (DU) of activity liberates 1 g/h of reducing sugar, expressed as glucose, under the conditions of the assay.
enzyme to produce 1 μmol of panose per hour at pH 4.5 and 35°C, different amounts of acarbose (0-160 μg) were added from a stock solution of 3.6 mg acarbose/mL prepared in 0.025 M acetate buffer, pH 4.5. A final volume of 0.1 mL was attained by addition of 0.025 M acetate buffer. After 30 min incubation at 35°C, 0.1 mL of a 20% (w/v) maltose solution was introduced as before. The presence of transglucosylase was observed by following the formation of panose, which is a primary reaction product synthesized by TG from maltose, by HPLC. The second assay for determining TG activity in the glucoamylase pools was that described by Benson et al. This method was developed based on the authors' observation that α-Me-glucoside was hydrolyzed slowly by transglucosylase and was not hydrolyzed by glucoamylase. The glucose released in the reaction mixture was quantified using the glucose oxidase method.

Electrophoretic procedures Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 0.5-mm thick 10% gel was carried out as described in LKB Application Note 320. Samples were prepared in sample buffer and either maintained at 60°C for 5 min with 0.1% by weight dithiothreitol or held at 30-100°C, at 10°C increments, for 5 min without dithiothreitol. Electrophoresis was performed at pH 8.8, 10°C, at a constant voltage of 250 V. The gel was prerun for 3 h at 150 V and constant voltage. The protein was visualized using a silver stain method.

Disc gel electrophoresis (DISC-PAGE) was conducted following the method of Davis, using a vertical 4% polyacrylamide gel. Constant power of 11 W was employed, and gels were stained either with silver or
Coomassie Brilliant Blue G-250<sup>85</sup>.

Isoelectric focusing (IEF) in a 0.2- or 0.5-mm thick horizontal slab gel was carried out as outlined in the LKB instruction brochure for the Ultramold gel casting kit<sup>86</sup>. Two carrier ampholytes, Pharmacia (Uppsala, Sweden) Pharmalyte pH 2.5-5 and pH 3-10, were employed. Electrophoresis was run at 10°C for either 6 h with ampholyte pH 2.5-5 or 3 h with ampholyte pH 3-10 by applying a constant voltage of 1800 V. Protein was visualized with either Coomassie Brilliant Blue G-250 or silver stain.

**Purification of Glucoamylase**

All operations described here were performed at 4°C, unless otherwise specified, by Zivko Nikolov.

A 20-g sample of Miles (Clifton, NJ) Diazyme 160, designated DGA, was dissolved in 200 mL distilled water, shaken for 30 min, filtered through Whatman #4 filter paper, and centrifuged at 10,000 x g for 15 min. The supernatant was treated at room temperature with ammonium sulfate to 80% saturation, centrifuged for 15 min at 10,000 x g, and the resulting precipitate was redissolved in 50 mL of 0.025 M citrate-phosphate buffer, pH 8.0. The solution was desalted by passage through a 600-mm x 35-mm i.d. Sephadex G-25 column (Pharmacia) using the same buffer as an eluent with a flow rate of 4 mL/min. The glucoamylase fraction was loaded on a 350-mm x 35-mm i.d. DEAE-cellulose column (Sigma, St. Louis, MO, fine mesh, Lot 71F-0040) and eluted at 1 mL/min with a decreasing linear pH gradient as described previously<sup>14</sup>. The glucoamylase II pool (DGAII) was collected from the column at volumes from 1965 mL to 2400 mL (pH 6.4 to pH 6.2), while glucoamylase I (DGAI) eluted from 3165 mL to 3600 mL (pH 4.3
to pH 3.9). The two pools were each desalted by passage at 4 mL/min through the Sephadex G-25 column described above, using a distilled water eluent, and concentrated with an Amicon (Danvers, MA) 402 ultrafiltration cell under 4 atm N₂ pressure to a final volume of 70 mL. The DGAI and DGAII pools were rechromatographed on the DEAE-cellulose column following the procedure of Lineback et al.⁵ The pools of rechromatographed DGAI and DGAII were desalted and concentrated as described above and then stored at -20°C. The extent of purification and the yield of each step are summarized in Table 4.

Table 4. Purification of glucoamylase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total volume, mL</th>
<th>Protein conc., mg/mL</th>
<th>Total protein, mg</th>
<th>Total activity, U</th>
<th>Specific activity, U/mg</th>
<th>Activity yield, %</th>
</tr>
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<tbody>
<tr>
<td>Crude</td>
<td>123</td>
<td>12.3</td>
<td>1570</td>
<td>12,400</td>
<td>7.9</td>
<td>100.0</td>
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<tr>
<td>Desalted (NH₄)₂SO₄ ppt.</td>
<td>114</td>
<td>3.9</td>
<td>1020</td>
<td>9,950</td>
<td>9.8</td>
<td>80.0</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DGAI</td>
<td>435</td>
<td>0.72</td>
<td>313</td>
<td>3,070</td>
<td>9.8</td>
<td>24.7</td>
</tr>
<tr>
<td>DGAII</td>
<td>435</td>
<td>0.50</td>
<td>217</td>
<td>2,670</td>
<td>12.3</td>
<td>21.5</td>
</tr>
<tr>
<td>DEAE-cellulose rechromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DGAI</td>
<td>605</td>
<td>0.42</td>
<td>255</td>
<td>2,550</td>
<td>10.0</td>
<td>20.5</td>
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<tr>
<td>DGAII</td>
<td>420</td>
<td>0.36</td>
<td>153</td>
<td>2,050</td>
<td>13.4</td>
<td>16.5</td>
</tr>
</tbody>
</table>

Another purification run with a new Diazyme glucoamylase preparation specially prepared by Miles (Elkhart, IN) and donated by Dr. J. Shetty was
performed by a somewhat different procedure. Because the new glucoamylase preparation, designated as NGA, was more pure, the ammonium sulfate precipitation step was eliminated. A 0.1-g sample of NGA in 5 mL of appropriate buffer was loaded on a 800-mm x 26-mm i.d. Fractogel HW-55F (MCB, Gibbstown, NJ) gel permeation column and eluted at 1 mL/min with either 0.025 M citrate-phosphate buffer, pH 8.0, or 0.05 M acetate buffer, pH 4.5. Because of the limited capacity of the gel permeation column, two additional runs were required to generate a sufficiently large glucoamylase sample for further purification. Concentration and buffer exchange of the fractions containing glucoamylase activity from the Fractogel HW-55F column was conducted with the Amicon 402 ultrafiltration cell. A 20-mL glucoamylase sample (0.3 g of protein) was added to a Fractogel DEAE-650 S column (850 mm x 26 mm i.d.) and eluted at 1 mL/min by applying a decreasing linear pH gradient as before. Glucoamylase II was eluted at volumes from 3390 mL to 3600 mL (pH 5.5 to 4.8), while glucoamylase I was eluted from 3825 mL to 3975 mL (pH 4.4 to 3.8). No other major protein peak except for the two glucoamylase forms eluted from the Fractogel DEAE-650S column. The two glucoamylase pools, NGAI and NGAII, were desalted and concentrated by ultrafiltration with the Amicon 402 cell and stored at -20°C.

Purity of Glucoamylase Preparations

Homogeneity of the four glucoamylase pools, DGAI, DGAII, NGAI, and NGAII, was tested by three methods. Disc gel electrophoresis revealed that DGAI contained two minor impurities, one slower-traveling than the main band and second moving with the dye front. The DGAII pool was
contaminated only with DGAI, which was subsequently removed by chromatography on the Fractogel DEAE-650S column by applying a decreasing linear pH gradient from 7.0 to 3.5. The NGAII pool appeared to be homogeneous by DISC-PAGE, while NGAI contained a band that was slower-traveling than the main band.

SDS-electrophoresis of NGAI and DGAI in the presence of dithiothreitol showed three bands that were of higher molecular weight and one band that moved slightly faster than NGAII and DGAII. NGAII showed two bands of substantial molecular weight and a third band that traveled just in front of the major band. DGAII also showed high molecular weight fragments and a band in front of the major band, along with a faint band traveling the same distance as DGAI. If the protein samples were subjected to SDS-electrophoresis without dithiothreitol, high molecular weight bands appeared in greater amounts as the protein mixture was heated to higher temperatures, implying that these bands were artifacts of heating. It is interesting to note that no band corresponding to NGAII appeared in the NGAI sample except when NGAI was treated with dithiothreitol, suggesting an artifact of treatment. Also, there appeared to be no transglycosylase present in any of the glucoamylase samples.

The third method of testing homogeneity was by isoelectric focusing. When pH 3-10 ampholyte was employed, NGAI and DGAI had very faint bands at pI 3.9 using both staining techniques, while NGAII and DGAII both appeared to be composed of two very closely separated bands with the Coomassie Blue stain. Those appeared as one band with silver staining because of overloading. NGAI, NGAII, DGAI, and DGAII focused at a pI of approximately
3.55 to 3.6. The transglucosylase sample, which is only visible with silver staining, had two bands at pI 4.3 and 4.5 and a glucoamylase band at pI 3.55. Both NGA and DGA have these two bands, but neither is present in NGAI, DGAI, NGAI, or DGAII. When pH 2.5-5.0 ampholyte was employed, NGAI and DGAI both focused as single bands at pI 3.45, while NGAI had a major band at pI 3.58 and a minor band at pI 3.45. DGAII had three bands of similar intensity at pIs of 3.42, 3.50, and 3.58, and a very faint band at pI 3.45. All glucoamylase samples were visualized with silver staining. Both GAI samples appeared to be single proteins, while NGAI and DGAII, especially the latter, seemed to exhibit micro-heterogeneity. NGAI contained a protein with the same pI as NGAI, but the SDS-PAGE results did not show any NGAI present in the NGAI sample, supporting the idea of micro-heterogeneity. Another interesting note is that all of the proteins focused within 0.16 pH unit.

The effect of acarbose on glucoamylase and transglucosylase is illustrated in Fig. 2. At a concentration of 0.0525 mM acarbose, glucoamylase was 99% inhibited, while transglucosylase retained 96% of its activity, which confirms the results of Shetty and Marshall. The experiments to determine the presence of transglucosylase were performed at 0.15 mM acarbose, which is well above the 0.0525 mM used above, insuring that GA was completely deactivated. Three glucoamylase samples, Diazyme 160, NGAI, and DGAII, were tested for TG activity. All three showed no panose formation, even after 14 h of incubation under the conditions of the assay.
Figure 2. Effect of acarbose concentration on inhibition of glucoamylase (GA) and transglucosylase (TG) in the presence of 10% maltose at 35°C and pH 4.5. 

[GA] = 3.5 x 10^{-3} mM

[TG] = 3 x 10^{-3} mM
As mentioned above, there were protein bands of pI 4.5 present in the two crude glucomylase samples, NGA and DGA, that were similar to that of TG. Samples of 2.25 mg DGA and NGA were submitted to semi-preparative isoelectric focusing from pH 3 to 10 on a 2-mm thick gel. The protein was loaded and allowed to focus at 20 W constant power for 3 h, at which time a small segment was stained for protein by Coomassie Brilliant Blue G-250 to insure proper focusing. The gel from the anode to pH 3.6, 5 mm, was sliced and extracted with 2 mL of 0.025 M acetate buffer, pH 4.5. From pH 3.6 to 5.0, 1.5-mm thick x 90-mm wide segments were cut and extracted with 1 mL of 0.025 M acetate buffer, pH 4.5. All the segments were checked for protein using the Bio-Rad protein assay. Protein was found in all of the DGA gel segments, while NGA tested positive from the anode to pH 3.6 and only in the first segment after pH 3.6. The gel segments from the anode to pH 3.6 were ultrafiltered to a concentration of 1.0 mg/mL and a volume of 100 μL. The two pools were found to be void of transglucosylase activity under the conditions of the assay.

The second assay for TG, the hydrolysis of α-Me-glucoside, was inconclusive because of the small amount of glucose that was produced, which may have come about from the hydrolytic action of glucomylase.

In conclusion, the electrophoresis results indicate that the NGAI, DGAI, NGAII, and DGAII are not homogeneous but contained minor bands that may have been formed during the fermentation process, enzyme preparation, or sample pretreatment. NGAI was free of NGAII, while NGAII was free of NGAI by SDS-PAGE. The DGAI was of the same purity as NGAI, while DGAII had smaller molecular weight compounds not found in
Another important criterion of purity was the absence of transglucosylase, which was verified by isoelectric focusing from pH 3 to 10 and by lack of action during acarbose studies.

**Kinetics and Subsite Mapping of Glucoamylases I and II**

**Substrates**

The following substrates were used during the kinetic studies. Kojibiose (2-0-α-D-glucopyranosyl-D-glucose) was purchased from Koch-Light Laboratories Ltd. (Colnbrook, Berks, England). Maltose (4-0-α-D-glucopyranosyl-D-glucose) was purchased from J. T. Baker (Phillipsburg, NJ). Isomaltose (6-0-α-D-glucopyranosyl-D-glucose), panose (6-0-α-D-glucopyranosyl-4-0-α-D-glucopyranosyl-D-glucose), maltotriose (4-0-α-D-glucopyranosyl-maltose), isomaltotriose (6-0-α-D-glucopyranosyl-isomaltose), maltotetraose (4-0-α-D-glucopyranosyl-maltotriose), maltopentaose (4-0-α-D-glucopyranosyl-maltotetraose), maltohexaose (4-0-α-D-glucopyranosyl-maltopentaose), and maltoheptaose (4-0-α-D-glucopyranosyl-maltohexaose) were purchased from Sigma. α,β-Trehalose (α-D-glucopyranosyl-β-D-glucopyranoside), nigerose (3-0-α-D-glucopyranosyl-D-glucose), isomaltotetraose (6-0-α-D-isomaltotriose), isomaltopentaose (6-0-α-D-glucopyranosyl-isomaltotetraose), and isomaltohexaose (6-0-α-D-glucopyranosyl-isomaltopentaose) were produced and purified as discussed in Appendix II.

**Analytical Procedures**

The reactions were performed in 0.3-, 1-, 3-, or 5-mL Reacti-Vials (Pierce, Rockford, IL), agitated and maintained at the desired
temperature, 25, 35, 45, or 55°C, in a thermostatted water bath. A stock solution of the desired substrate, which was dissolved in 0.05 M acetate buffer, pH 4.5, was diluted appropriately to obtain the specified concentration. Enzyme was added at zero time. Sample volumes ranging from 0.04 to 0.4 mL were taken at specified time intervals and quenched by pipetting the sample into a concentrated volume of TRIS, pH 7.1, to a final volume of 0.5 mL and a nominal TRIS concentration of 1 M.

**Glucose determination** The glucose concentration in the samples was estimated with the glucose oxidase method of Fleming and Pegler. To the quenched sample was added 1 mL glucose oxidase reagent. The mixture was maintained at 35°C for 1 h, and then 2 mL of 7 M HCl were added. The optical density was measured at 525 nm.

**Enzyme activity** Glucoamylase activity was checked periodically by measuring glucose production at 55°C with 4% maltose substrate in 0.05 M acetate buffer at pH 4.5. Glucose concentration was determined at regular intervals by the same glucose oxidase method used for the kinetic runs. One unit (1 U) of enzyme activity was defined as being the amount of enzyme required to cleave 1 μmol of α-1,4 linkages per minute under the conditions of the assay.

**Product analysis** The analysis of reaction products from the hydrolysis of panose were followed by HPLC using a 4.6-mm i.d. x 250-mm long Supelcosil LC-NH₂ column (Supelco, Inc., Bellefonte, PA) eluted with 68/32 CH₃CN/H₂O (v/v) at room temperature and a flow rate of 1.25 mL/min using an ISCO Model 2350 pump. Elution of carbohydrates was monitored with an Erma (Erma Optical Works, Ltd., Tokyo, Japan) ERC-7510 refractive
index detector. Volumes of 10 μL were injected with a Rheodyne (Cotati, CA) Model 7125 injector. Reactions were performed at pH 4.5 and 21°C and 45°C, and samples were taken at 5- and 13-min intervals. At both time intervals the amounts of glucose and maltose produced were equivalent.

**Calculational procedures** The Wilkinson method was used to provide the values of $K_m$ and $V_m$ of the Michaelis-Menten equation. The quantity $k_o$ was determined by dividing $V_m$ by the molar enzyme concentration.

The calculation of the subsite affinities is outlined in Appendix I.

**Experimental Data**

The results of the hydrolytic rate studies of the di- and trisaccharides at different pHs and temperatures with GAI and GAII are presented on Tables 5 through 14. The kinetic results for the maltooligosaccharides and isomaltooligosaccharides are presented on Tables 15 through 18, while the subsite mapping results are presented on Table 19.
### Table 5. Kinetic values obtained for GAI on α,β-trehalose

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp., °C</th>
<th><em>k_0</em>, s(^{-1})</th>
<th><em>K_m</em>, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>35</td>
<td>0.305 ± 0.016(^a)</td>
<td>224.7 ± 26.1</td>
</tr>
<tr>
<td>4.5</td>
<td>25</td>
<td>0.126 ± 0.003</td>
<td>113.3 ± 5.8</td>
</tr>
<tr>
<td>4.5</td>
<td>35</td>
<td>0.305 ± 0.008</td>
<td>120.1 ± 7.4</td>
</tr>
<tr>
<td>4.5</td>
<td>45</td>
<td>0.814 ± 0.008</td>
<td>146.3 ± 3.3</td>
</tr>
<tr>
<td>5.5</td>
<td>35</td>
<td>0.199 ± 0.009</td>
<td>75.9 ± 9.8</td>
</tr>
</tbody>
</table>

\(^a\)Standard deviation.

### Table 6. Kinetic values obtained for GAI on kojibiose

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp., °C</th>
<th><em>k_0</em>, s(^{-1})</th>
<th><em>K_m</em>, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>35</td>
<td>0.087 ± 0.001(^a)</td>
<td>138.1 ± 3.6</td>
</tr>
<tr>
<td>4.5</td>
<td>25</td>
<td>0.032 ± 0.001</td>
<td>129.2 ± 8.3</td>
</tr>
<tr>
<td>4.5</td>
<td>35</td>
<td>0.086 ± 0.002</td>
<td>126.8 ± 7.3</td>
</tr>
<tr>
<td>4.5</td>
<td>45</td>
<td>0.241 ± 0.008</td>
<td>143.5 ± 13.0</td>
</tr>
<tr>
<td>5.5</td>
<td>35</td>
<td>0.058 ± 0.001</td>
<td>99.2 ± 2.5</td>
</tr>
</tbody>
</table>

\(^a\)Standard deviation.
Table 7. Kinetic values obtained for GAI on nigerose

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp., °C</th>
<th>( k_0 ), s(^{-1})</th>
<th>( K_m ), mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>35</td>
<td>0.164 ± 0.008( ^a )</td>
<td>55.1 ± 7.4</td>
</tr>
<tr>
<td>4.5</td>
<td>25</td>
<td>0.055 ± 0.001</td>
<td>33.1 ± 1.9</td>
</tr>
<tr>
<td>4.5</td>
<td>35</td>
<td>0.116 ± 0.005</td>
<td>33.2 ± 4.2</td>
</tr>
<tr>
<td>4.5</td>
<td>45</td>
<td>0.329 ± 0.010</td>
<td>37.1 ± 2.9</td>
</tr>
<tr>
<td>5.5</td>
<td>35</td>
<td>0.078 ± 0.001</td>
<td>26.4 ± 1.2</td>
</tr>
</tbody>
</table>

\( ^a \) Standard deviation.

Table 8. Kinetic values obtained for GAI on maltose

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp., °C</th>
<th>( k_0 ), s(^{-1})</th>
<th>( K_m ), mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>35</td>
<td>4.94 ± 0.10( ^a )</td>
<td>1.39 ± 0.07</td>
</tr>
<tr>
<td>4.5</td>
<td>25</td>
<td>2.13 ± 0.03</td>
<td>0.55 ± 0.02</td>
</tr>
<tr>
<td>4.5</td>
<td>35</td>
<td>5.02 ± 0.19</td>
<td>1.23 ± 0.14</td>
</tr>
<tr>
<td>4.5</td>
<td>45</td>
<td>10.60 ± 0.27</td>
<td>1.74 ± 0.12</td>
</tr>
<tr>
<td>4.5</td>
<td>55</td>
<td>19.27 ± 0.43( ^b )</td>
<td>-----</td>
</tr>
<tr>
<td>5.5</td>
<td>35</td>
<td>3.54 ± 0.10</td>
<td>0.96 ± 0.07</td>
</tr>
</tbody>
</table>

\( ^a \) Standard deviation.
\( ^b \) Obtained from glucoamylase activity determination with 4% maltose.
Table 9. Kinetic values obtained for GAI on isomaltose

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp, °C</th>
<th>$k_0$, s$^{-1}$</th>
<th>$K_m$, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>35</td>
<td>$0.154 \pm 0.005^a$</td>
<td>$34.25 \pm 2.89$</td>
</tr>
<tr>
<td>4.5</td>
<td>25</td>
<td>$0.059 \pm 0.003$</td>
<td>$24.27 \pm 2.74$</td>
</tr>
<tr>
<td>4.5</td>
<td>35</td>
<td>$0.152 \pm 0.005^b$</td>
<td>$21.03 \pm 1.48^b$</td>
</tr>
<tr>
<td>4.5</td>
<td>45</td>
<td>$0.385 \pm 0.011$</td>
<td>$25.45 \pm 1.66$</td>
</tr>
<tr>
<td>5.5</td>
<td>35</td>
<td>$0.072 \pm 0.002$</td>
<td>$15.01 \pm 1.35$</td>
</tr>
</tbody>
</table>

*Standard deviation.

^Average of two experiments.

Table 10. Kinetic values obtained for GAI on panose

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp., °C</th>
<th>$k_0$, s$^{-1}$</th>
<th>$K_m$, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>35</td>
<td>$3.99 \pm 0.09^a$</td>
<td>$15.35 \pm 0.92$</td>
</tr>
<tr>
<td>4.5</td>
<td>25</td>
<td>$2.42 \pm 0.04$</td>
<td>$9.88 \pm 0.46$</td>
</tr>
<tr>
<td>4.5</td>
<td>35</td>
<td>$4.79 \pm 0.21$</td>
<td>$9.00 \pm 1.04$</td>
</tr>
<tr>
<td>4.5</td>
<td>45</td>
<td>$11.99 \pm 0.13$</td>
<td>$13.16 \pm 0.35$</td>
</tr>
<tr>
<td>5.5</td>
<td>35</td>
<td>$4.33 \pm 0.05$</td>
<td>$13.48 \pm 0.37$</td>
</tr>
</tbody>
</table>

*Standard deviation.
Table 11. Kinetic values obtained for GAI on isomaltotriose

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp., °C</th>
<th>( k_0 ), s(^{-1})</th>
<th>( K_m ), mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>35</td>
<td>0.614 ± 0.009(^a)</td>
<td>23.50 ± 0.87</td>
</tr>
<tr>
<td>4.5</td>
<td>25</td>
<td>0.200 ± 0.002</td>
<td>11.99 ± 0.61</td>
</tr>
<tr>
<td>4.5</td>
<td>35</td>
<td>0.533 ± 0.020</td>
<td>11.16 ± 1.02</td>
</tr>
<tr>
<td>4.5</td>
<td>45</td>
<td>1.317 ± 0.040</td>
<td>10.51 ± 0.92</td>
</tr>
<tr>
<td>5.5</td>
<td>35</td>
<td>0.314 ± 0.006</td>
<td>9.81 ± 0.39</td>
</tr>
</tbody>
</table>

\(^a\)Standard deviation.

Table 12. Kinetic values obtained for GAII on maltose

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp., °C</th>
<th>( k_0 ), s(^{-1})</th>
<th>( K_m ), mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>25</td>
<td>2.27 ± 0.14(^a)</td>
<td>1.07 ± 0.18</td>
</tr>
<tr>
<td>4.5</td>
<td>35</td>
<td>5.19 ± 0.26(^b)</td>
<td>0.99 ± 0.13(^b)</td>
</tr>
<tr>
<td>4.5</td>
<td>45</td>
<td>10.44 ± 0.32</td>
<td>1.41 ± 0.13</td>
</tr>
<tr>
<td>4.5</td>
<td>55</td>
<td>22.66 ± 3.34(^c)</td>
<td>-----</td>
</tr>
</tbody>
</table>

\(^a\)Standard deviation.
\(^b\)Average of two experiments.
\(^c\)Obtained from glucoamylase activity determination with 4% maltose.
### Table 13. Kinetic values obtained for GAII on maltotriose

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp., °C</th>
<th>$k_0$, $s^{-1}$</th>
<th>$K_m$, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>35</td>
<td>$13.89 \pm 0.97^a$</td>
<td>$0.346 \pm 0.076$</td>
</tr>
<tr>
<td>4.5</td>
<td>25</td>
<td>$8.73 \pm 0.35$</td>
<td>$0.183 \pm 0.024$</td>
</tr>
<tr>
<td>4.5</td>
<td>35</td>
<td>$21.04 \pm 0.89$</td>
<td>$0.363 \pm 0.040$</td>
</tr>
<tr>
<td>4.5</td>
<td>45</td>
<td>$42.14 \pm 1.27$</td>
<td>$0.514 \pm 0.044$</td>
</tr>
<tr>
<td>5.5</td>
<td>35</td>
<td>$14.72 \pm 0.81$</td>
<td>$0.395 \pm 0.061$</td>
</tr>
</tbody>
</table>

*Standard deviation.

### Table 14. Kinetic values obtained for GAII on isomaltotriose

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp., °C</th>
<th>$k_0$, $s^{-1}$</th>
<th>$K_m$, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>25</td>
<td>$0.26 \pm 0.02^a$</td>
<td>$17.28 \pm 3.00$</td>
</tr>
<tr>
<td>4.5</td>
<td>35</td>
<td>$0.45 \pm 0.03$</td>
<td>$9.34 \pm 1.13$</td>
</tr>
<tr>
<td>4.5</td>
<td>45</td>
<td>$1.55 \pm 0.08$</td>
<td>$10.46 \pm 1.69$</td>
</tr>
</tbody>
</table>

*Standard deviation.
Table 15. Rate parameters for GAI-catalyzed hydrolysis of maltooligosaccharides at pH 4.5 and 35°C

<table>
<thead>
<tr>
<th>DP</th>
<th>$k_o$, s$^{-1}$</th>
<th>$k_o$(obs)</th>
<th>$K_m$, mM</th>
<th>$k_o$(calc)</th>
<th>$K_m$(calc)</th>
<th>$k_o/K_m$ s$^{-1}$mM$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5.02 ± 0.19$^a$</td>
<td>1.00</td>
<td>1.23 ± 0.14</td>
<td>1.00</td>
<td>4.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.02$^b$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15.11 ± 0.59</td>
<td>1.00</td>
<td>0.275 ± 0.031</td>
<td>1.00</td>
<td>54.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.13</td>
<td></td>
<td>0.276</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30.95 ± 1.01</td>
<td>1.14</td>
<td>0.153 ± 0.013</td>
<td>1.14</td>
<td>202.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>27.13</td>
<td></td>
<td>0.134</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>30.27 ± 0.87</td>
<td>0.94</td>
<td>0.096 ± 0.007</td>
<td>0.93</td>
<td>315.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32.36</td>
<td></td>
<td>0.103</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>29.14 ± 0.88</td>
<td>0.92</td>
<td>0.082 ± 0.006</td>
<td>0.91</td>
<td>355.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31.38</td>
<td></td>
<td>0.091</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>33.90 ± 1.16</td>
<td>0.99</td>
<td>0.083 ± 0.006</td>
<td>0.99</td>
<td>408.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34.19</td>
<td></td>
<td>0.084</td>
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<td></td>
</tr>
</tbody>
</table>

$^a$Standard deviation.

$^b$Calculated from eqs. 23 and 24 in Appendix I.
Table 16. Rate parameters for GAI-catalyzed hydrolysis of isomaltooligosaccharides at pH 4.5 and 35°C

<table>
<thead>
<tr>
<th>DP</th>
<th>$k_0$, s$^{-1}$</th>
<th>$k_0$(obs)</th>
<th>$K_m$, mM</th>
<th>$K_m$(obs)</th>
<th>$k_0/K_m$, s$^{-1}$mM$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.162 ± 0.005$^a$</td>
<td>0.94</td>
<td>21.03 ± 1.54</td>
<td>0.99</td>
<td>0.0072</td>
</tr>
<tr>
<td></td>
<td>0.162$^b$</td>
<td></td>
<td>21.1$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.533 ± 0.020</td>
<td>0.64</td>
<td>11.16 ± 1.02</td>
<td>0.69</td>
<td>0.0478</td>
</tr>
<tr>
<td></td>
<td>0.828</td>
<td></td>
<td>16.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.820 ± 0.026</td>
<td>1.16</td>
<td>13.99 ± 0.92</td>
<td>1.24</td>
<td>0.0586</td>
</tr>
<tr>
<td></td>
<td>0.701</td>
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<td>11.25</td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>0.975 ± 0.015</td>
<td>1.07</td>
<td>11.43 ± 0.45</td>
<td>1.15</td>
<td>0.0853</td>
</tr>
<tr>
<td></td>
<td>0.905</td>
<td></td>
<td>9.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.896 ± 0.027</td>
<td>0.90</td>
<td>9.46 ± 0.77</td>
<td>0.95</td>
<td>0.0947</td>
</tr>
<tr>
<td></td>
<td>0.989</td>
<td></td>
<td>9.89</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Standard deviation.

$^b$Calculated from eqs. 23 and 24 in Appendix I.
Table 17. Rate parameters for GAI-catalyzed hydrolysis of maltooligosaccharides at pH 4.5 and 35°C

<table>
<thead>
<tr>
<th>DP</th>
<th>$k_0$, $s^{-1}$</th>
<th>$k_0$(obs)</th>
<th>$K_m$, mM</th>
<th>$K_m$(obs)</th>
<th>$k_o/K_m$, $s^{-1}$mM$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5.19 ± 0.26$^a,b$</td>
<td>1.05</td>
<td>0.993 ± 0.13$^b$</td>
<td>0.99</td>
<td>5.24</td>
</tr>
<tr>
<td></td>
<td>5.24$^c$</td>
<td></td>
<td>0.940$^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>21.04 ± 0.89</td>
<td>1.27</td>
<td>0.362 ± 0.040$^b$</td>
<td>1.37</td>
<td>58.1</td>
</tr>
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<td>16.35</td>
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<td>0.266</td>
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<td></td>
</tr>
<tr>
<td>4</td>
<td>29.34 ± 1.04</td>
<td>1.04</td>
<td>0.157 ± 0.018</td>
<td>1.10</td>
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<tr>
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<td>0.142</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>29.14 ± 0.66</td>
<td>0.79</td>
<td>0.092 ± 0.009</td>
<td>0.84</td>
<td>316.7</td>
</tr>
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<td>36.77</td>
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<td>0.109</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>28.39 ± 0.88</td>
<td>0.77</td>
<td>0.075 ± 0.007</td>
<td>0.82</td>
<td>378.5</td>
</tr>
<tr>
<td></td>
<td>36.77</td>
<td></td>
<td>0.091</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>27.64 ± 1.22</td>
<td>0.66</td>
<td>0.061 ± 0.009</td>
<td>0.71</td>
<td>453.1</td>
</tr>
<tr>
<td></td>
<td>41.70</td>
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<td>0.087</td>
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<td></td>
</tr>
</tbody>
</table>

$^a$Standard deviation.
$^b$Average of two experiments.
$^c$Calculated from eqs. 23 and 24 in Appendix I.
<table>
<thead>
<tr>
<th>DP</th>
<th>$k_0$, s$^{-1}$</th>
<th>$k_0$ (obs)</th>
<th>$K_m$, mM</th>
<th>$K_m$ (obs)</th>
<th>$k_0/K_m$</th>
<th>s$^{-1}$mM$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.134 ± 0.004$^a$</td>
<td>1.00</td>
<td>22.93 ± 1.54</td>
<td>1.00</td>
<td>0.0058</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.133$^b$</td>
<td></td>
<td>22.90$^b$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.452 ± 0.003</td>
<td>0.72</td>
<td>11.64 ± 0.18</td>
<td>0.72</td>
<td>0.0388</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.627</td>
<td></td>
<td>16.19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.622 ± 0.009</td>
<td>1.22</td>
<td>12.97 ± 0.47</td>
<td>1.22</td>
<td>0.0480</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.508</td>
<td></td>
<td>10.63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.797 ± 0.010</td>
<td>1.15</td>
<td>10.54 ± 0.33</td>
<td>1.15</td>
<td>0.0756</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.691</td>
<td></td>
<td>9.15</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>6</td>
<td>0.759 ± 0.009</td>
<td>1.03</td>
<td>9.26 ± 0.29</td>
<td>1.03</td>
<td>0.0820</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.738</td>
<td></td>
<td>8.99</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Standard deviation.  
$^b$Calculated from eqs. 23 and 24 in Appendix I.
Table 19. Subsite structures of GAI and GAII with the malto- and isomalto-oligosaccharides at pH 4.5 and 35°C

<table>
<thead>
<tr>
<th>Subsite no. (i)</th>
<th>Subsite Affinities, kJ/mol</th>
<th>Maltooligosaccharides</th>
<th>Isomalto-oligosaccharides</th>
<th>GAI</th>
<th>GAI</th>
<th>GAI</th>
<th>GAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0.33</td>
<td>-2.09</td>
<td>-5.60</td>
<td>-3.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>20.60</td>
<td>21.86</td>
<td>15.26</td>
<td>15.02</td>
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</tr>
<tr>
<td>3</td>
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<td>6.66</td>
<td>6.14</td>
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<td>4.85</td>
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</tr>
<tr>
<td>4</td>
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<td>3.35</td>
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</tr>
<tr>
<td>5</td>
<td></td>
<td>1.13</td>
<td>1.34</td>
<td>0.96</td>
<td>1.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.29</td>
<td>0.46</td>
<td>0.25</td>
<td>0.21</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.38</td>
<td>0.46</td>
<td>----</td>
<td>----</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$k_{int} \ s^{-1}$  
64.2  136.0  9.09  4.02

°The subsites are numbered from the nonreducing end of a productively bound substrate molecule.

Subsite Mapping

Maltooligosaccharides The binding affinities of the active sites of A. niger glucoamylases I and II appear to be similar, except for a slight difference in subsite 1, where GAI has a small positive affinity and GAII has a slight negative affinity. The second subsite of both forms has the largest affinity for glucosyl residues; thereafter the affinities gradually decrease (Fig. 3). The binding energy of the first subsite is thought to be composed of two parts, a change in Gibbs free energy of the glucosyl residue binding to the first subsite and the energy required to induce transformation of the glucosyl residue from the chair to the half-chair form, the transition state analogue. Ohnishi et al. found
Figure 3. Histogram of subsite affinities of GAI and GAIi acting on the maltooligosaccharides
by fluorescence spectrophotometry that gluconolactone, which is in the half-chair form, binds strongly to the first subsite, while glucose preferentially binds to the second subsite.

The subsite affinities of GAI and GAIi are very similar to those found in the literature for glucoamylases from a variety of fungal sources (Table 2). The subsite maps of the major forms from A. awamori and A. saitoi are almost identical to that found for the major form, GAI, in this study. The value of $k_{int}$ for A. awamori and A. saitoi are 47.9 s$^{-1}$ and 43.0 s$^{-1}$, respectively, while $k_{int}$ for GAI measured in this work is 64.2 s$^{-1}$, indicating that the active sites of the major forms from A. awamori, A. saitoi, and A. niger are kinetically very similar. The minor form, GAIi, is similar to the glucoamylase from Rh. niveus in that it has a larger $k_{int}$ and an overall negative affinity in subsite 1. The binding affinity of the second subsite for GAIi from A. niger is 5-16% larger than others reported (Table 2).

The quantity $k_{int}$ is the rate constant for hydrolysis of a productive complex and is assumed to be independent of chain length. Its values for GAI and GAIi are 64.2 s$^{-1}$ and 136.0 s$^{-1}$, respectively, a two-fold difference. The quantity $k_{int}$ and the affinity of subsite 1 are derived from a plot of $\exp (A_{n+1}/RT)$ versus $1/(k'_o)^n$, where $n$ is both subsite number and substrate chain length (Figs. 4 and 5). The value of $k_{int}$ is the reciprocal of the $x$-intercept or, alternatively, is the slope divided by the $y$-intercept. In the case of GAI and GAIi acting on the maltooligosaccharides, the $y$-intercepts are $-1.14 \pm 0.16$ and $-0.44 \pm 0.39$ and the slopes are $73.24 \pm 1.75$ s and $59.85 \pm 4.20$ s.
Figure 4. Plot of $\exp(A_n+1/RT)$ versus $1/k_o$ for GAI acting on the maltooligosaccharides
Figure 5. Plot of $\exp(A_{n+1}/RT)$ versus $1/k_o$ for GAI acting on the maltooligosaccharides.
respectively. Varying the y-intercept by one standard deviation above and below its mean value causes $k_{\text{int}}$ to vary from 55.90 to 75.48 s$^{-1}$ for GAI and 72.11 to 1,197 s$^{-1}$ for GAII. Comparing Fig. 4 and 5, it is obvious that the maltooligosaccharide kinetic data of GAI adheres to the model, while the points for GAII deviate from linearity, indicating that there is either a larger amount of experimental error associated with the GAII kinetic data or that the model is not representing the kinetic behavior of the enzyme.

The determination of $k_{\text{int}}$ is also sensitive to slight changes in $K_m$ for maltose, the uppermost point in Fig. 5. If the $K_m$'s of GAI and GAII for maltose are varied by one standard deviation above and below their mean values, 1.09 to 1.37 and 0.86 to 1.12 mM, respectively, the values of $k_{\text{int}}$ will vary from 82.8 to 54.7 s$^{-1}$ for GAI and 164 to 83 s$^{-1}$ for GAII.

The affinity of subsite 1 is also calculated from the y-intercept. A variation of a single standard deviation above and below the mean value of the y-intercept (-1.31 to -0.99) will yield values of $A_1$ from -0.08 to 0.67 kJ/mol, with a mean value of 0.33 kJ/mol for GAI. The same manipulation with GAII gives a range of the y-intercept of -0.83 to -0.05 and values of $A_1$ from -0.48 to -7.42 kJ/mol, with a mean value of -2.09 kJ/mol. Thus it is apparent that $k_{\text{int}}$ and the affinity of subsite 1 are subject to large variations based on experimental error. Even though values of $k_{\text{int}}$ for GAI and GAII are different numerically, they are within the ranges that are obtained by varying the y-intercept by one standard deviation from its mean, suggesting that the difference between $k_{\text{int}}$ for GAI and GAII is not major. In the case of subsite 1 there appears to be a
statistically significant difference between the values for GAI and GAII.

The ability of a model to predict experimental parameters is an indication of how well that model represents the reaction system. The ratios of $K_m^{(obs)}/K_m^{(calc)}$ and $k_o^{(obs)}/k_o^{(calc)}$ indicate how well this model conforms to the experimental data. For the case of GAI-catalyzed hydrolysis of the maltooligosaccharides, the observed and calculated values are in close agreement. For GAII-catalyzed hydrolysis of the maltooligosaccharides, however, the ratios of the observed to calculated values of the kinetic parameters deviate to a greater extent from 1, indicating that the model is not representing the reaction system as well as with GAI. Despite this, the subsite binding energies for the two enzyme forms acting on the maltooligosaccharides are similar. The experimental values of $k_o$ and $K_m$ for GAI and GAII for $G_2$, $G_4$, $G_5$, and $G_6$ are statistically the same, while those for $G_3$ and $G_7$ vary significantly. The discrepancies in the maltotriose data are unexpected and at this point cannot be explained.

Sergeev and Firsov subslite-mapped the major glucoamylase from A. awomori at five different temperatures ranging from 25°C to 50°C, and found that as the temperature decreased the experimental values deviated from those predicted by the model. They determined that $k_{int}$ for maltose was less than $k_{int}$ for the other maltooligosaccharides by the amount $\exp(-\Delta G_{int}/RT)$. The values of $\Delta G_{int}$ and the subsite affinities are listed in Table 20. The subsite affinities determined by Sergeev and Firsov are different from those reported by others (Table 4), especially for subsite 3, which has an affinity lower than that reported in any other study of
glucoamylase subsite structure, and for subsite 2, which has an affinity larger than any reported.

Table 20. Subsite structure of glucoamylase with the maltooligosaccharides at different temperatures from A. awamori with a change in $k_{\text{int}}$

<table>
<thead>
<tr>
<th>Temp., °C</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>$k_{\text{int}}$, s$^{-1}$</th>
<th>$\Delta G_{\text{int}}$, kJ/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.3</td>
<td>3.23</td>
<td>24.38</td>
<td>0.38</td>
<td>4.06</td>
<td>0.80</td>
<td>0.50</td>
<td>20.34</td>
<td>4.75</td>
</tr>
<tr>
<td>32.0</td>
<td>2.94</td>
<td>24.57</td>
<td>0.41</td>
<td>3.90</td>
<td>0.66</td>
<td>0.44</td>
<td>28.35</td>
<td>4.87</td>
</tr>
<tr>
<td>37.0</td>
<td>2.72</td>
<td>24.70</td>
<td>0.43</td>
<td>3.78</td>
<td>0.55</td>
<td>0.40</td>
<td>44.83</td>
<td>4.95</td>
</tr>
<tr>
<td>41.8</td>
<td>2.52</td>
<td>24.83</td>
<td>0.46</td>
<td>3.67</td>
<td>0.45</td>
<td>0.36</td>
<td>76.39</td>
<td>5.03</td>
</tr>
<tr>
<td>50.0</td>
<td>2.16</td>
<td>25.04</td>
<td>0.50</td>
<td>3.48</td>
<td>0.28</td>
<td>0.29</td>
<td>134.39</td>
<td>5.18</td>
</tr>
</tbody>
</table>

The subsites are numbered from the nonreducing end of a maltooligosaccharide in a productive complex.

Svensson et al. found that the first 512 amino acids of GAI were the same as the first 512 amino acids of GAI starting from the N-terminus. Obviously the active site of each form is located in this region. GAI has an additional 104 amino acids on the C-terminus end which have been found important in the binding of raw starch. Since the active site of both enzymes is composed of the same amino acids, one would expect the subsite binding energies for both forms to be the same, which in general is true. However, it is possible that the 104 amino acids on the C-terminus of GAI could have a small effect on the binding of the maltooligosaccharides, warranting the slight difference between GAI and GAI.

**Isomaltooligosaccharides** The active sites of GAI and GAII show similar binding affinities for the isomaltooligosaccharides, as
illustrated in Fig. 6. The first subsite for both forms has a more negative binding affinity for an α-1,6-linked nonreducing glucosyl residue than for an α-1,4-linked residue. The negative affinity indicates an overall unfavorable binding of the glucosyl residue. The second subsite has a high positive affinity, but it is 25% less than the affinity associated with an α-1,4-linked substrate. The third subsite has, on the average, 27% less binding affinity than that for the maltooligosaccharides. The fourth, fifth, and sixth subsites show very little affinity for α-1,6-linked glucosyl residues.

The ratios of $k_0$ for $iG_n$ and $G_n$ are listed in Table 21 for substrates of the same chain length. Regardless of chain length, the ratio is between 0.021 to 0.033 for both GAI and GAIi, similar to what has been reported in the literature for glucoamylase 12, 17, 23, 32.

<table>
<thead>
<tr>
<th>DP</th>
<th>GAI</th>
<th>GAIi</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.030</td>
<td>0.025</td>
</tr>
<tr>
<td>3</td>
<td>0.033</td>
<td>0.021</td>
</tr>
<tr>
<td>4</td>
<td>0.026</td>
<td>0.021</td>
</tr>
<tr>
<td>5</td>
<td>0.032</td>
<td>0.027</td>
</tr>
<tr>
<td>6</td>
<td>0.030</td>
<td>0.026</td>
</tr>
</tbody>
</table>

The values of $k_{int}$ for GAI and GAIi acting on the isomaltooligosaccharides are 9.09 and 4.06 s$^{-1}$, 14.0% and 3.0% of $k_{int}$ determined for the maltooligosaccharides, respectively (Figs. 7 and 8). These values do
Figure 6. Histogram of subsite affinities of GAI and GAII acting on the isomaltooligosaccharides
Figure 7. Plot of \( \exp(A_{n+1}/RT) \) versus \( 1/k_o \) for GAI acting on isomaltooligosaccharides.
Figure 8. Plot of $\exp(A_{n+1}/RT)$ versus $1/k_0$ for GAI acting on the isomaltooligosaccharides

slope = 0.91 ± 0.10 s
y-intercept = -0.23 ± 0.38
not come as a surprise, even though the α-1,6 bond being hydrolyzed in the isomaltooligosaccharides is through a primary hydroxyl group while the preferred α-1,4 bond in the maltooligosaccharides is through a secondary hydroxyl group. Though the bond to the primary hydroxyl has one more degree of freedom of rotation, it still cannot allow the isomaltooligosaccharides to assume the same configuration that is associated with the α-1,4 linkage.

### Binding Mechanisms

These values of $k_{\text{int}}$ and $k_0$ suggest that nonproductive binding of the isomaltooligosaccharides is occurring to a greater degree than that of maltooligosaccharides. Differences in binding between the maltooligosaccharides and isomaltooligosaccharides have been reported by others. Ohnishi et al.\(^{91}\) determined by fluorescence spectrophotometry that gluconolactone is a noncompetitive inhibitor of isomaltose. Earlier Ohnishi et al.\(^{88}\) showed that gluconolactone, which is postulated as a transition state analogue, binds preferentially to the first subsite, and that isomaltose binds to subsites 2 and 3. The binding of isomaltose there is not unexpected, based on the individual subsite binding energies. The second and third subsites of both GAI and GAII determined for the isomaltooligosaccharides have the highest binding energies and thus the greatest affinities for glucosyl residues, while the binding affinity of the first subsite is negative. There is more difference between the sums of the affinities for subsite 2 and subsite 3, on one hand, and subsite 1 and subsite 2, on the other, for the isomaltooligosaccharides then for the maltooligosaccharides, indicating that nonproductive binding of isomalto-
oligosaccharides is more energetically favorable.

Ohnishi et al.\textsuperscript{92} also investigated the hydrolysis of the maltooligosaccharides in the presence of gluconolactone and found the inhibition of glucoamylase by gluconolactone was of the mixed type, indicating the nonreducing end of the maltooligosaccharides can bind to the second subsite. Tanaka et al.\textsuperscript{93} investigated the hydrolysis of maltose in the presence of gluconolactone by stopped-flow kinetics and observed that both gluconolactone and maltose bind by a two-step mechanism, as illustrated in eq. 5. Tanaka et al.\textsuperscript{93} determined that gluconolactone binds transiently to subsite 2 in the bimolecular process and relocates to subsite 1 to form the more stable \( \text{ES}^* \) complex. The binding of maltose follows the same mechanism:

\[
\begin{align*}
E + S & \overset{k_{+1}}{\longrightarrow} ES \overset{k_{+2}}{\longrightarrow} \text{ES}^* \\
& \overset{k_{-1}}{\longleftarrow} \quad \overset{k_{-2}}{\longleftarrow}
\end{align*}
\]

as does the binding of the maltooligosaccharides.\textsuperscript{94} In both cases Tanaka et al.\textsuperscript{93,94} observed that ES is a loosely bound complex, while \( \text{ES}^* \) is more tightly bound. Also, the dissociation constant of ES, \( K_{-1} = k_{-1}/k_{+1} \), and \( k_{+2} \) were almost independent of chain length, whereas \( k_{-2} \) decreased with increasing DP. Tanaka et al.\textsuperscript{94} suggested that because \( K_{-1} \) is independent of the chain length, the only initial binding that was occurring was between the nonreducing-end glucosyl residue of the substrate molecule and subsite 2. The binding of the substrate to the other subsites did not
occur until the substrate had relocated over the catalytic site. The standard Gibbs free energy change for the fast bimolecular process, the formation of ES, was calculated to be 20 kJ/mol, which is close to the affinity of subsite 2.

The binding scheme of Tanaka et al.93,94 and the inhibition studies of Ohnishi et al.88,91 explain the differences between the rates of hydrolysis for the malto- and isomaltooligosaccharides for GAI. The nonreducing glucosyl residue of the isomaltooligosaccharides preferentially binds to subsite 2 in a nonproductive complex and will not as easily relocate to the first subsite as will that of the maltooligosaccharides.

In conclusion, the subsite model is able to predict the kinetic constants adequately, but the ratios of $K_m^{(obs)}/K_m^{(calc)}$ and $k_o^{(obs)}/k_o^{(calc)}$ do in some cases vary significantly from unity. The greatest deviation occurs with G$_3$ and iG$_3$ for both GAI and GAII, where $K_m$ and $k_o$ are over- or underpredicted by as much as 37%.

**Hydrolysis Kinetics**

Since the subsite binding affinities of glucoamylase I and II for both the malto- and isomaltooligosaccharides were virtually the same, the hydrolysis studies were performed with GAI, except for a few reactions that were carried out before the subsite mapping studies were conducted.

The Michaelis-Menten constants, $K_m$ and $k_o$, were determined for GAI acting on five disaccharides, α,β-trehalose, isomaltose, kojibiose, maltose, and nigerose, and two trisaccharides, panose and isomaltotriose, at three different temperatures and pHs (Tables 5-11). Similar but less
complete experimentation was conducted on maltose, maltotriose, and isomaltotriose with GAII (Tables 12-14). The kinetic constants were determined for two reasons: 1) to gain a better understanding of the action of glucoamylase on substrates with different glucosidic linkages and to expand the fundamental knowledge of glucoamylase, and 2) to be able to model the condensation reactions of glucoamylase. The seven sugars studied with GAI were observed by Nikolov\textsuperscript{14} to be condensation products produced by GAI and GAII from concentrated glucose solutions.

The $K_m$ is a measure of the binding efficiency of the substrate. The higher the $K_m$, the less strong the binding. Kojibiose and $\alpha,\beta$-trehalose have the highest $K_m$'s, while maltose and maltotriose have the lowest. Panose and isomaltotriose have lower $K_m$'s than any of the disaccharides except maltose, since they can interact with one additional enzyme subsite. It is interesting to note that glucoamylase is able to accommodate a wide variety of substrates whose three-dimensional structures are greatly different than the preferred $\alpha$-1,4-linked materials.

The value of $K_m$ for the different substrates are plotted versus temperature in Fig. 9. Assuming that $K_m$ is an equilibrium constant, we can determine the heats of reaction for the binding of the substrate to the enzyme from the following equation:

$$\frac{d(ln K_m)}{dT} = \frac{\Delta H^o}{RT^2}$$

(6)

By assuming that $\Delta H^o$ is constant over the temperature range investigated, eq. 6 can be integrated:
Figure 9. Plot of log $K_m$ versus $1/T$ for $\alpha,\beta$-trehalose (○), kojibiose (□), nigerose (○), maltose (●), isomaltose (△), maltotriose (▲), panose (○), and isomaltotriose (■) at pH 4.5. Solid lines: GAI; dashed lines: GAI I.
\[ \ln K_m = \frac{\Delta H^o}{RT} \]  (7)

If \( \ln K_m \) is plotted versus the reciprocal of the absolute temperature, the slope will equal the heat of reaction of binding. These are presented for all of the oligosaccharides along with literature values on Table 22.

The heat of reaction for the binding of maltoligosaccharides to the enzyme is more exothermic than that of other linkages, indicating that the binding of the \( \alpha-1,4 \)-linked substrates is energetically favored. The values of Sergeev and Firsov\(^8\) increase with DP, while those in this work appear to show no obvious trend. The binding of \( \alpha,\beta \)-trehalose and panose also is energetically favored, but to a lesser degree than that of the maltooligosaccharides. The binding of kojibiose, nigerose, and isomaltose is affected very little by temperature, while isomaltotriose is slightly endothermic in its binding.

Values of \( pK_m \), \( \log k_o \), and \( \log k_o/K_m \) are plotted versus pH for the seven oligosaccharides used with GAI (Figs. 10-16). The highest \( k_o \) occurs between pH 3.5 and 4.5 for all substrates except nigerose, which appears to have an optimum near pH 3.5. Variations in these values caused by changes in pH are attributed to the different dissociation states of the components of the system\(^9\). A model to explain the effects of pH on enzyme dissociation was proposed by Michaelis and Davidsohn\(^10\), and it and its extensions are presented in Appendix III.
Table 22. Standard heats of reactions of binding for substrates hydrolyzed by GAI

<table>
<thead>
<tr>
<th>Substrate</th>
<th>This work</th>
<th>Sergeev and Firsov$^{89}$</th>
<th>Thomas$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha,\beta$-Trehalose</td>
<td>$-10.04 \pm 3.31$</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Kojibiose</td>
<td>$-4.10 \pm 3.30$</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Nigerose</td>
<td>$-4.48 \pm 2.56$</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Maltose</td>
<td>$-45.61 \pm 9.57$</td>
<td>$-27.82 \pm 1.85$</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>$-10.79 \pm 4.40^b$</td>
<td></td>
<td>-----</td>
</tr>
<tr>
<td>Isomaltose</td>
<td>$-1.81 \pm 4.12$</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>$-40.84 \pm 6.91^b$</td>
<td>$-28.37 \pm 2.64$</td>
<td>-----</td>
</tr>
<tr>
<td>Panose</td>
<td>$-11.09 \pm 10.95$</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>$5.15 \pm 0.19$</td>
<td>$20.08 \pm 16.21^b$</td>
<td>-----</td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>-----</td>
<td>$-39.46 \pm 3.9$</td>
<td>$-55.02 \pm 8.42^b$</td>
</tr>
<tr>
<td>Maltopentaose</td>
<td>-----</td>
<td>$-46.07 \pm 4.28$</td>
<td>$-38.20 \pm 19.06^b$</td>
</tr>
<tr>
<td>Maltohexaose</td>
<td>-----</td>
<td>$-47.15 \pm 6.98$</td>
<td>-----</td>
</tr>
</tbody>
</table>

$^a$Values determined by Gregory Thomas as part of an Iowa State ChE 490 project.
$^b$Values determined using GAII.

By applying the rules outlined by Dixon to Figures 10-16, the $pK_e$ and $pK_{es}$ values ($K_{e1} = [EH^-][H^+]/[EH^2]$, $K_{e2} = [E^2^-][H^+]/[EH^-]$, $K_{es1} =$ $[EHS^-][H^+]/[EH_S]$, $K_{es2} = [ES^2-][H^+]/[EHS^-]$) can be determined. The plots consist of just three points, so it was only possible to determine the approximate ranges for the $pK$s (Table 23).

The range of values of $pK_{e1}$ and $pK_{e2}$ are the same for all the substrates except for $\alpha,\beta$-trehalose, which is expected since they are the dissociation constants for the free enzyme. The values for $pK_{e1}$ and $pK_{e2}$.
Figure 10. Effect of pH on $K_m$, $k_o$, and $k_o/K_m$ for $\alpha,\beta$-trehalose at 35$^\circ$C. Ranges here and on the following plots are the standard deviations.
Figure 11. Effect of pH on $K_m$, $k_o$, and $k_o/K_m$ for kojibiose at 35°C
Figure 12. Effect of pH on $K_m$, $k_o$, and $k_o/K_m$ for nigerose at 35°C
Figure 13. Effect of pH on $K_m$, $k_o$, and $k_o/K_m$ for maltose at 35°C
Figure 14. Effect of pH on $K_m$, $k_o$, and $k_o/K_m$ for isomaltose at 35°C
Figure 15. Effect of pH on $K_m$, $k_o$, and $k_o/K_m$ for panose at 35 °C
Figure 16. Effect of pH on $K_m$, $k_o$, and $k_o/K_m$ for isomaltotriose at 35°C
Table 23. The dissociation constants for GAI acting on seven oligosaccharides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$pK_{e1}$</th>
<th>$pK_{e2}$</th>
<th>$pK_{es1}$</th>
<th>$pK_{es2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>α,β-Trehalose</td>
<td>2.5 - 3.75</td>
<td>NE</td>
<td>NE or ≤ 2</td>
<td>5.25 - 5.75</td>
</tr>
<tr>
<td>Kojibiose</td>
<td>2.5 - 3.75</td>
<td>5.25 - 6.5</td>
<td>NE or ≤ 2</td>
<td>5.25 - 5.75</td>
</tr>
<tr>
<td>Nigerose</td>
<td>2.5 - 3.75</td>
<td>5.25 - 6.5</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>Maltose</td>
<td>2.5 - 3.75</td>
<td>5.25 - 6.5</td>
<td>NE or ≤ 2</td>
<td>5.25 - 5.75</td>
</tr>
<tr>
<td>Isomaltose</td>
<td>2.5 - 3.75</td>
<td>5.25 - 6.5</td>
<td>NE or ≤ 2</td>
<td>5.25 - 5.75</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>2.5 - 3.75</td>
<td>5.25 - 6.5</td>
<td>NE or ≤ 2</td>
<td>5.25 - 5.75</td>
</tr>
<tr>
<td>Panose</td>
<td>2.5 - 3.75</td>
<td>5.25 - 6.5</td>
<td>2.5</td>
<td>&gt; 6.4</td>
</tr>
</tbody>
</table>

*NE = No effect.
*UD = Undetermined.

Bracket the values of 2.9 and 5.9, respectively, which Hiromi et al. determined for a glucoamylase from *Rh. delemar* acting on panose and maltose. The value of $pK_{e1}$ is similar to that of a carboxyl group, while the value of $pK_{e2}$ suggests an imidazole group. However, Hiromi et al. determined that the second dissociable group of *Rh. delemar* was instead a carboxyl group, based on the premise that the $pK$ of the second group can be decreased if it is close to a negatively charged group, such as the first carboxyl group. Hiromi et al. substantiated this by determining the $pK$s of the two groups in a medium of lower dielectric constant. Lowering the dielectric constant of the medium will suppress the dissociation of a carboxyl group since this involves charge separation. The $pK$ of an imidazole group would not be affected in a medium of lower dielectric constant because there is no separation of charge. Hiromi et al. found that the $pK$ indeed decreased, indicating that charge separation was
occurring. They also determined that the heat of dissociation of both groups was 6.3 kJ/mol, the value of the heat of dissociation of carboxyl groups in proteins, indicating that both groups are carboxyls. Furthermore, Svensson et al.\textsuperscript{63} found that four active-site carboxyl groups in glucoamylase were protected by acarbose, further supporting a high value for a carboxyl pK\textsubscript{e2}. Accepting therefore that both dissociable groups are carboxyls, their active forms at the optimum pH of glucoamylase are COO\textsuperscript{−} and COOH, respectively.

The values of pK\textsubscript{es1} and pK\textsubscript{es2} are similar for α,β-trehalose, kojibiose, maltose, isomaltose, and isomaltotriose, indicating in those cases that substrate binding influences the dissociation of both groups in the same fashion. Nigerose, an α-1,3-linked disaccharide, and panose, an α-1,6/α-1,4-linked trisaccharide, appear to affect the dissociation of the two groups differently. With nigerose, a plot of log k\textsubscript{o} versus pH is monotonic over the entire pH range investigated, yielding pK\textsubscript{es} values lower than those of the other substrates, and indicating that the enzyme-nigerose complex increases the dissociation of the proton in both groups. With panose, both pK\textsubscript{es} values increase, suggesting that panose is binding to the active site in a configuration different from the other substrates.

Comparing the values of pK\textsubscript{e1} to pK\textsubscript{es1} indicates that substrate binding increases the dissociation of the first carboxyl group. This group may stabilize a carbonium ion intermediate, as does Asp 52 of lysozyme\textsuperscript{97}. Furthermore, Ohnishi et al.\textsuperscript{88} has proposed that D-gluconolactone is a transition intermediate in glucoamylase-catalyzed
hydrolysis, suggesting a mechanism similar to lysozyme. Substrate binding has little effect on the dissociation of the second carboxyl group, suggesting that it may be involved in hydrogen bonding.

The effect of temperature on the rate coefficient $k_o$ follows the typical Arrhenius relationship, as illustrated in Fig. 17. The activation energy for each oligosaccharide is listed on Table 24.

The activation energies for substrates not containing $\alpha$-1,4 linkages are close to each other, with an average value of 73.6 kJ/mol. The activation energies of maltose for GAI and maltose and maltotriose for GAI are appreciably lower, with an average value of 61.4 kJ/mol, which agree with those determined by Sergeev and Firsov and Thomas. The lower value indicates that less energy is required to overcome the barrier of hydrolysis for an $\alpha$-1,4 linkage than for other linkages. The exception is panose, which has an activation energy similar to the maltooligosaccharides, though its $\alpha$-1,6 rather than its $\alpha$-1,4 linkage is hydrolyzed. HPLC of the panose reaction mixture at pH 4.5 and 25°C and 45°C demonstrated that maltose and D-glucose, rather than D-glucose alone, accumulated upon hydrolysis. While the rate of hydrolysis of the $\alpha$-1,6 linkage is 3% that of the $\alpha$-1,4 linkage when one compares isomaltose with maltose and isomaltotriose with maltotriose, the $k_o$ of panose is actually 28.3% that of maltotriose. This indicates that the $\alpha$-1,4 bond on the reducing end of panose increases the stability of the enzyme-substrate complex and decreases the energy normally required for the hydrolysis of the $\alpha$-1,6 linkage.

The $k_o$'s of panose at various conditions are very close to those of maltose, but the $K_m$'s of panose are on the average ten times larger. One
Figure 17. Plot of $k_0$ versus $1/T$ at pH 4.5 for $\alpha,\beta$-trehalose (○), kojibiose (□), nigerose (○), maltose (●), isomaltose (Δ), maltotriose (▲), panose (●) and isomaltotriose (■). Solid lines: GAI; dashed lines: GAlI.
Table 24. The activation energies of GAI- and GAII-catalyzed hydrolysis of seven different oligosaccharides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activation Energy, kJ/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GAI</td>
</tr>
<tr>
<td>(\alpha,\beta)-Trehalose</td>
<td>73.5 ± 3.6(^a)</td>
</tr>
<tr>
<td>Kojibiose</td>
<td>79.6 ± 2.4</td>
</tr>
<tr>
<td>Nigerose</td>
<td>70.4 ± 8.1</td>
</tr>
<tr>
<td>Maltose</td>
<td>59.9 ± 2.1</td>
</tr>
<tr>
<td>Isomaltose</td>
<td>73.9 ± 1.0</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>-----</td>
</tr>
<tr>
<td>Panose</td>
<td>63.1 ± 6.5</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>74.3 ± 0.3</td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>-----</td>
</tr>
<tr>
<td>Maltopentaose</td>
<td>-----</td>
</tr>
<tr>
<td>Maltohexaose</td>
<td>-----</td>
</tr>
</tbody>
</table>

\(^a\)Standard deviation.
\(^b\)Determined by Gregory Thomas as part of an Iowa State ChE 490 project.

explanation is that panose may form more nonproductive complexes at lower initial concentrations than maltose, thus acting as a competitive inhibitor against itself. It was suggested earlier that the \(\alpha-1,6\)-linked isomaltooligosaccharides formed more nonproductive than productive complexes. A similar situation exists when \(\alpha,\beta\)-trehalose and isomaltose are compared. The \(k_o\) of \(\alpha,\beta\)-trehalose is larger than the \(k_o\) of isomaltose, but the \(K_m\) of \(\alpha,\beta\)-trehalose is at least five times larger than the \(K_m\) of isomaltose under similar conditions. It can again be postulated that \(\alpha,\beta\)-trehalose preferentially binds nonproductively at low initial
concentrations. Another explanation is that because α,β-trehalose is nonreducing, it has a glucosyl residue linked both α and β to an aglycon glucosyl residue. Depending on the binding of α,β-trehalose, a glucosyl residue with either an α- or a β-linkage would be in the first subsite. The latter would not be hydrolyzed and would act as a competitive inhibitor. Hiromi et al. determined the inhibition constants of 180 mM and 100 mM for the β-linked disaccharides cellobiose and gentiobiose, respectively. These $K_i$'s indicate that neither sugar is a strong inhibitor, suggesting that the β-glucosyl residue is not binding in the first subsite of glucoamylase, and supporting the hypothesis that the nonproductive binding is occurring at subsite 2.

Conclusions

Glucoamylase I and II from A. niger were separated by ion-exchange chromatography and found to be free of transglucosylase by an acarbose inhibition study and by three electrophoretic techniques, SDS-PAGE, DISC-PAGE, and IEF. Glucoamylase I and II were determined by the same electrophoretic techniques to be pure, but were found to be microheterogeneous.

The subsite affinities of the active site of glucoamylase I and II were calculated with the malto- and isomaltooligosaccharides. GAI and II had the same binding affinities for the maltooligosaccharides, except for subsite 1, where GAI had an affinity of 0.33 kJ/mol while GAlI had an affinity of -2.06 kJ/mol. The values of $k_{int}$ for GAI and II were 64.2 and 136 $s^{-1}$, respectively. The subsite maps of GAI and II with the isomalto-
oligosaccharides were similar to those of the maltooligosaccharides, except the equivalent subsite affinities for the former were approximately 25% less than for the latter. In addition, the affinities of subsite 1 for GAI and GAIII were more negative than the affinities of subsite 1 generated with the maltooligosaccharides. The values of $k_{\text{int}}$ for GAI and II were 9.09 and 4.06 s$^{-1}$, respectively. The conclusion of the subsite mapping studies was that GAI and GAIII were kinetically very similar or identical.

Hydrolysis rate studies were performed with GAI and to small extent with GAIII. The Michaelis-Menten constants, $K_m$ and $k_o'$, were determined for five disaccharides, $\alpha,\beta$-trehalose, kojibiose, nigerose, maltose, and isomaltose, and for two trisaccharides, panose and isomaltotriose, at different temperatures and different pHs with GAI. With GAIII $K_m$ and $k_o$ were determined for maltose, maltotriose, and isomaltotriose at different temperatures and for maltotriose at different pHs.

The quantity $K_m$ for the $\alpha$-1,4-linked substrates was temperature-dependent, and had a large exothermic standard heat of reaction for the binding of the substrate to the enzyme compared to other substrates. Substrates composed of links other than $\alpha$-1,4 had low $\Delta H^o$s, indicating that binding was not significantly temperature-dependent. Isomaltotriose had a small positive $\Delta H^o$, indicating that the binding was endothermic.

The effect of pH on $K_m$ and $k_o$ was modeled assuming that two dissociable groups were present in the active site. The pKa$s$ of both groups were similar to that of carboxyls found in proteins. Their forms at the optimum pH of glucoamylase are COO$^-$ and COOH, respectively. The
binding of substrate reduced the pK of first carboxyl by at least 1 pK unit, while the pK of the second carboxyl remained unchanged.

The effect of temperature on $k_o$ followed the typical Arrhenius relationship. The activation energy of the $\alpha$-1,4-linked substrates averaged 61.4 kJ/mol, while that for substrates not containing $\alpha$-1,4 linkages averaged 73.6 kJ/mol. The exception was panose, which had an activation energy similar to that of the maltooligosaccharides. Apparently the presence of the $\alpha$-1,4 linkage stabilizes the enzyme-substrate complex, reducing the energy barrier normally required to hydrolyze the $\alpha$-1,6 linkage.
CHAPTER 3.

PURIFICATION OF AN ENDOXYLANASE FROM THE ACTINOMYCETE CHAINIA

Introduction

The presence of xylan-degrading enzymes was inferred before the turn of the 20th century when Hoppe-Seyler in 1889 observed the evolution of gas from a wood xylan suspension inoculated with river mud organisms. Since then, many investigators have identified various fungal, bacterial, and marine microorganisms possessing the ability to hydrolyze xylan. A review of biological degradation of xylan was presented by Pigman.

Xylanases as whole have not been studied in as great detail as cellulases and amylases. The main reason is the products from a xylan hydrolysis are much more heterogeneous, including D-xylose, L-arabinose, D-uronic acids, and various forms of arabinouronooxylooligosaccharides, while the hydrolysis of cellulose and starch can yield D-glucose as the major product. Therefore the economic benefits of xylan hydrolysis are less.

Xylanases are much more diverse than the amylases or the cellulases. The differences are caused by the structural and chemical heterogeneity of xylan. Though starch is branched and cellulose is partially crystalline, they are at least composed of one monomer, which is not the case for xylan. Because of the specificity of most enzymes, one would expect there to be many different types of xylanases. The work of a number of groups, reviewed by Reilly, has resulted in the categorization of at least four
different types of xylanases:

1) \(\beta\)-Xylosidases, which hydrolyze short xyloooligosaccharides to D-xylose and exhibit transferase activity.

2) Exo-xylanases, which attack xyloooligosaccharides from the reducing end to form single products, are less active on short xyloooligosaccharides than on long ones, usually invert configuration, and show little transferase activity. Currently a few D-xylose-producing exo-xylanases have been tentatively identified, but there is no known xylobiose-producing exo-xylanase corresponding to \(\beta\)-amylase or cellobiohydrolase.

3) Endo-xylanases of two types:
   a) Those that are unable to cleave L-arabinosyl-initiated branch points, are active on xyloooligosaccharides as short as xylotriose and occasionally xylobiose, and produce oligosaccharide fragments of various sizes.
   b) Those that hydrolyze branch points as well as \(\beta\)-1,4-D-xylosyl bonds, and produce mainly xyloooligosaccharides of various sizes.

Only a discussion of endo-xylanases will be presented here; interested readers should refer to Reilly\(^{100}\) for a discussion of the others.

Endo-hydrolases as a class do not engage in endwise attack of polymers but instead cleave bonds throughout the molecule. Attack is often thought to occur randomly, but there are a number of factors that regulate the hydrolysis. The subsite structure and specificity of the enzyme will dictate the product distribution. This was seen by Robyt and
French\textsuperscript{101} for an $\alpha$-amylase isolated from \textit{Bacillus subtilis}. When the enzyme was reacted with amylopectin, two specific low molecular weight oligosaccharides, maltotriose and maltohexaose, were formed. Robyt and French determined that this $\alpha$-amylase had a active center of nine subunits with the catalytic site located between the third and fourth subsite from the reducing end. Also, Robyt and French\textsuperscript{102} proposed that $\alpha$-amylases acted by a multiple attack mechanism. The enzyme forms a complex with the substrate and catalyzes the first cleavage, then one of the fragments moves over the cleavage point and hydrolysis again occurs. In this manner, the enzyme may catalyze the hydrolysis of several bonds before it dissociates and forms a new active complex with another substrate molecule.

Another factor that affects the attack of endo-hydrolases is the structure of the substrate. Takenishi and Tsujisaka\textsuperscript{103} isolated an endo-xylanase from \textit{A. niger} van Tieghem that hydrolyzed $X_2A$ ($3^1-\alpha$-L-arabinosylxylobiose) to L-arabinose and xylobiose and $X_3A$ ($3^1-\alpha$-L-arabinosylxylotriose) to L-arabinose, xylotriose, xylobiose, and D-xylose. Their enzyme exhibited a greater affinity for 8-1,4-D-xylosidic linkages near an $\alpha$-1,3-L-arabinosylxylosyl branch point. Fournier et al.\textsuperscript{104}, on the other hand, isolated an endo-xylanase from \textit{A. niger} that was active on soluble and insoluble xylan only if arabinosyl-initiated branches were first cleaved by mild acid hydrolysis. The enzyme was active on a xylooligosaccharide mixture of $X_5-X_9$, but was inactive on xylotetraose, xylotriose, and xylobiose, while the xylanase of Takenishi and Tsujisaka was active on xylooligosaccharides of DP 3 or greater.
The enzyme that will be investigated in this project is an endo-xylanase from the actinomycete genus *Chainia*. The isolation of *Chainia* NCL 82-5-1, its microbiological properties, and its production of glucose and xylose isomerases have previously been reported\(^{105,106}\). This strain also elaborates xylanase activity, with the highest levels occurring when it is grown on xylan\(^{107}\). The xylanase activity was partially isolated by ethanol precipitation and appeared by polyacrylamide gel electrophoresis at pH 4.3 to be from one protein\(^{107}\).

The xylanase was brought in semi-purified form to this laboratory from the National Chemical Laboratory in India by K. B. Bastawde, and was further purified by him using DEAE-cellulose, Fractogel TSK DEAE-650S, and Sephadex G-50-50 column chromatography. Preliminary characterization showed it to be homogeneous by isoelectric focusing in the pH range 7-9 and by SDS-gel electrophoresis, with an isoelectric point between pH 8 and 8.5. The enzyme had a molecular weight between 5000-6000 daltons by SDS-gel electrophoresis, Sephadex G-50-50 column chromatography, and amino acid analysis, making it apparently the smallest enzyme yet described. Activity was highest in a 30-min assay on xylan at pH 5.0 and almost 65°C, and was unchanged after 10 min at pH 6.0 and 60°C. The endo-xylanase was active on xylooligosaccharides as small as X\(^4\), and the rates increased with the chain length. Also, when the enzyme was incubated with X\(^8\), X\(^9\) began to form between 10 and 20 min, and after 45 min X\(^{10}\) appeared, suggesting the possibility of condensation or transxylosylation activity. It is not known if X\(^9\) or X\(^{10}\) are indeed linear or branched compounds.

The objectives of this study are to complete the characterization of
the enzyme. This includes experimentation to find a more sure purification procedure, suitable for cruder preparations than those employed by Bastawde, and to determine a more precise molecular weight using gel filtration, SDS-PAGE, and amino acid sequencing. This work, when completed, will be the subject of a joint publication \(^{108}\).

The next section will review work that has already been performed by others in the field.

**Previous Work**

Endo-xylanases have been studied for many years in this laboratory. Five from *A. niger* which do not produce L-arabinose from xylan were purified to homogeneity and characterized \(^{104,109-112}\). The first endo-xylanase from *A. niger* had a pI of 6.7, a molecular weight of 20,800, and a maximum activity, in a 20-min assay, between pH 4 and 6 at a temperature of 55°C \(^{109}\). The enzyme hydrolyzed larchwood xylan to smaller xylooligosaccharides, with xylobiose and D-xylose the predominant products. It was subsite-mapped and had an active site five subunits long with the catalytic site between second and third subsite from the reducing end \(^{112}\).

The second and third endo-xylanases were isozymes with isoelectric points of pH 8.6 for xylanase I and 9.0 for xylanase II \(^{110}\). Both enzymes had molecular weights of 13,000 daltons and similar amino acid profiles, with pH optima for activity of 6.0 for I and 5.5 for II, while optimal pHs for stability were 5.0 and 6.0, respectively. They both hydrolyzed larchwood xylan more rapidly than insoluble branched xylan, but converted each substance to the same extent. Also, both endo-xylanases had low activity on linear xylooligosaccharides and no activity on debranched
insoluble xylan. The low molecular weight products from either soluble or insoluble xylan with either enzyme in decreasing order were tri- and pentasaccharides, followed by equal amounts of di-, tetra- and hexa-saccharides. The major products on either soluble or insoluble xylan had chain lengths greater than DP 10.

The fourth A. niger endo-xylanase had a pI of 4.5, a molecular weight of 14,000 daltons, and was much more active on soluble than insoluble xylan. As with the two isozymes previously discussed, no D-xylose or L-arabinose was produced. The optimal pH for activity was 4.9, while that for stability was pH 5.6. There was high activity on xyloooligosaccharides from DP 5-9, but not on xylobiose, xylotriose, and xylotetraose. The fifth endo-xylanase from A. niger had a pI of 3.65, a molecular weight of 28,000 daltons, was active on soluble xylan, on insoluble xylan only after arabinosyl-initiated branch points were removed, and on xyloooligosaccharides longer than xylotetraose. The main hydrolysis products of soluble and insoluble xylan were oligosaccharides of intermediate length, especially the tri- and pentasaccharides.

Endo-xylanases have been isolated from many other organisms besides Aspergillus. Though most are produced by eucaRyotes, bacteria are also known to excrete xylanases. Horikoshi and Atusakawa purified an alkalophilic Bacillus endo-xylanase with highest activity between pH 5.5 and pH 9 at 60°C. It had a pI of 6.3 and produced mainly X₂, X₃, and X₄ from xylan. Four alkalophilic Bacillus strains were also found to have xylanase activity. Two strains, W1 and W3, produced two very similar xylanases that were different from the two similar enzymes
produced by strains W2 and W4. Xylanase I from strain W1 had a pI of 8.5 and a molecular weight of 21,500, while xylanase I from strain W3 had a pI of 8.3 and a molecular weight of 22,500. Both had an optimal temperature of 65°C, a sharp pH optimum at 6.0, and produced X₂, X₃, X₄, and X₅ but no D-xylose from xylan. Xylanase II from strain W1 had a pI of 3.6 and a molecular weight of 49,000, while xylanase II from strain W3 had a pI of 3.7 and a molecular weight of 50,500. Both were most active within a pH range of 7.0 to pH 9.5 at 70°C, and produced X₁, X₂, X₃, and X₅ from xylan. All four enzymes had transferase activity. Honda et al. characterized two endo-xylanases from an alkalophilic Bacillus. Xylanase A had a molecular weight of 43,000 daltons, an optimal pH range of 6 to 10 at 70°C, and was still active at pH 12. Xylanase N was 16,000 daltons, with a narrower pH optimum of 6-7 at 70°C. Both enzymes had transxylosylase activity and gave xylobiose and larger oligosaccharides from xylan hydrolysis. Esteban et al. isolated two endo-xylanases and a β-xylosidase from Bacillus circulans WL-12. Endo-xylanases A and B were 85,000 and 15,000 daltons and had pH optima of 5.5 to 7 and pIs of 4.5 and 9.1, respectively. Xylanase A produced X₁, X₂, X₃, and X₄, while xylanase B produced X₂, X₃, and X₄ from xylan. Bernier et al. isolated and characterized an endo-xylanase from Bacillus subtilis that had pH and temperature optima of 5.0 and 50°C, a molecular weight of 32,000, and hydrolysis products of X₁, X₂, and X₃ from xylan. Bacillus pumilus excreted an endo-xylanase of molecular weight 22,384 with pH and temperature optima of 6.5 and 50°C, respectively. The xylanase hydrolyzed larchwood xylan to produce X₂-X₄, and converted X₃ to X₂ and X₄, as well
as \( X_4 \) to \( X_2 \), \( X_3 \), \( X_4 \), and higher oligosaccharides, indicating transxylosylase activity.

Endo-xylanases are present in other bacterial organisms. Berenger et al.\(^{122}\) purified three xylanases from *Clostridium stercorarium* with molecular weights of 41,000, 73,000, and 64,000 daltons, pIs of 4.53, 4.43, and 4.39, and carbohydrate contents of 19%, 3%, and 4%, respectively. All three were immunologically reactive toward each other and yielded mostly \( X_2 \) and \( X_3 \) and small amounts of \( X_1 \) and \( X_4 \) from xylan. Lee et al.\(^{123}\) screened 20 species of *Clostridium* and found 17 with xylanolytic activity. Two *Clostridium acetobutylicum* strains, NRRL B527 and ATCC 824, each excreted endo-xylanases with pH optima of 5.8 and 6.0, respectively, but with a common temperature optimum of 65°C. Neither strain was able to grow on xylan. Ristroph and Humphrey\(^{124}\) characterized a crude endo-xylanase preparation from a thermophilic bacterium of the genus *Thermomonospora*. The xylanase preparation had pH and temperature optima of 5.5-7.7 and 80°C, respectively, and produced \( X_2 \), \( X_3 \), and \( X_4 \) from xylan. Ohkoshi et al.\(^{125}\) purified three xylanases from an alkalophilic *Aeromonas* species. The three enzymes, L, M, and S, had pH and temperature optima of 7-8, 6-8, and 5-7, and 50, 50, and 60°C, respectively. Their molecular weights were 145,000, 37,000 and 23,000 daltons, respectively, and they produced \( X_2-X_4 \) from xylan. All three enzymes were stable to pH 10, with xylanase L stable to pH 12. *Ruminococcus flavefaciens*\(^{126}\) and *Bacteroides succinogenes*\(^{127}\) also possess xylanase activity, but no characterization studies were conducted.
Members of the actinomycete genus *Streptomyces*, which is of commercial importance in the antibiotic industry, also excrete xylanases. Iizuka and Kawaminami\(^{128}\) isolated an endo-xylanase from *Streptomyces xylophagus* that was most active and stable around pH 6.2 and 50°C in a 15-min assay with xylan. It initially produced xylobiose and larger oligosaccharides from xylan, but yielded finally xylobiose and D-xylose. Kusakabe *et al.*\(^{129}\) purified a similar xylanase from *Streptomyces* that had optima at pH 6 and 60°C. The endo-xylanase had a molecular weight of 40,500 daltons, an isoelectric point at pH 7.3, and produced D-xylose and xylobiose from xylan after originally forming xylooligosaccharides of intermediate size. Also, the enzyme yielded xylobiose and no D-xylose from xylotriose, suggesting transxylosylase activity\(^{130}\). Later work determined the identity of various arabinoxylosyl products from arabinoxylan\(^{131,132}\). Sreenath and Joseph\(^{133}\) purified three *Streptomyces exfoliatus* xylanases, one which might have been an exo-hydrolase, and found they had activity optima between pH 5.5 and 7.0 and near 50°C. Nakajima *et al.*\(^{134}\) described a *Streptomyces* endo-xylanase of 43,000 daltons with an isoelectric point at pH 6.9 and highest activity at pH 5.5 and 55°C. It did not debranch arabinoxylan, could slowly attack \(X_2\) and cleaved xylan to \(X_2\), \(X_3\), and \(X_4\). In a departure from the properties of the enzyme of Kusakabe *et al.*\(^{129}\), it had decreasing Michaelis constants and increasing maximum rates on substrates of increasing length.

Kinetic parameters for only seven endo-xylanases have been determined (Table 25). Working with endo-xylanases from *A. niger*, Takenishi and Tsujisaka\(^{135}\) estimated \(K_m\) and \(V_m\) for xylotriose to xylopentaose, while
Biely et al. calculated $V_m/K_m$ for $X_3$ to $X_8$, and Meagher et al. determined $K_m$ and $V_m$ values for xylotriose through xylooctaose. Kubackova et al. found $K_m$ and $V_m/K_m$ for $X_3$ to $X_6$ for an endo-xylanase from *Trametes hirsuta*, and Biely et al. determined $V_m/K_m$ for $X_3$ to $X_8$ for a *Cryptococcus albidus* endo-xylanase. Kusakabe et al., using an endo-xylanase from *Streptomyces*, calculated $K_m$ and $V_m$ for $X_3$ to $X_7$. Finally, Nakajima et al. determined $K_m$ and $V_m$ for $X_3$-$X_8$ with a *Streptomyces* endo-xylanase. Biely et al. subsite-mapped their endo-xylanases, and reported only the ratio $V_m/K_m$. Therefore, for comparison purposes, the ratio $V_m/K_m$ is presented for all cases.

Table 25. Summary of kinetic parameters from endo-xylanases acting on pure xyloooligosaccharides

<table>
<thead>
<tr>
<th>DP</th>
<th>$K_m^{a}$</th>
<th>$V_m^{b}$</th>
<th>$V_m/K_m^{c}$</th>
<th>$V_m/K_m^{c}$</th>
<th>$V_m/K_m^{c}$</th>
<th>$V_m/K_m^{c}$</th>
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</thead>
<tbody>
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<td>0.0022</td>
<td>0.0011</td>
<td>0.7</td>
<td>0.0111</td>
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<td>4</td>
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<td>0.321</td>
<td>0.0752</td>
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<td>0.0775</td>
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<tr>
<td>5</td>
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<td>6</td>
<td>2.29</td>
<td>0.686</td>
<td>0.2996</td>
<td>4.79</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>7</td>
<td>1.14</td>
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<td>0.5360</td>
<td>7.47</td>
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<td>---</td>
</tr>
<tr>
<td>8</td>
<td>0.54</td>
<td>0.589</td>
<td>1.0907</td>
<td>7.68</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

$^{a}$mM.
$^{b}$Normalized to xylan as 1.
$^{c}$min/min' IU.
Table 25. (continued)

<table>
<thead>
<tr>
<th>DP</th>
<th>$K_m^a$</th>
<th>$V_{V_m}$</th>
<th>$V_{V_m}/K_m^c$</th>
<th>$K_m^d$</th>
<th>$V_{V_m}$</th>
<th>$V_{V_m}/K_m^e$</th>
<th>$K_m^f$</th>
<th>$V_{V_m}/K_m^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>22.1</td>
<td>0.0123</td>
<td>0.00056</td>
<td>28.3</td>
<td>0.135</td>
<td>0.0048</td>
<td>70</td>
<td>0.093</td>
</tr>
<tr>
<td>4</td>
<td>6.9</td>
<td>0.0654</td>
<td>0.0094</td>
<td>3.30</td>
<td>0.324</td>
<td>0.0982</td>
<td>14.6</td>
<td>0.826</td>
</tr>
<tr>
<td>5</td>
<td>18.5</td>
<td>0.2127</td>
<td>0.0115</td>
<td>1.40</td>
<td>0.342</td>
<td>0.2443</td>
<td>0.75</td>
<td>5.20</td>
</tr>
<tr>
<td>6</td>
<td>22.5</td>
<td>0.3435</td>
<td>0.0153</td>
<td>0.82</td>
<td>0.419</td>
<td>0.5110</td>
<td>0.60</td>
<td>6.80</td>
</tr>
<tr>
<td>7</td>
<td>23.6</td>
<td>0.5234</td>
<td>0.0222</td>
<td>0.62</td>
<td>0.572</td>
<td>0.9226</td>
<td>0.60</td>
<td>6.80</td>
</tr>
</tbody>
</table>

$^d$ umol/min/mg enz.

$^e$ mL/min/mg enz.

$^f$ Degree L/mol/min.

Purification of Endo-Xylanases from Chalnia

Analytical Procedures

Protein assay  Protein concentration was estimated using the Bio-Rad protein assay with bovine serum albumin (BSA) as a standard.

Determination of xylanase activity  Xylanase activity of the eluent fractions were determined at pH 6.0 and 50°C. Samples of 0.1 mL from each tube were mixed with 0.9 mL of a 2% nominal xylan solution. The xylan solution was prepared by suspending 2 g of oatspelt xylan (Koch-Light, Lot No. 90560) and 0.05 g NaN₃ in 100 mL of 0.025 M K₂HPO₄ buffer at pH 6.0. The suspension was stirred overnight at room temperature and centrifuged at 15,000 x g for 20 min to removed undissolved material. Another xylan solution was prepared in the same manner except it was a 5% solution from larchwood (Sigma, Lot No. 125C-00582) and was used for determining enzyme
activity.

Total xylanase activity from each purification step was determined at pH 6.0 and 50°C. A 5-mL Reacti-Vial was filled with 1.95 mL of 5% xylan solution, placed in a 50°C constant temperature water bath, and stirred continuously. The reaction was initiated by addition of 0.05 mL of enzyme solution. Samples of 0.2 mL were removed from the reaction mixture at certain time intervals and quenched by pipetting them into 0.8 mL of an aqueous 2% (by wt.) Na₂CO₃ solution. To this mixture was added 5 mL of ferricyanide reagent, 2 g Na₂CO₃, 0.5 g KCN, and 0.09 g K₃Fe(CN)₆ in 100 mL H₂O. The mixture was heated to 100°C for 10 min, cooled to room temperature and read at 420 nm with a Bausch & Lomb (Rochester, NY) 710 UV/VIS spectrophotometer.

Pyridylethylation Before SDS-PAGE the cysteine groups were derivatized with 4-vinylpyridine to avoid the formation of xylanase oligomers, which can occur when cysteines from other peptides form disulfide bonds. Also, the cysteines were derivatized prior to sequencing to improve detection.

Eleven μg of xylanase (35 IU) were dissolved in 1.0 mL of 5 mM NH₄NaCO₃ buffer, pH 8. Two μL of 2-mercaptoethanol were added and the reaction mixture was incubated at room temperature for 30 min, after which 6 μL of 4-vinylpyridine were added and the mixture was incubated an additional 90 min. The mixture was diluted with water to 1.5 mL and evaporated to dryness under vacuum by a Savant (Farmingdale, NY) Speedvac. The derivatized protein was resuspended in 100 μL water, submitted to SDS-PAGE, and sequenced by an Applied Bio-Systems (Foster City, CA) 420A
automated sequencer.

**Electrophoresis**  Horizontal ultra-thin SDS gel electrophoresis was performed with the Pharmacia (Piscataway, NJ) PhastSystem. The 0.5-mm thick gel was composed of a 13-mm long, 4.5% acrylamide stacking gel followed by a 32-mm long running gel, which consisted of an acrylamide gradient from 8 to 25%. Ten-μL samples of a 0.11 μg/μL solution of the derivatized and underivatized xylanase were added to 10 μL sample buffer as described by Laemmli and heated to 100°C for 5 min. One-μL samples were submitted to electrophoresis as outlined in Separation Technique File No. 120 for the PhastSystem. The gel was fixed and silver-stained automatically according to Heukeshoven and Dernick as outlined in Development Technique File No. 210.

Ultra-thin gel isoelectric focusing also was performed with the PhastSystem using a 40-mm long by 0.35-mm thick gel, which was composed of 5% acrylamide and focused from pH 3 to 9. One μL of 0.11 μg/μL solution containing 2% glycine was submitted to electrophoresis as outlined in Separation Technique File No. 200. The gel was fixed and silver-stained automatically according to Heukeshoven and Dernick as described in Development Technique File No. 210.

**Gel filtration chromatography**  The molecular weight of the xylanase was estimated by gel filtration chromatography using two different packing materials. The first method used Fractogel TSK HW-40S, which is a hydrophilic semi-rigid spherical gel manufactured from a vinyl-based polymer, with a fractionation range of 1,000 to 10,000 daltons. Samples were loaded onto a 26-mm i.d. x 845-mm long column and eluted with a 0.025
M Na₂HPO₄, pH 6.4, buffer at 0.39 mL/min. Protein elution was monitored continuously with an ISCO Model UA-5 absorbance monitor at 254 nm. Fractions were collected every 15 min, and xylanase activity was detected as described above. The second gel filtration chromatographic technique utilized Sephadex G-50-50, which is a dextran-based gel with a fractionation range of 1,500 to 30,000 daltons. Samples were loaded onto a 26-mm i.d. x 810-mm long column and eluted with a 0.025 M Na₂HPO₄ buffer, pH 6.4, at a flow rate of 0.35 mL/min. Once again protein was monitored at 254 nm, fractions were collected every 15 min, and xylanase activity was detected as described earlier.

Protein Purification

All steps were performed at 4°C unless stated otherwise.

The alcohol-precipitated xylanase, a gift from the National Chemical Laboratory, Pune, India, was dissolved in 0.025 M Na₂HPO₄ buffer, pH 6.4, at a concentration of either 0.05 or 0.08 g/mL and centrifuged at 48,000 x g for 20 min. The supernatant was loaded onto either a 26-mm i.d. x 200-mm long DEAE-cellulose column (Sigma, medium mesh) or a 26-mm i.d. x 850-mm long DEAE Fractogel-650S column, both equilibrated with 0.025 M Na₂HPO₄ buffer, pH 6.4. The flow rate in both cases was 1.0 mL/min and fractions were collected every 15 min. Xylanase activity eluted in the void volume, while contaminating protein and colored pigments were bound to the column. Xylanase activity was pooled and concentrated at an N₂ pressure of 50 psi using an Amicon ultrafiltration cell and a YM2 membrane, which has a molecular weight cutoff of 1000 daltons. The xylanase pool was reloaded onto either a 26-mm i.d. x 250-mm long or a 26-mm i.d. x
850-mm long DEAE-Fractogel column and eluted with the same buffer. Once again the xylanase activity eluted in the void volume and was concentrated by ultrafiltration. The DEAE columns were regenerated with 1 M NaCl in 0.025 M Na₂PO₄, pH 6.4.

The xylanase pool was further purified with a G-50-50 Sephadex gel filtration column as described above. Two xylanase peaks eluted and were concentrated by ultrafiltration. As the first pool accounted for only 20% of the total xylanase activity, it was not studied further.

The second pool, which contained the xylanase of lower molecular weight, was submitted to buffer exchange with 0.05 M sodium acetate buffer, pH 4.5, by ultrafiltration. It was then further purified by cation-exchange chromatography employing a 16-mm i.d. x 190-mm long CM-Trisacryl (LKB, Bromma, Sweden) column eluted with the same buffer at 0.5 mL/min until the absorbance returned to zero, indicating that all negatively charged proteins had eluted from the column. Then a linear gradient of 300 mL of either 0 to 0.2 or 0 to 0.5 M NaCl was started. The xylanase pool was collected, concentrated, and desalted by ultrafiltration.

Results and Discussion

Two similar purification schemes are summarized in Tables 26 and 27. In the first purification two different anionic exchange packing materials, DEAE-cellulose and DEAE-Fractogel, were used, while in the second, DEAE-Fractogel was used in two successive steps. DEAE-cellulose chromatography was used to remove the bulk of the contaminants, especially
Table 26. Summary of purification of *Chainia* xylanase starting with 2 g of alcohol precipitate

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total volume, mL</th>
<th>Total protein, mg</th>
<th>Total activity, IU</th>
<th>Activity yield, %</th>
<th>Specific activity, IU/mg</th>
<th>Cum. purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude after centrifugation</td>
<td>21.5</td>
<td>75.3</td>
<td>213</td>
<td>100</td>
<td>2.83</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>33.0</td>
<td>18.4</td>
<td>153</td>
<td>66.2</td>
<td>8.31</td>
<td>2.94</td>
</tr>
<tr>
<td>(200 mm length)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractogel TSK DEAE-650S chromatography (250 mm length)</td>
<td>23.5</td>
<td>1.83</td>
<td>168</td>
<td>78.8</td>
<td>91.5</td>
<td>32.3</td>
</tr>
<tr>
<td>Sephadex G-50 chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-I</td>
<td>15.0</td>
<td>0.66</td>
<td>28.0</td>
<td>13.1</td>
<td>42.5</td>
<td>15.0</td>
</tr>
<tr>
<td>X-II</td>
<td>33.0</td>
<td>0.079</td>
<td>109</td>
<td>51.2</td>
<td>1,400</td>
<td>390</td>
</tr>
<tr>
<td>CM-Trisacryl chromatography (0-0.5 M NaCl gradient)</td>
<td>6.6</td>
<td>---</td>
<td>54.2</td>
<td>25.4</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 27. Summary of purification of Chainia xylanase starting with 8.4 g of alcohol precipitate

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total volume, mL</th>
<th>Total protein, mg</th>
<th>Total activity, IU</th>
<th>Activity yield, %</th>
<th>Specific activity, IU/mg</th>
<th>Cum. purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude after centrifugation</td>
<td>163</td>
<td>316</td>
<td>866</td>
<td>100</td>
<td>2.74</td>
<td>1</td>
</tr>
<tr>
<td>Fractogel TSK DEAE-650S chromatography</td>
<td>24.5</td>
<td>4.21</td>
<td>664</td>
<td>74.8</td>
<td>157</td>
<td>57.3</td>
</tr>
<tr>
<td>(850 mm length)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractogel TSK DEAE-650S chromatography</td>
<td>16.5</td>
<td>0.76</td>
<td>191</td>
<td>21.6</td>
<td>251</td>
<td>92.0</td>
</tr>
<tr>
<td>(850 mm length)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G-50 chromatography</td>
<td>3.51</td>
<td>0.17</td>
<td>124</td>
<td>14.3</td>
<td>730</td>
<td>270</td>
</tr>
<tr>
<td>X-II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM-Trisacryl chromatography (0-0.2 NaCl gradient)</td>
<td>2.81</td>
<td>0.022</td>
<td>70.0</td>
<td>8.08</td>
<td>3,200</td>
<td>1,200</td>
</tr>
</tbody>
</table>
the colored pigments (the crude after centrifugation was a dark brown translucent liquid). The specific activity increased from 2.83 to 8.31 IU/mg after this step and then to 91.5 IU/mL after the DEAE-Fractogel step. In the second procedure the specific activity increased from 2.74 to 157 IU/mg after initial chromatography by DEAE-Fractogel (Table 27). When the xylanase pool was reloaded onto the same 850-mm long column, the further increase in specific activity, to 251 IU/mg, was not enough to warrant the 70% loss of enzyme activity, which was unexpectedly high for this step.

The next step was gel filtration with Sephadex G-50-50 (Fig. 18). Two peaks corresponding to 23,000 and 4,900 daltons contained xylanase activity, and were designated X-I and X-II, respectively. This step increased the specific activity of X-II from 91.5 to 1,400 IU/mg for the first purification and from 250 to 730 IU/mg for the second, and was very effective in separating X-I from X-II.

The final step of the purification of X-II was cation exchange chromatography with CM-Trisacryl, employing either of two different NaCl gradients at pH 4.5. The first separation, with a gradient from 0 to 0.5M, eluted one symmetric protein peak over a NaCl concentration from 0.0 to 0.16 M, which corresponded to two peaks of xylanase activity (Fig. 19). The second separation, with a gradient from 0 to 0.2 M, eluted two peaks of xylanase activity over a NaCl concentration from 0.08 to 0.14 M (Fig. 20), but a definite protein peak was not associated with enzyme activity. Both gradients were started after unbound proteins and a small amount of xylanase activity had finished eluting from the column. Although each
Figure 18. Elution pattern on Sephadex G-50-50 gel filtration
Figure 19. Elution pattern of X-II on CM-Trisacryl with a 0 to 0.5 M NaCl gradient
Figure 20. Elution pattern of X-II on CM-Trisacryl with a 0 to 0.2 M NaCl gradient
gradient eluted the xylanase peak over approximately the same range of NaCl concentrations, the 0 to 0.2 M gradient appears to have separated more protein away from the xylanase peak. Unfortunately a specific activity was not determined in the first purification, for which a 0.0 to 0.5 M gradient was used, to allow confirmation of this observation.

The molecular weight of the X-II preparation was determined by two different gel filtration chromatographic techniques. As mentioned above, X-II eluted from a Sephadex G-50-50 column at an elution volume equivalent to a molecular weight of 4,800 daltons (Fig. 21). With Fractogel HW-40S, X-II eluted at a volume equivalent to a molecular weight of 9,000 daltons (Fig. 21).

The molecular weight of X-II from CM-Trisacryl chromatography with the 0 to 0.2 M NaCl gradient was also determined by SDS-PAGE. The gel was constructed with a gradient from 8 to 25% polyacrylamide, and the system was standardized with molecular weight markers from 14,000 to 70,000 (Sigma, Cat. No. MW-SDS-70) and 2,500 to 17,000 daltons (Sigma, Cat. MW-SDS-17). The underivatized sample (Fig. 22) had a major band at 6000 daltons, two minor bands at 14,000 and 16,000 daltons and a medium-intensity band at 24,000 daltons. A 1 to 10 dilution of the same sample yielded two bands at approximately 6,000 and 7,000 daltons. The pyridylethylated sample had a major band at 8,000 daltons and four minor bands from 11,000 to 16,000 daltons. A 1 to 10 dilution of the this sample gave a major band at 8,000 daltons and a very faint band at 12,500 daltons. The derivatized sample did not have the 24,000-dalton band that was present in the underivatized sample, suggesting that it was an
Figure 21. Plot of MW versus $K_{av}$ for Sephadex G-50-50 (-----) and Fractogel HW-40S (-----) gel filtration chromatography. $K_{av} = (V_e - V_0)/(V_t - V_0)$ where $V_e$ is elution volume of the protein, $V_0$ is column void volume, and $V_t$ is total column volume. X-I (□) and X-II (○). Standards: (●), carbonic anhydrase (MW = 29,000), cytochrome C (MW = 12,400), and aprotinin (MW = 6,500).
Figure 22. Ultra-thin SDS-PAGE of X-II from column chromatography with a 0.0 to 0.2 M NaCl gradient. Lanes 1 and 8 are molecular weight markers, 14 to 70 kD. Lanes 2 and 7 are molecular weight markers, 2.5 to 16 kD. Lane 3 is a 1 to 10 dilution of the pyridylethylated sample. Lane 4 is a 1 to 10 dilution of underivatized sample. Lane 5 is the pyridylethylated sample. Lane 6 is the underivatized sample.
oligomer of the 6000-dalton band. Another interesting observation is that the major band of the pyridylethylated sample had a molecular weight 2,000 daltons larger than that of the underivatized sample. One explanation is that the major band of the underivatized sample may be more globular in shape, and thus would migrate further than its actual molecular weight would indicate. This would suggest that the protein was not completely denatured, which is also an explanation for why there are two bands in the 1 to 10 dilution of the underivatized sample. Another explanation is that a sufficient number of pyridylethyl groups have reacted with the protein to increase the molecular weight by 2,000. This seems unlikely, since according to amino acid analysis there are at the most two cysteine residues in the molecule.

Isoelectric focusing (not shown) of the xylanase sample from CM-Trisacryl column chromatography with a 0 to 0.2 M NaCl gradient yielded a major band at a pI greater than 8.9 and a minor band at pI 8.4.

Amino acid sequencing of X-II was attempted, but because of significant protein contamination and insufficient protein no conclusive results were obtained.

Another unusual aspect of the xylanase besides its low molecular weight was its high specific activity. The latter, for a yet incompletely purified sample, was estimated at 3,200 IU/mg, using a protein concentration measured with the Bio-Rad protein assay against BSA as a standard. If we assume the molecular weight as 8000 daltons, the rate coefficient $k_o$ is 424 s$^{-1}$. This value may be compared with the value of other exo- and endo-hydrolases (Table 28).
Table 28. Rate coefficient, $k_0$, for different exo- and endo-hydrolases

<table>
<thead>
<tr>
<th>Source and enzyme</th>
<th>$k_0$, s$^{-1}$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis $\alpha$-amylase</td>
<td>818</td>
<td>141</td>
</tr>
<tr>
<td>Chainia endo-xylanase</td>
<td>424</td>
<td>This work</td>
</tr>
<tr>
<td>Wheat bran $\beta$-amylase</td>
<td>262</td>
<td>142</td>
</tr>
<tr>
<td>Aspergillus niger endo-xylanase</td>
<td>112</td>
<td>143</td>
</tr>
<tr>
<td>Rhizopus delemar glucoamylase</td>
<td>110</td>
<td>41</td>
</tr>
<tr>
<td>Crytococcus albidus endo-xylanase</td>
<td>38.2</td>
<td>144</td>
</tr>
<tr>
<td>Trichosporan cutaneum endo-xylanase</td>
<td>27.8</td>
<td>145</td>
</tr>
<tr>
<td>Bacillus sp. 11-1S endo-xylanase</td>
<td>4.93</td>
<td>146</td>
</tr>
</tbody>
</table>

The value $k_0$ for the Chainia xylanase is similar to those found for other exo- and endo-hydrolases, but is high compared to other xylanases. This, along with its low molecular weight appears to make X-II a very unusual enzyme. Until now the lowest known molecular weight of an endo-xylanase is 13,000 daltons$^{110}$, much higher than the 5,000-9,000 daltons of this endo-xylanase from Chainia.

Conclusions and Recommendations

The actinomycete Chainia produces two endo-xylanases, X-I and X-II, with molecular weights of 23,000 and 5,000 to 9,000 daltons, respectively. X-II was purified by four chromatographic steps, but not to homogeneity. The final step, CM-Trisacryl chromatography, split X-II into two closely spaced peaks. X-II has a high specific activity compared to other xylanases and appears to have an isoelectric point at or above pH 8.9.
Amino acid sequencing of the protein was attempted, but because of contamination by other proteins and insufficient protein, it was not successful.

The small size and high specific activity make this endo-xylanase very unusual. To better understand the action of this enzyme, it would be advantageous to have its three-dimensional structure. This would require the enzyme in crystalline form, which in turn requires it to be highly purified. The following are suggested improvements to the previously discussed purification scheme:

1) Optimize conditions for CM-Trisacryl column chromatography by investigating shallower salt gradients, to determine if the two peaks with xylanase activity obtained from X-II can be separated.

2) Determine the pI of the xylanase, which will then in turn allow the enzyme to be purified by preparative isoelectric focusing or purification by semi-preparative SDS. These techniques following CM-Trisacryl column chromatography could purify the enzyme well enough for sequencing.

3) One of the advantages of X-II has been its high specific activity, which has allowed its detection by activity assays when its protein concentration was too low to be measured by UV absorbance. Another way of detecting the enzyme would be to produce an antibody specific to it. The antibody would allow the xylanase to be detected when it was no longer active, such as during semi-preparative SDS and reverse phase chromatography. The antibody might also be used for affinity chromatography, and would possibly allow purification of the enzyme in a single step. It could also be used to determine if there is any
cross-reactivity between X-I and X-II. This information would help to determine if the two xylanases are from different genes, if X-II is the result of proteolytic action on X-I, or if X-I is an aggregate of several X-II units.

4) Determination of the sequence is necessary for an exact molecular weight. Also, knowing the sequence would permit the synthesis of c-DNA so the xylanase could be produced at higher yields by recombinant DNA techniques.

Acknowledgments

The author would like to thank Dr. Louisa Tabatabai of the National Animal Disease Center, Ames, for her expertise in protein chemistry and her attempted sequencing of X-II, the National Chemical Laboratory of Pune, India, for supplying the crude xylanase used in this work, and the International Paper Company for its financial support.
APPENDIX I.

DERIVATION OF SUBSITE MAPPING MODEL

The following is a derivation of the subsite mapping model proposed by Hiromi\(^\text{66,67}\). The reaction scheme below is suggested for an enzyme, E, capable of forming productive, \( p \), and nonproductive, \( q \), complexes with an \( n \)-mer substrate \( S_n \):

\[
\begin{align*}
\text{E} + \text{S}_n &\rightarrow \text{ES}_{n,p} \\
\text{E} + \text{S}_n &\rightarrow \text{ES}_{n,q}
\end{align*}
\]

\[
\begin{align*}
K_{n,p} &\rightarrow \text{ES}_{n,p} & k_{\text{int}} &\rightarrow \text{E} + \text{P} \\
K_{n,q} &\rightarrow \text{ES}_{n,q}
\end{align*}
\]  

(1)

\( K_{n,p} \) and \( K_{n,q} \) are association constants and are defined by

\[
K_{n,j} = \frac{(ES_{n,j})}{(E)(S_n)}
\]

(2)

where \( j \) refers to the binding mode.

The standard free energy change \( \Delta G_{n,j} \) for the binding equilibrium is

\[
\Delta G_{n,j} = -RT\ln K_{n,j}
\]

(3)

where \( R \) and \( T \) are the gas constant and the absolute temperature, respectively. The standard free energy change \( \Delta G_{n,j} \) or the standard
affinity $-\Delta G_{n,j}$ can be divided into unitary and cratic parts\textsuperscript{147,148}. The standard affinity $-\Delta G_{n,j}$ is further defined by eq. 4:

$$-\Delta G_{n,j} = B_{n,j} - \Delta G_{\text{mix}}$$

(4)

where $-\Delta G_{\text{mix}}$ is termed the "cratic" part of the standard free affinity and arises from the nonspecific random mixing of solute species with solvent. It represents the difference in the free energy of mixing of two species, $E + S_n$, with water, and that of mixing a single species, $ES_n$, with water. For dilute solutions this is approximated by eq. 5:

$$-\Delta G_{\text{mix}} = RT\ln(1/55.6)$$

(5)

$B_{n,j}$ is the unitary part of the standard affinity and represents the net specific interaction between $S_n$ and $E$. $B_{n,j}$ as a first approximation is defined as the sum of the individual subsites that are covered in a certain binding mode:

$$B_{n,j} = \sum_{i}^{\text{cov.}} \Delta A_i$$

(6)

By combining eqs. 3 through 6 we arrive at eq. 7:

$$RT\ln K_{n,j} = B_{n,j} - RT\ln(1/55.6)$$

(7)

or
The Michaelis-Menten equation for $S_n$ is

$$v = \frac{V'(S_n)}{K_m + S_n}$$  \hspace{1cm} (9)$$

We can express $K_m$ and $V_m$ in terms of productive and nonproductive binding modes:\textsuperscript{149,150}:

$$\frac{1}{K_m} \sum_{j} K_{n,j} = \sum_{p} K_{n,p} + \sum_{q} K_{n,q}$$  \hspace{1cm} (10)$$

and

$$k_{o} = \frac{V_m}{e_o} = \frac{k_{int} \sum_{p} K_{n,p}}{\sum_{p} K_{n,p} + \sum_{q} K_{n,q}}$$  \hspace{1cm} (11)$$

and hence

$$\frac{k_{o}}{K_m} = \frac{V_m}{K_{m,e_o}} = k_{int} \sum_{p} K_{n,p}$$  \hspace{1cm} (12)$$
For glucoamylase, where \( p = 1 \), eq. 12 reduces to

\[
\frac{k_o}{K_m} = k_{\text{int}} K_n,1
\]  

(13)

Substituting eq. 8 with \( j = 1 \) into eq. 13 yields

\[
\frac{k_o}{K_m} = 0.018 k_{\text{int}} \exp(\Sigma A_1/RT)_{n,1}
\]  

(15)

This equation is used to evaluate \( A_3 - A_7 \) by solving eq. 14 for the \( n \) and \( n-1 \) cases when \( n \geq 3 \), as seen by eq. 15:

\[
\ln\left(\frac{k_o}{K_m}\right)_n - \ln\left(\frac{k_o}{K_m}\right)_{n-1} = \left(\Sigma A_1 - \Sigma A_1\right)/RT = A_n/RT
\]  

(15)

To evaluate \( A_1 \) and \( k_{\text{int}} \), it is assumed that only one productive (\( j = 1 \)) and one nonproductive (\( j = 2 \)) binding mode dominate all other binding modes. This assumption is supported by the fact that \( B_{2,\text{app}} \), which is defined below:

\[
B_{2,\text{app}} = RT\ln(1/K_m) + 10.3 \text{ kJ/mole} = 27.6 \text{ kJ/mol}
\]  

(16)

is appreciably higher than \( A_3 \), 6.15 kJ/mol, implying that either \( A_1 \) or \( A_2 \), or at least their sum, is greater than \( A_3 \). Thus, as a good approximation
it is safe to assume that substrate molecules will preferentially bind in either the \( j = 1 \) or \( j = 2 \) mode. Thus

\[
\frac{1}{K_m} = \sum_{n} K_{n,j} = K_{n,1} + K_{n,2} \quad (17)
\]

Substituting eq. 17 into eq. 11 for \( p = 1 \) gives eq. 18:

\[
k_o = \frac{k_{int}}{K_{n,1} + K_{n,2}} \quad (18)
\]

Eq. 18 can be written with eq. 7 as follows:

\[
\frac{k_{int}}{(k_o)n} - 1 = \frac{K_{n,2}}{K_{n,1}} = \exp(B_{n,2} - B_{n,1}) \quad (19)
\]

As seen from eq. 6

\[
B_{n,2} - B_{n,1} = A_{n+1} - A_1 \quad (20)
\]

Thus eq. 21 is defined as

\[
\exp(A_{n+1}/RT) = \left(\frac{k_{int}}{(k_o)n} - 1\right) \exp(A_1/RT) \quad (21)
\]
If \( \exp(A_{n+1}/RT) \) vs. \((1/k_0)^n\) is plotted, the vertical intercept is 
\(-\exp(A_1/RT)\), while the horizontal intercept is \(1/k_{\text{int}}\).

To solve for \(A_2\) we make use of eq. 17 as follows:

\[
\frac{1}{K_m} = K_{n,1} + K_{n,2} = 0.018\exp(A_2/RT) \exp[(A_1 + A_3 + \ldots + A_n)/RT] \\
+ 0.018\exp(A_2/RT) \exp[(A_3 + \ldots + A_n + A_{n+1})/RT]
\]

This equation can be solved for \(A_2\), since the other \(A_i\) values have already
been obtained. The values calculated for substrates with \(n = 2, \ldots, 6\) are
averaged to give \(A_2\).

Knowing the values of \(A_i\), it is now possible to calculate the rate
parameters \(K_m\) and \(k_0\). By substituting eq. 8 into eq. 17, \(K_m\) can be
determined (eq. 23):

\[
\frac{1}{(K_m)^n} = \frac{1}{0.018\exp[(\Sigma A_i)/RT]} + \frac{1}{0.018\exp[(\Sigma A_i)/RT]} \\
1^n + 1^n + 2^n
\]

while \(k_0\) is determined by substituting eq. 8 into eq. 18 to give eq. 24:

\[
k_{\text{int}} = \frac{0.018\exp[(\Sigma A_i)/RT]}{2^{n+1}} \\
k_0 = \frac{0.018\exp[(\Sigma A_i)/RT]}{2^n} \\
\frac{0.018\exp[(\Sigma A_i)/RT]}{2^{n+1}} + \frac{0.018\exp[(\Sigma A_i)/RT]}{2^n}
\]
APPENDIX II.

PRODUCTION AND PURIFICATION OF SUBSTRATES FOR GLUCOAMYLASE KINETICS

Introduction

This appendix describes the production of nigerose, the isomalto-oligosaccharides, and α,β-trehalose, all of which were needed for the hydrolytic rate studies of glucoamylase. The first two methods, the production of nigerose and the isomaltoooligosaccharides, were taken from the literature. An account of the third method, the production and purification of α,β-trehalose, has been accepted for publication in Carbohydrate Research and was a joint project between Dr. Frederick W. Parrish from the Southern Regional Research Center, Department of Agriculture, New Orleans, Louisiana, and myself. Dr. Parrish performed the chemical synthesis, purification by crystallization, and determination of melting points and optical rotation of α,β-trehalose. I performed the analytical and preparative chromatography and also determined the melting point and optical rotation.

Production and Purification of Nigerose

Introduction

The production of nigerose was accomplished in four steps. The first involved the isolation of nigeran from the cell wall of Aspergillus aculeatus as described by Gold et al.\textsuperscript{151} The second required the
fermentation of Pencillium melinii for the production of a mycodextranase as outlined by Tung et al. The third step was the enzymatic hydrolysis of nigeran into nigerose and nigerotetraose as described by Tung and Nordin. Finally, the nigerose was purified by charcoal and gel filtration chromatography.

Production of Nigeran

A. aculeatus QM 332, from the U.S. Army Natick Research and Development Laboratories, was grown aerobically for 48 h at 25°C in two 500-mL culture flasks with 200 mL of a synthetic medium as described elsewhere, except that 1 g/L of yeast extract (Difco Laboratories, Detroit, MI) was added. Two 7-L New Brunswick (Edison, NJ) fermentors with 5 L of the same medium were inoculated with 500 mL each of the starter culture, and the cells were allowed to grow at 25°C for 48 h. The mycelia were removed and washed with sterile water and resuspended in the same medium minus NH₄Cl, the inorganic nitrogen source. The resuspended cell mass was allowed to ferment for 5 days until the glucose, initially 4% by weight, was exhausted. The cell mass was harvested, washed, and then heated for 30 min at 100°C in water at a concentration of 100 g wet cells/L. The suspension was then submitted to suction filtration through a Whatman #4 filter. The filtrate was maintained at 12°C for several hours, resulting in the precipitation of nigeran. The nigeran, which is soluble at 100°C, was collected by centrifugation and precipitated three more times. The total yield from both fermentations was 36.59 g.
Production of Mycodextranase

P. melinii, QM 1931, from the U.S. Army Natick Research and Development Laboratories, was grown aerobically in a 500-mL culture flask containing 150 mL medium with 2.25% glucose (w/v) at 25°C. After two days all 150 mL were used to inoculate a 2-L Fernbach flask containing 1 L of the same medium, but with a glucose concentration of 1%. After 24 h of growth at 25°C, 1 g of nigeran in 100 mL of water was autoclaved, cooled, and added to the broth. The fermentation was manually controlled at pH 7.0 and stopped after 144 h, when the activity was 0.33 U/mL. Mycodextranase activity was determined as described elsewhere, with a unit being defined as the amount of enzyme needed to release 1 μmol of reducing power equivalent to glucose per minute. The enzyme was purified according to the procedure outlined by Tung et al. The culture medium was filtered through four layers of cheesecloth to remove the bulk of the cell mass. Two volumes of 95% ethanol at -20°C were gradually added to the filtrate, and the solution was allowed to stand overnight at 4°C. The supernatant was siphoned off, and the precipitate was collected by centrifugation at 5000 x g for 10 min at 4°C and redissolved in 20 mL of 0.02 M citrate-phosphate buffer, pH 4.2. Any insoluble material was removed by centrifugation. The 20 mL were loaded onto a 26-mm i.d. x 500-mm long SP-Sephadex C-25 column and eluted with 0.02 M citrate-phosphate buffer at 0.76 mL/min. The enzyme eluted in the void volume, with contaminating protein remaining adsorbed on the column. The pooled fractions were ultrafiltered to a volume of 5 mL. Buffer exchange was performed by diluting the concentrate to 50 mL with 0.02 M phosphate buffer, pH 7.1,
and ultrafiltering to 5 mL. This was repeated two more times. The 5 mL were loaded onto a 26-mm i.d. x 800-mm long Fractogel DEAE-650S column and eluted with 0.96 mL/min of a 2-L linear NaCl gradient, increasing from 0 to 1 M, in 0.02 M phosphate buffer, pH 7.1. The mycodextranase, which eluted at eluent volumes from 426 mL to 532 mL, was concentrated by ultrafiltration and submitted to buffer exchange by ultrafiltration with 0.05 M acetate buffer, pH 4.5, to a final volume of 40 mL. The amount of enzyme recovered from purification was 294 U, a yield of 81%.

**Enzymatic Hydrolysis of Nigeran**

Ten grams nigeran were suspended in 800 mL of 0.05 M acetate buffer, pH 4.5, and 3200 mL of water and were boiled until the solution was clear. The solution was allowed to cool to 50°C, and 50 U of mycodextranase were added. The reaction continued at 50°C until the solution was no longer turbid. The hydrolyzate was concentrated at 45°C under vacuum to a volume of 50 mL and centrifuged at 10,000 x g for 30 min to remove any residual nigeran. The concentrate was loaded on a 89-mm i.d. x 610-mm long column packed with a mixture of 50% (w/w) charcoal powder (Darco G-60, MCB, Cincinnati, OH) and 50% diatomaceous earth (Celite 560, Manville, Denver, CO) as described by Meagher. The charcoal column was eluted with a 36-L linear tert-butanol gradient from 0 to 3.5% (v/v) and then another 36-L gradient from 4 to 9% (v/v) at a flow rate of 430 mL/h. The hydrolysis and chromatography procedure was repeated two more times. Each enzyme hydrolysis yielded approximately 5 g nigerotetraose and 1.3 g nigerose. The total yield of nigerose for all three reactions was 4.0 g,
with a purity of 94-97%.

The nigerose was further purified by gel filtration using a 26-mm i.d. x 170-mm long column packed with Fractogel HW-40S and eluted at room temperature with water at 0.33 mL/min, as outlined by Nakakuki and Kainuma\textsuperscript{155}. Detection was achieved with a Waters R401 refractive index detector (Waters Associates, Milford, MA), and the sample load was 250 mg per run. After 16 runs, 2.34 g of nigerose were recovered, a yield of 58%.

**Production and Purification of Isomaltooligosaccharides**

**Introduction**

The production of isomaltooligosaccharides was performed by acid hydrolysis of dextran as outlined by Robyt\textsuperscript{156} followed by charcoal-Celite chromatography and gel filtration chromatography.

**Acid Hydrolysis of Dextran**

Forty grams of dextran (Sigma, industrial grade, average molecular weight 5-40 x 10^6 daltons) were suspended in 600 mL of water and brought to a boil. Once the dextran was dissolved, 120 mL of trifluoroacetic acid were added. The mixture was allowed to react for 1 h at 105°C, followed by neutralization with 6 M NaOH to pH 6.5 and centrifugation at 16,000 x g for 30 min to remove any solids. The supernatant was loaded on a 89-mm i.d. x 610-mm long charcoal-Celite column as described elsewhere\textsuperscript{154}, and was eluted with a linear n-butanol gradient from 0-2% over 40 L at a flow rate of 600 mL/h. Carbohydrate was detected by the phenol-sulfuric acid
The gradient was too steep and provided inadequate separation of the oligosaccharides, so the first eight pools were concentrated to dryness, redissolved in water, loaded onto the same column, and eluted with a 0-1% linear gradient of n-butanol. All other conditions were the same. This gradient provided good separation and yielded 1.06 g of isomaltotetraose, 1.29 g of isomaltopentaose, 1.18 g of isomaltohexaose, and 0.94 g of isomaltoheptaose. Isomaltotetraose and isomaltopentaose were further purified by gel filtration using Fractogel HW-40S, as described above, to 98% purity as determined by HPLC using a Bio-Rad HPX-42A strong acid ion exchange resin column in the Ag⁺ form. The HPLC column was 300 mm long and 7.8 mm i.d. and was maintained at 85°C; water flow was 0.4 mL/min, and injections were 20 μL. Samples were detected using an Erma ERC-7510 refractive index detector. Isomaltohexaose and isomaltoheptaose were already 98% pure after charcoal-Celite chromatography.

Production and Purification of α,β-Trehalose

Introduction

α,β-Trehalose [O-α-D-glucopyranosyl-(1→1)-β-D-glucopyranoside] was needed in order to confirm the identity of one of the reversion products of glucose catalyzed by glucoamylase, to determine the kinetics of its conversion to glucose with glucoamylase, and to examine its separation from α,α- and β,β-trehalose, as well as from other disaccharides, by HPLC. Mother liquors from a Koenigs-Knorr preparation of β,β-trehalose octaacetate were available, and since these are known to
contain α,β-trehalose octaacetate\textsuperscript{160}, it was decided to attempt the isolation of α,β-trehalose from this source. The use of chromatographic procedures for the separation and analysis of the various trehalose forms facilitated the isolation of unequivocally pure α,β-trehalose and the measurement of an accurate optical rotation and melting point for it.

**Experimental Procedures**

Thin layer chromatography (TLC) of acetylated compounds was performed on Brinkmann (Westbury, NY) silica gel with a mixture of diethyl ether and petroleum ether (2:1); detection of separated compounds was accomplished by spraying the TLC plates with 50% (v/v) aqueous sulfuric acid followed by heating at 110°C. Capillary gas chromatography of trimethylsilyl derivatives of sugars were performed as described by previous authors\textsuperscript{162}. Effluent from chromatographic separations was monitored for D-glucose with 3,5-dinitrosalicylic acid reagent\textsuperscript{163}, and for total carbohydrate with phenol-sulfuric acid\textsuperscript{157}. Optical rotation data were obtained with 2% solutions at 589 nm in a 1-mm tube with a Perkin-Elmer Model 241 automatic polarimeter. Melting points were determined using a Du Pont 1090 thermal analyzer. Carbon and \textsuperscript{1}H nuclear magnetic resonance (n.m.r.) were performed on α,β-trehalose dissolved in methanol-\textsubscript{d}\textsubscript{4} using a 300-MHz Nicolet NT300 n.m.r. spectrometer. Chemical shifts were based on tetramethylsilane. Samples were dried under vacuum and redissolved in methanol-\textsubscript{d}\textsubscript{4} three times.

2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide (1) (244 g, 0.60 mol) was prepared in crystalline form from anhydrous D-glucose (125 g, 0.69 mol) as described by Lemieux\textsuperscript{164} except that the recrystallization
step was omitted. 2,3,4,6-Tetra-O-acetyl-β-D-glucose (2) (87 g, 0.25 mol) was prepared from 1 (135 g, 0.33 mol) using active silver carbonate. Crude 1 (20.5 g, 0.05 mol) and 2 (17.4 g, 0.05 mol) were reacted with mercuric cyanide (12.5 g, 0.05 mol) to eventually give crystalline β,β-trehalose octaacetate (3) (10.1 g, 0.015 mol). The ether was evaporated from the mother liquor of the crystallization of 3 to give a syrup (27.5 g). The syrup was deacetylated with sodium methoxide (75 mg, 0.0014 mol) in 500 mL methanol at reflux for 30 min. The solution was concentrated in vacuo to a syrup which was dissolved in 100 mL water. The solution was applied to a 500 x 50 mm column of Fisher coconut charcoal (400 g, 50-200 mesh), and elution was performed with 2% (v/v) aqueous ethanol (2 L) followed by 10% ethanol (2 L). The 10% (v/v) aqueous ethanol effluent was concentrated at 40°C in vacuo to about 100 mL, and the concentrated solution was applied to a 200 x 20 mm column of 100-200 mesh Dowex-1 (OH⁻) resin which was eluted with 150 mL water. The nonreducing, aqueous solution that resulted was evaporated to a syrup at 40°C in vacuo, and the syrup was dissolved in 15 mL methanol. After being kept 16 h at -20°C, crystals (2.9 g, 0.0083 mol) of α,β-trehalose were obtained. A recrystallization yielded purer product. Alternatively, dried Dowex-1 eluent (250 mg) was further purified by passage through a 280 x 26 mm column of -400 mesh Bio-Rad Aminex AG1-X4 (OH⁻), which was eluted with water at 3 mL/min. The α,β-trehalose peak was pooled and concentrated at 50°C in vacuo to 100 mL using a 3-L single effect evaporator and was then freeze-dried to give 150 mg. Collection of the β,β-trehalose peak yielded 14 mg.
Results and Discussion

Compound 3 was obtained with a yield of 30%, which is comparable to the 31.5% yield reported previously\(^{160,162}\). Examination by TLC of the mother liquors from the preparation of 3 showed the presence of two compounds; the faster moving compound (R\(_f\) 0.67) had the same mobility as compound 2 while the slower moving component (R\(_f\) 0.40) had a mobility similar to that of compound 3 (R\(_f\) 0.47). The deacetylated mixture was analyzed by chromatographic\(^{162}\) and spectrophotometric techniques\(^{157,163}\) and contained 65% glucose, 2% \(\alpha,\alpha\)-trehalose, 31% \(\alpha,\beta\)-trehalose, and 2% \(\beta,\beta\)-trehalose. After removal of the glucose by Dowex-1 the mixture contained 3.8% \(\alpha,\alpha\)-trehalose, 81.8% \(\alpha,\beta\)-trehalose, 12.0% \(\beta,\beta\)-trehalose, and 2.4% unknown (Fig. 23). Thus, the composition of the reaction mixture prior to the isolation of crystalline 3 was roughly 45% 1 and 2, 1% \(\alpha,\alpha\)-trehalose octaacetate, 22% \(\alpha,\beta\)-trehalose octaacetate, and 32% \(\beta,\beta\)-trehalose octaacetate. Good agreement was found in the analytical data from four individual experiments.

The efficient separation of 3 from the corresponding \(\alpha,\beta\)-isomer by crystallization from ethereal solution is comparable to that of methyl tetra-O-acetyl-\(\beta\)-D-mannopyranoside from its \(\alpha\)-anomer\(^{167}\).

Helferich and Weis\(^{160}\) recognized that the mother liquor from the crystallization of 3 contained a considerable amount of compound 2, which they chose to remove as the benzylamine adduct prior to isolation of \(\alpha,\beta\)-trehalose octaacetate. We chose instead to remove D-glucose from the deacetylated mixture and to recover the trehaloses by chromatographic separation on coconut charcoal; this procedure was rapid and efficient.
Figure 23. Capillary gas chromatograms of trimethylsilylated samples of A) trehalose standard, B) trehalose mixture before anion exchange column chromatography, C) α,β-trehalose pool from anion exchange column chromatography, D) β,β-trehalose pool from anion exchange column chromatography. Chromatography was conducted on a 0.256-mm i.d. x 30-m long fused silica capillary column coated with DB-5 liquid phase using 220 mm/s He carrier gas. Oven temperature was 217°C until 31 min, when it was increased at 10°C/min to 250°C. Injector temperature was 270°C, detector temperature was 280°C, and sample size was 1.5 μL. Numerals denote min after injection.
Small amounts of D-glucose which contaminated the α,β-trehalose mixture were removed by passage through Dowex-1 (OH\(^-\)). In this manner, overall α,β-trehalose recovery after crystallization was 17%, higher than the 9% yield of Helferich and Weis\(^{160}\), but lower than the 40% yield of Sharp and Stacy\(^{168}\) using tetra-0-acetyl-β-D-glucopyranosyl fluoride and the 26% yield obtained by Marquez and Sotillo\(^{169}\) using 1, pentaacetyl-β-D-glucopyranoside instead of 2, and mercuric bromide instead of mercuric cyanide. The latter two articles did not report the amounts of α,α- and β,β-isomers which resulted under their conditions. Micheel and Hagel\(^{170}\) also obtained 3 and its α,β-isomer in yields of 9.7% and 18%, respectively, from 1 with mercuric cyanide in moist acetone.

α,β-Trehalose could also be separated from α,α- and β,β-trehalose and other impurities in the effluent from the Dowex-1 column by using a column filled with Aminex AG1-X4 (OH\(^-\)) resin (Fig. 24). Chittenden\(^{171}\) had previously separated α,α- from α,β-trehalose with Dowex-1-X2 (OH\(^-\)) (200-400 mesh), while Onishi and Karasawa\(^{172}\) separated all three trehaloses with Dowex-1-X4 (OH\(^-\)) resin (200-400 mesh), both using water as the column eluent. The order of elution reported by the latter was α,α-trehalose followed by α,β- and then β,β-trehalose, while Chittenden reported an elution order of α,α- and then α,β-trehalose. The elution order observed in this work is different than that of Onishi and Karasawa, with β,β-trehalose eluting before α,β-trehalose (Fig. 24). Liquid chromatography purified α,β- and β,β-trehalose to 99.5% or greater (Fig. 23).

The yield of α,β-trehalose using only chromatographic techniques was 16%, compared to 17% by crystallization. Crystallization is the better
Figure 24. Elution pattern of a 250-mg trehalose mixture from a 26-mm i.d. x 280-mm long AG1-X4 (OH⁻) anion exchange column eluted with 3 ml/min water. Hatching indicates pools of α,β- and β,β-trehalose.
choice for isolating gram quantities while column chromatography is very effective for smaller amounts.

Physical constants of $\alpha,\beta$-trehalose prepared by a variety of methods are considerably different. The product obtained by crystallization had m.p. $147^\circ C$ and $[\alpha]^2_{D2} +78^0$ (c 2, $H_2O$), while that prepared by liquid chromatography had m.p. $149^\circ C$ and $[\alpha]^2_{D2} +83.5^0$ (c 2, $H_2O$). These values differ from m.p. $80^\circ C$ and $[\alpha]^18_{D} +70^0$ (c 0.04, $H_2O$) of Sharp and Stacey$^{168}$, and from the values of m.p. $178-179^\circ C$ and $[\alpha]^2_{D} +136^0$ (c 5, $H_2O$) reported by Marquez and Sotillo$^{169}$. The optical rotation value of Marquez and Sotillo is approximately twice the $+70^0$ theoretically predicted by Hudson$^{173}$, while that of Sharp and Stacey is the same. When the supernatant form the crystallization of 3, which contained all three trehalose forms (Fig. 23B), was deacetylated, the glucose removed by charcoal and Dowex-1 chromatography, and a single instead of a double crystallization preformed, the product had m.p. $80^\circ C$ (decomp.) and $[\alpha]_{D2}^{20} +69.9$, similar to the values of Sharp and Stacy, suggesting that their $\alpha,\beta$-trehalose preparation may have been contaminated with the other trehalose forms.

The melting points and rotations of 3 [m.p. $181^\circ C$, $[\alpha]_{D}^{22} -17.5^0$ (c 2, CHCl$_3$)], and of $\beta,\beta$-trehalose [m.p. $138-140^\circ C$, $[\alpha]_{D}^{22} -40^0$ (c 2, $H_2O$)] were in good agreement with values published earlier$^{174}$.

$^{13}C$ n.m.r of the chromatographically purified $\alpha,\beta$-trehalose dissolved in methanol-$d_4$ yields 12 peaks, independent of those for methanol (Fig. 25). The two peaks of interest have chemical shifts of 105.18 and 101.99 ppm and represent $C_1$ atoms linked equatorially and axially to glycosidic oxygen, respectively. The eight peaks from 78.4 through 71.7 ppm
Figure 25. $^{13}$C n.m.r. spectrum of chromatographically purified $\alpha,\beta$-trehalose dissolved in methanol-$d_4$. 
represent the C-2 through C-5 atoms of the two glycosyl residues, while the two peaks at 63.23 and 63.08 ppm correspond to the two C-6 atoms.

$^1$H n.m.r. yields two doublets of nearly equal height and area at 5.09 and 4.45 ppm, corresponding to axial and equatorial protons attached to the two C-1 atoms (Fig. 26). The peaks at 4.90 and 4.92 ppm are attributed to glucosyl hydroxyl protons only partially exchanged with deuterium. The large signal at 3.3 ppm is assigned to the incompletely deuterated methyl group of the methanol-d$_4$. Integration of all other peaks indicates that there are a total of 12 protons attached to the C-2 through C-6 atoms of the two glucosyl residues.

The n.m.r. spectra of the purified preparation, as well as the knowledge that it is nonreducing and is composed exclusively of glucosyl residues, unequivocally establishes it as $\alpha,\beta$-trehalose.

Since analysis showed the presence of a considerable amount of 2, either unreacted material or that derived from 1, in the mother liquor from crystallization of 3, further study of the reaction using chromatographic analysis$^{158,162}$ of the products could lead to improved yields of both 3 and its $\alpha,\beta$-isomer. However, from results reported by Garegg et al.$^{175}$ on Koenigs-Knorr glycosylations, the use of mercuric bromide in combination with mercuric cyanide, instead of mercuric cyanide alone, would not be expected to alter significantly the relative amounts of the isomeric forms of the products.
Figure 26. $^1$H n.m.r. spectrum of chromatographically purified $\alpha,\beta$-trehalose dissolved in methanol-$d_4$. 
Acknowledgments

We thank Mr. Steven Eklund for conducting the $^{13}\text{C}$ and $^1\text{H}$ n.m.r. measurements and Professor Walter Trahanovsky for helping to interpret them.
APPENDIX III.

DISSOCIATION MODEL FOR AN ENZYME ASSUMING DIBASIC BEHAVIOR

The following model describes the effect of pH on the kinetic parameters of an enzyme, assuming the enzyme behaves as a dibasic acid. The derivation will follow that originally developed by Michaelis and Davidsohn\(^9\), further developed by Dixon and Webb\(^9\), and described by Cornish-Bowden\(^1\).

The pH behavior of the active site of an enzyme can be described as a dibasic acid, as shown in eq. 1:

\[
\begin{align*}
\text{HEH} & \quad \leftrightarrow \quad \text{EH}^- + \text{H}^+ \\
& \quad \leftrightarrow \quad \text{E}^2^- + 2\text{H}^+
\end{align*}
\]

(1)

The enzyme can be represented as a dibasic acid, HEH, with two non-identical acidic groups. Assuming that all species are in equilibrium, the concentration of each of the species can be expressed in terms of the hydrogen ion concentration, \(\text{H}^+\), and the dissociation constants:

\[
\begin{align*}
[\text{HE}^-] &= [\text{HEH}]K_{11}/[\text{H}] \\
[\text{EH}^-] &= [\text{HEH}]K_{12}/[\text{H}] \\
[\text{E}^2^-] &= [\text{HEH}]K_{11}K_{22}/[\text{H}]^2 = [\text{HEH}]K_{12}K_{21}/[\text{H}]^2
\end{align*}
\]

(2) \hspace{2cm} (3) \hspace{2cm} (4)
Examination of eq. 4 indicates that $K_{11}K_{22} = K_{12}K_{21}$.

If the total enzyme concentration, $e_0$, is the sum of all four species, [HEH], [EH⁻], [HE⁻], and [E²⁻], then

$$[\text{HEH}] = \frac{e_0}{1 + \frac{K_{11} + K_{12}}{H} + \frac{K_{11}K_{22}}{H^2}} \quad (5)$$

$$[\text{EH}^-] = \frac{e_0K_{11}/H}{1 + \frac{K_{11} + K_{12}}{H} + \frac{K_{11}K_{22}}{H^2}} \quad (6)$$

$$[\text{HE}^-] = \frac{e_0K_{12}/H}{1 + \frac{K_{11} + K_{12}}{H} + \frac{K_{11}K_{22}}{H^2}} \quad (7)$$

$$[\text{E}^2^-] = \frac{e_0K_{11}K_{22}/H^2}{1 + \frac{K_{11} + K_{12}}{H} + \frac{K_{11}K_{22}}{H^2}} \quad (8)$$

Evaluation of all four dissociation constants is not kinetically possible. The ratio of $[\text{EH}^-]/[\text{HE}^-] = K_{11}/K_{12}$, which is a constant, is independent of pH, thus any change in pH will not effect $[\text{EH}^-]$ without a proportional change in $[\text{HE}^-]$. The two species $[\text{EH}^-]$ and $[\text{HE}^-]$ must be treated as a single species, so eq. 1 may now be written as follows:

$$\text{EH}_2 \xleftarrow{K_1} \text{EH}^- \xrightarrow{K_2} \text{E}^2^-$$

(9)
where

\[ K_1 = \frac{[EH^-][H]}{[EH_2]} \]  \hspace{1cm} (10) \]

and

\[ K_2 = \frac{[E^{2-}][H]}{[EH^-]} \]  \hspace{1cm} (11) \]

Substituting \([EH^-] + [HE^-]\) for \([EH^-]\) into eqs. 10 and 11 and knowing the definitions of \(K_{11}, K_{12}, K_{21},\) and \(K_{22}\), we obtain

\[ K_1 = K_{11} + K_{12} \]  \hspace{1cm} (12) \]

\[ K_2 = \frac{K_{11}K_{22}}{K_{11} + K_{12}} \]  \hspace{1cm} (13) \]

Adding eqs. 6 and 7 and using eqs. 12 and 13, we arrive at eq. 14:

\[ [EH^-] = [EH^-] + [HE^-] = \frac{e_0}{H} \frac{K_2}{1 + \frac{K_1}{K_2} + \frac{f_{eh^-}}{H}} \]  \hspace{1cm} (14) \]

\(K_1\) and \(K_2\) are molecular dissociation constants, while \(K_{11}, K_{12}, K_{21},\) and \(K_{22}\) are group dissociation constants.

Equations for the two other species have the following form:

\[ [EH_2] = \frac{e_0}{H^2} \frac{f_{eh^-}K_2}{1 + \frac{K_1}{K_2} + \frac{f_{eh^-}}{K_2}} \]  \hspace{1cm} (15) \]
\[
\left[ E^{2-} \right] = \frac{e_0}{1 + \frac{K_1}{H^+} + \frac{K_{1,2}}{H^2}} = \frac{e_0}{f_{e^{2-}}}
\]

The dibasic ionization model can now be used to describe the pH effect on enzyme activity as illustrated in the following reaction scheme:

There are two species that will be treated as dibasic acids, the \(EH_2\) complex, composed of \(EH^2\), \(EH^-\), and \(E^{2-}\), with molecular dissociation constants \(K_{e1}\) and \(K_{e2}\), and the \(EH_2S\) complex, composed of \(EH_2S\), \(EHS^-\), and \(ES^{2-}\), with molecular dissociation constants \(K_{es1}\) and \(K_{es2}\). The model assumes that there is only one reacting species, \(EHS^-\), which is a good approximation based on the fact that enzyme activity often decreases as the pH is increased or decreased. Also, dissociation occurs rapidly compared to the breakdown and formation of the enzyme-substrate complex, so all ionic species with the same number of substrate residues attached are in equilibrium with each other. Finally, the reaction scheme is composed of only two steps, as is the Michaelis-Menten mechanism.
The maximum rate for the Michaelis-Menten equation is defined by

\[ V_m = k_{+2}e_0 \]  

(18)

where \( k_{+2} \) is a function of pH, and \( e_0 \) is the sum of all the different ionic forms of the enzyme complexes. The maximum rate for eq. 17 is defined as

\[ V_m = \tilde{k}_{+2}[EHS^-] \]  

(19)

where \( \tilde{k}_{+2} \) is independent of pH.

Adapting eq. 14 to \([EHS^-]\) and substituting into eq. 18 gives

\[ V_m = \frac{\tilde{k}_{+2}e_0}{1 + \frac{K_{es2}}{K_{es1}} + \frac{f_{ehs}}{e}} \]  

(20)

where \( K_{es1} \) and \( K_{es2} \) are the dissociation constants that affect the acid and alkaline side of the pH curve, respectively. Eqs. 18 and 19 were formulated assuming that the enzyme is saturated with substrate.

The Michaelis constant, \( K_m \), is defined as follows:

\[ K_m = \frac{k_{-1} + k_{+2}}{k_{+1}} \]  

(21)

The rate coefficients \( k_{+1}, k_{-1}, \) and \( k_{+2} \) are defined for all enzyme and enzyme-substrate complexes regardless of their dissociation states. The values of the coefficients, which are effected by pH, will be dependent on the concentration of the species associated with that rate coefficient,
such as \([ES_o] = [ES^2 - ] + [EHS^-] + [EH_2S]\) for the rate constant \(k_+^2\). Thus \(k_+^2\) would be composed of three parts based on the three different dissociation states, assuming all three species form products. In this case only the complex \(EHS^-\) reacts to form products. Adopting eq. 14 to \([ES_o]\) and \([E_o]\), where \([E_o] = [EH_2] + [EH^-] + [E^{2-}]\), we can define the rate coefficients as equal to a pH independent rate constant divided by the function \(f\), which describes the dissociation state of the species affecting that particular rate constant. Thus eq. 21 is now defined as

\[
\frac{k_+^1}{K_m} = \frac{f_{eh^-}^{-1} + f_{eh^-}^{2}}{f_{eh^-}} = \frac{k_+^2}{k_+^2 + k_+^1} \cdot \frac{f_{eh^-}^{-1}}{f_{eh^-}}
\]

(22)

where \(K_m\) is defined with respect to the particular ionic forms of the components and is independent of pH.

Dividing eq. 20 by eq. 22 gives an expression for \(V_m/K_m\) in terms of \(H\) and the molecular dissociation constants:

\[
V_m/K_m = \frac{\hat{V}_m/K_m}{(1 + H/K_{e1} + K_{e2}/H)}
\]

(23)

Inspection of eqs. 20, 22, and 23 reveal that \(V_m\) is a function of the dissociation of only EHS complexes, \(V_m/K_m\) is a function of the dissociation of only free enzyme complexes, and \(K_m\) is a function of both.
By taking the logarithms of eqs. 19, 22, and 23, we arrive at the following expressions:

\[
\log V_m = \log \frac{\tilde{k} e_0}{r_0} + p(1 + H/K_{es1} + K_{es2}/H) \tag{24}
\]

\[
\log K_m = \log \tilde{k}_m - p(1 + H/K_{e1} + K_{e2}/H) + p(1 + H/K_{es1} + K_{es2}/H) \tag{25}
\]

\[
\log \frac{V_m}{K_m} = \log \left( \frac{\tilde{V}_m}{\tilde{K}_m} \right) + p(1 + H/K_{e1} + K_{e2}/H) \tag{26}
\]

If \( \log V_m \) is plotted versus pH, three regions will appear if \( K_{es1} \) and \( K_{es2} \) are separated by 2 or 3 pH units. In the region where \( K_{es1} \) and \( K_{es2} \) are negligible compared to \( \log \tilde{k}_e e_0 \), eq. 24 reduces to \( \log V_m = \log \tilde{k}_e e_0 \) a horizontal straight line independent of pH. In the acidic region where \( K_{es2}/H \) is significantly less than 1, eq. 24 reduces to

\[
\log V_m = \log \tilde{k}_e e_0 + pH - pK_{es1} \tag{27}
\]

which represents a straight line with a slope of 1 intersecting the \( \log \tilde{k}_e e_0 \) line at a pH equivalent to \( pK_{es1} \). The same procedure can be used to calculate \( K_{es2} \). By applying the same logic, \( K_{e1} \) and \( K_{e2} \) can be determined from eq. 16 and a plot of \( \log V_m/K_m \) versus pH. Though eq. 25 is more complex, Dixon developed a set of rules to interpret the graphical representation of \( pK_m \) versus pH:

1) The graph of \( pK_m \) versus pH will consist of straight-line sections joined by short curved parts.
2) The straight-line portions have integral slopes, positive or negative.

3) Each bend indicates the pK of a dissociating group, and the straight line portions intersect at pH's equivalent to their pK's.

4) Each pK produces a one-unit change in slope.

5) Each pK of a group situated in the ES complex produces an upward bend, while the pK of a group corresponding to the free enzyme produces a downward bend.

An example of a Dixon plot is shown in Fig. 27.
Figure 27. Diagram illustrating the effects of pH on $K_m$, $k_o$, and $k_o/K_m$ and the determination of pKs.
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