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Construction of a Chimeric Thermostable Pyrophosphatase To Facilitate Its Purification and Immobilization by Using the Choline-Binding Tag

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The thermophilic inorganic pyrophosphatase (Pyr) from *Thermus thermophilus* has been produced in *Escherichia coli* fused to the C terminus of the choline-binding tag (ChB tag) derived from the choline-binding domain (ChBD) of pneumococcal LytA autolysin. The chimeric ChBD-Pyr protein retains its thermostable activity and can be purified in a single step by DEAE-cellulose affinity chromatography. Pyr can be further released from the ChBD by thrombin, using the specific protease recognition site incorporated in the C terminus of this tag. Remarkably, the ChB tag provides a selective and very strong thermostable noncovalent immobilization of ChBD-Pyr in the DEAE-cellulose matrix. The binding of choline or choline analogues, such as DEAE, confers a high thermal stability to this tag; therefore, the immobilized chimeric enzyme can be assayed at high temperature without protein leakage, demonstrating the usefulness of the ChB tag for noncovalent immobilization of thermophilic proteins. Moreover, ChBD-Pyr can be purified and immobilized in a single step on commercial DEAE-cellulose paper. The affinity of the ChB tag for this versatile solid support can be very helpful in developing many biotechnological applications.

Inorganic pyrophosphatases (EC 3.6.1.1.) (Pyr) are ubiquitous enzymes that hydrolyze inorganic pyrophosphate (PP_i) into two orthophosphates and that catalyze the exchange of oxygen between water and inorganic phosphate. These enzymes function as a thermodynamic trap in coupled reactions, shifting the equilibrium of pyrophosphate-generating biosynthetic reactions towards product formation (12). This property makes Pyr a very useful molecular tool for improving several in vitro processes. For instance, DNA and RNA synthesis are inhibited by small amounts of PP_i; therefore, the addition of Pyr could shift the equilibrium in favor of synthesis (2, 10). This is the case in PCR amplifications where PP_i is released each time DNA polymerase incorporates a deoxynucleoside monophosphate from a deoxynucleoside triphosphate (5). The putative use of Pyr to increase the efficiency of a PCR assay requires the isolation of a thermostable Pyr (32). In this sense, Pyr from *Thermus aquaticus* was one of the first thermostable enzymes of this family isolated and characterized (33). More recently, the genes encoding Pyr from *Thermoplasma acidophilum* (22), *Thermus thermophilus* (27), *Sulfolobus acidocaldarius* (7, 16), "*Aquifex aeolicus*" (8), and *Aquifex pyrophilus* (9) have been cloned and expressed in *Escherichia coli*. Furthermore, Pyr from *T. thermophilus* has been crystallized, which will facilitate further modifications to increase its biotechnological applications (29).

On the other hand, the use of functional tags to facilitate the purification and immobilization of proteins has been well documented in the literature (30), but there is little data about the fusion of tags to thermophilic proteins (18–20). Most likely, this lack of data is due either to the fact that thermophilic proteins can be partially purified by thermal treatments or that most of the tags described so far for such purposes are thermolabile. In this work, we have investigated the advantages that a novel tag developed by Sánchez-Puelles et al. (25) in the laboratory of J. L. García and P. García might offer. This tag was originally named C-LytA (25) but will referred to hereafter as choline-binding tag (ChB tag). The ChB tag is a 19-kDa protein which corresponds to the choline-binding domain (ChBD) of the major autolysin of *Streptococcus pneumoniae* (4). This domain is able to specifically bind to choline or its structural analogues (e.g., diethylaminoethanol or DEAE), allowing its purification by affinity chromatography in a single step using choline- or DEAE-containing supports (25). The ChBD has been studied extensively by biophysical and biochemical techniques (15), and its three-dimensional structure was recently elucidated (3). The ChBD has a novel solenoid fold consisting exclusively of β hairpins that stack to form a left-handed superhelix, known as a β spiral staircase.

Taking into account the peculiarities of the ChB tag, we have investigated the properties of a novel chimeric protein constructed by fusing this tag to the Pyr from *T. thermophilus*. This fusion has revealed novel and interesting biotechnological applications for a thermostable tag.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* DH5α (6) was used as the host strain. Plasmid pGEM-T easy vector (Promega) was used to clone PCR-amplified DNA fragments; other plasmids used were pCE17 (26), pCUZ1 (26), and pPCR21PYR (kindly provided by A. Haro). *E. coli* cells were cultured in Luria-Bertani broth containing ampicillin (100 μg/ml) at 37 or 24°C with aeration.

Molecular cloning and sequencing procedures. Recombinant DNA techniques were conducted by conventional protocols (24). PCR amplification reactions

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were performed with 0.5 U of *Taq* DNA polymerase (Biotools), 0.25 μM concentration of each synthetic primer, 25 μM concentration of each deoxynucleoside triphosphate, and the buffer recommended by the manufacturer. These amplifications were done in a Perkin-Elmer thermal cycler (GeneAmp PCR system 2400). The pyrophosphatase gene was initially amplified from pPCR21PYR with primers PYR5 (5′-CCATCGATCTAGCCCTTGTAGCGGGCGATGC-3′) and PYR3 (5′-AACTGCAGGCGAACCTGAAGAGCCTTCCCGTGG-3′) (the PstI restriction site is underlined) and cloned into pGEM-T to give rise to pPYRT1. PCR amplification of the thrombin recognition coding sequence, using pCE17 as the template, was achieved using primers Clytnco (5′-CATGCCATGGTATCAAATGCCTTTATCC-3′) (the NcoI restriction site is underlined) and Tromter (5′-CGGGATCCTTATTACTGCAGGGAGCCACGCGGAACCAGTTTTACTGTAATCAAGCCATCTGG-3′) (the PstI restriction site is underlined). This fragment was digested with NcoI-PstI and inserted into pCUZ1 previously digested with the same enzymes to generate pAPC60. To construct pAPC62, we digested pPYRT1 with PstI-SphI, and the *pyr* gene fragment was inserted into pAPC60 that had also been cut with PstI-SphI. DNA sequencing of recombinant clones was performed with the Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems) and an automatic sequencer (ABIPRISM model 377; Applied Biosystems), using double-stranded plasmids as templates and specific primers.

Enzymatic assay. Pyr was assayed by the methods of Lanzetta et al. (13) and Verhoeven et al. (33). The assay mixture contained 20 mM Tris-HCl buffer (pH 8.0), 1 mM PP_i, and 3 mM $MgCl_2$. This mixture was heated to 80°C, and the enzyme was added. After 5 min at 80°C, the reaction was stopped by placing the reaction mixture on ice. A 50-μl sample was mixed with 800 μl of a 1:3 mixture of 0.045% malachite green hydrochloride and 4.2% ammonium molybdate in 2 N HCl. After 1 min at room temperature, the reaction was stopped with 150 μl of 34% sodium citrate, and the A_{660} was measured 5 min later. One unit of activity is defined as the release of 1 μmol of phosphate per min at 80°C. The immobilized enzyme in filters or DEAE-cellulose matrix was assayed in a final volume of 10 ml with agitation.

Enzyme purification. The ChBD-Pyr fusion protein overproduced in *E. coli* DH5α(pAPC62) was purified on DEAE-cellulose essentially as previously described (25). Briefly, *E. coli* cells were grown at 24°C for 20 h, harvested by centrifugation, washed, resuspended in 20 mM Tris-HCl buffer (pH 8.0), and broken in a French pressure cell. The crude extract was clarified by centrifugation. The total soluble protein was loaded onto a DEAE-cellulose column equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The column was extensively washed with 20 mM Tris-HCl buffer (pH 8.0) containing 1.5 M NaCl, until no protein was detected in the eluate by the method of Bradford (1) or by measuring the A_{280}. ChBD-Pyr was eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 1.5 M NaCl and 2% choline or with the same buffer containing 1.5% Triton X-100. It is worth noting that the cultures were grown at 24°C to reduce the formation of inclusion bodies.

Enzyme immobilization. DEAE-cellulose paper filters, 2.3 cm in diameter (Whatman), inserted into a metallic filter holder (Millipore), were equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The enzyme sample was injected inside the holder, and the filter was extensively washed with 20 mM Tris-HCl buffer (pH 8.0) containing 1.5 M NaCl, until contaminant proteins were eliminated. The entire process was performed at room temperature. Next, the filter was withdrawn from the holder to measure the enzymatic activity bound to it. The pH stability assays were performed with 20 mM Tris-HCl buffer (pH 8.0, 7.5, and 7.0) containing 1.5 M NaCl or 20 mM sodium citrate buffer (pH 6.0 and 5.0) containing 1.5 M NaCl.

Analytical ultracentrifugation. Sedimentation velocity experiments were performed at 50,000 rpm and 20°C in an XL-A analytical ultracentrifuge (Beckman-Coulter) equipped with UV-visible light absorbance optics using double sector Epon-charcoal centerpieces. Differential sedimentation coefficient distributions, $c(s)$, were calculated by least-squares boundary modeling of sedimentation velocity data using the SEDFIT program (28). From this analysis, the sedimentation coefficients of all the sedimenting species were obtained. The latter were corrected for buffer composition using the SEDNTERP program (14) to obtain the corresponding standard values (water and 20°C).

Miscellaneous methods. Western blot analysis was performed with a polyclonal antiserum against C-LytA, used at a 1:1,000 dilution, as described previously (25). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% or 12.5% polyacrylamide) was performed as described by Laemmli (11), and protein bands were visualized by staining with Coomassie brilliant blue R250.

RESULTS

Production and purification of the chimeric ChBD-Pyr protein. To demonstrate the usefulness of the ChB tag for purifying and immobilizing thermostable proteins, we constructed a chimeric protein by fusing the ChB tag to the N terminus of Pyr. Moreover, to increase the versatility of the original ChB tag (25), encoded by plasmid pCUZ1, we cloned the gene fragment coding for the ChBD of LytA fused in frame to the fragment encoding the thrombin recognition site (LVPRGS) to construct plasmid pAPC60 (Fig. 1). Expression of this new ChB tag protein was confirmed by the presence of a band showing the expected molecular mass in a Western blot analysis (Fig. 2A). Next, we constructed pAPC62 and the chimeric ChBD-Pyr protein (314 amino acids; 35,420 Da) was overproduced in *E. coli* DH5α(pAPC62) cells. Crude extracts of these recombinant *E. coli* cells showed remarkable Pyr activity at 80°C, strongly suggesting that the ChBD-Pyr protein maintained its quaternary active structure.

The soluble ChBD-Pyr protein was completely retained in a DEAE-cellulose column. After the column was washed with a high-ionic-strength buffer to eliminate the contaminant proteins, the Pyr activity could be eluted with a buffer containing 2% choline, and a pure chimeric protein was observed by SDS-PAGE analysis (Fig. 2B). Nevertheless, despite this successful result, the yield of the purification process was unexpectedly lower than those obtained for other ChBD-fused proteins (25), suggesting that most of the ChBD-Pyr protein remained in the column. This hypothesis was confirmed by the following findings: the cellulose matrix showed high Pyr activity, and SDS-PAGE analysis of the protein retained in the support demonstrated the presence of the pure chimeric ChBD-Pyr protein (not shown). This result suggested that the new chimera had been retained in the matrix with higher affinity than other chimeric ChBD-fused proteins constructed so far, which were completely eluted from the matrix by 2% choline (17). Remarkably, this circumstance which did not favor the purification process can be very useful for immobilization purposes (see below).

Since it has been suggested that the high ionic concentration of the elution buffer might favor the formation of Pyr oligomeric complexes (7), we tried unsuccessfully to release the fusion protein by using buffers of lower ionic strength, different pHs, or even 6 M urea. However, more than 60% of the ChBD-Pyr protein was released in the presence of Tris-HCl buffer containing 1.5 M NaCl, 2% choline, and 1.5% Triton X-100 (Table 1). It is worth mentioning that the release was due specifically to the combined action of choline and Triton X-100, as ChBD-Pyr was not eluted when the detergent alone was added to the Tris-HCl buffer containing 1.5 M NaCl. This result suggested that ChBD-Pyr protein was bound mainly by specific interactions of the ChB tag with DEAE, but it is most likely that additional strong interactions were produced by multimerization or aggregation of Pyr moieties, creating a highly stable multipoint binding. Such aggregation could be eliminated or reduced in the presence of detergent.

Effect of thrombin in the ChBD-Pyr fusion. To determine whether the thrombin recognition site, placed between ChBD and Pyr, was accessible to the protease, we incubated the soluble purified chimeric enzyme with thrombin for 16 h at

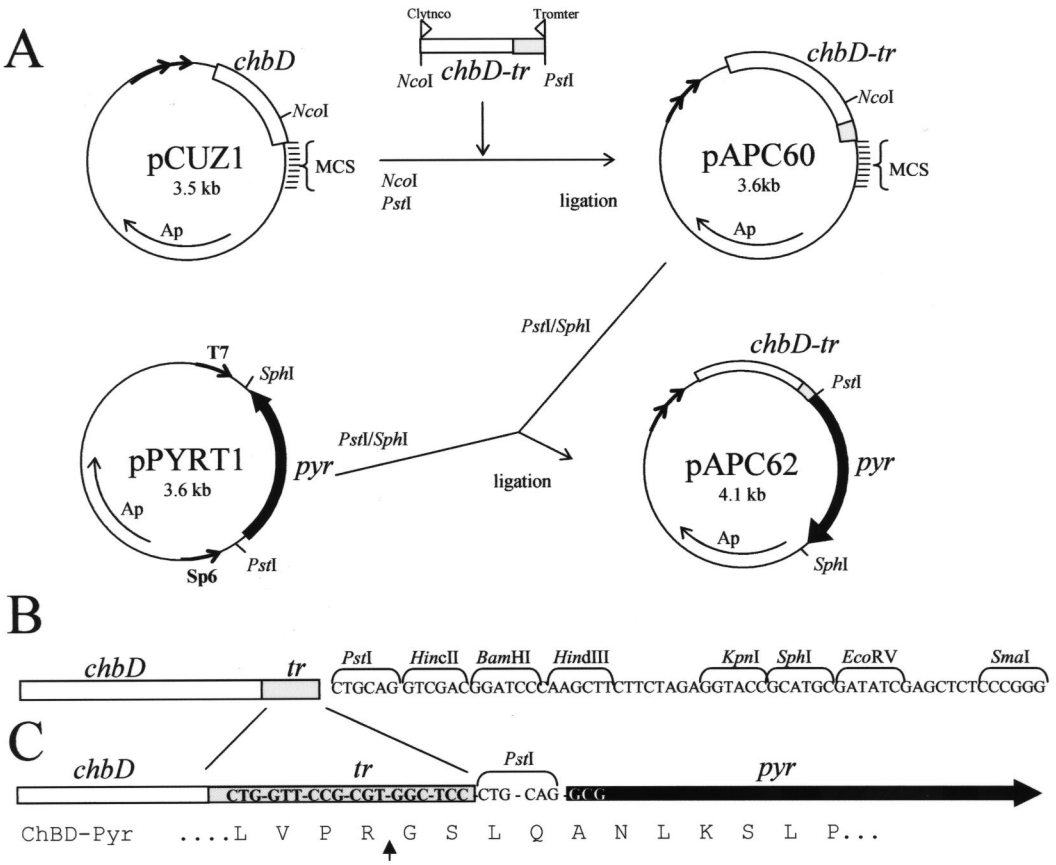

FIG. 1. Construction of pAPC62. (A) The *chbD* gene (white boxes), *pyr* gene (thick black arrows), thrombin recognition coding sequence (*tr*) (lightly stippled boxes), primers used for PCR amplification (triangles), promoters (→), and tandem *Plpp-lac* promoters (→→) are depicted. Abbreviations: MCS, multicloning site; Ap, ampicillin resistance gene. (B) Multicloning site sequence of pAPC60. (C) Partial sequence of the recombinant gene *chbD-tr-pyr* and the corresponding protein. The enzymatic cleavage site of thrombin is indicated by the small arrow.

22°C. Under these conditions, we did not observe the presence of any band different from that of the chimeric protein by SDS-PAGE analysis, suggesting that the thrombin site was protected by the tertiary and/or quaternary structure of the chimera. Therefore, taking advantage of the thermal stability of the Pyr protein, we investigated the effect of thermal denaturation of the fusion protein for 5 min at 70 and 100°C either in the presence or absence of choline in the incubation buffer. The results shown in Fig. 3 indicated that thrombin was able to release more than 50% of the Pyr protein only when thermal denaturation was performed in the absence of choline. Remarkably, choline appears to protect the ChBD against thermal denaturation and/or facilitate a rapid renaturation, i.e., differential scanning calorimetry of ChBD has revealed that its stability depends on the presence of choline showing a highly reversible thermal denaturation with a melting temperature of 75°C in the presence of 140 mM choline (15). Moreover, these results revealed that the ChB tag behaves as a highly thermostable protein in the presence of choline or choline analogues, and most likely, this behavior positively contributes to the global stability of the chimeric enzyme retained in the DEAE-support.

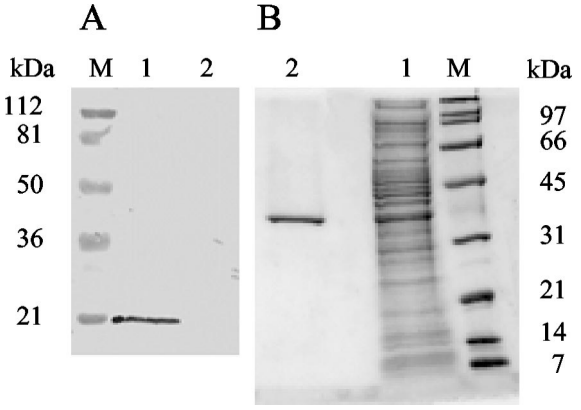

FIG. 2. Western blot analysis of the ChB tag and purification of ChBD-Pyr protein. (A) Western blot developed with anti-C-LytA serum. Lanes: 1, crude extract from *E. coli* DH5α(pAPC60); 2, crude extract from *E. coli* DH5α; M, prestained molecular mass markers. (B) SDS-PAGE analysis (12.5% polyacrylamide). Lanes: 1, soluble extract from *E. coli* DH5α(pAPC62); 2, ChBD-Pyr from DEAE-cellulose chromatography eluted with 2% choline buffer; M, standard molecular mass markers. The gel was stained with Coomassie blue. The positions of molecular mass markers (in kilodaltons) are indicated on the sides of the gels.

TABLE 1. Purification of inorganic pyrophosphatase from *T. thermophilus*

Fraction	Total U of enzyme activity (10^3)	Total protein (mg)	Purification (fold)	Yield (%)
Crude extract[a]	1,956	400	1	100
Fraction eluted with choline + Triton X-100	1,213	13.5	17.5	62

[a] Culture from 1 liter of *E. coli* DH5α(pAPC62).

Immobilization of ChBD-Pyr in alternative DEAE-cellulose supports. One of the main advantages that the use of the ChB tag offers is the existence of a great diversity of commercial supports that can be used to immobilize the ChBD-fused proteins. To gain new insights into the peculiar immobilization behavior of ChBD-Pyr, we have investigated the utility of DEAE-cellulose paper filters to immobilize the ChBD-Pyr fusion. Although there are several reports of the immobilization of proteins on different matrixes, not much work has been done on the use of DEAE-cellulose paper. For instance, this matrix has been suggested as a good support for developing simple and economical therapeutics and diagnostic kits using immobilized enzymes (21).

When a crude cell extract containing the recombinant ChBD-Pyr fusion was passed through a DEAE-cellulose filter, all the protein was retained in this support. After extensive washing of the column with buffer containing 1.5 M NaCl, the chimeric ChBD-Pyr was the single protein retained in this support, as confirmed by SDS-PAGE analysis. Remarkably, the retained protein was very active and could be easily assayed in its immobilized state. However, the specific activity of the chimeric enzyme retained in the paper filter (22,160 U/mg) was lower than that of the soluble enzyme (89,200 U/mg), as well as that of the immobilized enzyme in the particulate DEAE-cellulose support used for chromatographic purposes (95,900 U/mg). This is because the activity of the filter was determined using a typical batch assay without destroying and dispersing the filter matrix. Obviously, under these conditions, the enzymatic activity was not kinetically favored compared to a soluble enzyme or a perfectly disperse suspension of the enzyme bound to a particulate matrix, such as that used for chromatography. Nevertheless, the activity of the enzyme retained in the filter was still very high, and it can be useful for performing different types of assays.

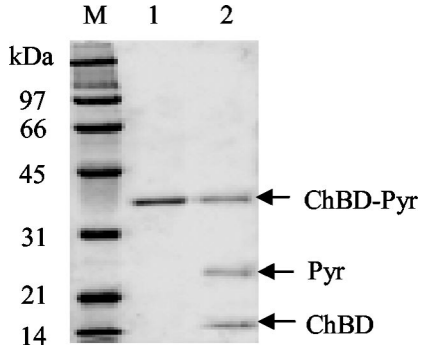

FIG. 3. Effect of thrombin on the ChBD-Pyr protein. SDS-PAGE analysis (10% polyacrylamide) of purified ChBD-Pyr protein, heated at 70°C for 5 min, and digested with thrombin. Lanes: 1, sample in the presence of 2% choline buffer; 2, sample in the absence of choline; M, standard molecular mass markers. The gel was stained with Coomassie blue. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel.

As in the case of the DEAE-cellulose chromatographic matrix, the chimeric protein retained in the filter could be released by the addition of a buffer containing 2% choline plus 1.5% Triton X-100, suggesting that the ChBD-Pyr was retained in the filter matrix by the same type of interactions. In this sense, the ChBD-Pyr protein was also strongly immobilized in the filter, since it was stable at different pHs (from 5 to 8) and after extensive incubation at 80°C or even at 100°C in 20 mM Tris-HCl at pH 8.0. In all these conditions, we could not detect the release of ChBD-Pyr protein from the filter, either by enzymatic or Western blot analyses, although at 100°C, the enzyme progressively lost its activity. These observations confirmed that the ChB tag bound to the DEAE molecules of the paper filter behaves as a thermostable protein, keeping its functional conformation, as occurred with the soluble protein in the presence of free choline (see above).

DISCUSSION

The heterologous production of thermophilic enzymes in mesophilic organisms, such as *E. coli*, has become very popular, because there are numerous advantages to this type of production on an industrial scale (34). However, there has been little effort to improve the production of thermophilic enzymes in combination with affinity tags that could facilitate their purification and immobilization. Procedures that utilize the affinities of biomolecules and ligands for the immobilization of enzymes are gaining increasing acceptance in the construction of sensitive enzyme-based analytical devices as well as for other applications (23). One example of a tag-fused thermophilic protein was created to facilitate heat-mediated activation of an affinity-immobilized *Taq* DNA polymerase (18). This polymerase was released from the matrix at the same time and activated by thermal denaturation of the fused tag.

In this study, we have explored and exploited the thermophilic properties of the ChB tag by fusing it to an important thermophilic enzyme, such as the Pyr from *T. thermophilus*. To do this, the ChB tag was improved by the following changes in two traits with respect to those of the original tag (25). (i) The 3′ end of the *chbD* gene cloned into pAPC60 is not truncated, as was the case for the tag encoded by pCUZ1 (25). This change increases the solubility of the ChB tag protein. (ii) The fusion of the thrombin recognition site to the C terminus of the tag allows the release of the native protein from the ChB tag. These advantages, together with the low cost of the chromatographic supports used to purify and immobilize the fused proteins, the high specificity of the binding process, and the versatility of elution buffers, make the ChB tag an attractive alternative compared to other affinity systems. In several aspects, the utility of the ChB tag is comparable to that of the well-known cellulose-binding domain (31), but the new tag provides additional properties and can be used with inexpensive matrixes other than cellulose.

The experimental results reported here on the purification and immobilization of the ChBD-Pyr fusion protein show the

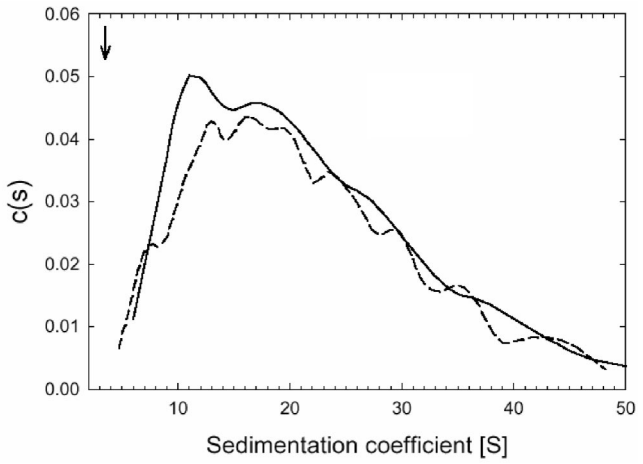

FIG. 4. Sedimentation coefficient distribution $c(s)$ of the ChBD-Pyr protein. Samples were equilibrated in 20 mM Tris-HCl buffer (pH 8.0) with either 1.5 M NaCl plus 2% choline (solid line) or 0.2 M NaCl (broken line). The arrow shows the theoretical sedimentation coefficient of a protein monomer (3.7S), assuming a spherical shape.

versatility of the ChB tag. Our results have shown that the ChB tag was not released from the DEAE-matrix after a thermal treatment, strongly suggesting that the interaction of the protein with the immobilized ligand mimics the situation observed in solution preserving the protein from a thermal denaturation. It is noteworthy that the protein retains its native conformation, since after the thermal treatment not only can it be released by choline but it can be reloaded onto a new DEAE-matrix after dialysis of the choline (not shown). Most likely the DEAE-matrix provides a three-dimensional interaction, with the ChBD occupying at the same time all their choline binding sites and, therefore, mimicking a high concentration of free choline.

Elucidation of the crystal structure of Pyr from *T. thermophilus* demonstrated that this protein mainly folds as a hexameric molecule (29). More recently, crystallographic analysis of the ChBD of the major LytA autolysin from *S. pneumoniae* has revealed that this tag can bind four molecules of choline per monomer (3). Thus, theoretically, the hexameric ChBD-Pyr protein can be adsorbed to the matrix through a three-dimensional multipoint interaction with multiple choline-binding sites.

Analytical ultracentrifugation analysis has demonstrated that the multimeric nature of the purified chimeric protein is maintained in the presence of different concentrations of NaCl or choline. Figure 4 shows the sedimentation coefficient distributions $c(s)$ of the ChBD-Pyr protein equilibrated in either 0.2 M NaCl or 1.5 M NaCl plus 2% choline obtained from sedimentation velocity. In both cases, it is clear that the protein sediments as polydisperse samples with a rather broad distribution of species much larger than that of the monomer (3.7S). It is interesting that whereas the aggregation state of Pyr should favor the stability of the immobilized ChBD-Pyr, in this case this property limits the specific elution of the protein by choline from the DEAE matrix. Nevertheless, we have observed that the addition of a mild detergent in the buffer improves its specific elution by choline. The detergent does not eliminate the specific DEAE-ChBD interaction, because the protein cannot be released only by the detergent, but it might relax the Pyr-Pyr oligomer interactions, decreasing the global binding affinity and allowing free choline to favorably compete with DEAE in the choline-binding site of ChBD. The dual behavior of this tag in the absence (thermally unstable) and presence (thermally stable) of choline is a novel property that might be exploited to activate or deactivate fused enzymes. Moreover, the results of proteolytic experiments performed with thrombin confirmed that the thermal stability of the fused ChB tag strongly depends on the presence of choline. It is not infrequent that the proteolytic site created in a tag to facilitate the further release of the native protein was hidden in the folded conformation of the fusion. Although this was the case for the ChBD-Pyr protein, we were able to overcome this problem, because the ChB tag is easily denatured in the absence of choline, making the thrombin site accessible to the protease.

Cellulose is an inexpensive, chemically inert material, which is safe for use even in food or pharmaceutical applications. Many cellulose matrixes, including paper filters with different properties, are available. Cellulose paper is an attractive matrix for enzyme immobilization mainly because of its highly suitable physical properties and its very low price. Cellulose membrane supports provide large surface areas for high enzyme loading without diffusional limitations, and the high hydrophilicity of cellulose can also favor the activity of many enzymes. Since the ChB tag fusions adsorb spontaneously to DEAE-cellulose paper from almost any solution, no pretreatment of the samples is required prior to the immobilization. Therefore, the immobilization method described in this work simplifies the process, allowing the purification and immobilization steps to be performed in a single filtration step. The ChB tag system could be very useful for performing biotransformations in the pharmaceutical and chemical industries, since it provides an immobilized enzyme in a solid support that can be very easily pulled out when the reaction has been accomplished. DEAE-cellulose paper offers the advantages of low cost, ease of immobilization and handling, long shelf-life, and no danger of microbial spoilage of the enzyme, since the paper strips are usually used once. Finally, the affinity of the ChB tag for DEAE-cellulose paper provides another interesting characteristic of the tag, since the soluble enzymes fused to ChBD can be easily and specifically removed from a reaction mixture by a simple filtration. This characteristic could be very useful for those reactions that can be performed in successive and independent steps.

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REFERENCES

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
2. **Dignam, J. D., and M. P. Deutscher.** 1979. Aminoacyl-tRNA synthetase stimulatory factors and inorganic pyrophosphatase. Biochemistry **18:**3165–3170.

3. **Fernández-Tornero, C., R. López, E. García, G. Giménez-Gallego, and A. Romero.** 2001. A novel solenoid fold in the cell wall anchoring domain of the pneumococcal virulence factor LytA. Nat. Struct. Biol. **8:**1020–1024.
4. **García, P., J. L. García, E. García, and R. López.** 1986. Nucleotide sequence and expression of the pneumococcal autolysin gene from its own promoter in *Escherichia coli*. Gene **43:**265–272.
5. **Gibson, N. J., C. R. Newton, and S. Little.** 1997. A colorimetric assay for phosphate to measure amplicon accumulation in polymerase chain reaction. Anal. Biochem. **254:**18–22.
6. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
7. **Hansen, T., C. Urbanke, V.-M. Leppänen, A. Goldman, K. Brandenburg, and G. Schäfer.** 1999. The extreme thermostable pyrophosphatase from *Sulfolobus acidocaldarius*: enzymatic and comparative biophysical characterization. Arch. Biochem. Biophys. **363:**135–147.
8. **Hoe, H. S., H. K. Kim, and S. T. Kwon.** 2001. Expression in *Escherichia coli* of the thermostable inorganic pyrophosphatase from the *Aquifex aeolicus* and purification and characterization of the recombinant enzyme. Protein Expr. Purif. **23:**242–248.
9. **Hoe, H. S., H. K. Kim, and S. T. Kwon.** 2002. Cloning, analysis, and expression of the gene for inorganic pyrophosphatase of *Aquifex pyrophilus* and properties of the enzyme. Mol. Cell **13:**296–301.
10. **Kent, R. B., and S. K. Guterman.** 1982. Pyrophosphate inhibition of rho ATPase: a mechanism of coupling to RNA polymerase activity. Proc. Natl. Acad. Sci. USA **79:**3992–3996.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
12. **Lahti, R.** 1983. Microbial inorganic pyrophosphatases. Microbiol. Rev. **47:** 169–179.
13. **Lanzetta, P. A., L. J. Alvarez, P. S. Reinach, and O. A. Candia.** 1979. An improved assay for nanomole amounts of inorganic phosphate. Anal. Biochem. **100:**95–97.
14. **Laue, T. M., B. D. Shah, T. M. Ridgeway, and S. L. Pelletier.** 1992. Interpretation of analytical sedimentation data for proteins, p. 90–125. *In* S. Harding, A. Rowe, and J. Horton (ed.), Analytical ultracentrifugation in biochemistry and polymer science. Royal Society of Chemistry, Cambridge, United Kingdom.
15. **Medrano, F. J., M. Gasset, C. López-Zumel, P. Usobiaga, J. L. García, and M. Menéndez.** 1996. Structural characterization of the unligated and choline-bound forms of the major pneumococcal autolysin LytA amidase. Conformational transitions induced by temperature. J. Biol. Chem. **271:**29152–29161.
16. **Meyer, W., R. Moll, T. Kath, and G. Schäfer.** 1995. Purification, cloning, and sequencing of archaebacterial pyrophosphatase from the extreme thermoacidophile *Sulfolobus acidocaldarius*. Arch. Biochem. Biophys. **319:**149–156.
17. **Moldes, C.** 2003. Desarrollo de nuevos sistemas para la producción de proteínas de fusión por fermentación. Ph.D thesis. Universidad Complutense, Madrid, Spain.
18. **Nilsson, J., M. Bosnes, F. Larsen, P. A. Nygren, M. Uhlen, and J. Lundeberg.** 1997. Heat-mediated activation of affinity-immobilized *Taq* DNA polymerase. BioTechniques **22:**744–751.
19. **Ong, E., N. R. Gilkes, R. C. Miller, A. J. Warren, and D. G. Kilburn.** 1991. Enzyme immobilization using a cellulose-binding domain: properties of a beta-glucosidase fusion protein. Enzyme Microb. Technol. **13:**59–65.
20. **Pessela, B. C., C. Mateo, A. V. Carrascosa, A. Vian, J. L. Garcia, G. Rivas, C. Alfonso, J. M. Guisan, and R. Fernandez-Lafuente.** 2003. One-step purification, covalent immobilization, and additional stabilization of a thermophilic poly-His-tagged beta-galactosidase from *Thermus* sp. strain T2 by using novel heterofunctional chelate-epoxy Sepabeads. Biomacromolecules **4:**107–113.
21. **Reddy, K. R. C., P. K. Srivastava, P. M. Dey, and A. M. Kayastha.** 2004. Immobilization of pigeonpea urease (*Cajanus cajan*) on DEAE-cellulose paper strips for urea estimation. Biotechnol. Appl. Biochem. **39:**323–327. (First published 31 October 2003; 10.1042/BA20030122.)
22. **Richter, O. M., and G. Schafer.** 1992. Cloning and sequencing of the gene for the cytoplasmic inorganic pyrophosphatase from the thermoacidophilic archaebacterium *Thermoplasma acidophilum*. Eur. J. Biochem. **209:**351–355.
23. **Saleemuddin, M.** 1999. Bioaffinity based immobilization of enzymes. Adv. Biochem. Eng. Biotechnol. **64:**203–226.
24. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Sánchez-Puelles, J. M., J. M. Sanz, J. L. García, and E. García.** 1992. Immobilization and single-step purification of fusion proteins using DEAE-cellulose. Eur. J. Biochem. **203:**153–159.
26. **Sánchez-Puelles, J. M., J. M. Sanz, J. L. García, and E. García.** 1990. Cloning and expression of gene fragments encoding the choline-binding domain of pneumococcal murein hydrolases. Gene **89:**69–75.
27. **Satoh, T., T. Samejima, M. Watanabe, S. Nogi, Y. Takahashi, H. Kaji, A. Teplyakov, G. Obmolova, I. Kuranova, and K. Ishii.** 1998. Molecular cloning, expression, and site-directed mutagenesis of inorganic pyrophosphatase from *Thermus thermophilus* HB8. J. Biochem. **124:**79–88.
28. **Schuck, P.** 2000. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. Biophys. J. **78:**1606–1619.
29. **Teplyakov, A., G. Obmolova, K. S. Wilson, K. Ishii, H. Kaji, T. Samejima, and I. Kuranova.** 1994. Crystal structure of inorganic pyrophosphatase from *Thermus thermophilus*. Protein Sci. **3:**1098–1107.
30. **Thorner, J., S. D. Emr, and J. N. Abelson (ed.).** 2000. Methods in enzymology, vol. 326. Applications of chimeric genes and hybrid proteins, part A. Gene expression and protein purification. Academic Press, Inc., San Diego, Calif.
31. **Tomme, P., A. Boraston, B. McLean, J. Kormos, A. L. Creagh, K. Sturch, N. R. Gilkes, C. A. Haynes, R. A. J. Warren, and D. G. Kilburn.** 1998. Characterization and affinity applications of cellulose-binding domains. J. Chromatogr. B **715:**283–296.
32. **Van der Horn, P. B., M. C. Davis, J. J. Cunniff, C. Ruan, B. F. McArdle, S. B. Samols, J. Szasz, G. Hu, K. M. Hujer, S. T. Domke, S. R. Brummet, R. B. Moffett, and C. W. Fuller.** 1997. Thermo sequenase DNA polymerase and *T. acidophilum* pyrophosphatase: new thermostable enzymes for DNA sequencing. BioTechniques **22:**758–762.
33. **Verhoeven, J. A., K. M. Schenck, R. R. Meyer, and J. M. Trela.** 1986. Purification and characterization of an inorganic pyrophosphatase from the extreme thermophile *Thermus aquaticus*. J. Bacteriol. **168:**318–321.
34. **Vieille, C., and G. J. Zeikus.** 2001. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol. Mol. Biol. Rev. **65:**1–43.