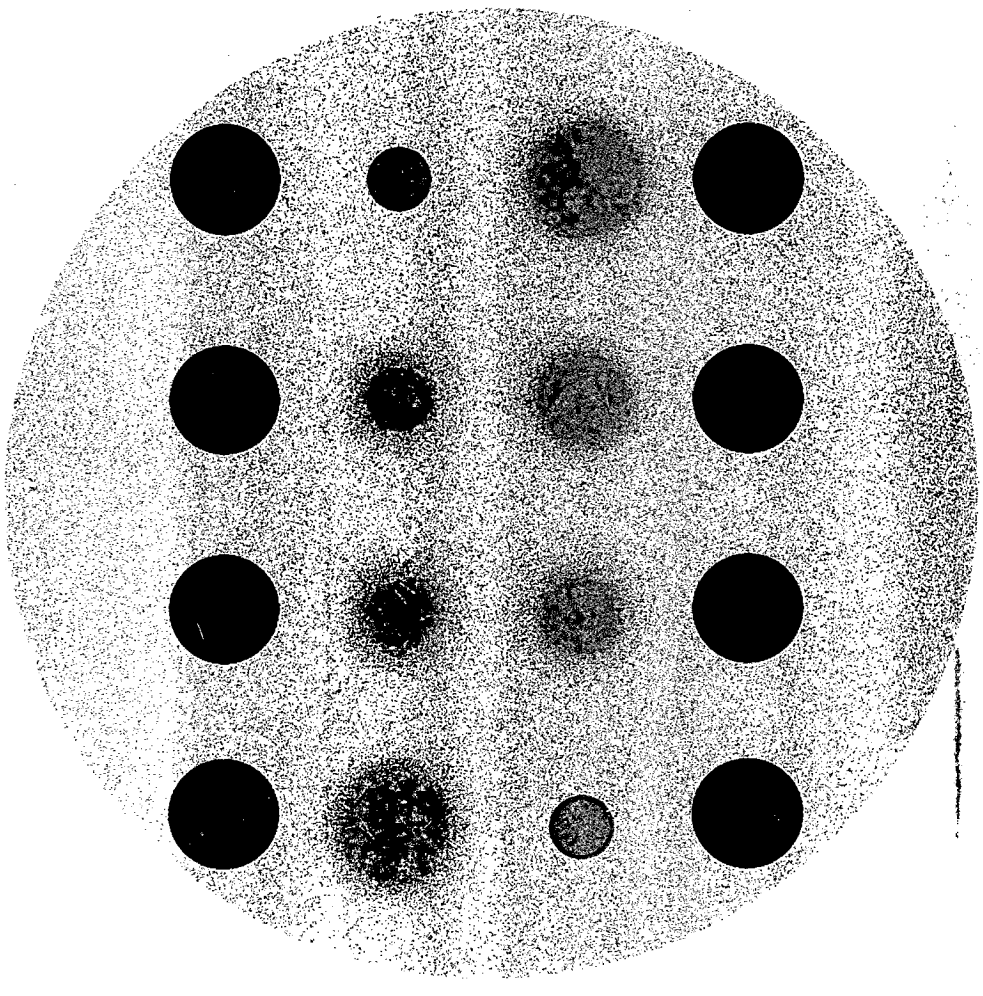
CHITIN HANDBOOK

Edited by
Riccardo A. A. Muzzarelli
and
Martin G. Peter



European
Chitin
Society

Chitin Handbook, R.A.A. Muzzarelli and M.G. Peter, eds.,
European Chitin Society. 1997. ISBN 88-86889-01-1

Overproduction of the recombinant chitinase A from *Serratia marcescens* in *E. coli*: purification, biochemical, and biophysical characterization

Constantinos E. Vorgias

Athens University, Biology Department,
Division of Biochemistry and Molecular Biology, Biochemistry Laboratory,
Panepistimiopolis-Kouponia, 15701 Athens, Greece,
Fax: +30-1-7257572, *e-mail: cvorgias@biology.db.uoa.gr*

A new fast and convenient procedure for purification of the *Serratia marcescens* chitinase A overproduced in *E. coli* is presented. The enzyme is characterized biochemically and biophysically. The thermal stability of chitinase A is investigated by protein melting experiments using Circular Dichroic Spectroscopy showing that the enzyme is rather stable at higher temperatures. The procedure described is suitable for preparation of larger quantities of chitinase A (>> 10 mg) that are required for structural and biophysical studies, as well as extensive biochemical analysis.

Materials and equipment

Chemicals: Bacto tryptone and bacto yeast from Difco; Ampicillin (Amp) from Sigma; Q-Sepharose and Phenyl-Sepharose from Pharmacia. All other chemicals, should be used in the highest available quality, as supplied by Merck, Sigma, or Serva. Glass- and plasticware are purchased from various local distributors.

Facilities for bacterial culture like shaker incubator, agar plates, an autoclave, a centrifuge with high volume capacity are required. For chromatography of chitinase A, glass columns and a conventional chromatography unit, consisting of a peristaltic pump, fraction collector, and optionally a continuous flow UV photometer with recorder. Analysis of the various protein fractions is performed by slab gel electrophoreses according to Laemmli (1970). For determination of the thermostability of the protein, a Circular Dichrograph is required.

Experimental procedure

Enzyme assay and protein determination: Chitinase A from *Serratia marcescens* is an exo-chitinase. Its enzymatic activity is measured conveniently by using *p*-nitrophenyl-*N,N'*-diacetyl-β-chitobioside [*p*-NP-$(GlcNAc)_2$] in 0.1 M phosphate buffer, pH 8.0, at 37°C. The release of *p*-nitrophenol is continuously monitored at 405 nm (E_{405} =18.5 $mmol^{-1} \cdot cm^{-1}$) using a 1 cm path length, temperature controlled cuvette. One unit of chitinase A activity is defined as the amount of enzyme that catalyzes the release of 1 $\mu mol \cdot min^{-1}$ of *p*-nitrophenol. Proteins are quantified by the method of Bradford (1976).

Cloning of the chiA *gene encoding chitinase A protein*: The initial isolation of *chi*A gene was carried by Jones *et al.* (1986) using classical molecular biology techniques (Sambrook *et al.*, 1989). The clone which is used for this protocol is a new construct, utilizing the original *chi*A gene and recloning it under the control of the oLpL operator and the promoter of bacteriophage λ. The constructed plasmid was named ppL*chi*A and introduced into the *E. coli* strain A2097 which harbours a defective λ prophage carrying a thermosensitive *c*I repressor. The final clone was named A5745 (Oppenheim *et al.*, 1990).

Cell growth and induction: For preparative purposes, 1 L of LB medium (Sambrook *et al.*, 1989) supplemented with 50 μg/mL of ampicillin (Amp) is inoculated with a single colony of the clone A5745 and grown overnight at 37°C in a shaker incubator. Next day, 4 L of LB medium with Amp are added to the culture. The LB medium is preheated at 42 - 43°C in order to raise the temperature of the culture to ca. 42°C and initiate the induction of chitinase A. The temperature of the incubator is adjusted to 37°C (practically, the temperature may reach 40°C) for the next 4 h.

Cell harvesting and analysis of induction products: The induced bacteria cell culture is removed from the shaker incubator and kept at room temperature. The bacteria are separated from the culture medium by low speed centrifugation, using e.g. a Sorvall centrifuge with a GSA rotor, at 4000 rpm, for 15 min at 4°C. Although a significant amount of chitinase A is secreted into the medium at 24 h after induction, the specific activity of the protein is lower as compared to the enzyme that is produced during the first 4 - 5 h of induction. Therefore, it is recommended to find an optimum between obtaining a lower amount of enzyme and its specific activity. This can be done in the following way (Fig. 1):

At the time intervals shown in Fig. 1, aliquots of 2 mL are withdrawn from the bacterial culture, 1 mL of which is used for determination of the cell density at OD_{600}. The remaining 1 mL is centrifuged in an Eppendorf centrifuge at full speed for 5 min at 4°C and the supernatant is carefully removed from the cell pellet, saved and kept at 4°C. The cell pellet is dissolved in sample buffer for SDS-PAGE at a final concentration of 0.1 OD_{600} / 10 μL sample buffer. The proteins in the supernatant are precipitated by addition of cold trichloroacetic

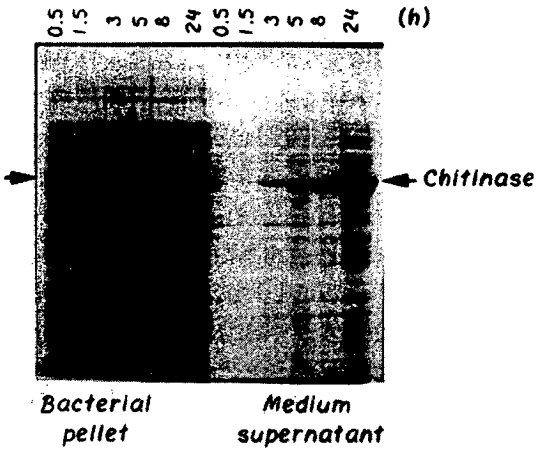


Figure 1. Electrophoretic analysis of the bacterial pellet and culture medium supernatant.

acid (TCA) to give a final concentration of 5 %, followed by full speed centrifugation in an Eppendorf centrifuge for 10 min. The pellet is washed twice with cold acetone in order to remove remaining TCA. Finally, the protein pellet is air dried and dissolved in sample buffer for SDS-PAGE. The volume of sample buffer for SDS-PAGE is adjusted according to the OD_{600} of the cell culture in order to obtain comparable amounts of cells and protein in the medium. If, for example, the cell density is 0.6 OD_{600}, the bacteria pellet as well as the protein pellet is dissolved in 60 µL sample buffer. For SDS-PAGE, 15 µL of each sample are loaded onto a medium size gel. In the experiment described here, it was found that the best source for enzyme isolation is obtained after 4 - 5 h heat induction. All further steps are carried out at 4°C.

Column chromatography

A 50 mL Phenyl-Sepharose CL-6B column is equilibrated by extensive washing (at least 500 mL) with 0.3 M ammonium sulfate in buffer A (20 mM Tris-HCl, pH 8.0, containing 0.1 mM phenylmethanesulfonyl fluoride) at a flow rate of 400 to 600 mL/h. Adjust the cell free medium supernatant to 0.3 M ammonium sulfate in buffer A. Take care to add the ammonium sulfate slowly and under continuous stirring. The sample volume (> 5 L) is loaded directly on the Phenyl-Sepharose CL-6B column. The column is then washed with 0.3 M ammonium sulfate in buffer A (usually 1 L) until the recorder shows the baseline.

Elution with a 1000 mL linear descending gradient of 0.3 M to 0 M ammonium sulfate at a flow rate of 200 mL/h is followed by further washing with 500 - 600 mL buffer A. Fractions of 10 mL are collected (Fig. 2). Chitinase A is eluted at the end of the descending gradient and during the first 200 - 250 mL of the final wash with buffer A. Fractions containing chitinase A are identified by 0.1 % SDS / 12.5 % PAGE and enzymatic activity as described above. Fractions are pooled according to the results of SDS-PAGE analysis and assay of enzyme activity. Usually, the first chitinase A fractions contain impurities while the latest fractions are almost pure. Impure chitinase A fractions can be repurified by column chromatography using the same procedure.

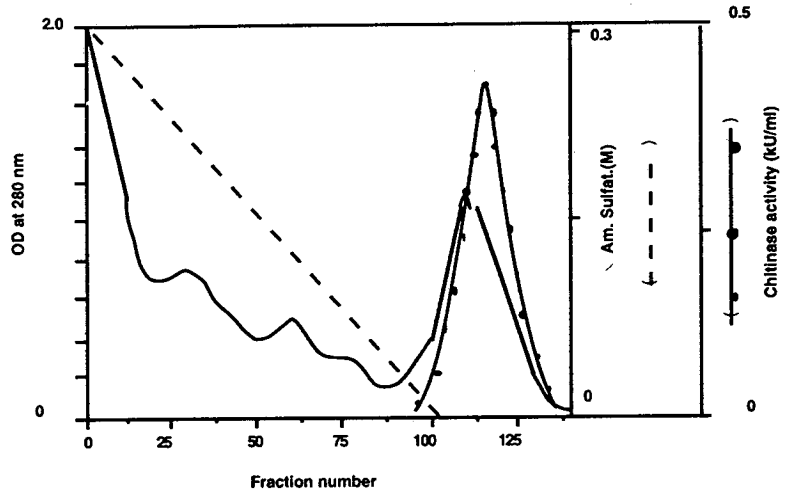


Figure 2. Chromatography of the bacterial culture supernatant on Phenyl-Sepharose CL-6B at pH 8.0 in buffer A. Chitinase A elutes at the end of the ammonium sulfate descending gradient as a broad symmetric peak.

Chitinase A is further purified by chromatography on a 10 - 20 mL Q-Sepharose column which is equilibrated with buffer A. This step can be done either with batches or with the entire volume of the enzyme solution obtained after chromatography on Phenyl-Sepharose CL-6B. The pooled chitinase A fractions are loaded onto the column. Impurities are bound to the column while the eluate contains highly pure chitinase A. Since the eluate has a rather large volume (200 - 300 mL), an Amicon ultrafiltration device equipped with a 30 kDa cut-off filter is used to concentrate the solution. During this procedure, ca. 10 % of the protein sticks to the membrane. In order to minimize protein loss in subsequent ultrafiltration steps, it is recommended to re-use the membrane which can be stored in a refrigrator under sterilized water / 20 % ethanol. In a typical

example, the specific enzyme activity is 530 U/mg and the overall yield is ca. 80 %.

Properties of chitinase A

The apparent molecular weight of chitinase A, as determined by SDS-PAGE is 62 kDa. This is in good agreement with the molecular weight expected from the gene sequence. It indicates that the leader sequence was removed during the secretion of chitinase A into the medium (Oppenheim *et al.*, 1990).

Catalytic activity: The reaction velocity is linear in the range between 20 and 100 µmol *p*-NP-$(GlcNAc)_2$ per 20 to100 ng of pure enzyme. In a standard protocol, an enzyme concentration of 30 ng/mL and 50 µM substrate is used. Under these conditions, the effect of pH and temperature on the activity of chitinase A can be examined. In 100 mM phosphate buffer, the optimum activity is found at pH between 7.5 and 8.5; optimum temperature: 52°C; K_m = 500 µM for *p*-NP-$(GlcNAc)_2$, as determined from a Lineweaver-Burk plot.

Iso-electric point: The pI of chitinase A is determined by isoelectric focusing (IEF) under non-denaturing conditions, using a Mono P (5X5HR) FPLC column (Pharmacia). The column is equilibrated with starting buffer (25 mM diethanolamine-HCl, pH 9.5). A protein sample of 0.5 mg is pre-equilibrated in starting buffer before loading it to the column. The column is developed with 13 mL of a buffer prepared by mixing 1 mL Pharmalyte 8-10.5 and 5.2 mL Polybuffer 96, and adjusting the pH to 7.0 with HCl, then filling up to 100 mL with distilled water. The column is run at a flow rate of 1 mL/min while fractions of 0.5 mL are collected. The pH of each fraction is measured using a Beckman SS-1 pH meter equipped with a microelectrode. The isoelectric focusing pattern of chitinase A under nondenaturing conditions reveals a major single peak for the enzyme with p_I = 8.6. Another small peak, containing ca. 5 % of the total protein, appears at pH ca. 8.2 - 8.4. This could be due to a yet undetermined post-translational modification.

Melting temperature: Circular Dichroism spectroscopy is used to follow the secondary structure of a protein under various conditions, in particular temperature dependence. The spectra are measured in an Auto Dichrograph Mark V (Jobin Yvon), using a 0.2 mm cuvette. Temperature control is achieved by means of an external water circulation device and a heating rate electronic controller. Spectra from 200 to 245 nm are recorded in intervals of 1.0 nm. Temperature dependent protein denaturation is followed by measuring the ellipticity at 220 nm wich is characteristic of the α-helical structure. The temperature gradient is adjusted to 0.5°C / min. Native and denatured forms are defined by the pre- and post-transition state baseline, respectively. The melting temperature, T_m, is defined by the inflexion point of the curve, indicating 50 % denaturation (Fig. 3).

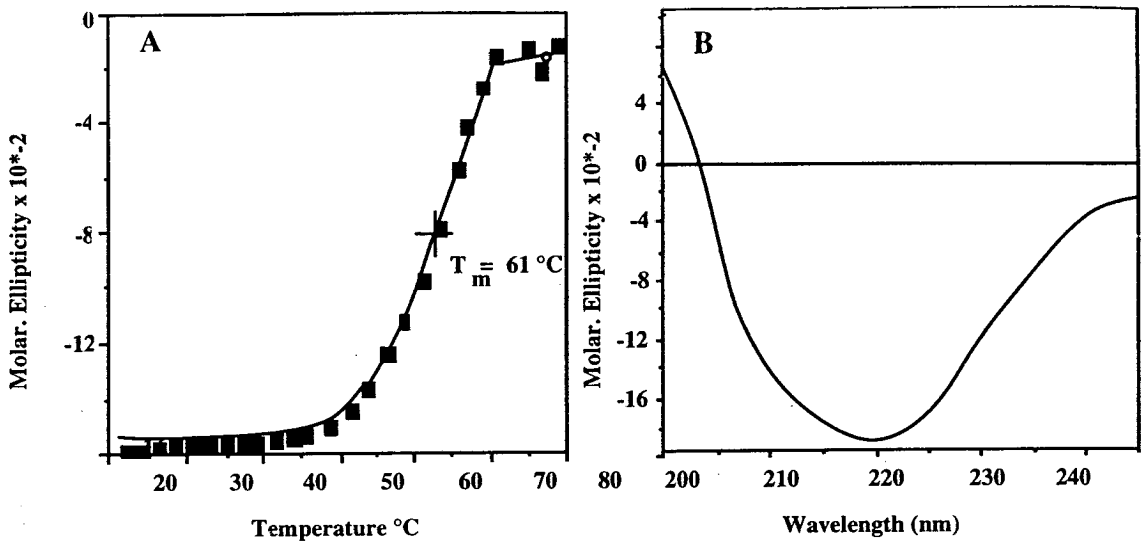


Figure 3. (A) Melting curve of chitinase A in 10 mM phosphate buffer, pH 8.0, at a protein concentration of 0.2 mg/mL. T_m is the mid-point of the low temperature and high temperature plateau. (B) Far-ultraviolet CD spectrum of chitinase A in 20 mM phosphate buffer, pH 7.0.

Advantages and limitations of the method

The protocol for purification of chitinase A is reasonably short, allowing the preparation of 20 to 50 mg of pure and active chitinase A in a time schedule of a few days. The protein should be treated as gently as possible. It can be used for various purposes like crystallization studies, biophysical and biochemical analysis, preparation of antibodies etc. The fact that the protein could be crystallized is another strong evidence that it is obtained in high purity.

A limitation of the method is seen in the fact that the Phenyl-Sepharose column can be used only once. Even after proper regeneration, the performance of the column is significantyl reduced.

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