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THE INFLUENCE OF CHITIN STRUCTURE ON ITS ENZYMATIC DEACETYLATION

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The possibility of producing chitosan by enzymatic deacetylation of chitin has been the subject of numerous investigations over the last twenty years, but to date no satisfactory method has been developed. In this paper the influence of chitin chain conformation and chitin particle crystallinity on the enzymatic deacetylation of chitin is investigated to determine the relative importance of these two factors on the process. It is shown that the high crystallinity of chitin is the main obstacle to converting chitin to chitosan by enzymatic deacetylation.

Keywords: chitin, chitin deacetylase, enzymatic deacetylation, structure, crystallinity, conformation

1. INTRODUCTION

Chitosan is conventionally produced by chemical deacetylation of chitin using concentrated NaOH solutions. This method is used for most industrial production of chitosan. In principle, chitosan could also be produced from chitin by enzymatic deacetylation using chitin deacetylase, which currently is the only enzyme known that is able to deacetylate the *N*-acetyl-**D**-glucosamine (GlcNAc) units of chitin or chitosan to the **D**-glucosamine (GlcN) units (Jaworska, 2012). This method has been intensively investigated since the late 1990s as it was thought that it might be possible to produce chitosan directly from chitin without the use of environmentally harmful reagents such as highly concentrated sodium hydroxide. However despite the many studies of the problem, it is still far from being resolved.

Chitin deacetylase is produced by micro-organisms, both as an intercellular enzyme (e.g. *Mucor rouxi*, *Absidia coerulea*, *Rhizopus stolonifer*) and as an extracellular enzyme secreted in the culture medium (eg.*Colletotrichum lindemuthianum*, *Gongronella butleri*, *Aspergillus nidulans*), Tsigos et al. (2000). Most of experiments with this enzyme were carried out using chitin oligomers or chitosan that were soluble in the buffer solution. The reaction was a homogeneous one, hence all the GlcNAc units in the oligomer substrates were available to the enzyme. The situation becomes more complicated when the substrate consists of insoluble particles of chitin and the process of enzymatic deacetylation is carried out as heterogeneous one. Chitin particles have crystalline structures in which the chains are either anti-parallel (α-chitin) or parallel (β-chitin) depending on the source. These crystalline structures are stabilised by intra- and inter-chain bonds (Rinaudo, 2006) which reduce the extent of swelling in water, so rendering the particles less accessible to chitin deacetylase than are the soluble oligomers. Hence only those few GlcNAc units that are exposed on the outer surface of the chitin particles can be deacetylated while the majority of the GlcNAc units are hidden inside the crystalline structure of the

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chitin particle. The configuration of chitin chains for α- and β-chitin and accessibility of the GlcNAc groups is also strongly connected with the degree of crystallisation of the particles.

The deacetylation rates of different forms of chitin indicate that chitins with low crystallinity (colloidal or amorphous) have higher rates of deacetylation than chitins with high crystallinity (Aye et al., 2006; Cai et al., 2006; Martinou et al., 1995; Tokuyasu et al., 1996). The data confirms that compact crystalline forms of chitin, where the GlcNAc units are less accessible to the enzyme, are less suitable for enzymatic deacetylation. But differences in deacetylation rate can be the result not only of differences of crystallinity, but also of differences in the specific surface area of chitin particles. A given weight of chitin can be characterised by the specific surface area of the particles or, more simply, by the particle size distribution of the chitin sample used, since for smaller particles the specific surface area will be larger and so the number of GlcNAc units located at the surface, and hence accessible to the enzyme, will also be larger, resulting in an increase in the rate of deacetylation.

We can state out that there are two main factors whose influence on chitosan deacetylation has to be resolved if a viable process for the production of chitosan by enzymatic deacetylation of chitin is to be developed. The first is that of the conformation of the chitin chains and their organisation within the chitin particles, i.e. parallel or antiparallel, the second is that of the crystallinity of the chitin particles. The aim of the current paper is to investigate which of these factors has the greater influence on the enzymatic deacetylation of chitin as the data presented in the literature is not sufficient to reach a definite conclusion. To resolve this, the enzymatic deacetylation of α- and β- chitins has been measured to determine the influence of the chitin chain conformation, and also the deacetylation of α-chitin particles with different crystallinities to determine the influence of crystallinity. Cai et al. (2006) and Jaworska et al. (2003) presented data comparing the crystallinity of chitins having different origins, and observed that α-chitin from shrimps has the highest crystallinity while α-chitin from fungal biomass has a much less crystalline structure. Hence a comparison of the rate of deacetylation of chitin from these two sources, having similarly sized particles, will demonstrate the influence of crystallinity on enzymatic deacetylation.

2. MATERIALS AND METHODS

2.1. Chitin

Samples of α-chitin from shrimp (Merck), β-chitin from squid pen (BioLog, Germany), and the fungal complex of chitin-glucan from *Absidia orchidis* NCAIM F00642 (as a residue after chitosan extraction from the fungal alkali insoluble fraction (Jaworska and Konieczna, 2001)) were used in the experiments.

The chitins and the chitin-glucan complex were washed several times with boiling water to neutral pH, dried (60 °C) and ground. For each sample the fraction of particles with diameter 25-50 μm was used.

2.2. Chitin deacetylase

The enzyme used in all experiments was separated from *Absidia orchidis* NCAIM F 00642 (this enzyme is not commercially available). The fungi were cultivated in a 7.0-L batch culture (26 °C, pH 5.5, YPG nutrient medium (Jaworska and Konieczna, 2001) and separated from the nutrient medium by centrifugation (6000 rpm, 20 min, 4 °C). Next, the biomass was frozen, then slowly thawed and homogenised, and the crude cell extract separated by centrifugation (6000 rpm, 20 min, 4 °C) and salted out with ammonium sulphate (80% saturation, overnight, 4 ° - 6 °C). The solution was dialysed

with HCl (pH 4.0) to remove ammonium sulphate (using a membrane module with a cut-off 10 kDa) and then concentrated by ultrafiltration. This enzyme solution, adjusted to pH 4.0 (optimal pH) with HCl, was used in all experiments (Jaworska 2012). The activity of the preparation was 61 U/mL (where 1U is the amount of the preparation solution that forms 1 μmol during 1 min of reaction with chitosan (with acetylation degree of 0.397) as a substrate, at 40 °C, pH 4.0.

2.3. Deacetylation of chitin/chitin-glucan complex

Experiments were carried out in a 1 L thermostated, mixed (150 rpm) reactor. The reactor was filled with 0.80 g of α- or β-chitin or chitin-glucan complex and 500 mL HCl (pH 4.0). The solution was thermostated to reach the temperature of 40°C and the reaction was started by adding 15 mL of the chitin deacetylase preparation (total activity of added preparation was 915 U). The concentration of acetic acid was measured before the deacetylation was started and at the end of the process (96 h). The reaction mixture was sampled (2 mL) and the reaction was stopped immediately by addition of 0.10 mL 1.0 M NaOH. Chitin was separated by centrifugation and the released acetic acid concentration in the clear supernatant solution was measured.

All experiments were performed in three repetitions with standard deviation of max. 12% for enzymatic deacetylation process and of max. 9% for experiments with methyl orange.

2.4. Analytical methods

The acetic acid concentration in the clear solution was analysed by the HPLC isocratic system (Varian ProStar 210) with HyperREZ XP Organic acid column (60°C) and HyperREZ XO Carbohydrate H^+ Guard Column, 0.0025M H_2SO_4 as eluent (0.5 mL/min), and a refractometer detector (Varian ProStar 350). The quantification limit was evaluated at 10 nmol/mL with a standard deviation of 8% of the mean value. The method was validated for acetic acid determination in chitosan-HCl (pH 4.0) solutions.

The concentration of $–NH_2$ groups at the surface of the chitin particles was measured by a modified spectrophotometric (VIS) method of Hartwig et al. (1994) using Methyl Orange (C.I. Acid Orange 52). In this method the dye forms bonds with $–NH_2$ groups at the surface of the chitin particles. The reaction is equimolar between dye and amine groups, so the concentration of amine groups can be calculated from the difference in dye concentration in the solution before and after the reaction. The reaction between dye and chitin particles was carried out at room temperature for 30 minutes to minimise penetration of the dye into the chitin particles, so that the change in concentration of the dye could be correlated with amine groups at the surface. The dye concentration was measured at $\lambda = 465$ nm and the concentration read from a previously prepared calibration curve.

Particle surface area was calculated on the basis of particle size distribution measured with Laser Diffraction Particle Size Analyzer, type LS 13 320 (Beckman-Coulter). The results were calculated as the mean of measurement of 5 samples read 3 times each.

3. RESULTS

The deacetylation process was followed by monitoring the changes in concentration of acetic acid, as it is one of the products of the reaction. It is formed in an equimolar reaction, so the concentration of GlcNAc units deacetylated can be easily correlated with the concentration of acetic acid liberated. As

the process of enzymatic deacetylation may be dependent on the conformation of the chitin chains and/or on particle crystallinity, chitins from different origins were used to investigate these effects.

3.1. Influence of conformation of chitin

Chitin chains can be organised either in α-chitin or β-chitin chains forming sheets of particles. The organisation of a single sheet influences the distances between sheets and so does the swelling process of a particle. Swelling will influence the possibility of the enzyme, chitin deacetylase, penetrate into the particle. Thus through comparing the deacetylation of these two types of chitin (α- or β- chitin) we can investigate the influence of chain conformation in chitin particles on the enzymatic process. Results showing the amount of liberated acetic acid are presented in Table 1 (the concentration at zero time was below the detection limit):

Table 1. Amount of acetic acid liberated after 96h enzymatic deacetylation

	α-chitin (shrimp)	β-chitin (squid)	α-chitin (fungal chitin-glucan complex)
Total amount	7.2 µmol	10.4 µmol	85.5 µmol
Specific concentration	8.897 nmol/ mg 0.154 nmol/ cm^2	12.843 nmol/ mg 0.208 nmol/ cm^2	106.4 nmol/ mg 3.360 nmol/ cm^2

Table 1 shows that similar amounts of acetic acid were liberated for both chitins samples, and although a slightly larger amount was observed in the case of β-chitin the difference is not significant. Similar differences in surface amine groups before and after deacetylation were detected (Table 2).

Table 2. Surface amine group concentration before and after enzymatic deacetylation

	α-chitin (shrimp)	β-chitin (squid)	α-chitin (fungal chitin-glucan complex)
Initial particles	4 nmol/mg 0.073 nmol/cm^2	2 nmol/mg 0.024 nmol/cm^2	None detected
Deacetylated particles (after 96 h)	4.5 nmol/mg 0.083 nmol/cm^2	4 nmol/mg 0.043 nmol/cm^2	5 nmol/mg 0.173 nmol/cm^2

The data in Table 2 confirms the previous observations of the amount of acetic acid liberated in the deacetylation process. The change in amine group concentration for α-chitin is nearly negligible while for β-chitin, although the increase is nearly double that for α-chitin, it is still very limited. An interesting fact is that the amounts of acetic acid liberated during the deacetylation process are higher than the measured changes in amine group concentration, by a factor of ~2 for α-chitin and of 3-5 for β-chitin. The quantitative difference between the measured increase in the concentration of amine groups and the amount of acetic acid liberated during enzymatic deacetylation may be explained by the fact that there are PO_4^{3-} anions in the solvent system for the Methyl Orange solution and these could compete with the dye anions for the protonated amine groups and reduce the percentage of amine groups detected.

Overall the data indicates that both structural forms of chitin are hardly deacetylated and the difference in liberated amine groups is marginal. This suggests that chain conformation, and its effect on the swelling of chitin, is a relatively unimportant factor in the enzymatic deacetylation of chitin.

3.2. Influence of crystallinity

Crystallinity is the second important parameter that could influence the deacetylation process. Because α- and β-chitin particles are characterised by similar levels of crystallinity (Rinaudo, 2006), it was necessary to find a source of chitin that has a different crystallinity. Fungal chitin fulfills this requirement as it can be separated from fungi cell walls as a chitin-glucan complex in which the chitin has the same anti-parallel α-conformation as shrimp chitin (Rinaudo, 2006) but a lower level of crystallinity (Jaworska et al., 2003). So comparing the enzymatic deacetylation of shrimp α-chitin and fungal α-chitin-glucan complex enables the influence of the crystallinity of the chitin particles on the deacetylation process to be investigated, Table 1. Such comparison is not a direct comparison as α-chitin-glucan complex contains usually only up to 20% of α-chitin (Reye et al., 1985), so the lower reaction rates and smaller amounts of liberated acetic acid could be expected, but it can show the tendency of the process.

The difference in liberated acetic acid can be easily observed. The total amount of AcOH liberated from fungal α-chitin is more than ten times higher that for shrimp chitin, Table 1. This difference is even more significant when we compare the specific concentration expressed per unit of particle surface or per unit of particle mass. The values for α-chitin-glucan complex could be even higher when recalculated per real content of chitin, but as the real content was unknown they were presented per total complex used in experiments. This difference is confirmed by determination of the number of amine groups liberated (Table 2). Comparison of the surface concentrations of amine groups shows a significant increase for fungal chitin that is nearly twice that for shrimp chitin. As in the study of the effect of chain conformation, the amount of liberated acetic acid suggests a much higher level of deacetylation than is indicated by measuring the change in amine group concentration at the surface, but there is no doubt that both measures clearly indicate that fungal α-chitin is more readily deacetylated than is shrimp α-chitin, confirming that substrate crystallinity is a major factor in controlling the enzymatic deacetylation of chitin.

4. DISCUSSION

Enzymatic deacetylation using chitin deacetylase, which is the only enzyme known that is able to hydrolyse the acetamido linkage in the N-acetylglucosamine units of chitin, has been proposed as an alternative method to the chemical deacetylation process currently used for the industrial production of chitosan. Although it has been investigated for several years there is still no successful solution. As chitin is insoluble in solvents suitable for chitin deacetylase the process has to be a heterogeneous one between the insoluble chitin particles and soluble enzyme. In such a situation the conformation of the chitin chains and the crystallinity of the chitin particles may play important roles. To investigate which factor is more important, chitins of different origins characterised by different chain conformations (α- and β-) and different crystallinities (shrimp α-chitin and a fungal α-chitin-glucan complex) were subjected to enzymatic deacetylation. Comparison of the acetic acid liberated and the increase in amine group concentrations showed that the chain conformation is relatively unimportant, but that the extent of crystallinity has a significant influence on the extent of deacetylation. This is in agreement with the observation of Win and Stevens (2001) who reported that heterophase enzymatic deacetylation of chitin particles resulted in only an insignificant reduction in the concentration of GlcNAc units in the polymer. This can be explained by comparing the size of chitin deacetylase and the inter-sheet spacing

in α-chitin particles. The size of chitin deacetylase appears to depend on its source and ranges from a molecular weight of 27 kDa for a sample isolated from *Aspergillus nidulans* (Alfonso et al., 1995), to a molecular weight of 110 kDa for a sample from *Rhizopus stolonifer* (El Ghaouth et al., 1992). As the distance between chitin chains is reported to be about 0.47 nm (Rinaudo, 2006), it is not possible for the enzyme to penetrate the particle's crystalline structure. In such a case it would be reasonable to assume that the lower the crystallinity of the chitin particles the greater would be the ease of enzymatic deacetylation. This assumption is confirmed by the data presented for shrimp and fungal chitin. Deacetylation of the polymer with lower crystallinity (the fungal α-chitin-glucan complex) gave a much higher amount for the liberated acetic acid and a higher amount for the amine groups produced, than did deacetylation of chitin with a higher crystallinity (shrimp α-chitin). This observation is in agreement with the investigation of Win and Stevens (2001) who decreased the acetylation degree of chitin particles by about 20% by reprecipitating the chitin prior to subjecting it to enzymatic deacetylation. So the current experiments and the literature reports confirm that the crystallinity of chitin particles is the main obstacle to enzymatic deacetylation of chitin and production chitosan with enzymatic method.

In such a situation two possible solutions suggest themselves: to reduce the particle size or to destroy the structure of chitin and change its crystallinity. The first solution, lowering the particle size to colloidal size, will not solve the problem as the process will still be carried out heterogeneously and the reaction will still only proceed at the surface of the particles. Having the particles of colloidal size will significantly increase the outer surface area per unit weight of particles so a larger number of GlcNAc will be accessible to the enzyme, but the enzyme will not be able to penetrate the particles. Also there will be limit to the extent of particle size reduction that will be beneficial from an economic point of view. The second solution is to find a solvent for chitin that is "enzyme friendly" and will not inactivate chitin deacetylase. In this case the reaction would be carried out homogenously so all GlcNAc units of the chitin chains would be accessible to the enzyme and this would allow chitin to be converted into chitosan using enzymes. Currently none of the solvents for chitin reported in the literature fulfill the "enzyme friendly" criterion, but recent investigations on ionic liquids as solvents for chitin seem to be extremely interesting. It has been shown (Jaworska et al., 2012) that some imidasolium ionic liquids are able to dissolve chitin and thereby destroy its crystallinity (Xie et al., 2006), but its effect on chitin deacetylase has not been examined. As the subject is quite new it needs further investigation, but it is possible that ionic liquids may open a new chapter in the chemistry and enzymology of chitin.

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