Enzymatic generation of chitooligosaccharides from chitosan using soluble and immobilized glycosyltransferase (Branchzyme®)

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ABSTRACT

Chitooligosaccharides possessing remarkable biological properties can be obtained by enzymatic hydrolysis of chitin. In this work, the chitosanase activity of soluble and immobilized glycosyltransferase (Branchzyme®) toward chitosan, as well as its biochemical characterization are described for the first time. This enzyme was found to be homotetrameric with a mol. wt. of 256 kDa, an isoelectric point of 5.3, and an optimal temperature range of between 50-60°C. It was covalently immobilized to glutaraldehyde-agarose with protein and activity immobilization yields of 67% and 17% respectively. Immobilization improved enzyme stability, increasing its half life five-fold, and allowed enzyme reuse for at least 25 consecutive cycles. The chitosanase activity of Branchzyme® on chitosan was similar for the soluble and immobilized forms. The reaction mixture was constituted by chitooligosaccharides with degrees of polymerization (DP) of between 2 to 20, with a higher concentration having degrees of polymerization of 3 to 8.

KEYWORDS: *chitooligosaccharides, COS, Branchzyme, immobilization, MALDI-TOF, HPLC-SEC, GC-FID*

INTRODUCTION

Chitin, an unbranched N-acetyl-D-glucosamine polysaccharide linked by β-(1-4) bonds, is widely distributed in nature as the principal component of exoskeletons of crustaceans and insects as well as of cell walls of fungi and some bacteria, and is regarded as the second most abundant natural polymer after cellulose.[1] Partial deacetylation (commonly by alkaline treatment) of chitin gives rise to chitosan, a cationic polysaccharide composed of units of glucosamine (2-amino-2-deoxy-D-glucose) and N-acetyl glucosamine (2-acetamido-2-deoxy-D-glucose) linked by β-(1-4) bonds. Due to its cationic character, chitosan presents a wide variety of physicochemical and biological properties, including antimicrobial, antioxidant, hypocholesterolemic and antihypertensive properties. These properties make it suitable for numerous applications in different fields, for instance, medicine (pharmacology, cosmetics), biotechnology, agriculture, the food industry (nutritional enhancement and food processing), the environment (waste and water treatment) and the textile industry.[2-3] In addition, its lack of toxicity and allergenicity, as well as its biocompatibility, biodegradability and bioactivity make it a very attractive substance for use in biomaterial design.

However, its poor solubility in solutions of neutral and basic pH hinders its use in many industrial applications. Therefore, there is considerable interest in improving chitosan solubility and different strategies have been developed, for instance the partial hydrolysis of chitosan to obtain low MW chitosan (LMWC) and chitooligosaccharides (COS), which are soluble in water. COS are defined as chitosan with a degree of polymerization (DP) less than 20 and MW of up to 3900 Da. Besides improved water solubility, COS are reported to possess remarkable biological properties, such as antibacterial and antitumor activity, as well as immune enhancing effects in animal health.[3-9]

Hydrolysis of chitosan can be carried out by chemical, physical and enzymatic methods. Chemical hydrolysis is performed at high temperatures under highly acidic conditions, mainly using HCl or HNO_2 and produces a large amount of glucosamine (chitosan monomer).[9,10] Due to the complexity of controlling the progress of the reaction, these treatments also result in the formation of secondary compounds that are difficult to remove.[9] By physical methods such as irradiation with low frequency ultrasound (20 kHz), partial depolymerization is obtained, reducing the average MW from 2000 kDa down to 450 kDa or from 300 kDa to 50 kDa; however, the reduction of MW is limited.[9]

Enzymatic methods, such as the use of chitosanases and non-specific enzymes seem to be generally preferable to chemical methods because the reaction is performed under more gentle conditions and the MW distribution of the product is more controllable.[4,11] The expensive cost of chitosanases limits their wide application on an industrial scale, even using immobilized enzymes.[4,12,13] On the other hand, non-specific enzymes are inexpensive, commercially available and have been used in the industry for years to produce COS with relatively low cost.[7] Pantaleone et al.[14] reported the hydrolytic susceptibility of chitosan to a wide range of enzymes, including glycanases, proteases, and lipases derived from bacterial, fungal, mammalian and plant sources.

Although different non-specific enzymes have been used to obtain COS from chitosan, due to the limited capacity of most hydrolytic enzymes there is still interest in finding new enzymes with better properties. Enzymatic chitosan hydrolysis can be performed either with soluble or immobilized enzymes,[10,15] in batch,[16,17] column or membrane reactors.[4,13] The advantages of using enzymes immobilized onto solid phase have been widely reported, including increased biocatalyst stability, reaction media free of enzyme at the end of the process, the opportunity to reuse the immobilized biocatalyst with

concomitant reduction of process costs, as well as the feasibility of continuous operation.[6]

Branchzyme® is a relatively inexpensive commercial preparation which contains a branching glycosyltransferase from *Rhodothermus obamensis* expressed in *Bacillus subtilis*. This enzyme catalyses the transfer of a segment of a 1,4-α-D-glucan chain to a primary hydroxyl group in a similar glucan chain to create 1,6-α-linkages, thereby increasing the number of branch points.[18,19]

Despite the difference between the glycosyltransferase activity and the chitosanase activity, Branchzyme® exhibits both of them. Nevertheless, to the best of our knowledge, it has not previously been used for chitosan hydrolysis. In this study, the biochemical characterization of the chitosanase activity of Branchzyme®, used in soluble form and immobilized onto glutaraldehyde-agarose, using chitosan as substrate, is described for the first time. Assays were carried out in order to determine the MW and the isoelectric point of the enzyme as well as the optimal temperature and pH conditions for chitosanase activity, and its kinetic parameters. Moreover, a comparative study of the performance of soluble and immobilized enzyme for COS production was performed, as well as characterization of the hydrolysis products.

MATERIALS AND METHODS

All results represent averages of at least three experiments.

Materials

Glucosamine, N-acetyl-glucosamine, LMWC (average MW of 130 kDa) and pullulan samples (0.3-800 kDa) were supplied by Sigma-Aldrich (St. Louis, MO). A mixture of chitosan oligomers from dimer to hexamer was acquired from Seikagaku Corporation (Tokyo, Japan). Sepharose 4B, PD-10 Sephadex G25 columns and MW markers from

2000 to 13.7 kDa were from General Electric (Buckinghamshire, UK). The commercial enzyme preparation Branchzyme® produced by cloning in *B. subtilis* was a generous gift from Novozymes (Dittingen, Switzerland). All other chemicals used were of analytical grade.

Enzyme immobilization onto glutaraldehyde-agarose

Glutaraldehyde-agarose containing 15 μmol of glutaraldehyde per g of suction-dried gel was prepared as described previously by Guisán et al.[20] Aliquots of 1 g of suction-dried glutaraldehyde-agarose gel were incubated with 10 mL of enzyme solution (previously gel-filtered on a PD-10 column, Sephadex G-25, to remove low molecular weight contaminants) containing 0.7 mg/mL of protein and 19 EU/mL in 0.1 M sodium phosphate buffer at pH 7.0 (immobilization buffer). The suspension was gently stirred at room temperature for 24 h. Then it was washed on a sintered glass filter with immobilization buffer and equilibrated with 40 mM sodium carbonate buffer at pH 10.0. The derivative was suspended in 1mg/mL sodium borohydride solution in 40 mM sodium carbonate buffer pH 10.0 at a ratio of 1 g of suction-dried gel: 14 mL of total volume. The mixture was gently stirred for 30 min at room temperature, washed with 50 mM sodium acetate buffer, pH 5.3 (activity buffer) and stored at 4 °C.

Characterization of soluble and immobilized enzyme

Molecular weight and isoelectric point of enzyme

SDS-PAGE electrophoresis and isoelectric focusing (ISO) analysis were done with PhastSystem equipment (Pharmacia LKB). SDS-PAGE was performed with Homo12.5 Phast Gels. The isoelectric point was determined using the broad pI calibration kit, run

on Phast Gel IEF 3-9. The proteins in the polyacrylamide gels were stained with Coomassie Brilliant Blue.

To confirm the MW of the enzyme and to determine its quaternary structure, size-exclusion chromatography (SEC) analysis was performed in an AKTA Purifier System (AKTA Purifier10, General Electrics), using the Superdex 200 10/300 GL column (GE Healthcare) following the manufacturer's instructions. The column was calibrated using the following set of proteins: blue dextran (MW>2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and ribonuclease (13.7 kDa).

Protein determination

The protein concentration of the soluble and immobilized enzyme was estimated by the bicinchoninic acid (BCA) assay[21] using Bovine Serum Albumin as standard. Protein concentration was expressed as mg/mL for soluble protein and mg protein per g of suction-dried gel for immobilized protein.

Enzymatic activity

The chitosanase activity of Branchzyme® (toward chitosan) was determined by measuring the rate of release of reducing sugars. The amount of reducing sugars formed was estimated by the dinitrosalicylic acid (DNS) method.[22] The oxidised dinitrosalicylic formed was quantified spectrofotometrically at 540 nm. In order to remove low MW reducing molecules which could interfere with the quantification of reducing sugars, the enzyme solution was previously gel filtered on a PD-10 column (Sephadex G-25). Aliquots of 160 μL of a suitably diluted enzyme solution were added to 1.0 mL of 20 mg/mL chitosan solution in 0.1 M acetic acid (final pH 5.3) and incubated at 50 °C. At regular intervals, aliquots of the reaction mixture were taken and the enzymatic reaction was stopped by heating at 100 °C for 5 min; the samples were analyzed for reducing

sugars by the DNS method. For the immobilized enzyme, activity was measured under identical conditions by incubating 100 mg of the gel derivative with 1.0 mL of 20 mg/mL chitosan solution in 0.1 M acetic acid at 50 °C under continuous stirring. One unit of chitosanase activity (EU) was defined as the amount of enzyme that catalyzes the formation of 1 μmol of reducing sugar (expressed as D-glucosamine) per hour under the above conditions. Enzymatic activity was expressed as EU/mL for the soluble enzyme. The gel bound activity was expressed as EU per g of suction-dried gel.

Effect of temperature and pH on soluble and immobilized enzyme activity

The optimum temperature was determined by assaying the chitosanase activity at pH 5.3 in a temperature range from 40 to 60°C as described above. Similarly, the optimum pH was determined by testing chitosanase activity at 60 °C in a pH range from 4.3 to 6.3.

Thermal stability of soluble and immobilized enzyme

Aliquots of enzyme solution or immobilized enzyme suspension containing 3 EU/mL in 0.1 M sodium acetate buffer pH 5.3 were incubated at 50 °C under gentle stirring. Aliquots were taken at regular intervals up to 6 days, and the residual activity was determined.

Reuse of the immobilized enzyme derivative

Aliquots of 1 g of suction-dried immobilized enzyme derivative containing 4.8 mg of protein/g were incubated with 10 mL of a 20 mg/mL chitosan solution in 0.1 M acetic acid (final pH 5.3) for 24 h at 50 °C. The enzymatic reaction was stopped by a simple filtration step. The immobilized derivative was washed with 30 mL of activity buffer (0.1 M sodium acetate pH 5.3) and reused. The capacity to produce reducing sugar was plotted against time; the capacity to produce reducing sugar was defined as follow:

[(mg/mL of glucosamine produced in each reuse) / (mg/mL of glucosamine produced in the first use)]*100

Determination of kinetic parameters (K_M and V_{Max})

The kinetic parameters were determined using varying concentrations of chitosan: 2.0 to 6.0 mg/mL in 0.1 M acetic acid (final pH 5.3). The K_M and the V_{Max} were determined by the Lineweaver-Burk method.[23]

Enzymatic hydrolysis of chitosan and fractionation of chitooligosaccharides

Chitosan was depolymerized using both soluble and immobilized enzyme. Aliquots of 19 μL of soluble enzyme (0.9 EU) or 50 mg of immobilized enzyme (1.6 EU) were incubated with 0.5 mL of 20 mg/mL chitosan solution in 0.1 M acetic acid (final pH 5.3) at 50 °C for different time periods. The enzyme reaction was stopped by heating at 100 °C for 5 minutes for the soluble enzyme or by filtration for the immobilized derivative. The aliquots were lyophilized, dissolved in 1% acetic acid and analyzed by SEC-HPLC.

In order to characterize chitosan and COS obtained by the hydrolysis, aliquots from reaction mixtures using both soluble and immobilized enzyme corresponding to 24h of incubation were treated as follows: the solution was filtered with 0.45 μm filters, precipitated with ethanol 90% (v/v) and centrifuged to separate low and high MW chitooligomers (L-COS and H-COS, respectively). The supernatant containing L-COS was vacuum-dried at 30°C, dissolved in water and then lyophilized. The pellet containing H-COS was washed with ethanol 90% (v/v) twice, dissolved in water and lyophilized.

Characterization of chitosan and chitooligosaccharides (COS)

Degree of deacetylation

The degree of deacetylation (DD) of chitosan and COS was determined by Fourier Transform Infrared Spectroscopy (FTIR) in a Perkin-Elmer spectrometer (Spectrum One). The degree of acetylation (DA) was calculated using the baselines and the equation proposed by Moore and Roberts: [24]

$$DA\ (\%) = ((A_{1655}/A_{3450})\times 100)/1.33$$

where A_{1655} is the intensity of the absorption band of amide I, used as the specific band for *N*-acetylation and A_{3450} is the intensity of the absorption of the hydroxyl group band, used as the reference band; 1.33 is the ratio of the absorbance at 1655 cm^{-1} to that of the absorbance at 3450 cm^{-1} for fully N-acetylated chitosan. DD was then calculated using the following equation: DD (%) = 100- DA (%).

Molecular weight determination

MW of chitosan and COS formed was determined by SEC-HPLC as described by Ruiz-Matute et al.[25] Aliquots of 50μL of chitosan or COS solutions (1 mg/mL) were injected into the chromatographic system. Commercial pullulan samples of different MW (0.3-800 kDa) were used to construct the calibration curve.

In addition, the lyophilized powder obtained from ethanol 90% (v/v) treatment, which contains high MW compounds (H-COS), was also characterized by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-ToF MS) on a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a pulsed nitrogen laser (λ = 337 nm, 3 ns pulse width, and 3 Hz frequency) and a delayed extraction ion source. Ions generated by laser desorption were introduced into a time of flight analyzer (1.3 m flight path) with an acceleration voltage of 25 kV, 94%

grid voltage, 0.025% ion guide wire voltage, and a delay time of 200 ns in the reflector positive ion mode. Mass spectra were obtained over the m/z range 500-5000. External mass calibration was applied using the monoisotopic $[M + H]^+$ values of des-Arg1 bradykinin and angiotensin I of Calibration Mixture 1, Sequazyme Peptide Mass Standards Kit; Applied Biosystems. 2,5-dihydroxybenzoic acid (>98%, Fluka) at 10 mg/mL in water was used as the matrix. The sample was diluted 100 times in water, and mixed with the matrix at a ratio of 1:4 (v: v). One μL of this solution was spotted onto a flat stainless-steel sample plate and dried in air before analysis.

Quantification of COS by gas chromatography with flame ionization detection (GC-FID)

Before analysis, the supernatant of the precipitation with ethanol 90% (v/v), containing 4 mg of lyophilized L-COS samples, was added to 0.4 mL of internal standard (I.S.) solution (0.5 mg/mL phenyl-β-glucoside). Afterwards the mixture was dried at 38-40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland).

The trimethylsilyl oximes (TMSO) obtained were analyzed by GC-FID on an Agilent Technologies 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA), using a ZHT-5 fused-silica capillary column (15 m × 0.25 mm i.d. × 0.1 μm film thickness). The nitrogen flow rate was 1 mL min^{-1}. The initial oven temperature was 150 °C and increased to 250 °C, then heated at 10 °C min^{-1} to 380 °C and held for 10 min. The injector and detector temperatures were 240 and 380 °C, respectively. Injections were made in the split mode (1:20).[26-27] Identification of TMSO derivatives of carbohydrates was carried out by comparison of their linear retention indices (I^T) with those of previously derivatized standard compounds, containing chitosan oligomers from dimer to hexamer (Seikagaku).

Statistical Analysis

Data were subjected to one-way analysis of variance (Tukey HSD Multiple Range Test) by applying the Statgraphic 4.0 program (Statistical Graphics Corp., Rockville, MD) for Windows. The significance of differences was defined as $p < 0.05$.

RESULTS AND DISCUSSION

Enzyme characterization

Molecular weight and isoelectric point determination of Branchzyme® enzyme

In order to establish the degree of purity of the enzyme preparation and to determine the MW of the enzyme, an SDS-PAGE electrophoresis was performed. As can be observed in Figure 1 the enzyme preparation was quite pure, exhibiting a main band with a MW close to 64 kDa. With the aim of determining the overall MW of the protein, SEC was performed. The native protein had an overall MW of about 256 kDa, suggesting that the quaternary structure of the enzyme is a homo-tetramer composed by monomers of 64 kDa. The isoelectric point of the enzyme was also determined obtaining a value of 5.3.

Enzyme immobilization on glutaraldehyde-agarose

Among the advantages of using immobilized enzymes are: an increase in enzyme stability, easy separation of the enzyme from other reaction components and the possibility of recycling the enzyme.[6,21,28] Enzymes with chitosanase activities have been immobilized onto a variety of supports using both covalent and non covalent methods with relatively good yields.[16,17,29] Nevertheless, in the case of chitosanases that use substrates with high MW, access to the active sites can be hindered when the enzyme is immobilized, reducing its apparent activity. Therefore, the success of the immobilization process should be evaluated not only by the amount of gel bound protein but also the gel bound activity. In this particular case, Branchzyme® was

immobilized onto glutaraldehyde-agarose. This immobilization strategy involves the superficial amine groups of the enzyme which react with the aldehyde groups of the support producing Schiff bases that are later reduced generating stable alkyl amine covalent bonds.[20,30] This results in a non-reversible covalent binding between the enzyme and the support, which generally results in some gain in the enzyme thermal stability.

The enzyme immobilization process was efficient since 67.4% of the applied protein was immobilized onto the support; however, the gel bound activity was reduced to 17.2% of the applied activity (Table 1). The low percentage of gel bound activity can be attributed to several factors, for instance diffusional restrictions due to the immobilization process exacerbated by the high MW of the substrate. Besides, it has been reported that reducing treatment at pH 10.0, necessary to block the excess reactive groups on the gel and to reduce the Schiff bases between the enzyme and the support, may possibly negatively affect gel-bound activity.[28]

Effect of pH and temperature on enzyme activity

Optimum enzyme temperature for the soluble and immobilized forms was determined. As showed in Figure 2 optimal temperature was 60 °C and 50 °C for the soluble and immobilized enzyme, respectively. However, taking into account the standard deviations this difference was not significant. The values found in literature for enzymes with chitosanase activity are diverse, ranging from 30 to 70 °C; [31, 32] in our case, the elevated value for the optimal temperature is in concordance with the fact that the enzyme is obtained from a thermophilic microorganism (*Rhodothermus obamensis).*

The effect of pH on enzyme activity was studied at 60 °C; Figure 3 shows that the optimal pH was 5.3 for both soluble and immobilized enzyme. At pH 6.2 the activity of the immobilized enzyme falls considerably in comparison with that of the soluble

enzyme. This could be due to the increase of chitosan viscosity at increased pH values, hindering its access to the active sites of the enzyme.

Kinetic parameters

Usually, the immobilization process may restrict the access of the substrate to the active site of the enzyme, because of diffusional restrictions which may result in an apparent lower activity of the derivative.[21,23] This effect is more pronounced in the case of macromolecular substrates such as chitosan. So, to further characterize this phenomenon, kinetic parameters of the soluble and the immobilized enzyme were studied in the conditions previously selected (pH 5.3 and 50 °C). K_M and V_{Max} values of the soluble and immobilized Branchzyme® were determined with the Lineweaver–Burk plot method.[23]

The K_M value determined for the soluble enzyme (4.0 mg/mL of chitosan, Table 2) was consistent with those found in the literature for chitosanases; e.g. Zeng and Zheng[12] reported that the chitosanase from *Penicillium* sp. ZDZ1 had a K_M of 2.61 mg/mL of chitosan determined at pH 5.0 and 50 °C. Liu and Xia [33] reported that the chitosanase activity exhibited by the cellulase from *Trichoderma viride* had a K_M of 10 mg/mL of chitosan determined at pH 5.2 and 60 °C.

The immobilization of Branchzyme® onto glutaraldehyde-agarose produced a two-fold increase in the apparent K_M value when using chitosan as a substrate, and a three-fold decrease in apparent V_{Max} (Table 2). Even though the K_M value was higher for the immobilized enzyme than for the soluble enzyme, this increase was not as pronounced as might have been expected;[21] probably the macromolecular nature of the substrate (chitosan) promotes diffusional limitations that prevent the access to the active site of the enzyme, even in its soluble form. In addition to this, the immobilization process may

induce conformational changes in enzyme structure which could contribute to the higher K_M, among other factors.

The decrease of the apparent V_{Max} due to the immobilization process is consistent with the inactivation previously observed, probably mainly caused by the high pH value during the reducing treatment negatively affecting gel-bound activity and the V_{Max} value. Nonetheless, the reducing treatment could not be avoided since it prevents leakage of the enzyme from the matrix. Another possible explanation for V_{Max} reduction would be that some percentage of the enzyme molecules was bound to the support through their active sites, contributing to the decrease in the apparent V_{Max}.

Thermal stability

The stability of biocatalysts is one of the key parameters that limits their industrial application, and enzyme stabilization is a central issue in biotechnology.[34] Therefore, in order to be suitable for different applications, an enzyme needs to be stable or must be stabilized. Immobilization on to solid carriers is perhaps the most frequently used strategy to improve the operational stability of biocatalysts.[35,36] In order to evaluate the stability of the enzyme at the selected operational temperature, both soluble and immobilized enzyme were incubated for 6 days at 50 °C and pH 5.3, and the residual activity was determined in each case.

Figure 4 shows the experimental plots of residual activity versus time at 50 °C for both immobilized and soluble enzyme. The half-life of the immobilized enzyme (2.5 days) was at least five times higher than that of the soluble enzyme (0.5 days). The better stability of the derivative immobilized onto glutaraldehyde-agarose may be due to the fact that glutaraldehyde is known to polymerize and, thus, is prone to introduce a number of cross links between the abundant lysyl groups on the enzyme and the aldehyde moieties on the matrix.[37] The introduction of cross links leads to a

rigidification of the tertiary structure and this is recognized as a general mechanism for stabilization of the native conformation.[38]

Another possible interpretation for this behavior is that the enzyme was attached to the support through a "critical area" for enzyme stability.[39] It has been reported that denaturation of a protein begins in a defined region of the protein called the "critical area", so when enzymes are immobilized through this area, their stability will be improved.[40]

Enzyme re-uses

One of the advantages of enzyme immobilization onto an insoluble support is the possibility of re-use of the enzymatic derivative for consecutive cycles of chitosan hydrolysis. In order to characterize the operational stability of the enzymatic derivative, the capacity to generate reducing sugars from chitosan was evaluated in a repeated batch process as detailed in the Material and Methods section. Figure 5 shows that the enzymatic derivative could be used to hydrolyze chitosan for 25 consecutive reuses retaining over 40% of its initial capacity. This result seems not to be in accordance with that obtained for thermal stability of the immobilized enzyme where its residual activity fell to 40% after 6 days (Figure 4). Yet operational stability assays are performed in the presence of the substrate (chitosan) which could exert a protective effect over the enzyme, increasing its stability.

LMWC and COS characterization

In order to characterize the COS obtained by chitosan enzymatic hydrolysis different studies were performed. The DD value for LMWC by FT-IR was 73% and no significant change was observed after the fractionation process. This behavior was in

agreement with the report by Il'ina and Varlamov [41] related to slight changes in chitosan deacetylation during enzymatic hydrolysis and isolation.

The characterization of the MW of commercial LMWC (substrate) and COS fractions obtained by enzymatic hydrolysis at different times up to 24 h (pH 5.3 and 50 °C) was performed by SEC-HPLC (Figure 6). LMWC eluted with a retention time of 17.7 min which corresponds to a MW of 130 kDa, according to the calibration curves made with pullulan standards. Enzymatic hydrolysis of chitosan could be observed using both the soluble and immobilized enzyme, evidenced by the change in the MW distribution of the reaction mixture with the course of time (Figure 6). After 24 hours, the chromatograms corresponding to soluble and immobilized enzyme showed a major peak that could be assigned to a range of MW between 0.65-0.90 kDa.

These results indicate that the hydrolysis product profiles for both soluble and immobilized enzyme were similar. So, to perform a deeper characterization of the COS obtained, the reaction mixtures corresponding to 24 h were treated with ethanol 90% (v/v), resulting in two fractions with different composition. The supernatant contained low MW oligosaccharides (L-COS) with a degree of polymerization (DP) ranging from 2 to 5. In this fraction DP3 oligosaccharides were found in the highest amounts. The precipitate contained the high MW fraction (H-COS) with DP from 5 to 20, and the highest concentrations were of DP 7 and 8.

The L-COS was analyzed by GC-FID and Figure 7 shows that the reaction mixture was composed of oligomers of DP from 2 to 5, without free monomers of glucosamine or N-acetyl-glucosamine. The results obtained with the soluble (Figure 7a) and immobilized (Figure 7b) enzyme revealed that the qualitative profile in both cases was similar. Nevertheless, the composition of fractions in each case was slightly different, being 17.8% of DP2, 49.9% of DP3, 26.8% DP4 and 5.5% of DP5 with soluble enzyme and

30.5% of DP2, 53.2% of DP3, 15.7% of DP4 and 0.6% of DP5 with immobilized enzyme.

The H-COS obtained, corresponding to the precipitate with 90% (v/v) ethanol, was analyzed by MALDI-ToF MS. As expected, this fraction was composed mainly of chitooligomers of DP 4 to 20 (Table 3; Figure 8); among them, COS of DP 7 and 8 were at the highest concentration. The analysis of the products obtained with soluble and immobilized enzyme (data not shown) again showed similar profiles. In both cases, these analyses revealed the occurrence of oligomers of glucosamine (GlcN) and N-acetyl-glucosamine (GlcNAc) with a ratio 3:1; the presence of GlcNAc was observed for chitooligomers with DP greater than 6.

Others authors employing MALDI-ToF for the characterization of COS mixtures found similar DPs and DP up to 11 using different enzymes with chitosanase activities.[10,15,42,43]

In summary, COS obtained by the depolymerization reaction of chitosan with Branchzyme® (soluble and immobilized) were complex mixtures of chitooligomers with DP between 2 and 20, among which L-COS with DP 2 and 3 and H-COS with DP 7 and 8 had the highest concentrations.

Additionally, depolymerization reaction performance after 24 h corresponding to reuses 12, 16 and 17 of the immobilized enzyme were analyzed by SEC-HPLC. The results were similar to those obtained in the first use of the derivative, showing that over repeated use of the immobilized enzyme the profile of the depolymerization reaction was maintained, with little change in the composition of the COS.

In conclusion, we are reporting for the first time the chitosanase activity of Branchzyme®. The immobilization of the enzyme onto glutaraldehyde-agarose was

successful since the loss of activity during the process was amply compensated for by the superior stability of the immobilized enzyme compared with the soluble form and also by the possibility of an elevated number of reuses of the immobilized derivative, with the same depolymerization performance on chitosan..

Furthermore the profile of the products obtained in the hydrolysis of chitosan is similar for both the soluble and the immobilized enzyme confirming that immobilization of this kind of chitosanase onto solid supports represents a useful alternative to the soluble enzyme.

Abbreviations Used:

COS: Chitooligosaccharides

LMWC: Low molecular weight chitosan

DD: Degree of deacetylation

DP: Degree of polymerization

MW: molecular weight

H-COS: high molecular weight chitooligomers

L-COS: low molecular weight chitooligomers

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FIGURE CAPTIONS

Figure 1. SDS-PAGE electrophoresis in 12.5% homogeneous Phast Gels. Lane 1: MW markers; Lane 2: Branchzyme®

Figure 2. Effect of temperature on soluble (▲) and immobilized (■) enzyme activity (mean ± SD, n = 3)

Figure 3. Effect of pH on soluble (▲) and immobilized (■) enzyme activity (mean ± SD, n = 3)

Figure 4. Thermal stability of soluble (▲) and immobilized (■) enzyme at 50°C (mean ± SD, n = 3)

Figure 5. Reuse of the immobilized enzyme (mean ± SD, n = 3)

Figure 6. Analysis of the performance of the depolymerization reaction with the soluble and immobilized enzyme at 0 h, 8 h and 24 h by SEC-HPLC.

Figure 7. CG-FID chromatograms of oligomer mixtures after 24 h of depolymerization (A) with soluble and (B) with immobilized enzyme.

Figure 8. MALDI-ToF MS spectrum of high MW chitooligomers (H-COS) obtained with soluble enzyme after 24 h of depolymerization (Table 3).

Table 1. Immobilization of the enzyme onto glutaraldehyde-agarose (mean ± SD, n = 3)

Gel-bound protein		Gel-bound activity		Gel-bound specific activity (EU/mg)
mg/g gel	%[a]	EU/g	gel %[b]	
4.8 ± 0.5	67.4 ± 11.4	33.2 ± 4.7	17.2 ± 4.6	6.9 ± 0.5

[a] Amount of immobilized protein as percentage of the amount of applied protein (protein immobilization yield).
[b] Amount of immobilized activity as percentage of the amount of applied activity (activity immobilization yield).
Soluble enzyme specific activity: 27.4 ± 3.5 EU/mg

Table 2. Kinetic parameters of soluble and immobilized enzyme (mean ± SD, n = 3)

Enzyme	K_M (mg chitosan/mL)	V_{Max} (μmol glucosamine/h.mg)
Soluble	4.0 ± 0.8	72.7 ± 7.0
Immobilized	8.3 ± 2.5	21.5 ± 4.6

Table 3. Assigned ion composition of MALDI-ToF MS spectra of high MW chitooligomers (H-COS) obtained with soluble enzyme (Figure 8).

m/z	*Types*	*DP*	*Ion composition*
848,8	$[M + Na]^+$	5	$(GlcN)_5$
1007,4	$[M + Na]^+$	6	$(GlcN)_6$
1146,1	$[M + H]^+$	7	$(GlcN)_7$
1169,2	$[M + Na]^+$	7	$(GlcN)_7$
1210,2	$[M + Na]^+$	7	$(GlcN)_6$-GlcNAc
1307,1	$[M + H]^+$	8	$(GlcN)_8$
1329,4	$[M + Na]^+$	8	$(GlcN)_8$
1345,7	$[M + K]^+$	8	$(GlcN)_8$
1371,6	$[M + Na]^+$	8	$(GlcN)_7$-GlcNAc
1413,4	$[M + Na]^+$	8	$(GlcN)_6$-$(GlcNAc)_2$
1468,2	$[M + H]^+$	9	$(GlcN)_9$
1533,4	$[M + Na]^+$	9	$(GlcN)_8$-GlcNAc
1574,5	$[M + Na]^+$	9	$(GlcN)_7$-$(GlcNAc)_2$
1695,3	$[M + Na]^+$	10	$(GlcN)_9$-GlcNAc
1735,4	$[M + Na]^+$	10	$(GlcN)_8$-$(GlcNAc)_2$
1777,7	$[M + Na]^+$	10	$(GlcN)_7$-$(GlcNAc)_3$
1854,5	$[M + Na]^+$	11	$(GlcN)_{10}$-GlcNAc
1897,5	$[M + Na]^+$	11	$(GlcN)_9$-$(GlcNAc)_2$
1939,3	$[M + Na]^+$	11	$(GlcN)_8$-$(GlcNAc)_3$
1981,5	$[M + Na]^+$	11	$(GlcN)_7$-$(GlcNAc)_4$
2058,2	$[M + Na]^+$	12	$(GlcN)_{10}$-$(GlcNAc)_2$
2100,4	$[M + Na]^+$	12	$(GlcN)_9$-$(GlcNAc)_3$
2142,7	$[M + Na]^+$	12	$(GlcN)_8$-$(GlcNAc)_4$
2219.0	$[M + Na]^+$	13	$(GlcN)_{11}$-$(GlcNAc)_2$
2261,2	$[M + Na]^+$	13	$(GlcN)_{10}$-$(GlcNAc)_3$
2302,8	$[M + Na]^+$	13	$(GlcN)_9$-$(GlcNAc)_4$
2381,1	$[M + Na]^+$	14	$(GlcN)_{12}$-$(GlcNAc)_2$
2422,7	$[M + Na]^+$	14	$(GlcN)_{11}$-$(GlcNAc)_3$
2464,5	$[M + Na]^+$	14	$(GlcN)_{10}$-$(GlcNAc)_4$
2584,3	$[M + Na]^+$	15	$(GlcN)_{12}$-$(GlcNAc)_3$
2625,3	$[M + Na]^+$	15	$(GlcN)_{11}$-$(GlcNAc)_4$
2667,2	$[M + Na]^+$	15	$(GlcN)_{10}$-$(GlcNAc)_5$
2743,8	$[M + Na]^+$	16	$(GlcN)_{13}$-$(GlcNAc)_3$
2786,8	$[M + Na]^+$	16	$(GlcN)_{12}$-$(GlcNAc)_4$
2828,3	$[M + Na]^+$	16	$(GlcN)_{11}$-$(GlcNAc)_5$
2905,9	$[M + Na]^+$	17	$(GlcN)_{14}$-$(GlcNAc)_3$
2947,8	$[M + Na]^+$	17	$(GlcN)_{13}$-$(GlcNAc)_4$
2989,3	$[M + Na]^+$	17	$(GlcN)_{12}$-$(GlcNAc)_5$
3149,9	$[M + Na]^+$	18	$(GlcN)_{13}$-$(GlcNAc)_5$
3311,3	$[M + Na]^+$	19	$(GlcN)_{14}$-$(GlcNAc)_5$
3473,3	$[M + Na]^+$	20	$(GlcN)_{15}$-$(GlcNAc)_5$

Figure 1

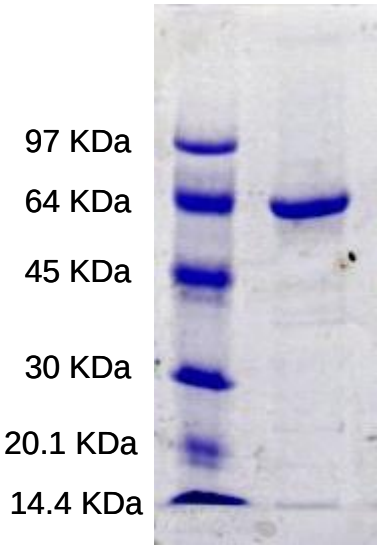


Figure 2

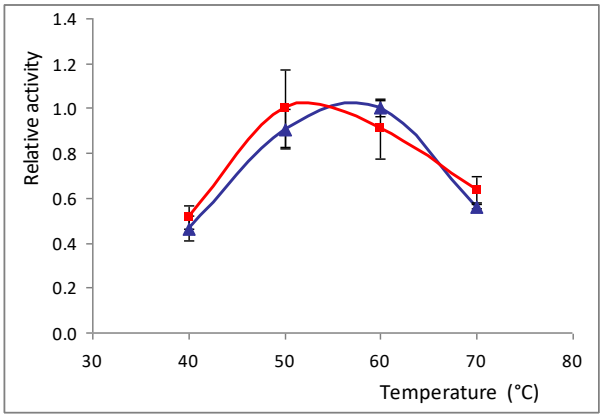

Relative activity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
30
40
50
60
70
80
Temperature (°C)

Figure 3

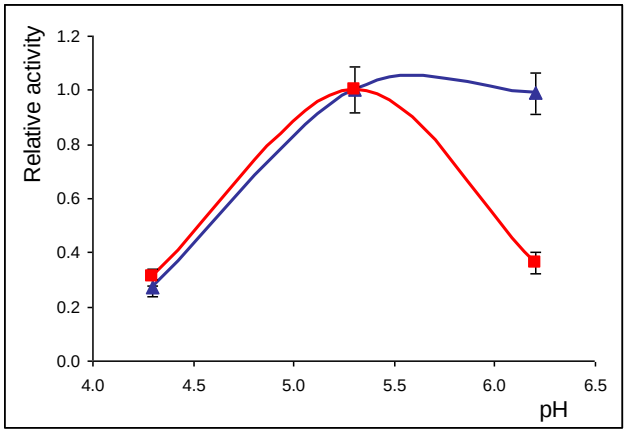


Figure 4

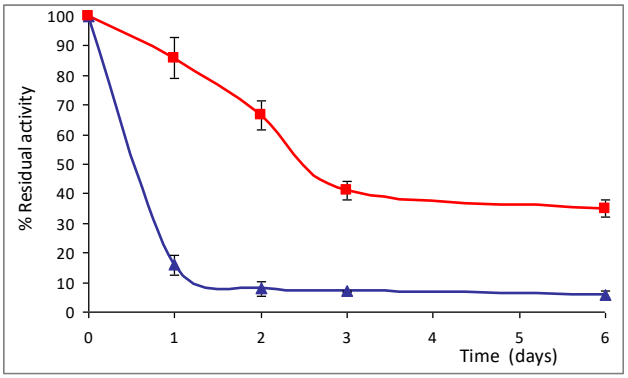

% Residual activity
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Time (days)

Figure 5

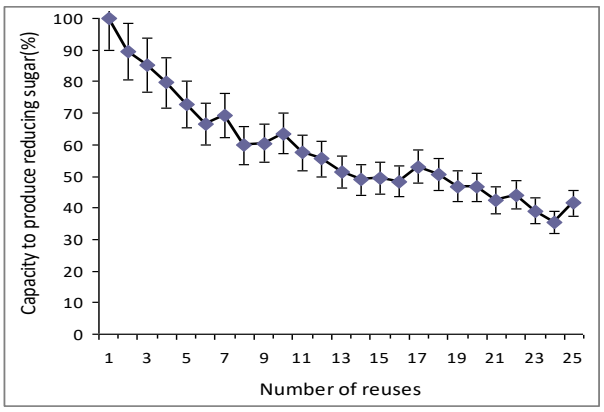
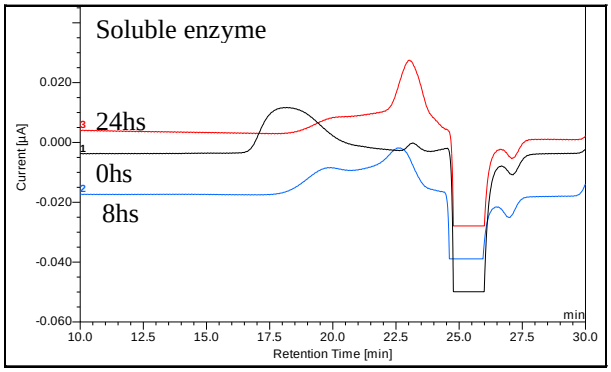

Capacity to produce reducing sugar(%)
100
90
80
70
60
50
40
30
20
10
0
1
3
5
7
9
11
13
15
17
19
21
23
25
Number of reuses

Figure 6


Soluble enzyme
24hs
0hs
8hs
Current [µA]
0.020
0.000
-0.020
-0.040
-0.060
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
30.0
min
Retention Time [min]

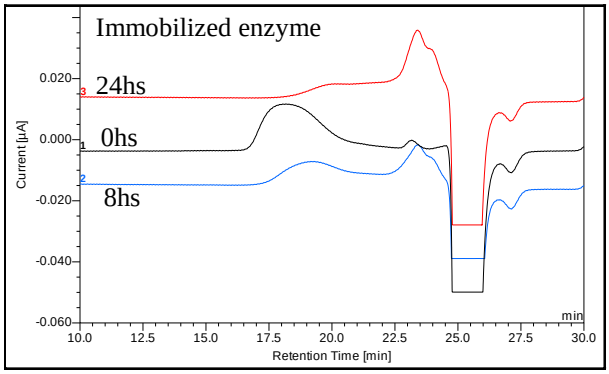

Immobilized enzyme
24hs
0hs
8hs
Current [µA]
0.020
0.000
-0.020
-0.040
-0.060
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
30.0
min
Retention Time [min]

Figure 7

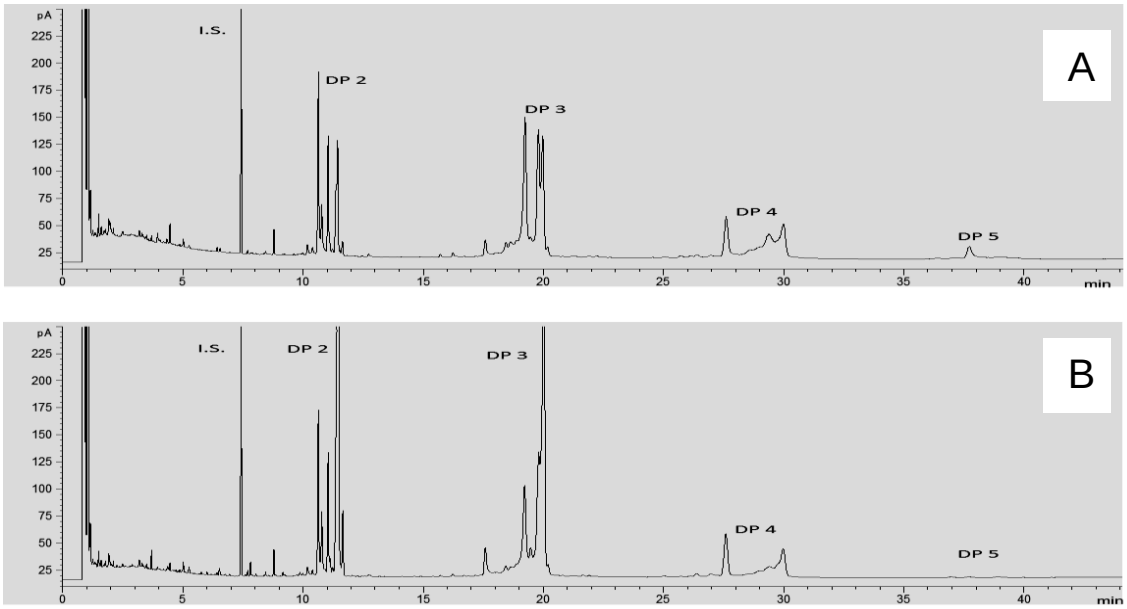
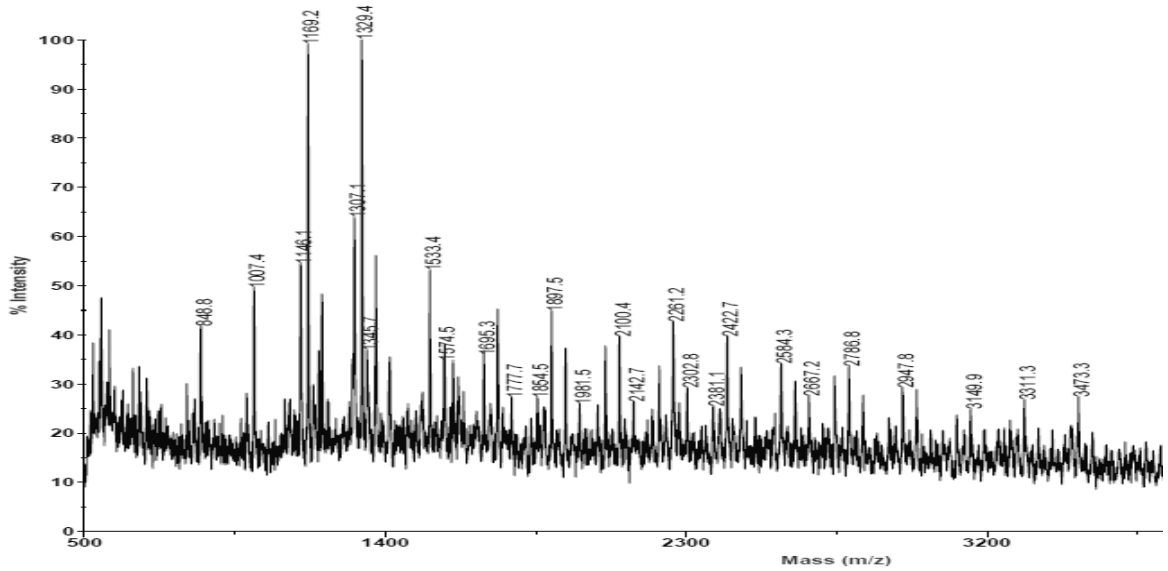


Figure 8


% Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
0
500
1400
2300
3200
848.8
1007.4
1146.1
1169.2
1307.1
1329.4
1345.7
1533.4
1574.5
1695.3
1777.7
1854.5
1897.5
1981.5
2100.4
2142.7
2261.2
2302.8
2381.1
2422.7
2584.3
2667.2
2786.8
2947.8
3148.9
3311.3
3473.3