ELSEVIER

High yield production of monomer-free chitosan oligosaccharides by pepsin catalyzed hydrolysis of a high deacetylation degree chitosan

Tomás Roncal,[a,*] Alberto Oviedo,[b,†] Iratxe López de Armentia,[b]
Laura Fernández[b] and M. Carmen Villarán[b]

[a]Unit of Sustainable Chemistry, Fundación LEIA C. D. T., Parque Tecnológico de Álava, Leonardo da Vinci 11,
01510 Miñano (Álava), Spain
[b]Unit of Food Science, Fundación LEIA C. D. T., Parque Tecnológico de Álava, Leonardo da Vinci 11, 01510 Miñano (Álava), Spain

Abstract—The high molecular weight of chitosan, which results in a poor solubility at neutral pH values and high viscosity aqueous solutions, limits its potential uses in the fields of food, health and agriculture. However, most of these limitations are overcome by chitosan oligosaccharides obtained by enzymatic hydrolysis of the polymer. Several commercial enzymes with different original specificities were assayed for their ability to hydrolyze a 93% deacetylation degree chitosan and compared with a chitosanase. According to the patterns of viscosity decrease and reducing end formation, three enzymes—cellulase, pepsin and lipase A—were found to be particularly suitable for hydrolyzing chitosan at a level comparable to that achieved by chitosanase. Unlike the appreciable levels of both 2-amino-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-glucose monomers released from chitosan by the other enzymes after a 20 h-hydrolysis (4.6–9.1% of the total product weight), no monomer could be detected following pepsin cleavage. As a result, pepsin produced a higher yield of chitosan oligosaccharides than the other enzymes: 52% versus as much as 46%, respectively. Low molecular weight chitosans accounted for the remaining 48% of hydrolysis products. The calculated average polymerization degree of the products released by pepsin was around 16 units after 20 h of hydrolysis. This product pattern and yield are proposed to be related to the bond cleavage specificity of pepsin and the high deacetylation degree of chitosan used as substrate. The optimal reaction conditions for hydrolysis of chitosan by pepsin were 40 °C and pH 4.5, and an enzyme/substrate ratio of 1:100 (w/w) for reactions longer than 1 h.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: Chitosan hydrolysis; Chitosan oligosaccharides; D-Glucosamine; N-Acetyl-D-glucosamine; Pepsin; Deacetylation degree

1. Introduction

The field of bioactive oligosaccharides, compounds having interesting functional properties and many uses, and their production from different raw materials is gaining considerable attention in recent years.[1] So far, the main interest has been focused on a few oligosaccharides, such as galactooligosaccharides, lactulose and fructooligosaccharides, and considerably less attention has been paid to the rest, including chitosan oligosaccharides.

Chitosan is a polysaccharide with linear structure constituted by a copolymer of β-(1→4)-linked 2-amino-2-deoxy-D-glucose (GlcN) and 2-acetamido-2-deoxy-D-glucose (GlcNAc) residues. It is obtained chiefly by homogeneous deacetylation of chitin with strong bases, rendering chitosans of different acetyl content or deacetylation degrees. Chitosan is a non-toxic, biodegradable biopolymer of high molecular weight with a fiber-like structure similar to that of cellulose. As a result of these properties, chitosan solutions show high viscosity. Contrary to chitin and owing to its acetyl groups removal, chitosan is readily dissolved in dilute acidic aqueous solutions below pH 6.0. Obviously, chitosan solubility depends on its deacetylation degree: the higher the

* Corresponding author. Fax: +34 945 298217; e-mail: tomasr@leia.es
† Present address: Hematopoietic Division, CIEMAT, Avda. Complutense 22, Ed.07-P.01-D.12, 28040 Madrid, Spain.

deacetylation degree, the higher the solubility. Above pH 7.0, chitosan solubility is low and precipitation or gelation tends to occur.

Although chitosan shows a number of interesting functional properties in different areas, the above mentioned features regarding its high molecular weight and viscosity of aqueous solutions, and the special conditions (acidic media) required to achieve solubilization in water, limit the best part of its potential uses.[2] In this respect, chitosan oligosaccharides, because of their shorter chain length, display a reduced viscosity and are soluble in aqueous media at pH values close to neutrality, which increases their bioavailability and opens a wide range of new potential applications.

Chitosan oligosaccharides are bioactive compounds with many uses in the fields of food, health and agriculture. They have been claimed to have a great number of effects and activities, including among others: prebiotic, antimicrobial, antitumoural, tissue recovery stimulation, antidiabetic, immunostimulant, antiinflammatory, calcium absorption acceleration, antimutagenic, antioxidant and activator of plant resistance towards insect and pathogen attack.[2,3]

Chitosan oligosaccharides can be obtained by chemical or enzymatic hydrolysis of the chitosan chains. Chemical hydrolysis is carried out by two alternative methods: acid hydrolysis with concentrated acids[4] or oxidative degradation with hydrogen peroxide.[5] Both methods have been applied successfully to chitosan degradation, which occurs almost quantitatively, but show some drawbacks,[2] including the difficulty to obtain low polymerization degree oligosaccharides because high polydispersity mixtures predominate, and to control the extent of hydrolysis, which frequently results in hydrolysates containing a high ratio of monosaccharides. In addition, the harsh reaction conditions required, such as elevated temperatures and high reagent concentrations, may cause environmental problems and often result in the formation of chemically modified oligosaccharides.

Alternatively to the aggressive chemical hydrolysis, chitosan may also be hydrolyzed in a milder way using enzymes. Enzyme catalyzed chitosan hydrolysis is more specific and allows a greater control of the extent of reaction and, therefore, of the product size. The specific enzymes intended to catalyze chitosan hydrolysis would be chitosanases.[6–8] These enzymes, however, show a reduced commercial availability and, as a consequence, are rather expensive, which limit their industrial use.

In the search for alternative enzymes to chitosanases, showing original specificities different from chitosan hydrolysis, but able to catalyze the hydrolysis of this polysaccharide and, most important, commercially available in great quantities and at reduced cost, several enzymes have been found to fulfil these requirements, including cellulases, hemicellulases, pectinases,

β-(1→3)(1→4)-glucanases, proteases, lysozyme and lipases.[9–13] These findings have opened the possibility of developing novel efficient and economically feasible industrial processes for hydrolyzing chitosan.

The use of chitosanases and some of the above enzymes in the production of chitosan oligosaccharides has been assessed at laboratory scale, using enzyme reactors both in batch and in continuous configurations.[8,14–17] In most cases, the resulting products show high molecular weight (>10 kDa) and, when low polymerization degree oligosaccharides have been obtained, their yield has been poor and with predominance of the smallest sized species (2–4 residues) and monomers.

In this paper, an efficient procedure for the production of low- and medium-size chitosan oligosaccharides, in high yields and almost free of monomers, starting from a high deacetylation degree chitosan as substrate and using pepsin, a low cost commercial enzyme, as catalyst is described. The products resulting from chitosan hydrolysis are expected to be suitable for most of the reported chitosan applications, where the large molecular weight of the polymer limits its use.

2. Results and discussion

2.1. Hydrolysis of chitosan by commercial enzymes

In an attempt to develop an efficient process for the production of chitosan oligosaccharides on a large scale, that is, with a good yield of low- and medium-chain length species and reduced levels of monomers, we studied the hydrolysis of chitosan catalyzed by several commercial enzymes previously reported to do it. The enzymes assayed included cellulase, hemicellulase, papain, bromelain, pepsin, protease type XIV from *Streptomyces griseus*, lysozyme and lipase A, with chitosanase as control. Unlike most of the previous studies of this kind, which used chitosan with deacetylation degrees ranging from 70% to 85% and resulted in high levels of dimers and monomers, our rationale was to use a very high deacetylation degree (93%) chitosan in order to avoid a too extensive hydrolysis of the polymer.

Except hemicellulase, all the enzymes assayed showed an appreciable activity under the standard reaction conditions, as determined by viscosimetry and reducing end assay. Results are shown in Table 1.

According to the viscosimetry assays, all enzymes, apart from lysozyme and papain, showed a chitosan hydrolyzing activity comparable, and even greater, to that of chitosanase, reaching a viscosity decrease higher than 80–85% in 20 h. The effects of enzyme action could be observed early, occurring the greatest viscosity decreases in the first hour of hydrolysis.

Reducing end formation was slower than viscosity decrease and, in general, paralleled to it, although both

Table 1. Chitosan hydrolysis catalyzed by different commercial enzymes[a]

Enzyme	Viscosity decrease (%)		Reducing ends (mM)	
	$t = 1$ h	$t = 20$ h	$t = 1$ h	$t = 20$ h
Bromelain	61	86	—	0.51
Lysozyme	28	41	—	0.20
Cellulase	69	85	0.34	3.11
Pepsin	80	89	0.43	3.32
Lipase A[b]	82	97	0.26	4.69
Papain	42	70	—	0.28
Protease type XIV	37	82	—	0.42
Chitosanase	65	96	0.41	5.46

[a] Enzyme activity was measured by the resulting decrease in viscosity and by reducing end formation, according to the standard method described in the text.

[b] Lipase A assays were carried out at pH 3.0, instead of standard pH (4.5).

measurements were not directly related in all the cases. The most notorious example of this was bromelain that, causing a viscosity decrease comparable to that of the most active enzymes, showed a significantly lower reducing end formation. After the first hour of hydrolysis, reducing end formation could only be detected for four of the assayed enzymes: cellulase, pepsin, lipase A and the control chitosanase. Again, these enzymes showed activities similar to that of chitosanase.

As a result of the fast viscosity decrease occurring at the beginning of hydrolysis, the change of this parameter with time was monitored in the first hour of reaction (Fig. 1). Most active enzymes were bromelain, cellulase, pepsin, lipase A and chitosanase. By far, pepsin showed the initial higher rates, reaching a viscosity decrease greater than 60% in only 10 min. All the enzymes showed similar reaction kinetics, with the main viscosity decrease taking place in the first 10–20 min.
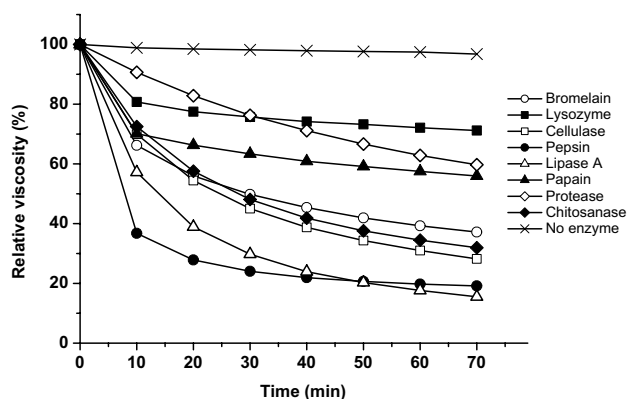

Figure 1. Time course of the viscosity decrease of a 1% (w/v) chitosan solution resulting from the chitosanase activity of different commercial enzymes. All the enzymes were assayed according to the standard procedure described in the text, except lipase A, which was assayed at pH 3.0. Chitosan solution viscosity is expressed relative to the starting value. A control reporting the viscosity variations in the absence of enzymes has also been included.
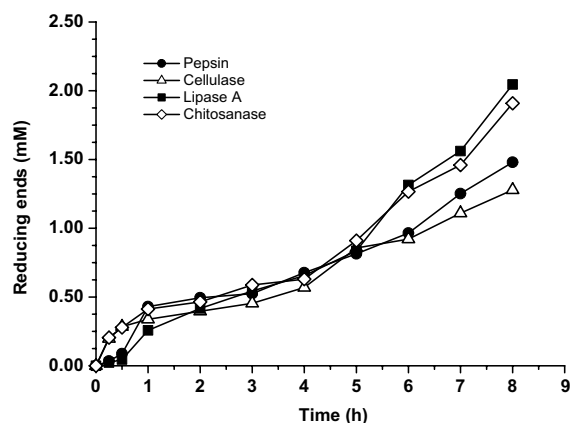

Figure 2. Time course of the reducing end formation from a 1% (w/v) chitosan solution treated with different commercial enzymes. All the enzymes were assayed according to the standard procedure described in the text, except lipase A, which was assayed at pH 3.0. Reducing ends are expressed as D-glucosamine equivalents.

These kinetics results from the predominant *endo*-type action of the enzymes: the hydrolysis of the initially large chitosan chains cause greater viscosity decreases than the subsequent degradation of their shorter hydrolysis products.

The four enzymes that showed the highest chitosan hydrolyzing activities (cellulase, pepsin, lipase A and chitosanase) were also studied for the formation of reducing ends throughout time (Fig. 2). In the first hour a relatively fast generation of reducing ends occurred, decreasing their formation rates in the following 3 h. Afterwards, hydrolysis rates increased again and were maintained rather constant up to 8 h. This behaviour was common for all the enzymes, with lipase A and chitosanase showing a higher reducing end releasing activity.

The initial fast generation of reducing end during the first hour of enzymatic hydrolysis of chitosan could be explained taking into account the four different types of glycosidic linkages found in chitosan and the cleavage specificities of the enzymes (see Section 2.2). An enzyme able to cleave two or more types of these linkages would likely show different affinities for each of them, so resulting in different cleavage rates. As a result, the initial fast generation of reducing ends would represent the preferential cleavage of bonds towards which enzymes show a higher affinity.

Therefore, three of the commercial enzymes assayed—cellulase, pepsin and lipase A—, when compared to chitosanase, were particularly suitable for hydrolyzing chitosan, as judged by their ability to reduce the viscosity of chitosan solutions and to release reducing ends from the polymer.

2.2. Analysis of hydrolysis products

The hydrolysis products resulting from the enzyme action upon chitosan were first divided into an insoluble

fraction and a soluble one following neutralization of the reaction mixture, as described elsewhere.[13] The insoluble fraction, containing low molecular weight chitosan, was separated and weighted after lyophylization. The soluble fraction, composed of a mixture of oligosaccharides and monomers, was separated by HPLC and the percent yield of monomers (GlcN and GlcNAc) and chitosan oligosaccharides was calculated based on their corresponding peak area. The calculated percent yield of each of these fractions is showed in Table 2.

Typical chromatograms of the soluble fraction showed, in addition to monomer peaks where present, three major peaks that could not be assigned to specific oligosaccharide species because they were composed of several unresolved peaks. However, a fraction obtained after passing the whole hydrolysate through an ultrafiltration membrane having a molecular weight cut-off of 5 kDa, corresponding to a polymerization degree of around 27–28 units, showed an HPLC profile similar to that of the soluble fraction, with the same three main peaks but the third extending to higher elution times (higher polymerization degrees). This would mean that the soluble fraction would probably contain a mixture of chitosan oligosaccharides with polymerization degrees of up to around 20 units.

The product distribution after a 20 h-hydrolysis showed some interesting features. Probably the most important was that both GlcN and GlcNAc monomers were not detected in pepsin-derived hydrolysates, in contrast with those of the other enzymes, which released appreciable levels of these species, ranging from 4.6% to 9.1% of the total product weight. This particular property of pepsin allowed it to produce a significantly higher quantity of chitosan oligosaccharides than the other enzymes: nearly 52% of the total products from pepsin hydrolysis were oligosaccharides, whereas the quantities released by rest of the enzymes did not exceed 46%. Finally, the yield of low molecular weight chitosans generated by the four enzymes ranged from 45% to 50%.

In order to add new elements to the above hydrolysis product analysis, through a careful examination of the results shown in Table 1, it was possible to make an approximate calculation of the average polymerization degree of the hydrolysis products taking into account three factors: the total weight of chitosan in the reaction mixture, the concentration of the reducing ends released, and the average molecular weight of the monomers (considering a molar ratio of 93% GlcN and 7% GlcNAc). According to this approach, after 20 h of hydrolysis, the average polymerization degree of chitosan degradation products would be 16.6, 17.6, 11.8 and 10.0, for pepsin, cellulase, lipase A and chitosanase, respectively.

All these results as a whole point out that the most suitable enzyme for hydrolyzing a high deacetylation degree chitosan in order to obtain oligosaccharides is pepsin, because it causes one of the greatest (and the fastest) viscosity decreases and reducing end releases, and produces the highest quantities of short- and medium-chain oligosaccharides, without hardly monomer generation. In addition, pepsin is the cheapest among the enzymes used in this study, which is very important for development of an economically feasible industrial process.

Unlike the results presented in this paper, in previous studies dealing with pepsin catalyzed chitosan hydrolysis the production of relatively high levels of monomers was reported.[13,18] The key that explains this difference is the high degree of deacetylation (93%) of chitosan substrate used in this work, which contrasts with the ~74% deacetylation degree chitosan employed in the other studies. Chitosan may contain four types of glycosidic linkages (-GlcN-GlcN-, -GlcN-GlcNAc-, -GlcNAc-GlcN- and -GlcNAc-GlcNAc-), the probability of the occurrence of each of these bonds depending on the deacetylation degree of the polymer. On the other hand, chitosan obtained by homogeneous deacetylation of chitin (such as chitosan used in this work) appears to contain its *N*-acetyl groups randomly distributed along the polysaccharide chains.[19] It is also known that pepsin is able to hydrolyze chitin,[20] which means that it acts on -GlcNAc-GlcNAc- bonds, and that chitosans with lower deacetylation degrees are more prone to hydrolysis by pepsin.[21] Furthermore, from the hydrolysis products pattern and its susceptibility to the action of hexosaminidase, which specifically releases GlcNAc from the non-reducing end, it has been suggested that pepsin cleaves chitosan at -GlcN-GlcNAc- and -GlcNAc-GlcNAc- linkages, resulting in products with GlcNAc in the non-reducing end.[13] Finally, the presence of monomers in the hydrolysis products has been also considered indicative of an *exo*-type action of pepsin,[13] complementary to its *endo*-type activity.

Taking in mind the above considerations, a likely explanation of the absence of monomers in the hydrolysis products obtained in this work can be found. As a result of the high deacetylation degree of the chitosan

Table 2. Product yield from enzymatic hydrolysis of chitosan for 20 h[a]

Enzyme	Monomers[b] (%)	COS[c] (%)	LMWC[d] (%)
Cellulase	4.6	46.1	49.3
Pepsin	n.d.[e]	52.2	47.8
Lipase A[f]	7.1	42.5	50.4
Chitosanase	9.1	46.3	44.6

[a] The percent yield of each of the fractions was measured after 20 h of hydrolysis according to the procedure described in the text.
[b] Monomers: sum of GlcN and GlcNAc.
[c] COS: chitosan oligosaccharides.
[d] LMWC: low molecular weight chitosan.
[e] n.d.: not detected.
[f] Lipase A assays were carried out at pH 3.0, instead of standard pH (4.5).

used, in which only 7% of the glycose residues is GlcNAc, the probability of the occurrence of -GlcNAc-GlcNAc- pairs in the polymer is very low (<0.5%) and, therefore, the vast majority of GlcNAc present is linked to GlcN. This means that almost all the hydrolytic events should occur at -GlcN-GlcNAc-linkages. In addition, following cleavage of this latter linkages, the reducing ends of the resulting products are not susceptible to further *exo*-type attack by pepsin and no GlcN is released. The only case where the monomer of GlcN could be obtained is by cleavage of a terminal GlcN-GlcNAc- pair present in the non-reducing end of the starting chitosan chains, a possibility present in a statistically very minoritory number of the relatively few initial chitosan chains, so that the amount of GlcN monomer potentially released would be extremely low. The absence of the other monomer, GlcNAc, at detectable levels can be explained by the same kind of reasoning. There are only two ways to generate GlcNAc monomers. One of them is similar to the case explained above: the *exo*-type cleavage of a terminal -GlcN-GlcNAc pair present in the reducing end of the starting chitosan chains. The other requires the presence of at least two contiguous GlcNAc residues anywhere in the chitosan chains. Again, both cases have, as we have previously explained, a low probability to occur. In conclusion, the high deacetylation degree of chitosan used in this work and the cleavage specificity of pepsin explain the extremely low levels of monomers released, that even remained under the detection threshold of the HPLC detector employed.

The hydrolytic action of pepsin on the high deacetylation degree chitosan resulted in a great yield of oligosaccharides (∼52%). The average polymerization degree of the hydrolysis products was around 16.6 units after 20 h of hydrolysis and this value is probably close to the minimum value reachable after the complete hydrolysis of the substrate. This statement is in good accordance with the theoretical value of around 14.3 units calculated taking into account the proposed specificity cleavage of pepsin and the degree of deacetylation of the chitosan substrate.

In previously published studies that used chitosans of lower deacetylation degrees, in addition to an appreciable yield of monomers, the average molecular weight of the main products after 5 h of hydrolysis was reported to be 4.6 kDa,[13] corresponding to a polymerization degree of around 25 units. This value can be compared with that obtained in the present study, where by this time the average size of the products was close to 68 units (value calculated from data shown in Figure 2, as previously explained), using in both cases the same enzyme/substrate ratio (1:100). Taking into account all these results and the fact that they used a chitosan having a deacetylation degree of 74%, it is likely that the complete hydrolysis of their substrate (surely not

reached by 5 h; see Fig. 2) would result in a hydrolysate with an average size significantly lower than that obtained in the present work.

2.3. Temperature and pH profiles of pepsin catalyzed chitosan hydrolysis

The effect of temperature and pH on chitosan hydrolysis by pepsin are depicted in Figure 3a and b, respectively. Activity increased with rising temperatures reaching a maximum at around 40–45 °C, and decreasing afterwards. This temperature optimum is in accordance with previously published data.[13,18,21] The enzyme showed a high thermal stability up to 45 °C (results not shown), where no losses in enzyme activity were observed after 1 h of incubation. At 50 °C the activity of pepsin decreased by 30% in 1 h. In addition, the peak activity of pepsin in chitosan degradation was measured at pH 4.5 which, again, agrees with reported values.[13,18,21] Activity was not measured at pH values higher that 5.0 as a result of the low solubility of chitosan and the reported irreversible inactivation of the proteolytic activity of pepsin occurring at pH values >6.0.[22] The
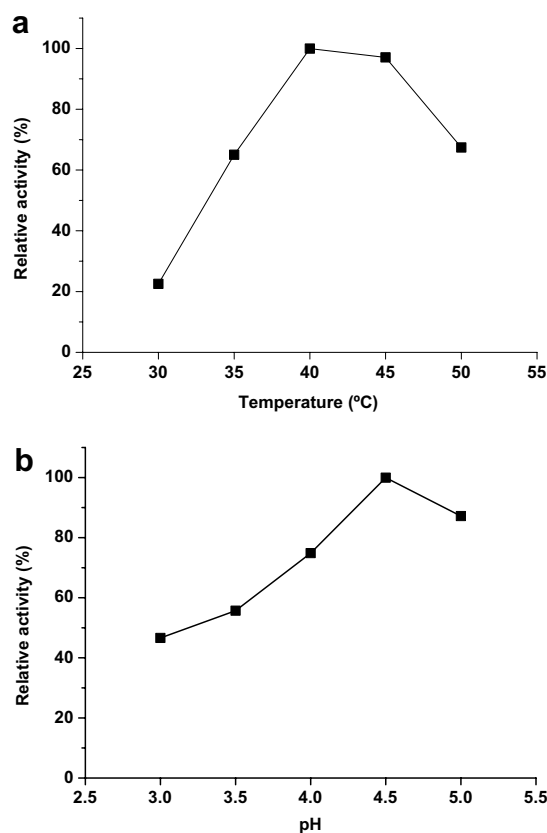

Figure 3. Chitosan hydrolyzing activity of pepsin as a function of incubation temperature (a) and pH (b). Activity was measured as the release of reducing ends after a 1 h-hydrolysis according to the standard procedure described in the text, and expressed in terms of relative activity with respect to the highest activity measured, which takes the value of 100.

optimum pH of the chitosanolytic activity of pepsin markedly differed from that of its proteolytic activity which although presenting some variability depending on the particular protein substrate and its native or denatured state, is generally accepted to be close to pH 2.0.[23]

2.4. Influence of the enzyme/substrate ratio on pepsin catalyzed chitosan hydrolysis

All the experiments so far carried out were performed at an enzyme/substrate ratio of 1:100 (w/w), which was the standard value adopted in order to compare the different enzymes tested. Apart from other parameters, the reaction rate depends on the relative concentration of enzyme (pepsin) and substrate (chitosan). The chitosan concentration was set at a maximum of 10 g L^{-1} throughout the study because of the extremely high viscosity of more concentrated chitosan solutions. Therefore, the reaction dependence on the enzyme/substrate ratio was determined by simply altering the pepsin concentration in the reaction medium at a fixed 10 g L^{-1} chitosan concentration.

Upon pepsin addition chitosan solution viscosity decreased at a rate directly related to enzyme concentration (Fig. 4). At 60 min, for enzyme/substrate ratios of 1:100 and higher the resulting viscosity was independent on this parameter, being stabilized in a value close to 20% of the initial one. At enzyme/substrate ratios lower than 1:100, the effect was not so pronounced and the viscosity continued to decrease slowly after 60 min. Therefore, from the above results it can be stated that for chitosan hydrolysis times higher than 1 h an enzyme/substrate ratio of 1:100 is enough to reach the maximum degree of hydrolysis.
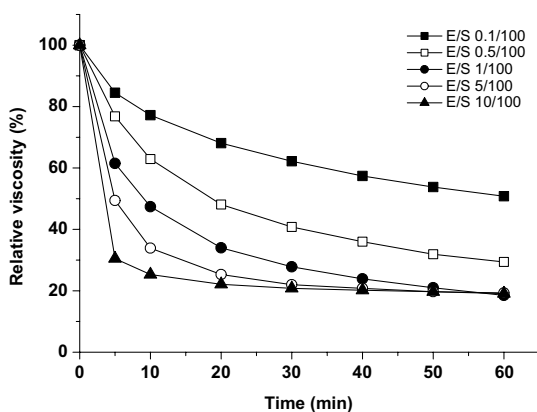
Consequently, a high yield of low- and medium-size chitosan oligosaccharides, with barely any monomer, can be produced by hydrolysis of chitosan catalyzed by a low cost commercial enzyme such as pepsin, by simply selecting a chitosan substrate with a high deacetylation degree (>90%). For optimal results, pepsin catalyzed hydrolysis should be carried out a 40 °C and pH 4.5, and at an enzyme/substrate ratio not greater than 1:100 for reactions longer than 1 h. These results open, therefore, the possibility of developing a feasible industrial process aimed at producing chitosan oligosaccharides on a large scale and useful for many applications.

3. Experimental

3.1. Enzymes

The following enzymes were purchased from Sigma–Aldrich and used without further purification: chitosanase from *S. griseus*, cellulase from *Trichoderma viride*, hemicellulase from *Aspergillus niger*, papain from papaya latex, bromelain from pineapple stem, pepsin from porcine gastric mucosa, protease type XIV from *S. griseus*, lysozyme from chicken egg white and lipase A (Amano) from *A. niger*.

3.2. Chitosan hydrolyzing activity assay

The reaction mixture of the standard assay contained 1% (w/v) chitosan (Sigma–Aldrich; deacetylation degree, 93%, according to the supplier) dissolved in 100 mM sodium acetate buffer, pH 4.5, and the corresponding enzyme in an enzyme/substrate ratio of 1:100 (w/w). The extent of chitosan hydrolysis, which was carried out at 40 °C, was measured by viscosimetry and/or reducing end formation assay.

3.3. Viscosimetry

The enzyme-catalyzed viscosity decrease of the highly viscous chitosan solutions along the reaction time was measured continuously with a Brookfield DV-II + viscosimeter. Measured values were normalized and plotted as the relative viscosity with respect to the initial value, taking an enzyme-free chitosan solution as control.

3.4. Reducing end assay

The concentration of reducing ends following chitosan hydrolysis was measured according to the method of Schales and Schales,[24] with a few modifications. After suitable time intervals, samples were withdrawn and the reaction stopped by heat inactivation of the enzyme in a boiling water bath for 5 min. Then, to a 1.5 mL

Figure 4. Influence of the enzyme/substrate ratio on pepsin catalyzed chitosan hydrolysis. Relative activity was measured as the decrease in viscosity of the chitosan solution, according to the standard procedure. Chitosan solution viscosity is expressed relative to the starting viscosity value. The enzyme/substrate ratio values assayed are shown in the inset.

sample 2 mL of a 0.5 g L^{-1} potassium ferricyanide solution in 0.5 M sodium carbonate were added. Samples were then protected from light by covering the test tubes with aluminium foil, and heated in boiling water for 15 min. After cooling at room temperature and centrifuging to remove precipitated chitosan, 2 mL of the supernatant were diluted with 1 mL of water, and the absorbance of the resulting solution was measured at 420 nm. The concentration of reducing ends was expressed as the concentration of reducing sugars equivalent to glucosamine from a calibration curve prepared with this monosaccharide as standard.

3.5. Hydrolysis products analysis

Initial fractionation of hydrolysis products was carried out according to Vishu Kumar and Tharanathan.[13] After stopping hydrolysis reaction by heat, samples were adjusted to pH 7.0 with 2 N NaOH, resulting in a precipitate of low molecular weight chitosan and a supernatant containing both chitosan oligosaccharides and monomers. The precipitate was collected by centrifugation, was lyophylized and weighted. The supernatant was analyzed by HPLC in an Applied Biosystems Series 1100 chromatograph using a Hypersil APS-2 column (4.6 × 250 mm; particle size, 5 μm). The analysis was carried out at 40 °C using a mobile phase of 3:2 MeCN–water 60:40 (v/v) and a flow rate of 0.8 mL min^{-1}, and the peaks were detected with a refractive index detector.

Acknowledgements

References

1. Crittenden, R. G.; Playne, M. J. *Trends Food Sci. Technol.* **1996**, *7*, 353–361.
2. Kim, S.-K.; Rajapakse, N. *Carbohydr. Polym.* **2005**, *62*, 357–368.
3. Jeon, Y.-J.; Shahidi, F.; Kim, S.-K. *Food Rev. Int.* **2000**, *16*, 159–176.
4. Horowitz, S.; Roseman, S.; Blumenthal, H. J. *J. Am. Chem. Soc.* **1957**, *79*, 5046–5049.
5. Chang, K. L.; Tai, M. C.; Cheng, F. H. *J. Agric. Food Chem.* **2001**, *49*, 4845–4851.
6. Cheng, C. Y.; Li, Y.-K. *Biotechnol. Appl. Biochem.* **2000**, *32*, 197–203.
7. Fukamizo, T.; Ohkawa, T.; Ikeda, Y.; Goto, S. *Biochim. Biophys. Acta* **1994**, *1205*, 183–188.
8. Kuo, C. H.; Chen, C. C.; Chiang, B. H. *J. Food Sci.* **2004**, *69*, 332–337.
9. Wu, G. J.; Tsai, G. J. *Fish. Sci.* **2004**, *70*, 1113–1120.
10. Yalpani, M.; Pantaleone, D. *Carbohydr. Res.* **1994**, *256*, 159–175.
11. Nordtveit, R. J.; Vårum, K. M.; Smidsrød, O. *Carbohydr. Polym.* **1994**, *23*, 253–260.
12. Muzzarelli, R. A. A.; Tomasetti, M.; Ilari, P. *Enzyme Microb. Technol.* **1994**, *16*, 110–114.
13. Vishu Kumar, B. A.; Tharanathan, R. N. *Carbohydr. Polym.* **2004**, *58*, 275–283.
14. Jeon, Y.-J.; Kim, S.-K. *Carbohydr. Polym.* **2000**, *41*, 133–141.
15. Kuroiwa, T.; Ichikawa, S.; Hiruta, O.; Sato, S.; Mukataka, S. *Biotechnol. Prog.* **2002**, *18*, 969–974.
16. Terbojevich, M.; Cosani, A.; Muzzarelli, R. A. A. *Carbohydr. Polym.* **1996**, *29*, 63–68.
17. Zhang, H.; Du, Y.; Yu, X.; Mitsutomi, M.; Aiba, S. *Carbohydr. Res.* **1999**, *320*, 257–260.
18. Vishu Kumar, B. A.; Varadaraj, M.; Tharanathan, R. N. *Biomacromolecules* **2007**, *8*, 566–572.
19. Varum, K. M.; Anthonsen, M. W.; Grasdalen, H.; Smidsrod, O. *Carbohydr. Res.* **1991**, *211*, 17–23.
20. Ilankovan, P.; Hein, S.; Ng, C.-H.; Trung, T. S.; Stevens, W. F. *Carbohydr. Polym.* **2006**, *63*, 245–250.
21. Tao, H.; Wei, W.; Mao, Y.; Zhang, S.; Xia, J. *Anal. Sci.* **2005**, *21*, 1057–1061.
22. Fruton, J.; Pepsin, S. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. 3, pp 119–164.
23. Schlamowitz, M.; Peterson, L. U. *J. Biol. Chem.* **1959**, *234*, 3137–3145.
24. Schales, O.; Schales, S. S. *Arch. Biochem.* **1945**, *8*, 285–292.