Overproduction of Thermus sp. β-Galactosidase in Escherichia coli and Preparation by Using Tailor-Made Metal Chelate Supports

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Received 22 October 2002/Accepted 7 January 2003

A novel thermostable chimeric β-galactosidase was constructed by fusing a poly-His tag to the N-terminal region of the β-galactosidase from Thermus sp. strain T2 to facilitate its overexpression in Escherichia coli and its purification by immobilized metal-ion affinity chromatography (IMAC). The poly-His tag fusion did not affect the activation, kinetic parameters, and stability of the β-galactosidase. Copper-iminodiacetic acid (Cu-IDA) supports enabled the most rapid adsorption of the His-tagged enzyme, favoring multisubunit interactions, but caused deleterious effects on the enzyme stability. To improve the enzyme purification a selective one-point adsorption was achieved by designing tailor-made low-activated Co-IDA or Ni-IDA supports. The new enzyme was not only useful for industrial purposes but also has become an excellent model to study the purification of large multimeric proteins via selective adsorption on tailor-made IMAC supports.

β-β-D-Galactosidases (EC 3.2.1.23) catalyze the hydrolysis of β-1,4-d-galactosidic linkages and are industrially useful for the hydrolysis of lactose in dairy products (15, 29) and as a tool for organic chemistry (2, 27, 38). Since catalysis at high temperatures has a number of recognized advantages, some β-galactosidases from thermophilic organisms have been characterized and used for these purposes (1, 11, 14, 17, 24–26, 28, 32, 35). Nevertheless, the production of these enzymes in thermophilic microorganisms is difficult to achieve at the industrial scale, and therefore the possibility of producing them in mesophilic microorganisms by using recombinant techniques has become very attractive (6, 9, 12, 23, 37). In this sense, we have demonstrated that the β-galactosidase (BgaA) of Thermus sp. strain T2 could be overexpressed in Escherichia coli (36). Further, we have investigated here the possibility of constructing a new fusion BgaA having a poly-His tag (hereafter referred to as “Htag-BgaA”) to facilitate the purification of the enzyme which should improve its potential biotechnological applications.

The use of immobilized metal-ion affinity chromatography (IMAC) to purify proteins fused with poly-His tags is becoming increasingly popular as a tool for simple and inexpensive productions of large quantities of pure industrial enzymes, although a proper design of purification protocols is necessary to achieve optimal results (4, 10, 13, 30). Natural and poly-His-tagged proteins are separated on IMAC-supports based on their differential adsorption properties, and thus, while poly-His-tagged proteins can be adsorbed to the matrix by the interaction of the tag with a single metal chelate, natural proteins require several metal chelate-protein interactions to become adsorbed (3, 34). Therefore, supports and conditions that favor multipoint interactions with natural proteins decrease the advantage of adsorption selectivity for poly-His-tagged proteins. Current versions of commercial supports are not always the best choice to perform a selective adsorption, since they usually promote many unspecific multipoint interactions with natural proteins. Furthermore, in the case of multimeric poly-His-tagged proteins, commercial supports may produce multisubunit interactions that are too strong, making difficult the final desorption of the enzyme. Hence, strategies favoring single-point adsorption such as those described in this work will be useful to prevent this problem.

MATERIALS AND METHODS

Materials. Cross-linked agarose 6% beads were generously donated by Hispanagar SA (Burgos, Spain). Iminodiacetic acid (IDA) was from Sigma (St. Louis, Mo.). Epichlorohydrin and imidazole were purchased from Merck (Darmstadt, Germany). IDA di-sodium salt monohydrate and CuSO4 were from Fluka (Buchs, Switzerland). NiCl2, ZnCl2, CoCl2, and other reagents for electrophoresis were obtained from Sigma. All other reagents were analytical grade.

Bacterial strains, plasmids, and growth conditions. E. coli MC1116 (8) was used for cloning and gene expression. Plasmids used were pBGT1 (36) and pTrHCisB (InVitrogen, Leek, The Netherlands). The native BgaA and the Htag-BgaA fusion protein were overproduced in E. coli MC1116(pBGT1) (36) and in E. coli MC1116(pBGt3) (see below), respectively. Briefly, cells were grown in 1 liter of Luria-Bertani medium (LB), supplemented with ampicillin (125 µg/ml), at 37°C with overnight shaking at 250 rpm (Infors, Bottmingen, Switzerland); harvested by centrifugation; resuspended in 50 ml of 50 mM sodium phosphate buffer, pH 7.0; and broken in a French pressure cell at 1,100 lb/in2. The crude extract was clarified by centrifugation. The total soluble protein produced in the crude extract was 375 mg with a total β-D-galactosidase activity of about 1.5 × 10⁶ U determined at pH 6.5 and 70°C using n-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate.

Molecular cloning and sequencing procedures. Recombinant DNA techniques were performed by means of conventional protocols (31). DNA sequencing was performed using PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems) and an Applied Biosystems model 377 automatic

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sequence, using double-stranded plasmids as templates and universal or specific oligonucleotides as primers. To check the correct assembly of the His-tag coding region, plasmid pBGT3 was sequenced using the primer OBGT-6. (5’/H11032-GACAGTAGCAGCGCACG-3’/H11032) located 250 bp downstream the bgaA start codon.

E. coli transformants were selected on LB agar plates supplemented with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) and ampicillin (125 μg/ml).

Preparation of supports. Epoxy-agarose supports were obtained by a modification of the procedures described elsewhere (18) using epichlorohydrin. Supports with low density of epoxy groups (containing around 12 μmol of epoxy groups/ml gel) (Eps-12 supports) were prepared as follows. Ten milliliters of cross-linked 6% agarose was washed thoroughly with distilled water, and the moist gel was suspended in 30 ml of 1.9 M NaOH containing 340 mg of NaBH4, 11.4 ml of acetic acid, and 5.7 ml of epichlorohydrin. The suspension was stirred for 4 h at 4°C and finally washed thoroughly with distilled water. The epoxy group content of the matrix was determined with sodium periodate after hydrolyzing the epoxy groups with 0.25 M sulfuric acid at 25°C for 30 min. The preparation of IDA supports was carried out according to a modification of the method previously described (19, 33). A 10-ml volume of epoxy-agarose was suspended in 10 ml of 0.1 M Na2CO3 buffer containing 0.9 g of IDA, adjusted to pH 11.0 with NaOH and gently stirred at 25°C for 12 h. The resulting IDA supports were then washed with distilled water. To prepare the metal chelate supports, the IDA supports were incubated in distilled water containing CuSO4 or ZnCl2 (5 mg/ml) or in 0.05 M sodium phosphate buffer (pH 6.0) containing 1.0 M NaCl plus NiCl2 or CoCl2 (5 mg/ml) (18). Finally, the supports were washed thoroughly with distilled water.

Enzymatic assays. β-Galactosidase assay was performed with ONPG at either 70 or 25°C in both 0.05 M sodium phosphate buffer (pH 7.0) and Noto 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, 3.33 mM sodium carbonate). ONPG was dissolved in 0.05 M sodium phosphate buffer (pH 7.0) and used at a final concentration of 13.3 mM. β-Galactosidase activity of permeabilized cells was expressed as nanomoles of o-nitrophenol (ONP) released per minute under the conditions described above. β-Galactosidase activity on lactose was deter-
mined by quantitative analysis of glucose release, using glucose (Trinder) reagent from Sigma Diagnostics. These assays were carried out in 0.1 M sodium phosphate buffer, pH 7.0, at 70°C, using 138 mM lactose as the substrate, and the activity was expressed as nanomoles of glucose released per minute. Protein concentration was estimated by the method of Bradford (5), using bovine serum albumin as the standard. The K_m was calculated using the method described elsewhere (7). The optimal pH of Htag-BgaA was determined with 0.1 M citric acid-sodium phosphate buffer (pH range, 3.0 to 6.0) and sodium phosphate buffer (pH range 7.0 to 9.0) (16). The optimal temperature for Htag-BgaA was determined by assaying the enzyme in buffer Novo at temperatures ranging from 40 to 97°C. The thermal stability of Htag-BgaA was studied by determining the remaining activity (at 70°C for 10 min) after preincubating the enzyme in the absence of substrate at 60, 70, 80, and 90°C for different periods of time.

Protein adsorption and purification. A volume of 20 ml of crude protein extract from E. coli (pBGT3) was mixed with 40 ml of Novo buffer and with 28 ml of metal-IDA support. The suspension was maintained at 25°C under very gentle stirring. Imidazole (25 mM final concentration) was also added to the mixture when required. Nonadsorbed proteins were eluted by washing the supports with Novo buffer with or without 25 mM imidazole. The adsorbed proteins were eluted with increasing concentrations of imidazole. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (21), using a precast 12% gradient gel (model Protean 16; Bio-Rad Laboratories, Richmond, Calif.). Gels were stained with Coomassie brilliant blue R-250. To analyze amount of proteins adsorbed to IMAC supports, a sample of the support was first boiled in the presence of 1% SDS (wt/vol) and 2% 2-mercaptoethanol (vol/vol) to desorb the proteins (4, 22). The activity of β-galactosidase was measured spectrophotometrically as described above. The amount of adsorbed proteins was analyzed by the method of Bradford (5) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Construction of the chimeric thermostable β-galactosidase Htag-BgaA. Plasmid pBGT3 encoding a chimeric Htag-BgaA protein resulting from the fusion of a poly-His tag to the N terminus of the BgaA was constructed as described in Fig. 1. E. coli MC1116(pBGT3) cells showed a blue phenotype in LB plates containing X-Gal and, in agreement with this observation, the permeabilized cells exhibited a high β-galactosidase activity when assayed at 70°C. On SDS-PAGE E. coli MC1116(pBGT3) cell extracts showed a novel protein of about 73 kDa that corresponds to the expected size of the chimeric Htag-BgaA enzyme (Fig. 2). This new protein accounted for 3 to 5% of the total protein (ca. 20 mg of β-galactosidase/liter of culture; 15 × 10^6 U/liter of culture). This means that E. coli MC1116(pBGT3) cells produced almost 3 orders of magnitude more β-galactosidase activity than that produced by Thermus (35). These results demonstrated not only that the new chimeric Htag-BgaA enzyme can be overproduced in E. coli in an active form but also that the chimera retains the thermostability of the native BgaA enzyme.

Biochemical characterization of the chimeric Htag-BgaA enzyme. Several biochemical properties of the Htag-BgaA enzyme were analyzed and compared with those of the native BgaA enzyme using crude extracts from E. coli MC1116(pBGT3) and E. coli MC1116(pBGT1), respectively. The optimal pH for both enzymes was very similar, showing the maximum activity at pH 6.5 (Fig. 3A). Both enzymes retained a high activity at pH 5.0 to 7.5, but surprisingly, Htag-BgaA showed a significant activity at pH 4.0, whereas BgaA was inactive at this pH. The K_m value of 3 mM calculated for Htag-BgaA at pH 6.5 and 70°C using ONPG as substrate was very close to that previously determined for BgaA (K_m, 4 mM) (36).

The analysis of the temperature-activity profile of BgaA and Htag-BgaA in the range 40 to 97°C at pH 6.5 revealed that both enzymes presented maximum activity between 80 and 90°C (Fig. 3B). In both cases, the activity decreased rapidly above 90°C, most likely due to thermal denaturation effects. These results revealed that the chimeric Htag-BgaA enzyme has almost the same thermal stability as the native BgaA enzyme.

When the thermal stability of both enzymes was studied we observed that their activities first increased during the first 20 min of incubation then decreased over longer incubation times at all temperatures tested (from 60 to 90°C). This effect is illustrated in Figure 3C, which shows the behavior of both enzymes when incubated at 80°C. The observed activation-inactivation phenomenon has been already described for other thermostable β-galactosidases and may most likely ascribed either to aggregation-dissociation processes or to conformational changes induced by the temperature (9, 28). Therefore, taking all these results together, a temperature of 70°C was chosen as the standard reaction temperature for testing the activity of the enzyme.

Purification of the Htag-BgaA enzyme on different chelate supports. To purify the new chimeric Htag-BgaA enzyme several chelate supports were made using different metals. The efficiencies of the IMAC supports were tested with the crude protein extract from E. coli MC1116(pBGT3). When the adsorption courses of the Htag-BgaA enzyme were analyzed on IDA supports chelated with Cu^{2+}, Co^{2+}, or Ni^{2+} we observed that the protein adsorption rate was higher with Cu^{2+} than with Co^{2+} or Ni^{2+}. Using the Cu-IDA support all the Htag-BgaA protein present in the crude extract was adsorbed in 10 min, whereas the Co-IDA or Ni-IDA matrices required 30 min for its complete adsorption.

Although the Cu-IDA support very quickly adsorbed the Htag-BgaA protein, this matrix also adsorbed most of the other contaminant proteins present in the crude extract, i.e., about 70% of the total protein present in the crude extract were adsorbed in 10 min. To improve the selectivity of the adsorption process, the protein adsorption was performed in the presence of 25 mM imidazole (3). As expected, the addi-
tion of a low concentration of imidazole provided a significant improvement on the selective binding of Htag-BgaA to the Cu-IDA support, since the chimeric enzyme was still fully adsorbed in this condition, but the adsorption of contaminant proteins only accounted for 10% of the total protein contained in the crude extract (Fig. 4A). It is worth mentioning that in the presence of 25 mM imidazole the complete adsorption of Htag-BgaA also occurred in only 10 min. After washing the matrix with 50 mM imidazole we observed that the Htag-BgaA enzyme still remained bound to the support (Fig. 4A), and hence this washing step allowed us to eliminate almost all the contaminant proteins adsorbed on the matrix. Finally, the Htag-BgaA was desorbed after washing the support with 100 mM imidazole (Fig. 4A and Table 1). Under these conditions, the separation of the active chimeric Hga-BgaA was achieved with a high purification factor and a very high yield.

Although the Cu-IDA support seemed to provide a very efficient method to purify Htag-BgaA, we observed that when the crude protein extract carrying the Htag-BgaA enzyme was maintained interacting with the Cu-IDA support for more than 20 min before the desorption step, the chimeric enzyme could not be desorbed from the support even at 200 mM imidazole (Fig. 5). This strong interaction of Htag-BgaA with the support was ascribed to a multisubunit adsorption of the enzyme and it represents a drawback for developing this purification protocol.

FIG. 3. Comparison of different biochemical properties of the chimeric Htag-BgaA and wild-type BgaA enzymes. (A) Effect of pH on enzyme activity. (B) Effect of temperature on enzyme activity. (C) Thermal stability of Htag-BgaA. Inactivation was performed by preincubating the enzyme at 80°C and pH 7 during different periods of time. Enzymatic assays were performed using ONPG as the substrate. Results of one experiment are given; values were reproducible in three separate experiments. Other specifications are described in Materials and Methods. Symbols: ■, Htag-BgaA; ▲, BgaA.

FIG. 4. Desorption of Htag-BgaA with imidazole from the Cu-IDA support. A crude extract from *E. coli* (pBGT3) containing Htag-BgaA enzyme was allowed to interact with the Cu-IDA support for 5 min or for 20 min and the enzyme was further desorbed from the support with different concentrations of imidazole. Symbols: ●, desorption of Htag-BgaA after 5 min of support-enzyme interaction; ■, desorption of Htag-BgaA after 20 min of support-enzyme interaction. Enzymatic assays were performed at 70°C and pH 6.5 using ONPG as the substrate. Results of one experiment are given; values were reproducible in three separate experiments. Other specifications are described in Materials and Methods.

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<th>TABLE 1. Purification of Htag-BgaA on a Cu-IDA support</th>
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<sup>a</sup> Enzyme activity was determined at 70°C and pH 6.5 using ONPG as the substrate. Results of one experiment are given; values were reproducible in three separate experiments.
at industrial scale, since short adsorption times are unsuitable for handling large industrial volumes.

On the other hand, we have observed that when the Htag-BgaA protein purified by the Cu-IDA support was stored at 4°C for 24 h, it lost more than 65% of activity. This effect is not due to a proteolysis of the enzyme or to stability problems since the Htag-BgaA in the crude extract remains fully active under these storing conditions. Such effect is more likely due to the presence of Cu²⁺ traces released from the Cu-IDA support. In fact, we have observed that Htag-BgaA is inactivated after incubation in the presence of 10 μM CuCl₂ (data not shown). Therefore, the enzyme inactivation agrees with the observed susceptibility of BgaA to Cu²⁺ ions (20).

When Co-IDA and Ni-IDA supports were used to absorb the chimeric enzyme, the immobilization rates of Htag-BgaA were slower than those found with Cu-IDA, but the adsorption of Htag-BgaA was more selective. Hence, whereas all Htag-BgaA was adsorbed in these supports, only 30% of total protein of the crude extract was bound. When the adsorption process was performed in the presence of 25 mM imidazole, the Htag-BgaA enzyme became the main protein adsorbed in the matrix (Fig. 4B). Then, to eliminate the contaminant proteins, the support was washed with 50 mM imidazole and the final desorption of the enzyme was achieved with 100 mM imidazole. Using this protocol, higher than 90% recovery of Htag-BgaA was found with a purification factor of 20 (Table 2). The specific activity of the purified Htag-BgaA determined at 70°C and pH 6.5 was about 900,000 and 30,000 U/mg using ONPG or lactose as substrates, respectively. In this case, the pure enzyme remained fully active after several days of storage at 4°C. Moreover, we did not observe the multipoint adsorption effect using Co-IDA or Ni-IDA supports since more than 90% of the Htag-BgaA activity was recovered even after several hours of interaction with the support. Therefore, these IMAC supports will allow the development of a process for the purification of Htag-BgaA at an industrial scale with large volumes of protein extracts, because with these forms of support the purification process does not depend on a short adsorption step.

In summary, the overproduction of a thermostable His-tagged β-galactosidase derived from the BgaA enzyme from _Thermus_ sp. strain T2 in a mesophilic microorganism has been achieved. The resulting Htag-BgaA protein retains the biochemical properties of the wild-type BgaA protein and can be quickly and easily purified by tailor-made IMAC supports under tailor-made conditions via a selective and single-subunit adsorption process that facilitates the desorption of the enzyme without detrimental effects on the enzyme activity or stability. The problems related to the multipoint adsorption of the enzyme on Cu-IDA supports and the inactivation by Cu²⁺ ions were solved by using Co³⁺ and Ni²⁺ in IMAC supports.

**ACKNOWLEDGMENTS**

We thank R. López for helpful comments. We thank Y. Koyama for sharing with us plasmid pOS103, the starting plasmid used to construct plasmids pBG1 and pBG3 used in this work. We especially thank Pedro Sebastiá, the Angolan ambassador in Spain, for his support of B. Pessela.

This work was supported by grant CORO70/94 from the Comunidad Autónoma de Madrid. We thank Hispanagar SA for its kind financial support. Benevides C. C. Pessela was the recipient of a Ph.D. fellowship from the government of Angola.

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