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Article

ESTIMATION OF THE MOLECULAR WEIGHT OF CHITOSAN BY PAGE

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Abstract

The estimation of the molecular weight of chitosan, a natural polymer consisting of molecules with masses ranging from 10 to 10^3 kDa, using polyacrylamide gel electrophoresis was investigated. The polyacrylamide gel was adapted for the migration of positive charged polymers. As application of electrophoresis of chitosan, time course of the hydrolysis of chitosan with hydrochloric acid and with some enzymes (chitinase, cellulase and α-amylase) used alone or in mixture, was studied. The preliminary results have shown that cellulase has a relatively small chitinolytic activity and that in the presence of cellulase and α-amylase, chitinase has an increased activity towards the glycosidic bounds of monomers from chitosan.

Keywords: polyacrylamide gel electrophoresis, chitosan hydrolysis, chitinase, α-amylase, cellulase.

1. Introduction

Chitosan is a polymer that is obtained by the deacetylation of chitin which is the second most abundant polysaccharide from the world after cellulose. Chitosan is a polymer formed

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from monomeric units of glucosamine (GA) and N-acetyl glucosamine (NAG) linked by glycosidic bonds in different ratio [1]. The number of monomeric units in a polymeric chain, i.e. the molecular weight, greatly depend on the natural source of chitin[2]. By chemical or enzymatic hydrolysis, shorter chains of oligosaccharides can be obtained. For chemical hydrolysis of chitosan, strong acids such as hydrochloric acid [3-6], phosphoric acid [7,8], chemical compounds like hydrogen peroxide [9-11] are used, sometime in ionic liquids [12-14]. Enzymatic hydrolysis of chitosan for the production of oligosaccharides involves enzymes like chitinase and chitosanase that are enzymes with a high specificity towards the chemical structure of chitosan. There are mentioned examples when other types of enzymes, like pectinases, cellulases, α-amylases, β-amylases[15], pepsin, proteases, lipases[16], lysozyme and even some tissue extracts from different organisms[3,16] can hydrolyze chitosan.

Although many papers have aimed to identify the degree of acetylation and molecular weight of chitosan by different methods this goal remains a challenge for research on chitin and chitosan.

In this work the determination of depolymerisation degree of chitosan by an electrophoresis procedure was studied.

The aim of this study was the determination of depolymerisation degree of chitosan by electrophoresis procedure. The paper of Audy and Asselin [3] was used as starting point, where some preliminary results regarding the migration of chitosan oligomers on polyacrylamide gel electrophoresis were presented. Mainly, PAGE is used for separation of proteins, but in this study it was investigated the migration in the electric field of charged chains of chitosan. As application, the time course of the hydrolysis reaction of chitosan in the presence of hydrochloric acid or of some enzymes was studied.

2. Materials and Methods

2.1. Reagents

Most of the reagents were purchased from Carl Roth or Sigma Aldrich: urea (Sigma Aldrich, #15604, acetic acid (Sigma Aldrich ,#27225) acrylamide (Fluka, #01700), N, N' – methylenebis acrylamide (Sigma Aldrich, #146072), N, N, N', N' – tetramethylethylenediamine (Sigma Aldrich, #T22500), coomassie brilliant blue R250 (Carl Roth, #3862.1), 2-propanol (Sigma Aldrich, #33539), brilliant green (Carl Roth, #0324.1), ammonium persulphate (Sigma Aldrich, #215589), sodium acetate (Carl Roth, #6773.1), sodium hydroxide (Sigma Aldrich, #30620) chitosan low molecular mass (Sigma Aldrich, #448869).

2.2. Enzymes

The enzymes used for hydrolysis of chitosan were: chitinase from *Streptomyces griseus* (Sigma Aldrich, #6137), cellulase from *Aspergillus niger* (Sigma Aldrich, #22178), α-amylase from bacteria (Merck, #1329).

2.3. Buffers and solutions

The following solutions were realized: *Chitosan solution:* 0.5% chitosan in 2% acetic acid, pH 4.6 adjusted with NaOH; *Running buffer*: 5% acetic acid; *Sample buffer*: 1,8 mL 10 M urea, 100 μL dH_2O, 100 μL 100% acetic acid, 2 mg green brilliant; *Staining solution*: 0.05% coomassie brilliant blue (CBB), 25% isopropanol, 10% acetic acid; *Destaining solution*: 10% acetic acid; *Enzymes solutions*: *chitinase solution*: 1mg/mL chitinase 0.2 U/mg in potassium phosphate buffer, 0.5M, pH 6, *cellulase solution*: 1mg/mL celluase 1.14 U/mg in sodium acetate buffer 0.1M, pH 4.5, *α-amylase solution*: 1mg/mL α-amylase 130 U/mg in H_2O.

2.4. Instruments

A Mini-protean III cell (Bio-Rad) electrophoresis system was used to run the electrophoresis at a constant voltage (80V per slab gel), using a Power PAC 300 source. The gels were realised using a casing gel mold and 8x10 cm glass plates.

For performing the hydrolysis reaction at various temperatures, the samples were incubated in a thermal cycler (Applied Biosystems).

2.5. Methods

2.5.1. Chitosan hydrolysis with hydrochloric acid

Portions of 0.5 g chitosan were hydrolysed in 10 mL of 37% hydrochloric acid at 70^0C for 123 hours. Samples were taken at various time intervals: 0, 2, 4, 8, 24, 29, 52, 76, 100 and 123 h. The reaction was stopped by keeping the samples at -20^0C until used in experiments PAGE.

2.5.2. Chitosan hydrolysis with enzymes

To 1 mL of 0.5% chitosan in 2% acetic acid, 200 μL of solution of each of the studied enzyme (chitinase, cellulase and α-amylase) was added and incubated at 37ºC, under constant

mixing (250 rpm). Combination of two or of all three enzymes were also used for hydrolysis of chitosan. From each reaction mixture, at define time intervals (0, 1, 2, 4, 6, 8, 24, 48 and 72 hours) samples were collected. To inactivate the enzymes, these samples were warmed at 100ºC in PCR thermoblock, for 10 minutes. The samples were kept at - 20ºC until analyzed by PAGE.

2.5.3. Acid acetic-urea PAGE

To separate the chitosan chains by electrophoresis in polyacrylamide gels, the starting point was the work of Audy and Asselin [3]. The acrylamide gels with 12% concentration were prepared as follows: 2.12 mL H_2O, 3.75 mL 7 M urea, 0.2 mL 5,5% acetic acid, 4 mL 30% acrylamide, 20 µL TEMED, 40 µL 20% APS were mixed and poured in the casing gel mold. Before loading on the gel, the samples were mixed 2:1 with sample buffer and boiled for 3 minutes. A volume of 5 µL of each sample was loaded in sample wells and the electrophoresis was run at 80 V, for about 55 minutes, until the dye reached near the edge of the gel.

2.5.4. Staining and destaining of the gels

For staining, the gels were immersed in the staining solution, boiled for few seconds (in microwave oven) and cooled at room temperature for 10 minutes, under slow shaking. Destaining was realized in 10% acetic acid, boiling the gels in microwave oven for 1-2 seconds and cooled under gentle shaking for 10 minutes. These step was repeated for few times to obtain clear gels with well colored bands.

3. Results and Discussions

The main aim of this study was to find a simple, rapid and reliable method to assess the degree of polymerization or the molecular weight of chitosan polymers. The selected method has to be fitted to study the hydrolysis of chitosan in chemical or enzymatic reactions. Among the studied method, the electrophoresis in polyacrylamide gels drew our attention as PAGE is chiefly used for separation of proteins, rarely for separation of nucleic acids and seldom for separation of other type of polymers. In fact, only one article was published on strictly the subject of separation of chitosan using PAGE[3], paper that was used as starting point in this study.

In Figure 1 there is presented the electrophoresis of a chitosan sample subjected to hydrolysis with hydrochloric acid. The first observation is that in contrast with the protein PAGE, the electrophoretic bands of chitosan are very wide. This is due to the fact that chitosan is a natural product (derived from chitin) and like the majority of the polysaccharides

do not have a unique molecular weight. The same sample of chitosan contains polymeric chains with the molecular mass ranging from 10 to 10^3 kDa, depending on the natural source of chitin[17].

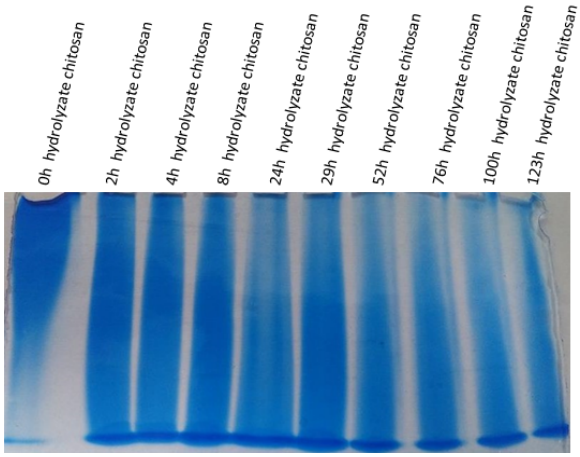

Figure 1. Acetic Acid – Urea Polyacrylamide Gel Electrophoresis of hydrolysed chitosan with HCl at different reaction time intervals.

In the first lane of electrophoresis presented in Figure 1 the band although is wide, proving that this chitosan sample contains chains having the molecular weight in a large range, do not contains polymeric chains with low molecular weight as the first lane is quite uncolored at its bottom. That means at the beginning of the hydrolysis experiment, the studied sample of chitosan contained only polymeric chains with relatively high molecular weights. Looking at the other lanes from the gel, one can see that as the hydrolysis time increases, the concentration of polymeric chains with low molecular weight increases too, as the bands become more colored in their lower part. This is a prove that PAGE can be a simple, rapid and convenient technique for the estimation of the hydrolysis reaction of chitosan.

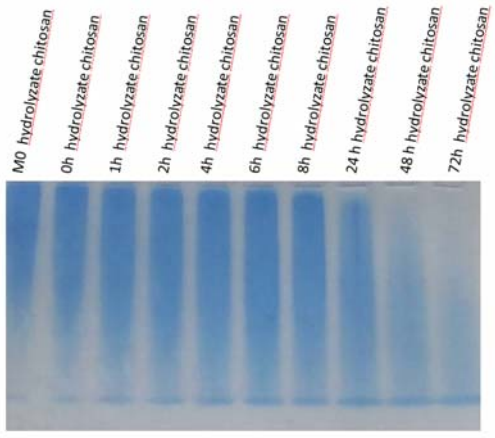

Figure 2. Acetic Acid – Urea Polyacrylamide Gel Electrophoresis of hydrolysed chitosan with chitinase at different reaction time intervals.

A similar electrophoresis pattern is obtained when the same type of chitosan is hydrolyzed with a commercial chitinase (Figure 2). On the first lane, corresponding to zero reaction time, the band is colored in the upper part and uncolored in the lower part, proving that the loaded sample contained polymeric chains with relatively high molecular weight.

As the reaction time increases, the representation of the polymeric chains tend to reverse, i.e. the lanes correspond to samples that contain less polymeric chains with high molecular weight and more small molecules, like oligochitosaccharides. From the lane corresponding to 72 hours of hydrolysis with chitinase, it becomes obvious that the sample has contained only small molecules and no long polymeric chains.

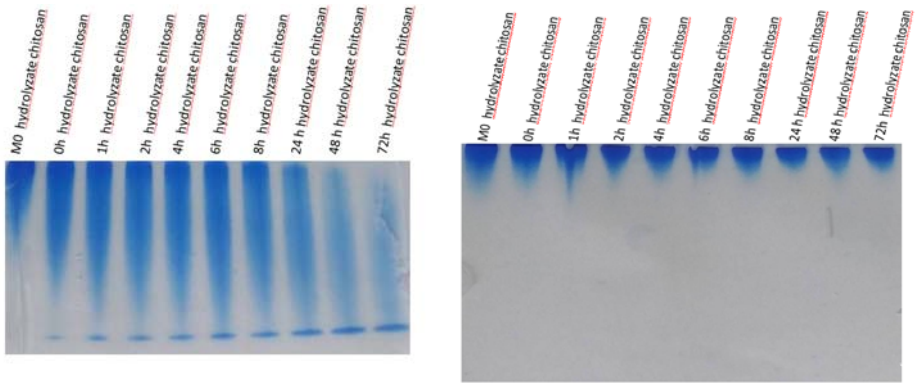

Figure 3. Acetic Acid – Urea Polyacrylamide Gel Electrophoresis of hydrolysed chitosan with a mixture of chitinase and α-amylase (left electrophoresis) and with α-amylase alone (right electrophoresis), at different reaction time intervals.

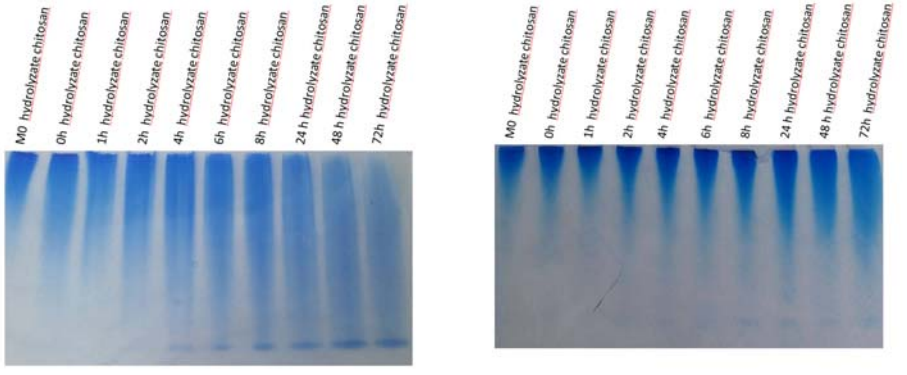

Figure 4. Acetic Acid – Urea Polyacrylamide Gel Electrophoresis of hydrolysed chitosan with a mixture of chitinase and cellulase (left electrophoresis) and with cellulase alone (right electrophoresis), at different reaction time intervals.

In another series of experiments it was studied the hydrolysis of chitosan with chitinase, α-amiylase and cellulase and combination of them. In Figure 3 the two electrophoresis present the reaction course of hydrolysis of chitosan with a mixture of chitinase and α-amylase and, for comparison the reaction course of hydrolysis of the same type of chitosan with α-amylase alone. From these two electrophoresis is clear that α- amylase do not hydrolyze chitosan, as the patterns of the lanes from the right electrophoresis, corresponding to the hydrolysis with α-amilase, do not change.

A different situation is when a similar experiment was carried out with cellulase (data presented in Figure 4). Although the mixture of chitinase and cellulase has a higher hydrolytic activity, as the right electrophoresis from Figure 4 shows, cellulase has a chitinolytic activity. In this stage of the research we cannot assign this chitinolytic activity to the cellulase, as the presence of a chitinase as an impurity of a chitinase cannot be excluded. Nevertheless, based on the results presented in Figure 4, the most plausible hypothesis is that cellulase can hydrolyse (with low activity) the glycosidic links between monomer units of chitosan.

To find out if the above assumption is correct, another experiment of hydrolysis of chitosan was realized with a mixture of all three enzymes: chitinase, cellulase and α-amylase (see Figure 5). As it was expected, the chitosan is hydrolyzed when it is mixed with these enzymes, with a higher velocity than when it was hydrolyzed only with chitinase or with binary mixture of chitinase and cellulase. Although this conclusion is based on a semi-quantitative estimation of the color intensities of the bands (specialized software Gel Anayser), it can be considered to be correct, if we take into account the observation that cocktails of hydrolytic enzymes have higher activities than one enzyme alone[18].

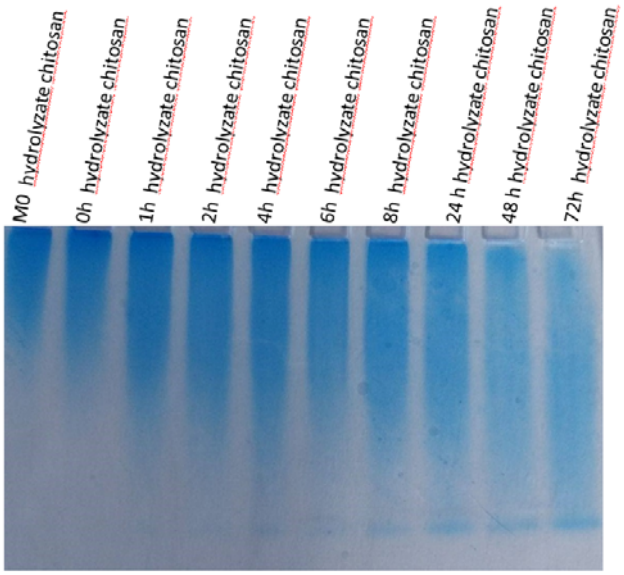

Figure 5. Acetic Acid – Urea Polyacrylamide Gel Electrophoresis of hydrolysed chitosan with a mixture of chitinase, cellulose and α-amylase, at different reaction time intervals.

The main drawbacks of the use of PAGE for estimation of the molecular weight of chitosan chains, as for any other polysaccharide bearing charged groups, is the fact that these natural polymers are synthetized by the organisms with chains with different lengths, sometime the range of the values of molecular weight range covering three or more degree of magnitude. Nevertheless, PAGE can be used to estimate the variation of the molecular mass of these polymers, as it is happen when the hydrolysis (depolymerization) reaction is studied.

4. Conclusions

A polyacrylamide gel electrophoresis was adapted to estimate the molecular weights of chitosan molecules and to study the reaction of hydrolysis of this polymer. The time course of hydrolysis of chitosan with hydrochloric acid, chitinase, α-amylase, cellulase, as well as with mixtures of these enzymes was examined.

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