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ARTICLE *in* BIOTECHNOLOGY PROGRESS · OCTOBER 2004

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A Simple Strategy for the Purification of Large Thermophilic Proteins Overexpressed in Mesophilic Microorganisms: Application to Multimeric Enzymes from *Thermus* sp. Strain T2 Expressed in *Escherichia coli*

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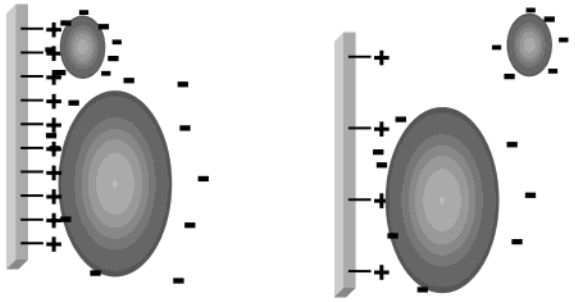
The heating of protein preparations of mesophilic organism (e.g., *E. coli*) produces the obliteration of all soluble multimeric proteins from this organism. In this way, if a multimeric enzyme from a thermophilic microorganism is expressed in these mesophilic hosts, the only large protein remaining soluble in the preparation after heating is the thermophilic enzyme. These large proteins may be then selectively adsorbed on lowly activated anionic exchangers, enabling their full purification in just these two simple steps. This strategy has been applied to the purification of an α-galactosidase and a *β*-galactosidase from *Thermus* sp. strain T2, both expressed in *E. coli*, achieving the almost full purification of both enzymes in only these two simple steps. This very simple strategy seems to be of general applicability to the purification of any thermophilic multimeric enzyme expressed in a mesophilic host.

Introduction

Recently, it has been shown that the mechanism of adsorption of proteins on ion exchanger supports (multipoint interactions between the protein and the support) permits the selective adsorption of large proteins compared to small ones (*1*). This was based on the fact that proteins only become adsorbed on the ionic chromatographic matrix by multipunctual interactions between several groups placed on the surface of the protein molecule and some groups located on the surface of the support (*1*). Then, only large enough proteins (that is, covering a large area of the support) could be adsorbed on the matrix when using very lowly activated supports (Scheme 1). Therefore, it may be possible to purify via one simple ionic adsorption any multimeric protein from any contaminant monomeric protein. However, to take full advantage of this, it is necessary to find cases where the only multimeric enzyme present in the enzyme preparation is the target protein. That may be the case of multimeric enzymes from a thermophilic organism expressed in a mesophilic host. These enzymes have a growing interest as a suitable solution for the lack of thermostability of mesophilic enzymes, mainly when the use of high temperatures is convenient (e.g., to prevent microbial contamination in food chemistry) (*2*−*6*). Moreover, as a result of the problem of the production in the native producers (use of high temperatures, nutrient requirements, productivity) (*7*, *8*), these enzymes are routinely expressed in mesophilic microorganisms (e.g., *E. coli*) (*9*−*11*).

Scheme 1. Adsorption of Large and Small Protein on Ionic Exchangers



The heating of the protein preparation from a mesophilic organism having the multimeric thermophilic enzyme is the conventional way of starting the purification of thermophilic enzymes expressed in a mesophilic host. This treatment precipitates many of the proteins of the host microorganisms, depending on the pH, ionic strength, etc. (*12*). It is possible that this treatment may destroy the quaternary structure of most of the mesophilic multimeric proteins, considering their fairly low stability under drastic conditions (*13*). If this was the

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10.1021/bp049785t CCC: $27.50
Published on Web 08/17/2004

case, the only multimeric enzyme present in the heated extract should be the thermophilic one.

As a model, this two-step strategy has been applied to the hexameric α- and the tetrameric β-galactosidases from *Thermus* sp. strain T2 overexpressed in *Escherichia coli*, both with industrial interest. (*2*, *14*−*21*).

Materials and Methods

Materials. Cross-linked 4% agarose beads (4BCL) were a gift from Hispanagar SA. (Burgos-Spain). α-Galactosidase and β-galactosidase from *Thermus* sp. strain T2 were produced as described elsewhere (*16*, *22*). *Escherichia coli* MC2508 (23) (relevant genotype *melA*; reference number 4926 of the Coli Genetic Stock Center, University of Yale) was used for cloning and expression studies for α-galactosidase production. The plasmids were pOS105 (*24*) (kindly provided by Dr. Y. Koyama) and pIN-III-lpp^{P5}-A3 (*25*). For β-galactosidase production, *E. coli* JM109 was used for cloning and gene expression. The plasmid and vectors used were the same as those used for the α-galactosidase cloning. In both cases, *E. coli* cells harboring plasmids were cultured in Luria−Bertani medium with sodium ampicillin (150 μg/mL) at 30 °C, harvested by centrifugation, resuspended in 50 mL of 50 mM sodium phosphate buffer, pH 7.0, and broken in a French press (*22*).

p-Nitrophenyl-α-D-galactopyranoside, ethylenediamine, and other reagents for electrophoresis were purchased from the Sigma Chemical Co. Sodium periodate was from Merck (Darmstadt, Germany). Molecular weight marker kit for gel filtration chromatography was from Sigma Chemical Co. All other reagents were analytical grade.

Preparation of MANAE-Agarose Supports. The protocol was similar to the previously described (*26*), but using glyoxyl agarose (*27*) with different activation degrees. Ten milliliters of agarose (4BCL) containing the desired amount of glyoxyl groups was suspended in 90 mL of 1 M ethylenediamine, pH 10.05, and the mixtures was gently stirred for 2 h. Then, 1 g of solid $NaBH_4$ was added, and the suspension was reduced for 2 h. The reduced gels, monoaminoethyl-*N*-aminoethyl agarose (MANAE), were filtered and washed with 100 mL of 0.1 M sodium acetate, 1 M NaCl at pH 5.0, with 100 mL of 0.1 M sodium bicarbonate buffer, 1 M NaCl at pH 10.0, and finally with 500 mL of deionized water. The full conversion of glyoxyl to MANAE groups permitted the concentration of amino groups to correspond to the concentration of glyoxyl groups on the support (*27*).

Determination of Enzyme Activity. *α-Galactosidase.* Activity was followed spectrophotometrically by the increase in the absorbance at 405 nm caused by the hydrolysis of *p*-NPG. The reaction medium was 13.3 mM *p*-NPG, dissolved in 50 mM sodium phosphate buffer, pH 7.0, at 25 or 65 °C (molar extinction coefficient, 10 310 L mol^{-1} cm^{-1} at pH 7.0). One unit of activity is defined as the amount of enzyme that hydrolyzed 1 μmol of substrate per min and per mg of protein under the assay conditions. Experiments were carried out at least in triplicate, and experimental error was never over ± 5%.

β-Galactosidase. Activity was followed spectrophotometrically by the increase in the absorbance at 405 nm caused by the hydrolysis of *o*-NPG. The reaction medium was 13.3 mM *o*-NPG, dissolved in Novo buffer, pH 6.5 (2.7 mM sodium citrate; 7.91 mM citric acid; 2.99 mM potassium biphosphate; 10.84 mM potassium phosphate; 19.43 mM potassium hydroxide; 4.08 mM magnesium chloride; 5.1 mM calcium chloride; and 3.33 mM sodium carbonate) at 25 °C. The β-galactosidase activity is defined as the amount of enzyme that hydrolyzed 1 μmol of substrate per min and per mg of protein under the assay conditions. Experiments were carried out at least by triplicate and experimental error was never over ± 5%.

Adsorption of Proteins on MANAE-Agarose Support. A volume of 2 mL of anionic exchanger support (1, 2.5, 5, 20, and 40 μmol of amino groups per mL of support) was suspended in 10 mL of protein solution (0.5 mg of protein per mL of 5 mM sodium phosphate buffer at pH 7.0 and 25 °C) to prevent diffusion limitations. During adsorptions, samples were withdrawn from the supernatant, and the suspension and enzyme activities were determined. After 24 h, the supports with the adsorbed enzymes were washed with an excess of distilled water and stored at 4 °C.

SDS−PAGE Analysis. Experiments of SDS−PAGE were performed as described by Laemmli (*28*) in a SE 250-Mighty small II electrophoretic unit (Hoefer Co.) using gels of 12% polyacrylamide in a separation zone of 9 cm × 6 cm and a concentration zone of 5% polyacrylamide. Gels were stained with Coomassie brilliant blue. Low molecular weight markers from Pharmacia were used (14,000−94,000 D).

Desorption of Proteins Adsorbed on MANAE-Agarose Supports. Two milliliters of adsorbed enzymes was suspended in 5 mL of 5 mM sodium phosphate buffer at pH 7.0. Different concentrations of NaCl were then added to increase the ionic strength, and samples were taken from the supernatant 30 min after addition of NaCl at room temperature (longer incubation times, up to 6 h, did not result in significant increments in the desorbed proteins). Desorbed proteins were checked via enzyme activity and/or Bradford's method (*29*). A reference solution with soluble enzyme was submitted to the same treatment to detect any possible effect of the NaCl upon activity of the enzyme. To ensure full desorption, in some instances the support was boiled in the presence of 9 M guanidine (*30*).

Incubation of Protein Crude Extract at High Temperature. *E. coli* extracts containing the target enzymes were incubated in 50 mM sodium phosphate buffer at pH 7.0 and 70 °C for 20 min, centrifuged, and assayed for both α- and β-galactosidase activity at 25 °C as described above.

Gel Filtration. Gel-filtration analysis was performed using a glass column packed with agarose 4BCL (column bed volume 100 mL). The eluting buffer used was 50 mM sodium phosphate buffer, pH 7.0. All separations were carried out at 25 °C with a flow rate of 0.5 mL/min employing an isocratic pump and detecting the absorbance at 280 nm. The column was equilibrated with 500 mL of 50 mM sodium phosphate buffer, pH 7.0. The eluted samples were collected in 1 mL aliquots, and the concentration of protein was determined by Bradford's method (*29*), using bovine serum albumin as the standard. The standard M_r of the different proteins was estimated from a calibration curve plotted using standard proteins (apoferritin from horse spleen, 443 kDa; catalase from bovine liver, 240 kDa; β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; PGA, 90 kDa; BSA, 67 kDa; carbonic anhydrase, 29 kDa; and lysozyme, 14 kDa). The elution volume of the apoferritin from horse spleen was between 55 and 60 mL; catalase from bovine liver, β-amylase, and alcohol dehydrogenase eluted from 68 to 75 mL respectively; BSA eluted at 80 mL; and finally carbonic anhydrase and lysozyme were eluted between 85 and 95 mL.

Table 1. Summary of Purification of α- and *β*-Galactosidases from *Thermus* sp. Strain T2 by Thermal Precipitation[a]

step	activity (U/mL)	protein concn (mg/mL)	specific activity (U/mg)	yield (%)	purification (fold)
crude extract of α-galactosidase	0.36	9.93	0.036	100	1
crude extract of α-galactosidase purified by thermal precipitation	0.29	3.8	0.076	80.6	2.1
crude extract of *β*-galactosidase purified by thermal precipitation	7.3	4.2	1.74	81	2.1

[a] Crude extract of enzyme was incubated at 70 °C and pH 7.0, during 20 min. After that, the clarified extract was centrifuge, and enzymatic activity was determined at 25 °C and pH 7.0 using *p*-NPG and *o*-NPG as the substrates for α- and *β*-galactosidases, respectively. Results of one experiment are given; values were reproducible in three separate experiment.

Results and Discussion

Effect of Thermal Treatment on Multimeric Enzymes from Mesophilic Organisms. The thermal treatment permitted improvement of the purity of the enzyme by a 2.1 factor, with around 80% recovery of the activity from the crude enzyme solution for both enzymes, α- and *β*-galactosidase (Table 1). This is a significant purification factor but evidently seemed to be quite short for the activity cost. Longer incubations of the extract at 70 °C did not improve the purity of any of both enzymes (data not shown). Higher temperatures or lower pH values make it possible to precipitate more proteins from *E. coli* but also presented a negative effect on the activity of the enzymes (results not shown). Thus, this heated extract (under very mild conditions and very likely under conditions that could be used for any thermohilic enzyme) was used for further experiments.

However, as explained in the Introduction, our goal was to check the effect of this treatment on the multimeric enzymes of the mesophilic host. Thus, a similar treatment was performed in the different *E. coli* strains used to harbor the genes of the α- and *β*-galactosidase (*16*, *22*). Figure 1 shows the gel filtration analysis of the untreated and thermally treated protein preparation (similar for the two strains, *E. coli* MC2508 and *E. coli* JM109). This chromatogram shows that the largest proteins contained in the crude were eliminated (precipitated or, perhaps, just destroying their multimeric structures). Thus, a similar treatment with the extract containing the thermophilic galactosidases left these enzymes as the only ones with a very large size. In fact, pure *β*- and α-galactosidases could be obtained just by gel filtration of this heated preparation, as the only large proteins (results no shown). Evidently, gel filtration may be of laboratory use, but it is not a simple industrial chromatographic process.

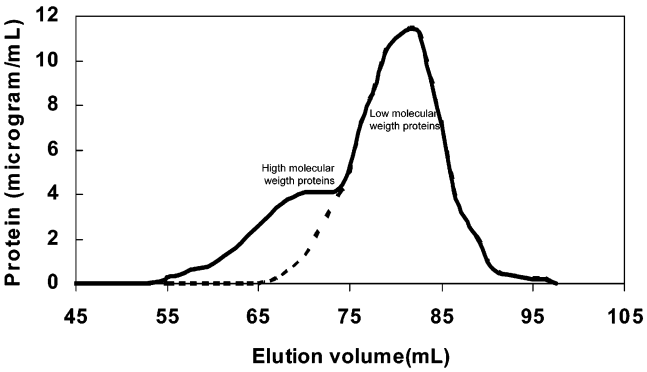

Figure 1. Gel filtration analysis of *E. coli* extracts. The sample analyzed was the extract before and after precipitation (purification) at 70 °C for 20 min and centrifugation. This sample was injected in a glass column containing 100 mL of 4BCL agarose. Flow rate was 0.5 mL/min. Other details are described in Methods: (—) crude extract before thermal precipitation; (- - -) crude extract after thermal precipitation.

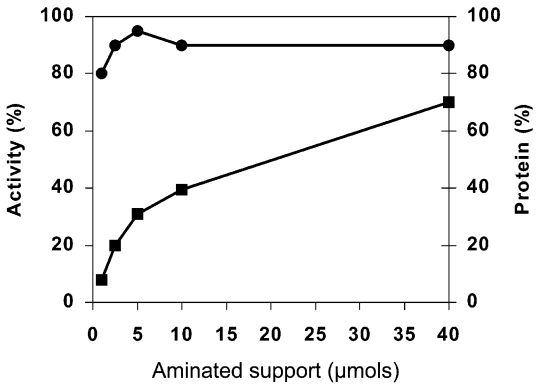

Figure 2. Adsorption of proteins and α-galactosidase on differently activated MANAE supports. Experiments were carried out as described in Methods: (◆) α-galactosidase activity; (■) total protein.

Adsorption of Thermally Treated Extract on Aminated Supports Having Different Activation Degrees. Aminated supports having different activation degrees (1, 2.5, 5, 20, and 40 *μ*mol per gram of support) were used in these experiments. It was possible to adsorb all of the α-galactosidase activity contained in the extract on the five assayed supports. After 1 h of incubation, over 95% of the total protein remained nonadsorbed in the lowly activated supports, whereas more than 70% was adsorbed on the more activated ones (Figure 2). Similar results were obtained with the *β*-galactosidase from *Thermus* sp. strain T2.

Moreover, desorption could also present some problems using the highly activated supports, because the adsorption strength may be very high (*1*). The supports with the adsorbed proteins containing α- and *β*-galactosidase were recovered, washed, and incubated in a growing concentration of NaCl. The results obtained with α-galactosidase using the highest activated support (40 *μ*mol of aminated groups/g of support) are shown in Figure 3A. In this case, some of the enzyme activity remained on the support even using 1 M NaCl, revealing the strong adsorption of this large protein on the support (having a large surface and, therefore, presenting many ionic bonds between each enzyme molecule and the support). However, using supports with a lower activation degree, the enzyme could be desorbed from the support at progressively lower concentration of NaCl (50−100 mM). Similar results were obtained with the *β*-galactosidase (results not shown).

Figure 3B shows the SDS−PAGE gels of the proteins desorbed from lowly activated supports using α-galactosidase. Only the band corresponding to the α-galactosidase could be visualized. Table 2 summarizes the results obtained with α-galactosidase on different activated supports. We found that the major recovery was obtained when the experiments were carried out with low activated supports (a 20-fold purification factor that yields an almost fully pure enzyme).

On the other hand, the crude containing the *β*-galactosidase from *Thermus* sp. strain T2 was offered to these supports after thermal treatment. When using supports

Table 2. Summary of Purification of α-Galactosidase from *Thermus* sp. Strain T2, by Adsorption on Different Activated MANAE-Agarose Supports[a]

step	activity (U/mL)	protein concn (mg/mL)	yield (%)	specific activity (U/mg)	purification (fold)
soluble extract	0.290	3.400	100	0.086	1
agarose-MANAE 1 μmol	0.240	0.086	83	2.000	22
agarose-MANAE 2.6 μmol	0.210	0.097	72	2.100	24
agarose-MANAE 5 μmol	0.200	1.100	70	0.180	2.1
agarose-MANAE 20 μmol	0.160	1.220	55	0.130	1.7
agarose-MANAE 40 μmol	0.144	1.040	50	0.140	1.8

[a] Details are described in Methods.

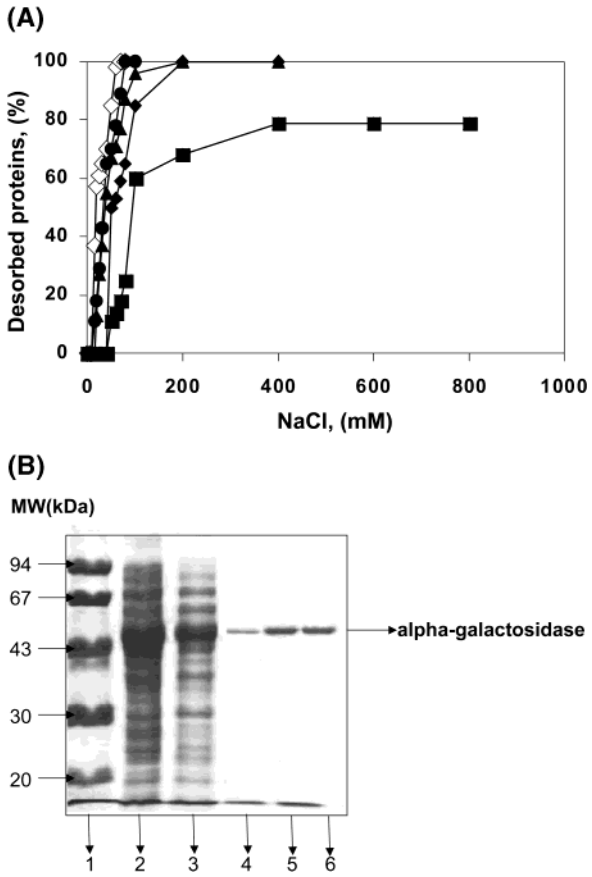

Figure 3. (A) Desorption of α-galactosidase from *Thermus* sp. strain T2 from different anionic exchangers (1, 2.5, 5, 20, and 40 μmols of amino groups/mL of support). Proteins adsorbed on different aminated supports were incubated at growing concentrations of NaCl at pH 7.0 as described in Methods: (◊) desorbed α-galactosidase from *Thermus* sp. strain T2 from 1 μmol of MANAE-agarose support; (●) desorbed α-galactosidase from *Thermus* sp. strain T2 from 2.5 μmol of MANAE-agarose support; (▲) desorbed α-galactosidase from *Thermus* sp. strain T2 from 5 μmol of MANAE-agarose support; (◆) desorbed α-galactosidase from *Thermus* sp. strain T2 from 20 μmol of MANAE-agarose support; (■) desorbed α-galactosidase from *Thermus* sp. strain T2 from 40 μmol of MANAE-agarose support. (B) SDS−PAGE gel of the proteins desorbed from supports activated with 1, 2.5, and 5 μmol/g of support after boiling the supports in the presence of SDS as described in Methods. Lane 1: low molecular marker. Lane 2: crude extract of α-galactosidase from *Thermus* sp. T2. Lane 3: extract of α-galactosidase from *Thermus* sp. T2 after thermal precipitation. Lane 4: proteins released from 1 μmol of aminated support. Lane 5: proteins released from 2.5 mmol of aminated support. Lane 6: Protein released from 5 μmol of aminated support.

with only 1 μmol of amino groups per gram of agarose, more than 80% of the β-galactosidase activity was adsorbed with less than 4% of total proteins, after 1 h of adsorption. Thus, the specific activity of the enzyme increased by a 21-fold factor (considering that the initial amount of the enzyme is just under 5%). Figure 4 shows

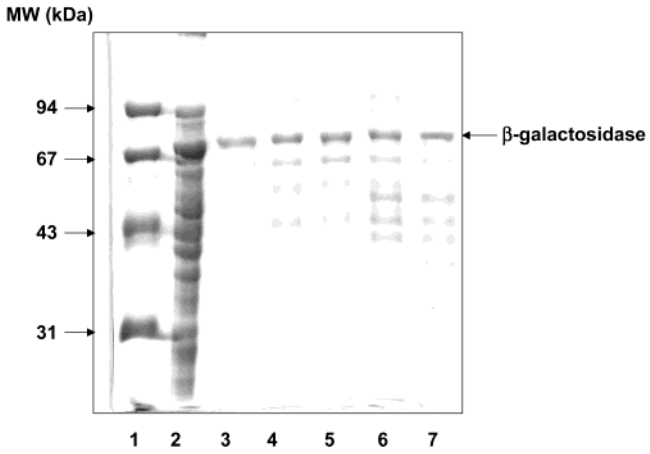

Figure 4. Analysis by SDS−PAGE of proteins adsorbed/desorbed on different aminated supports. Lane 1: molecular marker. Lane 2: crude extract of β-galactosidase from *Thermus* sp. strain T2 expressed in *E. coli*. Lane 3: proteins adsorbed on 1 μmol of aminated agarose support. Lane 4: proteins adsorbed on 2.5 μmol of aminated agarose support. Lane 5: proteins adsorbed on 5 μmol of aminated agarose supports. Lane 6: proteins adsorbed on 10 μmol of aminated agarose support, Lane 7: proteins adsorbed on 40 μmol of aminated agarose support. Experiments were performed as described in Methods.

the SDS−PAGE gel of this purified enzyme, where only some very minor contaminant enzymes could be found.

Adsorption of the crude extract without thermal treatment in lowly activated supports obviously permitted the adsorption of the target enzymes, but also many of the multimeric enzymes presented in the sample become adsorbed, decreasing the purification degree (*1*). Thus, the purification factor decreased to around 10 for both enzymes (that is, about 50% of the proteins are the target protein).

Conclusions

The results presented in this paper show a new strategy for the very easy purification of large multimeric thermophilic enzymes produced in a mesophilic host, based on the special sensibility of multimeric enzymes of the mesophilic hosts to the thermal treatment and the new strategy of a selective adsorption of large proteins in lowly activated anionic exchanger supports.

This strategy may be of general use for all thermophilic multimeric enzymes that may be adsorbed on any matrix via multipoint covalent attachment, the use of other more sophisticated techniques (e.g., addition of poly-His tags and use of IMAC chromatography, etc.) not being necessary.

Acknowledgment

This work was supported by grant O7G/002/2003 from the Comunidad Autónoma de Madrid and an UE-Project (MATIONES G5RD-CT-2002-00752). We specially thank the Angolan Government for a Ph.D. fellowship to Mr.

M. Filho and a Ph.D. fellowship from Universidad Industrial de Santander, Colombia for R. Torres. We thank Angel Berenguer for his help during the writing of this paper.

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Accepted for publication July 8, 2004.

BP049785T