Mechanoenzymatic breakdown of chitinous material to N-acetylglucosamine: the benefits of a solvent-less environment

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Abstract: Chitin is not only the most abundant nitrogen-containing biopolymer on the planet, but also a renewable feedstock too often treated as a waste. Current chemical methods to break down chitin typically employ harsh conditions, large volumes of solvent, and generate a mixture of products. Although enzymatic methods have been reported, they require a harsh chemical pre-treatment of the chitinous substrate and rely on dilute solution conditions that are remote from the natural environment of microbial chitinases, which typically consists of surfaces exposed to air and moisture. We report an innovative and efficient mechanoenzymatic method to hydrolyze chitin to N-acetylglucosamine monomer by using chitinases under the recently developed reactive aging (RAging) methodology, based on repeating cycles of brief ball-milling followed by aging, in the absence of bulk solvent. Our results demonstrate that the activity of chitinases increases several times by switching from traditional solution-based conditions of enzymatic catalysis to solvent-less RAging which operates on moist solid substrates. Importantly, RAging is also highly efficient for the production of N-acetylglucosamine directly from shrimp and crab shell biomass without any other processing except for a gentle wash with aqueous acetic acid.

Introduction

It is estimated that 6 to 8 million tons of crustacean shell waste is generated on an annual basis, and mostly discarded either into landfills or back into the ocean.[1] The recent search for renewable, non-fossil-based feedstocks has sparked interest in transforming this inexpensive, renewable resource into valuable products. Chitin, a linear β-(1,4)-linked polymer of N-acetylglucosamine (GlcNAc), is an important constituent of crustacean shells. It is also the most abundant nitrogen-containing biopolymer on Earth,[2] making it an attractive feedstock for production of drugs and agrochemicals, 80% of which involve nitrogen.[3] While methods for transforming GlcNAc into useful products (e.g. ethanolamine or nitrogen-containing furans) have been reported,[4,5] recovering high purity GlcNAc from chitinous biowaste remains a challenge. Notably, current methods to extract chitin from crustacean shells typically use NaOH to remove the proteins, followed by treatment with HCl to eliminate the calcium carbonate, and bleaching to decompose pigments.[6–9] Chemical hydrolysis of chitin into its monomer requires acidic conditions at high temperature and pressure, and yields a complex mixture of products together with a substantial amount of waste.[10–13] Therefore, it is clear that there is a pressing need for more sustainable means of fractionating chitinous biowaste and hydrolyzing chitin cleanly to GlcNAc.

While chitinases, i.e. enzymes that catalyze the hydrolysis of chitin into its monomer, offer an opportunity for clean processing of chitin (Figure 1), their use is hindered by the poor solubility of chitin, which mandates harsh pre-treatment of the chitinous material,[9,14–26] e.g. with HCl to form a colloidal chitin suspension,[27] or using phosphoric acid to generate swollen chitin.[28] Further, there is a need to improve chitinase activity and stability for biotechnological applications.[29,30]
Figure 1. Hydrolysis of chitin by chitinases to generate N-acetylglucosamine (GlcNAc).

The challenges of using chitinases in processing chitinous biomass are in contrast with the natural ability of these enzymes, secreted by bacteria and fungi, to degrade chitin of crustacean shells, insect exoskeletons, and fungi. Chitinases produced by soil microorganisms have evolved to catalyze chitin hydrolysis in an extracellular environment, on surfaces exposed to air moisture, i.e. in a moist environment. Consequently, the natural environment for chitinase activity is very different from the bulk aqueous solutions or suspensions used in chemical and industrial processing.[31–33] Mechanocatalysis, i.e. reactions induced or sustained by mechanical milling or shear, has recently emerged as a way to conduct chemical transformation in either complete absence of solvents or, in the case of liquid-assisted grinding (LAG),[34–36] in the presence of a small amount of a liquid which can enhance and direct reactivity while avoiding solubility limitations. It was recently shown that enzymes can retain activity under mechnochemical conditions,[37–42] and our group has reported that efficient mechanoenzymatic hydrolysis of cellulose can be achieved by repeating cycles of milling and aging (static incubation)[43–45] of physical mixtures of the solid substrate, lyophilized cellulase enzymes, and a small amount of water. This process, termed reactive aging (RAging), led to significantly higher glucose concentrations than previously reported methods and, importantly, without the need for biomass pre-treatment or the use of harsh chemicals.[46] As the amount of water in RAging is orders of magnitude lower than in conventional solution processes, and stoichiometrically comparable to that of the substrate, we speculated that RAging should provide a more suitable environment for chitinase activity compared to conventional biocatalysis.

We now report that RAging leads to several fold enhancement of chitinase efficiency compared to solution processes, achieving at least 40% cleavage without bulk solvents, aggressive reagents, or chitin pre-treatment. This mechanoenzymatic process yields a GlcNAC product that is 2-8 times more concentrated (>400 mM) than typical chitinase reactions conducted in solution, while generating little solvent waste.[16,22,47] Importantly, we also show that RAging is highly efficient for the production of GlcNAC directly from ground shrimp and crab shells, after a gentle wash with aqueous acetic acid. While these results demonstrate a significant improvement in enzyme-catalyzed processing of chitin and chitinous biomass, they also highlight liquid-assisted mechanocatalysis and aging as reaction environments of choice when developing processes for enzymatic reactions.

Results and Discussion

Chitinase mixture characterization

We sourced a commercial Aspergillus niger chitinase preparation sold as a lyophilized powder. A. niger is a terrestrial fungus widely prevalent on moist surfaces, both in residential and in natural areas. This microorganism secretes chitinases and other
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enzymes to digest biopolymers into easier-to-absorb smaller molecules. Based on the Bradford assay, it was established that the commercial chitinase powder has a total protein content of 2% (w/w).[48] SDS-PAGE analysis revealed a mixture of several proteins, ranging in molecular weight from 40-150 kDa, many of which corresponding to chitinases found on the carbohydrate-active enzyme (CAZy) database for A. niger (Figure S1).[49,50] The major constituent of this powder is maltodextrin, a common food additive and lyoprotectant, which accounts for ~70% (w/w) based on an enzymatic assay. The commercial chitinase mixture tested negative for chitosanase activity (Figure S2),[51] but was found to exhibit amylase activity (hydrolysis of α-(1,4)-glycosidic bonds, Figures S3, S4). Since amylases can hydrolyze maltodextrin to glucose,[52,53] we had to expect that upon incubation, the commercial enzyme mixture alone would produce some glucose (Figure S4).

Enzymatic activity is enhanced under mechanochemical conditions

The 3,5-dinitrosalicylic acid (DNS) assay measures reducing sugar concentration.[54] It was used to rapidly estimate the reaction progress, and a detailed characterization and quantification of the reaction products (HPLC-UV-MS) was performed at key steps along the study. The chitinase activity of this commercial powder was first evaluated in the presence of an aqueous solvent. Gentle shaking of a suspension of untreated, practical grade chitin (ca. 79% w/w purity)[55] and chitinase at a protein content of 0.1 mg/mL in water or buffer provided, after 7 days at 45˚C ca. 15 mM of reducing sugars (Figure 2), of which 0.84 ± 0.09 mM was GlcNAc (Figure S5).

Figure 2. Measuring chitinase activity in solution over a 7-day period. Insoluble chitin was suspended at a concentration of 10 mg/mL with 5 mg/mL chitinase powder (i.e. 0.1 mg/mL protein). This mixture was allowed to react with gentle shaking. Error bars are standard deviations, with n = 3 for each point.

Unless noted otherwise, all solvent-less experiments were performed using 200 mg of chitin and 0.7% by weight protein content. The enzyme loading is reported as the mass of protein to total solid in the reaction (substrate + enzyme preparation). Thus, a combination of 200 mg of chitin with 100 mg of chitinase powder (itself containing only 2% protein), yields a 0.7% (w/w) protein loading. For LAG, the samples were typically milled for 30 minutes in a Teflon assembly operating at 30 Hz, and aging was performed at 45˚C for up to 7 days. Water is an essential substrate for chitinase activity and was also used as the liquid additive in LAG.[34,38,46] The amount of water in LAG was optimized by varying the ratio of liquid volume to the weight of the reaction mixture ($\eta$)[34–36] from 0 to ca. 3 μL/mg, revealing that the yield plateaued at $\eta$ of ca. 1.7 μL/mg (i.e. 500 μL water per 300 mg reaction mixture, Figure 3A).[56] Such water loading is consistent with LAG conditions previously reported in mechanochemical reactions,[34–36] and was found to produce a fluffy paste - an initial texture that was also found optimal for the hydrolysis of cellulose by cellulases via RAging (Figure S6).[46]
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The effective chitin loading under these conditions is 400 mg/mL, which is, to our knowledge, 2-8 times higher than the most concentrated chitinase reactions reported.[16,22,47] Replacing water with a pH 6.0 sodium phosphate buffer (preferred buffer for this enzyme)[57] had a negligible effect on activity (Figure S2B). No hydrolysis was observed in the absence of water, without enzyme, or with inactivated enzyme (Figure S2C).

We next explored different enzyme loadings, with protein content of 0.1, 0.4, 0.7, or 1% (w/w) of dry weight. Interestingly, more enzyme did not necessarily translate into a higher yield (Figure 3B) and a chitinase loading of 0.7% (w/w) was found to be optimal. Finally, milling frequencies of 10 Hz, 20 Hz, and 30 Hz all gave similar enzyme activity, but a higher reproducibility was observed at 30 Hz (Figure S2D).[58]

Before investigating reactivity upon RAging, we also examined enzyme kinetics in a simple combination of one-time LAG followed by aging. As shown in Figure 3C, increasing LAG time beyond 5 minutes did not substantially increase the yield of reducing sugars. In contrast, the duration of subsequent aging had a considerable effect, as the rate of hydrolysis became negligible only after ~20 hours (Figure 3D), which also demonstrates that chitinase activity is retained upon mechanochemical LAG treatment. Although aging of the mixture at 55°C instead of 45°C gave a higher reaction rate over the first 12 hours, the resulting product was not as clean, with a change in colour and odor suggesting the onset of Maillard-like reactions.[59]

Next, we examined the exact nature of the chitinase reaction products, obtained under optimized conditions of LAG and aging (200 mg chitin, 0.7% w/w chitinase, 500 µL water; milling at 30 Hz for 30 min, followed by aging at 45°C for 7 days) (Figure S5B,E). As expected, a major component of the product mixture was glucose resulting from the hydrolysis of the

Figure 3. Optimizations of solventless chitinase reactions. (A) Adding 9–1000 µL H2O to 100 mg of the chitinase mixture (which corresponds to 2 mg of protein) and 200 mg of chitin, with milling at 30 Hz for 30 min, followed by aging at 45°C for up to 7 days. (B) Adding 0.1-1% w/w of protein (corresponding to 10-200 mg of chitinase mixture) to 200 mg of chitin and 500 µL of H2O, with milling at 30 Hz for 30 min, before aging at 45°C for up to 7 days. (C) Enzyme kinetics of milling at 30 Hz for up to an hour using 100 mg of chitinase mixture (i.e. 2 mg of protein), 200 mg of chitin, and 500 µL of H2O. (D) Enzyme kinetics of aging over a 96-hour period at room temperature, 45°C, or 55°C, after milling 100 mg of the chitinase mixture (i.e. 2 mg of protein), 200 mg of chitin, and 500 µL of H2O for 5 min at 30 Hz. Error bars are showing standard deviations, with n = 3 for each point.
lyoprotectant by amylases included in the commercial chitinase preparation. The major chitinase product was GlcNAc (56 ± 3 mM), followed by diacetyl-chitobiose (2GlcNAc: 27 ± 4 mM), and triacetyl-chitotriose (3GlcNAc: 16 ± 2 mM). No other soluble GlcNAc oligomers were detected. Comparing these results to solution-based reactions for the same chitin/enzyme ratio (200 mg of chitin and 2 mg of protein), reaction time and temperature, reveals that for this chitinase preparation, the in-solution reaction yields 3.7 mg of GlcNAc, while the solvent-less reaction generated 6.1 mg of GlcNAc, i.e. almost twice as much.

**Chitinase activity is further enhanced with RAging**

To investigate the behaviour of chitinase under RAging, i.e. multiple LAG and aging cycles, the duration of consecutive milling and aging segments were varied over multiple cycles, while keeping other parameters constant (Figure S7). During the first 10 hours, RAging was found to provide little benefit over simple aging of the milled reaction mixture. At longer reaction times, however, the repeated cycles of RAging provided increasingly higher yields of reducing sugars. For example, using optimized RAging conditions for 24 hours (2 cycles of 5 min milling and 12 h aging at 45˚C) afforded ca. 50% higher conversion compared to LAG combined with aging (Figure S7). Conducting the RAging process for 7.5 days (15 cycles) under the same conditions resulted in a reducing sugar concentration of 1100 ± 100 mM (Figure 4A).

Importantly, performing the identical RAging process without enzyme or with an inactivated enzyme produced no significant hydrolysis after 2.5 days, demonstrating the involvement of chitinase (Figure S8).

**RAging chitin with chitinase produces mainly GlcNAc**

Analysis of the chitin reaction products obtained by RAging revealed that GlcNAc was again the dominant chitinase product, reaching a concentration of 430 ± 80 mM after 20 cycles of RAging (Figure 4B,C), corresponding to a chitin hydrolysis yield >30%. It should however be noted that this number is likely to be an underestimation, as chitin is not a single linear chain, the purity of practical grade chitin is batch-dependent and, finally, chitin can contain deacetylated GlcN units (poorer chitinase substrate), which all makes the calculation of an exact yield impractical. In comparison, milling once followed by aging for 7-days yields 56 ± 3 mM GlcNAc (Figure S5). The GlcNAc concentration obtained under RAging is 2.4 times higher than any other reported GlcNAc concentration obtained from a chitinase reaction (up to 180 mM).[47] It is also orders of magnitude higher than that of a comparable in-solution reaction (0.017 mM, Figure S5), further illustrating the benefits of a solvent-less environment for chitinase activity. Concentrations of 2GlcNAc (5.3 ± 1.6 mM) and 3GlcNAc (7.5 ± 0.6 mM) obtained by RAging were minuscule compared to GlcNAc, and no other soluble GlcNAc oligomer was detected. The presence of deacetylated GlcNAc, i.e. glucosamine (GlcN), found at 10.5 ± 1.7 mM in the initial reaction mixture, increased to 25.0 ± 3.1 mM at the end of the enzymatic reaction (Figure 4C). The production of GlcN is likely to arise from the chitin polymer being partially deacetylated from the start (deacetylation is known to occur during industrial chitin purification) and released by chitinase during the process.[60]

In solution, chitinases often produce a mixture of different chitoooligosaccharides.[17,61–63] The data presented above however demonstrate that under solvent-less RAging conditions, GlcNAc accounts for >92% of the water-soluble degradation products of chitin. Moreover, for the same chitin/enzyme ratio, while the in-solution reaction produces 9 mg of GlcNAc, RAging yields 47 mg of GlcNAc, i.e. 12 times more (Table 1).

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<th>Table 1. GlcNAc produced from 200 mg of substrate and 2 mg of protein using various conditions.</th>
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[a] Shaking at 250 rpm and 45˚C for 7 days. Data from two separate experiments, each in triplicates. [b] RAging cycles of 5 min milling at 30 Hz and 12 h aging at 45˚C; a total of 20 cycles were used with chitin, and 10 for AcShrimp and AcCrab samples.

**Biomass hydrolysis is enhanced under solvent-less conditions**
Our early attempts at reacting untreated biomass with chitinases under solvent-less conditions showed negligible hydrolysis (Figure S9A). We first suspected that the high CaCO$_3$ content of the biomass (pH ~8) might impact hydrolysis by increasing the pH beyond the level tolerated by A. niger’s chitinase.$^{[57,64]}$ To verify this hypothesis, chitinase activity was tested on a mixture of chitin supplemented with 40% (w/w) CaCO$_3$ and a ~50% reduction in the rate of hydrolysis was observed compared to the reaction on chitin alone (Figure S9B). Furthermore, a chitinase reaction with untreated biomass in the
presence of concentrated buffer (1 M sodium phosphate, pH 6.0) to lower the pH also resulted in negligible hydrolysis (Figure S9A), indicating that pH alone does not fully account for the loss of activity.

Figure 4. Chitinase hydrolysis of chitin under RAgeing conditions. (A) The reaction mixture consisted of 100 mg of chitinase mixture (i.e. 2 mg of protein), 200 mg of chitin, and 500 µL of H2O, which underwent 20 cycles of 5 min milling + 12 h aging at 45°C. The arrows correspond to the points used for the iPLC chromatograms shown in B. (B) Chromatographic separation was achieved with an amino (NH2) reversed phase column. The standards trace shows the elution pattern of: (1) N-acetylglucosamine or GlcNAc, (2) diacetylchitobiose or 2GlcNAc, (3) triacetylchitotriose or 3GlcNAc, (4) tetraacetylchitotetraose or 4GlcNAc, and (5) pentaacetyl-chitopentose or 5GlcNAc. Each RAgeing cycle corresponds to 5 min milling at 30 Hz + 12 h aging at 45°C. (C) In situ concentration of the different chitin degradation products generated after 0, 5, 10, 15, and 20 cycles of RAgeing (5 min milling + 12 h aging). HPLC-UV-Mass was used, with or without derivatization, to quantify glucosamine, N-acetylglucosamine (GlcNAc), diacetyl-chitobiose (2GlcNAc), and triacetylchitotriose (3GlcNAc). Error bars are standard deviations, with n = 3 for each measurement.
In chitinous biomass, proteins are tightly wrapped around chitin nanofibrils, and multiple such assemblies are grouped within a CaCO₃ matrix to form fibers, which further assemble into stacked, twisted planes.⁶⁶ As a result, native biomass chitin is largely out of reach for chitinases. To improve the accessibility of chitin, we used diluted aqueous acetic acid (AcOH) to remove some of the CaCO₃, and partially denature the proteins. The chitin content of the recovered solid was assessed following a published solid-state nuclear magnetic resonance (ssNMR) protocol⁶⁷ and found to increase after the AcOH wash, from 39% to 57% (w/w) for shrimp, and from 31% to 54% (w/w) for crab (Figure S10).

The AcOH washed shrimp (AcShrimp) and crab shells (AcCrab) were found to be even better chitinase substrates than commercially purified chitin. One cycle of milling followed by aging for 3 days produced reducing sugar concentrations of 690 ± 30 mM for AcShrimp and 580 ± 30 mM for AcCrab (Figure 5), compared to 465 ± 19 mM for commercial chitin (Figure 3D). Washing commercial chitin with diluted aqueous AcOH (AcChitin) did not increase the yield of hydrolysis, consistent with the wash affecting the accessibility of chitin from biomass rather than altering the polymer itself or affecting enzyme activity (Figure S9B). Lastly, the activity of chitinase on biomass was compared with and without bulk solvent. In solution, 10 mg/mL of biomass and 0.1 mg/mL of protein were allowed to react at 45˚C with gentle shaking. After 7 days the reducing sugar concentration reached 12.5 ± 0.3 mM for AcShrimp and 10.2 ± 0.3 mM for AcCrab (Figure S11). This is 55 times less concentrated than the corresponding solvent-less reactions (one cycle of milling + aging). Moreover, calculating the total amount of product formed at the same enzyme/substrate ratio, the chitinase reaction in solution yielded 18 mg and 11.4 mg of GlcNAc from AcShrimp and AcCrab, respectively (Figure S5A, Table 1), while solvent-less milling once followed by aging afforded 33 mg and 31 mg of GlcNAc (Figure S5C,F), respectively, confirming that chitinase is more efficient under solvent-less conditions (Figure 6). Performing the same reaction using heat-inactivated chitinase gave no hydrolysis after 2.5 days, confirming that the observed chitin digestion is due to enzymatic activity (Figure S8).

RAGing on biomass produces sugar monomers

The optimal RAging parameters described above for chitin (several cycles of 5 min milling + 12 h aging), were next applied to AcShrimp and AcCrab. Here again, for the same overall reaction time, chitinase was more efficient with multiple cycles of milling and aging than with only one cycle. After 3 days of RAging, the reducing sugar concentration reached 810 ± 150 mM and 812 ± 120 mM for reactions with AcShrimp and AcCrab, respectively (Figure 6A,B). HPLC-UV-MS analysis of the RAging reactions (Figure 6C-F) revealed that chitinase degradation of AcShrimp and AcCrab predominantly yields the GlcNAc monomer. After 10 cycles of RAging, the concentration of GlcNAc in the mixture reached 462 ± 81 mM and 442 ± 66 mM for AcShrimp and AcCrab hydrolysis, respectively. Assuming a single linear chain of chitin, this corresponds to at least 41% and 42% hydrolysis of the AcShrimp and AcCrab chitin content, respectively (the chitin content of biomass was quantified using ¹³C ssNMR as previously reported;⁶⁷ Figure S10). For the same reasons as mentioned above, the yields are underestimates. Furthermore, these results were obtained at very low enzyme loading (0.7% w/w); larger enzyme loadings and/or longer reaction times would afford higher yields. Minor amounts of GlcN and 2GlcNAc were also produced, while 3GlcNAc was not detected. Overall, the GlcNAc produced by chitinase from AcShrimp and AcCrab, accounts for 83% and
90\%, respectively, of all water-soluble chitin-derived products. Comparing the amount of GlcNAc generated at the same biomass-to-protein ratio, and from the same protein preparation, for in-solution versus solvent-less RAging reactions, reveals a 3-5 times higher yield for the latter (Table 1). Overall, this mechanoenzymatic process is much more efficient and cleaner than the typical in-solution chitinase reactions, with the added benefits of avoiding the need for large solvent volumes, and minimal biomass pre-treatment needed.

Conclusions

Outside of their native biological context, enzymes are typically used by researchers and industry at highly dilute concentrations in an aqueous solvent. This is strikingly different from the conditions under which microbe-secreted enzymes, such as chitinases, have evolved to thrive in. We show here that both the efficiency and selectivity of chitinase are increased several times by switching from solution-based environments, traditionally used in enzymatic reactions,\textsuperscript{9,14-26} to RAging conditions that operate on moist solid substrates in the absence of bulk solvent media. Our current hypothesis is that aging is the closest to the natural environment of microbial chitinases (under which there is no need for an exceedingly high reaction rate), whereas RAging, with its periodic milling activation events, may further accelerate the reaction via mechanical effects and improved mixing.

The RAging methodology presented here for chitin depolymerization is very simple - the reaction mixture is left in an incubator for most of the time and periodically mixed - yet it yields almost exclusively the GlcNAc monomer as a highly concentrated product, compared to the dilute oligomeric mixtures often obtained in solution environments. Importantly, the RAging process requires no pre-treatment of chitin, and is also highly efficient for the degradation of chitinous biomass after a gentle wash with aqueous acetic acid to reduce the carbonate content. This methodology enabled the direct conversion of at least 40\%\textsuperscript{†} of its chitin content into the GlcNAc monomer. The benefits of RAging are further highlighted by comparing the substrate loading used and the concentration of obtained GlcNAc, to those of previously reported methods (Table S1).\textsuperscript{13-23} The typical substrate loading for RAging is at least one order of magnitude larger than for methods using bulk solvent, and even when considering studies on less recalcitrant and/or pre-treated chitin forms than the one used here, the GlcNAc yield in mg/mL is ca. 2-30 times superior.

Biocatalysis enables reactions that would otherwise require harsh reagents and conditions. Solvent-less mechanoenzymology is breaking the boundaries of traditional in-solution biocatalysis with the ability to reach extremely high substrate concentrations and yield highly concentrated products. Furthermore, the absence of solvent translates into a much smaller total reaction volume which facilitates manipulation while generating less waste, and enables reactions of poorly-soluble and recalcitrant substrates like chitin and cellulose.\textsuperscript{146} These advances may offer cost-effective sustainable strategies to transform waste into useful chemicals.
Experimental Section

Materials. Practical grade chitin and chitosan (≥75% deacetylated) from shrimp shells, 4-nitrophenyl N-acetyl-β-D-glucosaminide, 3,5-dinitrosalicylic acid, potassium sodium tartrate tetrahydrate, disodium hydrogen phosphate, sodium phosphate monobasic, NaNO₃, and Fmoc-OSu were purchased from Millipore Sigma (Oakville, ON, Canada). N-Acetylglucosamine was purchased from Alfa Aesar (Tewksbury, MA, USA).
This is the peer reviewed version of the following article: [Therien, J.P.D., Hammerer, F., Friščič, T., and Auclair, K. (2019). Mechanoenzymatic Breakdown of Chitinous Material to N-Acetylglucosamine: The Benefits of a Solventless US]. Diacetyl-chitobiose, triacetyl-chitotriose, tetraacetyl-chitotetraose, and pentaacetyl-chitopentaose were purchased from Megazyme (Chicago, IL, USA). Food grade Aspergillus niger chitinase was purchased from Creative Enzymes (Shirley, NY, USA). Calcium carbonate was from BDH Chemicals (VWR international, Radnor, PA, USA). Coomassie Brilliant Blue R-250 Dye, trimethylamine, and HPLC-grade solvents were purchased from Thermo Fisher (Waltham, MA, USA). Unless specified otherwise, water was from a MilliQ system with a specific resistance of 18.2 MΩcm at 25°C.

Biomass Preparation. Penaeus monodon (Black Tiger) shrimps were caught in Vietnam and shipped to a restaurant in Montreal, QC, Canada. The headless, frozen shrimps were peeled at the restaurant, and the frozen shells were transported to the laboratory. They were thawed in water to remove any remaining meat, the legs and the tails were also stripped off, before allowing the shells to dry on parchment paper in ambient air. The dry shells were either directly milled or stored at room temperature until milled.

Chionoecetes opilio (snow crab) were caught by Louisbourg Seafoods Limited (NS, Canada) between the months of April and October in the inshore/offshore Northwest Atlantic Ocean. The commercially desired legs and shoulders were recovered, and the waste bodies were sent to Cape Breton University (to Prof. S. MacQuarrie’s Lab) where they were left at room temperature to dry before shipping to McGill University and stored at -80°C until processing. The shells were stripped of any remaining meat, rinsed under water, and allowed to dry at room temperature overnight. The dry shells were either directly milled or stored at -80°C until milled.

Milling of the dry shrimp or crab shells was achieved from 2 g of dried shells, in a 25 mL stainless steel jar with a single 15 mm (11.7 g) stainless steel ball, at 30 Hz for 5 min. The resulting fine powder was stored at room temperature.

Washing of the milled shrimp or crab shells with diluted aqueous acetic acid consisted of adding 5 g of the powdered biomass to 30 mL of a vigorously stirring H₂O:AcOH (50:50) solution until the formation of bubbles ceased (~1 hour). The solid was collected by centrifugation (5 min at 1000 × g) and washed several times (until neutral pH) by resuspending in water (~45 mL), vortexing and collecting the solid by centrifugation. The sample was lyophilized using a Labconco (Kansas City, MO, USA) FreeZone 1 Liter benchtop freeze dry system.

DNS assay to quantify reducing sugars. The total amount of reducing sugar in reaction mixtures was quantified using the 3,5-dinitrosalicylic acid (DNS) reagent. The DNS reagent solution was prepared by first mixing DNS (1 g), Rochelle salt (potassium sodium tartrate, 30 g) in water (50 mL) and NaOH (20 mL of a 2M solution), before diluting to 100 mL in water. The solution was filtered through cotton and stored at 4°C for up to one month. The DNS solution was calibrated using N-acetylglucosamine dissolved in water over a range of 0.2 to 1.0 mg/mL (0.9-4.5 mM).

For measuring the concentration of reducing sugars, aliquots (10-30 mg) were collected in separate 1.5 mL microcentrifuge tubes and stored at -20°C until analysis. The aliquots were suspended in ice-cold water to a final chitin or biomass concentration of 10 mg/mL.

The microcentrifuge tubes containing the solid from the solvent-less reactions were suspended in ice-cold water then immediately placed into a boiling water bath for 30 min to inactivate the enzymes. For the in-solution enzymatic reactions, aliquots were collected in a microcentrifuge tube and kept at -20°C until assayed, at which point they were directly added to a boiling water bath for 30 min. Once cooled, the samples were mixed with a spatula to break up large solids and centrifuged at 21,100 × g for 5 min to pellet any insoluble material. The supernatant was stored at -20°C for further analysis (e.g. HPLC-MS, glucose content).

The amount of reducing sugar was quantified after mixing the prepared aliquot (200 µL, diluted 4-8× or not) to the DNS solution (100 µL), allowed to boil for 5 min, before transferring 200 µL into the well of a clear-bottom 96-well microtiter plate. Absorbance was measured at 540 nm using a Molecular Devices SpectraMax i3x (San Jose, California, USA) microplate reader with pathcheck enabled.

In order to determine the percent hydrolysis, chitin was assumed to be a single linear chain (even though it is not) to allow estimation of the amount of GlcNAc that would be produced from its complete hydrolysis. As such, the complete hydrolysis of 200 mg practical grade chitin (79% pure) would generate 0.78 mmol of GlcNAc. The chitin content of the biomass was determined using ssNMR (see below). The complete hydrolysis of 200 mg of shrimp, AcOH-treated shrimp, crab, or AcOH-treated crab would lead to 0.38 mmol, 0.56 mmol, 0.31 mmol, and 0.53 mmol GlcNAc, respectively.

In-solution chitinase reactions. The substrate (~10 mg suspended to a 10 mg/mL concentration) and A. niger chitinase (5 mg of chitinase mixture/mL) were mixed in water or buffer (100 mM sodium phosphate buffer, pH 6.0). Sodium azide (0.02% w/v) was added to prevent the growth of microorganisms. The reaction was allowed to proceed on a rotary shaker (250 rpm) at 45°C for 7 days, with monitoring over time. Reaction aliquots were directly stored at -20°C until analysis.

Solvent-less chitinase reactions. Milling experiments were performed in 10 mL Teflon jars containing two 7-mm stainless steel balls (1.37 g each) shaken using a FTS1000 shaker mill (Form-Tech Scientific, Montréal, QC, Canada). Static incubation (aging) involved placing the samples into a Thermo Fisher MaxQ 480R HP incubator (Waltham, MA, USA) set to the desired temperature. Unless noted otherwise, water was added immediately before milling. The reaction aliquots collected weighed 10-30 mg. All experiments were performed at least in triplicate. The optimum conditions used chitinase (100 mg enzyme preparation, corresponding to 2 mg of total protein), chitin or biomass (200 mg), and
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The milling frequency was optimized under the conditions mentioned above but at frequencies of 10, 20, or 30 Hz, each for 30 min. To optimize the amount of water, volumes of 0, 250, 500, 750, or 1000 µL were added to either 10 or 100 mg of chitinase mixture (corresponding to 0.5 to 5 mg of protein), with a constant 200 mg of chitin. Finally, to optimize the enzyme loading, 10, 50, 100, or 200 mg of the chitinase mixture (corresponding to 0.5, 2.5, 5, and 10 mg of protein) was used with 200 mg of chitin and 500 µL of water.

Aging kinetic experiments were performed under the optimal conditions mentioned above after milling for 5 min.

R Aging reactions involve multiple cycles of milling and aging. Preliminary experiments used variable milling (5-15 min) and aging (1-12 h) periods. The optimized RAging conditions consisted of repeating cycles of milling for 5 min and aging at 45°C for 12 h, for up to 20 cycles (10 days).

To investigate the effect of calcium carbonate found in biomass on enzyme activity, chitin was supplemented with 40% (w/w) CaCO3 (200 mg total) was reacted with water (500 µL) in the presence of chitinase (2 mg of protein).

The chitosanase activity in the commercial chitinase mixture was evaluated using chitosan (200 mg), chitinase (2 mg of protein) and water (500 µL).

Buffer (500 µL, 100 mM sodium phosphate, pH 6.0) was occasionally tested as a replacement for water in the degradation of purified chitin. Since biomass is naturally quite basic, the buffer used with biomass was more concentrated (1 M sodium phosphate buffer, pH 6.0) than the one used with chitin.

For every reaction data, several control reactions were performed, for example missing each reaction constituent separately. When inactivated enzyme was used, it was prepared as followed. Chitinase (2 mg of protein, corresponding to 100 mg of enzyme preparation), was dissolved in water (500 µL). The container was immersed in a boiling water bath for 30 min. Once cooled down, it was added to the desired reaction mixture.

HPLC-UV-MS analysis to quantify GlcNAc monomer and oligomers. This analytical method was adapted from a previously published protocol.[14] Mono- and poly- saccharides were identified and quantified using an HPLC-UV-MS system equipped with quaternary pump, autosampler, multiwavelength UV-Vis detector, and ES mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), coupled to a Zorbax 70Å NH2 Column (150 mm × 4.6 mm, 5 µm, Agilent Technologies). The mobile phase consisted of water:acetonitrile, with the ratio linearly changing from 20:80 to 40:60 over 60 min, at a flow rate of 1 mL/min. UV detection was set to 205 nm.

The following commercial compounds were used for identification and quantification (via calibration curves) of the reaction components: N-acetylglucosamine (GlcNAc), diacetyl-chitobiose (2GlcNAc), triacetyl-chitotriose (3GlcNAc), tetraacetyl-chitotetraose (4GlcNAc), and pentaacetyl-chitopentaose (5GlcNAc). All samples were dissolved in methanol and centrifuged for 5 min at 21,100 × g to remove any large insoluble material. Finally, a 0.22 µm nylon filter (Chromspec, Brockville, ON, Canada) was used to remove any fine particle. The area under the peak was used to calculate the concentration.

HPLC analysis to quantify glucosamine. Glucosamine monomers and oligomers were first derivatized using a previously published method.[68] Fmoc-OSu was used as follows to derivatize the compounds. Reaction aliquots (10 µL) were first mixed with 76 mM triethylamine in water before addition of Fmoc-OSu (100 µL of 5 mg/mL solution in acetonitrile). The mixture was sonicated at 50°C for 30 min, before dilution in a 50:50 water:acetonitrile solution containing 0.05% TFA (860 µL), and filtration using a PTFE membrane (Chromspec, Brockville, ON, Canada).

The derivatives were identified and quantified by HPLC using a Zorbax RX-C18 column (150 mm × 4.6 mm, 5 µm, Agilent Technologies). The mobile phase consisted of a water:acetonitrile mixture containing 0.05% TFA, starting with a linear 70:30 gradient for 12 min, ramped up to 0:100 over the next 5 min, and kept at 0:100 for an additional 5 min, using a flow rate of 0.8 mL/min. UV detection was at 265 nm.

Protein characterization. SDS-PAGE used a 4–20% acrylamide gradient Mini-PROTEAN® TGX™ Precast Protein Gel with either 4 µL of the Amersham low molecular weight ladder for SDS-PAGE or 15 µL of the chitinase powder suspended in 1x Laemmli buffer at a concentration of 10 mg/mL. The gel was stained with a Coomassie blue solution and imaged with a Bio-Rad ChemiDoc MP imaging system (Hercules, California, USA).

Proteinase concentration was determined using the commercial Thermo Scientific Pierce Coomassie Plus (Bradford) Assay Kit.

Chitinase activity was periodically measured based on a previously published protocol,[69] to evaluate any loss in activity. Briefly, the commercial chitinase solid mixture was dissolved in water to generate a 10 mg/mL solution and 25 µL was added to sodium phosphate buffer (200 µL, pH 7.0) in a 96-well clear bottom microtiter plate. Following incubation at 37°C for 15 min, a DMSO solution of 4-nitrophenyl N-acetyl-β-D-
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glucosaminide (pNP-GlcNAC, 25 µL of a 10 mM stock in DMSO) was added. Immediately after, absorbance at 410 nm was measured every 20 sec for 15 min. Enzyme activity was estimated from the initial rate, using a calibration curve of p-nitrophenol (pNP). Chitinase activity with this assay was typically around 250 U per gram of solid mixture, where 1 U of enzyme releases 1 µmol of pNP per minute. No significant loss in activity was observed over several months of periodic testing.

**Quantification of the percentage of chitin in biomass samples.** This analytical method was adapted from a previously published protocol.[67] The chitin content of all biomass samples was determined using solid-state $^{13}$C NMR (ssNMR) at 100 MHz using a Varian VNMRS spectrometer equipped with a 4 mm double-resonance Varian Chemagnetics T3 Probe (now Agilent, Santa Clara, CA). The sample (33–37 mg) was centre-packed into a 3.5 mm rotor and spun at 8 kHz. Chitin standards were prepared by mixing practical grade chitin with bovine serum albumin (BSA) to 25%, 50%, 75%, or 100% w/w. A practical chitin purity of 79% was assumed based on the literature,[55] translating into 20, 39, 59, and 79% w/w as the final chitin percent weight.

The $^{13}$C ssNMR MultiCP parameters were 10 periods of 1 msec CP at about 60 kHz rf field, alternating with delay periods of 1 sec. The recycle delay was 3 sec. An echo was performed at the end of the MultiCP experiment with echo time of one rotor period (250 µsec). A total of 512 scans were acquired for each sample with the exception of the 25% chitin sample, which required a total of 2560 scans. The integrals of the peaks were scaled according to the number of transients acquired. $^1H$ 90° pulses were 2.4 µsec long and $^{13}C$ pulses were 3.7 µsec long. The spectra were referenced to the C=O resonance of α-glycine at 175.7 ppm. A chitin calibration curve was prepared by plotting the percent of chitin (w/w) over the integral of the NMR signal (100% chitin was set to have an integral of 1) divided by the mass loaded in the rotor.

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**Conflicts of Interest**

Some of the herein presented work is a part of the patent application US 62/465,443 filed 1 March 2017.

**Keywords:** Biocatalysis • Biomass • Mechanoenzymatic • Solvent-less • R Aging

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