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Characterization and solvent engineering of wheat β-amylase for enhancing its activity and stability

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ABSTRACT

The kinetic and thermodynamic parameters of wheat β-amylase (WBA) were characterized and various additives were evaluated for enhancing its activity and thermostability. WBA activity was examined by neocuproine method using soluble starch as substrate. The Michaelis constant (K_m) and molecular activity (k_{cat}) were determined to be 1.0 ± 0.1 % (w/v) and 94 ± 3 s^{-1}, respectively, at pH 5.4 and at 25°C. The optimum reaction temperature (T_{opt}) for WBA activity was 55°C and the temperature (T_{50}) at which it loses half of the activity after 30-min incubation was 50 ± 1°C. Modifications of the solvent with 182 mM glycine and 0.18% (w/v) gelatin have increased the T_{50} by 5°C. Glycerol, ethylene glycol, dimethylformamide (DMF) and dimethyl sulfoxide have also slightly enhanced the thermostability plausibly through weakening the water structure and decreasing the water shell around the WBA protein. Ethanol and DMF activated WBA by up to 24% at 25°C probably by inducing favorable conformation for the active site or changing the substrate structure by weakening the hydrogen bonding. Its half-life in the inactivation at 55°C was improved from 23 to 48 min by 182 mM glycine. The thermodynamic parameters indicate that WBA is thermo-labile and sufficient stabilization was achieved through solvent modification

with additives and that the heat inactivation of WBA is entropic-driven. It is suggested that WBA could be applied more widely in starch-saccharification industries with employing suitable additives.

Keywords: activation; additives; β-amylase; thermostablility; solvent engineering, wheat

1. Introduction

β-Amylase hydrolyses the α-1, 4-glucan bonds in amylosaccharide chains from the non-reducing ends and generates maltose. It has considerable application in the production of high maltose syrups together with starch debranching enzymes and α-amylases. β-Amylase has been well characterized in higher plants [1-3] and micro-organisms [4-7]. However, the well-characterized β-amylases are neither active nor stable at higher temperatures > 65^{o}C [8]. In many findings, α-amylase is more stable compared to β-amylase of the same origin [9-11]. Enzymes may easily be denatured by slight change of the environmental conditions such as temperature, pressure, pH, and ionic strength [9]. Nevertheless, stabilization of the enzymes could be achieved in several ways: screening for more stable ones (favorably from thermophiles and extremophiles), chemical modification, site-directed mutagenesis, immobilization and solvent engineering or modifying the enzyme reaction conditions with stabilizing additives [12-17]. In solvent engineering, selection of appropriate additives is dependent on the nature of the enzyme and there are no established rules to select effective additives for improving enzyme functions [18]. The thermostabily of β-amylase has been substantially enhanced by modifying the solvent with additives [18, 19].

Different amylases give oligosaccharides with specific lengths of end products. For this reason, amylases with unique properties need to be studied for various applications in starch-saccharification for production of food and bio-ethanol [20]. Unlike soybean, barely and sweet potato, wheat was not the common source of β-amylase for starch-saccharification so far. However, the sources of β-amylase supply has drastically changed due to the escalating prices of the major sources like soybean. Wheat β-amylase (WBA) is prepared from wheat bran, which is an industrial by-product in the production of wheat starch and gluten. It is a cheaper alternative source of β-amylase for industries. Nevertheless, it is lower in thermostability as compared with β-amylases of other crops and microbes. For instance, the optimum temperature (T_{opt}) of Clostridium thermosuiphurogenes β-amylase is 75^{o}C [8]; the temperature at which it loses half of its activity after 30-min incubation (T_{50}) of barely β-amylase is 56.8^{o}C and that of soybean β-amylase is 63^{o}C [21] while the T_{opt} and T_{50} after 30-min incubation of WBA are 55 and 50^{o}C, respectively (Data obtained in this study).

Therefore, enhancing the activity and thermostability of WBA has an excellent prospect for starch-saccharification industries. In this study, we used a commercially-available WBA preparation, Himaltosin, without further purification

because it is already purified from other protein contaminants and utilized industrially. In this paper, we describe the kinetic and thermodynamic properties of WBA and improvement in its activity and thermostability via solvent engineering using various additives. This suggests that WBA would be likely applicable to a wide range of starch-saccharification industries.

2. Materials and methods

2.1. Materials

Himaltosin GS (Lot 2S24A), a commercial preparation of WBA, was purchased from HBI Enzymes (Osaka, Japan). This preparation was filtered with a Millipore membrane filter (Type HA; pore size: 0.45 μm) and used without further purification. According to the manufacturer, the Himaltosin preparation contains 90% starch as a stabilizer, and almost all of the protein is β-amylase and α-amylase was not detected. Himaltosin was suspended to 20 mM sodium acetate buffer (pH 5.4) at 25^{o}C to be 0.3 mg/ml. In this paper, this buffer was hereinafter referred to as buffer A. The WBA protein content was expected to be 0.03 mg/ml in the suspension and it was

followed by filtration with the Millipore membrane filter. However, the protein concentration in the filtrate was less than 10% of the expected content, suggesting that > 90% of the WBA protein was remained with starch on the filter. The WBA concentration was determined spectrophotometrically in buffer A using the absorptivity value (*A*) of 1.40 ± 0.02 at 281 nm with a 1.0-cm light-path for the WBA solution at the concentration of 1.0 mg/ml [22]. The molecular mass of 57.5 kDa for WBA was used to determine the molar concentration of WBA (see sections 2.3 and 3.1). Under the standard condition in this study, the concentration of WBA in the enzyme-reaction solution was set to 15.0-30.0 nM. The starch concentration due to the stabilizer starch (0.027%, w/v) was completely removed by filtration with the Millipore membrane filter. Soluble starch (Lot M7H1482) as substrate and maltose (Lot M1F7568) as standard for the activity assay were obtained from Nacalai Tesque (Kyoto, Japan). The substrate has a weight-average molecular weight of 1.0×10^6 according to the manufacturer, and thus the average degree of polymerization of the glucose unit is estimated to be 6,000. Neocuproine-HCl (2, 9-dimethyl-1, 10-phenanthroline, Lot 032K2533) as coloring reagent B in the neocuproine method was from Sigma (St. Louis, MO, USA). Coloring reagent A (0.38 M Na_2CO_3, 1.8 mM $CuSO_4$, and 0.2 M glycine) in the neocuproine method and all other chemicals were purchased from Nacalai Tesque. All enzyme

127 reactions were carried out in buffer A, pH 5.4.

128

129 *2.2. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*

130

131 SDS-PAGE was performed in a 10% polyacrylamide gel under reducing
132 conditions [23]. The Himaltosin preparation was suspended in buffer A to the
133 concentrations of 1, 2, and 5% (w/v). The solution was filtered with a Millipore
134 membrane filter (Type HA) and reduced by treatment with 10 mM dithiothreitol (DTT).
135 The solution was applied to SDS-PAGE with a constant voltage of 150 V for 80 min.
136 Proteins were stained with Coomassie Brilliant Blue R-250. The molecular-mass marker
137 kit consisting of rabbit muscle phosphorylase *b* (97.2 kDa), bovine serum albumin (66.4
138 kDa), hen egg albumin (44.3 kDa), and hen egg white lysozyme (14.4 kDa) was a
139 product of Takara Bio (Otsu, Japan).

140

141 *2.3. Measurement of enzyme activity*

142

143 Various initial concentrations of the soluble starch substrate [0.00, 0.09, 0.45,
144 0.90, 1.13, 1.35, 1.80, 2.02, 2.25, 2.70, 2.93, and 3.15% (w/v)] in the reaction solution

were prepared in buffer A at 25°C. The WBA solution in the same buffer was filtered with a Millipore membrane filter (Type HA) and kept in ice water for immediate use. The various concentrations of starch were hydrolyzed by WBA (30.0 nM) for 0, 2.5, 5.0, 7.5, and 10.0 min at 25°C. The reaction was stopped by adding 300 μl of 0.1 M NaOH into 100 μl of the enzyme-reaction solution. The amount of the reducing sugar in the enzyme-reaction solution was determined by the neocuploine method as follows [24]. Reagent A and reagent B, 250 μl each, were mixed with 50 μl of the enzyme-reaction solution, boiled for 8 min, and diluted with 550 μl of water after cooling in ice water. The activity was measured at 450 nm using a Beckman-Coulter DU 800 spectrophotometer (Batavia, IL, USA) [25, 26]. The enzyme activity was determined by measuring the velocity (v) of reducing sugar production, and the reaction velocity was analyzed by the Michaelis-Menten kinetics. The maximum velocity (V_{max}) and Michaelis constant (K_m) were obtained from the v vs. the substrate concentration ([S]) plots using KaleidaGraph 4.1 (Synergy Software, Reading, PA, USA). The molecular weight of WBA is 57,500 [27] and was used for the evaluation of the molecular activity (k_{cat}) (see section 3.1).

2.4. The optimum temperature

Starch solution (900 μl) in buffer A was mixed with the WBA solution (0.150 μM; 100 μl) in the same buffer for 0, 2.5, 5.0, 7.5, and 10.0 min at 25°C after incubation at 15, 25, 35, 45, 55, 65, and 75°C in a water bath for 10 min. The initial concentrations of starch and WBA in the reaction solution were 1.80% and 15.0 nM, respectively. The reaction was stopped by adding 300 μl of 0.1 M NaOH into the enzyme-reaction solution (100 μl). Then the enzyme activity was determined by the neocuproine method (see above). The optimal reaction temperature at which WBA exhibited the highest activity was referred to as the optimal temperature (T_{opt}).

2.5. Thermal inactivation of WBA

The substrate and WBA solutions were prepared in buffer A. The enzyme solution was incubated at 25, 35, 45, 55, and 65°C for 10, 20, and 30 min and cooled at 25°C for 3 min in a water bath. The substrate solution (2.00%, w/v; 900 μl) was mixed with the heat-treated WBA solution (0.300 μM; 100 μl) at 25°C and incubated for 0, 2.5, 5.0, 7.5, and 10.0 min. The initial concentrations of substrate and enzyme in the reaction solution were 1.80% and 30.0 nM, respectively. The activity was assayed as

aforementioned by the neocuproine method and the first-order rate constant k of the thermal inactivation was determined assuming pseudo-first order kinetics by plotting ln (v/v_o) against the heat-treatment time (t) (Eq. 1), where v is the initial reaction velocity of the enzyme with heat treatment at each incubation temperature and v_o is that obtained without heat treatment and at 25°C. The activation energy E_a of the thermal inactivation was obtained by the Arrhenius plot (Eq. 2), and the standard Gibbs energy difference of activation for thermal inactivation ($\Delta G^{o\ddagger}$), the standard enthalpy difference of activation ($\Delta H^{o\ddagger}$), and the standard entropy difference of activation ($\Delta S^{o\ddagger}$) were obtained from the Eyring plot according to Eqs. 3 and 4 [24, 28].

$$\ln (v/v_o) = k\,t \quad (1)$$

$$\ln k = -(E_a/R)(1/T) \quad (2)$$

$$\Delta G^{o\ddagger} = -RT[\ln hk/k_B T] \quad (3)$$

$$\ln (hk/k_B T) = (\Delta H^{o\ddagger}/RT) + (\Delta S^{o\ddagger}/R) \quad (4)$$

where k_B, h, and R are the Boltzmann, Plank, and gas constants, respectively. T is temperature in Kelvin.

2.6. Activation and thermostablization of WBA using additives

The WBA solution in buffer A was mixed and incubated with equal volume of various additives in the same buffer at 25, 45, 55, and 65^{o}C in a water bath for 30 min before hydrolyzing soluble starch. The initial concentrations of WBA and substrate in the reaction solution were 30.0 nM and 1.80%, respectively. The additive concentrations in the reaction solution were: 45.5, 91, 182, and 364 mM glucose, NaCl, and glycine; 45.5 and 91 mM L-arginine; 0.9, 1.8, 3.6, and 45.5 mM of L-aspartate; 45.5 and 91 mM of L-cysteine and glutathione (GSH); 0.18 and 0.45% (w/w) gelatin; 0.91, 1.82, and 5.5% (w/w) ethanol and 2-methyl-2-butanol (2M2B); 0.45 and 2.7% (w/w) dimethyl sulfoxide **(**DMSO) and dimethylformamide (DMF); and 0.91 and 5.5% glycerol, ethylene glycol (EG), and β-mercaptoethanol (βME). Their effects on activation and thermal stabilization of WBA were examined. The enzyme-additive mixture solution (0.20 ml) was diluted with water (0.55 ml), and the enzyme activity was measured by the neocuproine method.

3. Results

3.1. Kinetic parameters of WBA

The WBA preparation, Himaltosin, shows a single band in SDS-PAGE with molecular mass of 57.5 kDa (Fig. 1). This value is in good agreement with those (54.0-64.2 kDa) so far reported [22, 27]. In this paper, we used 57.5 kDa for the molecular mass of WBA. This indicates that the Himaltosin preparation is composed of solely β-amylase as a protein component, although it contains 90% (w/w) starch as a stabilizer. Substantially, there was no starch carried over from the stabilizer starch into the reaction solution as examined by the starch-iodine reaction. Therefore, the enzyme preparation was treated as WBA without further purification.

(Fig. 1)

The initial velocity (v) of the WBA-catalyzed hydrolysis of different concentrations of soluble starch was examined in buffer A at 25^{o}C at the enzyme concentration of 30.0 nM. The dependence of v on substrate concentration exhibited the Michaelis-Menten profile (Fig. 2). The K_m, V_{max}, and k_{cat} were determined to be 1.0 ± 0.1 % (w/v); 2.8 ± 0.1 μM s^{-1}, and 94 ± 3 s^{-1}, respectively, by fitting the experimental data to the Michaelis-Menten equation.

(Fig. 2)

3.2. Thermal inactivation of WBA

WBA at the concentration of 0.300 μM was treated thermally as described in the Material and methods section, and the WBA concentration in the enzyme-reaction solution was fixed to 30.0 nM. The enzyme activity was decreased with the progress of the heat treatment at every temperature examined (Fig. 3). The enzyme activity in the hydrolysis of soluble starch was evaluated by measuring the initial velocity (v) in the same buffer at 25°C. The v value measured in the 0-min incubation at each temperature was designated as v_o. The relative activity (v/v_o) observed after incubation at various temperature decreased progressively with increasing the incubation time. The semi-log plots of v/v_o against incubation time showed linear relationship at the respective incubation temperatures (Fig. 3), indicating that the thermal inactivation process of WBA follows the first-order kinetics. The first-order rate constant (k) at the indicated incubation temperature was evaluated from the slope of the plot.

(Fig. 3)

253

254 The activation energy E_a value of the thermal inactivation of WBA in buffer A was 36 ±

255 1 kJ mol^{-1} from the slope of the Arrhenius plot (Fig. 4). The $\Delta G^{o\ddagger}$, $\Delta H^{o\ddagger}$, and $T\Delta S^{o\ddagger}$

256 values for the thermal inactivation were found to be 90 ± 1, 33 ± 1, and -59 ± 1 kJ mol^{-1}

257 respectively, at 25°C from the slope of the Eyring plot (Fig. 5). As E_a is defined

258 theoretically as $\Delta H^{o\ddagger} + RT$, the $\Delta H^{o\ddagger}$ value is calculated to be 34 ± 1 kJ mol^{-1}, which is

259 in good agreement with the value obtained from the Eyring plot.

260

261 (Fig. 4)

262 (Fig. 5)

263

264 *3.3. The optimum temperature of WBA*

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266 WBA activity was measured at various reaction temperatures (Fig. 6). The

267 maximal activity in starch hydrolysis was obtained at 55°C, being the optimal

268 temperature (T_{opt}) of this enzyme in buffer A, pH 5.4.

269

270 (Fig. 6)

3.4. The T_{50} of WBA

The heat inactivation of WBA was studied by incubating the enzyme at various temperatures for 30 min before hydrolyzing the substrate. The enzyme activity was observed to decline with heat treatment even at moderate temperatures (45 and 55^{o}C). The T_{50} of WBA, which is the temperature at which the enzyme loses half of its activity with thermal treatment for 30 min was determined to be 50 ± 1^{o}C (Fig. 7) by plotting the residual activities (%) against temperature of incubation. WBA has lost 30% of its activity at 45^{o}C, 76% at 55^{o}C, and 95% at 65^{o}C after 30 min of incubation.

(Fig. 7)

3.5. Effect of additives on WBA activity

The catalytic activity of WBA in starch hydrolysis was examined in the presence of various additives at 25^{o}C without heat treatment. Majority of the evaluated additives did not enhance the enzyme activity (Fig. 8). However, it is noted that ethanol

289 at the concentration of 0.91-5.5%, w/w (or 0.20-1.20 M) enhanced the activity by 24%
290 and lower concentration (1.8%) of DMF by 11%. On the other hand, the activity was
291 not much affected with the addition of NaCl and glycine up to 364 mM; and DMF,
292 DMSO, and EG up to 5.5%. The addition of cysteine up to 91 mM and aspartate up to
293 45.5 mM showed no substantial effect on the activity, and gelatin (0.18-0.45%, w/w)
294 had no effect either. The other additives inhibited the activity to varying degrees.
295 Especially, the activity was reduced to almost 10% with the addition of 0.91-5.5%, w/w
296 (or 0.11-0.71 M) βME; and to zero with 45.5-91 mM arginine. The activity decreased
297 with increasing the glucose and GSH concentrations, and 50% of the activity was lost
298 with 182 mM glucose; and 75% was with 91 mM GSH.
299
300 (Fig. 8)
301
302 *3.6. Effect of additives on the thermostabilty of WBA*
303
304 The rate of starch hydrolysis by heat treated WBA was examined in modified
305 solvent by various additives. Glycine was found to be the best in improving the thermal
306 stability of WBA followed by gelatin. The T_{50} of WBA was improved by 6^{o}C with 182

307 mM glycine and by 4°C with 0.18% (w/w) gelatin (Fig. 9). The half-life times of the
308 enzyme were enhanced by 25 min and 17 min with glycine and gelatin, respectively, at
309 55°C (Table 1). It should be noted that the enzyme activities remained after the thermal
310 treatment at 45°C was 97% and 92% in the presence of 0.18% (w/w) gelatin and 182
311 mM glycine although it was only 75% in the absence of additives. Aspartate (45.5 mM)
312 and DMSO (5.5%, w/w) had no effect on the stability of WBA. βME (0.91%); ethanol
313 (0.91%); NaCl (182 mM); and arginine (45.5 mM) slightly decreased the stability of
314 WBA, and the T_{50} values were 46-47°C. Glucose (182 mM), cysteine (46 mM), and
315 GSH (91 mM) considerably decreased the stability with the T_{50} values of 35-45°C.
316
317 (Fig. 9)
318 (Table 1)
319
320 **4. Discussion**
321
322 In general, depending on the nature and concentration of the additives, they
323 affect the protein conformation in: (a) screening effect, where the electrostatic repulsion
324 between similarly charged groups of proteins is reduced by cosolvent ions; (b)

325 solvophobic effect, where ion pair formation occurs, favoring protein folding; and (c)
326 modification of water structure leading to hydrophobic interactions in proteins [10, 29].
327 The catalytic activity and thermostability of various enzymes were considerably
328 enhanced through solvent engineering [17-19, 30, 31].

329 The Michaelis constant (K_m = 1.0 ± 0.1 %, w/v) and molecular activity (k_{cat} =
330 94 ± 3 s^{-1}) of WBA was evaluated at pH 5.4 and at 25^{o}C using soluble starch as
331 substrate (Fig. 2). Similarly, the K_m of glutenin-adsorbed WBA was reported to be
332 0.15% (w/v) using soluble starch substrate [32], indicating that WBA (which is free in
333 the reaction solution) used in our study has lower affinity to soluble starch as compared
334 with WBA adsorbed on glutenin. The other available reports on the kinetic parameters
335 of WBA were using maltotriose, maltoheptaose, amylodextrin, and amylopectin
336 substrates under various reaction conditions [27, 33]. The K_m values of plant β-amylases
337 have been reported to be in the range of 0.2-0.7 % (w/v) for soluble starch. The value
338 obtained in this study is apparently higher than those. However, the kinetic parameters
339 for WBA and other plant β-amylases have not yet examined under the same reaction
340 conditions using the same soluble starch preparation. Therefore, it is not suitable to
341 discuss the enzyme functions by comparing the kinetic parameters so far reported. In
342 this paper, we have reported the kinetic parameters (K_m and k_{cat}) of WBA obtained under

343 the fixed conditions with the WBA and soluble starch preparations in the same lot. It
344 should be noted that the activity and stability of WBA are affected easily by additives
345 added in the reaction solution. This means that the kinetic parameters of WBA must be
346 evaluated with the data collected from the experiments conducted carefully under the
347 same conditions.

348 Thermodynamic parameters for the heat inactivation of WBA showed that the
349 $T\Delta S^{o\ddagger}$ value (59 ± 1 kJ mol^{-1}) was greater than the $\Delta H^{o\ddagger}$ value (33 ± 1 kJ mol^{-1}), and
350 thus it can be inferred that the heat inactivation of the enzyme is entropic-driven. The
351 optimum reaction temperature (T_{opt}) of WBA is 55°C (Fig. 6). It has been reported that
352 the T_{opt} of Sorghum bicolor cv β-amylase is 50°C [11, 20, 34], while α-amylase from
353 the same cereal crop has T_{opt} of 70°C corresponding with the fact that β-amylases in
354 most of the cases are lower in thermostability than their respective α-amylases of the
355 same origin [10, 11]. The T_{50} of WBA was determined to be 50 ± 1°C (Fig. 6), which is
356 lower than those of soybean β-amylase, 63.2°C, and of barely β-amylase, 56.8°C [21].

357 In the present study, we have examined the effects of additives on the kinetic
358 parameters and thermostability of WBA. The additives are supposed to have effects on
359 the structures of WBA, soluble starch substrate, and bulk water. For example, when we
360 observed decrease in activity by the addition of an additive, there might be some

361 reasons considered such as inhibition of the enzyme by the additive, conformational
362 changes of the enzyme and/or starch substrate by the additive, etc. Therefore, strict
363 interpretation of the molecular effects of the additives on the activity and stability of
364 WBA seems to be difficult, and further studies must be needed. Thus in this paper we
365 tried to present the effects of the additives without describing the molecular-mechanistic
366 insights of the cause for the effects, although some possible comments have been made
367 with references to other enzymes. The molecular-mechanistic study for the effects of the
368 representative additives is underway.

369 Ethanol and low concentration of DMF have enhanced the activity of WBA by
370 up to 24% (Fig. 8). The activation of acetylcholinesterase (AchE) by ethanol was
371 reported and explained as that it non-competitively alters the hydrophobic-interaction
372 site and subsequently induces favorable conformation to the active center [35]. However,
373 higher concentration of ethanol had a destabilization effect on WBA (Fig. 9B). This
374 agrees with a finding that a high concentration of ethanol (> 800 mM) can perturb the
375 structure of water around hydrophilic area of AchE causing instability to the
376 conformation of the enzyme [35]. This might be the case for the effects of ethanol on
377 WBA. These effects should be considered also from the viewpoint of solvent polarity
378 (see below). The effects of various alcohols on the enzyme structure and activity have

379 been extensively studied with thermolysin, a thermophilic and halophilic
380 metalloproteinase produced by Bacillus thermoproteolyticus. Thermolysin is
381 remarkably activated and stabilized by neutral salts such as NaCl, and is inhibited by
382 increasing concentration of alcohols and the degree of inhibition is dependent on the
383 size of alcohols [36]. It is interesting to note that alcohol such as 2-methyl-1-propanol
384 (2MP) which binds tightly to the active site inhibits thermolysin strongly, whereas the
385 alcohols with the sizes larger or smaller than 2MP bind weakly to the active site and
386 inhibit thermolysin weakly [37]. This suggests that the enzyme activity could be
387 controlled intentionally using additives with suitable sizes. These lines of evidence have
388 provided information for the optimal conditions for thermolysin-catalyzed synthesis of a
389 precursor of the sweetener, aspartame [14]. As shown with thermolysin to find the
390 optimal conditions for its inhibition by alcohols, it should be possible to find the optimal
391 conditions for industrial application of WBA by examining the effects of alcohols on the
392 activity and stability of WBA by changing systematically the size of alcohols.

393 Contrary to the effect of ethanol, the T_{50} of WBA is slightly improved by
394 polyols, indicating that polyols are WBA stabilizer. Our finding agrees with reports that
395 solvent modification by sugars and polyols had marked thermal stabilization in various
396 enzymes [9, 30]. Polyols were explained to preferentially be excluded from the surface

layer of the protein and form a water shell around the protein, so that the protein is preserved and the conformation becomes more rigid or stable [9, 30]. Similar mechanism of stabilization by polyols was also reported for organic polar solvents (DMSO and DMF) [31], and actually polar organic solvents have conferred a slight thermostabilization to WBA. However, sugars, which are classified also as polyols, interestingly had no stabilizing effect on WBA in our study.

The activation and thermostabilization of WBA were tested using different additives with various values of empirical parameters of solvent polarity, $E_T(30)$ [38] In particular, ethanol ($E_T(30)$ = 217 kJ mol^{-1}), DMSO (189 kJ mol^{-1}), DMF (183 kJ mol^{-1}), glycerol (238 kJ mol^{-1}), EG (235 kJ mol^{-1}), βME (224 kJ mol^{-1}), and 2M2B (172 kJ mol^{-1}) were examined at different temperatures. According to the results, the half-life time of WBA was enhanced at 55^{o}C with ethanol. It is suggested that thermostabilization by additives could be induced by reducing the degree of water-solvation or deformation of a water shell around the protein. However, a solvent 2M2B with a low $E_T(30)$ value decreased the activity and stability of WBA (Figs. 8B and 9B), suggesting that the effects of additives on the enzyme activity and stability are complicated and that the effect on the water-solvation might not be the main one [39].

Glycine was the most favorable among the evaluated additives in stabilizing

WBA followed by gelatin (Table 1) and the stabilization effects of various additive concentrations were presented in Fig. 9. It should be noted that the thermostability of WBA in the presence of favorable additives such as glycine and gelatin is almost comparable with that of soybean β-amylase widely used in starch-saccharification industry. This suggests that WBA could be applied more widely in food and bio-ethanol industries with employing suitable additives. A good empirical correlation between the relative stabilizing effects of glycine with the change in solvent-accessible hydrophobic surface area of the folded protein was reported [40]. The possible protective effect of gelatin on penicillinase was stated that it combines with enzyme and form a thermostable complex [41]. The molecular mechanism of glycine and gelatin on stabilizing WBA should be examined in the next step from these lines.

Arginine was found to destabilize WBA in our study. Similarly, chloroperoxidase was confirmed to be inactivated by arginine mainly by the binding of a guanidinium group with the catalytic site [42]. This inactivation effect might be the same as that given by the denaturant, guanidine hydrochloride; namely, arginine might cleave the hydrogen bonds in the protein structure and increase the solubility of hydrophobic residues of the protein. On the other hand, arginine is known to work effectively in recovering human matrix metalloproteinase 7 from inclusion bodies [43].

This suggests that arginine promotes unfolding the misfolded protein structure to lead it into the correctly folded form. While arginine seems to show multiple effects on protein stability depending on target proteins, it could be a good tool to increase the stability of WBA by selecting suitable conditions.

We tried to modify thiol groups of WBA using reducing agents like βME and GSH, whereas they destabilized WBA. This is because they attack disulfide bonds and expose proteins to heat denaturation and, hence reduces their thermostability [44]. Plausible involvement of thiol groups in the catalytic activity of amylases has been suggested, although it is not known currently with WBA. The effects of βME and GSH should be considered from this point in the next step.

Various concentrations of NaCl exhibited destabilizing effect on WBA in our study. Three possible reasons were suggested for salt-induced inactivation or destabilization effect on enzymes: (a) break weak hydrogen-bonds and disrupt the protein conformation; (b) attract water molecules and the enzyme coagulates by protein-protein hydrophobic interaction; and (c) high concentration of salt makes the enzyme more likely to bind with the salt ions instead of the substrate by electrostatic interaction [45]. However, high concentration of neutral salts remarkably improves thermolysin activity [14, 15]. The catalytic activity of thermolysin is enhanced by 4

451 times with the substitution of the active-site zinc with cobalt and further exponentially
452 by NaCl up to 13-15 times [29].

453 It is well known that enzyme activity is controlled by the factors of enzyme
454 structure and reaction environment. The structural factors and environmental factors are
455 sometimes independent and sometimes closely connected. With their optimal
456 combination, the optimal catalytic activity should be realized. Protein engineering
457 (namely, site-directed mutagenesis and chemical modification) is a tool for changing the
458 enzyme structure in a predictable and precise manner to effect a change on the catalytic
459 process. Since the enzyme is even improved in only one side of a reaction, any changes
460 in the rest of the reaction may also alter the catalytic process [46]. Solvent engineering
461 is a powerful tool in rational control of enzyme activity. In reality, both approaches are
462 still somewhat difficult to confirm their effects, whereas they have been used
463 successfully to alter the protein properties [47]. At last, it should be reminded that the
464 protein-engineered enzymes are strictly limited to use for food processing in many
465 countries in order to avoid unpredictable harms. Thus solvent engineering must be an
466 inevitable alternative to find the optimal conditions from the viewpoints of enhancing
467 enzyme catalytic efficiency, guaranteeing safeness of the products, reducing the costs
468 for production, etc. When the enzyme activity in its industrial application is increased

by solvent engineering, the cost of the additive should be considered in the total cost, although it is generally much cheaper than that of the enzyme, and the improved enzyme activity and stability by the additive should decrease the enzyme amount needed and thus shortened the reaction time, which results in decreasing the running costs, utility, labor, etc.

5. Conclusion

From the thermodynamic parameters evaluated in this study, it is eminent that WBA is not stable at temperatures higher than 55^{o}C. The thermal stability of WBA was improved by the addition of glycine and gelatin explicably through convening conformation of the enzyme and reducing the interaction of the protein with the solvent. Polyols and organic polar solvents (DMSO and DMF) also conferred slight stability to the enzyme while some evaluated additives have exhibited destabilizing effect. The thermodynamic parameters indicate that WBA is thermo-labile and sufficient stabilization was achieved by solvent engineering with additives and that the heat inactivation of WBA is entropic-driven. On the other hand, it was shown that WBA activity was enhanced by the addition of ethanol and DMF persuasively by altering the

hydrophobic interaction and inducing favorable conformation to its active center. It is suggested that WBA would be applicable to a wide range of saccharification industries such as food and bio-ethanol production with employing suitable additives.

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611

612 **Figure legends**

613

614 **Fig. 1.** SDS-PAGE of the Himaltosin preparation. Molecular marker proteins (lane 1);
615 Himaltosin preparation: 1% (w/v) (lane 2), 2% (w/v) (lane 3), and 5% (w/v) (lane 4).
616 The experimental conditions were given in the text (Materials and methods).

617

618 **Fig. 2.** Dependence of the initial reaction velocity (v) in the hydrolysis of soluble starch
619 catalyzed by wheat β-amylase (WBA) on substrate concentration. The hydrolysis was
620 carried out in 20 mM sodium acetate buffer (buffer A), pH 5.4, at 25^{o}C. The WBA
621 concentration in the reaction solution was 30.0 nM.

622

623 **Fig. 3.** Progress of the decrease in WBA activity with the time of thermal treatment.
624 WBA (0.300 μM) was treated at 25, 35, 45, 55, and 65^{o}C for 0-30 min in buffer A, pH
625 5.4. WBA activity in the hydrolysis of soluble starch was evaluated by measuring the
626 initial velocity (v) in the same buffer at 25^{o}C (see Materials and methods). The WBA
627 concentration in the enzyme-reaction solution was 30.0 nM. The v value measured in
628 the 0-min incubation at each temperature was designated as v_o. The logarithm of the
629 relative activity [log (v/v_o)] was plotted against the thermal-treatment time. The markers

are (temperature in ^{o}C): 25, o; 35, ◊; 45, Δ; 55, □; and 65, ●. From these semi-log plots, the first-order rate constant for the thermal inactivation of WBA was evaluated at the specified temperature of the thermal treatment.

Fig. 4. Arrhenius plot of WBA for the first-order rate constant (*k*) of thermal inactivation. The rate constants were evaluated from the semi-log plots as shown in Fig. 3 obtained by thermal treatment of the enzyme at various temperatures. The reaction conditions were given in the text (Materials and methods).

Fig. 5. Eyring plot of WBA for the first-order rate constant (*k*) of thermal inactivation. The rate constants were evaluated as described in the legend of Fig. 4.

Fig. 6. Effect of temperature on the initial velocity (*v*) of WBA in hydrolyzing soluble starch. The enzyme reaction was carried out at the temperature indicated. The initial concentrations of starch and WBA in the reaction solution were 1.80% and 15.0 nM, respectively. The optimal temperature T_{opt} was determined to be 55^{o}C.

Fig. 7. Effect of thermal inactivation of WBA on the enzyme activity. The activity was

assayed in buffer A (pH 5.4) in hydrolyzing starch after incubation at various temperatures indicated for 30 min. The reaction conditions are given in the text (Materials and methods). The T_{50} was determined to be 50 ± 1°C.

Fig. 8. The effects of various concentrations of additives on the activity of WBA at 25°C. Symbols for additives (mM): (A) Gly, o; NaCl, ◊; Asp, □; Cys, Δ; glucose, ●; GSH, ◆; and Arg, ▲. Symbols for additives (%, w/w): (B) ethanol, o; DMF, ◊; DMSO, Δ; EG, □; gelatin, ●; glycerol, ◆; BME, ▲; and 2M2B, ∇. The relative activity (%) of the enzyme obtained without additive was set as 100%.

Fig. 9. Effects various additives on the thermostabilization of WBA after incubation with the additives at various temperatures for 30 min. Symbols for concentrations (mM) of additives: (A) buffer, o; 182 glycine, ◊; 45.5 Arg, □; 45.5 Asp, Δ; 182 NaCl, ●; 182 glucose, ◆; 45.5 Cys, ▲; and 91 GSH, ■. Symbols for concentrations (%, w/w) of additives: (B) buffer, o; 5.5 EG, ◊; 0.18 gelatin, Δ; 5.5 glycerol, □; 0.91 βME, ●; 2.7 DMF, ◆; 0.91 ethanol, ▲; 5.5 DMSO, ■; and 5.5 2M2B, ∇. Low concentrations of some additives were evaluated because of their solubility limit in buffer A (pH 5.4) at 25°C. The residual activity of WBA without additive was depicted in broken lines.

Table 1. Effects of selected additive concentrations on thermostablization of wheat β-amylase (WBA) after incubation at 55°C with the additives for 30 min prior to starch hydrolysis

Additives	T_{50} (°C)	k (min^{-1})	$t_{1/2}$ at 55°C (min)	$\Delta G^{o\ddagger}$ (kJ mol^{-1})
none	50 ± 1	0.030 ± 0.004	23 ± 2	90 ± 1
0.18% gelatin	54 ± 1	0.017 ± 0.003	40 ± 1	92 ± 1
0.45% gelatin	54 ± 1	0.018 ± 0.001	39 ± 2	92 ± 1
91 mM glycine	54 ± 1	0.017 ± 0.003	41 ± 1	92 ± 1
182 mM glycine	56 ± 2	0.014 ± 0.006	48 ± 3	92 ± 1

T_{50} is the temperature at which WBA loses half of it activity after 30 min of incubation; k is the first-order rate constant of the thermal inactivation; $t_{1/2}$ (at 55°C) is the time at which WBA loses half of its activity at 55°C; and $\Delta G^{o\ddagger}$ is the standard Gibbs energy difference of activation. Each value is a mean of triplicate analysis ± standard deviation. The initial concentrations of starch and WBA in the reaction solution were 1.80% and 30.0 nM, respectively.

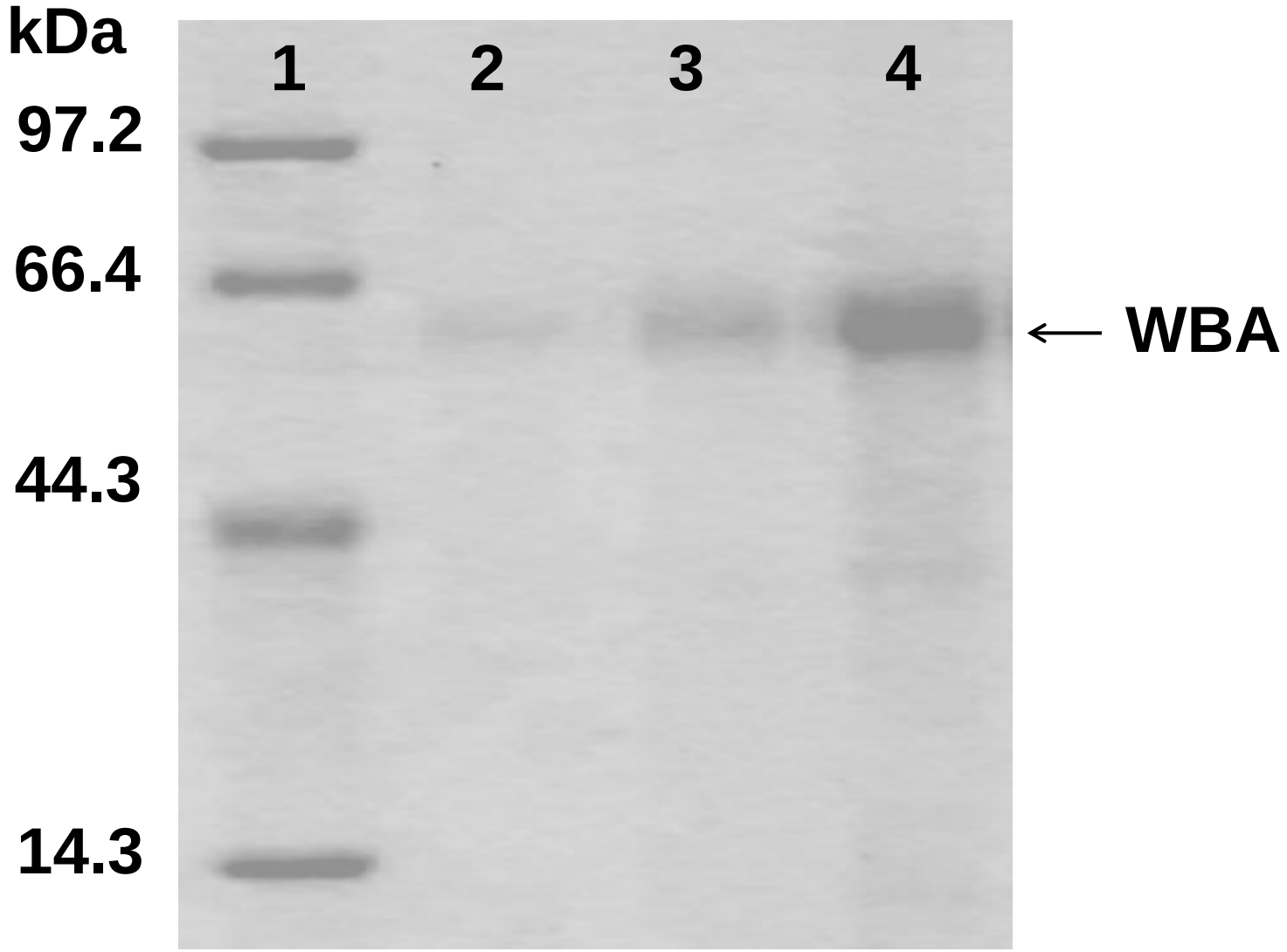

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Fig. 1

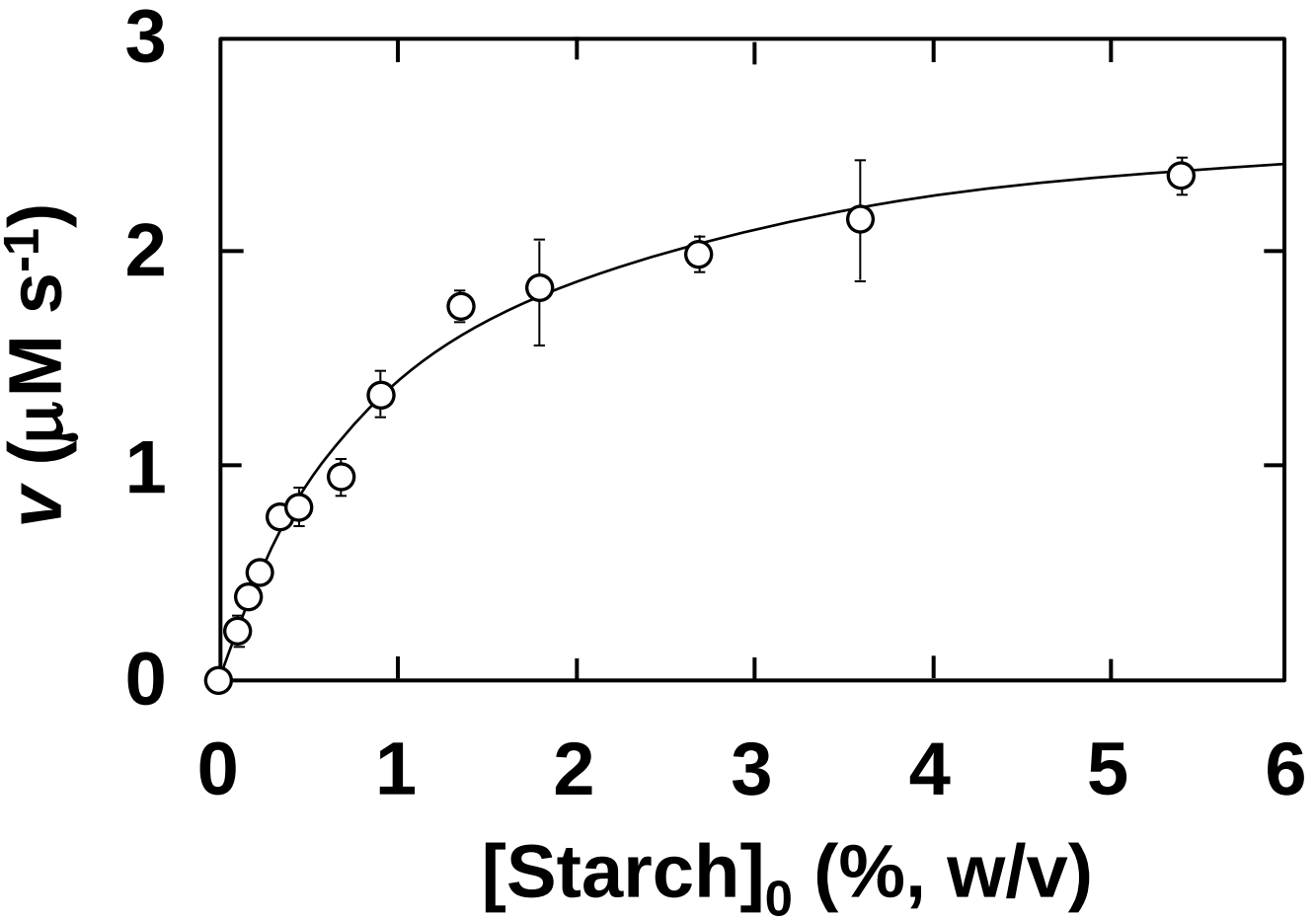

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Fig. 2

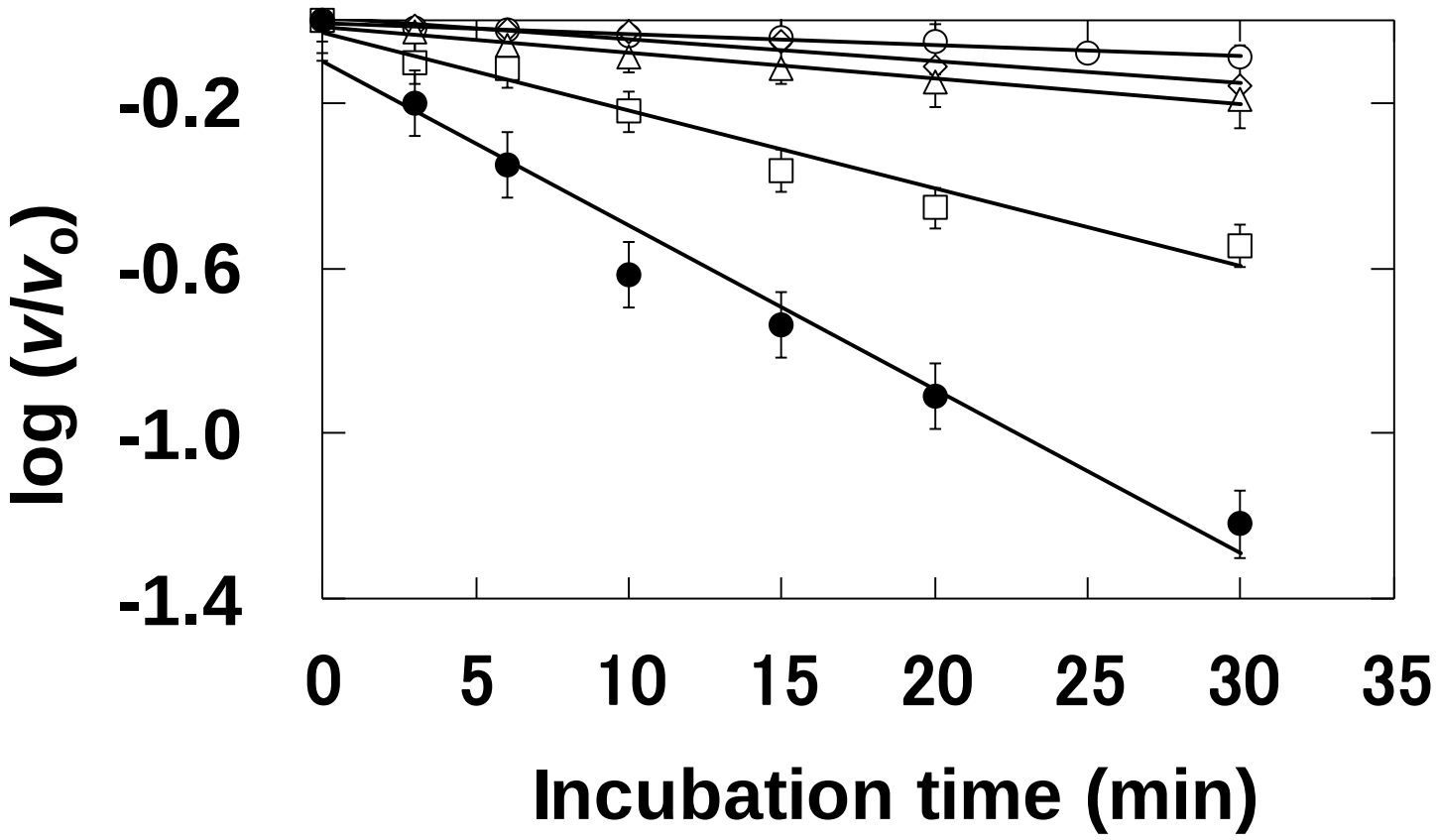

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Fig. 3

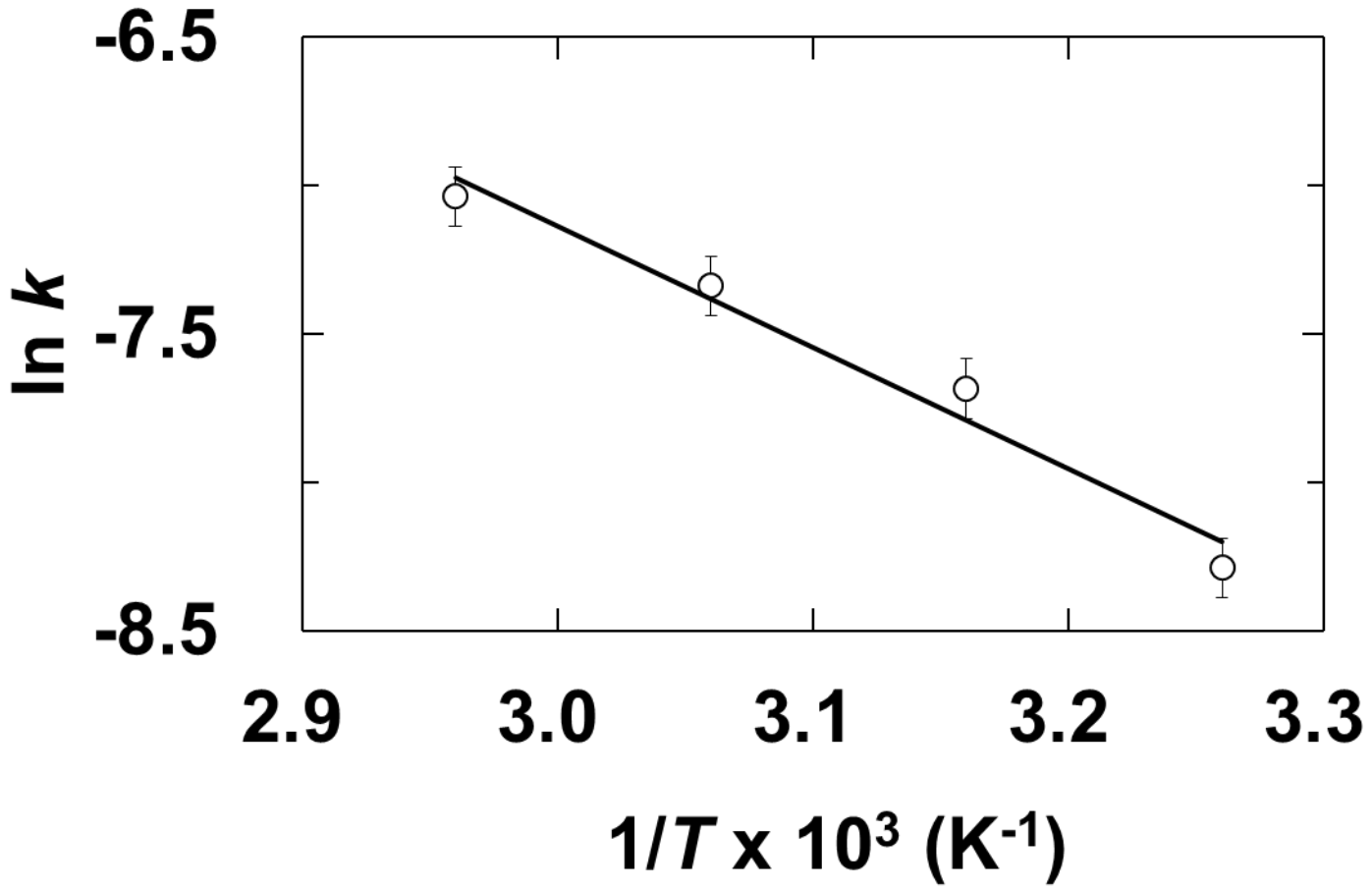

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Fig. 4

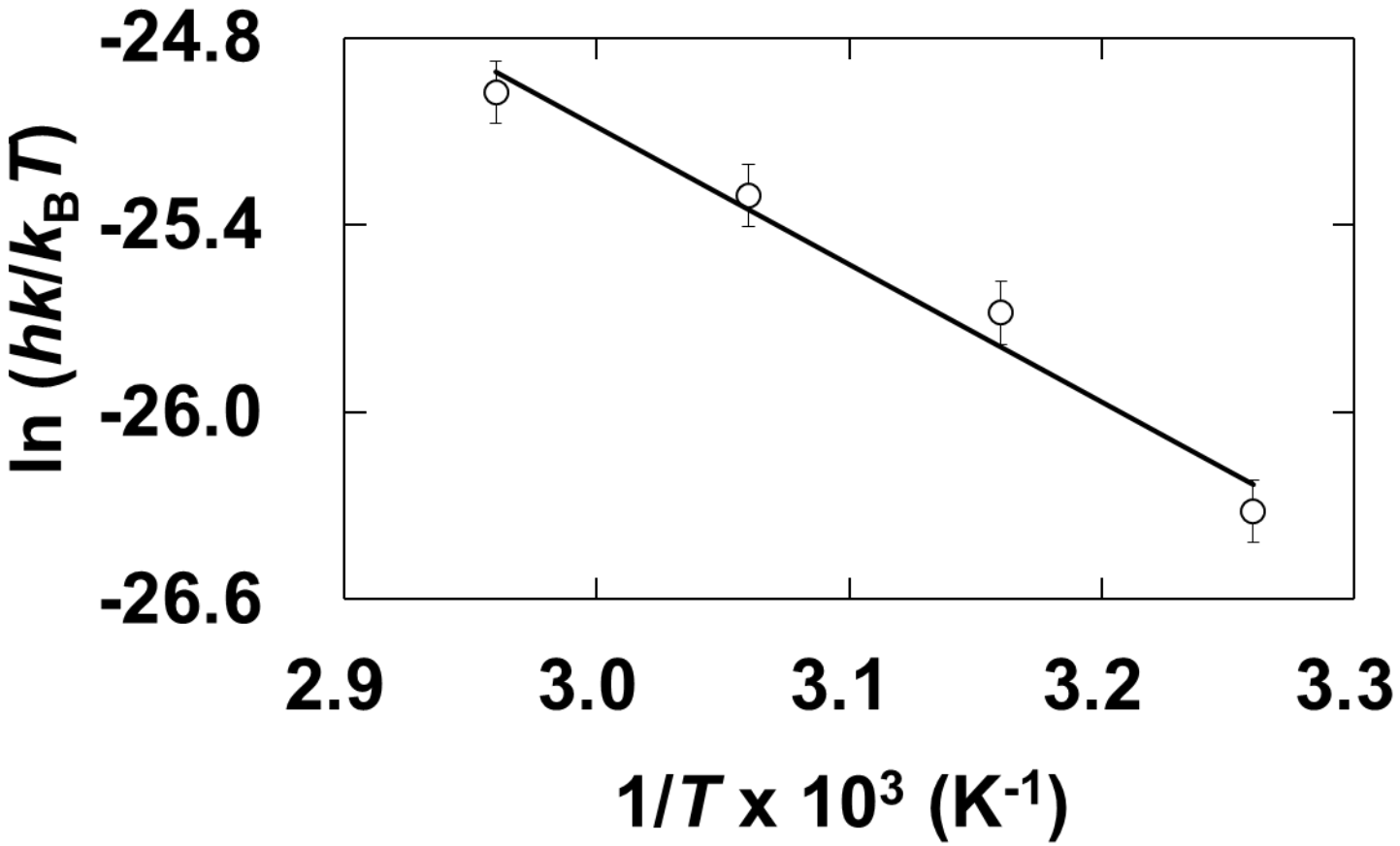

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Fig. 5

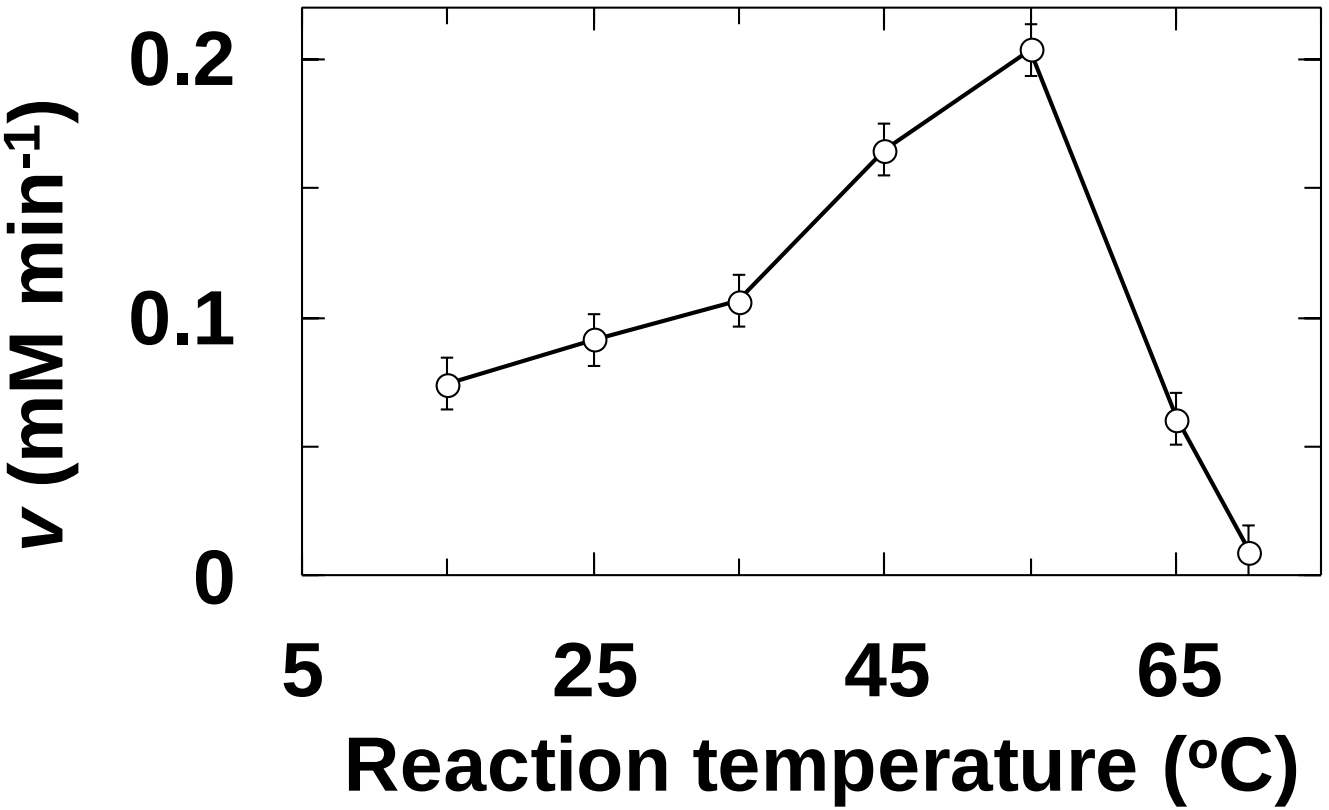

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Fig. 6

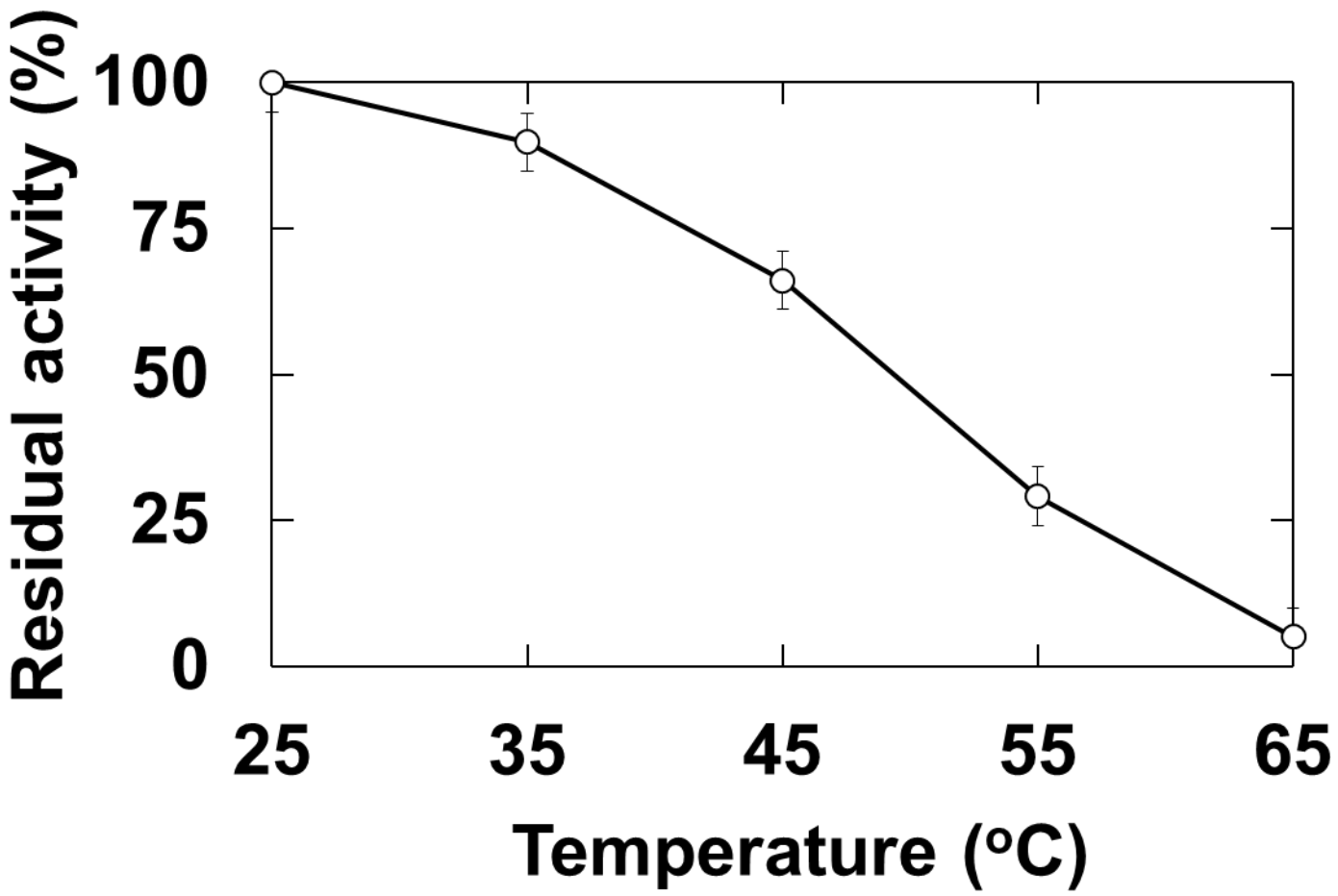

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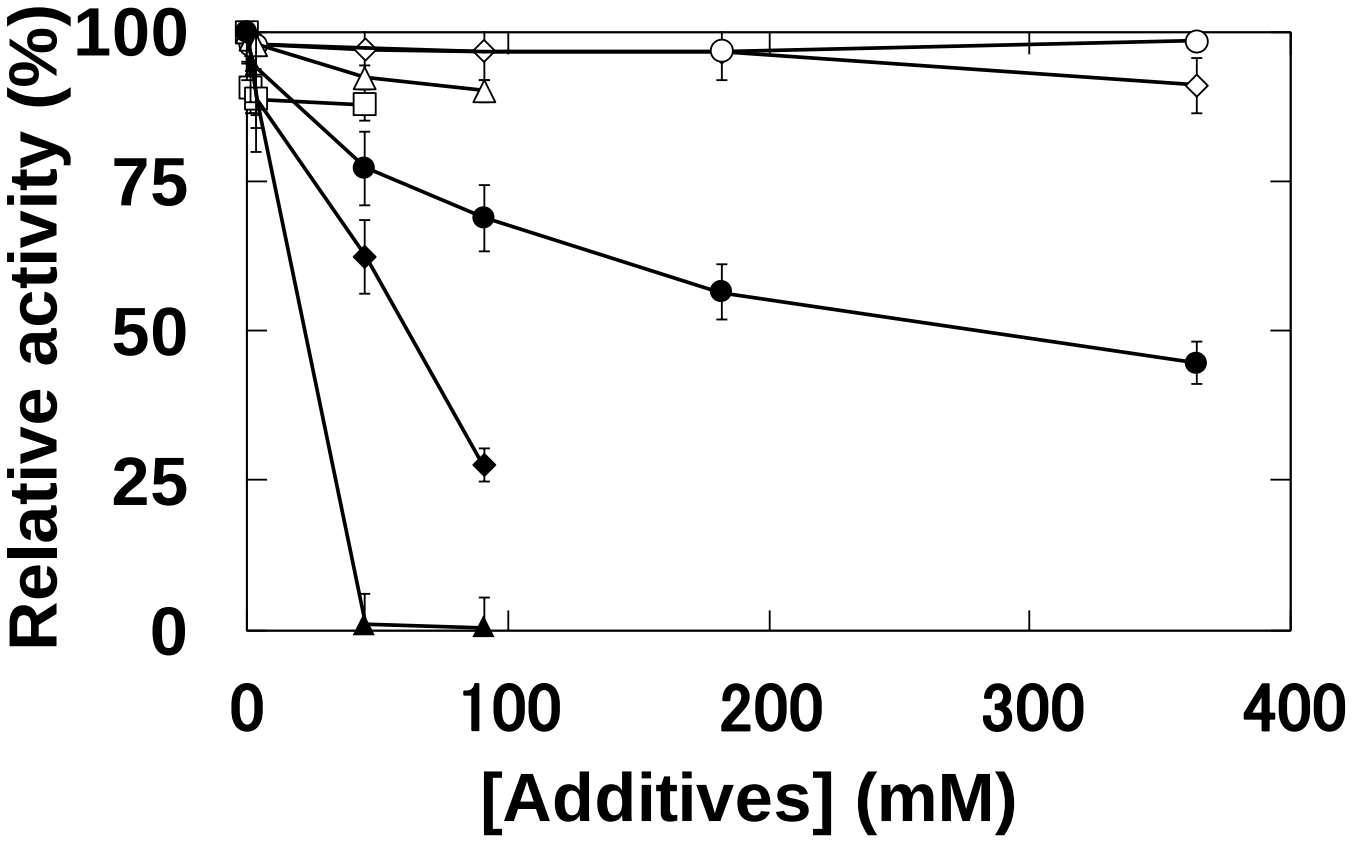
Fig. 7


Relative activity (%)
100
75
50
25
0
0
100
200
300
400
[Additives] (mM)

Top

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Fig. 8A

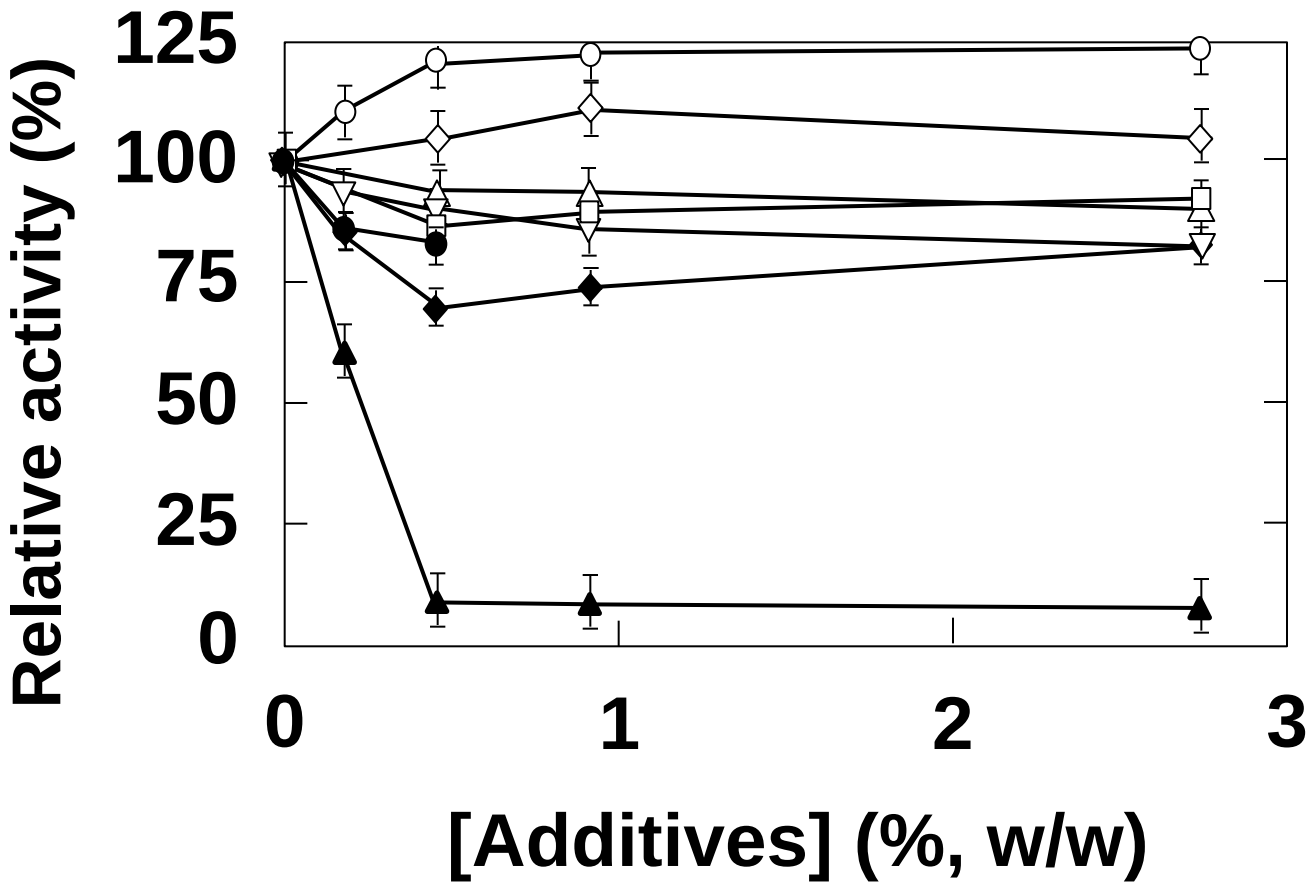

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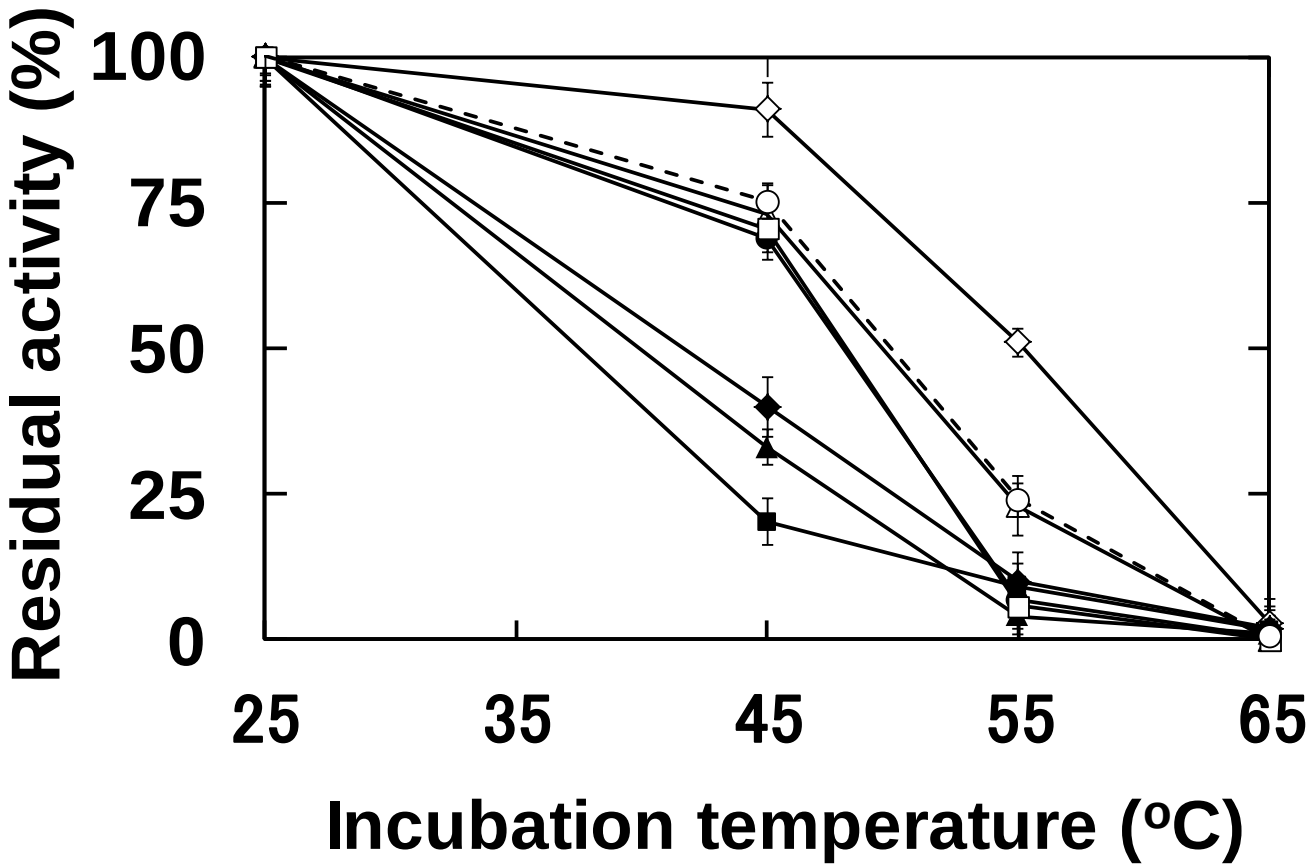
Fig. 8B


Residual activity (%)
100
75
50
25
0
25
35
45
55
65
Incubation temperature (°C)

Top

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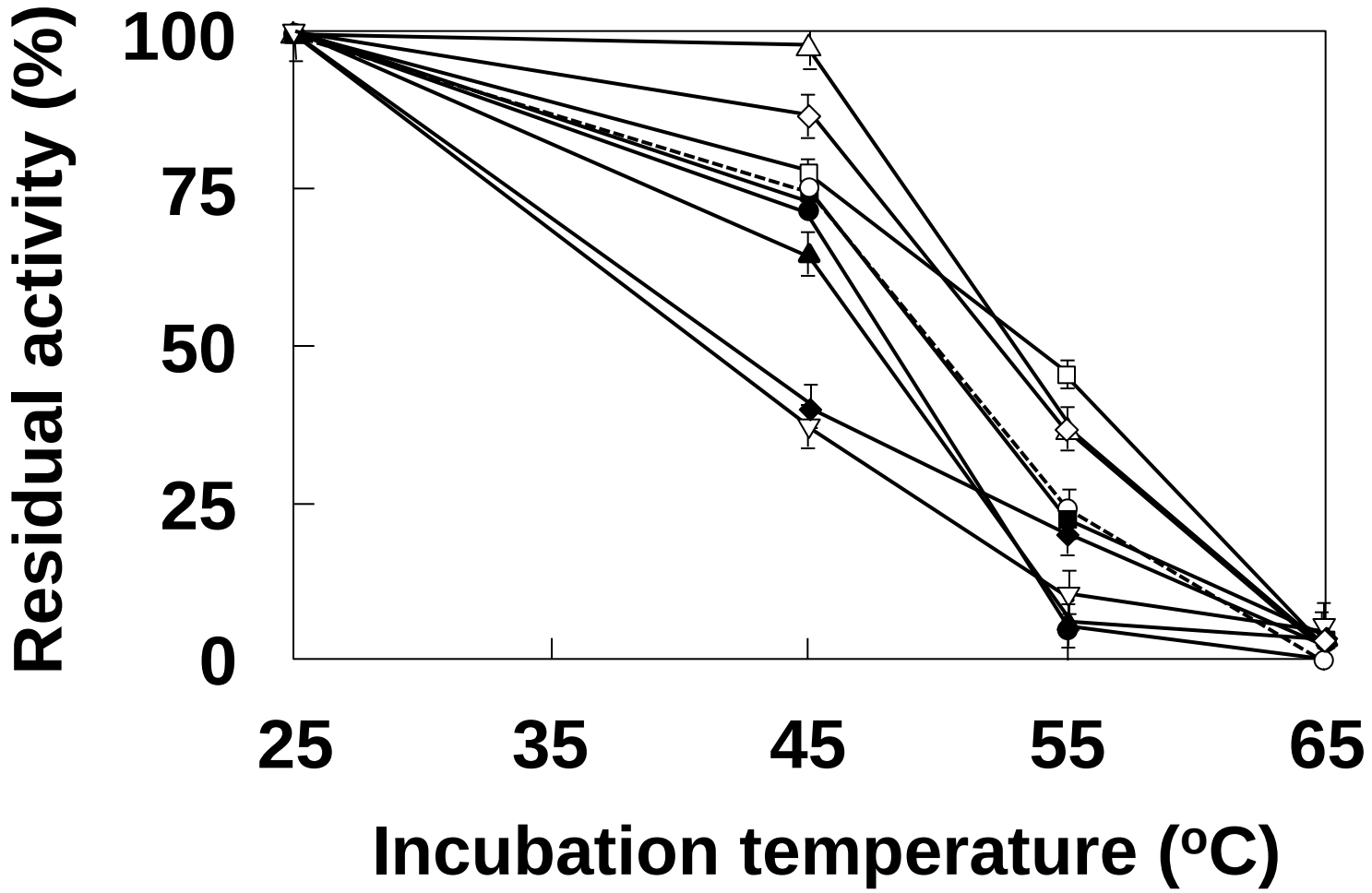
Fig. 9A


Residual activity (%)
100
75
50
25
0
25
35
45
55
65
Incubation temperature (ºC)

Top

⇧

Fig. 9B