Chitosan Hydrolysis by Non-Specific Enzymes

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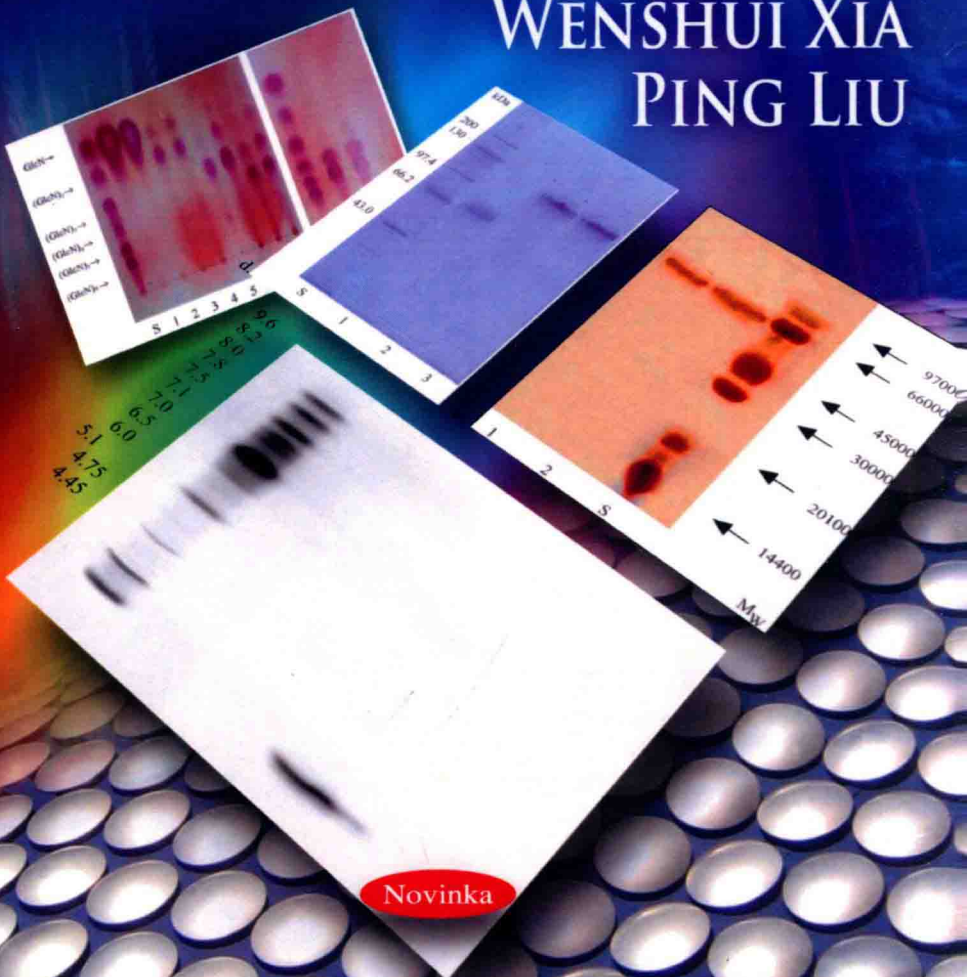

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ABSTRACT

Chitosan, the only alkaline polysaccharide of β-1,4 linked N-acetyl-glucosamine and glucosamine,could be hydrolyzed by many non-specific enzymes such as cellulase, protease, and lipase, especially cellulase, which show high activity on chitosan. The hydrolytic mechanisms of these non-specific enzymes have been received growing attentions.

The focus of this chapter was the characterizations and hydrolyzing mechanism of the non-specific enzymes toward chitosan choosing the three typical non- specific enzymes: cellulase, lipase and papain as objects. We have studied the enzymatic characteristics, purification, product analysis, glycoside bond cleavage, active sites and gene cloning of these enzymes to expatiate their non-specific hydrolysis mechanism. From these, we obtained two bifunctional enzymes with chitosanolytic activity from commercial cellulase and lipase, respectively, and one chitosanase from papain.The three purified enzymes were the main reasons for the non-specific chitosanolytic hydrolysis of cellulase,lipase and papain, respectively. Moreover, It is identified that the bifunctional enzyme with chitosanolytic and cellulolytic activity(CCBE) from cellulase (*T.viride*) is identified as a cellobiohydrolase I with exo-β-D-glucosaminidase activity belonging to glycosyl hydrolysase 7 family.The enzmye with chitinase and chitosanase activity (CNBE)from lipase (*A. oryzae*) is the exo-β-D -glucosaminidase with N-acetyl-chitobiosidase activity belonging to glycosyl hydrolysase 18 family. Both of the two enzymes are novel and first reported in chitosanase families. Besides, the active sites and gene expression analysis of CCBE indicated that their dual activities originated from two distinct catalytic domains; while the two active sites overlapped partially.

Keywords: chitosan, cellulase, lipase, papain, chitosanase, non-specific enzyme, purification, characterization, gene cloning, expression

CONTENTS

Chapter 1

INTRODUCTION

Chitosan, a linear polysaccharide composed of 2-acetamido-2-deoxy-β-D-glucose (N-acetylglucosamine, GlcNAc) and 2-amino-2-deoxy-β-D-glucose (D-glucosamine, GlcN) with various contents of these two monosaccharides, is a partial deacetylated derivative of chitin,which is the second most abundant polysaccharide on earth (after cellulose) and largely available in the exoskeletons of invertebrates and the cells of fungi [1]. Chitosan could be hydrolyzed enzymatically and chemically for the preparation of low molecular weight chitosans (LMWC) and chitooligosaccharides (COS). Enzymatic preparation methods captured a great interest due to safe and non-toxic concerns. Recently, chitosanolytic enzymes are increasingly gaining importance as LMWC and COS exhibit many outstanding biological properties, such as antimicrobial effect[2-8,18], antitumor and anticancer effect[2,7,9-11,18], antioxidant effect[2,7,13,14], immunostimulant effects[2,7,15-18], Antiatherosclerotic effects[18-20], Angiotensin I converting enzyme (ACE) inhibitory activity [21,22] and so on, which show innumerable applications in biomedical, pharmaceutical, agricultural, biotechnological and food fields[2,7,18].

In general, for preparation of COS and LMWC, chitosan could be degraded enzymatically by specific chitosanases and non-specific enzymes. The specific enzyme for chitosan hydrolysis has been found in a wide range of organisms, including bacteria [23, 24], fungi [25, 26] and plants[27]. Most of these chitosanases were characterized as endo-type and they split β-1,4 glycosidic linkages in chitosan in a random way to form chitosan oligosaccharides. But the utility of these specific chitosanases in hydrolysis is limited because of its cost and unavailability in bulk quantities. With regard to the non-specific hydrolysis on chitosan, in 1992, Pantaleone etc first found chitosan could be hydrolyzed by many kinds of enzymes such as cellulases,proteinases, pectinases, and lipases,

which first raised the derivation of non-specificity[53].Since then, a number of non-specific commercial enzymes have been reported for their ability to degrade chitosan at a level comparable to that achieved by specific chitosanases, and have been used for preparation of chitooligosaccharides or low molecular weight chitosan from chitosan hydrolysis, such as cellulase[5,8,9,10,28-31], pectinase [32-36], pepsin[37], papain[38-42], amylase[43] and lipase[44-48], especially cellulases. The reactions are of interest as these commercial enzymes, especially food grade enzymes, have been used in food industries for decades and are safe and relatively inexpensive. Another advantage of non-specific chitosanolysis is the production of low molecular weight chitosan in higher yields due to their low specificity or nonspecificity [49].

However, few researches have been reported on the mechanism of these non-specific enzymatic processes, and even then controversial views exist. On the one hand, Kittur et al.[33] first reported that a multiple functional pectinase isoform from *Aspergillus niger* could be responsible for chitosan degradation by pectinases,but on the other hand, some authors have purified hetero chitosanases or/and chitinases from several commercial proteases,which were charged with their chitosanolytic activity [51-53].Whereas,no reports have been focused on non-specific hydrolysis of cellulases and lipases before, although their utility were popular in chitosan hydrolysis [5,8,9,10,28-31,43] and several bifunctional cellulase-chitosanases have been reported to be secreted from bacteria [58,79,87].

To make clear the non-specific mechanism for chitosan hydrolysis, taking the three typical non-specific enzymes: cellulase, lipase and papain as objects, we have been working on this research in detail and systematically for several years and have got a great progress. This chapter aims to the characterization and non-specific hydrolysis mechanism of these enzymes toward chitosan based on our research, which may provide a contribution for the development of chitin science.

Chapter 2

CHARACTERIZATION OF CHITOSAN HYDROLYSIS BY NONSPECIFIC ENZYMES

2.1. CHARACTERIZATION OFCHITOSAN HYDROLYSIS BY NON-SPECIFIC CELLULASES

Cellulase constitutes a complex enzymatic system responsible for the degradation of cellulose substances into glucose. It is obtained from bacteria and fungus, mainly from fungus such as *Trichoderma*, *Aspergillus*, *Penicillium*, and *Acremonium*. Especially, many commercial celulases are produced by *Trichoderma viride* and *T. reesei*. Cellulase is one of the two enzymes firstly applied in non-specific hydrolysis of chitosan [53, 54]. There have been many reports on the characteristics of chitosan hydrolysis by cellulases. Through investigating the optimum temperature and pH, pI, hydrolyzing kinetics, change of viscosity and distribution of products of cellulase on chitosan, we found that cellulases from different sources all show chitosanase activity, although their properties differ. In our previous research, the effects of temperature and pH, hydrolyzing kinetics, DD and metal ions on chitosan hydrolysis by a commercial cellulase from *T.viride* were studied in detail, the results showed that the optimal conditions of chitosan hydrolysis were at 60℃ and pH 5.2.The enzyme activity was increased by the addition of Na^{+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, while it was strongly inhibited by the addition of Ag^{2+},Cu^{2+}, Hg^{2+} and glucose. It still retained 60.5% of its original activity at 60℃ for 1h.its best substrate was colloid chitosan with 90%DD. Meanwhile, product analysis indicated that this commercial cellulase hydrolyzes chitosan in both endo-split and exo-split manners, which could cleave the GlcN-GlcN bond as well as GlcNAc-GlcN bond [29].

Based on our study and compared to the reference, the characterizations of chitosan hydrolysis by non-specific cellulases reported were summarized as follows:

(1) Temperature and pH

The effects of temperature on an enzyme-catalyzed action are mainly embodied in two respects: one is the increase of the reaction rate because a higher temperature accelerates molecular collisions between the enzyme and the substrate; the other is the inactivation of the enzyme for a higher temperature denatures the enzyme. The optimum temperature of cellulase acting on chitosan was in the ranges of 30-70 ℃. The optimal temperature of cellulase secreted by fungus on chitosan was between 50-60 ℃, higher than that from bacteria, which was often lower than 40 ℃.

The pH affects not only the dissociation behavior of the substrate but also the space structure and dissociation state of the active group in the enzyme, therefore, pH is among the most significant factors affecting the enzyme-catalyzed reaction. The variations in optimum pH of its chitosanolytic activity between the sources were not obvious. Most of the optima were focused in the range 5.0-7.0, few in particular: the optimum pH for a commercial cellulasewere 4.0 [31], while *Streptomyces griseus* cellulase acting on chitosan had optimum pH at 8.0 [55].

(2) Molecular Weight

Most of the cellulases that show chitosanolytic activity have the apparent molecular mass in the range of 23-55kDa, which is consistent with that of specific chitosanases. But there are also few exceptions such as: the molecular weights of those from *T. viride* [29, 56] and *T. reesei* PC-3-7[57] are 66kDa and 97kDa, respectively.

(3) Reduction of the Viscosity

Almost all the cellulases from different sources can decrease the viscosity of chitosan extensively in a short time; more than 60% of viscosity was reduced in the early stage (30 min or 1h) of the reaction, which suggestes that the cellulases

possess the chitosanolytic activities of the endo-type manner. Pantaleone et al found three cellulases derived from *A. niger*, *T. viride*, and *T.reesei* enable to result in a 99% reduction of the viscosity [53, 54]. Several researchers at home also found the similar characteristics [8-10, 28-31].

The distribution of the reaction products confirms that most of cellulases from various sources show an endo-acting nature, they predominantly liberate a dp2-10 mixture, made up of dimers, trimers and oligomers from chitosan, though there are few in exo-type, for instance, the bifunctional enzyme purified from a commercial cellulase [29,56] and from *T.reesei*[57]showed an exo-β-D-glucosaminidase activity on chitosan.

(4) Kinetic Parameters

The Km and Vmax values reported for cellulases on chitosan differ from organism to organism and from those on cellulase [56] which means such chitosanolytic cellulases have different affinity with chitosan and cellulose ----two substrates with high similarity in structures: for Km value is a index reflecting the affinity of enzyme and substrate. From this, we assume that the cellulases with dual activities probably have two substrate binding domains or different binding sites were involved in a single binding domain for chitosan and cellulose, which needs further research.

(5) Metal Ions

Generally speaking, metal ions are not involved in the catalytic activities of chitosanases and cellulases[43,55,56,58,59], but they activate or inhibit their activity. The activities of these chitosanases and cellulases could be inhibited by heavy metal ions Ag^{+}, Hg^{2+}, Cu^{2+}, which form coordinate bonds with the side chain group of the enzyme, resulting in the change of protein conformation hence inactivating the enzyme. While Mg^{2+}, Mn^{2+} etc could enhance the molecular conformational stability, accelerating the catalysis[60], hence, enzymes incubated with Mg^{2+} etc generally show higher catalytic activity compared to the native (measuring under the same condition). The chitosanase activity of cellulases were inhibited strongly by Ag^{+}, Hg^{2+} etc heavy metals, and stimulated by Mg^{2+}, Mn^{2+}, which is similar to its cellulolytic activity[29,56,58].These phenomenon indicates that the active sites for the two activities of cellulase have some similarities.

2.2. CHARACTERIZATION OF CHITOSAN HYDROLYSIS BY NON-SPECIFIC LIPASE

Lipases (EC3.1.1.3) are glycerol ester hydrolases, they can catalyze th hydrolysis of triacyglycerols into free fatty acids, partial acylglycerols ar glycerol [63].Lipase was found to exert chitosanolytic activity. Althoug Pantaleone et al first found that the lipase from two *A. niger* and many glucanas showed highest non-specific chitosanolytic activity,much more than that protease[53], there are still few reports on chitosanolysis of lipases up to now [4 61, 62]. Muzzarelli et al firstly took a detail research about the chitosan hydrolys by lipase. They found that the wheat germ lipase preparation was very active depolymerizing chitosan and modified chitosans dissolved in slightly acid aqueous solutions. This enzyme preparation was effective in drastically loweri the viscosity of chitosan, MP-chitosan, and NCM-chitosan solutions within a fe minutes. Typically, the viscosity was lowered to 35% of the initial value upon 1 min contact with lipase at 25°C. Measurements taken in the lipase concentrati range 4.5 mg/L -0.9 g/L showed a logarithmic dependence of the initial veloci on the enzyme concentration. N-Carboxymethyl chitosan was an even bett substrate, with the initial velocity over double that for chitosan [44]. Since then, 2001,Shin et al studied the degradation of chitosan with the aid of lipase fro *Rhizopus japonicus* for the production of soluble chitosan,finding that the lipa could degrade chitosan to water-soluble chitosan with Mw between 30 kDa ~ kDa at its optimal temperature of 40℃[62]. Many researchers at home also fou that lipase could degrade chitosan effectively, the reaction did not follow t classic Michaelis-menten equation,and the optimum temperature were in a ran of 40℃~55℃,while the optimal pH differ significantly, such as pH 5[64],pH [46] and pH5.0~5.5[47].However, there has been no report on qualitative analys of chitosan oligomers resulted from the chitosan hydrolysis aided by lipase.

In our recent study, we investigated the effects of a commercial lipase fro *A.oryzae* on chitosan hydrolysis with different conditions such pH,temperature,degree of deacetylation (DD) and molecular weights, me ions,viscosity reduction, and qualitatively analyzed COS products using kine analysis,thin layer chromatography (TLC) and HPLC methods[48].

The optimal temperature and pH of the lipase was 55℃for all chitosa Considering that DD and distribution of N-acetyl groups significantly affected t properties of chitosan solution [65], in our research, it was found that when fo chitosans with various degrees of deacetylation were used as substrates, the lipa showed higher optimal pH toward chitosan with higher DD,among pH4.5-4.8.T enzyme exhibited highest activity to chitosans which were 82.8% and 73.2

deacetylated, indicating that the presence of GlcNAc residues in the chitosan molecules is important for the enzyme to exhibit chitosanolytic activity and the enzyme recognized not only the GlcN residues, but also the GlcNAc residues in the substrate. In addition, it can be seen that when chitosans with the same DD were used as the substrates, chitosans with lower molecular mass were more susceptible to be hydrolyzed by the enzyme than those withhigher DP (degree of polymerization).Kinetics experiments show that chitosans with DD of 82.8% and 73.2% which resulted in lower Km values(3.588mg/mL and 3.754mg/mL, respectively) had stronger affinity for the lipase and the lipase action on all chitosans obeyed the Michaelis-Menten kinetics (data not shown).

Time course analysis of chitosan hydrolysis with the aid of lipase exhibited that there was a sharp decrease in viscosity of the mixture during the early hydrolysis stage at both 37℃and 55℃ temperatures, although there was a very weakly marked difference in viscosity;besides,the chitosan hydrolysis carried out at 37℃produced larger quantity of COS (chitooligosaccharides) than that at 55℃ when the reaction time was longer than 6 h, and COS yield of 24 h hydrolysis at 37 ℃was 93.8%. Hydrolysis of chitosans with different DD produced the same series of products,viz., glucosamine and chitosan oligomers with DP from 2 to 6 and above. This means that chitosans with different degrees of deacetylation did not result in different products.and the lipase acted on chitosan in both exo- and endo-hydrolytic manner. According the dynamics of COS and chitosan hydrolysis, it can be seen as follows: at the early stage of hydrolysis, the lipase cleaved glycosidic bonds in both endo- and exo-mode, and GlcN was first produced, and COS with higher DP were then produced. During this time, viscosity of the mixture decreased dramatically. Then the lipase hydrolyzed the oligomers mainly in an exo-mode, therefore, the oligomers gradually disappeared, the amount of GlcN increased, and the viscosity of the action mixture almost remained the same. Moreover, the FT-IR analysis of the chitosan hydrolysates suggested that the lipase had loose substrate specificity, which didn't change the side-chain conformation and degree of deacetylation of the products during chitosan hydrolysis,which is not similar with that from papain[49].This result is useful in the direction of production of COS. If the objective is to obtain oligomers with higher DP, the reaction time should be strictly controlled in order to avoid the production of large quantity of monomers; on the other hand, this lipase can be used to prepare the chitosan monomer, glucosamine, which is widely used to relieve arthritic complaints [66].

2.3. Characterization of Chitosan Hydrolysis by Non-Specific Papain

Many proteases such as papain [38-42,53], pepsin[37], trypsin[67], bromelain[53,68], ficin[50,53] etc ,all can degrade chitosan,among these,papain was found to depolymerize chitosan efficiently [53]. It's been reported that papain degraded chitosan under a optimal conditions of pH 3.0-4.0 and temperature of 40-50 °C ,although different papains were used in different research, and the high molecular weight of chitosan with a Mw of $4\text{-}5\times10^5$ were degraded by papain to produce the LMWC and COS in a range of 10^3-10^4.

Muzzarelli et al [69] studied chitosan deploymerization by papain in the form of its lactate salt at acidic pH values by papain. The results showed that this enzymatic process did not obey a simple kinetic model; the initial velocity was, however, strongly enhanced by high substrate concentration, while the temperature had little influence. A viscosity decrease as high as 94% could be obtained in 1 h with free papain at pH 3.2 and 50°C. Initial velocity for a $19g.L^{-1}$ solution at 25°C and pH 3.2 was 110 mPa .s . min^{-1}. Chitosans with average molecular weights in the range $4\text{-}7 \times 10^5$ could be easily deploymerized to highly polydisperse chitosans. Modified chitosans were also depolymerized, though with lower initial velocities. Once immobilized on chitin, papain could be used repeatedly to deploymerize chitosan lactate salt with no observable loss of activity. The data are of value for the production of chitooligomers of medical and biotechnological interest.While Yalpani[54] found that at lower concentration(0. 5% -1%) , the concentration of substrate did not affect the chitosan hydrolysis by papain and papain could be repeatedly used at least 10 times when immobilized on chitin or chitosan with no evident lost of its chitosanolytic activity.Under the optimum conditions: NaAc–HAc buffer system, pH 4.0, temperature 45 °C, immobilized papain was used to prepare chitosan oligomers, After 24 h reaction with stirring, the yield of chitosan oligomers with molecular weight under 10,000 was 49.55% to total substrate, while that of between 600 and 2000 was 11.07%. The results showed immobilized papain could depolymerize chitosan successfully, and separating the product by ultrafiltration membrane was feasible. An amount of 1mg free papain and immobilized papain could produce 2.57 and 17.34 mg chitosan oligomers, respectively. The results indicated that the depolymerization efficiency of immobilized papain was obviously higher than that of free papain[70].

Terbojevich et al[42]studied the molecular parameters of chitosan depolymerize with the aid of papain,finding that the chitosan with the highest degree of polymerization were preferred and its Mw,viscosity and RG values were all lowered. The papain mainly cleaved the GlcN-GlcNAc bond in chitosan. However, Su et al reported that papain could split the GlcNAc-GlcN as well as

GlcN-GlcN bond and produced the mixture of LMWC and COS with DP of 2-6[38]. Kumar found the similar results using papain from Papaya latex and protease from *Streptomyces griseus* under each optimal conditions [49],Low molecular weight chitosans (LMWC) of different molecular weight (4.1–5.6kDa), which were obtained by the depolymerization of chitosan using papain ,could be tested by Scanning electron microscopy (SEM) ,circular dichroism (CD), FT-IR and solid-state CP-MAS 13C-NMR data,while LMWC with different Mw could be isolated by ultrafiltration and Sephadex G-50 gel filtration.

Chapter 3

MECHANISM OF NON-SPECIFIC ENZYMES TOWARD CHITOSAN

Considering there are many reports on the characterization of chitosan degradation by these non-specific enzymes,while their mechanisms are still controversial,we studied the mechanism of these non-specific chitosan hydrolysis taking the typical cellulase,lipase and papain as objects.Using two purification systems: one is column chromatography combining with ultrafiltration, ion exchange, hydrophobic interaction chromatography and gel filtration and the other is gel electrophoresis,we have found that the non-specific hydrolysis of these enzymes toward chitosan were due to the existence of the bifunctional enzymes with chitosanolytic activity in cellulase and lipase[56,71] or hetero chitosanases in papain[72] (seen in Table1). Bifunctional enzymes with chitosanolytic activity was focused much attention since its novelty and contravention to the traditional enzymological theory----one enzyme shared one or one kind of catalytic activity.

With regard to the conception of multiple-functions, it has been presented for about two decade's years. Multiple substrate specificities of a number of glycanases and glycosidases have been known in literature. β-1,4-Glucanases with hydrolyzing activity on mannan has been reported from *T.reesei*[73]. β-Glycosidases (designated as BgIA and BgIB) from *Bacillus* sp. GLI have been shown to cleave α- and β-linkages in p-nitrophenyl (pNP)-glycosides and positional isomers of β-1,4-glycopyranosyl linkages [74]. The product of BgIB gene was also identified as a gellan degrading enzyme. Pectinase with chitosanolytic activity have been reported from *A.niger* [33]. Some bifunctional enzymes with chitosanolytic and cellulolytic activity have been also reported from the Prokaryotes [58, 75-83].