GLYCEMIC RESPONSE TO CORN STARCH MODIFIED WITH CYCLODEXTRIN GLYCOSYLTRANSFERASE AND ITS RELATIONSHIP TO PHYSICAL PROPERTIES

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Abstract

Corn starch was modified with cyclodextrin glycosyltransferase (CGTase) below the gelatinization temperature. The porous granules with or without CGTase hydrolysis products may be used as an alternative to modified corn starches in foods applications. The amount and type of hydrolysis products were determined, containing mainly β-cyclodextrin (CD), which will influence pasting behavior and glycemic response in mice. Irregular surface and small holes were observed by microscopic analysis and differences in pasting properties were observed in the presence of hydrolysis products. Postprandial blood glucose in mice fed gelatinized enzymatically modified starch peaked earlier than their ungelatinized counterparts. However, in ungelatinized enzymatically modified starches, the presence of β- CD may impede the orientation of amylases slowing hydrolysis, which may help to maintain lower blood glucose levels. Significant correlations were found between glycemic curves and viscosity pattern of starches.

Keywords: corn starch; glucose response; enzymatic modification; CGTase; cyclodextrins.

Abbreviations

AUC: area under the curve

CD: cyclodextrin

CGTase: cyclodextrin glycosyltransferase

CGT-NW: unwashed enzymatically modified sample with cyclodextrin glycosyltransferase

CGT-W: washed enzymatically modified sample with cyclodextrin glycosyltransferase

G: glucose

GI: Glycemic index

N: Native corn starch

1. Introduction

Starch is one of the most important glycemic carbohydrates that provides great part of the energy requirement for humans through diet. Starch modification leads to structural changes of the granule varying also properties like pasting, gelling, digestibility and absorption. Enzymatic modification of starch has been proposed as a very attractive way to modulate those properties. Enzymatic modification under gelatinization temperature is less common but it may provide useful properties for some food applications. CGTase is an endoenzyme member of the α-amylase family or glycosyl hydrolase family 13. It catalyzes the cleavage of an interior α-glycosidic bond producing oligosaccharides that subsequently cyclize into cyclodextrins (CDs). The most common CDs are α-, β-, and γ-CD consisting of six, seven, and eight glucose monomers, respectively. CDs have circular conformation in which the glucose units are arranged in a circle with the hydrophilic side facing out, which confers its solubility in water. An internal hydrophobic cavity is formed that can enclose inclusion complexes of hydrophobic compounds [1]. Some CDs may sequester cholesterol and reduce its absorption, apart from other applications as food preservatives [2].

Moreover, enzymatic modification of starches has been proposed to reduce the glycemic index (GI) and to improve the quality characteristics of the foods. The GI is defined as the area under the curve of the blood glucose concentration in the 2 h period immediately following consumption of a fixed amount of digestible carbohydrate compared to the *in vivo* digestibility of

white bread as a standard [3]. The rate of carbohydrate digestion and absorption affects blood glucose concentration and is of high interest because of its impact on diabetes and other metabolic diseases. Diabetics may benefit from low glycemic index (GI) foods as low GI diets protects against development of type 2 diabetes in general population [4]. For instance the treatment of banana starch with pullulanase reduced the available starch [5], or α-amylase treatment of corn starch reduced postprandial glycemic response in rats [6]. Moreover, the addition of CDs to soluble starch also contributes to lower the GI, owing the action of γ-CD [7]. Therefore, starch state is crucial to the GI value and the role of some CDs has been reported, but there is no information about the impact of CGTase modified starch and the resulting hydrolysis products on the GI. Further, animal studies offer the possibility to study the GI concept in a near lifelong perspective. Hence, it is on our particular interest to study the effects of enzymatic modification by CGTase on corn starch samples and the possible health benefits of containing CDs and modify the GI in food formulation application. The present study evaluated the effect of enzymatic modification of corn starch by CGTase, under specific sub-gelatinization conditions, focused on the glycemic properties in mice and their relationship to physical properties.

2. Materials and methods

2.1. Materials and reagents

Corn starch samples were generously supplied by Huici Leidan (Navarra, Spain). Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) (Toruzyme® 3.0 L) of food grade was provided by Novozymes (Bagsværd, Denmark). Chemical reagents from Sigma-Aldrich (Madrid, Spain) were of analytical grade.

2.2. Methods

2.2.1. Samples preparation

Corn starch (10.0 g) was suspended in 50 mL of 20 mM sodium phosphate buffer at pH 6.0. Modified starches were prepared by adding CGTase (0.32 U of CGTase/g starch). Samples were kept in a shaking water bath (50 rpm) at 50 ºC for 48 h. 50 mL of water were added to the

suspensions and homogenized with a Polytron Ultraturrax homogenizer IKA-T18 (IKA works, Wilmington, DE, USA) for 1 min at speed 3. Samples were centrifuged for 15 min at 7,000×*g* and 4 ºC. The starch pellets were washed with 50 ml of water and centrifuged again with the same conditions. Supernatants were pooled and boiled in a water bath for 10 min to inactivate the enzyme. To assess the role of the water soluble hydrolysis products, two enzymatically treated corn starches were prepared, washed enzymatically treated corn starch (CGT-W) and the enzymatically treated washed corn starch with hydrolyzates added back (CGT-NW). CGT-W sediments were freeze-dried and kept at 4 ºC for further analyses. CGT-NW contained CGT-W and the water soluble compounds from heated and dried washings. The CGT-NW was also freeze-dried and kept at 4 ºC for further analyses. Native corn starch (N) without treatment was used as the control sample.

2.2.2. CDs and oligosaccharides quantification by High Performance Anion Exchange Chromatography.

Supernatants containing released hydrolysis compounds were freeze-dried and oligosaccharides and CDs were detected by HPAEC through a CarboPac PA-100 column (250 mm × 4 mm), coupled to a pulsed amperometric detector (Dionex). The flow rate was 1.0 mL/min and the injection volume 10 μL. Using solutions A (water), B (1 mol/L NaOH), C (1 mol/L $C_2H_3NaO_2$), and D (water), the following running profile was applied: time zero, 46.25% A, 5% B, 2.5% C, 46.25% D; 25 min, 42.5% A, 5% B, 10% C, 42.5% D; 1 min, 35% A, 15% B, 15% C, 35% D; 3 min, 33% A, 15% B, 19% C, 33% D; 5 min, 28.5% A, 15% B, 28% C, 28.5% D; 1.5 min, 18.5% A, 15% B, 48% C, 18.5% D. For the identification and quantification of each compound, standards of known concentrations were previously analyzed [8]. Analysis was carried out at least in duplicate.

2.2.3. Scanning electron microscopy (SEM)

Corn starch powders, held to specimen holders with cuprum tape, were coated with gold in a vacuum evaporator (JEE 400, JEOL, Tokyo, Japan). Samples were scanned at 10 kV

105 accelerating voltage with a SEM (S-4800, Hitachi, Ibaraki, Japan). The microstructure analysis
106 was performed using image analysis software (Image-Pro Plus 7.0, Media Cybernetics, USA) in
107 the Central Service for Experimental Research of the Universidad de Valencia.
108 *2.2.4. Pasting properties*
109 The pasting properties were determined with a rapid visco analyzer (RVA) (model 4500, Perten
110 Instruments, Hägersten, Sweden) using the Approved Methods of the American Association of
111 Cereal Chemists (AACC International, 1997), although 2 g of starch were used. Viscosity was
112 recorded using Thermocline software for Windows (Newport Scientific Pty. Limited). Assays
113 were carried out in triplicate.
114 *2.2.5. Determination of postprandial glucose by glucose tolerance test (GTT)*
115 Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) were used for the GTT.
116 The mice were housed individually in an environmentally controlled room (20–22 °C, 60%
117 relative humidity, 12-h alternating light:dark cycle). Mice were acclimatized and had *ad libitum*
118 access to water and mouse chow diet (LabDiet, PMI International; protein, 239 g/kg; fat, 50
119 g/kg; non-nitrogenous substances, 487 g/kg; crude fiber, 51 g/kg; ash, 70 g/kg; energy, 17
120 MJ/kg; and sufficient amounts of minerals and vitamins for healthy maintenance). Samples
121 tested included glucose (G), native corn starch (N), washed corn starch treated with CGTase
122 (CGT-W) and non-washed corn starch treated with CGTase (CGT-NW). The physiological
123 effects of CGTase modified starches on postprandial glucose were assessed. Starch samples were
124 administered by gavage. Starches were suspended in water, 20% (w/v) for ungelatinized samples
125 and 10% (w/v) for gelatinized samples, due to their high viscosity produced after heating,
126 according to Ayala et al [9]. Starches were gelatinized by immersion in a boiling water bath for
127 15 min. Determination of postprandial glucose was performed on 6 mice once a week for 4
128 weeks. The samples were administered using a 4x4 Latin square design. Mice were fasted for 4
129 hours prior to starch administration and administered 100 µl/g body weight of a suspension.

Blood glucose levels were measured from the tail vein at 0, 15, 30, 60, and 120 min after sample administration using a OneTouch Ultrameter (LifeScan Inc., Milpitas, CA, USA).

Statistical analysis

Experimental data were statistically analyzed for analysis of variance (ANOVA) using Statgraphics Centurion XV software (Bitstream, Cambridge, N). When analysis of variance indicated significant *F* values, multiple sample comparisons were also performed by Fisher's least significant differences (LSD) test to differentiate means with 95% confidence ($P<0.05$). Data was also evaluated using Pearson correlation coefficients to establish relationship among variables.

3. Results and Discussion

3.1. CDs and oligosaccharides in hydrolysates

Oligosaccharides and CDs content in the starch samples are presented in Table 1. The N and CGT-W did not contain hydrolysis products. The analysis confirms the removal of the water soluble CGTase hydrolysates. The CGT-NW samples contained several oligosaccharides, with a large amount of maltose followed by maltotriose and glucose. CGTase cleaves α-1,4-glycosidic bonds in the inner part of a polysaccharide chain, leading to CDs and oligosaccharides with different degrees of polymerization through disproportionation reactions [10]. α-CD and β-CD were found in CGT-NW samples, with predominance of β-CD, likely due to the reaction conditions. Blackwood & Bucke [11] reported that β-CD was the main hydrolysis compound, although the release of α-CD could be favored by adding organic solvents. In the early stage and at low enzyme concentrations CGTase produces predominantly α-CD but with prolonged reaction time or high enzyme dosage, the amount of the other CDs exceeds that of α-CD [12]. CDs have been very useful in food formulations to encapsulate flavors, protect against oxidative degradation, and sequestrate cholesterol [2].

3.2. Microstructure of starch

155 Scanning electron micrographs of native and enzymatically modified starches are presented at
156 low magnification (x2000) in Fig.1 (see in Electronic Supplementary material) and high
157 magnification (x3500) in Fig.2. Changes in microstructures are readily evident. The native starch
158 granules have flat polygonal faces, straight edges and smooth surface with no evidence of cracks
159 or holes in the surface (Fig 1a and 2a). Enzymatically modified starch granules (Fig 1b and c; 2b
160 and c) have a distinctly different surface microstructure. After 48 hours of enzymatic treatment,
161 shapeless structures appear where edges and corners were no longer visible. Native starch
162 granules have a unique semi-crystalline supramolecular structure with concentric layers of
163 amorphous, made up of amylose, and crystalline regions, manly composed of amylopectin,
164 radiating from the hilum. It might be expected that treatment with CGTase at 50 ºC promotes
165 hydrolysis of the amorphous areas of granules, leading to internal fissures whereas the crystalline
166 lamellas are more resistant to hydrolysis. Small and randomly distributed porous starches were
167 obtained when corn starch was treated with CGTase at sub-gelatinization temperature because
168 starch in the interior is more susceptible to enzyme action than the outer part of the granules.
169 This mode of degradation differs from those exhibited by the action of other amylase enzymes
170 on corn starch granules that yield bigger and open holes [13]. This interior hydrolysis confirms
171 the unique CGTase degradation mechanism of hydrolysis activity and transglycosylation
172 reaction. At lower magnification, (Fig 1c), some hydrolysis products can be observed from the
173 action of the enzyme, not present in CGT-W samples (Fig. 1b) because those samples were
174 washed. The action of the enzyme and the presence/absence of hydrolysis products are validated
175 by SEM micrographs confirming the existence of hydrolysis products recovered from the
176 supernatant.

177 *3.3. Pasting properties*

178 RVA curves are plotted in Figure 3. Pasting properties of the enzymatic treatment were observed
179 when compared to native starch (Table 2, see in Electronic Supplementary Material). Treated
180 starches underwent the action of the enzymatic treatment and also possible rearrangement due to

annealing during the preparation process under sub-gelatinization temperature [9]. The CGT-NW treated starch showed significant lowering of the peak time indicating either earlier granule swelling during heating or an effect due to hydrolysis products. The CGT-NW starch also had higher breakdown, indicating lower stability of the samples, and lower viscosity at the end point of the cooling cycle, likely due to steric impediment of the hydrolysis products on the amylose crystallization. The setback viscosity predicts the degree to which the starch polymers are able to form networks resulting in the formation of gels that have higher viscosity as the starch paste cools. The setback was very low for CGT-NW compared to N and CGT-W samples. The oligosaccharides and CDs from the hydrolysis of amylose might physically interfere with the reorganization (gelling) process [14]. The final viscosity of the CGT-W sample was lower than the N sample probably because it contained less amylose to form the gel or viscous paste after gelatinization and cooling. Similar results have been described by Gujral and Rosell [15] when adding CGTase to wheat starch suspensions. They also observed a decrease in peak and final viscosity of the wheat starch suspension. Trough viscosity were higher for N and CGT-W compared to the CGT-NW samples because they were more stable during gelatinization and their peak and final viscosities were higher. Due to CGTase activity hydrolysis products were formed and these products were present in CGT-NW samples resulting in higher hydrolysis percentage at 95 ºC and even higher at 50 ºC when comparing to N or CGT-W samples. The differences in pasting behavior between CGT-W and CGT-NW are attributed to the presence of β-CD and oligosaccharides in the CGT-NW samples. Similar results were observed by addition of β-CD to cereal starches that caused early granule swelling, decreased onset of pasting temperature and decreased peak viscosity, likely due to its ability to form CD-lipid complex [2]. β-CD and oligosaccharides may disrupt formation of amylose-lipid complexes and increase the swelling and solubility of starch granules during gelatinization [16]. Viscosity differences have been linked with the GI of carbohydrates. Guar gum also decreased blood serum glucose in rats during

the first month of the experiment [17], suggesting that the viscosity of cereal carbohydrates may affect blood glucose and insulin responses.

3.4. Glycemic response

Glycemic response is a numerical index that reflects the overall rate of absorption of glucose from the digestive tract into the blood and transfer of glucose from the blood into peripheral tissues. Jenkins et al. [3] reported that the rate of food digestion was an important determinant of glycemic response. There was a significant difference ($P<0.05$) in blood glucose level at 15 min. between glucose and the nongelatinized N or CGTase modified starches (Fig.4). As expected, glucose had the shortest time to peak and the highest peak because it is rapidly absorbed compared to all starches. Glucose also had the most rapid decrease in blood level suggesting rapid uptake by tissues. All starches had a time to peak of about 30 min, later than glucose, attributed to the slower hydrolysis of amylopectin. The crystalline regions of amylopectin resist the conformational changes necessary for enzymatic hydrolysis. CGT-W had a slightly higher peak viscosity at 30 min compared to the other two starches. SEM had shown that starch granules incubated with amylolytic enzymes at 50 ºC results in changes in the amorphous areas, leading to granules with more ordered internal structure. Hydrolysis of the amorphous regions may lead to more internal surfaces that are accessible to enzyme attack and may be the reason that CGT-W samples seem to be more rapidly digested. CGT-NW had the lowest peak maximum and appeared to be more slowly digested. The presence of CDs may impede the orientation of amylases slowing hydrolysis. Weselake, Hill [18] showed that β-CD inhibits hydrolysis of starch by α-amylase and intestinal α-glucosidase enzymes resulting in a longer period for absorption. This result suggests that CDs and oligosaccharides decrease the rate of absorption and may help to maintain lower blood glucose levels. Glucose concentration from all samples returned to the base line level 2 hrs after the gavage samples. The area under the incremental glucose response curves (AUCs) were calculated from 0 to 2 h post feeding. No significant differences were found between samples. There is a trend where AUC for the CGT-NW starch appears to be lower than

232 the CGT-W or N starches; this may be due to the rapid hydrolysis and absorption into the blood
233 stream of the hydrolysis products but slower uptake by peripheral tissues due to β-CD inhibitory
234 action. The time to peak of gelatinized starches were shorter and about the same as for glucose
235 (Fig. 5, see in Electronic Supplementary Material). Starch gelatinization resulted in more rapid
236 digestion as shown by the decrease in time to peak from 30 min for ungelatinized to about 17-27
237 min for gelatinized starches. When starch samples are subjected to extremely boiling
238 temperatures water is absorbed, the granule swells and some amylose is leached, transforming
239 the semicrystalline granules to an amorphous state that increases accessibility to enzymes [8].
240 These results are similar to Collings et al. [19] findings, who also found that the response of
241 serum glucose to glucose monohydrate and cooked starch were very similar, while that to raw
242 starch was significantly less. No significant differences in AUC were found between starch
243 samples. Correlation between pasting properties and insulinemic responses in mice were
244 analyzed. For ungelatinized samples, there was a negative correlation between setback values
245 and glucose concentration at 2 h (r=-1.0, P< 0.05). Greater values of setback viscosity were
246 related to lower values of glucose at 2 hrs. For gelatinized samples there were positive
247 correlations between hydrolysis at 50 ºC and glucose concentration at 60 min (r=0.84, P< 0.05)
248 and hydrolysis at 95 ºC and glucose concentration at 60 min (r=0.81, P< 0.05). A negative
249 correlation was also found between peak time and glucose concentration at 60 min (r=-0.85, P<
250 0.05); setback and glucose concentration at 60 min (r=-0.88, P< 0.05), and finally trough and
251 glucose concentration at 60 min (r=-0.8218, P< 0.05). These correlations support the idea that
252 the different patterns of the glycemic curves are related to the viscosity of the samples.

253 **4. Conclusions**

254 Enzymatic modification at sub-gelatinization temperatures of corn starch by CGTase with or
255 without hydrolysis products offers new possibilities to the modern food industry and the
256 enormous variety of food products looking for healthy properties. Porous starches were obtained
257 by CGTase treatment, which manage to have different pasting behavior and diverse glycemic

response owing to the presence of ß-CD that inhibits the α-amylase action. Significant correlations were found between glycemic curves and viscosity pattern of starches. Overall, CGTase enzymatically modified corn starch may fill a niche in the diverse uses of starches.

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Compliance with Ethical Standards

All authors declare that they have no conflict of interest. All animal procedures were approved by the Animal Care and Use Committee, Western Regional Research Center, USDA, Albany, CA, USA. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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Figure captions

Fig 1 Scanning electron micrograph of corn starch samples treated enzymatically (b and c) and native corn starch sample (a). Magnification 2000×. Native corn starch (a); CGTase washed sample, CGT-W (b); CGTase non-washed sample, CGT-NW (c) (arrows show the hydrolysis products) (**in Electronic Supplementary Material**)

Fig 2 Scanning electron micrograph of corn starch samples treated enzymatically (b and c) and native corn starch sample (a). Magnification 3500×. Native corn starch (a); CGTase washed sample, CGT-W (b); CGTase non-washed sample, CGT-NW (c). Arrows indicate the presence of hydrolysis products

Fig 3 RVA profiles of enzymatically treated corn starches. Legends: Native corn starch, N (■); CGTase washed sample, CGT-W (□); CGTase non-washed sample, CGT-NW (Δ)

Fig. 4 Glucose tolerance in mice gavage with ungelatinized samples. The samples studied were glucose, G (●); native corn starch, N (■); CGTase washed sample, CGT-W (□) and CGTase non-washed sample, CGT-NW (Δ). (A) Glucose tolerance tests (GTT) were performed in the fasting state. (B) Area under the curve (AUC) values. Data are expressed as mean *n* = 6/group

Fig. 5 Glucose tolerance in mice gavage with gelatinized samples. The samples studied were glucose, G (●); native corn starch, N (■); CGTase washed sample, CGT-W (□) and CGTase non-washed sample, CGT-NW (Δ). (A) Glucose tolerance tests (GTT) were performed in the fasting state. (B) Area under the curve (AUC) values. Data are expressed as mean *n* = 6/group.

Table 1 Monosaccharides, oligosaccharides and cyclodextrins released by CGTase from corn starch, expressed in mg 100 g^{-1} of starch.

	Glucose	Maltose	Maltotetriose	Maltotetraose	Maltopentaose	α-CD	β-CD	γ-CD
CGT-NW	2,961	4,025	3,097	<dl	0,604	3,884	6,039	<dl
CGT-W	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl

<dl means under detection limit. CGT-NW: CGTase non-washed sample; CGT-W: CGTase washed sample.

Fig.2

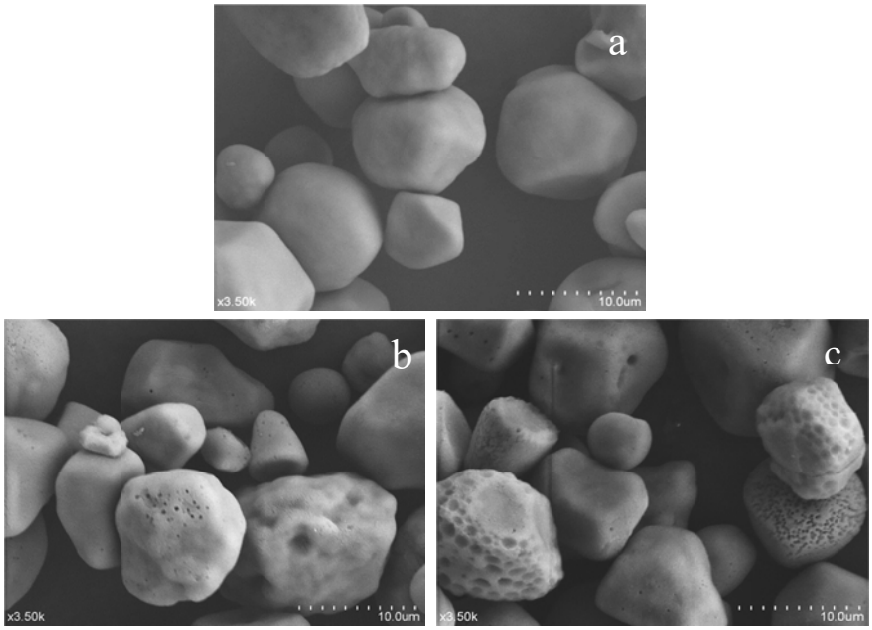

a
x3.50k
10.0um
b
x3.50k
10.0um
c
x3.50k
10.0um

Fig.3

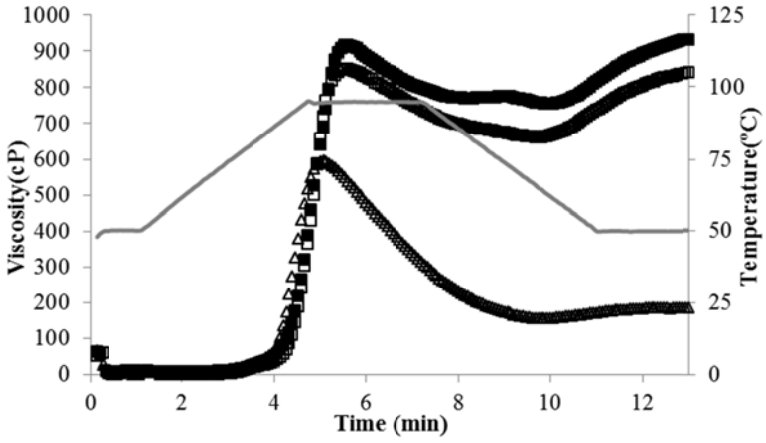
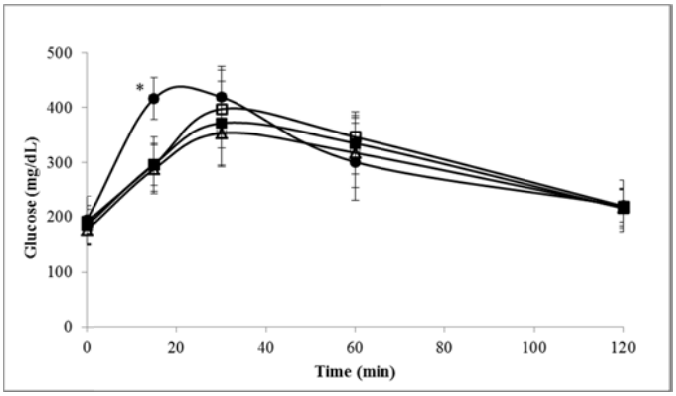

Viscosity(cP)
Temperature(°C)
Time (min)
0
100
200
300
400
500
600
700
800
900
1000
0
25
50
75
100
125
0
2
4
6
8
10
12

Fig.4

A.


Glucose (mg/dL)
Time (min)
*
0
100
200
300
400
500
0
20
40
60
80
100
120

B.

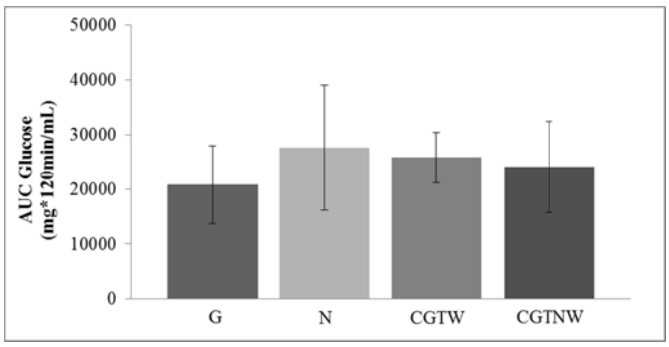
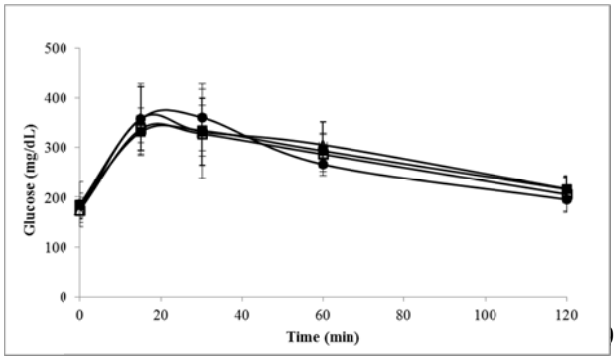
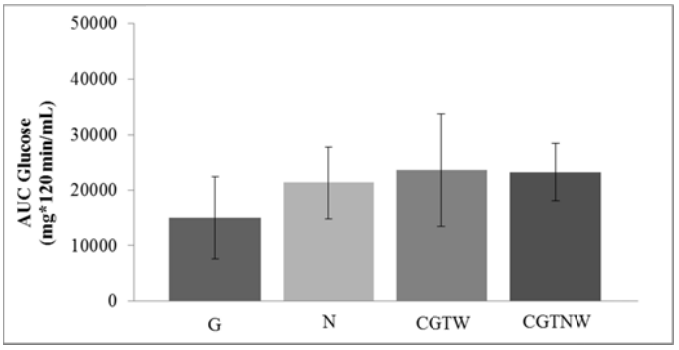

AUC Glucose
(mg*120min/mL)
0
10000
20000
30000
40000
50000
G
N
CGTW
CGTNW

Fig.5

A.


Glucose (mg/dL)
500
400
300
200
100
0
0
20
40
60
80
100
120
Time (min)

B.


AUC Glucose
(mg*120 min/mL)
50000
40000
30000
20000
10000
0
G
N
CGTW
CGTNW