Purification and characterization of the recombinant chitin degrading enzymes, Chitinase A and Chitobiase from *Serratia marcescens*

Constantin E. Vorgias*, Ivo Tews, Anastassis Perrakis, Keith S. Wilson and Amos B. Oppenheim¶

European Molecular Biology Laboratory (EMBL), c/o DESY, Notkestrasse 85, 2000 Hamburg 52, Germany. Tel: +49-40-89902118, Fax: +49-40-89902149. ¶ Department of Molecular Genetics, P.O.B. 1172, The Hebrew University-Hadassah Medical School, Jerusalem 91010, Israel. *Corresponding author

---

Summary

Convenient new procedures for the purification of the *Serratia marcescens* chitin degrading enzymes Chitinase A and Chitobiase overproduced in *E. coli* are presented. The procedures are short and facilitate the purification of both recombinant enzymes in considerable amounts, high purity and high specific activity. The purified enzymes have been characterized biochemically and biophysically. The thermostability of both enzymes was investigated by protein melting experiments using circular dichroic spectroscopy and differential scanning calorimetry. Chitinase A has remarkable heat stability compared to Chitobiase.

Chitinase A, Chitobiase, *Serratia marcescens*, thermostability, overexpression, purification.

Introduction

Chitin is a polysaccharide homopolymer of *N*-acetyl-D-glucosamine in β(1->4) linkage. It is one of the major structural components of the arthropod exoskeleton and of the cell wall of almost all fungi (1). Bacteria produce enzymes that hydrolyze chitin which is degraded in two sequential steps and used as energy source. In the first step an endochitinase (E. C. 3. 2. 1. 14) randomly hydrolyzes the glycosidic linkages of chitin producing multimers of GlcNAc among which *N, N´*-diacetylchitobiose is predominant. In the second step Chitobiase (E. C. 3. 2. 1. 30) converts these multimers to GlcNAc (2 - 4). The production of Chitinase A by higher plants serves as defense mechanism against fungal pathogens. The way in which Chitinase A, as well as other pathogen-related proteins, contribute to pathogen resistance is under intensive investigation (5 - 12). During the last decade renewed interest on chitin degrading enzymes has led to the isolation of genes coding for Chitinases and Chitobiases from a variety of organisms (13). The primary structure, expression, cellular localization and antifungal activity of most of the Chitinases isolated has been characterized. However, the properties of these enzymes at the biochemical and structural level remain to be examined. We are interested in determining the 3-D structure of both chitin degrading enzymes. A source of relatively high amounts of pure and well characterized protein is necessary. Both Chitinase A and Chitobiase from *Serratia marcescens* have been cloned. The characterization of the clones

is described in (14 - 16). Previously we described the crystallization of Chitinase A (17) and Chitobiase (18). The aim of this paper is to present purification protocols for the preparation of highly pure enzymes overproducsed in *E. coli* and their biochemical and biophysical characterization.

Materials and Methods

Materials

Bacto tryptone and bacto yeast were purchased from Difco Labs. The antibiotics used for the bacterial culture were from Sigma. Chromatographic media were from Pharmacia. All other chemicals used for this study were in the highest available quality from Merck, Sigma and Serva and used according to the specifications of the manufacturer.

Cell growth, induction and harvesting

The construction of individual *E. coli* clones A5745 and A5441 which produce Chitinase A and Chitobiase, respectively, is described in (15, 16). The cells were collected by low speed centrifugation. In the case of Chitinase A the supernatant medium was used while in the case of Chitobiase the cell pellet was stored frozen until used.

Purification of recombinant Chitinase A

All further procedures were carried out at 0 to 4 °C, unless otherwise specified. In a routine preparation a 5 litre bacterial culture of the A5745 clone was centrifuged at 6000 x g for 10 min and the supernatant was saved. The supernatant was adjusted to 0.3 M ammonium sulfate, 20 mM Tris-HCl pH 8.0 and 0.1 mM PMSF (buffer A). The solution was directly applied on a Phenyl-Sepharose column equilibrated with 0.3 M ammonium sulfate in buffer A and loaded at a flow rate of 400 to 600 ml/h. Bound Chitinase A was eluted with a 250 ml linear descending gradient of ammonium sulfate at a flow rate of 60 ml/h and 10 ml fractions were collected. The column was further washed with buffer A. Fractions containing Chitinase A activity were analyzed by 0.1% SDS - 12.5% PAGE and pooled. The first Chitinase A fractions were eluted from the column with other impurities while the latest fractions were very pure. Impure Chitinase A fractions were repurified using the same procedure. Highly pure Chitinase A was recovered in the flow through of a Q-Sepharose column in buffer A. The protein was concentrated by Amicon ultrafiltration.

Purification of recombinant Chitobiase

The recombinant Chitobiase was purified from the A5441 *E. coli* clone. The enzyme was found to be secreted into the periplasm (16, 19). The cells were suspended in 3 to 5 ml per g cell paste in buffer B (100 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.5 M Sucrose, 0.1mM PMSF, 1 mM DTT). 100 µl of 2 mg/ml lysozyme solution per ml of suspension were added and the mixture incubated on ice for 20 min. An equal volume of ice-cold water was added and kept on ice for the next 20 min. Finally the extract was adjusted to 5 mM $MgCl_2$ and centrifuged at 15000 x g for 20 min. The supernatant contained the periplasmic fraction of the *E. coli* cells. Chitobiase was precipitated between 55 and 85% saturated ammonium sulfate. The protein was dissolved in a large volume of buffer C (10 mM Na-phosphate pH 8.0, 1 mM EDTA, 0.1 mM PMSF, 1 mM DTT) and the Chitobiase enriched fraction was directly applied on a S-Sepharose FF, equilibrated in buffer C. Bound protein was eluted with a 15 to 20 column volume 0 to 0.5 M NaCl gradient in buffer C, at a flow rate of 60 ml/h and 10 ml fractions were collected. The Chitobiase activity assay was used to select and combine the proper fractions which were also checked by 0.1% SDS - 12.5% PAGE. Chitobiase fractions were concentrated on a Mono-S column using FPLC.

Isoelectric focusing

Both purified enzymes were analyzed by isoelectric focusing (IEF) under non-denaturing conditions. A Mono P (5X5HR) FPLC column was used according to the manufacturer's instructions. Briefly, the Mono P column was equilibrated in starting

buffer (25 mM diethanolamine-HCl pH 9.5) and 0.5 mg of protein sample pre-equilibrated in starting buffer was applied to the column. The Mono-P column was developed with 13 ml Polybuffer 96. The column was run at 1 ml/min flow rate and 0.5 ml fractions were collected. The pH of each fraction was measured using a Beckman SS-1 pHmeter equipped with a microelectrode.

Assay for Chitinase A and Chitobiase activity

Although Chitinase A form *Serratia marcescens* is a exochitinase we have determined its enzymatic activity by measuring the hydrolyis of the synthetic substrate pN-$(GlcNAc)_2$ in 0.1 M phosphate buffer at pH 8.0, at 37 °C. The enzymatic activity of Chitobiase was determined by measuring the hydrolysis of pNP-NAGA under the same conditions. The release of p-nitrophenol was continuously monitored at 405 nm (ε_{405} =18.5 $mmol^{-1}$ cm^{-1}) using a 1 cm path length temperatted cuvette. One enzyme unit was defined as the amount of enzyme that catalysed the release of 1 µmol p-nitrophenol per min under the conditions described above.

Thermal denaturation experiments

The thermal denaturation of Chitinase A and Chiotobiase was measured using CD and DSC spectroscopy. The CD spectra were measured in an AUTO DICHROGRAPH mark V, (JOBIN YVON, Division D'Instruments S.A). The sample was connected to an external LKB thermostat. Denaturation was followed by measuring the ellipticity at 220 nm, characteristic of α-helices in the protein, as a function of the temperature. The temperature increase was adjusted to 0.5 °C per min. DSC measurements were carried out in the MC-2 Ultrasensitive Scanning Calorimeter (Microcal Inc. MA, USA) at a heating rate of 0.5 °C per min. In both measurements the protein was in 20 mM phosphate pH 7.0, 1 mM EDTA at a concentration between 1.2 and 2.5 mg/ml.

Other methods

The protein concentration was determined by the Bradford method as described in (20). 0.1% SDS - 12.5% PAGE was run according to the Laemmli system (21) and stained with Coomassie Blue G-250.

Results and Discussion

Purification of Chitinase A overproduced in *E. coli*

In a routine preparation about 5 litre induced *E. coli* cells harbouring the ppL*chi*A plasmid were heat induced for 4 to 5 h. During that induction period Chitinase A is the major protein secreted into the medium. Prolonged induction resulted the appearance of several other proteins and partial degradation of Chitinase A. The cell free culture medium was adjusted to 0.3 M ammonium sulfate and the pH to 8.0 with Tris-HCl. The medium was applied on a Phenyl-Sepharose column to which the Chitinase A was bound, and eluted as a symmetric peak at the very end of a 0.3 to 0 M ammonium sulfate descending gradient. The next and final purification step was a Q-Sepharose column at pH 8.0. Under the conditions described in methods, Chitinase A was run through the column leaving several impurities bound to it. The resulting protein is more than 90% homogeneous as shown by 0.1% SDS - 12.5% PAGE in Figure 2 (lane A) and IEF (data not shown). Each purification step was monitored by 0.1% SDS - 12.5% PAGE and determination of the enzymatic activity. The specific activity was measured to be 530 U/mg and the overall yield is about 80%.

Purification of Chitobiase produced in *E. coli*

E. coli cells harbouring the pCBII plasmid were grown at 30 °C and the Chitobiase was constitutively expressed. The protein was localised in the periplasm of the *E. coli* cells (16). Therefore, the cells were resuspended in a hypertonic buffer, the cell wall was digested with lysozyme and after a centrifugation the resulting supernatant contained the

periplasmic fraction (19). The supernatant was further fractionated by ammonium sulfate. Chitobiase was precipitated between 55 and 85% saturated ammonium sulfate. This fraction contained 38% of the total periplasmic proteins and 80% of the total recovered Chitobiase activity. The next and final purification step was a S-Sepharose column at pH 8.0. Under the conditions described in methods, Chitobiase was bound to the column and eluted via a salt gradient between 250 and 300 mM NaCl as shown in Figure 1. The Chitobiase peak contained 50% of the ammonium sulfate precipitable activity. This fraction was used as the Chitobiase fraction because of its high specific activity. The prepared protein is more than 90% pure as shown by 0.1% SDS - 12.5% PAGE in Figure 2 (lane B) and IEF (data not shown).

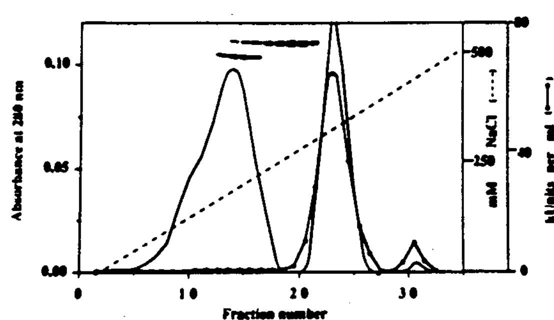

Figure 1. Column chromatography profile of the bacterial periplasmic extract after ammonium sulfate fractionation, enriched in Chitobiase and applied on a S-Sepharose column at pH 8.0 in buffer C. The Chitobiase was eluted between 200 and 300 mM NaCl as the major symmetric peak (fractions 19-26, for details see methods).

Enzymatic properties of the purified Chitinase A and Chitobiase

The purified enzymes were further characterized by determining the catalytic properties for the substrates used to determine the enzymatic activity. Chitinase A activity was measured as described in methods using the synthetic substrate *p*N-$(GlcNAc)_2$.

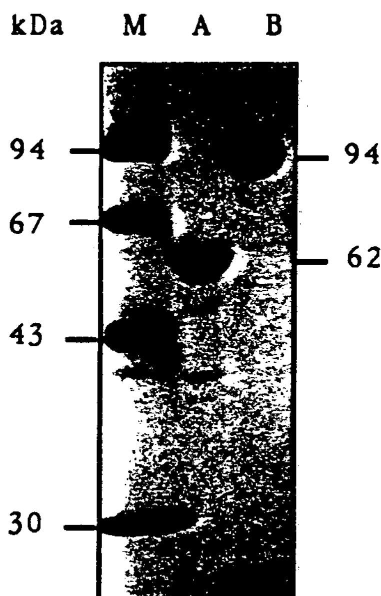

Figure 2. 0.1% SDS - 12.5% PAGE of the purified enzymes Chitinase A (A) Chitobiase (B), and molecular weight markers (M) (for details see methods). The apparent molecular weight of both enzymes was calculated to be 62,000 and 94,000 daltons, respectively. Chitinase A is in a good agreement with the expected molecular weight from the gene sequence. This indicates that during the secretion of Chitinase A into the medium the leader peptide was removed (15). The same or similar mechanism may have removed the leader sequence of Chitobiase during the translocation of the recombinant enzyme from the cytoplasm to the periplasm of *E. coli*.

The velocity of the degradation of this substrate is in the range of 20 to 100 µM per 20 to 100 ng pure enzyme. Therefore we used as assay conditions an enzyme concentration of 30 ng/ml and 50 µM substrate. Under these conditions the effect of pH

and temperature on the activity of Chitinase A were examined. The optimum activity was found in 100 mM phosphate buffer at pH between 7.5 and 8.5. 52 °C was the optimum temperature for activity. A K_m for *p*N-$(GlcNAc)_2$ of 500 µM was determined according to the method of Lineweaver and Burk in the concentration range described above.
Chitobiase activity was measured as described in methods using as substrate *p*NP-NAGA in the range of 20 to 150 µM for 20 to 150 ng pure enzyme. We used as assay conditions 40 ng/ml enzyme concentration and 70 µM for the substrate. The pH optimum in 100 mM phosphate buffer was between 7.5 and 8.5. 42 °C was the optimum temperature for Chitobiase. The K_m value for pNP-NAGA was estimated as 165 µM.

Isoelectric focusing

IEF was performed using a Mono P column as described in methods. The isoelectric focusing pattern of Chitinase A and Chitobiase under nondenaturing conditions revealed a major single peak for each enzyme. The isoelectric points are 8.6 for Chitinase A and 7.1 for Chitobiase.

Protein melting experiments

Figure 3 presents the melting curves for Chitinase A and Chitobiase. Assuming that the unfolding process follows a two-state mechanism, native and denatured forms were defined the pre- and post-transition state baseline, respectively. T_m, melting temperature was defined the temperature value where 50% of the denaturation was reached. Preliminary experiments have shown that the experimental determination of the T_m depended on the denaturation rate. A denaturation rate of 0.5 °C per min was found to be the best in terms of reliability and reproducibility. Using the DSC, T_m values of 51 °C for Chitobiase and 59 °C for Chitinase A were measured, which are in reasonable agreement with the CD values.

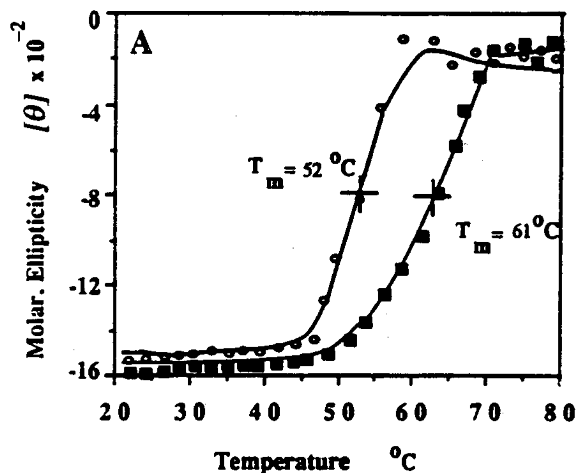

Figure 3. Melting curves of Chitinase A (o) and Chitobiase (■) in 10 mM phosphate at pH 8.0 at protein concentration of 2 mg/ml. The melting of both enzymes was monitored at 220 nm at different temperatures with automated recording. T_m is the mid-point of the low temperature and high temperature plateaus.

The aim of this study was the development of a rapid and efficient purification scheme for crystallization studies. The efficiency of the purification protocols has led to the successful crystallization of both enzymes (17, 18). We are now proceeding with three-dimensional analysis of the two proteins.

Acknowledgments

We thank P. M. Padas for helping in running the CD experiments. Prof. Dr. J. F. Holzwarth for free access to the calorimeter of Fritz-Haber Institut of Max-Planck Society and R. Clarke for helping us to measure and evaluate the DSC spectra.

Abbreviations

GlcNAc, *N*-acetyl-D-glucosamine; CD, circular dichroism; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide-gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; T_m, melting temperature; *p*N-$(GlcNAc)_2$, *p*-Nitrophenyl-β-D-*N,N'*-Diacetylchitobiose; *p*NP-NAGA, *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine; DSC differential scanning calorimetry; IEF, isoelectric focussing.

References

1. Muzzarelli, R. A. (1977) Chitin. Pergamon Press, New York, pp 155-181.
2. Jeuniaux, C (1966) Methods Enzymol. **8**, 644-650.
3. Agrawal, K. M. L and Bahl, Om. P. (1972) Methods Enzymol. **28**, 720-728.
4. Bahl, Om. P. and Agrawal, K. M. L. (1972) Methods Enzymol. **28**, 728-734.
5. Chet. I., Cohen, E. and Elster, I. (1986) in: Muzzarelli, R., Jeuniaux, C.and Gooday, G. W (eds) Chitin in nature and technology. Plenum Press, N. York, pp. 237-240.
6. Boller T. (1986) in: Muzzarelli, R., Jeuniaux, C.and Gooday, G. W (eds) Chitin in nature and technology. Plenum Press, N. York, pp. 223-231.
7. Legrand, M., Kauffman, S., Geoffroy, P. and Fritig, B. (1987) Proc. Natl. Acad. Sci. **84**, 6750-6754.
8. Kombrink, E., Schroder, M. and Hahlbrock, K. (1988) Proc. Natl. Acad. Sci. **85**, 782-786.
9. Nasser, W., de Tapia, M., Kauffman, S., Montasser-Kouhsari, S. and Burkard, G. Plant Mol. Biol. **11**, 529-538.
10. Joosten, M. H. A. J. and de Wit, P. J. G. M. (1989) Plant Physiol. **89**, 945-951.
11. Ordentlich A., Elad, Y. and Chet, I. (1988) Soil Biol. Biochem. **49**, 747-751.
12. Takayanagi, T., Ajisaka, K., Takiguchi, Y. and Shimahara, K. (1991) Biochem. Biophys. Acta **1078**, 404-410.
13. Collinge, D. B., Kragh, K. M., Mikkelsen, J. D., Nielsen, K. K., Rasmussen, U. and Vad, K. (1993) Plant J. **3**, 31-40.
14. Shapira, R., Ordentlich, A., Chet. I. and Oppenheim. A. B. (1989) Phytopathology **79**, 1246-1249.
15. Oppenheim, B. A., Hirsch, Y., Koby, S. and Chet, I. (1990) in: Biologicals from Recombinant Microorganisms and Animal Cells. White, M. D., Reuveny, S. and Shafferman, A.(eds). Proc. of the 34th Ohoto Conference, Eliat Israel.
16. Kless, H., Sitrit, Y., Chet, I. and Oppenheim, A. B. (1989) Mol. Gen. Genet. **217**, 471-473.
17. Vorgias, C. E., Kingswell, A. J., Dauter, Z. and Oppenheim, A. B. (1992) J. Mol. Biol. **226**, 897-898.
18. Tews, I., Dauter, Z., Oppenheim, A. B. and Vorgias, C. E. (1992) J. Mol. Biol. **228**, 696-697.
19. Jannatipour, M., Soto-Gil, R. W., Childers, L. C.and Zyskind, J. W. (1987). J. Bacteriol. **169**, 3785-3791.
20. Bradford, M. (1976) Anal. Biochem. **72**, 248-254.
21. Laemmli, U. K. (1970) Nature **227**, 680-685.