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ENGINEERED BirA FOR *IN VITRO* BIOTINYLATION

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A major attraction to use *Bacillus subtilis* as an expression host for heterologous protein production is its capability to secrete extracellular proteins into the culture medium. To take full advantage of this system, an efficient method of recovering the target protein is crucial. For secretory proteins which cannot be purified by a simple scheme, *in vitro* biotinylation using biotin ligase (BirA) offers an effective alternative for their purification. Availability of large amounts of quality BirA can be critical for *in vitro* biotinylation. We report here the engineering and production of an E. coli BirA and its application in the purification of staphylokinase, a fibrin-specific plasminogen activator, from the culture supernatant of *B. subtilis* via *in vitro* biotinylation. BirA was tagged with both a chitin binding domain and a hexahistidine tail to facilitate both its purification and its removal from the biotinylated sample. We show in this paper how, in a unique way, we solved the problem of protein aggregation in the *E. coli* BirA production system to achieve a yield of soluble functional BirA hitherto unreported in the literature. Application of this novel BirA to protein purification via *in vitro* biotinylation in general will also be discussed. Biotinylated staphylokinase produced in the study not only can act as an intermediate for easy purification; it can also serve as an important element in the creation of a blood clot targeting and dissolving agent.

"Designer affinity purification" of proteins (1), a strategy which involves fusing the target protein with an affinity tag to facilitate its purification, is an attractive approach for efficient purification of target proteins from a crude preparation. Many affinity tags have been developed for this purpose, among which glutathione S-transferase (GST) (2), P-galactosidase (3), maltose binding protein (4), and biotin acceptor domains (5, 6) are more popular. Since some of these proteins tags are relatively large, it is not uncommon for them to contribute to more than 50% of the molecular mass of the protein fusions and create some undesirable side effects. For example,

large tags can occasionally cause solubility problem during protein production and adversely affect the conformation and biological activity of the target proteins (7, 8). Moreover, use of these tags usually requires post-purification tag removal by chemical or enzymatic means which can be a challenging, time-consuming and costly process and which may not be compatible with the target protein (1). For these reasons, small tags including His-tag (9), strep-tag (10) and biotinylation tag (11, 12) are preferred choices. Strep-tags I and II are short peptides (9 amino acids in length) that can bind selectively to streptavidin (Kd -10' M). Biotinylation tags, identified through screening of a peptide library (11, 12, 13), are peptide tags (13-15 amino acids) that can be biotinylated enzymatically using the *E. coli* biotin ligase (BirA). Since the affinity of both his- and strep-tags to their affinity matrices is not particularly high, presence of contaminants is a common problem. This shortcoming can be avoided by the use of the biotinylation tag since biotin binds to monomeric avidin (14, 15) or nitro-avidin (16) with a much higher affinity (Yd 10' to 10' M). A systematic comparison of the use of these three tags to purify a rat neurotensin receptor expressed in *E. coli* demonstrates that biotinylation tag provides the highest efficiency and purity (17).

Recombinant proteins with biotinylation tags have commonly been biotinylated *in vivo* using endogenous BirA or co-overproduced BirA (6, 17, 18, 19). This approach may not be desirable for some applications as *in vivo* biotinylation has a number of drawbacks. First, incomplete biotinylation of the target proteins because of limited cellular resources (BirA and ATP) is a common occurrence (18). Limitation caused by BirA deficiency could sometimes be overcome by coexpressing birA (17); however, limitation from depletion of intracellular ATP is more difficult to address. In the biotinylation reaction, BirA uses ATP to introduce the biotin moiety to the lysine residue on the biotinylation tag (20). Thus, all events involved in producing the biotinylated protein fusions *in vivo* (production of BirA, production of the target protein, enzymatic biotinylation) are highly energy-demanding processes. This may explain why in some cases, coexpression of BirA could only partially improve the biotinylation efficiency with still a large amount of the target proteins remaining unbiotinylated (17, 18). A second drawback with *in vivo* biotinylation is the presence of endogenous biotinylated proteins. For example, *E. coli* has one (biotin carboxyl carrier protein or BCCP, a subunit of acetyl-CoA carboxylase)(5) and *B.*

subtilis has two such biotinylated entities (BCCP and pyruvate carboxylase)(21). Although these biotinylated proteins are present as a tiny fraction of the total intracellular proteins, the prospect of having these contaminants in an otherwise highly pure sample is definitely undesirable. Finally, *in vivo* biotinylation has a serious limitation: it cannot be used to effectively biotinylate secreted proteins since both BirA and ATP are intracellular. Thus, this technique cannot be used to exploit the full advantages of secretory production of heterologous proteins such as ease of protein recovery, reduced cell toxicity and absence of intracellular biotinylated contaminants as mentioned earlier. It may be desirable to avoid these limitations by carrying out biotinylation *in vitro*.

For *in vitro* biotinylation, availability of BirA can be a critical factor. This is particularly true if large scale protein purification via biotinylation is the goal. In this paper, we describe the production and characterization of an engineered BirA which contains a C-terminal His-tag and an N-terminal chitin binding domain (CBD). Both tags facilitate the purification of this engineered version of BirA. In addition, the N-terminal CBD also allows the rapid removal of BirA from the biotinylation mixture after the completion of the reaction. This engineered BirA was applied to biotinylate a secretory recombinant protein (staphylokinase) from Bacillus subtilis. The recombinant staphylokinase was tagged with a biotinylation peptide. We show that, by coupling *in vitro* biotinylation with subsequent affinity purification using monomeric avidin, we have been able to recover a large amount of active staphylokinase in high purity.

Construction of pET-BirA-His

Plasmid pET-BirA-His is an expression vector to produce BirA with a C-terminal hexahistidine tag in *E. coli* using the T7 promoter system. *E. coli* BirA was amplified by PCR with *E. coli* genomic DNA as the template and synthetic oligonucleotides ECBIRAF (5' GGGAATTCTAAGGATAACACCGTGCCACTG 3') and ECBIRAB (5' GGAAGCTT TTGTTTTTCTGCACTACGCAGGG 3') as the forward and backward primers, respectively. The amplified product carried an *Eco*RI site at the 5' end and a *Hind*III site at the 3' end. The 970-bp fragment was digested with *Eco*RI/*Hind*III and inserted in frame to pET-29b (Novagen, USA) to give pET-BirA-His.

Construction of pET-CBD-BirA-His

This vector allows the production of CBD-BirA-His in *E. coli*. The gene encoding a chitin binding domain (22) was amplified from the pCYBI plasmid carrying the CBD of chitinase Al (New England BioLabs, Canada) using the forward primer CBDF (5' CCCATATGACGACAAATCCTGGTGTATCC 3') and the backward primer CBDB (5' CCAGATCTTGAAGCTGCCACAAGGCAGGAAC 3'). The 165-bp amplified product was then digested by *Nde*I and *Bgl*II and inserted into pET-BirA-His. The resulting plasmid, designated pET-CBD-BirA-His, was transformed to *E. coli* BL21(DE3) (Novagen, USA) for expression studies.

Construction of pSAKPFB

This is a *B. subtilis* vector for secretory production of staphylokinase (SAK) containing a C-terminal biotinylation peptide (PFB). This vector used a strong and constitutively expressed promoter (P43) to drive the transcription and a B. subtilis levansucrase signal peptide to direct the secretion. The biotinylation tag was fused translationally to secretory staphylokinase in the following manner. The sequence encoding PFB was fused in frame to the 3'-end of the *sak* gene in pSAK-K1, a pWB980-based vector in B. subtilis (23), by PCR with pSAK-K1 as the template, 5' CAAGCAACAGTATTAACC 3' as the forward primer and 5' CCAAGCTTATCGATGATTCCAAACCATTTTTTGTGCAT CAAGAATATGATGAAGGGATCCAGAGCCACTAGTAGATCC 3' as the backward primer. The backward primer encodes a 15-amino acid peptide with the amino acid sequence LHHILDAQKMVWNHR (11). The amplified 578-bp fragment was digested with *Hind*III and used to replace an equivalent fragment from *Hind*III digested pSAK-K1. The resulting plasmid, designated pSAKPFB, was transformed to *B. subtilis* WB800, an eight-protease deficient strain (24) and the transformants screened for the right orientation of the insert.

Cell growth

E. coli BL21[pET-CBD-BirA-His] was grown at 30°C in Luria broth (1% tryptone, 0.5% yeast extract, 0.2% NaCl) containing 30 μg/ml kanamycin to 150 klett units in a shake flask. IPTG was then added to a final concentration of 0.1 mM and growth continued for 5-10 hours. Cell density was measured using a Klett-Summerson photoelectric colorimeter with a green filter (Klett Mfg. Co., USA). *B. subtilis* WB800[pSAKPFB] was cultivated in super-rich medium (25) containing 10 pg/ml of kanamycin at 37°C in a shake flask. Cells were harvested at 5-6 hours after inoculation.

Purification of BirA

Cells of *E. coli* BL21 [pET-CBD-BirA-His] were harvested by centrifugation at 10,000 x g for 5 min at 4°C. Cell pellet was resuspended in lysing buffer, disrupted with French press and the crude lysate was separated into the soluble and insoluble fractions by centrifugation (20,000 x g for 20 min). CBD-BirA-His in the soluble fraction was purified by either of two schemes: metal chelation chromatography or chitin affinity chromatography. For metal chelation chromatography, the lysing buffer contained 15 mM imidazole, 0.5M NaCl, 0.1% Triton X, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 20 mM Tris-HCl, pH 8.0. His.Bind Quick 900 cartridges (Novagen, USA) charged with Ni^{2+} were used as the affinity matrix. CBD-BirA-His was eluted stepwise with increasing imidazole concentrations (60 mM, 250 mM, 1 M imidazole) according to the manufacturer's suggestions. For chitin afflinity chromatography, cells were lysed in buffer containing 1M NaCl, 1 mM EDTA, 0.1% Triton X, 5 mM β-mercaptoethanol, 1 mM PMSF and 20 mM sodium phosphate, pH 7.0. The soluble cellular fraction was loaded to a column packed with chitin beads (New England BioLabs, Canada) equilibrated in lysing buffer. After washing with 5-10 column volumes of lysing buffer followed by 20 mM sodium acetate, pH 5.5, CBD-BirA-His was eluted with 20 mM acetic acid, pH 3.0.

In either scheme, fractions containing pure CBD-BirA-His (confirmed by SDS-PAGE) were pooled, concentrated and buffer-changed to a storage solution containing 50 mM imidazole, 50 mM NaCl, 5% glycerol and 5 mM β-mercaptoethanol, pH 6.8, using Ultrafree-4

centrifugation tubes (Millipore Corporation, USA). Pure CBD-BirA-His was quantified by its absorbance at 280 run using a molar extinction coefficient of 68420 M^{-1} cm^{-1} (26).

Purification of SAK-PFB

Culture supernatant of *B. subtilis* WB800[pSAKPFB] was separated from the cells by centrifugation at 10,000 x g for 10 min at 4°C. SAK-PFB was precipitated with ammonium sulfate to 65% saturation at 4°C, desalted by dialysis, concentrated to appropriate volume and buffer-changed to 10 mM Tris-HC1, pH 8.0, using Ultrafree-4 centrifugation tubes (Millipore Corporation, USA). The sample was then biotinylated at 30°C for 4 hours to overnight, using CBD-BirA-His. The reaction mixture contained 50 mM bicine (pH 8.3), 10 mM ATP, 10 mM magnesium acetate, 50 µM biotin, and for every ml of final mix, 500 µg of SAK-PFB and 5 µg of purified *E. coli* CBD-BirA-His. Following the reaction, the sample was mixed with a small amount of chitin beads to remove CBD-BirA-His. After a simple centrifugation to remove the chitin beads, the sample was passed over a column containing Sephadex G-25 (Amersham Pharmacia Biotech, Canada) to remove the excess biotin. Biotinylated SAK-PFB was separated the unbiotinylated proteins by passing the sample over a monomeric avidin agarose column (Pierce, USA). Bound biotinylated SAK-PFB was eluted by competition with 2 mM d-biotin. Pure biotinylated SAK-PFB, was quantified by its absorbance at 280 nm using a molar extinction coefficient of 22,900 $M^{-1}cm^{-1}$ (26) for calculation.

Determination of the activity of purified CBD-BirA-His

The activity of purified CBD-BirA-His was compared with that of a wild type *E. coli* BirA available from a commercial source (Avidity, USA) using an ELISA method (13). In this assay, maltose binding protein-AviTag fusion (MBP-AviTag, Avidity, USA) was used as the substrate. AviTag is a peptide tag for efficient biotinylation (11). MBP-AviTag was adsorbed to the wells of a Reacti-bind maleic anhydride activated polystyrene strip plate (Pierce, USA). Biotinylation was carried out at 30°C with different amounts of enzymes and different reaction times. The reaction mixture contained 50 mM bicine (pH 8.3), 10 mM ATP, 10 mM magnesium acetate, 50

μM biotin and BirA from different sources. Biotin ligated to the AviTag was detected by its interaction with streptavidin-horseradish peroxidase (Pierce, USA) using 1 step slow TMB-ELISA (3,3',5,5'-tetramethylbenzidine, Pierce) as the color development reagent. A standard curve of biotinylation reaction was established using known quantities of fully biotinylated MBP-AviTag (Avidity, USA). Readings were taken at end point at 450 nm using a Bio-Tek CERES 900 plate reader (Bio-Tek Instruments, Inc., USA).

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometric analyses

Protein samples in 25 mM ammonium acetate and the matrix solution of sinapinic acid were mixed on the MALDI plate and analyzed on a Perseptive Biosystems (Framingham Mass.) Voyager-DE STR Mass spectrometer equipped with a pulsed nitrogen laser operated at 337 nm in a linear mode. The mass spectrometer was previously calibrated with apomyoglobin (horse skeletal) m/z 16952.56 and its dimer m/z 33905.12. These analyses were done in Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Canada.

Other methods

Vent DNA polymerase (New England BioLabs, Canada) was used for all DNA amplification reactions. The sequence of all PCR products was confirmed to be free of PCR errors by nucleotide sequencing based on the dideoxy method using a T7 sequencing kit from Amersham Pharmacia Biotech, Canada. SDS-polyacrylamide gel electrophoresis followed standard procedure based on the Laenimli system. Western blot was done on a nitrocellulose membrane using 4-chloro- 1 -naphthol (Bio-Rad, Canada) as the color development reagent. SAK activity was determined by radial caseinolysis assay on plasminogen-skim milk agarose plate (27).

Results

Production of *E. coli* CBD-BirA-His using the pET expression system.

In this study, an IPTG-induced expression of BirA in a pET-29b based vector was used for intracellular production of *E. coli* CBD-BirA-His in BL21(DE3). CBD-BirA-His (with both CBD- and His-tags) produced migrated as a 40-kDa protein on the SDS gel (Fig. 1). The presence of the His-tag was found to complicate the production because, at a growth temperature of 30°C, 90% of CBD-BirA-His accumulated as inclusion bodies (Fig. 1A, lanes 1 and 2). In contrast, over 80% of CBD-BirA (no His tag) produced under the same cultivation condition was in the soluble form (data not shown). To address the solubility problem, different measures were taken. These include lowering the growth temperature from 30°C downwards, lowering the salt concentration in the culture medium, varying the IPTG levels, and modifying the cellular osmotic environment with the use of sorbitol and betaine during cell growth (28). These measures at best yielded marginal improvement with still more than 70% of CBD-BirA-His present as insoluble aggregates. However, supplementing the culture medium with 10-20 μM biotin not only enhanced the growth rate of the culture (data not shown) but also conspicuously promoted solubility of CBD-BirA-His with about 40% of the protein in the soluble fraction (Fig. 1 A, lanes 3 and 4; Fig. 1 B, lanes 1 and 2). Moreover, whereas temperature lowering by itself did not effectively solve the problem of inclusion body formation, a measure combining biotin supplementation and temperature lowering (25°C, post-induction) enhanced BirA solubility significantly. Typically, 70-90% of BirA produced under this condition was in the soluble form (Fig 1B, lanes 3 and 4), amounting to about 100 mg of soluble CBD-BirA-His per liter of culture.

Engineered *E. coli* BirA could be purified with simple manipulations

CBD-BirA-His was equipped with two tags: a 6-amino-acid histidine tag preceded by an 8-amino-acid linker and a 53-amino-acid chitin binding domain followed by an 18-amino-acid linker. These tags allow rapid purification of the protein by either scheme: metal chelation or chitin affinity chromatography. Fig. 2A shows the purification of CBD-BirA-His using a Ni^{2+} chelation column. CBD-BirA-His bound to the column effectively with essentially no loss in the flow-through fractions (lane 2). Reasonably pure fractions (over 80% purity) were recovered by

elution with imidazole (lanes 3-5). CBD-BirA-His in these fractions could be further purified to over 95% purity by repeatedly reloading the purified CBD-BirA-His to the Ni^{2+} chelation column. The chitin affinity scheme was more efficient. CBD-BirA-His bound to the chitin column with high affinity and great specificity with no CBD-BirA-His detectable in the flow-through and washes (Fig. 2B, lanes 2 and 3). A single-column operation was usually adequate to recover CBD-BirA-His with over 95% purity (Fig. 2B, lane 4). Chitin affinity chromatography, however, has a major drawback. About 40-50% of the CBD-BirA-His tended to be retained on the column and could not be recovered even with extensive washes and elutions at low pH. Despite this drawback, we have been able to recover 1.5-2 mg of highly pure CBD-BirA-His from 100 ml of shake flask culture using the chitin column, representing an overall recovery yield of 15-20%. The recovery rate with the metal chelation scheme (involving three cycles of Ni' chelation column) to purify CBD-BirA-His with over 95% purity is similar.

Purified engineered BirA demonstrated high biological activity

Activity of CBD-BirA-His was determined by its ability to biotinylate maltose binding protein tagged with a short biotinylation peptide designated AviTag (11) in an ELISA study. With unbiotinylated MBP-AviTag as the substrate using parameters (amount of enzyme used and reaction time) that ensured a linear rate of enzymatic reaction, the activity of CBD-BirA-His purified from either scheme was found to be 50% more active than that of the natural *E. coli* BirA from a commercial source (Table 1).

Table 1. Activity of BirA from different sources

Source of BirA	Specific Activity	Relative Activity
Metal chelation[1]	40.2	1.48
Chitin affinity[2]	42.7	1.58
Commercial[3]	27.1	1

Activity of BirA was determined by ELISA method (13) using unbiotinylated MBP-AviTag (Avidity, USA) as the substrate. Specific activity of BirA is defined as ng biotinylated MBP-

AviTag formed per min per μg of enzyme at 30°C. [1]CBD-BirA-His purified by metal chelation chromatography. [2]CBD-BirA-His purified by chitin affinity scheme. [3]Wild type *E. coli* BirA obtained from a commercial supplier (Avidity, USA). Data represent the average of two independent trials.

This shows that the BirA engineered, produced and purified using our purification scheme is of high quality. The presence of His-tag has little effect on the biological activity of the purified enzyme as CBD-BirA and CBD-BirA-His exhibited similar specific activities on biotinylation of MBP-AviTag (data not shown). The readiness of CBD-BirA-His to biotinylate proteins with a biotinylation tag was also demonstrated in a Western blot analysis (Fig. 3). Two test proteins were used as examples: MBP-AviTag and staphylokinase tagged with another biotinylation tag designated PFB. Probing with streptavidin-horseradish peroxidase showed biotinylation of both proteins with BirA (Fig. 3B, lanes 1 and 2).

Engineered BirA is active in a fairly broad pH range

The pH activity profile of CBD-BirA-His was established with an ELISA study similar to the one used for the determination of its biotinylation activity. Different reagents were used to provide buffering capacity for a broad pH range (see legend to Fig. 4). MBP-AviTag was used as the substrate. Fig. 4 shows that CBD-BirA-His had a pH optimum around 6.5. It retained a fairly high activity at pH 5.5-8.3, but the activity dropped substantially at either ends. This information would be useful for one to tailor an optimal condition for *in vitro* biotinylation with this enzyme. To our knowledge, the pH activity profile of natural *E. coli* BirA has not been systematically studied before.

Secretory production of staphylokinase-PFB from B. subtilis

To explore the possibility of purifying a secretory fusion protein carrying a biotinylation tag from a *B. subtilis* culture supernatant via *in vitro* biotinylation using the engineered BirA, staphylokinase (SAK), a very promising blood clot dissolving agent (29), was used as a model

system. A 15-amino-acid biotinylation tag (PFB) was added to the C-terminal end of SAK containing an 18-amino-acid C-terminal linker sequence [$(GSTSG)_3SGS$]. Addition of the linker and the biotinylation tag did not affect the secretory production yield of SAK-PFB since SAK with or without PFB was produced at a comparable level (Fig. 5A, lanes 1 and 2). When analyzed by SDS-PAGE, SAK-PFB showed an apparent molecular mass of 21 kDa. The calculated molecular mass of SAK-PFB is 18,862 Da. To confirm that the intact form of SAK-PFB was produced from B. subtilis, the molecular mass of SAK-PFB was determined by MALDI-TOF mass spectrometry. The observed molecular mass matched closely with the expected value and was determined to be 18,861.22 Da (data not shown).

Functional SAK-PFB could be purified via *in vitro* biotinylation using the engineered BirA

After concentrated from the culture supernatant, SAK-PFB was biotinylated *in vitro* using purified CBD-BirA-His. The rate of biotinylation depends, among other variables, on the amount of enzyme used for the reaction. As SAK-PFB is fairly stable, biotinylation could be carried out using varying amounts of enzyme from several hours to overnight with no apparent adverse effect. Biotinylated SAK-PFB, with an apparent molecular mass of 21.5 kDa on the SDS gel, migrated more slowly than its unbiotinylated counterpart (Fig. 5A, lane 3 vs. lane 2, Fig. 6, lane 2 vs. lane 1). This allows us to easily monitor the extent of biotinylation. In all biotinylation runs attempted so far, over 95% biotinylation of SAK-PFB could be achieved as demonstrated by the absence of any significant amount of SAK-PFB in the flow-through or washes of the monomeric avidin agarose column (Fig. 6, lanes 3 and 4). The completion of biotinylation was also demonstrated by the MALDI-TOF mass spectrometric analysis. The peak with the expected molecular mass corresponding to the unbiotinylated form of SAK-PFB disappeared completely in the biotinylated sample while a new peak with the expected molecular mass corresponding to the biotinylated form appeared (data not shown). Biotinylated SAK-PFB could be effectively purified using a monomeric avidin agarose column with remarkable specificity (Fig. 6, lanes 5-7). We have been able to recover about 450 μg of highly pure SAKPFB from a crude sample containing 600 μg of SAK-PFB on a single column, representing an overall yield of 75%. SAK-PFB purified by this method showed full biological activity as compared with both the

unbiotinylated form and the natural, untagged SAK on a plasminogen assay ml of *B. subtilis* culture.

Discussion

To capture the full advantages of *in vitro* biotinylation, a ready source of easily purified, high quality BirA is needed. In this study, we addressed this concern by engineering an *E. coli* BirA with a different tag at each end (CBD-BirA-His). These tags enable easy recovery of the protein by simple column manipulations. Use of the His-tag allows a one-step recovery of large amounts of reasonably pure BirA, while use of the CBD enables, again, a single-column recovery of a lesser quantity of ultrapure BirA. These two grades of BirA can be found useful in different applications. For example, reasonably pure BirA can be used to biotinylate a crude extract (such as the secreted fraction) as other contaminants can be removed later via the monomeric avidin step. On the other hand, ultrapure BirA is critical in the biotinylation of pure proteins (such as affinity-purified single chain antibodies). Besides the tag advantage, the production yield and quality of our engineered BirA compare favourably with the literature data. By supplementing the medium with biotin and lowering the post-induction temperature to 25°C, the soluble CBD-BirA-His reached a level of 100 mg per liter of culture. This level is double the amount of GST-BirA reported previously (30). Moreover, the specific activity of CBD-BirAHis was found to be more than that of the natural BirA from a commercial source. In one study involving GST-BirA (30), thrombin was applied to cleave off GST from the fusion and the resulting BirA showed a comparable activity similar to that of the wild type BirA. In another case (19), GST-BirA, used uncleaved, was shown to retain biotin ligase activity but the specific activities of the fused and non-fused versions were not studied.

Several interesting and important observations were made during the development of the engineered BirA. First, supplementation of biotin in the culture medium could help reduce the formation of inclusion bodies. Biotin was commonly included in the culture medium in *in vivo* biotinylation studies involving the *E. coli* system since *E. coli* has been shown to uptake biotin via an active transport mechanism (31). In those studies, biotin served mainly as one of the

substrates for BirA in the biotinylation reaction. Our observation in this work suggests that being a substrate, biotin can also possibly enhance the proper folding of BirA in favour of soluble protein formation. Second, presence of small tags at both ends of BirA does not materially affect the biological activity of BirA as a biotin ligase. We designed two small affinity tags for the BirA: a 53-amino acid chitin binding domain and a 6-amino acid His-tag. The engineered BirA, used as such, demonstrated a higher specific activity than that of the natural BirA (from a commercial source). This shows that the engineered BirA retained good biological activity through the purification procedure and, unlike some large tags, can be used uncleaved. Third, although CBD-BirA could be produced as a soluble enzyme in large quantities, addition of a short C-terminal His-tag severely reversed the situation with the problematic formation of inclusion aggregates. This shows that the use of small tags does not guarantee that the system will work as expected. Even if the tags do not affect biological activity of the target protein, complications like protein insolubility during production can arise and have to be addressed accordingly.

Two interesting observations were also made during the purification of the biotinylated proteins. Occasionally, we detected a biotin-BirA complex in Western blot probed with streptavidin-horseradish peroxidase even though the sample had been boiled in the presence of SDS before loading to the SDS-polyacrylamide gel. This complex is likely to be the tight entity ($Kd = 7 \times 10^{-11}$) formed between BirA and biotinoyl-5'-AMP, an intermediate in the biotinylation reaction carried out by BirA (32). The presence of this complex means that postbiotinylation removal of BirA is necessary not only when pure target protein is involved but also when crude sample is used for biotinylation. The installation of the N-terminal CBD in CBDBirA-His allows rapid removal of BirA by the use of chitin beads. In the purification of SAKPFB, CBD-BirA-His was removed by chitin bead treatment in a simple centrifugation step to avoid the potential problem of contamination. Thus, the tags on CBD-BirA-His facilitate not only purification of CBD-BirA-His but also removal of CBD-BirA-His from the postbiotinylation reaction mixture. Another interesting observation is that the biotinylated protein exhibited a small mobility shift on the SDS gel. This has a practical application for the biotinylation of small target proteins as one

may be able to monitor the extent of biotinylation, easily by SDS-PAGE. This method worked well for SAK-PFB with a molecular mass of 19 kDa.

In vitro biotinylation offers a general tool to affinity purify secretory proteins not only from *E. coli* but also from other organisms such as *B. subtilis*. This approach is most valuable for the purification of proteins (e.g. staphylokinase) which cannot be recovered by other affinity purification methods and which require multiple chromatographic steps for their purification. As demonstrated in this study, addition of the biotinylation tag to staphylokinase affected neither the production yield nor the biological activity of staphylokinase and intact SAK-PFB could be produced as confirmed by mass spectrometric analysis. This system works best when the target protein has a high-level expression, the fusion is stable, and protease activity is absent. The high efficiency biotinylation achieved with our SAK-PFB study may be attributed to the remarkable secretory yield of SAK in *B. subtilis* (over 100 mg/l in a shake flask) (3 3), the stability of SAK-PFB, and the use of an eight-protease deficient strain which has been shown to dramatically enhance the yield (24) and stability (unpublished data) of some secretory proteins in B. subtilis. The high efficiency biotinylation, coupled with the high capacity of monomeric avidin with its exceptional affinity and specificity to biotin, contributes to a remarkable recovery of quantitative amounts of distinctly pure staphylokinase. This approach can be applied to other secretory proteins from *B. subtilis*.

Besides protein purification, the homogeneous biotinylated products made possible by the highly selective, site-specific action of CBD-BirA-His on the biotinylation tag offers many other applications. They serve as agents in immunoassays, drug delivery, imaging and targeting (34, 3 5, 36, 37). Biotinylated proteins can also be immobilized in an orientation-specific manner (38) to generate protein or antibody biochips for surface plasmon resonance based biosensor measurements (39, 40), active electronic microchips for biomolecule detection and quantification (41), and high density protein microarrays for high throughput proteomics studies (42).

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Figure legends Fig. I

Effects of (A) biotin and (B) growth temperature on the distribution of CBD-BirA-His in the intracellular fractions of *E. coli*. (A) Cultures were grown at 30°C throughout. Lanes 1 and 2: no biotin added to culture medium. Lanes 3 and 4: biotin added at 12 μM to culture medium. (B) Growth medium contained 12 μM biotin for all samples. Lanes 1 and 2: cultures grown at 30°C throughout. Lanes 3-6: cultures grown at 30°C and shifted to 25°C post IPTG induction. Lanes 1-4: *E. coli* BL2l(DE3)[pET-CBD-BirA-His]; Lanes 5 and 6: negative control, *E. coli* BL21(DE3)[pET29b]. Samples were analyzed on a 10% SDS-polyacrylamide gel and stained by Coomassie blue. M: molecular weight marker; S: soluble fraction; I: insoluble fraction. Arrow indicates CBD-BirA-His.

Fig. 2

Purification of *E. coli* CBD-BirA-His using (A) Ni^{2+} column and (B) chitin affinity column. (A) Lane 1: crude lysate. Lane 2: column flow-through. Lanes 3: eluates (60 mM imidazole). Lane 4: eluate (250 mM imidazole). Lane 5: eluate (μM imidazole). (B) Lane 1: crude lysate. Lane 2: column flow-through. Lane 3: pooled washes (2-column volumes). Lane 4: eluate. Samples were analyzed on a 10% SDS-polyacrylamide gel. M: molecular weight marker. Arrow indicates CBD-BirA-His.

Fig. 3

Protein biotinylation using CBD-BirA-His purified on chitin affinity column, (A) Coomassie blue-stained gel. (B) Western blot probed with streptavidin-horseradish peroxidase using 4-chloro- 1 -naphthol (Bio-Rad, Canada) as the color development reagent. Samples were analyzed on a 12% SDS gel. M: molecular weight marker. Lane 1: MBP-AviTag (Avidity) as the substrate; Lane 2: SAK with 15-mer biotinylation peptide tag as the substrate. Biotinylation reaction was carried out at 30°C for 2 hours using 1 μg of the substrate, 50 ng of CBD-BirA-His and other components in the reaction mixture as described in Materials and Methods.

Fig. 4

pH profile of engineered *E. coli* CBD-BirA-His. 100 ng of unbiotinylated MBP-AviTag (Avidity, USA) was coated on the wells of Reacti-bind maleic: anhydride activated polystyrene strip plate (Pierce, USA) to act as the substrate. Reaction mixture contained 10 mM ATP, 10 mM magnesium acetate, 50 μM biotin and 10 ng CBD-BirA-His purified by chitin affinity chromatography. Biotinylation reaction was carried out at 30°C for 20 min. Bound biotin was detected by streptavidin-horseradish peroxidase (Pierce) with 1-step slow TMB-ELISA (Pierce) as the color development reagent. The following buffers were used at 50 MM to provide buffering capacity for a pH range 2.5 - 11: glycine (2.5), NaAc (4.5), MES (5.5), BIS-TRIS (6.5), TRIS-HCl (7.5), bicine (8.3, 9), CAPS (10, 11). Data represent the average of three independent trials.

Fig. 5

Staphylokinase activity as determined by the radial caseinolysis assay. (A) Coomassie blue-stained SDS gel showing SAK produced by *B. subtilis*. Amounts of samples loaded on the lanes were normalized to cell density. M: molecular weight marker. Lane 1: natural, untagged SAK produced by WB800[pSAKP] (33). Lane 2: unbiotinylated SAK-PFB produced by WB800[pSAKPFB]. Lane 3: purified biotinylated SAK-PFB produced by WB800[pSAKPFB]. Lane 4: negative control WB800[pWB980]. (B) SAK activity was estimated using the top agarose plasminogen-skim milk plate method. The amounts of SAK in the individual wells were identical to those in the corresponding lanes shown in (A). Picture was taken at 10 hours after incubation at 37°C. Nuinbers 1-4 correspond to the numbering in (A).

Fig. 6

Purification of SAK-PFB from the culture supernatant of *B. subtilis* WB800[pSAKPFB] by *in vitro* biotinylation and monomeric avidin agarose chromatography. Samples were analyzed on a

12% SDS polyacrylamide gel and stained by Coomassie blue. M: molecular weight marker. Lane 1: ammonium sulfate precipitate before biotinylation. Lane 2: ammonium sulfate precipitate after biotinylation. Lane 3: column flow-through. Lane 4: 1-column volume wash. Lanes 5 and 6: eluate. Lane 7: concentrated pure SAK-PFB.

WHAT IS CLAIMED IS:

1. A polypeptide comprising a biotin ligase having a C-terminal His-tag and an N-terminal chitin binding domain.
2. An isolated, purified or recombinant DNA which encodes for a a biotin ligase having a C-terminal His-tag and an N-terminal chitin binding domain.
3. An isolated, purified or recombinant DNA which is substantially homologous to the DNA of claim 2.
4. An expression vector comprising the DNA molecule of claim 2 or 3.
5. A host cell comprising the expression vector of claim 4.
6. A method of producing and recovering a protein comprising the steps of:

   (a) culturing cells which express and secrete the protein tagged with a biotinylation peptide;
   (b) biotinylating the protein with a biotin ligase as claimed in claim 1;
   (c) separating the biotinylated protein by passing over an avidin column;
   (d) eluting the the biotinylated protein.

7. The method of claim 6 wherein the protein is staphylokinase produced by *B. subtilis*.

Fig. 1

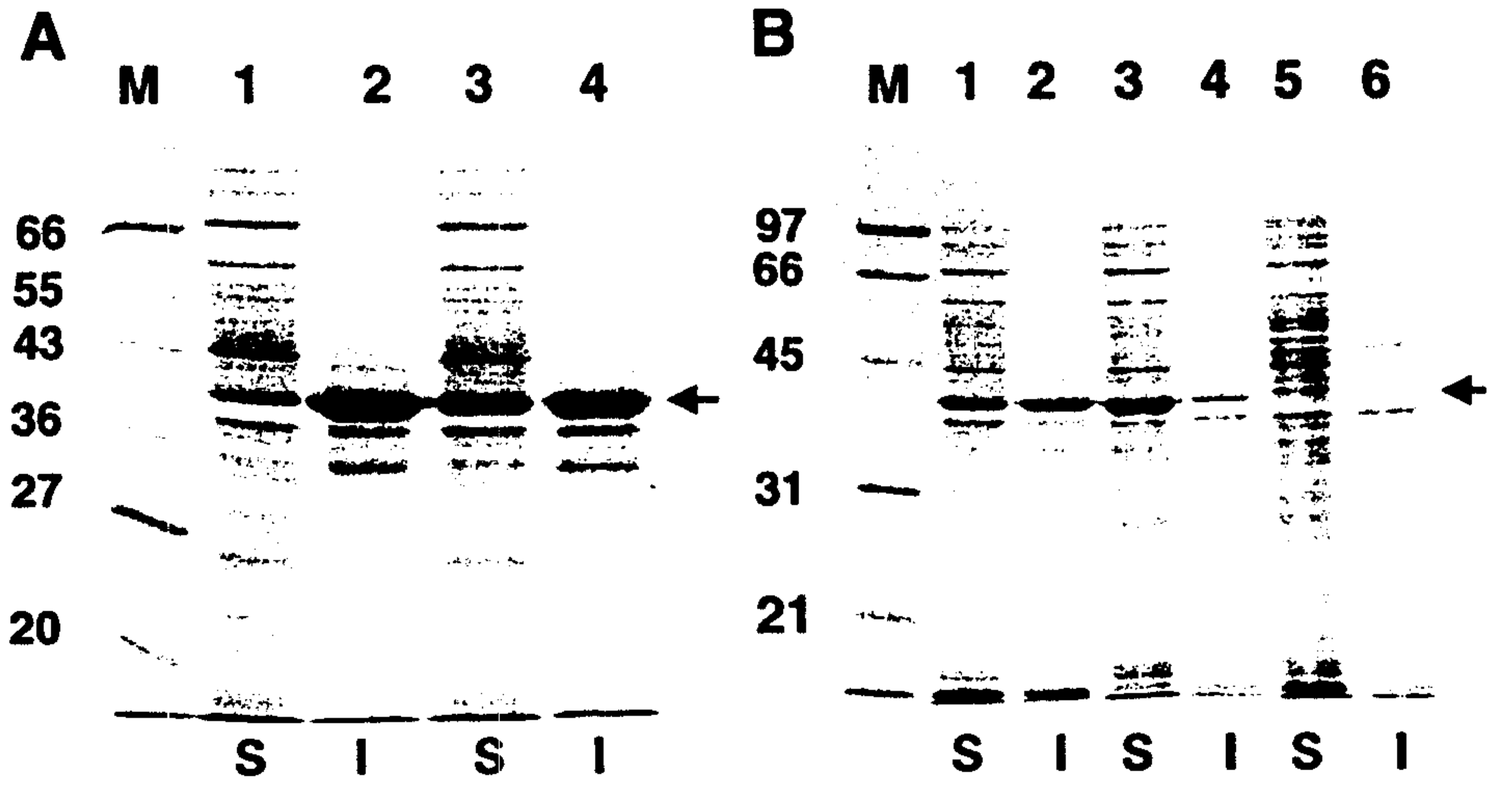
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Fig. 2

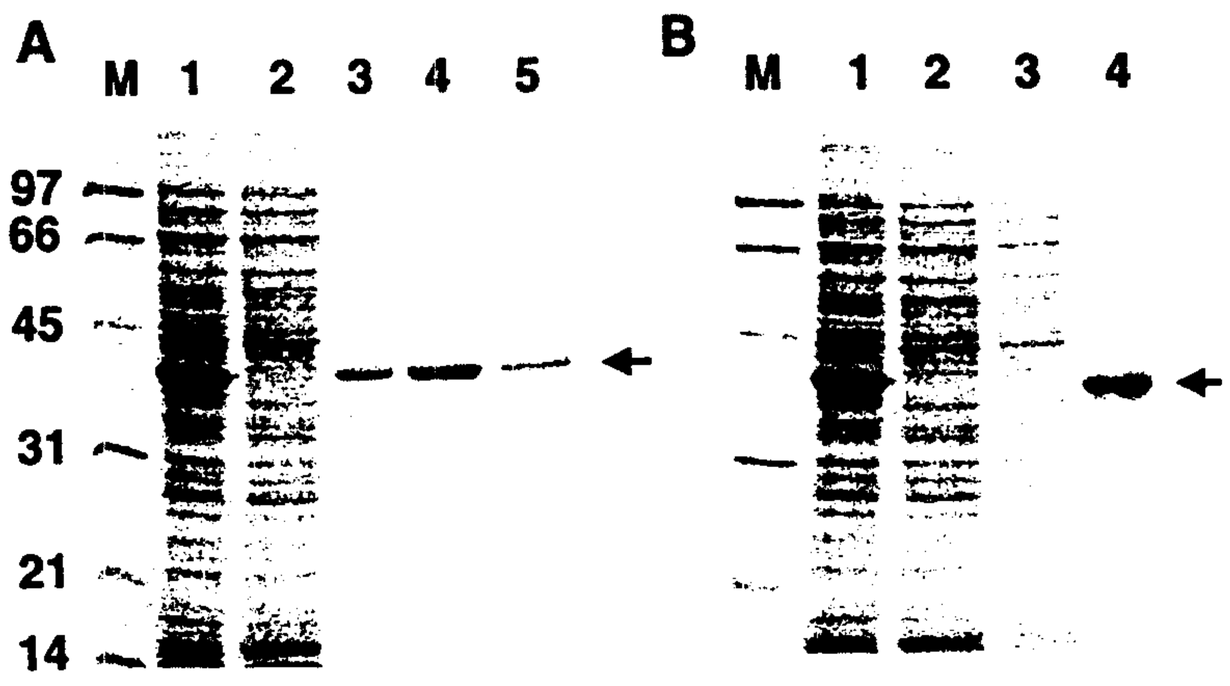
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Fig. 3

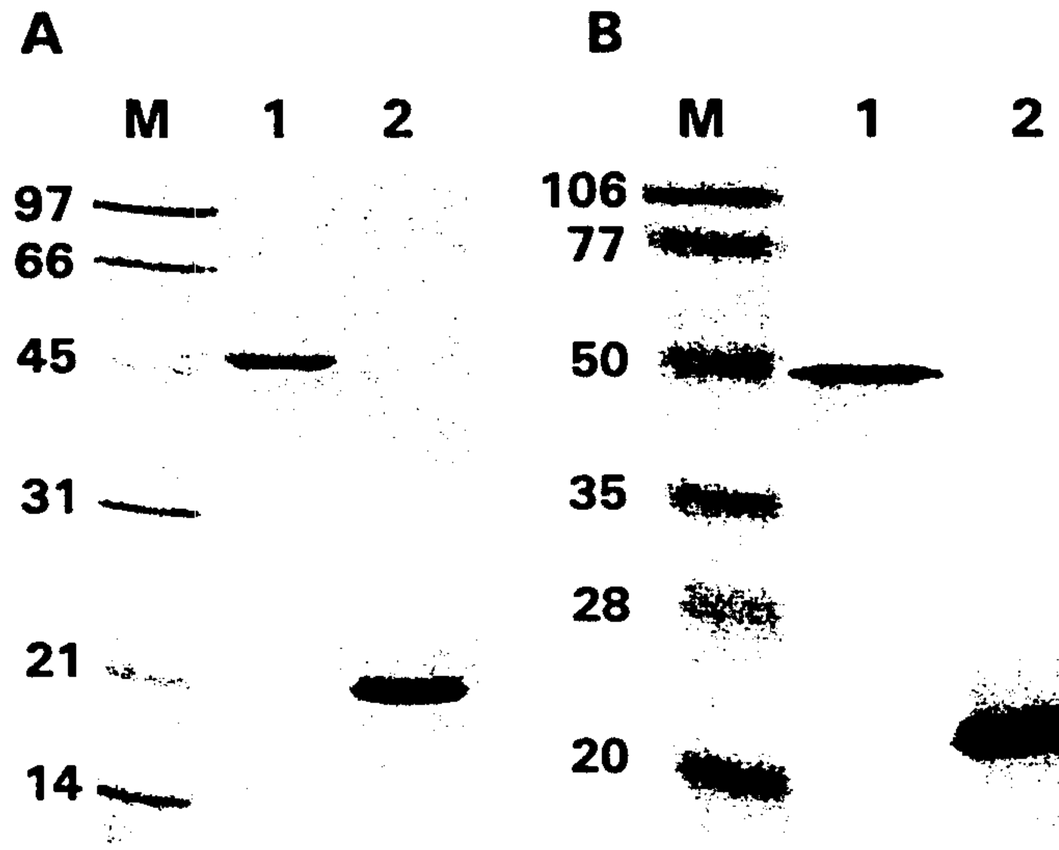

Fig. 4

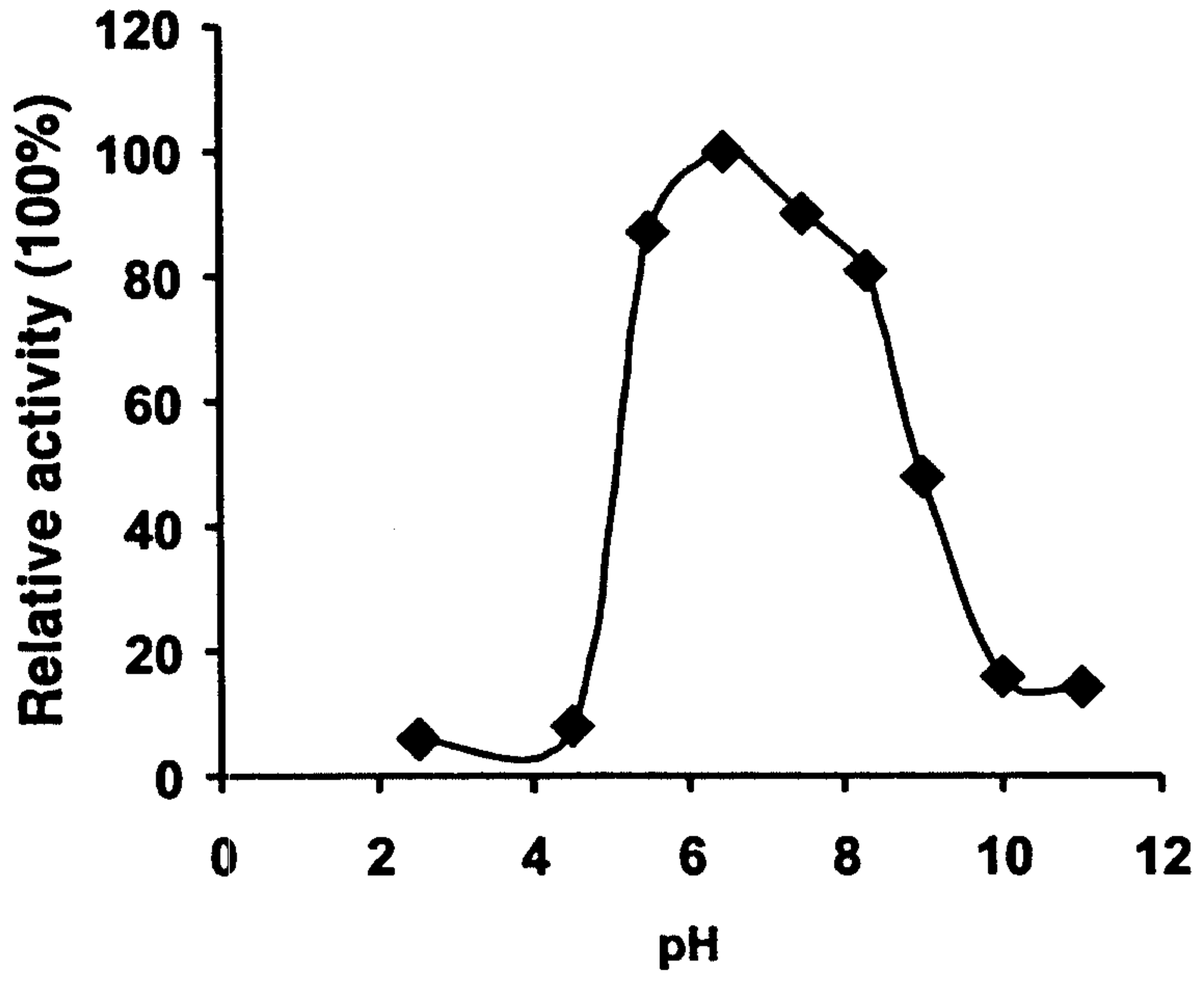

Fig. 5

Fig. 6