1 Enzyme and Microbial Technology
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3 **Kinetic and thermodynamic analysis of the inhibitory effects of maltose, glucose, and related**
4 **carbohydrates on wheat β-amylase**
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23 Abbreviations: BacBA, *Bacillus* β-amylase, BBA, barley β-amylase; EI, enzyme-inhibitor
24 complex; p*I*, isoelectric point; SBA, soybean β-amylase; WBA, wheat β-amylase
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26

ABSTRACT

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.

49 **1. Introduction**

50

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

61 The inhibition kinetics of β-amylase by glucose [7], maltose [7, 8], and cyclohexa-amylose
62 [9, 10] were examined. However, the binding sites of these inhibitors have not been established at
63 the subsite level. The inhibition type by glucose, cyclohexa-amylose, and maltose on SBA was pH
64 dependent [6]. Barley β-amylase (BBA), SBA, and WBA are well characterized. The degrees of
65 thermo-stability defined by T_{50}, which is the temperature at which enzymes lose 50% of their
66 activity after 30-min incubation, are 57°C for BBA, 63°C for SBA, and 50°C for WBA [11, 12].

67 Most recently, we have reported the effects of additives (carbohydrates, amino acids, organic
68 solvents, proteins, detergents, etc.) on the activity and stability of WBA [12]. It is interesting to note
69 that 182 mM glycine and 0.18% gelatin stabilize WBA by increasing its T_{50} by 5°C and that ethanol
70 and dimethylformamide (DMF) increase WBA activity by 24%, although most additives have no
71 effects on stability and have decreasing effect on the activity. Among the additives examined,
72 arginine, 2-mercaptoethanol, glucose and maltose inhibited WBA strongly [12]. These lines of
73 evidences suggest that the stability and activity of WBA could be changed by modification of the
74 enzyme reaction system or solvent engineering.

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

83

84

85 **2. Materials and methods**

86

87 *2.1. Materials*

88

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

97 The WBA concentration was determined spectrophotometrically in 20 mM sodium
98 acetate buffer, pH 5.4 (hereinafter designated as buffer A) at 25°C using the absorptivity value (A)
99 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
100 mg/ml [12, 13]. The molecular mass of 57.5 kDa for WBA was used to determine the molar
101 concentration of WBA [4]. The substrate has a weight-average molecular weight of 1.0 x 10^{6}
102 according to the manufacturer, and thus the average degree of polymerization of the glucose unit

103 is estimated to be 6,000. Soluble starch (Lot M7H1482), maltose (Lot M1B6462), glucose (Lot
104 M3G8543), potassium iodide (Lot V1P5303), cellobiose (Lot M2G9713), and other chemicals
105 were purchased from Nacalai Tesque (Kyoto, Japan) and iodine solution (Lot CEM7810),
106 acarbose (Lot LAQ5872), difructose anhydride (Lot TLM1444), α,α-1,1-trehalose (Lot
107 PEH6208; hereinafter designated as simply trehalose), and 1-deoxynojirimycin (Lot DCL2444)
108 were from Wako Pure Chemical (Osaka, Japan).

109

110 *2.2. Inhibition of WBA by glucose, maltose, and other carbohydrates*

111

112 WBA (1.6 μM) and various concentrations of glucose, maltose, and other carbohydrates
113 were prepared in buffer A (pH 5.4) at 25°C. The WBA solution (100 μl) was pre-incubated with
114 100 μl of the carbohydrates solutions at the initial concentrations of 0, 0.15, 0.31, 0.62, 1.23, and
115 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,
116 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,
117 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,
118 0.120, and 0.150 M acarbose; and 0.001, 0.002, 0.005, and 0.009 M 1-deoxynojirimycin for 5 min
119 at 25°C before reacting with the substrate. The soluble starch solution (0.69%, w/v; 450 μl) was
120 mixed with the mixture (200 μl) of WBA and the carbohydrates and was hydrolyzed for 5 min in

121 buffer A at 25°C. Therefore, the WBA and soluble starch concentrations in the initial conditions of
122 the enzyme reaction were 0.25 μM and 0.69 %, w/v, respectively. The reaction was stopped by
123 mixing the reactants (50 μl) with 4.5 ml of 1 mM potassium iodide (KI) prepared in 0.1 N HCl.
124 The absorbance was measured at 580 nm using a Beckman-Coulter DU 800 spectrophotometer
125 (Batavia, IL, USA) [6, 14]. Iodine staining is not preferable method for precise kinetic studies.
126 Nevertheless, it was not possible to use the common product measurement methods such as
127 neocuproine, DNS (dinitrosalicylic acid), and Somogi-Nelson [15] methods which are based on
128 the determination of the reducing ends of the products because some of the carbohydrates
129 examined possess reducing ends. The 50% inhibitory concentrations (IC_{50}) of the carbohydrates
130 are their concentrations giving 50% of the activity observed in the absence of the carbohydrates.
131
132 *2.3. Reversibility of WBA inhibition by glucose and maltose*
133
134 Reversibility of the WBA inhibition by glucose and maltose was examined through
135 dialysis and dilution techniques. In dialysis, 1.6 μM of WBA in buffer A was filtered through
136 Millipore membrane filter (Type HA; pore size: 0.45 μm) and kept in ice for immediate use. The
137 filtered WBA solution (1 ml) was incubated for 5 min with 1 ml of buffer A, or 1 ml of 2 M
138 glucose or 0.5 M maltose prepared in buffer A. The WBA-glucose or WBA-maltose mixtures

139 were dialyzed using a dialysis membrane (Wako Chemicals, Kyoto) against buffer A 100-times
140 the volume of the mixtures; the dialyzing buffer was changed 3 times with 2-h intervals at 4°C.
141 Soluble starch (0.82%, w/v) was hydrolyzed by WBA for 10 min at 25°C. The reaction rate of
142 substrate hydrolysis was determined using iodine staining method and the reaction rates of the
143 dialyzed WBA in the presence of glucose and maltose were compared with the reaction rates in
144 the absence of the inhibitors.

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

156

157 *2.4. Temperature dependence of the inhibitor constants (K_i) for the inhibition of WBA by glucose*

158 *and maltose.*

159

160 Various initial concentrations of carbohydrates in the enzyme reaction: 0.08, 0.15, 0.23,

161 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

162 buffer A at 15, 25, 35, and 45°C. Various initial concentrations of soluble starch: 0.35, 0.52, and

163 0.69% (w/v) were hydrolyzed by 0.32 μM WBA pre-incubated for 5 min with various

164 concentrations of glucose or maltose at each temperature. The initial reaction rates (v) of starch

165 hydrolysis were determined from the reaction progress over 0.5-4.0 min and the reactant (50 μl)

166 was stopped by adding 4.5 ml of the 1 mM iodine solution. The K_i values at each temperature

167 were determined from the Dixon plots [17]. The enthalpy changes (ΔH) of the dissociations of the

168 WBA-glucose and WBA-maltose complexes were determined from the van't Hoff plots [18],

169 while the Gibbs energy changes (ΔG), and entropy changes (ΔS) were determined from Eqs. 1 and

170 2 [18, 19].

171 $$\Delta G = -RT \ln K_i \quad (1)$$

172 $$\Delta S = (\Delta H - \Delta G)/T \quad (2)$$

173 where R is the universal gas constant and T is temperature in Kelvin

174

175 *2.5. pH-Dependence of K_i.*

176

177 Various initial concentrations of soluble starch: 0.35, 0.69, 1.04, 1.38, 1.73, and 2.08%

178 (w/v) were prepared in 20 mM glycine-HCl buffer (pH 3.0), 20 mM sodium acetate buffer (pH

179 5.4; buffer A), and 20 mM borate buffer (pH 9.0) at 25°C following the method previously

180 reported [20]. WBA (0.32 μM) in the respective buffers at each pH was pre-incubated with 0.31,

181 and 0.62 M glucose, and 0.15, and 0.31 M maltose for 4 min at 25°C. The soluble starch solution

182 (450 μl) varying in concentration was mixed with 100 μl of the pre-incubated WBA-glucose or

183 WBA-maltose mixtures at pH 3.0, 5.4, and 9.0 at 25°C. The initial reaction rates (v) were

184 determined from the reaction progress over 5 min. The absorbance was measured at 700 nm using

185 a Beckman-Coulter DU 800 spectrophotometer. The inhibition type and $K_{\rm i}$ values at each pH was

186 determined from Hanes-Woolf plot [21]. The $K_{\rm i}$ values were calculated using Eqs. 3 and 4 for

187 competitive and uncompetitive inhibitors, respectively [22].

188

189 $$K_{\rm i} = [{\rm I}]/ ((K_{\rm m,app}/K_{\rm m}) -1) \quad (3)$$

190 $$K_{\rm i} = [{\rm I}]/ ((V_{\rm max}/V_{\rm max,app}) -1) \quad (4)$$

191

192 where, $K_{\rm m}$ and $V_{\rm max}$ are the Michaelis constant and maximum reaction rate observed in the absence

193 of inhibitors; $K_{m,app}$ and $V_{max,app}$ are those in the presence of inhibitors; and [I] is inhibitor
194 concentration.
195
196 **3. Results**
197
198 *3.1. Inhibition of WBA by glucose, maltose, and other carbohydrates.*
199
200 The rate of soluble starch hydrolysis by WBA was measured from the decrease in
201 absorbance at 580 nm as described in the materials and methods. The catalytic activity of WBA was
202 reduced by increasing the concentrations of maltose or other carbohydrates (Figs. 1). The inhibitory
203 activities of fructose, sucrose, trehalose, difructose
204 (α-D-fructofuranose-β-D-fructofuranose-2',1:2,3'-dianhydride shown in supplementary Fig. 8),
205 cellobiose, and 1-deoxynojirimycin were comparatively weak. On the other hand, acarbose and
206 maltose strongly inhibited WBA activity followed by glucose (Fig. 2A). The inhibition by acarbose,
207 maltose, and glucose were significant with the IC_{50} values of 0.06 ± 0.01, 0.22 ± 0.09, and 1.41 ±
208 0.17 M, respectively (Fig. 2A). The IC_{50} values were not attained by the other carbohydrates
209 examined at the concentrations of their maximal solubility (Fig. 2B). The maximum degrees of
210 inhibitions by 1-deoxynojirimycin, difructose, trehalose, sucrose, fructose, and cellobiose were

211 around 30%, and the IC_{50} values were estimated to be > 9 mM, > 0.3 M, > 2 M, > 0.5 M, > 2 M,

212 and 0.4 M, respectively. The IC_{50} values were the values of $[I]_o$ at which the fractional activity of

213 WBA becomes 0.5 as shown in Fig. 2.

214

215 (Fig. 1)

216 (Fig. 2)

217

218 *3.2. Reversibility of WBA inhibition by maltose and glucose.*

219

220 The reversibility of WBA inhibition by glucose and maltose was studied through both dialysis

221 and dilution methods. The activity of WBA in the absence and presence of inhibitors was measured

222 using blue value method ($\Delta A_{580\ nm}$) after dialysis. The activity of WBA in buffer A (control) was

223 taken as 100%, and the reversibility in the presence of inhibitors after dialysis were calculated

224 relative to the activity of the control (Table 1). The activity of WBA was completely restored

225 through dialysis.

226 In the dilution method, the inhibitor concentrations were diluted 10 times and the effect of

227 dilution was examined following the method described previously [16]. The activity of WBA in the

228 presence of 18 mM maltose was 86% relative to the activity observed in the absence of inhibitors.

229 When 180 mM maltose was diluted to 18 mM, the activity recovered to 89%, and hence the

230 reversibility was 103 ± 6%. In the same manner, various concentrations of glucose and maltose

231 were studied and complete reversibility was observed through dilution (Table 2).

232

233 (Table 1)

234 (Table 2)

235

236 *3.3. Temperature dependence of K_i.*

237

238 The temperature-dependence of the K_i values of glucose and maltose at 15, 25, 35, and 45$^\mathrm{o}$C

239 were estimated at pH 5.4 using the Dixon plots shown in the supplementary Figs. 4 and 5. The K_i

240 values of both inhibitors increased slightly with increasing temperature, indicating that the

241 inhibitory activity of the inhibitors decrease with increasing temperature in the range of 15-45$^\mathrm{o}$C.

242 The thermodynamic parameters: the Gibbs energy change (ΔG), enthalpy change (ΔH), and entropy

243 change (ΔS) of the dissociation of the WBA-glucose or WBA-maltose complexes were determined

244 at various temperatures (Table 3). The van't Hoff plots of the K_i values of WBA inhibition by

245 glucose and maltose with the slope - $\Delta H/R$ were shown in Fig. 3.

246

247 (Fig. 3)

248 (Table 3)

249

250 *3.4. pH-Dependence of K_i.*

251

252 The inhibition types and K_i values of WBA inhibition by glucose and maltose at 25°C were

253 evaluated at pH 3.0, 5.4, and 9.0 using the Hanes-Woolf plots shown in the supplementary Figs. 6

254 and 7. Here, we should note the difference between the inhibitory mechanisms of the competitive

255 inhibition and uncompetitive inhibition. In the case of competitive inhibition, the inhibitor (I) binds

256 only to the enzyme (E) to form the enzyme-inhibitor complex (EI) but does not bind to the

257 enzyme-substrate complex (ES). In the case of uncompetitive inhibition, it binds only to the ES

258 complex to form the enzyme-substrate-inhibitor (ESI) ternary complex but does not bind to E. Thus,

259 the EI complex is not formed in uncompetitive inhibition, whereas the ESI complex is not formed in

260 competitive inhibition. In other words, it binds to the active site of the enzyme in competitive

261 inhibition, but binds to the secondary binding site other than the active site in uncompetitive

262 inhibition. Glucose and maltose bind to the active site of WBA at pH 3.0 and 5.4, and bind to the

263 secondary binding site of WBA at pH 9.0. In competitive inhibition, K_m increases and V_{max} remains

264 constant while both the K_m and V_{max} values decrease in uncompetitive inhibition. It is interesting to

265 note that the K_i values obtained at pH 5.4 for both glucose and maltose are slightly larger than at pH
266 3.0 and 9.0, suggesting that the inhibitory effects of glucose and maltose on WBA at pH 5.4 (which
267 is the optimal pH of WBA) are lower than at pH 3.0 and 9.0, at which the enzyme activity is
268 extremely low. The K_i values determined by the Hanes-Woolf plot and Dixon plot for the respective
269 inhibitors at pH 5.4 and 25°C are in good agreement. The K_i values of glucose and maltose in the
270 WBA inhibition are considered to be the dissociation constant (K_d) values for the WBA-glucose and
271 WBA-maltose complexes. Thus, the ΔG values of the dissociations of the WBA-glucose and
272 WBA-maltose complexes can be determined from K_i values at each pH (Table 4).
273
274 (Table 4)
275
276 **4. Discussion**
277 *4.1. Inhibition of WBA.*
278
279 The catalytic activity of WBA was noticeably inhibited in a dose-dependent manner by acarbose,
280 maltose and glucose. β-Amylases from other crops such as soybean [6], sweet potato [8], and alfalfa
281 [14] were reported to be inhibited by glucose and maltose. In the present study, other sugar
282 analogues and sugar derivatives were systematically selected to evaluate their inhibitory effects on

283 WBA, among which, only acarbose containing acarviosin (α-amylase inhibitor) and maltose moiety
284 was found to expressively inhibit WBA. However, this IC_{50} value of acarbose is very big in view of
285 pharmaceutical industries. The well-known α-amylase inhibitor, acarbose was reported to
286 competitively inhibit barley BA [23]. Acarbose analogues, containing cellobiose and lactose
287 structures were potent competitive inhibitors of β-glucosidase, and the lactose analogues were
288 uncompetitive inhibitors of β-galactosidase [24]. On the other hand, fructose, sucrose, trehalose,
289 difructose, 1-deoxynojirimycin, and cellobiose did not show substantial inhibition on WBA.
290 However, the maximum solubility of cellobiose is very low even at high temperature and hence,
291 difficult to evaluate its inhibitive power.

292 The respective IC_{50} and K_i values of maltose are 0.22 ± 0.09 and 0.12 ± 0.03 M and that of
293 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
294 0.034 ± 0.0 M for maltose and 0.32 ± 0.08 M for glucose were reported in SBA inhibition [6]. This
295 shows that maltose is stronger inhibitor than glucose of not only WBA but also of SBA. From the
296 crystal structure study of maltose and glucose binding of BacBA, it was stated that glucose binds to
297 subsite 1 and maltose binds to subsites 1 and 2 of the active site [2]. This attests that maltose binds
298 at two subsites of BacBA at a time while glucose binds at only a single subsite. Therefore, the molar
299 energy of maltose binding to the active site may be given as the sum of the molar energy of glucose
300 moieties binding to subsites 1 and 2, while the molar energy of glucose binding is that to subsite 1.

301 Hence, the inhibition of maltose is stronger than that of glucose. This is not always true if more than
302 one maltose molecules that bind different binding sites or subsites are considered. In such cases, the
303 binding affinities of the second binding site or subsites are enhanced in positive co-operativity and
304 inhibited in negative co-operativity. By considering the binding modes of glucose and maltose by
305 WBA to be similar to that of BacBA, we can estimate the molar binding energy of the glucose
306 moieties to subsites 1 and 2 separately from Table 4. The binding energy of the glucose moiety to
307 subsite 1 would be -3.43 and -2.33 kJ/mol at pH 3.0 and 5.4, respectively, and those to subsite 2
308 would be -1.81 and -2.20 kJ/mol. From these values, it can be seen that the molecular binding
309 energy to subsite 1 is much lower in magnitude at pH 5.4 than at pH 3.0, although that to subsite 2
310 is higher at pH 5.4 than at pH 3.0, and the molar binding energy of maltose to the active site of
311 WBA is slightly smaller at pH 5.4 than at pH 3.0 as shown in Table 4.
312

313 *4.2. Reversibility of maltose and glucose inhibition.*
314

315 Inhibition of WBA by glucose and maltose was completely reversible. We can also infer from
316 reports [6, 8, 14] that the inhibition was reversible indicating that the molecular liaison between
317 WBA and the sugars is not covalent. The reversibility of WBA inhibition by glucose and maltose
318 was examined by dialysis. The dialysis was conducted at 4°C to avoid the activity loss, and WBA

319 activity was completely reinstated (Table 1). In dialysis, enzymes are restored from the easily

320 dissociable, non-covalently formed enzyme-inhibitor complexes [25]. The reversibility of WBA

321 activity was also confirmed through dilution technique. Similar dilution principles were reported on

322 the reversibility study of the inhibition of thermolysin [16] and neuraminidase [26] by alcohols.

323 Contrary to the dialysis method, low concentration of the inhibitor remains in the reaction after

324 dilution. Due to this fact, reversibility was calculated relative to the initially low inhibitor

325 concentration the same as after 10 times dilution. It was explained that the drastic activation of

326 neuraminidase activity after dilution could be because of the change in the conformation and

327 hydrated state of the enzyme, in addition to the change in the viscosity of the reaction medium [26].

328

329 *4.3. Temperature dependence of K_i.*

330

331 The inhibitor constant (K_i) values of EI complex dissociations were affected by the change in

332 reaction temperature which influences the molecular activities of the solvent, enzyme, and

333 inhibitors. It also affects the structure of the protein, which entails change in enzyme-inhibitor

334 binding or dissociation [19, 27]. The K_i values increased with temperature (Table 3) which agrees

335 with reports on various enzymes and inhibitors showing an increase in K_i with temperature [16, 18,

336 19, 27]. The K_i values at various temperatures were determined from the Dixon plots in this study

337 following the method previously reported [17, 28]. Kitagishi *et al.* [27], have recommended that

338 inhibitor concentrations close to the K_i values should be used in order to determine the K_i values

339 accurately and in conditions where $[E]_o << [I]_o$, the K_i values can be obtained from the Dixon

340 plot. Hence, the concentration of enzyme is much less than the inhibitor concentrations in our study.

341 Temperature variation exerted significant influence on the thermodynamic parameters of

342 the EI complexes dissociations (Table 3). The positive ΔH values of the dissociations of the

343 WBA-glucose and WBA-maltose complexes indicate that the dissociations were endothermic. The

344 ΔG, a state function reaction change of the system determines the relative importance of the

345 enthalpy and entropy terms as driving forces behind a particular reaction [29]. The positive values

346 of ΔG indicate non-spontaneous dissociations of the EI complexes. Since the enthalpy terms are

347 greater in magnitude than the entropy terms, the EI dissociations are enthalpy-driven. The ΔG

348 values were found to decrease with increasing temperature. The source of the energy demand for

349 the dissociations is the partial hydrogen-bond breakage in the system [30]. The bindings of glucose

350 and maltose to the active site of BacBA are mainly by hydrogen-bonds from the crystallographic

351 study [2]. It can be elucidated from these facts that there is hydrogen-bond cleavage with the

352 dissociations of the WBA-glucose or WBA-maltose complexes. In general, the hydrogen-bond

353 dissociation energies are 20 kJ mol^{-1} but it can span more than two orders of magnitude (about 8 -

354 100 kJ mol^{-1}) depending on the nature of the interactions and environments [30]. The ΔG required

355 for the dissociation of the WBA-maltose complex is almost twice as that of the WBA-glucose

356 complex (Tables 3 and 4), which is in good agreement with the structural compositions of the

357 inhibitors.

358

359 *4.4. pH-Dependence of* K_i.

360

361 The inhibition type of WBA by both glucose and maltose was affected by pH. Both sugars

362 behaved as competitive inhibitors at pH 3.0 and 5.4 at 25°C but exhibited uncompetitive type of

363 inhibition at pH 9.0. Nomura *et al*. [6] reported that the inhibition type on SBA by maltose and

364 cyclohexa-amylose was competitive at pH 5.4 but it was mixed-type by glucose at pH 5.4 (I binds

365 both E and ES to form EI and ESI complexes), and competitive by all the sugars at pH 8 and form

366 solely EI complexes. Change of the inhibition type depending on pH was also reported in aspartic

367 protease [31] and pepsin [32]. The isoelectric point (p*I*) value of WBA was reported to be 5.8 [33].

368 The pK_{e1} (where the ionizable group 1 of SBA is deprotonated) and pK_{e2} (where the ionizable group

369 2 of SBA is protonated) were 3.41 and 8.09 with p*I* value of 5.63 [34, 35]. This is to indicate that at

370 pH 3.0, WBA has a net positive charge. In this state, both glucose and maltose bind to the active site.

371 At pH 9 when the net charge of WBA becomes negative, the sugar molecules bind the secondary

372 binding sites on the EI complex and behaved as uncompetitive inhibitors. According to the subsite

373 model of amylases [4, 36], the inhibition type depends on the binding site of the inhibitors [37].

374 The K_i values of the dissociations of the WBA-glucose and WBA-maltose complexes were
375 also pH dependent (Table 4). The pH-dependences of K_i in various enzymes and inhibitors were
376 reported with the changes in K_i being different depending on the response of particular enzymes to
377 the pH changes [6, 17, 27, 31, 32]. This phenomenon apparently suggests that pH either induces
378 conformational or change in ionization states of binding residues of an enzyme. Our result in Table
379 4 shows that the inhibitive power of the inhibitors declines with increasing the activity of the
380 enzyme at optimum pH.

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

388

389

390 **5. Conclusions**

391

392 The end products of starch hydrolysis, maltose and glucose inhibit WBA dose-dependently and

393 the interaction is reversible. The catalytic activity of WBA is more strongly inhibited by acarbose
394 than by maltose and glucose. The inhibitor constant (K_i) of glucose and maltose are temperature
395 dependent. The dissociations of the WBA-glucose and WBA-maltose complexes are endothermic
396 and enthalpy-driven. Both glucose and maltose bind the active site when WBA is protonated at pH
397 3.0 and 5.4 and to the secondary binding sites when its net charge becomes negative at pH 9.0. A
398 change in pH sways the active site integrity in binding the inhibitors or induces a conformational
399 change to WBA and hence, affects the type of inhibition. Therefore, both the temperature and pH of
400 starch hydrolysis operations require careful manipulation to minimize end-product inhibition in
401 starch-saccahrifying industries.
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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. Panle 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.

	Control (buffer)	0.36 M glucose	0.09 M maltose
v before dialysis	0.034	0.020	0.017
(Relative activity)	(100%)	(59%)	(49%)
v after dialysis	0.030	0.030	0.029
(Relative activity)	(100%)	(98%)	(96%)

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.

	$[I]_o$			Reversibility (%)
	0.02 M	**0.18 M**	**0.18 M →0.02 M**	
Glucose	82 ± 11	74 ± 6	85 ± 3	104 ± 3
Maltose	86 ± 3	26 ± 12	89 ± 2	103 ± 6
	$[I]_o$			
	0.03 M	**0.27 M**	**0.27 M →0.03 M**	
Glucose	97 ± 7	68 ± 6	99 ± 4	103 ± 12
Maltose	86 ± 8	15 ± 1	90 ± 17	106 ± 18

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.

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

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

ΔH 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 ΔG and ΔS 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.

		pH		
		3.0	5.4	9.0
Glucose	K_i (M)	0.25 ± 0.03	0.39 ± 0.03	0.21 ± 0.03
	ΔG (kJ mol^{-1})	3.43 ± 0.13	2.33 ± 0.15	3.86 ± 0.34
	Inhibition type	competitive	competitive	uncompetitive
Maltose	K_i (M)	0.12 ± 0.04	0.16 ± 0.03	0.11 ± 0.04
	ΔG (kJ mol^{-1})	5.24 ± 0.13	4.53 ± 0.07	5.46 ± 0.10
	Inhibition type	competitive	competitive	uncompetitive

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.

0.7
0.6
0.5
0.4
0.3

$A_{580\ nm}$

0 1 2 3 4

Reaction time (min)

Top

Fig. 1A

0.7
0.6
0.5
0.4
0.3

$A_{580\ nm}$

0 1 2 3 4

Reaction time (min)

Top

Fig. 1B

Fractional activity

0.8

0.4

0

0 1 2 3 4

Log $[I]_o$

Top

Fig. 2A

Fractional activity

0.8

0.4

0

0 1 2 3 4

Log $[I]_o$

Top

Fig. 2B

-0.5

-1.5

-2.5

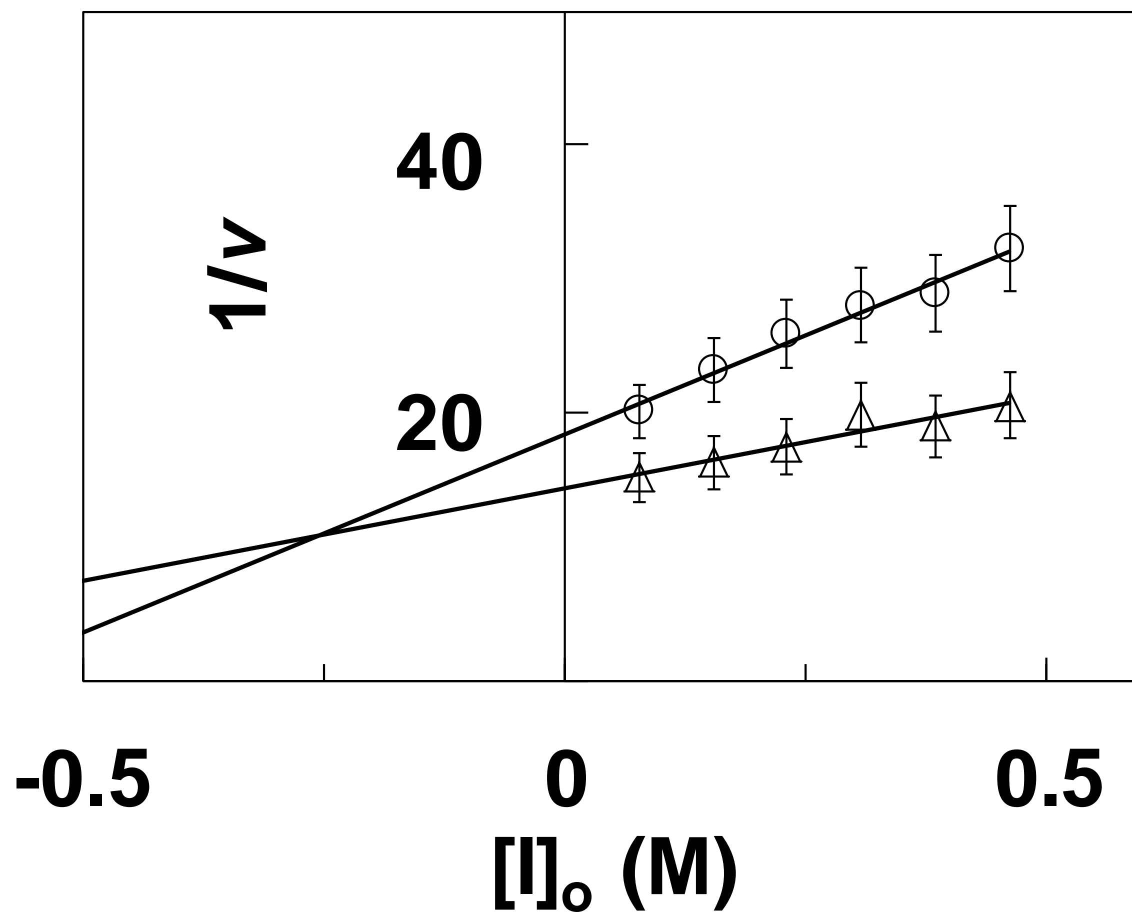
ln K_i

3.1 3.2 3.3 3.4 3.5

$1/T \times 10^3$ (K^{-1})

Top

Fig. 3



Top

⇧

Fig. 4A

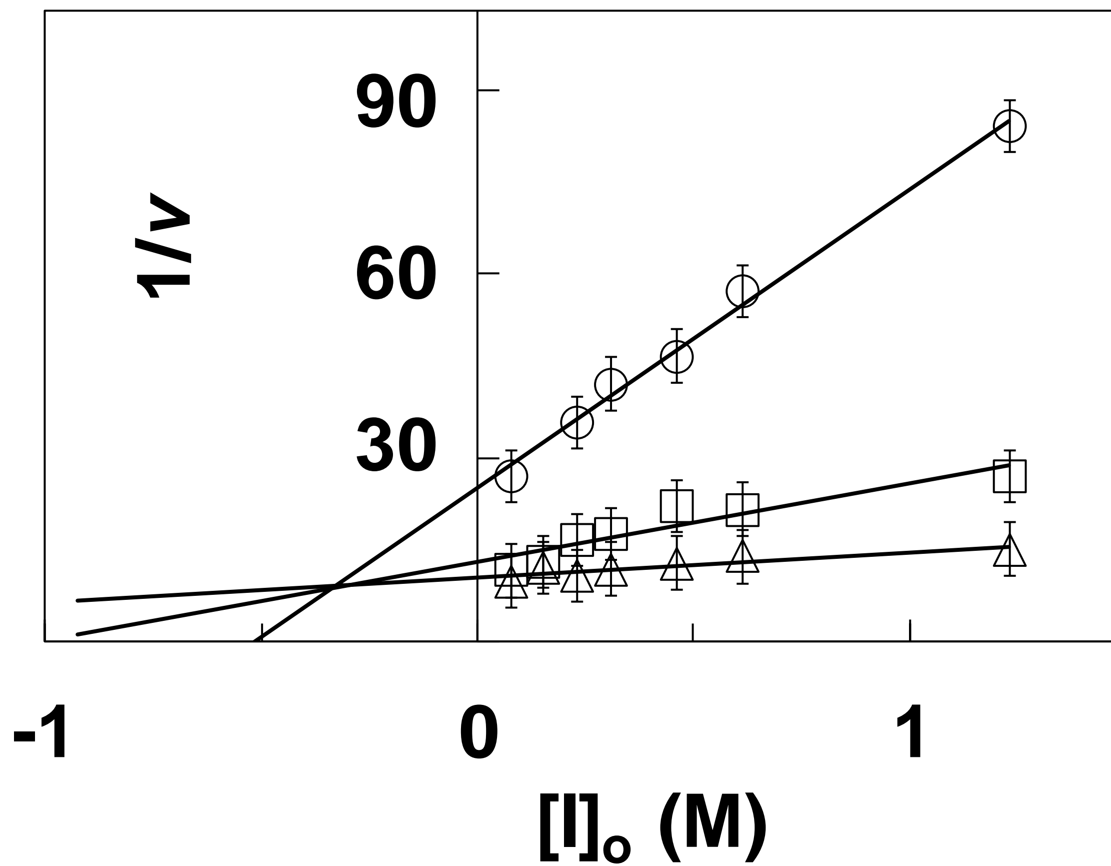


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Fig. 4B

1/v

40

20

-0.5 0 0.5

$[I]_o$ (M)

Top

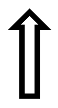


Fig. 4C

40

1/v

20

-0.5

0

0.5

$[I]_o$ (M)

Top

Fig. 4D

40

1/v

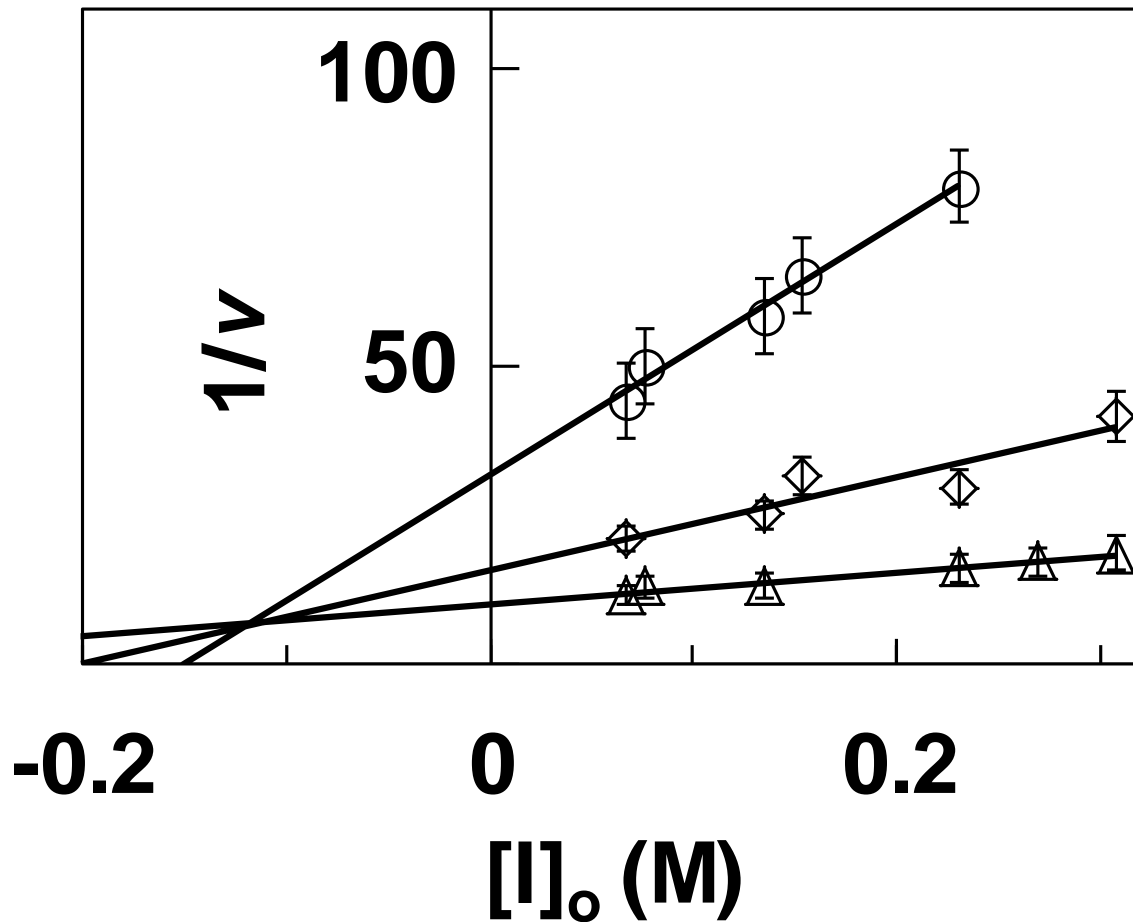
20

-0.3 -0.1 0.1 0.3

$[I]_o$ (M)

Top

Fig. 5A



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⇑

Fig. 5B

90

60

1/v

30

-0.3 0 0.3

$[I]_o$ (M)

Top

Fig. 5C

40

1/v

20

-0.3 -0.1 0.1 0.3

$[I]_o$ (M)

Top

Fig. 5D

[S]/v

60

30

-1 0 1 2

$[S]_o$ (%, w/v)

Top

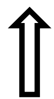


Fig. 6A

[S]/v

60

30

-1 0 1 2 3

$[S]_o$ (%, w/v)

Top

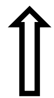


Fig. 6B

[S]/v

90

60

30

-1 0 1 2

[S] (%, w/v)

Top

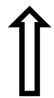


Fig. 6C

60

40

20

$[S]/v$

-1 0 1 2

$[S]_o$ (%, w/v)

Top

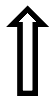


Fig. 7A

60

40

20

[S]/v

-1 0 1 2

$[S]_o$ (M)

Top

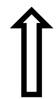


Fig. 7B

120
80
40
[S]/v
-1 0 1 2
$[S]_o$ (%, w/v)

Top

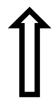
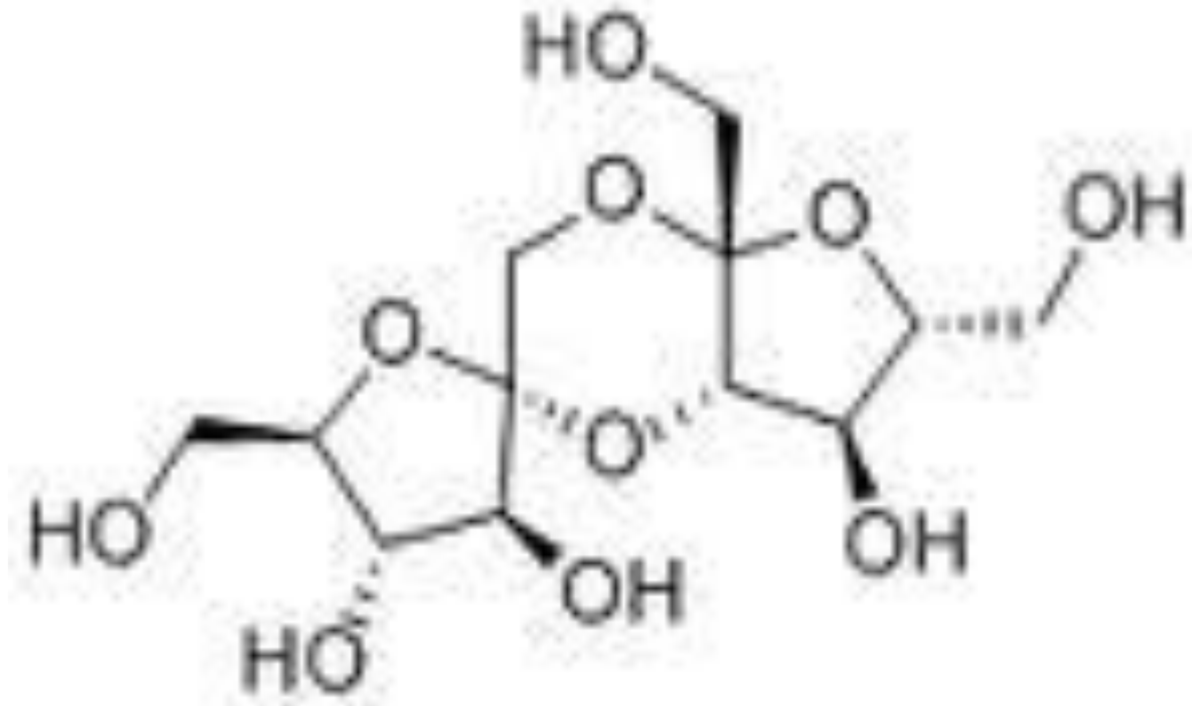


Fig. 7C



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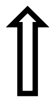

Fig. 8