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Cyclodextrin Formation Initiated by Enzyme Debranching Reaction On Amylopectin Branch Chain Of Tapioka

Amran Laga

Study Program of Food Science and Technology Faculty of Agriculture, Hasanuddin University
Kampus Unhas Tamalanrea, Jln Perintis Kemerdekaan km. 10, Makassar 90245

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ABSTRACT

Degradation of starch by the glucosyltransferase enzyme (CGTase) to produce the primary product of chain splitting undergoes an intramolecular reaction without the participation of water molecule. From this process, α-1,4-Linked cyclic product, known as cyclodextrins, are formed. The aim of the research was to cut amylopectin branch in order to produce one straight chain, to optimize cyclic reaction formation cyclodextrin by CGTase. The research was devided into 3 stages; (1) debranching enzyme concentration estimation (5,10,15,20, and 25 unit/gram) and the length of otimum reaction to produce straight chain for 5 hours which sample was taken each hour, (2) reaction length time estimation to form cyclodextrin in order to use debranching products (straight chain) as substrates, the reaction length for 360 minutes and sample taken each for 30 minutes, and (3) the best substrate concentration for straight chain (20-40% w/v) to produce cyclodextrin. The result showed that enzyme concentration treatment and optimal length reaction will produce straight chain with enzyme concentration of 14 units/gram for 3 hours and straight chain product of 20 units/gram for 1 hour with straight chain product of 83.5%. The optimum length of reaction for cyclodextrin formation from amylose produced from the de-branching process was 240 minutes. The amount of cyclodextrin produced was 143.45 g/L with conversion value of 47.81% at 30% (w/v) substrate concentration. Highest yield of cyclodextrin (154,28 g/L) and conversion value of 44.08% was obtained at 35% (w/v) substrate concentration.

Keywords: amylopectin, debranching, CGTase, cyclodextrin

INTRODUCTION

Cyclodextrin is oligosaccharide product non-reduction of modified starch with ring-formation and made through cycling reaction by CGTase activation (Cyclodextrin glycosil transferase) (Szejtli, 1988; Schmid, 1989; Tankova, 1998). Based on glucose formation, cyclodextrin was divided into: α-siklodekstrin (6 unit glucose), β-siklodekstrin (7 unit glucose) dan γ-siklodekstrin (8 unit glucose) (Szejtli, 1988 & Tankova, 1998). Cyclodextrin with ring formation can be seen at Figure 1.

CGTase mechanism is to catalyze cyclodextrin formation according to Schmid (1988) which started by 8-10 formation (or more than glucose number that formed cyclodextrin) glucose unit of starch molecules.The reaction is strated from glucose branch from non-reduction. Cyclodextrin has unique characteristics, which can be used in such industries as pharmacy, cosmetics, food, flavour, agriculture and chemical industries (Szejtli, 1988).

*Telp: +6281342665494
Email: amranlaga@yahoo.co.id

Tapioca is one of starch source to be used as cyclodexrin production. Tapioca contains high content of amylopectin 82-84% (Laga, 2008). High amylopectin caused limitation of tapioca usage as substrate to produce cyclodextrin. High amylopectin content will cause high pasta viscosity and also low CGTase activity towards branch chain. This caused by CGTase cycle activity on straight chain molecules (amilosa) is relatively higher compared to branch chain molecule (amylopectin) (Whistler *et al*., 1984).

Therefore, to optimize cyclodextrin formation from tapioca, amylopectines straight chain components needed to be cut as small chains before CGTase addition for cycle process to form cyclodextrin. The branch cutting of side chain branch (α-1.6) can produce straight component, lower pasta viscosity. This research was done to: (1) branch cutting of amylopectin to produce optimize straight chain catalyses by CGTase in cyclodextrin production, (2) know the length of time for cyclodextrin reaction to utilize the debranching products as substrates, (3) optimize substrate concentration estimation in the cyclodextrin production.

METHODS

Materials and Equipments. Materials used for the research were of pullulanase and CGTase (Toruzyme™ 3,0 L) from Novo Nordisk, tapioca flour, soluble starch , glucose standard, amylose standard, Phenolic 5%, DNS, H_2SO_4, NaOH 0,1 N, Iod, KI, Kalium-Natrium Tartrat , Pb asetat, $CaCl_2$ and $CaCO_3$.

Equipments used were shaker incubator (Barnstead/Lab-line Max^Q 4000), spektropho-tometer (UV/VIS spectrophotometer SP-3000 Plus OPTIMA JAPAN), pH meter (Dakton pH 510 Series), micropipet (Eppendorf Adjustable Volume Pipettors), sentrifugator (Heraeus Labofuge A), hot plate (Hot plate Stirerrer CB 302 Stuart), oven (Memmert) and glasswares.

Procedure. The research was devided into 3 stages; (1) enzyme debranching concentration estimation and the length of optimum reaction to produce straight chain for 5 hours which sample was taken each hour, (2) reaction length time estimation to form cyclodextrin in order to use the debranching products (straight chain) as substrates, and (3) the best substrate concentration for straight chain to produce cyclodextrin.

Concentration debranching enzyme (pullulanase) and optimum reaction time to produce straight chain. The research started by making tapioca starch for 40% w/v concentration, added Ca^{2+} ($CaCl_2$) for 12 ppm and acidity degree on pH 5. Suspension were then distributed in the flasks 250 mL with volume 150 mL. Pullulanase were then added onto suspension for 10, 15, 20, and 25 (unit/g substrate). Suspension were then heated or gelatinized with stirred to achieve 75^{0}C, then the temperature was then cooled to 50^{0}C. The reaction was maintained at 50^{0}C for 5 hours and sample was taken every one hour.

The parameter observed was amilosa value (as straight chain) IRRI methods (1971) and amylopectin value from starch differences with the lowest amilosa value (Apriyantono *et al*., 1989). The data was done in 2 variables, variable A; enzyme concentration and variable B was reaction time length. The experiment was done in 2 replicates. If there are differences in between, data were further analyzed with BNJ test.

Reaction length time estimation to form cyclodextrin in order to use debranching products (straight chain) as substrates. Debranching substrate (straight chain) preparation was done by enzyme concentration treatment and the best time length produced at the first stage. Debranching product which

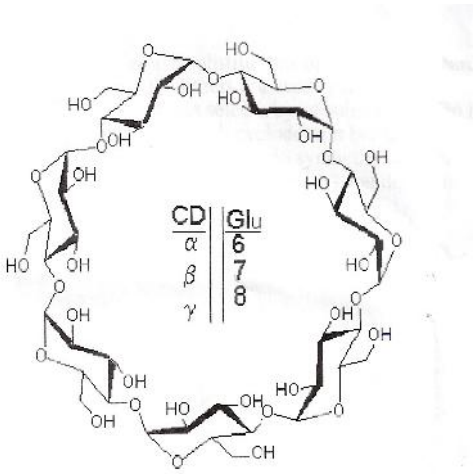

Figure 1. Structures of â-cyclodextrin (Szejtli *et al*, 1988)

was done by adding some substrate with 30% w/v concentration. Substrate was then suspended into phosphat buffer pH 6 (0.2 M) then CGTase 100 unit/ gram was added onto substrate and ethanol 10% (v/v). The ethanol usage was to prevent reversed reaction (Blackwood & Bucke, 2000). The reaction was done in shaker incubator at 60^{0}C with the stirrer speed of 200 rpm for 360 minutes. The sample was taken every 30 minutes.

The parameter observed including cyclodextrin Kitahata method (1988), reduction sugar (DNS method), and conversion value. The research was done by experimental method with 3 times repetition. The best temperature and time length was estimated by data plotting.

The best Substrate concentration estimation of straight chain on cyclodextrin formation. Substrate debranching (straight chain) preparation was done by using enzyme concentration treatment and the best reaction time length which was gained on the first treatment. The debranching product was used as base product which concentration of substrate of; 20, 25, 30, 35 and 40% w/v.

The debranching product was then suspended on phosphate buffer pH 6 (0.2 M), then CGTase was added 100 unit/gr of substrate and ethanol 10% v/v. The reaction was done in shaker incubator at 60^{0}C with the stirrer speed of 200 rpm for 360 minutes. The length of reaction of sample was taken from the best result of second stage experiment.

The parameter observed including cyclodextrin Kitahata method (1988), reduction sugar (DNS method) and conversion value. The research was done by experimental method with 2 times repetition. If there

were any significat result showed, the data was then analyzed with BNJ test.

RESULT AND DISCUSSION

Pullulanase concentration estimation and Optimized Debranching time length on Straight Chain formation (Amylose). Amylopectin. The amylopectin result showed that during dibranching reaction, the higher enzyme concentration (5-25 unit/ gram), the lower amylopectin concentration (Figure 2). The longer debranching reaction, (1-5 hours) showed the decreasing amylopectin value (Figure 2). Debranching enzyme is a specific enzyme that cut α-1.6 D-glycosidic of amylopectin (strach), glicogen and pullulan (Hamilton *et al*., 2000).

Statistical analysis indicates that enzyme concentration, length of reaction and its interaction significantly affect the breakdown of side branch. Further analysis on the effect of the interaction indicates debranching was optimal at 15 units of enzyme concentration with reaction time of 3 hours. Under these conditions, the residual amylopectin was 16%. The use of 20 units enzyme concentrtation resulted in 16.5% residual amylopectin with 1 hour of reaction time. These values indicate that the debranching of amylopectin under the above conditions was relatively efficient with the degree of hydrolysis reached 80% and 79.37% of the total amylopectin initially present in the substrate. This is in accordance with the opinion of Vasanthan & Bhatty (1996) who indicated that the efficiency of a reaction can be measured based on substrate reduction during reaction.

Amylose. The result from analysis of straight chain (amylose) from enzyme concentration treatment, showed that pullulanse usage with concentration of 5 unit/gram with straight chain resulted realtifity small

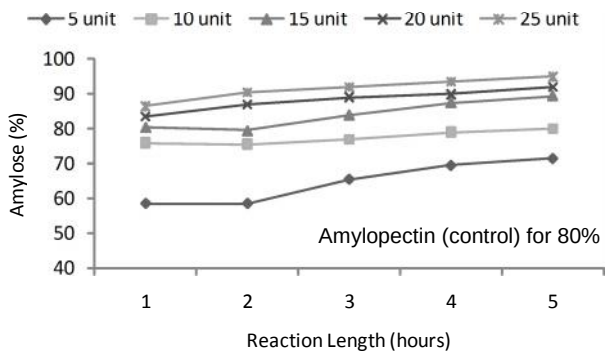

Figure 2. Amylopectin change pattern of tapioca starch of enzyme concentration and enzyme reaction

with the average of 64.7% during debranching for 1-5 hours (Figure 3). The increasing of enzyme concentration for 10, 15, 20 & 25 unit showed straight chain increasing by 77,5%, 88,2%, 88,3% and 91,5% respectively. BNJ test results between enzyme concentration showed the significance differences from one to another except 15 and 20 unit/gram was not significant.

The reaction length showed the reaction time from 1, 2, 3, 4 and 5 hour were tend to increase straight chain products of 77%, 78,2% 81,5%, 83,9% and 85,6% respectively. Significant test showed one treatment to the other was significant, except for 1 and 2 hours was not dignificant.

The straight chain reaction (amylose) from different interaction of enzyme concentration for reaction time, showed optimized treatment for enzyme concentration for 15 unit/gram with the reactiion time of 3 hours and enzyme consentration for 20 unit/gram for 1 hour. Straight chain reaction from both combination for both treatments each for 84% and 83,5%.

Debranching reaction resulted amylopectin components from pullulanase reaction were straight chain componen (amylose). According to Yakobayashi (1988), that debranching enzyme hydrolise starch the of α-1.6 glicosidic chain of starch (amylopectin) or glycogen and result straight chain of amylose as below:

$$\underset{\text{(amylopectin, glicogen)}}{\alpha\text{-}1,4{:}1,6 \text{ glukan branch} + nH_2O} \xrightarrow[\text{debranching}]{\text{enzim}} \underset{\text{(short\&long chain)}}{(n+1)\alpha\text{-}1,4 \text{ glukan}}$$

Amylose component is a polimer arranged from glucose monomer, one of glucose unit among one another connected from α-1.4 to form straight chain (Whisler & Daniel, 1985). Amylose polimerized degree from 500-5000 (Doelle *et al*., 1992; Klucinec *et al*., 1999). Amylopectin components have side chains for α-1.6 D glikosidic, the length of the chain are 20-25 unit glucose, from one another that connected by α-1.4 D glikosidic (Pomeranz, 1985).

The length of reaction estimation of optimized cyclodextrin by using debranching products as substrate. Debranching product usage as substrate from the beginning of reaction until 90th minute, showed cyclodextrin formation relatively small, 15.55 g/L at 30 minutes and 31.96 g/L at 90 minutes. On the same time, big amount of reduction sugar of 19,55 g/L at 30 minutes and 32,35 g/L at 60 minutes (Figure 4).

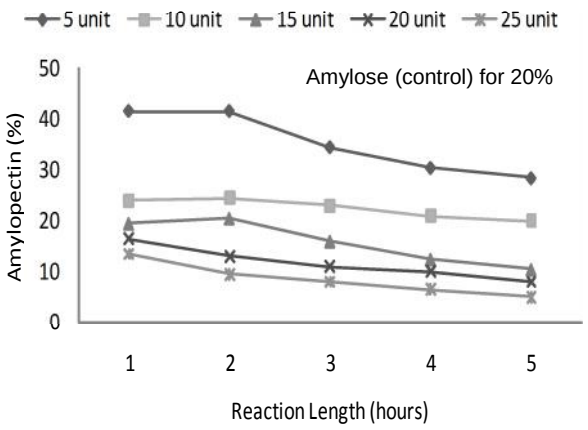

Figure 3. Amylose formation pattern of topioca starch of enzyme concentration and enzyme reaction.

The phenomena caused by cyclic process at cyclodexrin formation from debranching units products with relatively short chain. The average of each branch chain of tapioca amylopectin was around 20-25 units of glucose (Pomeranz, 1985). β-siklodekstrin (7 units glucose) making process of branch units chain may undergo cyclic process 2-3 times, short chain components could not be cycled. Short chain fragments would not raised reduction sugar contents on medium.

Cyclodextrin formation on bigger form at 150 to 240 minutes (101,35-143,45 g/L) at the same time with reduction sugar may increase even small amount (Figure 4). The phenomena showed at the particular time-length, the formation of cyclodextrin formed from starch molecules (the main branch), which will form relatively small fragments of reduction sugar. The particular condition may trigger CGTase activity on one direction, therefore cyclic reaction formation could be optimized.

Cyclodextrin achieved after 240 minutes reaction time tend to decrease, on 270 minutes for 141,65 g/L and 360 minutes for 138,77 g/L. Decreasing cyclodextrin condition, reduction sugar, and conversion value were constant (Fig 4). The decreasing was tend to happenned of cyclodexrin was at 240 minutes, will caused reaction of disproporsionation before cyclic reaction happenned.

Disproporsionation reaction was one of CGTase activities to connect the small chains to be long chains. According to Schmid (1989), if disproporsionation long chain optimized, then CGTase cyclic reaction happenned. Cyclodextrin resulted from the beginning of substrate from G2 (maltosa) and G3 (maltotriosa) at certain time will be reduced. It happens due to characteristics of G2 and G3 as aceptor that caused cyclodextrin easily decomposed (Kitahata, 1988).

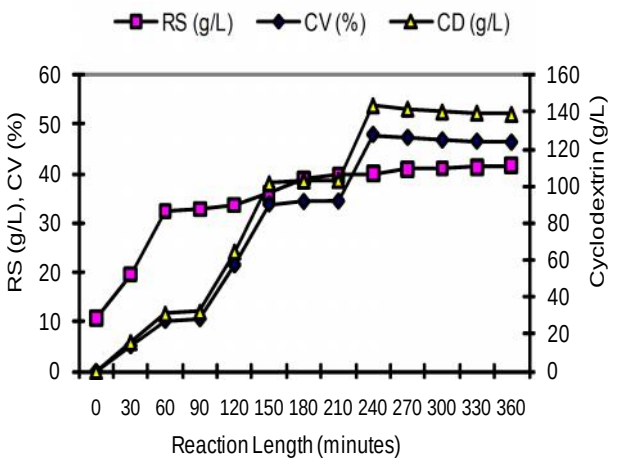

Figure 4. Profile of reduction sugar (RS) invertion, cyclodextrin (CD), and conversion value (CV) through the reaction.

The result of analysis during the reaction showed that the highest cyclodextrin (143,45 g/L) achieved at 240 minutes reaction time with conversion value of 47,81% on substrate usage of 30% w/v. For that reason, the second stage of experiments was conducted.

The best estimation of Straight Chain substrate concentartion for Cyclodextrin formation. The best debranching sugar concentration estimation to produce cyclodextrin was done using the best treatment from first stage and second. The treatment used for debranching process was pullulanase enzyme usage for 20 unit/gram with 1 hour reaction time. On the production stage, cyclodexrin time to optimize the reaction was 240 minutes.

Reduction sugar value and Dextrose Equivalent. The purpose of reduction sugar analysis was to find out the total reduction sugar during cyclodexrin formation, since reduction sugar on certain concentration could inhibit cyclodexrin formation. Chemical analysis on samples during reaction reveals that the increase in substrate concentration tends to increase the formation of reduction sugar (Fig. 5). Therefore, there is a direct relation between the production of cyclodextrin and the formation of reduction sugar as a side product of the process.

Dextrose equivalent (DE) value tends to fluctuate as subsrate concentration was increased. At 20% (w/v) substrate concentration, DE value was 15,89% while at 25% and 30% (w/v) the DE value decreased to 13,96% and 13,32% respectively. Increasing substrate concentration further to 35% and 40% (w/v) resulted in an increase the DE value to 17,55% and 18,99% .

Starch cyclic activity for formation of cyclodextrin will result in short chain components that may not be

converted to cyclodextrin. Short chain accumulation will further result in the increasing of sugar reduction value. According to Kitahata (1988) & Tankova (1998), cyclodextrin formation from substrate (starch or maltoligosaccharides) through cyclic reaction CGTase formed simple sugar (short chain molecules).

Cyclodextrin and conversion value. Analysis on cyclodextrin yield suggested that the yields tend to vary based on substrate concentration. Increasing substrate concentration tended to increase cyclodextrin yields. An exception to this is the yield obtained at 40%(w/v) substrate concentration which was slightly lower than the yield obtained at 35%(w/v) substrate concentration. Conversion of substrate into cyclodextrin also indicated the same trend (Fig. 6). Under the experimental condition employed, the highest cyclodextrin yield (154.28 g/L) was obtained at 35% (w/v) substrate concentration. The corresponding conversion value was 44.08%. On the other hand, the highest conversion value (47.82%) and the corresponding cyclodextrin yield of 143.45 g/L was obtained at 30% (w/v) substrate concentration. These results reveal that, with respect to the degree of conversion, the use of de-branching product as substrate for cyclodextrin production was optimum at 30% (w/v) concentration. However, for maximum cyclodextrin yield, the concentration can be increased to 35% (w/v).

Higher substrate concentration of 40% w/v caused lower formation of cyclodextrin. It shows that substrate concentration above 35% w/v was not efficient. The phenomena shows that substrate concentration above 35% as the enzyme steady state concentration. Vasanthan & Bhatty, (1996), previously indicated that low substrate concentration can lower overall enzyme activity since there is a lack of substrate for the active site of the enzymes to attach to. Consequently, the enzymes compete for the available substrate thus reducing their activity. On the other hand, excessively high substrate concentration can hinder conversion process thus reducing the yield of the final product.

Other factors causing the decrease in the cyclodextrin produced at 35% (w/v) substrate concentration were the increase in the viscosity of the feed and the increase in the formation of reduction sugar. The increase in the feed viscosity can act to decrease the interraction between enzymes and substrates, thus reducing the rate of cyclization reaction. This is in accordance with what has been suggested by Lee & Kim, (1991), who suggested that enzymatic reaction is difficult at high starch concentration since suspension viscosity can significantly increase during liquefaction process. In addition, at high substrate concentration, initial reduction sugar concentration in the feed is relatively high which can act as an acceptor that partially inhibit cyclization reaction in cyclodextrin formation. In this case, reduction sugar can act as an acceptor whenever DE value exceeded 20%. Under this condition, the reduction sugar can induce *coupling* reaction which will open-up the cyclodextrin structure followed by disproporsionation process. The net result is a decrease in cyclodextrin yield (Schmid, 1989).

It is evident from the above results that the use of high concentration substrate tend to decrease cyclodextrin yield. This phenomenon is similar to findings reported by Mattsson *et al*., (1991), which indicate reduction in conversion (from 70% to 48.83%) when substrate concentration was increased to 30%. The same phenomenon was also reported by Lee & Kim (1991) from their work using non-liquefied corn

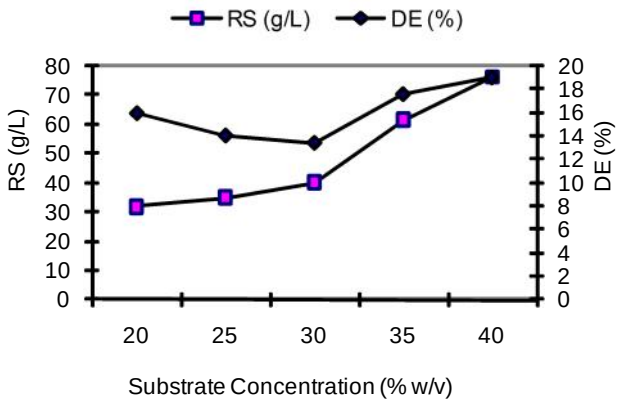

Figure 5. Substrate concentration relationship towards reduction sugar (RS) and dextrose equivalent (DE) of cyclodextrin formation reaction

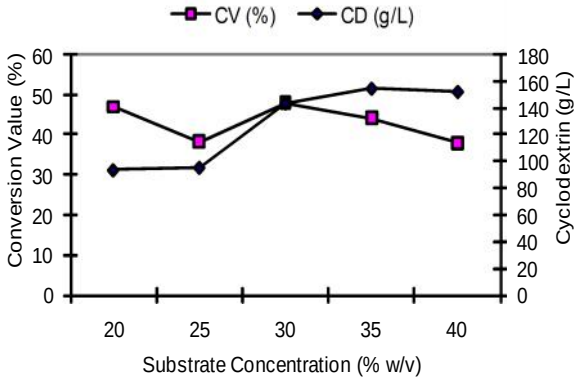

Figure 6. The relationship between substrate towards cyclodextrin and conversion value

starch. In their work, maximal cyclodextrin yield of 50 g/L was achieved when using 15% substrate concentration; slightly higher than the yield obtained at 20% substrate concentration.

CONCLUSION

The de-branching of tapioca using pullulanase was optimal at 15 unit/g enzyme concentration and reaction time of 3 hours. Under this condition, the degree of hydrolysis was 84%. At 20 unit/g enzyme concentration, the degree of hydrolysis achieved was down to 83.5% with one hour reaction time.

The optimum length of reaction for cyclodextrin formation from amylose produced from the de-branching process was 240 minutes. The amount of cyclodextrin produced was 143.45 g/L with conversion value of 47.81 % at 30% (w/v) substrate concentration. Highest yield of cyclodextrin (154,28 g/L) and conversion value of 44.08% was obtained at 35% (w/v) substrate concentration.

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