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Products from microwave and ultrasonic wave assisted acid hydrolysis of chitin

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ABSTRACT

Hydrolysis of α-chitin in concentrated hydrochloric acid under elevated temperature is a general preparation of a nutrapharmaceutical glucosamine hydrochloride (GlcN.HCl). In this study, the microwave and ultrasonic wave assisted acid hydrolysis of shrimp shell α-chitin are investigated with an aim to improve the reaction rate and selectivity. Microwave heating shortens the hydrolysis time from 120 min in the conventional heating process to 12 min to afford GlcN.HCl in 57% yield. Sonication can be used to assist chitin dissolution in 38% HCl prior to the hydrolysis at 120 °C for 120 min to produce GlcN.HCl in 62% yield. The selective hydrolysis of glycosidic bond of chitin is achievable at 30 °C for 4 h to give *N*-acetyl glucosamine (GlcNAc) in 37% yield.

1. Introduction

As the second most abundant and renewable natural polysaccharide, chitin is considered as an unlimited source of nitrogen containing organic compounds. It is the structural constituent of the exoskeletons of marine and terrestrial arthropods as well as cell walls of fungi and yeasts (Muzzarelli, 2012; Muzzarelli et al., 2012). Frozen and processed seafood industries generate marine biomass byproduct containing billions of tons of chitin each year. There will be huge economic and environmental benefits if these marine biomass wastes are efficiently utilized (Pillai, Paul, & Sharma 2009; Rinaudo, 2006; Sakai, 1995). One of the brightest applications of chitin is as a raw material for production of the amino sugar, glucosamine (GlcN). GlcN has been proven effective in numerous scientific trials for easing osteoarthritis pain (D′ Ambrosio, Casa, Bompani, Scali, & Scali, 1981; Gladman & Farewell, 1999; Hauselman, 2001; Mankin, Brandt, & Shulman, 1986; Matheson & Perry, 2003), and has long been prescribed as a safe nutraceutical alternative to nonsteroidal anti-inflammatory drugs (NSAIDs) for osteoarthritis pain and inflammation treatment (Da Camara & Dowless, 1998; Ishiguro, Kojima, & Poole, 2002; Todd, 2002; White & Stegemann, 2001). This has led to worldwide consumption of large amounts of a great variety of over-the-counter GlcN. Most of GlcN products for arthritis are usually prepared from the hydrochloride salt (GlcN.HCl) which is industrially produced by the acid hydrolysis of chitin using concentrated hydrochloric acid. GlcN.HCl also has potential applications in cosmetics (Szego & Makk, 1982), antiviral drugs (Floc'h & Werner, 1976; Rashad, Hegab, Abdel-Megeid, Micky, & Abdel-Megeid, 2008), anti-cancer (Chesnokov, Sun, & Itakura, 2009), wound healing (Mackay & Miller, 2003), and as a substrate in the synthesis of glycoproteins (Menon, Mayor, Ferguson, Duszenko, & Cross, 1988) and glycolipids (Mayor et al., 1990). The yield of GlcN.HCl obtained from the acid hydrolysis of chitin is depended on reaction conditions including acid concentration, acid to solid ratio, and reaction time (Ingle, Vaidya, & Pai, 1973; Kamasastri & Prabhu, 1961). In general, concentrated (38%, w/w) hydrochloric acid solution is required for solubilizing the solid chitin and high yield of glucosamine hydrochloride is obtained by refluxing this acidic solution at a temperature about 100 °C for at least 90 min (Gandhi and Laidler, 2002; Mojarrad, Nemati, Valizadeh, Ansarin, & Bourbour, 2007; Novikov, 2004). Under this condition, both amidic and anhydroglucosidic bonds are hydrolyzed to simultaneously affect the deacetylation and depolymerization. An alternative to the acid hydrolysis of chitin is an enzymatic hydrolysis which is used for chitin degradation to chito-oligosaccharides and/or its monomer, *N*-acetyl-D-glucosamine (GlcNAc) (Klaikherd, Jayanta, Boonjawat, Aiba, & Sukwattanasinitt, 2004; Pichyangkura, Kudan, Kuttiyawong, Sukwattanasinitt, & Aiba, 2002; Sashiwa et al., 2003; Sukwattanasinitt, Zhu, Sashiwa, & Aiba, 2002). Despite low selectivity between depolymerization and deacetylation, acid hydrolysis is still attractive mainly due to its cost effectiveness compared to the enzymatic hydrolysis.

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Ultrasonic wave and microwave irradiations have recently become useful energy sources in various chemical processes (Capelo-Martinez, 2009; Kidak & Ince, 2006; Srogi, 2006; Mats & Kristofer, 2006; Mutyala et al., 2010). The benefits of these irradiations in chemical processes include faster reaction rate, higher product yield and reduced energy consumption. Due to the lack of research in hydrolysis of chitin under these irradiations, we therefore decided to investigate the products from ultrasonic wave and microwave assisted acid hydrolysis of chitin and the condition optimization for greater product yields as reported herein.

2. Materials and methods

Shrimp chitin flakes (~0.4 mm^2) were obtained from Taming Enterprises (Thailand). Concentrated HCl (38%, w/w) and activated charcoal were purchased from Merck (Germany).

2.1. Hydrolysis of chitin by microwave heating

38% HCl (50 mL) was pre-warmed by conventional microwave oven (Samsung, M183GN) at 850 watts (W) for 30 s. Shrimp chitin (30 g; chitin/acid ratio = 1:2, w/w) was added quickly into the pre-warmed acid. After 1 min, when chitin was fully submerged, the microwave irradiation was continued for the designated period of time. After stopping the irradiation, the resulting slurry was allowed to cool to room temperature and filtered to obtain a brown precipitate containing GlcN.HCl. All reactions were performed in triplicates.

2.2. Purification of GlcN.HCl

The crude precipitate was dissolved in distilled water (30 mL/10 g initial chitin), stirred for 30 min with activated charcoal (20 mg/10 g initial chitin). The decolorized solution was stirred at room temperature for 30 min. After the removal of activated charcoal and insoluble residue by filtration, the clear filtrate was evaporated under reduced pressure to recover GlcN.HCl as light yellow solid. The solid was then dispersed in absolute ethanol (10 mL/10 g initial chitin), stirred for 30 min at room temperature and the slurry was then filtered. The white solid obtained was dried under vacuum for 24 h to yield pure GlcN.HCl. For purity determination by acid-base titration, GlcN.HCl solution prepared by dissolving GlcN.HCl salt in Milli-Q water (~0.01 M) was titrated with a standardized NaOH solution (~0.01 M) using phenolphthalein as the indicator. NaOH solution was standardized by potassium hydrogen phthalate (KHP) solution (~0.01 M) using phenolphthalein as the indicator. All titrations were performed in triplicates.

2.3. Hydrolysis with ultrasonic wave treatment

Chitin (10 g divided into 5 portions) was added portion wise into 38% HCl (50 g) immersed in a controlled temperature ultrasonic bath (Elmasonic S30H, 50/60 Hz, 275 W, England). The addition of chitin was performed while the acid was sonicated at designated temperature. Each chitin portion was added after complete dissolution of the previous portion was observed. Typically, all portions could be completely dissolved within 30 min. The chitin solution was allowed to stir at controlled temperature for a designated period of time and the product was monitored and isolated as described below.

2.4. Determination of degree of hydrolysis

The hydrolysate was neutralized with NaOH solution (10%, w/w) and was then centrifuged at 2000 rpm for 20 min. The supernatant was removed and the solid residue was washed with DI water (40 mL) and ethanol (95%, 40 mL). The solid was dried under vacuum and the degree of hydrolysis was calculated from 100 × (mass of starting chitin – mass of residue)/mass of starting chitin.

2.5. Monitoring of hydrolysis reaction by ESI-MS

The hydrolysate (2 mL) was pipetted into absolute ethanol (25 mL) at each time interval. The resulting cloudy mixture was kept in a refrigerator at 4 °C overnight and then centrifuged at 2000 rpm for 20 min. The precipitate was collected by decanting the liquid supernatant off. The precipitate was dissolved in DI-water (4 mL), added with activated charcoal (4 mg) and stirred for 30 min. The mixture was filtered through a filter paper (Whatman No. 1) and then a 0.45 μm PTFE filter. The filtrate volume was adjusted to 5 mL by DI-water in a 5 mL volumetric flask and analyzed by an electrospray ionization mass spectrometer (ESI-MS). The solution sample (~1 ppm, each 1.5 mL) was injected into the mass spectrometer using the following injection and ionization parameters; *i.e.* the voltage at capillary, extractor and RF lens were 40 kV, 3 V and 0 V, respectively. The cone voltage was 30 and 35 V for GlcN and GlcNAc, respectively. Under MS scan mode, these parameters were adjusted to give the highest signals corresponding to glucosamine (GlcN) and *N*-acetyl glucosamine (GlcNAc). GlcN was detected as the signal of $[GlcNH.H_2O]^+$ at $m/z = 162$ and GlcNAc was detected as the signal of $[GlcNAcH.H_2O]^+$ at $m/z = 204$. The relative abundance of these signals was plotted *vs* the hydrolysis time.

2.6. Purification of GlcNAc

The brown slurry obtained from the hydrolysis was diluted with 95% ethanol (40 mL) to precipitate a part of impurities. The ethanolic slurry was centrifuged at 2000 rpm for 20 min to remove the remaining chitin and impurities. The obtained filtrate was evaporated and the residue was dispersed in 10 mL of water and filtered to remove the water insoluble residue. The filtrate was then evaporated by rotary evaporator to dryness. The pH of supernatant was adjusted from pH ≤ 1 to neutral by filtering through $NaHCO_3$ powder (40 g). The neutral supernatant was then concentrated to 1/3 of volume by rotating evaporator and dropped into absolute ethanol (50 mL) while stirring to form a cloudy suspension and left in a refrigerator at 4 °C overnight to complete the precipitation. The precipitate was separated by centrifugation at 2000 rpm and dried in desiccators under vacuum to afford solid A. The supernatant was concentrated to 1/3 of volume by rotating evaporator and dropped into cool absolute ethanol (50 mL) to form cloudy suspension. The precipitate (solid B) was collected by centrifugation at 2000 rpm. The supernatant was decolorized by stirring with activated charcoal for 45 min, filtered off to yield a clear solution that was concentrated in a rotating evaporator and then freeze-dried to provide a white solid (solid C).

2.7. ^1H NMR spectroscopy

^1H NMR spectra of the product samples, GlcN.HCl and GlcNAc standards were acquired from the deuterium oxide (D_2O) solutions on Varian Mercury 400 NMR spectrometer at 400 MHz.

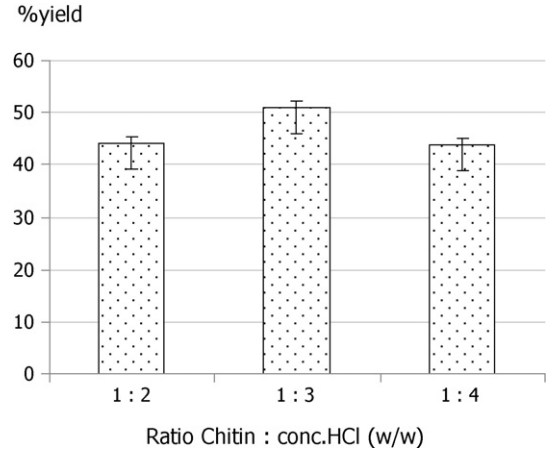

Fig. 1. GlcN.HCl yield obtained from chitin hydrolysis in 38% HCl with microwave irradiation power of 850 W for 12 min at various chitin/acid ratios. The plots are the average values from three replicates with the error bars representing the maximum and minimum values.

3. Results and discussion

3.1. Chitin hydrolysis under microwave heating

First, we have investigated the effect of chitin/38% HCl ratio on the yield of GlcN.HCl. As illustrated in Fig. 1, the optimum chitin/38% HCl ratio is 1:3, w/w where GlcN.HCl can be obtained ~50% yield. The amount of HCl used affected the yield of GlcN.HCl in two manners. With lower amount of 38% HCl (1:2 ratio), significant amount of insoluble chitin was observed after the hydrolysis. Though the reaction was done in reflux conditions, some HCl escaped from the reaction mixture leading to inadequate acidity to dissolve chitin in the initial state of the hydrolysis. When higher amount of 38% HCl (1:4 ratio) was used, less GlcN.HCl precipitate was obtained after allowing the reaction mixture to cool to room temperature due to dilution effect.

Fig. 2 shows a time course of GlcN.HCl yield obtained from the chitin hydrolysis with 38% HCl under microwave irradiation. The isolated yield of GlcN.HCl increased with the hydrolysis time up to 12 min. The GlcN.HCl yield dropped slightly as the irradiation time was extended beyond 12 min, probably due to the decomposition of GlcN.HCl product. The optimum irradiation time for hydrolysis is thus 12 min.

To study the effect of heat transfer in this microwave assisted chitin hydrolysis, the reaction was conducted with and without

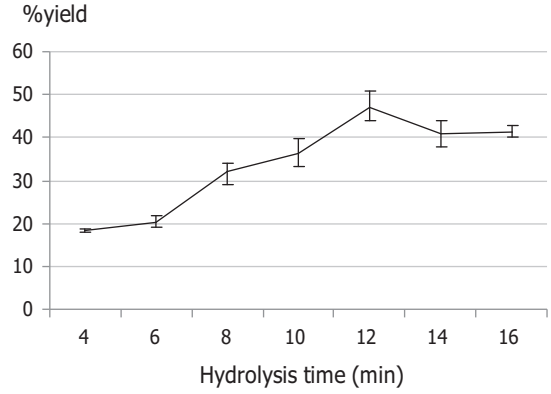

Fig. 2. GlcN.HCl yield obtained from chitin hydrolysis in 38% HCl (1:1, w/w) using microwave irradiation of 850 W at various irradiation times. The plots are the average values from three replicates with the error bars representing the maximum and minimum values.

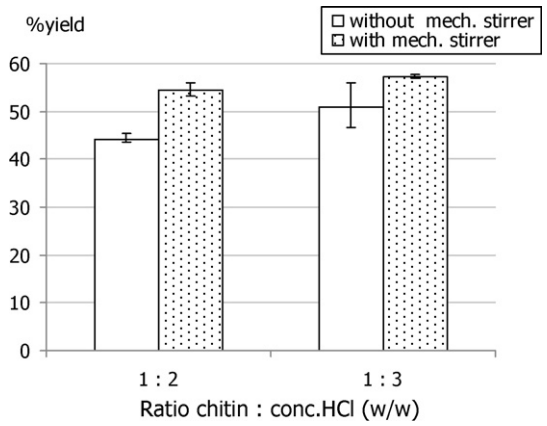

Fig. 3. GlcN.HCl obtained from chitin hydrolysis with microwave irradiation of 850 W for 12 min with and without mechanical agitation. The plots are the average values from three replicates with the error bars representing the maximum and minimum values.

stirring. Use of a mechanical stirrer improved the product yield by 5–10% (Fig. 3). Under the optimum 1:3 chitin/38% HCl ratio, GlcN.HCl was obtained in 57% yield with mechanical stirring. This yield is comparable to conventional heating reported in literatures but within much shorter time (Gandhi & Laidler, 2002; Ingle et al., 1973; Kamasastri & Prabhu, 1961; Mayor et al., 1990). Since agitation of the reaction mixture can increase the product yield, the faster reaction is likely to be a result of the faster heating rate rather than the superheating or selective heating. Microwave can thus speed up and reduce the energy consumption in the acid hydrolysis of chitin to produce GlcN.HCl but it cannot improve product yield.

White crystalline powder of GlcN.HCl was obtained by using the decolorization-precipitation method (Gandhi & Laidler, 2002). The isolated GlcN.HCl product obtained from the hydrolysis shows the same ^{1}H-NMR spectrum pattern (Fig. S1) as the standard GlcN.HCl (Fluka Chemicals, Ltd., ≥99% HPLC). The acid-base titration also confirmed the percent purity of GlcN.HCl above 99%.

3.2. Chitin hydrolysis with pre-sonication

As described in the previous section that there was no selectivity between glycosidic and amido bonds in the hydrolysis of chitin either by microwave or conventional heating and only GlcN.HCl was obtained as the final product. It is important to note that chitin is dissolved in 38% HCl very slowly at room temperature that a solution with concentration higher than 5% (w/w) cannot be obtained within acceptable time, *viz.* <1 h, without warming the acid (~60 °C). In this part of study, we used ultrasonic wave to accelerate the dissolution of chitin at low temperature to obtain acidic chitin solution with appreciable concentration. The hydrolysis of chitin is expected to proceed with selectivity that should allow a preparation of GlcNAc.

With sonication, 30 g of chitin was completely dissolved in 50 mL 38% HCl within 30 min even at 20 °C. After the dissolution

Table 1
Degree of hydrolysis of chitin (10 g) in HCl (50 g) without and with sonication.

Temperature	Without sonication[a]		With sonication[b]	
	Residue (g)	% hydrolysis	Residue (g)	% hydrolysis
20 °C	9.7	3	8.8	12
40 °C	4.3	57	2.0	80
60 °C	0.9	91	0.3	97

[a] Time = 120 min.
[b] Total time = 120 min with 30 min of sonication.

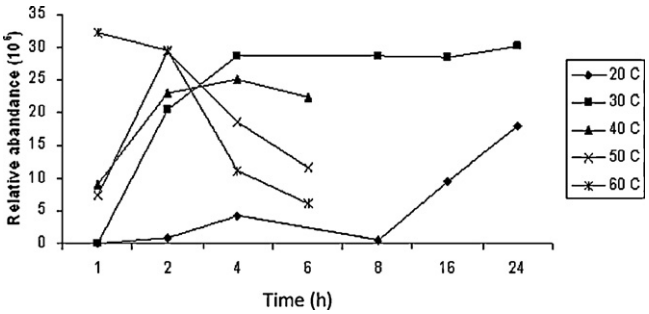

Fig. 4. MS signal intensities of GlcNAc during chitin hydrolysis in 38% HCl (1:5, w/w) at various temperature and times with 30 min pre-sonication.

of chitin by sonication at designated temperatures, the solutions were allowed to be stirred at controlled temperature for additional 90 min. For comparison, another similar set of chitin/38% HCl mixtures were allowed to stirred without pre-sonication at the same designated temperature for 120 min. The degree of hydrolysis, determined from the ratio of water soluble portion to the amount of initial chitin, was significantly higher with the sonication pretreatment (Table 1). At 20 °C, about 12% of chitin was hydrolyzed with pre-sonication while only 3% of hydrolysis was observed without the sonication pretreatment. At 40 °C, the sonication process improved the degree of hydrolysis from 57% to 80%. However, high degree of hydrolysis (>90%) could be obtained at the temperature 60 °C with and without sonication. The results confirmed that after the dissolution of chitin by sonication process, the hydrolysis of chitin readily proceeded even at the temperature below 40 °C.

To study the products obtained from the chitin hydrolysis, the hydrolysates at various temperature were monitored by an electrospray ionization mass spectrometer (ESI-MS). The MS spectra of the hydrolysate samples typically showed two major peaks corresponding to GlcN at $m/z = 162$ ($[GlcNH.H_2O]^+$) and GlcNAc at $m/z = 204$ ($[GlcNAc.H_2O]^+$) (Fig. S2). First the GlcNAc peak was monitored at various hydrolysis temperature and the signal abundance was plotted *vs* the hydrolysis time as shown in Fig. 4. At low hydrolysis temperature of 20 °C, GlcNAc started to appear after 8 h and increased slowly thereafter. Using hydrolysis temperature of 30 °C, GlcNAc yield reached the maximum at 4 h and became constant afterward. At 40 °C, the production of GlcNAc also reached the maximum at 4 h but gradually dropped afterward. For the hydrolysis temperature of 50 °C, GlcNAc yield reached the maximum within 2 h which then dropped quickly afterward. Similarly, the hydrolysis at 60 °C gave the maximum GlcNAc yield within the first hour which then dropped quickly. We hypothesize that the yield drop is caused by the deacetylation of GlcNAc. We monitored both GlcN and GlcNAc MS peaks obtained from the hydrolysis at 40 °C and found that the GlcN peak increased sharply after 4 h at the expense of GlcNAc peak (Fig. 5). The results confirmed that the deacetylation

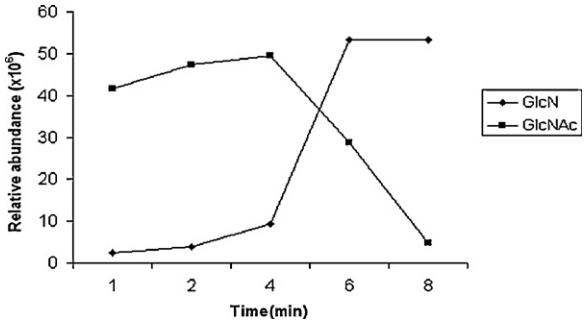

Fig. 5. MS signal intensities of GlcNAc and GlcN during chitin hydrolysis in 38% HCl (1:5, w/w) at 40 °C with 30 min pre-sonication.

Table 2
Purity and recovery mass of solid A, solid B and solid C from fractional precipitation.

Hydrolysis conditions	Solid A		Solid B		Solid C		GlcNAc yield (%)
	% purity	% mass	% purity	% mass	% purity	% mass	
30 °C, 4 h	75	27	77	13	>95	37	65
40 °C, 2 h	65	27	73	30	>95	16	55
40 °C, 3 h	69	20	67	28	>95	33	64
50 °C, 2 h	67	30	75	20	92	10	43
60 °C, 1 h	71	55	81	21	92	5	60

was responsible for the decrease of GlcNAc yield upon prolonged hydrolysis.

The crude GlcNAc product was purified by fractional precipitation in absolute ethanol followed by decolorization as described in Section 2.6. The purity was measured by ESI-MS against the GlcNAc standard. The purities and recovery masses of the precipitate fractions (solid A, B and C) are summarized in Table 2. The solid C which was the last precipitate fraction showed the highest purity. It is important to note that the low temperature (30 or 40 °C) hydrolysis of chitin afforded higher GlcNAc purity and recovery mass of solid C. With the hydrolysis at 30 °C for 4 h, the total GlcNAc yield of 65% was determined in the crude product which could be isolated by fractional precipitation to give 37% yield of high purity product (>95% pure). The high purity of GlcNAc was also confirmed by comparing the ^1H NMR spectrum of the product with standard GlcNAc (Fig. S3).

4. Conclusion

The acid hydrolysis of α-chitin with 38% HCl to GlcN.HCl can be accelerated by microwave irradiation. The reaction rate acceleration is likely to be a result of the faster heating rate rather than the superheating or selective heating. The hydrolysis gave GlcN.HCl comparable to the conventional heating within 12 min which is much shorter than 90 min normally required. Sonication brought about the dissolution of chitin in HCl even at 20 °C that provides a method for selective acid hydrolysis of chitin at low temperature to produce GlcNAc. This is the first report of microwave and ultrasonic wave assisted acid hydrolysis of chitin in the preparation of amino monosaccharide, GlcN.HCl and GlcNAc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.04.064.

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