Effects of Temperature and Additives on the Thermal Stability of Glucoamylase from Aspergillus niger

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Introduction

Study of the protein folding/unfolding process is favorable for elucidating the structure-function relationship of proteins, which further helps us to predict a specific mutation site or region more precisely for improving its stability against the denaturants, high temperature, and extreme pH of media [23, 26]. Various techniques, including circular dichroism (CD), fluorescence spectroscopy, Fourier-transform IR spectroscopy, UV-Vis spectroscopy, dynamic light scattering (DLS), and differential scanning calorimetry (DSC), were applied to investigate the protein folding/unfolding process [14, 22, 28, 29, 32]. Among them, CD offers information on the secondary structure, and is thus widely used for evaluating the conformation and stability of proteins in various environmental conditions [15]. Moreover, the conformational changes of proteins on the tertiary-structure level are examined via UV spectroscopy and fluorescence spectroscopy [1, 10]. DLS determines the hydrodynamic diameter based on the fluctuations of light-scattering intensity with time, and provides useful information on the quaternary structure of proteins and the extent of aggregation [30].

Glucoamylase (E.C. 3.2.1.3), known as amyloglucosidase or γ-amylase, is a biocatalyst capable of hydrolyzing the α-1,4-glycosidic linkage in raw or soluble starch and related oligosaccharides [8, 24, 36]. The major application of glucoamylase is to catalyze starch saccharification to produce glucose for food and fermentation industries. The enzymes, the majority produced by the filamentous fungi Aspergillus and Rhizopus, display low transglycosylation activity and near 100% glucose is obtained from starch.
hydrolysis [19, 20, 25].

Industrial application of glucoamylase requires stability during preparation and storage, especially under harsh conditions, including high temperature, extreme pH, and denaturants. Acidic and alkaline conditions fail to influence the conformation of glucoamylase from A. awamori nakazawa, which was detected by CD technique [23]. As reported recently, the acid-denatured state of glucoamylase at pH 1.0 presents the molten globule-like state; furthermore, polyols induced both secondary and tertiary structural changes in the acid-denatured state of the enzyme [33, 34]. The systematic research related to temperature-induced conformational change of glucoamylase has not been reported yet.

In our previous study, the two thermostable glucoamylases GAM-1 and GAM-2 were purified, which possessed different molecular masses, glycosylation, and thermal stability [18]. The thermal inactivation of glucoamylases GAM-1 and GAM-2 from A. niger B-30 were further investigated in the present research. CD spectroscopy, fluorescence spectroscopy, DLS technique, and Native-PAGE were performed to detect the conformational changes induced by temperature. Additionally, sorbitol and trehalose served as additives to improve the thermal stability of the enzymes. Our present findings provide experimental evidence for the commercial preparation and industrial applications of glucoamylases GAM-1 and GAM-2.

Materials and Methods

Purification of GAM-1 and GAM-2 and Activity Assay

The two different molecular mass glucoamylases GAM-1 and GAM-2 from A. niger B-30 were produced by fermentation. The purification and glucoamylase activity assay were completed as described previously [18, 21].

Thermal Inactivation of GAM-1 and GAM-2

The thermal inactivation of GAM-1 and GAM-2 was studied by incubating the enzyme solution (basic buffer) at the indicated temperatures (60°C, 70°C, and 80°C), and aliquots were removed at specific time points and refrigerated immediately. The residual activity was determined, and the activity of a sample without incubation served as 100%.

The effects of sorbitol and trehalose (Sigma, USA) on glucoamylase were studied by incubating the enzyme solution (basic buffer) in the presence of 1 M sorbitol or 1 M trehalose. As described previously, the thermal inactivation of the samples were determined.

The inactivation rate of the enzyme was calculated by a first-order expression: In (residual activity %) = -kt. The k values (inactivation rate constant or first-order rate constant) were calculated from a plot of ln (residual activity %) versus t at a particular temperature. The half-lives (t_(1/2)) were calculated with the equation t_(1/2) = ln2/k [5]. The thermal inactivation energy (∆G) was calculated: ∆G = RTln(k_0T/h) - RTlnk [27]. All experiments were conducted in triplicate, and the results are shown as mean values.

Native-PAGE

The purified glucoamylases GAM-1 and GAM-2 in the absence and presence of 1 M sorbitol or 1 M trehalose were first incubated at 60°C, 70°C, 80°C, or 90°C for 10 min, respectively, and then applied to a 10% PAGE at 4°C. After running, the gel was incubated in basic buffer at 4°C for 1 h, and then immersed in 2% soluble starch in basic buffer incubating at 60°C for 1 h and developed with 0.1% I and 1.5% KI. In the control, another gel was run under the identical conditions and developed with 0.25% Coomassie Brilliant Blue R-250 for protein visualization.

CD Spectral Measurement

The CD spectra of the purified GAM-1 and GAM-2 in the absence and presence of 1 M sorbitol or 1 M trehalose were recorded in the range of 190–260 nm at an interval of 0.1 nm with a JASCO J-810 spectropolarimeter (Japan) equipped with a temperature controlling unit. For samples in the absence of additives, the temperature was increased step-by-step from 25°C to 90°C. For samples in the presence of additives, the temperature was set at 25°C and 80°C. At each temperature for measurement, the solution was maintained for 10 min before scanning. The values of the scan rate, response, and band width were 100 nm/min, 0.25 sec, and 1.0 nm, respectively. The fractional contents of the secondary structure elements were calculated from far-UV CD spectra by the Dichroweb software package with the CDSSTR algorithm [13, 31].

UV Absorption Spectroscopy

The UV spectra were measured using the Shimadzu UV-2550 spectrophotometer (Japan) with a temperature controlling unit. GAM-1 and GAM-2 were incubated at different temperatures for 10 min and then the absorbance at 280 nm was recorded. The basic buffer was taken as the baseline.

Fluorescence Spectral Measurement

The fluorescence spectra of the purified GAM-1 and GAM-2 in the absence and presence of 1 M sorbitol or 1 M trehalose incubated at different temperatures ranging from 30°C to 90°C for 10 min were measured with a Shimadzu RF-5301 fluorescence spectrophotometer (Japan) equipped with a temperature controlling unit. The fluorescence spectra were measured from wavelengths 310 to 400 nm, with the excitation wavelength at 295 nm. The glucoamylase, sorbitol, and trehalose were dissolved in basic buffer.

DLS Measurement

The particle size of the purified GAM-1 and GAM-2 in the
absence and presence of 1 M sorbitol or 1 M trehalose were measured under different temperatures using the Zetasizer Nano ZS90 instrument (Malvern Instruments, UK). The samples were centrifuged at 18,000 \( \times g \) for 20 min and filtered through a 0.22 \( \mu \)m micropore film. All the data reported are the mean values of 20 runs. In addition, the temperature trend of DLS measurements was determined at 5°C intervals from 25°C to 90°C.

**Kinetic parameters**

The \( K_m \) and \( V_{max} \) values for the purified glucoamylases GAM-1 and GAM-2 in the absence and presence of 1 M sorbitol or 1 M trehalose were determined by incubating the soluble starch solution with different concentrations ranging from 0.5 to 4 mg/ml in basic buffer at 60°C, and a Lineweaver-Burk plot was employed.

**Results and Discussion**

**Thermal Inactivation Analysis of GAM-1 and GAM-2**

The thermal inactivation of GAM-1 and GAM-2 without additives was firstly detected. GAM-1 and GAM-2 were stable at 60°C. At 70°C, both GAM-1 and GAM-2 displayed different levels of thermal inactivation. The inactivation rate constants (k, \( \text{min}^{-1} \)) of GAM-1 and GAM-2 were 0.0153 and 0.0032, and the half-lives (\( t_{1/2} \), min) were 45 and 216, respectively (Fig. 1A). The thermal inactivation energies of GAM-1 and GAM-2 were 96.4 and 103.8 kJ, respectively. The results suggested that GAM-2 possesses better thermal stability than GAM-1.

Several additives, including sorbitol, trehalose, mannitol, glycerol, glycine, and sucrose, were applied to stabilize the thermal stability of GAM-1 and GAM-1. Among these stabilizers, sorbitol and trehalose were approved to be more effective (data not shown). Sorbitol and trehalose, known as excellent protein additives, are widely used in studies of microbial enzymes [2, 4, 7, 9]. Both sorbitol and trehalose increased the catalytic activity and intrinsic fluorescence intensity of *Pseudomonas cepacia* lipase [2]. The sorbitol was proved to stabilize the structural changes of \( \alpha \)-amylase [4]. Meanwhile, Devaraneni *et al.* [7] found the presence of sorbitol and trehalose at pH 2.5 to help in the retention of the structure of yeast hexokinase, using spectroscopy and calorimetry. Dong’s work confirmed that trehalose was not only a protein stabilizer, but also a protein aggregation suppressor for both native and denatured protein molecules [9]. Sorbitol is a small watersoluble molecule that can potentially interact with the protein. Hydrogen bonding of sorbitol with the protein surface is considered as the most important interaction. The addition of sorbitol to the enzyme could decrease the dielectric constant of its medium and increase the strength of hydrogen bonds, since the formation of hydrogen bonds between molecules of sorbitol and protein is stronger than that of water-protein bonds. Therefore, the stability of protein increases in the presence of sorbitol [2, 4, 7]. In contrast, trehalose increases the viscosity and surface tension, which leads to the creation of a preferential hydration site on the protein surface, and enters into the lattice structure of the water surrounding the protein and stabilizes the protein structure [2, 7, 9].

However, the effects of the two protein additives on glucoamylase have not been reported. To examine the protective effect of the additives on the thermal stability of
GAM-1 and GAM-2, 0.2–1 M additives were applied in our preliminary experiments, and 1 M sorbitol or trehalose displayed strong thermostability (data not shown). The hydrolytic capability of the enzymes was significantly retained at elevated temperatures in the presence of both additives, especially in the presence of trehalose (Fig. 1B). At 80°C, the apparent half-life of GAM-1 increased up to 9.7 min and 37.4 min by adding 1 M sorbitol and 1 M trehalose, respectively, comparing with the control group ($t_{1/2}$, 2.7 min). The apparent half-life of GAM-2 increased to nearly 12.0 min and 70.0 min by adding 1 M sorbitol and 1 M trehalose, respectively, comparing with the control sample ($t_{1/2}$, 2.8 min) (Fig. 1B).

Secondary Structure Analysis of GAM-1 and GAM-2 by Far-UV CD
Changes in the far-UV CD spectra of GAM-1 and GAM-2 at different temperatures are shown in Fig. 2. The far-UV CD spectra of GAM-1 and GAM-2 at 25°C (pH 4.6) exhibited two negative peaks at 210 and 218 nm and a positive peak at 194 nm (Figs. 2A and 2B), suggesting the existence of $\alpha$-helix-rich proteins, which was consistent with a previous study [35]. When the temperature was raised to 70°C, the intensity of the positive and negative CD bands of GAM-1 decreased gradually (Fig. 2A); meanwhile, little changes of the far-UV CD spectra of the GAM-2 were noted (Fig. 2B). The secondary structure contents analysis was conducted via CDSTR algorithm, where 44.8% $\alpha$-helix, 29.5% $\beta$-sheet, 9.4% $\beta$-turn, and 16.3% random structures were noted in native GAM-1; comparatively, the native GAM-2 contained 61.0% $\alpha$-helix, 22.4% $\beta$-sheet, 10.2% $\beta$-turn, and 6.3% random structures (Figs. 2C and 2D). The similar CD spectra ranging from 25°C to 60°C indicated there are little changes on the secondary structures of both enzymes. Exceeding 60°C, the $\alpha$-helical structure reduced with temperature increment, whereas, the contents of $\beta$-sheet, $\beta$-turn, and random structure increased gradually. The $\alpha$-helical content of GAM-1 and GAM-2 reduced to 9.7% (Fig. 2C) and 4.1% (Fig. 2D) at 70°C. The two proteins were inactivated partially, indicating that heating promotes helix unfolding, which is often correlated with activity loss. The results were consistent with a previous report [3]. Therefore, we speculated the stability and proportion of $\alpha$-helix played

![Fig. 2. Far-UV CD analysis of the secondary structure of glucoamylase.](image-url)

(A) CD spectra of GAM-1 at the indicated temperature for 10 min. (B) CD spectra of GAM-2 at the indicated temperature for 10 min. (C) The distribution of secondary structure of GAM-1. (D) The distribution of secondary structure of GAM-2.

important roles in the thermal stability of glucoamylases. The stability of α-helices showed an important role in the thermostability of glucoamylase [16]. In addition, the thermostability of glucoamylase was also improved by enhancing the stability of α-helices using substitution of amino acid residues [17].

Tertiary Structure Analysis of GAM-1 and GAM-2 by Fluorescence and UV Absorption Spectroscopies

The conformational changes of GAM-1 and GAM-2 on the tertiary level were monitored by steady-state fluorescence and UV absorption spectroscopies under different temperatures. The fluorescence emission intensity of GAM-1 and GAM-2 decreased with temperature increase (Fig. 3). At 60°C, GAM-1 showed 2 nm red shift (Fig. 3A), but no significant red shift of GAM-2 in the maximum wavelength was observed (Fig. 3B). The tertiary structure of GAM-1 changed at 60°C; however, the tertiary structure of GAM-2 was stable. At 70°C, GAM-2 showed red shift, indicating the Trp residues of GAM-2 were exposed to the polar environment, and the tertiary structure began to change.

The results suggested that the tertiary structure of GAM-2 was more stable than GAM-1. Both the enzymes exhibited a maximal UV absorption at 280 nm (Fig. 3C). At 60°C, the absorption intensity of GAM-1, but not GAM-2, increased. The absorption intensity of GAM-2 began to increase at 70°C.

Quaternary Structure Analysis of GAM-1 and GAM-2 by DLS

The temperature-induced changes in the quaternary structure of GAM-1 and GAM-2 are shown in the supplementary material (Fig. S1). The average hydrodynamic radius ($R_h$) of GAM-1 and GAM-2 was 8.3 and 7.6 nm at 25°C, respectively. The $R_h$ was stable below 60°C; however, the $R_h$ of GAM-1 and GAM-2 increased to 37 and 14 nm when the temperature was elevated to 60°C. Such alterations may be related to the generation of some oligomers and dimers. Above 60°C, exponential increases in $R_h$ were observed, suggesting the aggregation of both GAM-1 and GAM-2. When the temperature reached 75°C, a gradual decrease in intensity of GAM-1 was noted,

**Fig. 3.** Fluorescence spectra and UV absorption spectra of glucoamylase.
(A) Fluorescence spectra of GAM-1 at the indicated temperature for 10 min. (B) Fluorescence spectra of GAM-2 at the indicated temperature for 10 min. (C) UV absorption spectra of GAM-1 and GAM-2 at 25°C for 10 min. (D) UV absorption of GAM-1 and GAM-2 at 280 nm at the indicated temperature for 10 min.
suggesting the flocculation process caused by thermal denaturation. The intensity of GAM-2 decreased at 80°C. The results from DLS confirmed the quaternary structure of GAM-2 was more stable than GAM-1. Although the conformational changes were noted at 60°C, confirmed by CD, fluorescence spectrum, DLS, and thermal inactivation detection, the inactivation of GAM-1 was not observed. The conformational changes failed to cause the denaturation of the activity center or the changes were reversible.

**Effects of Additives on Secondary Structure of GAM-1 and GAM-2**

To evaluate the effects of sorbitol or trehalose on the secondary structures of GAM-1 and GAM-2, far-UV CD measurements were applied under various conditions. CD spectra at 25°C of GAM-1 and GAM-2 with or without 1 M sorbitol or trehalose displayed strong peaks of negative ellipticity at 210 and 218 nm, and a positive peak at 194 nm (Figs. 4A and 4C). The selected additives showed no

![Fig. 4](image.png)

**Table 1.** Effects of additives on the contents of secondary structural elements of GAM-1 and GAM-2.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>GAM-1</th>
<th>GAM-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic buffer</td>
<td>1 M sorbitol</td>
<td>1 M trehalose</td>
</tr>
<tr>
<td>α-Helix (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25°C</td>
<td>44.8</td>
<td>45.0</td>
</tr>
<tr>
<td>80°C</td>
<td>28.7</td>
<td>42.0</td>
</tr>
<tr>
<td>β-Strand (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25°C</td>
<td>29.5</td>
<td>28.8</td>
</tr>
<tr>
<td>80°C</td>
<td>32.2</td>
<td>32.0</td>
</tr>
<tr>
<td>β-Turn (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25°C</td>
<td>9.4</td>
<td>10.2</td>
</tr>
<tr>
<td>80°C</td>
<td>17.0</td>
<td>9.3</td>
</tr>
<tr>
<td>Random (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25°C</td>
<td>16.3</td>
<td>16.0</td>
</tr>
<tr>
<td>80°C</td>
<td>22.0</td>
<td>17.1</td>
</tr>
</tbody>
</table>
obvious effect on the secondary structure of GAM-1 and GAM-2 at native state. At 80°C, the CD spectra of GAM-1 and GAM-2 decreased in the intensities of the positive and negative peaks, and the α-helix contents of GAM-1 and GAM-2 decreased from 44.8% to 28.7% and from 61% to 45%, respectively. Both 1 M sorbitol or 1 M trehalose possessed protective effects on GAM-1 and GAM-2 stability, where the α-helix contents only decreased to 3% and 1% (Figs. 4B, 4D; Table 1). Sorbitol and trehalose counteract the temperature-perturbing actions, which have beneficial effects on the secondary structures of glucoamylase.

The effects of selected additives on the contents of enzyme secondary structure were not observed at 30°C [4]. However, the opposite results were found in that the secondary structure of lipase was changed by additives [2]. The different effects of sorbitol or trehalose on the secondary structure may be due to the different types of enzyme. Interestingly, different research groups agree that the presence of additives inhibits conformation changes, maintain the conformation stability, and improve the thermostability.

**Effects of Additives on Fluorescence Intensity of GAM-1 and GAM-2**

Fluorescence emission studies were performed as a function to study the changes in tertiary structure of GAM-1

\[ \text{Fig. 5. Effects of sorbitol or trehalose on fluorescence spectra of GAM-1 and GAM-2.} \]

The samples were treated at 25°C for 10 min (black line). The samples were treated at 70°C for 10 min (red line). The samples were treated at 80°C for 10 min (green line). The samples were treated at 90°C for 10 min (blue line). (A) GAM-1 in basic buffer. (B) GAM-1 in basic buffer containing 1 M sorbitol. (C) GAM-1 in basic buffer containing 1 M trehalose. (D) GAM-2 in basic buffer. (E) GAM-2 in basic buffer containing 1 M sorbitol. (F) GAM-2 in basic buffer containing 1 M trehalose.
and GAM-2 with or without 1 M sorbitol or 1 M trehalose at different temperatures. The results from the fluorescence spectra were similar to CD spectra (Fig. 5). Our data were consistent with a previous study [11].

Effects of Additives on Polydispersity of GAM-1 and GAM-2

The effects of the additives on protein according to the changes of the secondary structure and tertiary structure were commonly detected via CD spectrum and fluorescence spectrum; however, few studies related to DLS were reported. GAM-1 and GAM-2 present with or without additives under various temperatures were monitored by DLS. The $R_h$ values of all the samples remained the same initially, indicating that no aggregation occurred from 25°C to 55°C. The $R_h$ of GAM-1 and GAM-2 started to increase at above 60°C. However, the $R_h$ of GAM-1 and GAM-2 in the presence of 1 M sorbitol or 1 M trehalose started to increase at 65°C or 70°C (Figs. 6A and 6B). The particle size distribution of GAM-1 and GAM-2 present with or without additives at 65°C further confirmed the selected additives effectively prevent glucoamylases against the heat-induced aggregation (Figs. 6C and 6D). In industrial application, an extra purification process is required to remove the additives, which enhances production cost. Further experiments will be performed in our group to immobilize enzymes on different matrixes.

Analysis of Protective Effect of Additives by Native-PAGE

Native-PAGE is one of the most powerful techniques for studying the composition and structure of native proteins, since the conformation and biological activity of proteins remain intact during this technique [6]. To further elucidate the effects of additives on the activity and conformation of glucoamylases, GAM-1 and GAM-2 present with or without 1 M sorbitol or 1 M trehalose were incubated in a water bath under different temperatures, and then all samples were applied to 10% Native-PAGE. At 60°C, the enzymes were not denatured, indicated by the band sizes not being changed. At 70°C, only the band size of GAM-1 diminished, suggesting that the thermal stability of GAM-2 was higher than GAM-1 (Figs. 7A and 7B). At 80°C, both additives...

![Fig. 6.](image)

Fig. 6. Effects of sorbitol and trehalose on thermal aggregates of GAM-1 and GAM-2 at indicated temperatures for 10 min. (A) The temperature trends of the hydrodynamic radius of GAM-1 and its additives complex. (B) The temperature trends of the hydrodynamic radius of GAM-2 and its additives complex. (C) DLS size distribution of GAM-1 and its additives complex. (D) DLS size distribution of GAM-2 and its additives complex. Symbols: square, samples in basic buffer; circle, samples in basic buffer containing 1 M sorbitol; triangle, samples in basic buffer containing 1 M trehalose.
weakened the band size diminish of both GAM-1 and GAM-2 further, which confirmed that the additives could effectively prevent glucoamylases against thermal denaturation.

On comparing the effects of thermal inactivation and conformational changes, trehalose was more efficient than sorbitol. Trehalose and sorbitol bonding with solvent molecules by hydrogen increased the viscosity and surface tension of the protein solution, leading to the creation of preferential hydration sites on the protein surface. Trehalose contained more hydroxyl groups than sorbitol, which may cause larger viscosity and stronger surface tension.

Effects of Additives on Kinetic Parameters of GAM-1 and GAM-2

The $K_m$ of GAM-1 decreased from 5.60 to 2.66 mg/ml in 1 M sorbitol and 0.89 mg/ml in 1 M trehalose. For GAM-2, the $K_m$ decreased from 7.80 mg/ml to 2.98 mg/ml and 1.49 mg/ml (Table 2). In addition, the $k_{cat}/K_m$ value of GAM-1 in 1 M sorbitol and 1 M trehalose increased 1.99-fold and 3.80-fold, respectively, and for GAM-2, the value increased 1.1-fold and 2.08-fold, respectively. The results suggested that the glucoamylases in the presence of sorbitol or trehalose caused a higher substrate affinity and catalytic efficiency. Enzyme substrate interactions involve conformational shifts from one microstate to another with altered surface area, and this process was apparently affected by the stabilizing solutes [12]. A stabilizing solute may limit the mobility of enzyme domains, leading to lower apparent $K_m$ values. The reaction rate decrement by sorbitol and trehalose may be related to the reduction of the molecule’s motion [12].

Our present study systematically represented the effects of sorbitol or trehalose on the conformational changes of glucoamylases GAM-1 and GAM-2. The thermal stabilities of GAM-1 and GAM-2 were enhanced by additives. The possibility of stabilizing the glucoamylases GAM-1 and GAM-2 against thermal inactivation in the presence of sorbitol or trehalose would be of great interest for

Table 2. Kinetic parameters of GAM-1 and GAM-2 in the absence and presence of additives.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_m$ (mg/ml)</th>
<th>$V_{max}$ (mg/ml/min)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (ml/mg/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAM-1 Basic buffer</td>
<td>5.60</td>
<td>6.50</td>
<td>1,840</td>
<td>328.6</td>
</tr>
<tr>
<td>1 M Sorbitol</td>
<td>2.66</td>
<td>4.50</td>
<td>1,736</td>
<td>652.6</td>
</tr>
<tr>
<td>1 M Trehalose</td>
<td>0.89</td>
<td>3.10</td>
<td>1,111</td>
<td>1248.3</td>
</tr>
<tr>
<td>GAM-2 Basic buffer</td>
<td>7.80</td>
<td>9.27</td>
<td>2,053</td>
<td>263.2</td>
</tr>
<tr>
<td>1 M Sorbitol</td>
<td>2.98</td>
<td>3.91</td>
<td>865</td>
<td>290.7</td>
</tr>
<tr>
<td>1 M Trehalose</td>
<td>1.49</td>
<td>3.70</td>
<td>817</td>
<td>548.3</td>
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</table>
industrial preparation, storage, and application, which is important to make the two glucoamylases employ better in different industries.

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References


