Hydrolysis of Cassava Starch/Chitosan and Their Mixtures in Subcritical Water Media

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ABSTRACT

Starch widely available in agricultural and food by-products can be converted by hydrolytic depolymerisation into a valuable compound, like dextrin. The objective of this study was to understand the effect of pressure and temperature on reaction mechanisms of cassava starch, chitosan and their mixtures at subcritical water (SCW) conditions. A factorial design, including temperature (75, 100, 125 and 150ºC) and pressure (50, 85, 120 and 155 bar), was first investigated. Then, different chitosan/starch ratios (0, 0.025, 0.05, 0.075, 0.01 and 0.15 g chitosan/g starch) were evaluated in subcritical water media at 100 ºC and 85 bar. The hydrolysis was carried out using a subcritical fluid reaction system. Hydrolysates produced were characterized based on amylose content, reducing ends, particle size, molecular weight and zeta potential. A maximum amylose production of 27.1% from cassava starch was obtained at 75 ºC and 155 bar, which was almost twice the amylose content of native cassava starch (16.9%). A significant decrease was observed at 150 ºC and pressures investigated. No glucose was detected in the starch hydrolysate, indicating that amylose/amylopectin was depolymerized into dextrins. Overall, an increase of reducing ends from 1.6 to 17.3 mg glucose equivalent/g starch was observed. The depolymerisation was also confirmed by the decrease of starch molecular weight from 1243.5 (native starch) to 79.9 kDa after SCW treatment. A lower degree of depolymerisation of chitosan was obtained compared to starch. For chitosan hydrolysate, the molecular weight decreased from 205 (untreated chitosan) to 24.6 kDa, and the particle size decreased from 2.23 μm to 308.3 nm. In addition, the increase of zeta potential (-3 to -4 mV) to 2.11 mV after mixing with chitosan demonstrated the interaction between starch and chitosan. Therefore, SCW is a potential technology for dextrin production, promoting reactions between starch and chitosan.

1. INTRODUCTION

Dextrins, which are widely used in food, nutraceutical, and pharmaceutical industries, can be produced from the hydrolysis of polysaccharides [1,2]. Starch, one of the most abundant carbohydrates in biomass, is a polysaccharide consisting of glucose monomers connected with β- (1 → 4) and α- (1 → 6) linkages. There are two different forms of starch; amylose with a linear structure, and amylopectin with a more branched structure. Starch hydrolysates typically used in the industry including maltodextrin and glucose are produced by acid hydrolysis, requiring huge amounts of solvents and raising pollution issues. The enzymatic process yields many specific products, but it can be slow and last up to 36 h [3], besides involving the costs of obtaining the enzymes and purifying the product.

Chitosan, *N*-deacetylated derivative of chitin, is the second major source of natural polymers after cellulose. The high molecular weight of chitosan, which results in a poor solubility at neutral pH values and high viscosity aqueous solutions, limits its potential uses in the fields of food, health and agriculture. Similarly, water soluble chitosan can be produced by acidic or enzymic degradation of the chitosan polymer chain. The enzymic process is generally preferable because the product distribution can be readily controlled. It also minimizes alterations in the chemical nature of the reaction product. A series of commercially

enzymes including chitinases, chitosanases, glucanases, lipases and some proteases are available [4]. But, the high cost associated reduces the application of enzymatic methods in the industry.

Recently, polysaccharide hydrolysis under subcritical water in the absence of additives captured a great interest, as the process requires only water; consequently, neutralization and desalination processes can be eliminated [5]. A few studies have shown the possibility of biomass conversion to produce glucose from starch. Starch from sweet potato was decomposed in a batch reactor at hydrothermal conditions in the absence of a catalyst [6]. Glucose yields were negligible at temperatures below 180 ºC. However, the degradation product (5-hydroxymethylfurfural) from glucose appeared after 10 min at 240 ºC [6]. In a similar study on hydrolysis of sweet potato starch, Miyazawa and Funazukuri [7] reported a glucose yield of just 4% after 15 min at 200 ºC and unspecified pressure. A higher glucose production (53%) was obtained when the medium was acidified with CO_2, and the amount of glucose released increased linearly with increasing CO2 concentration from 0-0.32g [7]. All these studies focused on the glucose production therefore higher temperatures were used. Similarly to the starch, chitosan hydrolysis in subcritical water is not reported. One study used sub- and supercritical water as a pretreatment before the enzymatic degradation of chitin. The yield of *N*,*N*′-diacetylchitobiose at 400 °C for 1 min was up to 37%, compared to 5% without the pretreatment [8]. Another study used sub- and supercritical water to purify the chitosan from crab shell by removing protein. They also found that the average molecular weight of pure chitin decreased from 760 kDa to 0.9 kDa after 2 min at 400 ºC and the distance between chitin chains increased due to weakening of hydrogen bonds, promoting enzymatic degradation [9].

To date, there is no study on subcritical water hydrolysis of starch or chitosan at 75-150 ºC to produce valued-added products, like dextrin and low molecular weight chitosan. Therefore, the objective of this study was to understand the effect of pressure and temperature on reaction mechanisms of cassava starch, chitosan and their mixtures at subcritical water conditions.

2. MATERIALS AND METHODS

2.1 Materials

Cassava starch was provided by CbPAK Tecnologia (Rio de Janeiro, Brazil). Chitosan (75-85% deacetylated) with medium molecular weight of 190-310 kDa was purchased from Sigma Aldrich (Oakville, ON, Canada). Gallic acid (ACS reagent, ≥ 99.5%) was acquired from Sigma Aldrich (Oakville, ON, Canada). Purified water from a Milli-Q system (Millipore, Bellerica, MA, USA) was used.

2.2 Methods

2.2.1. Production of hydrolysates

Hydrolysis were carried out using the subcritical fluid reaction system (US patent PCT/CA 2014/000432) shown in Figure 1. For each experiment, known amounts of water, cassava starch, chitosan, 1wt% gallic acid solution and their mixtures were first preloaded inside the reactor with a volume of 270 mL. Then, the reactor was connected to the system and filled with Milli-Q water using a HPLC pump. After closing all valves in the system, the mixture inside the reactor was homogenized by a double helix stirrer for 5 min. After the desired temperature and pressure were reached, the reactor was hold at these conditions for 10 min for reaction. The cooling process was performed right after lowering the temperature to 50 ºC. Then, the reacted starch and starch/chitosan mixture solution were unloaded and precipitated with 99% ethanol (1:4 v/v), the resulting suspensions were passed through a

Buchner funnel. The precipitates were dried at room temperature for 48h and ground, then passed through a 100-mesh sieve. The supernatants were also collected for reducing sugar analysis by HPLC. The reacted chitosan solutions were freeze dried for further analysis.

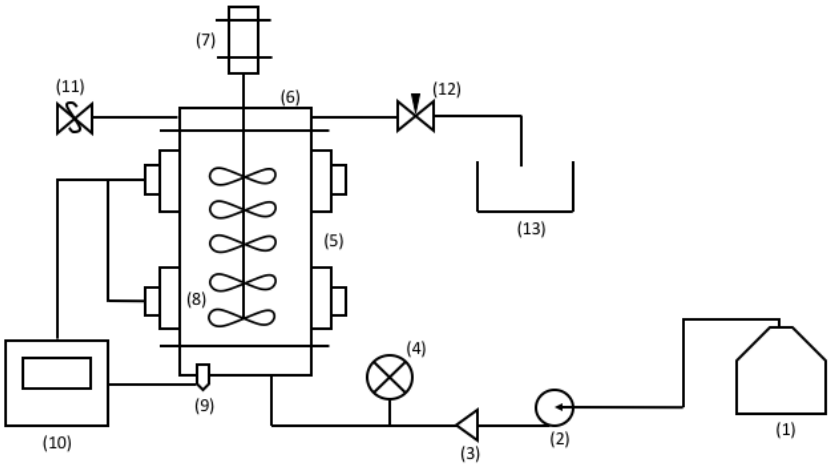


Fig. 1. Subcritical fluid reaction system: (1) Solvent reservoir, (2) Pump, (3) One-way valve, (4) Pressure gauge, (5) Band heaters, (6) Pressurised fluid reaction vessel, (7) Motor stirrer controlled by the control panel, (8) Stirrer, (9) Thermocouple, (10) Temperature controller, (11) Safety valve, (12) Back pressure regulator, and (13) Sample collection.

A factorial design, including temperature (75, 100, 125 and 150ºC) and pressure (50, 85, 120 and 155 bar), was first investigated. Then, different chitosan/starch ratios (0, 0.025, 0.05, 0.075, 0.01 and 0.15 g chitosan/g starch) were evaluated in subcritical water media at 100 ºC and 85 bar.

2.2.2. Hydrolysate characterization

The hydrodynamic diameter, zeta potential and average molecular weight of hydrolysates in solution were measured using a Malvern Zetasizer Nano-ZS instrument (Malvern, Worcestershire, UK). For a typical determination, solutions were prepared by dissolving the starch in DMSO and chitosan in Milli-Q water at the concentration of 0.1 mg/mL. All measurements were performed in triplicate.

The reducing end assay was carried out as described by Imoto and Yagishita [10]. Briefly, 0.6 mL of a 0.5 M sodium carbonate solution containing 0.5 g/L potassium ferricyanide was mixed with 450 mL of the sample. The mixture was heated for 15 min at 100 ºC and absorbance was determined at 420 nm.

For amylose content determination [11], starch (0.1 g) was dissolved by heating in DMSO (10 mL) for 15 min at 85 °C. When dissolved, this solution was then diluted to 25 mL in a volumetric flask with Milli-Q water. An aliquot (1 mL) of this solution was then diluted with 50 mL of water, 5 mL of a solution of iodine (0.0025 mol/L) in potassium iodide (0.0065 mol/L) added with mixing and the absorbance was read at 600 nm.

Reducing sugars in the starch hydrolysate were determined by HPLC (Agilent, Santa Clara, CA, USA) using a SUPELCO Pb column operating at 70°C, with a water flow rate of 0.6 mL/min and a refractive index detector.

The pH of the hydrolysates right after the reaction was measured using a pH meter (Fisher Scientific Accumet Basic XL20, Waltham, MA, USA).

2.2.3. Statistical analysis

The experiments were done at least in duplicates. R studio software was used to

conduct analysis of variance (ANOVA). Tukey's test was used to identify significant difference at $p<0.05$ between means of each sample.

2. RESULTS AND DISCUSSION

Figure 2 shows the amylose production of cassava starch treated with SWC at 75 to 150 ºC and 50 to 155 bar. Cassava starch usually has amylose values ranging from 16% to 20% [12]. The amylose content of native cassava starch used in this study was 16.9%. At 75 ºC, the amylose content increased with the increasing pressure from 13.4% to 27.1%. At 100 and 125 ºC, the yield of amylose peaked at 85 bar, reaching 22.4% and 17.3%, respectively. When higher pressures were used, the amylose production significantly reduced to less than 1%. Severe decrease was observed at 150 ºC at all pressures investigated. Under the SCW conditions, starch molecule undergoes depolymerisation through debranching at the β-(1 → 4) glycosidic bonds or decomposing within the chain between α- (1 → 6) glycosidic bonds. At a temperature of 75 ºC, long amylose/amylopectin chains were broken into short chain amylose, so the increased amylose yield was observed. With the subsequence depolymerisation at high temperatures and pressures, short chains of amylose were further degraded to dextrins.

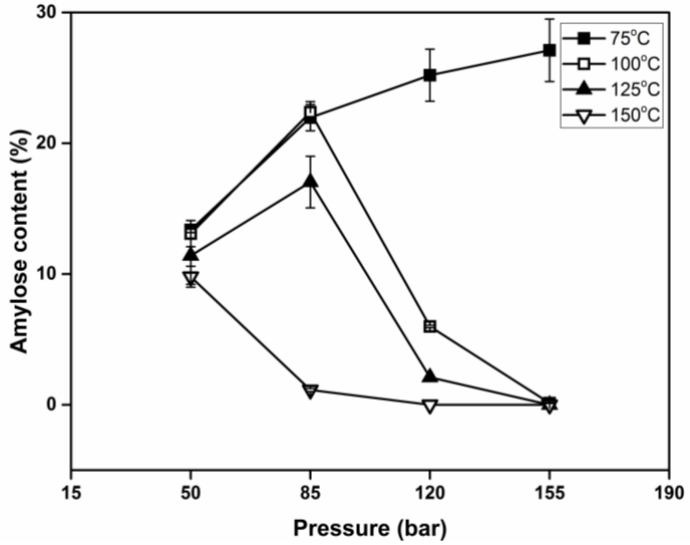


Figure 2. Amylose content in pure cassava starch after SWC treatment at 75-150 ºC and 50-155 bar.

The starch hydrolysate production was further demonstrated by the reducing end analysis as shown in Figure 3. In constant with the amylose content observed in starch hydrolysates, the decreased amylose concentration and increased reducing end production indicated the formation of low molecular weight fragments from further hydrolysis at high temperatures (100-150 ºC) and pressures (85-155 bar). The depolymerization was less significant at 75 ºC. At a pressure of 155 bar, the amounts of reducing end produced were twice compared with the reducing end formed at 50 bar and temperatures investigated. Moreover, the effect of temperature was more pronounced, where the reducing end yield increased around five times at 150 ºC compared with the yield at 75 ºC. The highest yield (38.6 mg glucose equivalent/g starch) was obtained at 150 ºC and 155 bar. However, no reducing sugar ($DP \leq 6$) was detected by HPLC in the starch hydrolysate at all conditions investigated (data not shown), indicating the production of low molecular weight dextrins instead of monosaccharides. This result is in agreement with the previous study performed by Nagamori and Funazukuri [6], where glucose yield was negligible at temperatures lower than 180 ºC.

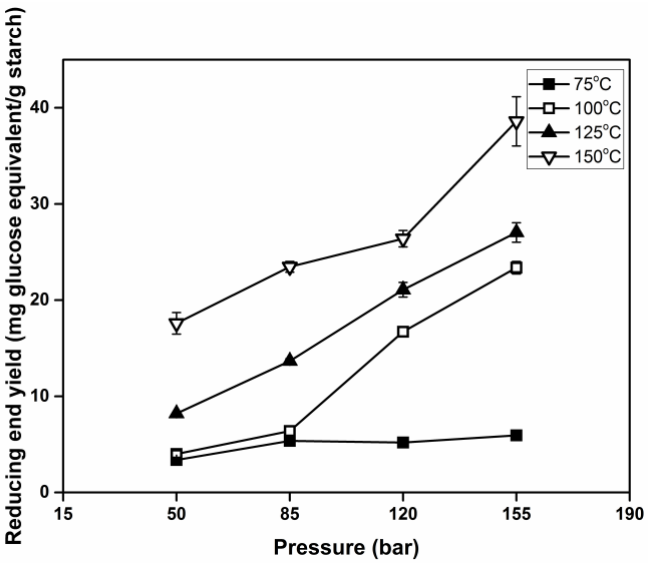


Figure 3. Reducing end yield of cassava starch after SWC treatment at 75-150 ºC and 50-155 bar.

The average molecular weight of native cassava starch after SWC treatment is shown in Figure 4. The molecular weight distributions of the starch hydrolysates shifted to lower molecular weights by increasing temperature and pressure. Starch average molecular weight decreased to almost half at all temperatures investigated with the increasing pressure from 50 to 155 bar. The most effective reduction of molecular weight from 513.5 kDa at 50 bar and 75 ºC to 172.5 kDa occurred at 50 bar and 150 ºC. Within 10 min of reaction, the average molecular weight was reduced from 1243.5 kDa (native cassava starch) to 79.8 kDa, which is much lower than that reported by Van der Veen et al. [13], who produced hydrolysed corn starch with molecular weight of 441 kDa at 110 ºC for 8h from the native corn starch with a molecular weight of 2286 kDa.

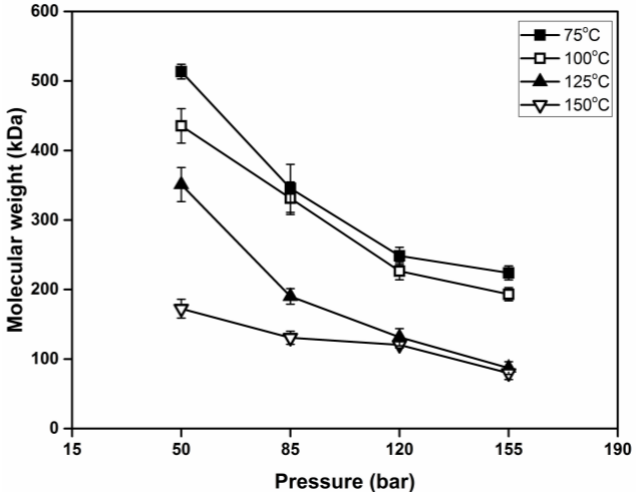
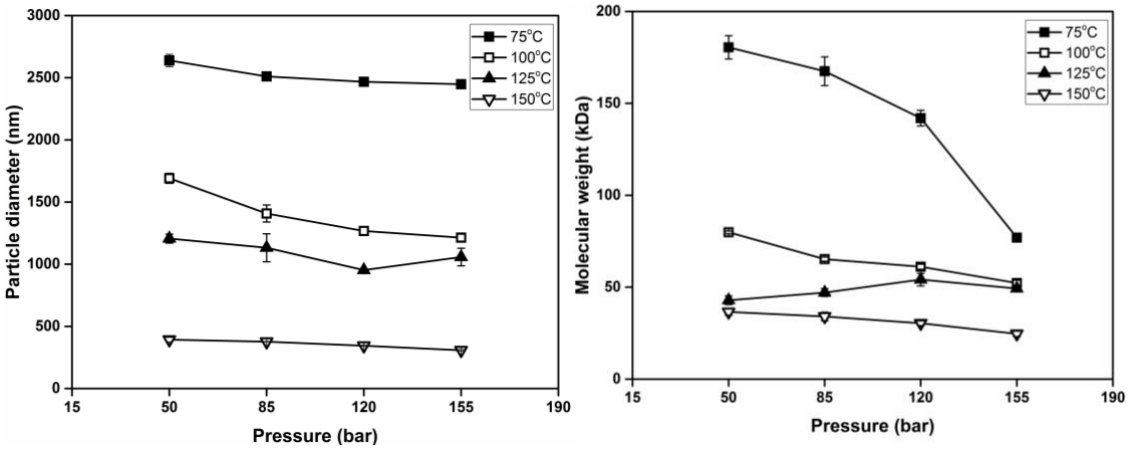


Figure 4. Molecular weight of pure cassava starch after SWC treatment at 75-150 ºC and 50-155 bar.

Particle size and molecular weight of pure chitosan after SCW treatment are shown in Figure 5. There was no significant difference of the particle size between untreated chitosan (2428.5 nm) and chitosan treated at 75 ºC (2510.5-2448.5 nm). Chitosan particle was reduced to one tenth the untreated value at the highest temperature of 150 ºC. SCW showed a had pronounced effect on chitosan hydrolysis than starch hydrolysis. The average molecular weight of untreated chitosan was around 205 kDa. At the lowest temperature (75 ºC) used, a dramatic reduction from180.5 to 77 kDa was found with the increasing pressure from 50 to 155 bar. More than twice of reduction was obtained at temperatures over 100 ºC, as gallic

acid favored chitosan hydrolysis in SCW. Also, Vårum et al. [14] reported that the activation energy for hydrolysis of the two deacetylated (D-D) glycosidic linkage was higher than the activation energy for hydrolysis of the two acetylated units (A-A) and an acetylated and a deacetylated unit (A-D) glycosidic linkage. As starch has similar non-acetylated (D-D) glycosidic linkages, it required more energy to be hydrolysed. The untreated chitosan used in this study had a relative crystallinity of 19.22%, while the native cassava starch had a higher relative crystallinity of 24.38%, indicating that chitosan was easier to be depolymerized due to the relative high level of amorphous regions. Linear chains of starch molecule are constrained under hydrogen bonds in crystal structures, the amorphous part was preferentially degraded because the easiness of attacking. Tian et al. [15] reported that in the crystal region, chitosan was depolymerized by debranching of the layers, while the amorphous portion was depolymerized by penetrating through the loose matrix requiring less energy.

The effect of pressure on particle size and molecular weight was less significant, especially above 100 °C. The role of pressure was mainly to retain water in liquid phase, while temperature accelerated the reaction kinetics in addition to affecting vapor–liquid equilibrium.



(a) (b)

Figure 5. (a) Particle size and (b) molecular weight of pure chitosan after SWC treatment at 75-150 °C and 50-155 bar.

The molecular weight of starch/chitosan complex increased with the increasing ratio of chitosan in the complex (Table 1). Starch is naturally negatively charged as -3/-4 mV, the increase of zeta potential to 2.11 mV after mixing with chitosan demonstrated the interactions between starch and chitosan. Specifically, the high levels of H^+ and OH^- at subcritical conditions facilitates acid- or base-catalyzed reactions. The relatively high density combined with the SCW high dissociation constant favors ionic reactions. In this study, ions produced (HO_3^+ and OH^-) at subcritical conditions activated the -OH groups in starch and chitosan, -COOH groups from gallic acid were partially dissociated, while HO_3^+ protonated the amino group to $-NH_3^+$, promoting the reactions between starch and chitosan through hydrogen bonds. In addition, part of non-dissociated gallic acid formed ester linkages after reacting with $-CH_2OH$ groups of starch and chitosan hydrocolloids; while dissociated gallic acid with COO-moiety bonded ionically with protonated amino groups (NH_3^+) of chitosan, establishing electrostatic interactions capable of forming complexes. Similar results were reported when murta leaf extract (rich in phenolic acids) was incorporated into corn starch/chitosan blend at 70 °C, phenolics formed electrostatic interactions with chitosan. Ester linkages and hydrogen

bonds were also formed between phenolic acids in murta leaf extract and chitosan/starch blends [16].

Table 1. Molecular weight and zeta potential of chitosan/starch mixture after SWC treatment at 100 ºC and 85 bar.

Chitosan/starch ratio (g/g)	0	0.025	0.05	0.075	0.1	0.15
Molecular weight (kDa)	331.4±23.4^{d}	950.0±5.6^{c}	955.0±17.7^{bc}	1216.0±36.7^{abc}	1511.0±78.5^{ab}	1656.0±31.1^{a}
Zeta potential (mV)	-3.85±0.30^{d}	0.08±0.01^{c}	0.15±0.01^{c}	0.29±0.02^{bc}	0.63±0.08^{b}	2.11±0.00^{a}

Data shown as mean±standard deviation (n = 3).
$^{a–d}$ Different lowercase letters in the same column indicate significant differences ($p < 0.05$).

4. CONCLUSIONS

The use of subcritical water technology offers an innovative green process to control hydrolysis of starch and chitosan. Cassava starch were hydrolysed to short chain amylose at 75-100 ºC, followed by further depolymerisation to small fragments. However, no reducing sugar was detected by HPLC under the conditions investigated. Chitosan experienced a more effective depolymerisation than starch due to its high amorphous level. Therefore, SCW is a potential technology for dextrin production. Furthermore, starch/chitosan complex were produced in SCW by enhancing cross-linking between starch and chitosan molecules. SCW promotes reactions between starch and chitosan for other applications in the food and nutraceutical industry.

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