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Kinetic and thermodynamic analysis of the inhibitory effects of maltose, glucose, and related carbohydrates on wheat β-amylase

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Abbreviations: BacBA, Bacillus β-amylase, BBA, barley β-amylase; EI, enzyme-inhibitor complex; pI, isoelectric point; SBA, soybean β-amylase; WBA, wheat β-amylase
Inhibition of wheat β-amylase (WBA) by glucose and maltose was studied by kinetics and thermodynamics. The inhibitory effects of fructose, difructose, sucrose, trehalose, cellobiose, acarbose, and 1-deoxynojirimycin on WBA were also evaluated. The half maximal inhibitory concentrations (IC_{50}) of acarbose, maltose and glucose were 0.06 ± 0.01 M, 0.22 ± 0.09 M, and 1.41 ± 0.17 M, respectively. The inhibitor constant (K_i) and the thermodynamic parameters such as changes in Gibbs energy (ΔG), enthalpy (ΔH), and entropy (ΔS) of the dissociation reactions of the WBA-glucose and WBA-maltose complexes were temperature and pH-dependent. The dissociation reactions were endothermic and enthalpy-driven. Both glucose and maltose behaved as competitive inhibitors at pH 3.0 and 5.4 at a temperature of 25°C with respective K_i values of 0.33 ± 0.02 M and 0.12 ± 0.03 M. In contrast, both sugars exhibited uncompetitive inhibition at pH 9 at a temperature of 25°C with K_i values of 0.21 ± 0.03 M for glucose and 0.11 ± 0.04 M for maltose. The pH-dependence of the inhibition type and K_i values indicate that the ionizing groups of WBA influence drastically the interaction with these carbohydrates. This evidence enables us to consider temperature and pH in the WBA-catalyzed hydrolysis to manipulate the inhibition by end-product, maltose, and even by glucose.

Keywords: glucose; inhibition; maltose; product inhibition; wheat β-amylase.
1. Introduction

β-Amylase [EC 3.2.1.2] is an exo-enzyme that catalyzes the cleavage of α-1,4-glucosidic bonds of polysaccharides and liberates maltose from the non-reducing end. It is a member of family 14 of the sequence-based classification of glycoside hydrolases [1]. β-Amylases are found only in higher plants and certain bacteria, and there are some differences between bacterial and plant β-amylases in their ability to bind and hydrolyze raw starch [2]. This binding aptitude is credited to a starch-binding domain located at the C-terminus of the sequence [3]. The subsite affinities of β-amylases were evaluated in wheat β-amylase (hereinafter designated as WBA) by Kato et al. [4] and in soybean β-amylase (SBA) by Suganuma et al. [5]. According to their reports, subsite 1 has the highest affinity to a glucose unit of the substrate among the five evaluated subsites and it plays an important role in the activity of the enzyme.

The inhibition kinetics of β-amylase by glucose [7], maltose [7, 8], and cyclohexa-amylose [9, 10] were examined. However, the binding sites of these inhibitors have not been established at the subsite level. The inhibition type by glucose, cyclohexa-amylose, and maltose on SBA was pH dependent [6]. Barley β-amylase (BBA), SBA, and WBA are well characterized. The degrees of thermo-stability defined by $T_{50}$, which is the temperature at which enzymes lose 50% of their activity after 30-min incubation, are 57°C for BBA, 63°C for SBA, and 50°C for WBA [11, 12].
Most recently, we have reported the effects of additives (carbohydrates, amino acids, organic solvents, proteins, detergents, etc.) on the activity and stability of WBA [12]. It is interesting to note that 182 mM glycine and 0.18% gelatin stabilize WBA by increasing its $T_{50}$ by 5°C and that ethanol and dimethylformamide (DMF) increase WBA activity by 24%, although most additives have no effects on stability and have decreasing effect on the activity. Among the additives examined, arginine, 2-mercaptoethanol, glucose and maltose inhibited WBA strongly [12]. These lines of evidences suggest that the stability and activity of WBA could be changed by modification of the enzyme reaction system or solvent engineering.

In the present study, we describe the interaction of WBA with glucose and maltose by inhibition kinetics. The temperature-dependence and pH-dependences of the inhibitor constants ($K_i$) and thermodynamic parameters for the dissociation constants of the WBA-glucose and WBA-maltose complexes are shown. In addition to the inhibitory effects of glucose and maltose as the direct end-products of starch hydrolysis, the inhibition of WBA by fructose, difructose, sucrose, trehalose, cellobiose, acarbose, and 1-deoxynojirimycin were also examined. This study will provides valuable information on the end-product inhibition of WBA in the process of starch hydrolysis and also on the specific interaction of WBA with various related carbohydrates.
2. Materials and methods

2.1. Materials

Himaltosin GS (Lot 2S24A), a commercial preparation of WBA, was purchased from HBI Enzymes (Osaka, Japan). WBA was purified from the Himaltosin preparation according to the method described previously [12] to a homogeneous state as judged by polyacrylamide-gel electrophoresis (SDS-PAGE) with a molecular mass of 57.7 kDa. The preparation is composed of only WBA as a protein component [12]. There was no starch carried over from the stabilizer starch contained in the Himaltosin preparation into the purified WBA preparation as examined by the starch-iodine reaction. Therefore, the WBA preparation was treated as WBA without further purification.

The WBA concentration was determined spectrophotometrically in 20 mM sodium acetate buffer, pH 5.4 (hereinafter designated as buffer A) at 25°C using the absorptivity value (A) of 1.40 ± 0.02 at 281 nm with a 1.0-cm light-path for the WBA solution for a concentration of 1.0 mg/ml [12, 13]. The molecular mass of 57.5 kDa for WBA was used to determine the molar concentration of WBA [4]. The substrate has a weight-average molecular weight of 1.0 x 10^6 according to the manufacturer, and thus the average degree of polymerization of the glucose unit
is estimated to be 6,000. Soluble starch (Lot M7H1482), maltose (Lot M1B6462), glucose (Lot M3G8543), potassium iodide (Lot V1P5303), cellobiose (Lot M2G9713), and other chemicals were purchased from Nacalai Tesque (Kyoto, Japan) and iodine solution (Lot CEM7810), acarbose (Lot LAQ5872), difructose anhydride (Lot TLM1444), $\alpha,\alpha$-1,1-trehalose (Lot PEH6208; hereinafter designated as simply trehalose), and 1-deoxynojirimycin (Lot DCL2444) were from Wako Pure Chemical (Osaka, Japan).

2.2. Inhibition of WBA by glucose, maltose, and other carbohydrates

WBA (1.6 $\mu$M) and various concentrations of glucose, maltose, and other carbohydrates were prepared in buffer A (pH 5.4) at 25°C. The WBA solution (100 $\mu$l) was pre-incubated with 100 $\mu$l of the carbohydrates solutions at the initial concentrations of 0, 0.15, 0.31, 0.62, 1.23, and 2.33 M glucose; or 0.15, 0.31, 0.62 M maltose; 0.15, 0.31, 0.46, 1.17, and 1.75 M fructose; 0.04, 0.08, 0.12, 0.15, and 0.24 M difructose; 0.15, 0.31, 0.46, and 0.62 M sucrose; 0.15, 0.31, 0.46, 0.62, 1.11, and 1.66 M trehalose; and 0.14, 0.28, and 0.42 M cellobiose; 0.004, 0.040, 0.080, 0.120, and 0.150 M acarbose; and 0.001, 0.002, 0.005, and 0.009 M 1-deoxynojirimycin for 5 min at 25°C before reacting with the substrate. The soluble starch solution (0.69%, w/v; 450 $\mu$l) was mixed with the mixture (200 $\mu$l) of WBA and the carbohydrates and was hydrolyzed for 5 min in
buffer A at 25°C. Therefore, the WBA and soluble starch concentrations in the initial conditions of the enzyme reaction were 0.25 µM and 0.69 %, w/v, respectively. The reaction was stopped by mixing the reactants (50 µl) with 4.5 ml of 1 mM potassium iodide (KI) prepared in 0.1 N HCl. The absorbance was measured at 580 nm using a Beckman-Coulter DU 800 spectrophotometer (Batavia, IL, USA) [6, 14]. Iodine staining is not preferable method for precise kinetic studies. Nevertheless, it was not possible to use the common product measurement methods such as neocuproine, DNS (dinitrosalicylic acid), and Somogi-Nelson [15] methods which are based on the determination of the reducing ends of the products because some of the carbohydrates examined possess reducing ends. The 50% inhibitory concentrations (IC₅₀) of the carbohydrates are their concentrations giving 50% of the activity observed in the absence of the carbohydrates.

2.3. Reversibility of WBA inhibition by glucose and maltose

Reversibility of the WBA inhibition by glucose and maltose was examined through dialysis and dilution techniques. In dialysis, 1.6 µM of WBA in buffer A was filtered through Millipore membrane filter (Type HA; pore size: 0.45 µm) and kept in ice for immediate use. The filtered WBA solution (1 ml) was incubated for 5 min with 1 ml of buffer A, or 1 ml of 2 M glucose or 0.5 M maltose prepared in buffer A. The WBA-glucose or WBA-maltose mixtures
were dialyzed using a dialysis membrane (Wako Chemicals, Kyoto) against buffer A 100-times the volume of the mixtures; the dialyzing buffer was changed 3 times with 2-h intervals at 4°C. Soluble starch (0.82%, w/v) was hydrolyzed by WBA for 10 min at 25°C. The reaction rate of substrate hydrolysis was determined using iodine staining method and the reaction rates of the dialyzed WBA in the presence of glucose and maltose were compared with the reaction rates in the absence of the inhibitors.

In the dilution method, the concentration of WBA 10-times higher than that described above was incubated with buffer A or with buffer A containing glucose or maltose at the initial concentrations of 18 mM and 0.18 M. After pre-incubation for 5 min, the WBA mixture with 0.18 M of glucose or maltose was 10 times diluted to 18 mM glucose or maltose with buffer A. The concentrations of the inhibitors (glucose and maltose) in the buffer were adjusted to attain the mentioned concentrations after dilution and 0.14 μM WBA. The relative activities in the presence of the carbohydrates were calculated relative to the activity in the absence of the carbohydrates and the reversibility (%) of the 10 times diluted forms was calculated relative to the same lower concentrations of the carbohydrates before dilution [16]. The same method was repeated to check the reversibility of WBA inhibition in the presence of various concentrations of glucose or maltose at pH 5.4, 25°C.
2.4. Temperature dependence of the inhibitor constants ($K_i$) for the inhibition of WBA by glucose and maltose.

Various initial concentrations of carbohydrates in the enzyme reaction: 0.08, 0.15, 0.23, 0.31, 0.38, and 0.46 M glucose; 0.07, 0.08, 0.12, 0.15, 0.18, and 0.23 M maltose were prepared in buffer A at 15, 25, 35, and 45°C. Various initial concentrations of soluble starch: 0.35, 0.52, and 0.69% (w/v) were hydrolyzed by 0.32 µM WBA pre-incubated for 5 min with various concentrations of glucose or maltose at each temperature. The initial reaction rates ($v$) of starch hydrolysis were determined from the reaction progress over 0.5-4.0 min and the reactant (50 µl) was stopped by adding 4.5 ml of the 1 mM iodine solution. The $K_i$ values at each temperature were determined from the Dixon plots [17]. The enthalpy changes ($\Delta H$) of the dissociations of the WBA-glucose and WBA-maltose complexes were determined from the van’t Hoff plots [18], while the Gibbs energy changes ($\Delta G$), and entropy changes ($\Delta S$) were determined from Eqs. 1 and 2 [18, 19].

$$\Delta G = -RT \ln K_i \quad (1)$$

$$\Delta S = (\Delta H - \Delta G)/T \quad (2)$$

where $R$ is the universal gas constant and $T$ is temperature in Kelvin.
2.5. pH-Dependence of $K_i$

Various initial concentrations of soluble starch: 0.35, 0.69, 1.04, 1.38, 1.73, and 2.08% (w/v) were prepared in 20 mM glycine-HCl buffer (pH 3.0), 20 mM sodium acetate buffer (pH 5.4; buffer A), and 20 mM borate buffer (pH 9.0) at 25°C following the method previously reported [20]. WBA (0.32 μM) in the respective buffers at each pH was pre-incubated with 0.31, and 0.62 M glucose, and 0.15, and 0.31 M maltose for 4 min at 25°C. The soluble starch solution (450 μl) varying in concentration was mixed with 100 μl of the pre-incubated WBA-glucose or WBA-maltose mixtures at pH 3.0, 5.4, and 9.0 at 25°C. The initial reaction rates ($v$) were determined from the reaction progress over 5 min. The absorbance was measured at 700 nm using a Beckman-Coulter DU 800 spectrophotometer. The inhibition type and $K_i$ values at each pH was determined from Hanes-Woolf plot [21]. The $K_i$ values were calculated using Eqs. 3 and 4 for competitive and uncompetitive inhibitors, respectively [22].

$$K_i = [I]/ ((K_m,app/K_m) -1)$$  \hspace{1cm} (3)

$$K_i = [I]/ ((V_{max}/V_{max,app}) -1)$$  \hspace{1cm} (4)

where, $K_m$ and $V_{max}$ are the Michaelis constant and maximum reaction rate observed in the absence
of inhibitors; $K_{\text{m,app}}$ and $V_{\text{max,app}}$ are those in the presence of inhibitors; and $[I]$ is inhibitor concentration.

### 3. Results

#### 3.1. Inhibition of WBA by glucose, maltose, and other carbohydrates.

The rate of soluble starch hydrolysis by WBA was measured from the decrease in absorbance at 580 nm as described in the materials and methods. The catalytic activity of WBA was reduced by increasing the concentrations of maltose or other carbohydrates (Figs. 1). The inhibitory activities of fructose, sucrose, trehalose, difructose ($\alpha$-D-fructofuranose-$\beta$-D-fructofuranose-2',1:2,3'-dianhydride shown in supplementary Fig. 8), cellobiose, and 1-deoxynojirimycin were comparatively weak. On the other hand, acarbose and maltose strongly inhibited WBA activity followed by glucose (Fig. 2A). The inhibition by acarbose, maltose, and glucose were significant with the $IC_{50}$ values of $0.06 \pm 0.01$, $0.22 \pm 0.09$, and $1.41 \pm 0.17$ M, respectively (Fig. 2A). The $IC_{50}$ values were not attained by the other carbohydrates examined at the concentrations of their maximal solubility (Fig. 2B). The maximum degrees of inhibitions by 1-deoxynojirimycin, difructose, trehalose, sucrose, fructose, and cellobiose were
around 30%, and the $IC_{50}$ values were estimated to be $> 9$ mM, $> 0.3$ M, $> 2$ M, $> 0.5$ M, $> 2$ M, and 0.4 M, respectively. The $IC_{50}$ values were the values of $[I]_0$ at which the fractional activity of WBA becomes 0.5 as shown in Fig. 2.

(Fig. 1)

(Fig. 2)

3.2. Reversibility of WBA inhibition by maltose and glucose.

The reversibility of WBA inhibition by glucose and maltose was studied through both dialysis and dilution methods. The activity of WBA in the absence and presence of inhibitors was measured using blue value method ($\Delta A_{580\text{ nm}}$) after dialysis. The activity of WBA in buffer A (control) was taken as 100%, and the reversibility in the presence of inhibitors after dialysis were calculated relative to the activity of the control (Table 1). The activity of WBA was completely restored through dialysis.

In the dilution method, the inhibitor concentrations were diluted 10 times and the effect of dilution was examined following the method described previously [16]. The activity of WBA in the presence of 18 mM maltose was 86% relative to the activity observed in the absence of inhibitors.
When 180 mM maltose was diluted to 18 mM, the activity recovered to 89%, and hence the reversibility was 103 ± 6%. In the same manner, various concentrations of glucose and maltose were studied and complete reversibility was observed through dilution (Table 2).

(Table 1)

(Table 2)

3.3. Temperature dependence of $K_i$.

The temperature-dependence of the $K_i$ values of glucose and maltose at 15, 25, 35, and 45°C were estimated at pH 5.4 using the Dixon plots shown in the supplementary Figs. 4 and 5. The $K_i$ values of both inhibitors increased slightly with increasing temperature, indicating that the inhibitory activity of the inhibitors decrease with increasing temperature in the range of 15-45°C. The thermodynamic parameters: the Gibbs energy change ($\Delta G$), enthalpy change ($\Delta H$), and entropy change ($\Delta S$) of the dissociation of the WBA-glucose or WBA-maltose complexes were determined at various temperatures (Table 3). The van't Hoff plots of the $K_i$ values of WBA inhibition by glucose and maltose with the slope - $\Delta H/R$ were shown in Fig. 3.
3.4. *pH*-Dependence of $K_i$

The inhibition types and $K_i$ values of WBA inhibition by glucose and maltose at 25°C were evaluated at pH 3.0, 5.4, and 9.0 using the Hanes-Woolf plots shown in the supplementary Figs. 6 and 7. Here, we should note the difference between the inhibitory mechanisms of the competitive inhibition and uncompetitive inhibition. In the case of competitive inhibition, the inhibitor (I) binds only to the enzyme (E) to form the enzyme-inhibitor complex (EI) but does not bind to the enzyme-substrate complex (ES). In the case of uncompetitive inhibition, it binds only to the ES complex to form the enzyme-substrate-inhibitor (ESI) ternary complex but does not bind to E. Thus, the EI complex is not formed in uncompetitive inhibition, whereas the ESI complex is not formed in competitive inhibition. In other words, it binds to the active site of the enzyme in competitive inhibition, but binds to the secondary binding site other than the active site in uncompetitive inhibition. Glucose and maltose bind to the active site of WBA at pH 3.0 and 5.4, and bind to the secondary binding site of WBA at pH 9.0. In competitive inhibition, $K_m$ increases and $V_{max}$ remains constant while both the $K_m$ and $V_{max}$ values decrease in uncompetitive inhibition. It is interesting to
note that the $K_i$ values obtained at pH 5.4 for both glucose and maltose are slightly larger than at pH 3.0 and 9.0, suggesting that the inhibitory effects of glucose and maltose on WBA at pH 5.4 (which is the optimal pH of WBA) are lower than at pH 3.0 and 9.0, at which the enzyme activity is extremely low. The $K_i$ values determined by the Hanes-Woolf plot and Dixon plot for the respective inhibitors at pH 5.4 and 25°C are in good agreement. The $K_i$ values of glucose and maltose in the WBA inhibition are considered to be the dissociation constant ($K_d$) values for the WBA-glucose and WBA-maltose complexes. Thus, the $\Delta G$ values of the dissociations of the WBA-glucose and WBA-maltose complexes can be determined from $K_i$ values at each pH (Table 4).

4. Discussion

4.1. Inhibition of WBA.

The catalytic activity of WBA was noticeably inhibited in a dose-dependent manner by acarbose, maltose and glucose. $\beta$-Amylases from other crops such as soybean [6], sweet potato [8], and alfalfa [14] were reported to be inhibited by glucose and maltose. In the present study, other sugar analogues and sugar derivatives were systematically selected to evaluate their inhibitory effects on
WBA, among which, only acarbose containing acarviosin (α-amylase inhibitor) and maltose moiety
was found to expressively inhibit WBA. However, this $IC_{50}$ value of acarbose is very big in view of
pharmaceutical industries. The well-known α-amylase inhibitor, acarbose was reported to
competitively inhibit barley BA [23]. Acarbose analogues, containing cellobiose and lactose
structures were potent competitive inhibitors of β-glucosidase, and the lactose analogues were
uncompetitive inhibitors of β-galactosidase [24]. On the other hand, fructose, sucrose, trehalose,
difructose, 1-deoxynojirimycin, and cellobiose did not show substantial inhibition on WBA.
However, the maximum solubility of cellobiose is very low even at high temperature and hence,
difficult to evaluate its inhibitive power.

The respective $IC_{50}$ and $K_i$ values of maltose are 0.22 ± 0.09 and 0.12 ± 0.03 M and that of
glucose are 1.41 ± 0.17 and 0.33 ± 0.02 M at 25°C, pH 5.4 (Fig. 2 and Table 3). The $K_i$ values of
0.034 ± 0.0 M for maltose and 0.32 ± 0.08 M for glucose were reported in SBA inhibition [6]. This
shows that maltose is stronger inhibitor than glucose of not only WBA but also of SBA. From the
crystal structure study of maltose and glucose binding of BacBA, it was stated that glucose binds to
subsite 1 and maltose binds to subsites 1 and 2 of the active site [2]. This attests that maltose binds
at two subsites of BacBA at a time while glucose binds at only a single subsite. Therefore, the molar
energy of maltose binding to the active site may be given as the sum of the molar energy of glucose
moieties binding to subsites 1 and 2, while the molar energy of glucose binding is that to subsite 1.
Hence, the inhibition of maltose is stronger than that of glucose. This is not always true if more than one maltose molecules that bind different binding sites or subsites are considered. In such cases, the binding affinities of the second binding site or subsites are enhanced in positive co-operativity and inhibited in negative co-operativity. By considering the binding modes of glucose and maltose by WBA to be similar to that of BacBA, we can estimate the molar binding energy of the glucose moieties to subsites 1 and 2 separately from Table 4. The binding energy of the glucose moiety to subsite 1 would be -3.43 and -2.33 kJ/mol at pH 3.0 and 5.4, respectively, and those to subsite 2 would be -1.81 and -2.20 kJ/mol. From these values, it can be seen that the molecular binding energy to subsite 1 is much lower in magnitude at pH 5.4 than at pH 3.0, although that to subsite 2 is higher at pH 5.4 than at pH 3.0, and the molar binding energy of maltose to the active site of WBA is slightly smaller at pH 5.4 than at pH 3.0 as shown in Table 4.

4.2. **Reversibility of maltose and glucose inhibition.**

Inhibition of WBA by glucose and maltose was completely reversible. We can also infer from reports [6, 8, 14] that the inhibition was reversible indicating that the molecular liaison between WBA and the sugars is not covalent. The reversibility of WBA inhibition by glucose and maltose was examined by dialysis. The dialysis was conducted at 4°C to avoid the activity loss, and WBA
activity was completely reinstated (Table 1). In dialysis, enzymes are restored from the easily
dissociable, non-covalently formed enzyme-inhibitor complexes [25]. The reversibility of WBA
activity was also confirmed through dilution technique. Similar dilution principles were reported on
the reversibility study of the inhibition of thermolysin [16] and neuraminidase [26] by alcohols.
Contrary to the dialysis method, low concentration of the inhibitor remains in the reaction after
dilution. Due to this fact, reversibility was calculated relative to the initially low inhibitor
concentration the same as after 10 times dilution. It was explained that the drastic activation of
neuraminidase activity after dilution could be because of the change in the conformation and
hydrated state of the enzyme, in addition to the change in the viscosity of the reaction medium [26].

4.3. Temperature dependence of $K_i$.

The inhibitor constant ($K_i$) values of EI complex dissociations were affected by the change in
reaction temperature which influences the molecular activities of the solvent, enzyme, and
inhibitors. It also affects the structure of the protein, which entails change in enzyme-inhibitor
binding or dissociation [19, 27]. The $K_i$ values increased with temperature (Table 3) which agrees
with reports on various enzymes and inhibitors showing an increase in $K_i$ with temperature [16, 18,
19, 27]. The $K_i$ values at various temperatures were determined from the Dixon plots in this study
following the method previously reported [17, 28]. Kitagishi et al. [27], have recommended that inhibitor concentrations close to the $K_i$ values should be used in order to determine the $K_i$ values accurately and in conditions where $[E]_o << [I]_o$, the $K_i$ values can be obtained from the Dixon plot. Hence, the concentration of enzyme is much less than the inhibitor concentrations in our study.

Temperature variation exerted significant influence on the thermodynamic parameters of the EI complexes dissociations (Table 3). The positive $\Delta H$ values of the dissociations of the WBA-glucose and WBA-maltose complexes indicate that the dissociations were endothermic. The $\Delta G$, a state function reaction change of the system determines the relative importance of the enthalpy and entropy terms as driving forces behind a particular reaction [29]. The positive values of $\Delta G$ indicate non-spontaneous dissociations of the EI complexes. Since the enthalpy terms are greater in magnitude than the entropy terms, the EI dissociations are enthalpy-driven. The $\Delta G$ values were found to decrease with increasing temperature. The source of the energy demand for the dissociations is the partial hydrogen-bond breakage in the system [30]. The bindings of glucose and maltose to the active site of BacBA are mainly by hydrogen-bonds from the crystallographic study [2]. It can be elucidated from these facts that there is hydrogen-bond cleavage with the dissociations of the WBA-glucose or WBA-maltose complexes. In general, the hydrogen-bond dissociation energies are 20 kJ mol$^{-1}$ but it can span more than two orders of magnitude (about 8 - 100 kJ mol$^{-1}$) depending on the nature of the interactions and environments [30]. The $\Delta G$ required
for the dissociation of the WBA-maltose complex is almost twice as that of the WBA-glucose complex (Tables 3 and 4), which is in good agreement with the structural compositions of the inhibitors.

4.4. pH-Dependence of $K_i$.

The inhibition type of WBA by both glucose and maltose was affected by pH. Both sugars behaved as competitive inhibitors at pH 3.0 and 5.4 at 25°C but exhibited uncompetitive type of inhibition at pH 9.0. Nomura et al. [6] reported that the inhibition type on SBA by maltose and cyclohexa-amylose was competitive at pH 5.4 but it was mixed-type by glucose at pH 5.4 (I binds both E and ES to form EI and ESI complexes), and competitive by all the sugars at pH 8 and form solely EI complexes. Change of the inhibition type depending on pH was also reported in aspartic protease [31] and pepsin [32]. The isoelectric point (pI) value of WBA was reported to be 5.8 [33].

The $pK_{e1}$ (where the ionizable group 1 of SBA is deprotonated) and $pK_{e2}$ (where the ionizable group 2 of SBA is protonated) were 3.41 and 8.09 with pI value of 5.63 [34, 35]. This is to indicate that at pH 3.0, WBA has a net positive charge. In this state, both glucose and maltose bind to the active site. At pH 9 when the net charge of WBA becomes negative, the sugar molecules bind the secondary binding sites on the EI complex and behaved as uncompetitive inhibitors. According to the subsite
model of amylases [4, 36], the inhibition type depends on the binding site of the inhibitors [37].

The $K_i$ values of the dissociations of the WBA-glucose and WBA-maltose complexes were also pH dependent (Table 4). The pH-dependences of $K_i$ in various enzymes and inhibitors were reported with the changes in $K_i$ being different depending on the response of particular enzymes to the pH changes [6, 17, 27, 31, 32]. This phenomenon apparently suggests that pH either induces conformational or change in ionization states of binding residues of an enzyme. Our result in Table 4 shows that the inhibitive power of the inhibitors declines with increasing the activity of the enzyme at optimum pH.

In starch-saccharifying industries, maltose and glucose are continuously produced from the enzymatic starch hydrolysis process. Hence, studying the molecular interactions of these end-products and β-amylase is worthwhile to search for suitable ways of enhancing production. This study thus, provides valuable information on the end-product inhibition of WBA, which has direct implication on the efficiency and cost of production. Mechanisms of reducing the inhibitory actions of glucose and maltose can be suggested like, continuous removal of the end-products from the reaction.

5. Conclusions

The end products of starch hydrolysis, maltose and glucose inhibit WBA dose-dependently and
the interaction is reversible. The catalytic activity of WBA is more strongly inhibited by acarbose than by maltose and glucose. The inhibitor constant ($K_i$) of glucose and maltose are temperature dependent. The dissociations of the WBA-glucose and WBA-maltose complexes are endothermic and enthalpy-driven. Both glucose and maltose bind the active site when WBA is protonated at pH 3.0 and 5.4 and to the secondary binding sites when its net charge becomes negative at pH 9.0. A change in pH sways the active site integrity in binding the inhibitors or induces a conformational change to WBA and hence, affects the type of inhibition. Therefore, both the temperature and pH of starch hydrolysis operations require careful manipulation to minimize end-product inhibition in starch-saccharifying industries.
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[31] Xie D, Gulnik S, Collins L, Gustchina E, Suvorov L, Erickson JW. Dissection of the pH dependence of inhibitor binding energetics for an aspartic protease: direct measurement of the


Figure legends

**Fig. 1.** Inhibition of WBA activity by various concentrations of glucose and maltose in the hydrolysis of soluble starch. The initial concentrations of WBA and soluble starch in the reaction solution were 0.25 μM and 0.82%, w/v, respectively. Panel A, inhibition by glucose. The initial concentrations of glucose were: ○, 0; ◇, 0.15; Δ, 0.31; □, 0.62; ●, 1.23; ◆, 2.33 M. Panel B, inhibition by maltose. The initial concentrations of maltose were: ○, 0; ◇, 0.15; Δ, 0.31; □, 0.62 M of maltose. WBA and carbohydrates (glucose or maltose) were pre-incubated for 5 min before reaction in 20 mM sodium acetate buffer (buffer A) at pH 5.4, 25°C. The progress of the reaction was followed by measuring the absorbance at 580 nm after staining the reaction solution by KI and the initial reaction rate was evaluated from the slope of the progress curve. The activity of WBA was considerably inhibited by both sugar inhibitors in dose-dependent manner.

**Fig. 2.** Inhibition of WBA by various carbohydrates in the hydrolysis of soluble starch. Dependence of the relative activity of WBA on the logarithmic concentrations of various carbohydrates was shown. Panel A, Carbohydrates: ●, acarbose; ♦, maltose; and ▲, glucose. Panel B, Carbohydrates: ●, 1-deoxynojirimycin; ♦, difructose; ▲, trehalose; □, sucrose; ■, fructose; and ○, cellobiose. The enzyme reaction was done in buffer A at pH 5.4 and 25°C. WBA activity observed in the absence of carbohydrates was set to the relative activity of
1.0. The $IC_{50}$ of the inhibitors are the concentrations corresponding to the midpoint of the relative activities.

**Fig. 3.** van’t Hoff plots of the inhibitor constants ($K_i$) of WBA-glucose and WBA-maltose dissociations at various temperatures and pH 5.4. The symbols: ○, maltose; and ◊, glucose. The slope of the plot gives -$\Delta H/R$. 
Table 1. The initial velocity and relative activity of WBA in the hydrolysis of soluble starch in the presence of glucose and maltose before and after dialysis.

<table>
<thead>
<tr>
<th></th>
<th>Control (buffer)</th>
<th>0.36 M glucose</th>
<th>0.09 M maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td>$v$ before dialysis</td>
<td>0.034</td>
<td>0.020</td>
<td>0.017</td>
</tr>
<tr>
<td>(Relative activity)</td>
<td>(100%)</td>
<td>(59%)</td>
<td>(49%)</td>
</tr>
<tr>
<td>$v$ after dialysis</td>
<td>0.030</td>
<td>0.030</td>
<td>0.029</td>
</tr>
<tr>
<td>(Relative activity)</td>
<td>(100%)</td>
<td>(98%)</td>
<td>(96%)</td>
</tr>
</tbody>
</table>

The initial concentrations of WBA and soluble starch in the enzyme reaction solution were 0.14 μM and 0.82%, w/v, respectively. The activity of WBA pre-incubated with only control (buffer A) was considered as 100% and the activities in the presence of inhibitors were calculated relative to the control.
Table 2. The relative activity and reversibility of WBA pre-incubated with various concentrations of glucose and maltose in hydrolysing soluble starch before and after 10 times dilution.

<table>
<thead>
<tr>
<th></th>
<th>[I]₀</th>
<th>Reversibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.02 M</td>
<td>0.18 M</td>
</tr>
<tr>
<td>Glucose</td>
<td>82 ± 11</td>
<td>74 ± 6</td>
</tr>
<tr>
<td>Maltose</td>
<td>86 ± 3</td>
<td>26 ± 12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>[I]₀</th>
<th>Reversibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.03 M</td>
<td>0.27 M</td>
</tr>
<tr>
<td>Glucose</td>
<td>97 ± 7</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>Maltose</td>
<td>86 ± 8</td>
<td>15 ± 1</td>
</tr>
</tbody>
</table>

The initial concentrations of WBA and soluble starch in the enzyme reaction solution were 1.6 μM and 0.82%, w/v, respectively. The activity of WBA in the absence of inhibitors was taken as 100%.

Relative activity is the activity relative to the activity in the absence of inhibitors while reversibility is relative to the activity of the same lower inhibitor concentrations before dilution as the diluted forms. The values are mean ± SD, each experiment was repeated three times.
Table 3. The temperature-dependence of the inhibitor constant ($K_i$) and thermodynamic parameters of the EI complexes dissociations in the inhibition of WBA by glucose and maltose at pH 5.4.

<table>
<thead>
<tr>
<th></th>
<th>Temperature(K)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>288</td>
<td>298</td>
<td>308</td>
<td>318</td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_i$ (M)</td>
<td>0.26 ± 0.05</td>
<td>0.33 ± 0.02</td>
<td>0.38 ± 0.03</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>$\Delta G$ (kJ mol$^{-1}$)</td>
<td>3.22 ± 0.97</td>
<td>2.74 ± 0.51</td>
<td>2.47 ± 0.69</td>
<td>2.29 ± 0.93</td>
</tr>
<tr>
<td>*$\Delta S$ (J mol$^{-1}$ K$^{-1}$)</td>
<td>30.73 ± 2.95</td>
<td>31.31 ± 2.85</td>
<td>31.17 ± 2.76</td>
<td>30.75 ± 2.67</td>
</tr>
<tr>
<td>$T\Delta S$ (kJ mol$^{-1}$)</td>
<td>8.85 ± 0.85</td>
<td>9.33 ± 1.06</td>
<td>9.60 ± 0.74</td>
<td>9.78 ± 0.96</td>
</tr>
<tr>
<td><strong>Maltose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_i$ (M)</td>
<td>0.11 ± 0.05</td>
<td>0.12 ± 0.03</td>
<td>0.14 ± 0.04</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>$\Delta G$ (kJ mol$^{-1}$)</td>
<td>5.28 ± 0.91</td>
<td>5.24 ± 0.77</td>
<td>5.03 ± 1.09</td>
<td>4.53 ± 0.92</td>
</tr>
<tr>
<td>*$\Delta S$ (J mol$^{-1}$ K$^{-1}$)</td>
<td>24.41 ± 3.58</td>
<td>23.72 ± 3.29</td>
<td>23.64 ± 3.57</td>
<td>24.47 ± 2.96</td>
</tr>
<tr>
<td>$T\Delta S$ (kJ mol$^{-1}$)</td>
<td>7.03 ± 1.03</td>
<td>7.07 ± 0.98</td>
<td>7.28 ± 1.10</td>
<td>7.78 ± 0.94</td>
</tr>
</tbody>
</table>

*the values are in J mol$^{-1}$ K$^{-1}$

$\Delta H$ was determined from van't Hoff plot [ln $K_i$ vs. $1/T$ (K$^{-1}$)]; where the slope of the graph equation gives -$\Delta H/R$ and 12.07 kJ mol$^{-1}$ for inhibition by glucose, and 12.31 kJ mol$^{-1}$ for inhibition by maltose. Whereas $\Delta G$ and $\Delta S$ were calculated as described in the experimental procedures.
Table 4. The pH-dependence of the inhibitor constants ($K_i$), the Gibbs energy change, and inhibition types for the dissociations of EI complexes in the inhibition of WBA by glucose and maltose at 25°C.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.0</td>
<td>5.4</td>
<td>9.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>$K_i$ (M)</td>
<td>0.25 ± 0.03</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>$\Delta G$ (kJ mol$^{-1}$)</td>
<td>3.43 ± 0.13</td>
<td>2.33 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>Inhibition type</td>
<td>competitive</td>
<td>competitive</td>
</tr>
<tr>
<td>Maltose</td>
<td>$K_i$ (M)</td>
<td>0.12 ± 0.04</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>$\Delta G$ (kJ mol$^{-1}$)</td>
<td>5.24 ± 0.13</td>
<td>4.53 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Inhibition type</td>
<td>competitive</td>
<td>competitive</td>
</tr>
</tbody>
</table>

The values are mean ± SD, each experiment was done in triplicates. The types of inhibition by glucose and maltose are from the Hanes-Woolf plots at various temperatures shown in supplementary Figs. 6 and 7.
Supplementary Data

Legend for supplementary figures

Fig. 4. Dixon plots of the inhibitor constants ($K_i$) of WBA-glucose complex dissociation at various temperatures. Reciprocals of the initial reaction rates in the hydrolysis of soluble starch were plotted against the glucose concentrations. Panels A, B, C, and D indicate reaction temperatures at 15, 25, 35, and 45°C, respectively at constant pH 5.4. The initial concentrations of soluble starch in the enzyme reaction: ○, 0.17%; □, 0.35%; and Δ, 1.38%, w/v, at 25°C (Panel A); and ○, 0.35%; □, 0.52%; and Δ, 0.69% at 15, 35 and 45°C (Panels B, C, and D). The initial concentration of WBA was 0.32 µM. WBA was pre-incubated for 5 min with increasing concentrations of glucose and catalyzed various concentrations of soluble starch.

Fig. 5. Dixon plots of the inhibitor constants ($K_i$) of WBA-maltose complex dissociation at various temperatures. Reciprocals of the initial reaction rates in the hydrolysis of soluble starch were plotted against the glucose concentrations. Panels A, B, C, and D indicate reaction temperatures at 15, 25, 35, and 45°C, respectively at pH 5.4. The initial concentrations of soluble starch in the enzyme reaction: ○, 0.17%; □, 0.35%; and Δ, 1.38%, w/v, at 25°C (Panel A); and ○, 0.35%; □, 0.52%; and Δ, 0.69% at 15, 35 and 45°C (Panels B, C, and D). The initial
concentration of WBA was 0.32 μM. WBA was pre-incubated for 5 min with increasing concentrations of glucose and catalyzed various concentrations of soluble starch.

**Fig. 6.** Hanes-Woolf plots of the initial reaction rates (v) in the presence and absence of glucose at pH 3.0, 5.4 and 9.0. The pH of the reaction: A, 3.0; B, 5.4; and C, 9.0. The initial concentrations of glucose in the enzyme reaction solution: ○, 0; □, 0.31; and Δ, 0.62 M. The inhibition types are competitive at pH 3.0 and 5.4 whereas uncompetitive type at pH 9.0, 25°C.

**Fig. 7.** Hanes-Woolf plots of the initial reaction rates (v) in the presence and absence of maltose at pH 3.0, 5.4 and 9.0. The pH of the reaction: A, 3.0; B, 5.4; and C, 9.0. The initial concentrations of glucose in the enzyme reaction solution: ○, 0; □, 0.31; and Δ, 0.62 M. The inhibition types are competitive at pH 3.0 and 5.4 whereas uncompetitive type at pH 9.0, 25°C.

**Fig. 8.** The structure of difructose anhydride III (DFA). Difructose anhydride (DFA) or α-D-fructofuranose-β-D-fructofuranose-2′,1:2,3’-dianhydride is the smallest cyclic disaccharide consisting of two fructose residues.
Fig. 2B

Fractional activity vs. Log $[I]_0$
Fig. 3

\[ \ln K_i = \frac{1}{T} \times 10^3 \ (K^{-1}) \]
Fig. 4A

Top

\[ \frac{1}{v} \] vs. \([I]_o \) (M)

-0.5 0 0.5

1/v

\([I]_o \) (M)

40

20
Fig. 4B
Fig. 4C
Fig. 4D
Fig. 5B
Fig. 5C
Fig. 5D
Fig. 6B
Fig. 6C
Fig. 7A
Fig. 7B

The diagram shows a plot with the x-axis labeled as $(S) / (M)$ and the y-axis labeled as $(M)$. The x-axis ranges from 0 to 60, and the y-axis ranges from 0 to 2. The diagram includes data points represented by different symbols, such as circles, squares, and triangles, corresponding to different values of $(S) / (M)$. The lines on the graph represent linear relationships between the variables.
Fig. 7C