Kinetic Inhibition of Human Salivary alpha-Amylase by a Novel Cellobiose-Containing Tetrasaccharide

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Objective: *The aim of the study was to evaluate the inhibitory kinetics of a novel cellobiose-containing tetrasaccharide on human salivary α-amylase (HSA).*

Material and Method: *Synthesis of cellobiose-containing tetrasaccharide was catalyzed by Paenibacillus sp. A11 CGTase using β-CD as a donor and cellobiose as an acceptor under the optimal conditions. The reaction mixture was analyzed by HPLC and a cellobiose-containing tetrasaccharide obtained was studied for its inhibitory kinetics.*

Results: *In vitro activity of human salivary α-amylase showed the optimum pH and temperature at 7.0 and 37°C, respectively. The effects of metal ions, protective chemicals and saccharides on α-amylase activity, they were found that 10 mM concentration of $CaCl_2$ and NaCl enhanced the enzyme activity. In contrast, the enzyme activity was significantly inhibited by 10 mM of $HgCl_2$, α-cyclodextrin (α-CD) and synthetic cellobiose-containing tetrasaccharide. Chemicals often used as protective substance for enzyme such as β-mercaptoethanol, EDTA or used as fungicide during enzyme purification (NaN_3) had no effect on the activity of this enzyme. As a cellobiose-containing tetrasaccharide was shown to have a pronounce inhibition on α-amylase activity. Its inhibition kinetic was performed and found that cellobiose-containing tetrasaccharide was a competitive inhibitor with a K_i value of 7.89 μM.*

Conclusion: *Inhibition kinetic of a cellobiose-containing tetrasaccharide on α-amylase activity was competitive type with K_i value of 7.89 μM. In addition, these results will be a basic knowledge in controlling α-amylase actions that have influence on blood glucose level of trial animal and human further.*

Keywords: *Beta-cyclodextrin (β-CD), Cellobiose, Enzyme inhibitor, Human salivary α-amylase (HSA), Tetrasaccharide*

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α-Amylase (EC 3.2.1.1) catalyzes the hydrolysis of the α (1→4) glucan linkages in polysaccharides of starch, amylose, amylopectin, glycogen and various maltodextrins. The α (1→6) bond is not hydrolyzed. α-Amylases are produced from a diverse variety of organisms such as bacteria, fungi, plants and animals[1-4]. Two kinds of α-amylases are produced by many mammals, salivary α-amylase from the parotid gland and pancreatic α-amylase from the pancreas. The digestion of food starch begins with salivary α-amylase in the mouth and then this digestion is stopped by the lower pH in the stomach. As soon as the food travels from the stomach into the small intestine, it is neutralized. Then, the digestion of starch is completed by an α-amylase from the pancreas secreted into the small intestine. For the structure of human salivary α-amylase (HSA), it consists of 496 amino acid residues, one calcium ion and one chloride ion and folds into three domains: A, B and C. The central N-terminal domain A serves as a scaffold on which two flexible domain B (complex loop) and domain C (independent domain) are placed. The starch-binding site of HSA, a deep cleft at the interface between domains A and B, consist of six subsites, (-4) through (+2). The interactions between the substrate and HSA at subsites (-1) and (+1), wherein the cleavage occurs, are very well conserved among many α-amylases[5-7]. Presently, α-amylases also have important industrial uses in the modification of starch in food materials and in the industrial production of maltodextrins. They are of interest in the clinical analysis of some diseases such as pancreatitis[8,9]. Inhibitors of α-amylases have applications in modifying and controlling α-amylase action that have medical applications such as the

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influence on blood glucose, serum insulin and starch loading tests in animals and human[10]. In addition, inhibition of human salivary α-amylase can prevent oral diseases including dental caries, periodontal disease and tooth loss. Due to oral bacteria, they utilize the starch hydrolyzing activity to obtain their nutrients. Localized acid production by bacteria, the metabolism of maltose generated by human salivary α-amylase, can lead to the dissolution of tooth enamel, a critical step in dental caries formation.

In a previous paper, the authors reported the synthesis of a novel cellobiose-containing tetrasaccharide using β-cyclodextrin (β-CD) as glucosyl donor and cellobiose as acceptor by the transglycosylation reaction of *Paenibacillus* sp. A11 CGTase[11]. In the present study, the authors report the kinetic inhibition of human salivary α-amylase by a tetrasaccharide containing cellobiose.

Material and Method

Materials and enzymes

Potato soluble starch (molecular mass 296 kDa), β-CD donor, cellobiose acceptor and 3,5-dinitrosalicylic acid were purchased from Sigma-Aldrich Chemical Co., MO, USA. Synthetic amylose AS-10 with an average molecular mass of 10 kDa was obtained from Nakano Vinegar Co., Ltd. (Aichi, Japan). Lyophilized power of human salivary α-amylase (Product number A1031) was purchased from Sigma-Aldrich Chemical Co., MO, USA. *Paenibacillus* sp. A11 CGTase (EC 2.4.1.19) was purified by starch adsorption and DEAE-Toyoperl 650M chromatography column (Tosoh Corporation, Tokyo, Japan)[12]. The enzyme had a specific activity of 5,000 U/mg as determined by its dextrinizing activity[12]. The other common chemicals were of reagent grade.

Synthesis of cellobiose-containing tetrasaccharide

Paenibacillus sp. A11 CGTase (64 U/ml) was added to 50 ml reaction mixture, containing 2% (w/v) of β-CD donor and 0.5% (w/v) of cellobiose acceptor in 50 mM sodium acetate buffer, pH 6.0 at 30°C for 2 h[11]. After stopping the reaction by boiling at 100°C for 5 min, the reaction mixture was analyzed by HPLC (Shimadzu model LC31) using a Spherisorb-NH_2 column (0.46 x 25 cm) and detected by RI detector. Prior to injection, the sample was filtered through 0.45 mm membrane filter. The eluent was a 3:1 (v/v) ratio of acetonitrile: water and the flow rate was 2 ml/min. The tetrasaccharide product (PC2) at retention time (R_t) 4.42 min was collected and lyophilized for further kinetic inhibition studies.

Human salivary α-amylase activity assay and protein concentration

The α-amylase activity was assayed in 0.5 ml reaction mixture that contained 0.2% (w/v) potato soluble starch in 0.2 M phosphate buffer, pH 7.0 and diluted enzyme (50 µl, 48 µg/ml). After incubation at 37°C for 5 min, the reducing sugar formed was measured by using the 3,5 dinitrosalicylic acid method[13].

One unit (U) of α-amylase was defined as the amount of enzyme that produced 1 µmol of reducing sugar as glucose in 1 min per ml of reaction at 37°C.

The protein concentrations were determined according to Bradford[14], using bovine serum albumin (BSA) as standard.

Effect of pH on the α-amylase activity

The α-amylase was incubated with 0.2% (w/v) potato soluble starch at different pHs and the enzyme was assayed by the 3,5 dinitrosalicylic acid method. The 0.1 M of acetate, phosphate, Tris-HCl and glycine-NaOH were used as reaction buffers for pH 3.0-5.0, 5.0-7.0, 7.0-9.0 and 9.0-11.0, respectively. The result was expressed as a percentage of the relative activity. The pH at which maximum activity was observed was set as 100%.

Effect of temperature on the α-amylase activity

The α-amylase was assayed by the 3,5 dinitrosalicylic acid method[13] at various temperatures from 4 to 90°C. The result was expressed as a percentage of the relative activity. The temperature at which maximum activity was observed was set as 100%.

Effect of metalions, protective chemicals and saccharides on the α-amylase activity

The α-amylase (50 µl, 48 µg/ml) was incubated in 10 mM phosphate buffer, pH 7.0 at 37°C for 30 min in the presence of various metal ions, protective chemicals and saccharides at the final concentration of 10 mM. Total reaction mixture was 100 µl. Only 50 µl of reaction mixture was withdrawn for the assay of residual activity by the 3, 5 dinitrosalicylic acid method as described. The residual activity was compared with the control condition and reported as a percentage of the relative activity.

Enzyme inhibition by a cellobiose-containing tetrasaccharide

The α-amylase (50 µl, 48 µg/ml) in 10 mM

phosphate buffer, pH 7.0 was mixed with various concentrations of synthetic amylose AS-10 and a fixed concentration of the tetrasaccharide in a final volume of 0.5 ml. The reaction mixture was incubated at 37°C for 1 min. The residual activity was assayed by the 3,5 dinitrosalicylic acid method. The velocity (V) was determined and expressed as unit of enzyme.

From all data, the plots of reciprocal initial velocities ($1/V_o$) against reciprocal substrate concentrations (1/[S]) at several concentrations of inhibitor were made for kinetic mechanism analysis. The inhibition constant (K_i) was calculated using the equation[15]:

$$\text{Slope of competitive inhibitor} = \frac{\{1 + [I]\} K_m}{K_i \quad V_{max}}$$

Where [I] is inhibitor concentration (mM), K_m is Michaelis-Menten constant (mM) and V_{max} is maximum velocity (μmole/min).

Results

Tetrasaccharide product analysis by high performance liquid chromatography (HPLC)

To synthesize the cellobiose-containing tetrasaccharide, 64 U/ml of *Paenibacillus* spp. A11 CGTase was added to 50 ml reaction mixture, containing 2% (w/v) of β-CD donor and 0.5% (w/v) of cellobiose acceptor at pH 6.0, 30°C for 2 h. The transfer products obtained were then isolated and analyzed by HPLC. The HPLC profiles of the products when using β-CD as the glucosyl donor for cellobiose in the coupling reaction, are shown in Fig. 1. At 0 h, two peaks with retention times of 3.27 and 5.94 min were the cellobiose and β-CD substrates, respectively. After 2 h, the peaks of both substrates were reduced and two peaks of transfer products with retention times of 3.81 and 4.42 min, respectively, were produced. The product peak (PC2) at retention time (R_t) 4.42 min was identified to be the cellobiose-containing tetrasaccharide by the mass spectrometer and ^{1}H and ^{13}C-NMR spectrometer in the previous report[11]. Therefore, in this study, the transfer product with R_t of 4.42 min (PC2) was collected and lyophilized for further studies.

Effect of pH and temperature on the α-amylase activity

The α-amylase was incubated with 0.2% (w/v) potato soluble starch at different pHs. In this study, the 0.1 M of acetate, phosphate, tris-HCl and glycine-NaOH were used as reaction buffers for pH 3.0-5.0, 5.0-7.0, 7.0-9.0 and 9.0-11.0, respectively. Activities at different pHs are shown in Fig. 2A. Optimum pH of this α-amylase was at pH 7.0. The enzyme showed 50-70% of the activity at pH 5.0-6.0 and 8.0-9.0 while 30% of the activity was observed at pH 11.0. At pH 3.0-4.0, the activities of enzyme were lower than 20%.

The optimum temperature of the enzyme was investigated by incubating the reaction mixture at various temperatures from 4 to 90°C. The enzyme showed the highest activity at 37°C which was defined

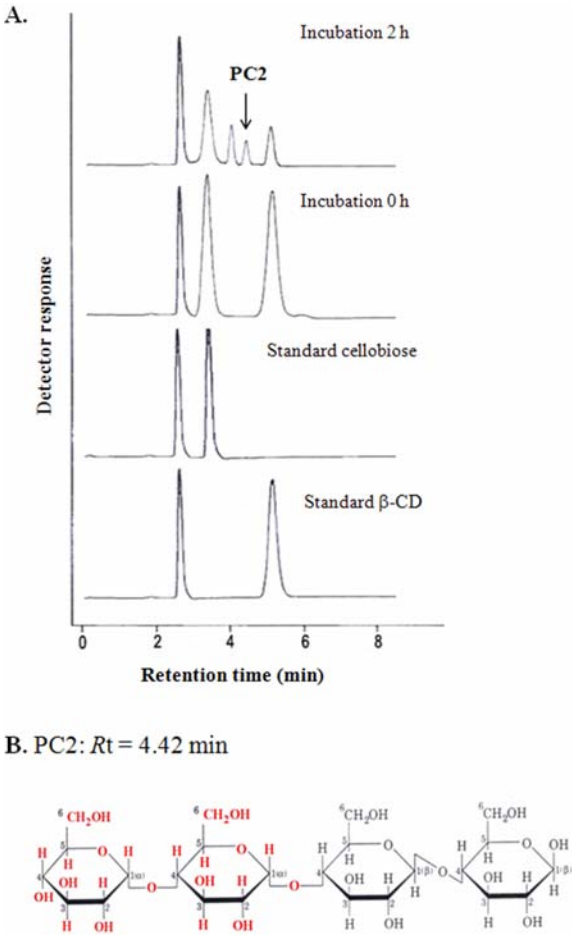

Fig. 1 Tetrasaccharide product analysis by HPLC (A.) HPLC chromatograms of standard β-CD, standard cellobiose and the reaction mixtures of the *Paenibacillus* sp. A11 CGTase-catalyzed coupling reaction at 0 and 2 hrs. The reaction conditions were: 2.0% (w/v) β-CD, 0.5% (w/v) cellobiose, 64 U/ml CGTase at 30°C, pH 6.0 for 2 h. The peak of PC2 transfer product was indicated by an arrow. (B) The proposed structure of PC2 by mass spectrometry and NMR analysis[11].

as 100% activity. At 70 and 80°C, 40 and 20 percents of the activity still remained (Fig. 2B).

Effect of metalions, protective chemicals and saccharides on the α-amylase activity

The effects of metal ions, protective chemicals and saccharides on α-amylase activity are summarized in Table 1. The enzyme was completely inhibited by 10 mM concentration of Hg^{2+} and Ag^{+} while Ca^{2+} and Na^{+} were shown to be enzyme activator. Chemicals often used as protective substance for enzyme such as β-mercaptoethanol, EDTA or used as fungicide during enzyme purification (NaN_3) had no effect on the activity of this enzyme. The saccharides, α-CD, maltose and cellobiose had no effect on enzyme activity except, a novel cellobiose-containing tetrasaccharide significantly inhibited α-amylase activity.

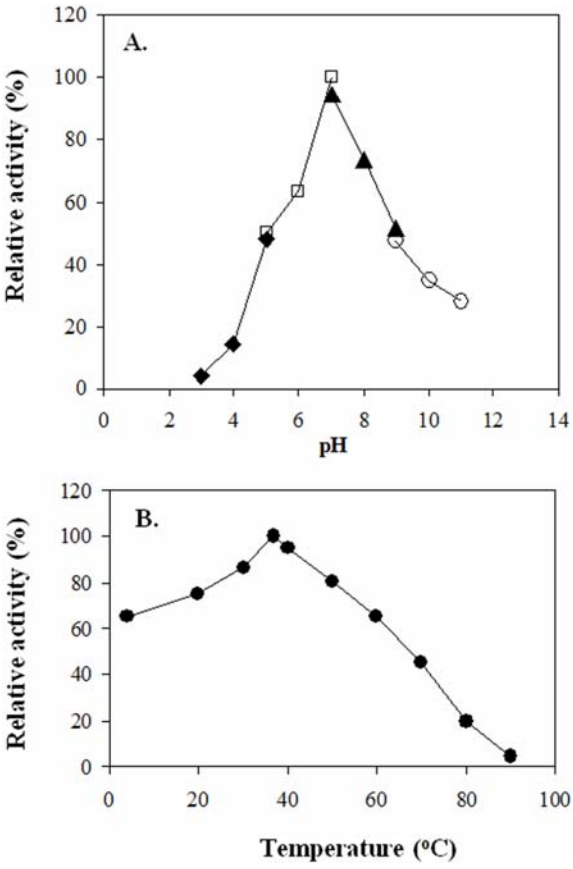

Fig. 2 Effect of pH and temperature on the α-amylase activity (A) Buffers used: 0.1 M acetate buffer, pH 3.0-5.0 (◆); 0.1 M K-phosphate buffer, pH 5.0-7.0 (□); 0.1 M Tris-HCl buffer, pH 7.0-9.0 (▲) and 0.1 M glycine-NaOH buffer, pH 9.0-11.0 (○). The highest activity was defined as 100%. Each point represents as a mean value of triplicate assays (B) The activity was measured at various temperatures as described. The highest activity was defined as 100%. Each point represents as a mean value of triplicate assays

Kinetic inhibition of the α-amylase by a cellobiose-containing tetrasaccharide

A Lineweaver-Burk plot of the initial velocities versus variable concentrations of the inhibitor at fixed different concentrations of substrate is shown in Fig. 3. It was found that a cellobiose-containing tetrasaccharide was also a strong competitive inhibitor for human salivary α-amylase with a K_i value of 7.89 x 10^{-3} mM.

Discussion

α-amylase is one of the major secretory products of the pancreas and salivary glands in human, playing an important role in digestion of starch to maltose and glucose. In normal persons, glucose obtained from α-amylase action in which was metabolized further by insulin action. However, for diabetic, the control of α-amylase action with enzyme inhibitor is important on glucose level in blood. Various α-amylase inhibitors have previously been studied such as acarbose[16] and α-acarviosinyl-(1→4)-α-D-glucopyranosyl-(1→6)-D-glucopyranosylidene-spiro-thiohydantoin (PTS-G-TH) inhibitors[5]. An understanding of how these inhibitors bound to the enzyme should provide a rational basis for the development of

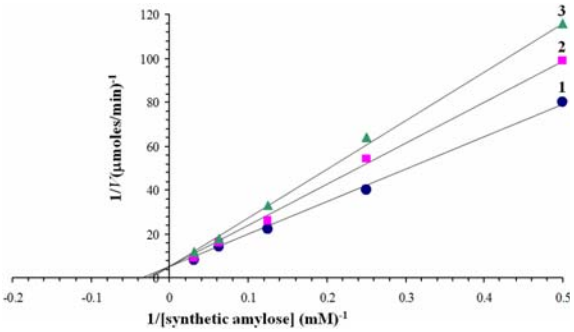

Fig. 3 α-amylase inhibition pattern by a cellobiose-containing tetrasaccharide. A Lineweaver-Burk plot for the human salivary α-amylase reaction with various concentrations of a cellobiose-containing tetrasaccharide as followed; no inhibitor (line 1), $2.5x10^{-3}$ mM a cellobiose-containing tetrasaccharide (line 2) and $5.0x10^{-3}$ mM a cellobiose-containing tetrasaccharide (line 3). Each point represents as a mean value of triplicate assays

new molecules with an increased affinity and specificity for human salivary α-amylase. For this paper, the authors are interested in studying the role of a novel tetrasaccharide [glucose (α1→4) glucose (α1→4) cellobiose] which had a molecular mass of 666 on activity of human salivary α-amylase[11]. The other cellobiose oligosaccharides have been reported to beneficially use in the food, feed, pharmaceutical and cosmetic industries[17,18]. They could be applied as a substitute sugar or drug for diabetics, to improve the intestinal microflora (prebiotic), or to prevent dental caries from inhibition of starch metabolism in the oral cavity.

In the present study, α-amylase from human salivary showed the optimum pH of 7.0 which was corresponding to the other previous reports[16,19]. For the buffers effects, at pH 7.0, Tris-HCl showed low enzyme activity while potassium phosphate was more appropriate (Fig. 2A) and has been chosen to use in further study. The optimum temperature of human salivary α-amylase was at 37°C which was similar to *Aspergillus oryzae*, *Bacillus amyloliquefaciens* and porcine pancreatic α-amylases[16]. However, the unique optimum temperature of 45°C was observed with α-amylase from *Clostridium acetobutylicum* ATCC 824[20].

When human salivary α-amylase was tested for the effects of metalions, protective chemicals and saccharides on enzyme activity, it was found that the enzyme was inhibited by metal ions such as Ag^+ and Hg^{2+} but stimulated by Ca^{2+}. The response of α-amylase to metal ions seemed to follow a similar pattern to those of other α-amylases[20,21]. Regarding the effects of metal ions on α-amylase action, Paquet et al[20] reported that 1 mM concentration of sulfhydryl oxidant metals (Hg^{2+}; an-S-Hg-S-bridge, $Ag^+$and Cu^{2+}) totally inhibited enzyme activity. Yoon et al[16] and Levitzki et al[19] found that both Ca^{2+} and Cl^- were necessary for enzyme activity and stability. The chemicals often used as protective substance for enzyme such as β-mercaptoethanol, EDTA or used as fungicide during enzyme purification (NaN_3) had no effect on the activity of this enzyme. The saccharides, α-CD, maltose and cellobiose, had slightly effect on a-amylase activity while a cellobiose-containing tetrasaccharide significantly inhibited activity of this α-amylase (Table 1). The K_i value of human salivary α-amylase on cellobiose-containing tetrasaccharide was 7.89 x 10^{-3} mM. Other examples of α-amylase inhibitors, Yoon et al[16] reported that acarbose was a mixed inhibitor for human salivary α-amylase with a K_i value of 1.27 x 10^{-3} mM while Kandra et al[5] reported that PTS-G-TH was a mixed non-competitive inhibitor with that of 8.45 x 10^{-3} mM when used amylose as substrate. So, when compared to the potency of the inhibitors in the human salivary α-amylase inhibition, it was found that the cellobiose tetrasaccharide was less effective inhibitor than acarbose, but it was still better than PTS-G-TH.

In summary, the present study clearly showed

Table 1. Effects of metal ions, protective chemicals and saccharides on α-amylase activity

Compound*	Final concentration	Relative activity (%)
None	-	100
Metal ions		
$CaCl_2$	10 mM	158
NaCl	10 mM	130
$AgNO_3$	10 mM	0
$HgCl_2$	10 mM	0
Protective chemicals		
β-mercaptoethanol	10 mM	105
EDTA	10 mM	100
NaN_3	10 mM	99
Saccharides		
α-CD	10 mM	96
Maltose	10 mM	95
Cellobiose	10 mM	97
Cellobiose-containing tetrasaccharide	10 mM	22
	50 mM	0

*Incubation with 50 µl of 48 µg/ml enzyme at 37°C, pH 7.0 for 30 min

that the cellobiose tetrasaccharide had effect on human salivary α-amylase activity as an inhibitor. This result can lead to basic knowledge in controlling the α-amylase action that has influence on blood glucose and serum insulin in human.

Conclusion

In conclusion, the human salivary α-amylase activity on potato soluble starch was optimal at pH 7.0 and 37°C. The activity of the HSA was stimulated by $CaCl_2$ and NaCl and was inhibited by $HgCl_2$, α-CD and synthetic cellobiose-containing tetrasaccharide. For the cellobiose-containing tetrasaccharide inhibitor, the inhibitory kinetic was studied and the result showed that the cellobiose-containing tetrasaccharide could inhibit the human salivary α-amylase in type of a competitive inhibition with a K_i of 7.89 μM.

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Potential conflicts of interest

None.

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จลนศาสตร์ในการยับยั้งเอนไซม์แอลฟา-อะมีเลสจากน้ำลายมนุษย์ของสารชนิดใหม่เททระแซ็กคาไรด์ที่มีเซลโลไบโอสเป็นองค์ประกอบ

ประกานต์ ฤดีกุลธำรง, จารุณี ควรพิบูลย์

วัตถุประสงค์: *จุดประสงค์ของการศึกษาเพื่อประเมินค่าการยับยั้งทางจลนศาสตร์ของเอนไซม์แอลฟา-อะมีเลสจากน้ำลายมนุษย์ด้วยเททระแซ็กคาไรด์ชนิดใหม่ที่มีเซลโลไบโอสเป็นองค์ประกอบ*

วัสดุและวิธีการ: *สังเคราะห์เททระแซ็กคาไรด์ชนิดใหม่ที่ประกอบด้วยเซลโลไบโอสด้วยการเร่งปฏิกิริยาของเอนไซม์ไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรส โดยการใช้บีตา-ไซโคลเดกทรินซ์เป็นสับสเตรทตัวให้และเซลโลไบโอสเป็นสับสเตรทตัวรับภายใต้เงื่อนไขที่เหมาะสม สารผลิตภัณฑ์ที่ได้จากการสังเคราะห์ด้วยเอนไซม์จะถูกแยก และวิเคราะห์ชนิดของสารด้วยเครื่อง HPLC และนำเฉพาะเททระแซ็กคาไรด์ชนิดใหม่ที่ได้ไปใช้ศึกษาจลนศาสตร์การยับยั้งของเอนไซม์*

ผลการศึกษา: *เอนไซม์แอลฟา-อะมีเลสในน้ำลายมนุษย์แสดงการเร่งปฏิกิริยาสูงสุดที่พีเอช และอุณหภูมิ 7.0 และ 37 องศาเซลเซียส ตามลำดับการศึกษาผลกระทบการทำงานของเอนไซม์ต่อสภาวะที่มีไอออนของโลหะ สารเคมีป้องกันเอนไซม์ และน้ำตาลพบว่าที่ความเข้มข้น 10 มิลลิโมลาร์ของแคลเซียมคลอไรด์ และโซเดียมคลอไรด์ช่วยเพิ่มการทำงานของเอนไซม์ได้ดีขึ้น ในทางตรงกันข้ามการทำงานของเอนไซม์ถูกยับยั้งอย่างมีนัยสำคัญเมื่อเติม 10 มิลลิโมลาร์ของเมอร์คิวริคคลอไรด์ แอลฟา-ไซโคลเดกซ์ทริน และเททระแซ็กคาไรด์สังเคราะห์ที่มีเซลโลไบโอสเป็นองค์ประกอบ สำหรับสารเคมีในกลุ่มที่ทำหน้าที่ป้องกันเอนไซม์ เช่น บีตา-เมอแคปโตเอทธานอล อีดีทีเอ หรือสารที่ใช้ฆ่าเชื้อราระหว่างการทำเอนไซม์ให้บริสุทธิ์ เช่น โซเดียมเอไซด์พบว่าไม่มีผลกระทบใดๆ ต่อการทำงานของเอนไซม์นี้ และจากการศึกษาจลนศาสตร์การยับยั้งของเอนไซม์ต่อเททระแซ็กคาไรด์ที่มีเซลโลไบโอสเป็นองค์ประกอบ พบว่าเททระแซ็กคาไรด์เป็นตัวยับยั้งเอนไซม์แอลฟา-อะมีเลสแบบแข่งขันให้ค่าคงที่การยับยั้งเท่ากับ 7.89 ไมโครโมลาร์*

สรุป: *จลนศาสตร์การยับยั้งของเททระแซ็กคาไรด์ที่มีเซลโลไบโอสต่อการทำงานของเอนไซม์แอลฟา-อะมีเลสเป็นชนิดการยับยั้งแบบแข่งขันโดยมีค่าคงที่การยับยั้งเท่ากับ 7.89 ไมโครโมลาร์ นอกจากนี้ ผลการทดลองที่ได้จะเป็นความรู้พื้นฐานในการศึกษาการควบคุมการทำงานของเอนไซม์แอลฟา-อะมีเลส ต่อระดับกลูโคสในเลือดของสัตว์ทดลองและมนุษย์ต่อไป*