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Development of an enzymatic method for site-specific incorporation of desthiobiotin to recombinant proteins in vitro

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Abstract

To extend the (strept)avidin-biotin technology for affinity purification of proteins, development of reusable biochips and immobilized enzyme bioreactors, selective immobilization of a protein of interest from a crude sample to a protein array without protein purification and many other possible applications, the (strept)avidin-biotin interaction is better when reversible. A gentle enzymatic method to introduce a biotin analog, desthiobiotin, in a site-specific manner to recombinant proteins carrying a biotinylation tag has been developed. The optimal condition for efficient in vitro desthiobiotinylation catalyzed by *Escherichia coli* biotin ligase (BirA) in 1–4 h has been established by systematically varying the substrate concentrations, reaction time, and pH. Real desthiobiotinylation in the absence of any significant biotinylation using this enzymatic method was confirmed by mass spectrometric analysis of the desthiobiotinylated tag. This approach was applied to affinity purify desthiobiotinylated staphylokinase secreted by recombinant *Bacillus subtilis* to high purity and with good recovery using streptavidin-agarose. The matrix can be regenerated for reuse. This study represents the first successful application of *E. coli* BirA to incorporate biotin analog to recombinant proteins in a site-specific manner.

Keywords: BirA; Biotin ligase; Desthiobiotinylation; Desthiobiotin; Biotinylation tag; Recombinant proteins; Reversible binding; Affinity purification; Avidin; Streptavidin; Mass spectrometry

Because of the exceptionally strong interaction between avidin/streptavidin and biotin, the avidin/streptavidin-based technology offers a wide range of applications from biomolecule immobilization, capture, detection, to quantification [1–3]. However, depending on the nature of the applications, the tight binding of biotin to avidin/streptavidin can also be a problem. For example, this technology is unsuitable for reversible affinity purification of biotinylated biomolecules and for the development of biochips with the possibility of removing a particular set of the immobilized biomolecules in a reversible manner. To overcome these limitations, various technological advancements have been developed and can be grouped into two different approaches. The first approach is to modify avidin/streptavidin molecules so that the resulting molecules show lower affinities toward biotin. This leads to the development of nitro-avidin [4,5] and monomeric avidin [6,7]. Either modification has its drawback. Even under optimal conditions, nitration of avidin is maximally ~70% complete [4]. This results in a hybrid tetrameric avidin with an average of only 3 avidin subunits modified [8,9]. As for monomeric avidin, the traditional method for preparing monomeric avidin-agarose is a relatively tedious and expensive process which requires denaturation and renaturation [6,7]. Monomerization of matrix-bound avidin through this procedure is not always complete. Furthermore, restoration of monomeric avidin to the high-affinity state has been observed [10]. The second approach is to use biotin analogs rather than biotin to interact with avidin/streptavidin. The most commonly used analog is 2-iminobiotin [11,12]. Iminobiotinylated ligands bind to avidin/streptavidin matrix

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under alkaline conditions (pH 10) and can be eluted off from the matrix under acidic conditions (pH 4). This method can only be applied to those proteins that are insensitive to these pH changes. The use of desthiobiotin (also known as dethiobiotin) as an alternative biotin analog offers a gentle method for affinity purifying desthiobiotinylated biomolecules since they can be eluted off from the avidin/streptavidin matrix by competition with desthiobiotin [13].

The only method currently available to generate desthiobiotinylated proteins is through chemical modifications which involve modifications of amino groups (N-terminal amino group and lysine side chain) in the proteins [13,14]. This chemical method suffers from several drawbacks. First, modification of the N-terminal amino group of certain proteins/peptides and the critical lysine residues which serve as catalytic or substrate binding residues in enzymes can inactivate these proteins [15–21]. Second, desthiobiotinylation is not specific since any surface-exposed lysine residues in a protein can be the target sites. Third, it is not easy to precisely control the number of desthiobiotin per desthiobiotinylated protein. Depending on the modification conditions and the nature of the target proteins, an average of 4–6 desthiobiotin molecules per desthiobiotinylated protein can be common [13]. This complicates certain potential applications. For example, for affinity purification of desthiobiotinylated proteins using avidin/streptavidin matrix, the presence of multiple desthiobiotin molecules per desthiobiotinylated protein can reduce the recovery of the desthiobiotinylated proteins because of the avidity effect [13]. Moreover, multiple desthiobiotinylated proteins are not suitable for the preparation of highly functional and reproducible protein microarrays [22,23] since immobilization of the chemically desthiobiotinylated proteins (with more than one lysine residues) in a consistent and specific orientation on the protein chip would be impossible.

All the above-noted limitations with desthiobiotinylation can be addressed if desthiobiotin can be efficiently introduced to a single specific site on the protein. To achieve this objective, we explore the possibility of using the *Escherichia coli* biotin ligase (BirA)[1] as a molecular tool to introduce desthiobiotin to a 15-amino acid biotinylation tag (designated PFB, peptide for biotinylation) located at the C-terminus of our model protein staphylokinase-PFB (SAK-PFB) [24,25], a potent blood clot dissolving agent. An 18-amino acid linker sequence is inserted between the C-terminal end of SAK and the PFB sequence to ensure that the PFB tag is exposed and that the presence of this tag does not interfere with the folding of SAK (Fig. 1). BirA has been shown previously to efficiently introduce biotin to a specific lysine residue on the PFB tag [24,25]. In this paper, we demonstrate that BirA is capable of using desthiobiotin as the substrate to carry out site-specific desthiobiotinylation of SAK-PFB. Desthiobiotinylated SAK-PFB can be affinity purified on the streptavidin-agarose column which can be regenerated for further rounds of protein purification.

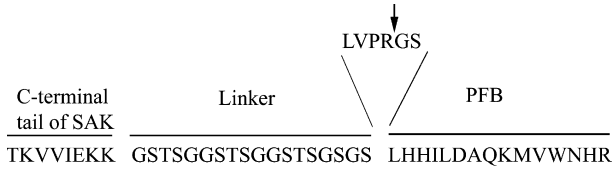

Fig. 1. Sequence features of the C-terminal region in SAKPFB and SAK-T-PFB. SAK-T-PFB differs from SAKPFB by the presence of a 6-amino acid thrombin cleavage sequence inserted between the linker sequence and the PFB. The arrow marks the thrombin cleavage site. SAK, staphylokinase; PFB, peptide for biotinylation.

Materials and methods

Construction of Bacillus subtilis expression vector for overproducing SAK-T-PFB

Plasmid pSAK-T-PFB is a derivative of pSAKPFB [24] which is a pWB980-based vector [26] for secretory production of staphylokinase (SAK) containing a C-terminal biotinylation tag (PFB). A nucleotide sequence encoding a 6 amino-acid peptide (LVPRGS) with a thrombin cleavage site was inserted between the nucleotide sequences encoding the C-terminal end of the linker and the N-terminal end of PFB (Fig. 1) in pSAKPFB to generate pSAK-T-PFB. Production of SAK-T-PFB is under the control of the *B. subtilis* P43 promoter for transcription and the levansucrase (SacB) signal sequence for secretion. To introduce the thrombin cleavage site, pSAKPFB was used as the template in PCR. The primers used for this amplification were SAKTPFB F (5′-GTGGATCCTTAGTGCCGAGAGGTTCCCTT CATCATATTCTTGATG-3′, the underlined region indicates the nucleotide sequence encoding the thrombin cleavage sequence) and PUB19NCOB (5′-GATACAA CTTTCTTTCGCCTG-3′). The amplified 655-bp fragment was digested with *Bam*HI and *Nco*I and used to replace an equivalent fragment from *Bam*HI-*Nco*I-digested pSAKPFB to generate pSAK-T-PFB.

[1] *Abbreviations used*: BirA, biotin ligase; CBD, chitin-binding domain; CDB-BirA-His, biotin ligase with an N-terminal chitin-binding domain and a C-terminal histidine tag; HABA, 4-hydroxyazobenzene-2-carboxylic acid; HRP, horseradish peroxidase; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; PFB, peptide for biotinylation; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.1% Tween 20; SAK, staphylokinase; SAK-PFB, staphylokinase with a C-terminal biotinylation tag; SAK-T-PFB, staphylokinase with a thrombin cleavage site between staphylokinase and its C-terminal biotinylation tag; SELDI-TOF MS, surface-enhanced laser desportion/ionization-time of flight mass spectrometry.

Purification of SAK-PFB via desthiobiotinylation

SAK-PFB was prepared from the culture supernatant of *B. subtilis* WB800[pSAKPFB] following the method previously reported [24] but with modifications. Instead of biotinylation, desthiobiotinylation was used to purify the protein. The reaction mixture contained 50 mM bicine (pH 9.0), 10 mM ATP, 10 mM magnesium acetate, 5 mM desthiobiotin, 40 μM SAK-PFB, and 0.45 μM *E. coli* CBD-BirA-His. All chemicals were purchased from Sigma except for desthiobiotin (biotin free as tested by strep-tag applications) which was purchased from IBA GmbH (US Distribution Center). After 3 h to overnight at 30 °C, CBD-BirA-His was removed by mixing with a small amount of chitin beads (New England Biolabs). These beads were then removed by a brief centrifugation. The sample was dialyzed extensively to remove the excess desthiobiotin. Desthiobiotinylated SAK-PFB was recovered by passing the sample over a streptavidin-agarose column (Novagen). After washing with several column volumes of PBS (100 mM sodium phosphate, 150 mM sodium chloride, pH 7.2), bound desthiobiotinylated SAK-PFB was eluted by 50 mM desthiobiotin. The eluted sample was dialyzed extensively to remove desthiobiotin. Pure desthiobiotinylated SAK-PFB was then concentrated by ultrafiltration and quantified by its absorbance at 280 nm using a molar extinction coefficient of 22,900 $M^{-1} cm^{-1}$ [27] for calculation.

To regenerate the streptavidin-agarose column, the column was washed with PBS followed by several column volumes of PBS containing 4-hydroxyazobenzene-2-carboxylic acid (HABA, Sigma). The column is ready for reuse after equilibration with PBS. Tris-based buffer (100 mM Tris–HCl, 150 mM sodium chloride, pH 8.3) can replace PBS in column regeneration.

Determination of the extent of desthiobiotinylation of SAK-PFB

An ELISA method was used to determine the extent of desthiobiotinylation of SAK-PFB [28]. Desthiobiotinylated SAK-PFB was adsorbed to the wells of a Nunc-Immuno MaxiSorp module (Nalge Nunc International) at 100 ng/well and left overnight at 4 °C. After blocking the nonspecific sites with 1% bovine serum albumin in PBST (PBS containing 0.1% Tween 20), desthiobiotin ligated to the biotinylation tag was detected by its interaction with horseradish peroxidase-conjugated streptavidin (HRP-streptavidin, Pierce). After washing three times with PBST, the amount of HRP-streptavidin retained on the wells was measured using 1-Step Turbo TMB (Pierce) as the substrate according to the manufacturer's instruction. Color development at end point was determined at 450 nm using a microplate reader (CERES 900, Bio-Tek Instrument). The experiment was repeated independently three times. A standard curve of desthiobiotinylation was established using known quantities of pure desthiobiotinylated SAK-PFB.

Preparation of SAK-T-PFB for surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS)

SAK-T-PFB was produced by secretion from *B. subtilis* WB800[pSAK-T-PFB] and purified following the procedure of Szarka et al. [18]. Pure SAK-T-PFB was biotinylated or desthiobiotinylated for 3.5 h at 30 °C. For biotinylation, the reaction mixture contained 50 mM bicine (pH 9.0), 10 mM ATP, 10 mM magnesium acetate, 100 μM biotin, 40 μM SAK-T-PFB, and 0.45 μM CBD-BirA-His. Desthiobiotinylation was carried out in a similar way except that desthiobiotin (5 mM) was used in place of biotin. Thrombin digestion of SAK-T-PFB was used to generate the peptides containing the PFB tag for SELDI-TOF mass spectrometry (Ciphergen Biosystems) analysis. One microgram of biotinylated/desthiobiotinylated SAK-T-PFB was digested with 0.2 NIH unit of human thrombin (Sigma, 1 NIH unit = 0.324 ± 0.073 μg thrombin) in 10 μl of 25 mM ammonium bicarbonate, pH 8.0. After 2 h at 37 °C, the reaction was stopped by freezing the sample at −20 °C. As a control, unmodified SAK-T-PFB was also thrombin-digested in a parallel reaction.

SELDI-TOF mass spectrometry

Peptides released from thrombin-digested SAK-T-PFB (modified or unmodified) were analyzed by SELDI-TOF mass spectrometry [29,30]. Thrombin-digested sample (1.5 μl) was spotted on a preactivated H4 protein chip, air-dried, and washed briefly with 5 μl of deionized water. Two 0.5-μl aliquots of a saturated solution of α-cyano-4-hydroxy cinnamic acid in 50% acetonitrile and 0.5% trifluoroacetic acid were applied to each spot which was then air-dried. The chip array was analyzed in a PBS (Protein Biosystem) II ProteinChip Reader (Ciphergen Biosystems). Mass spectrum for each sample was obtained by averaging at least 100 laser shots for each spot using the ProteinChip Software 3.1. Only those peaks with intensity having signal-to-noise ratio >5 were identified as true protein peaks. The ProteinChip Reader was calibrated using somatostatin (1637.9), dynorphin (porcine, 2147.5), and ACTH (human, 2933.5) in the All-in-1 peptide molecular weight standard mix (Ciphergen Biosystems).

Other methods

Vent DNA polymerase (New England Biolabs) was used for DNA amplification. The sequence of the PCR product was confirmed to be free of amplification errors by nucleotide sequencing performed at the University

Core DNA and Protein Services, University of Calgary, Calgary, Alberta, Canada. CBD-BirA-His was purified from *E.coli* BL21(DE3)[pET-CBD-BirA-His] as described previously [24]. Western blotting was done on a nitrocellulose membrane using 4-chloro-1-naphthol (Bio-Rad Laboratories) as the color development agent. Signals on the Western blot were quantified using the approach previously described [31].

Results

Establishment of the optimal conditions for desthiobiotinylation

The structure of desthiobiotin differs in a number of respects from biotin. While desthiobiotin retains the uredio ring of biotin, it misses the 5-member sulfur-containing thiophene ring (Fig. 2). As well, unlike biotin which has the pentanoic acid attached to the thiophene ring, a hexanoic acid is attached directly to the uredio ring in desthiobiotin. It is unsure how these structural differences would affect the efficiency for desthiobiotin to serve as a substrate for BirA. While the less bulky structure of desthiobiotin suggests that it could be a potential substrate candidate for *E. coli* BirA, the absence of the thiophene ring may weaken interactions between desthiobiotin and BirA. Therefore, the first step in this study is to determine whether desthiobiotin can serve as a substrate for BirA and to establish the optimal conditions for desthiobiotinylation by systematically varying several key parameters including substrate concentrations and reaction time. The engineered version of BirA (CBD-BirA-His) which has an N-terminal chitin-binding domain and a C-terminal histidine tag was used in this study [24]. SAK-PFB carrying a unique C-terminal biotinylation tag was selected as the model protein to evaluate the desthiobiotinylation process. This protein has been shown in a previous study to be biotinylated efficiently by BirA [24]. The degree of desthiobiotinylation was monitored in an ELISA assay with horseradish peroxidase-conjugated streptavidin (HRP-streptavidin) as the probe. The three substrates in the desthiobiotinylation reaction are ATP, desthiobiotin, and SAK-PFB. With ATP at an excess (10 mM) and SAK-PFB at 50 μM, the extent of desthiobiotinylation was monitored by varying the concentration of desthiobiotin. Fig. 3A shows that desthiobiotin at 1 mM was required to drive the reaction to 95% completion within 4 h. Further increase of the desthiobiotin concentration to 4 mM could drive the reaction to 100% completion. BirA concentration was kept constant at 0.45 μM.

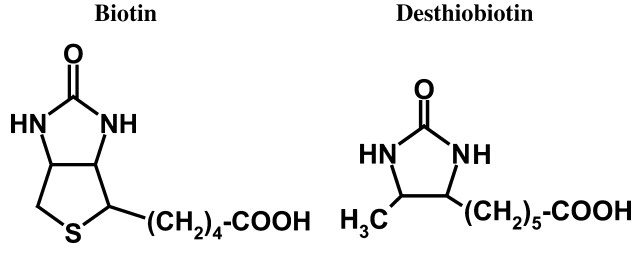

Fig. 2. Structural features of biotin and desthiobiotin. Biotin has a nitrogen containing 5-member ring (uredio ring) fused to a sulfur-containing 5-member ring (thiophene ring). A 5-carbon pentanoic acid is attached directly to the thiophene ring. Desthiobiotin misses the thiophene ring and a 6-carbon hexanoic acid is attached directly to the uredio ring.

The concentration of SAK-PFB has been found to critically affect the efficiency of biotinylation in a preliminary study. In this study, we found that similar to the biotinylation reaction, the rate of desthiobiotinylation was also dependent on the concentration of SAK-PFB. With a 4-h reaction time and the desthiobiotin concentration at 4 mM, the degree of desthiobiotinylation completion was almost linearly proportional to the concentration of SAK-PFB from 10 to 40 μM (Fig. 3B). Higher SAK-PFB concentrations could further speed up the desthiobiotinylation process (Fig. 3C). With SAK-PFB concentration at 50 μM, the reaction could be completed in 3 h. In contrast, with 20 μM SAK-PFB, desthiobiotinylation was only 60% complete in a 3.5-h reaction time.

pH effects on efficiency of desthiobiotinylation

All the above-noted desthiobiotinylation reactions were carried out at pH 8.3, a condition commonly used in the BirA-mediated biotinylation process in vitro. To determine whether this pH would be optimal for desthiobiotinylation, the pH activity profile for the BirA-mediated desthiobiotinylation was determined. SAK-PFB proteins desthiobiotinylated under different pH were quantified by Western blot probed with HRP-streptavidin. A pH of 9 was found to be the best with pH 8.3 very close to the optimal (Fig. 3D). To minimize potential complications such as incomplete protein transfer during Western blotting, the same set of desthiobiotinylated SAK-PFB proteins was immobilized to an ELISA plate and probed with HRP-streptavidin. The pH profile obtained was essentially the same as the one determined by Western blot (data not shown).

Efficiency of biotinylation versus desthiobiotinylation

With the establishment of the optimal condition for desthiobiotinylation, it would be interesting to compare the efficiency of the two BirA-mediated reactions: biotinylation versus desthiobiotinylation. With SAK-PFB at 50 μM, ATP at 10 mM and BirA at 0.45 μM, biotinylation was found to be much more efficient than desthiobiotinylation (Fig. 4). Biotinylation was complete in 1.5 h using a biotin concentration of 100 μM. In contrary,

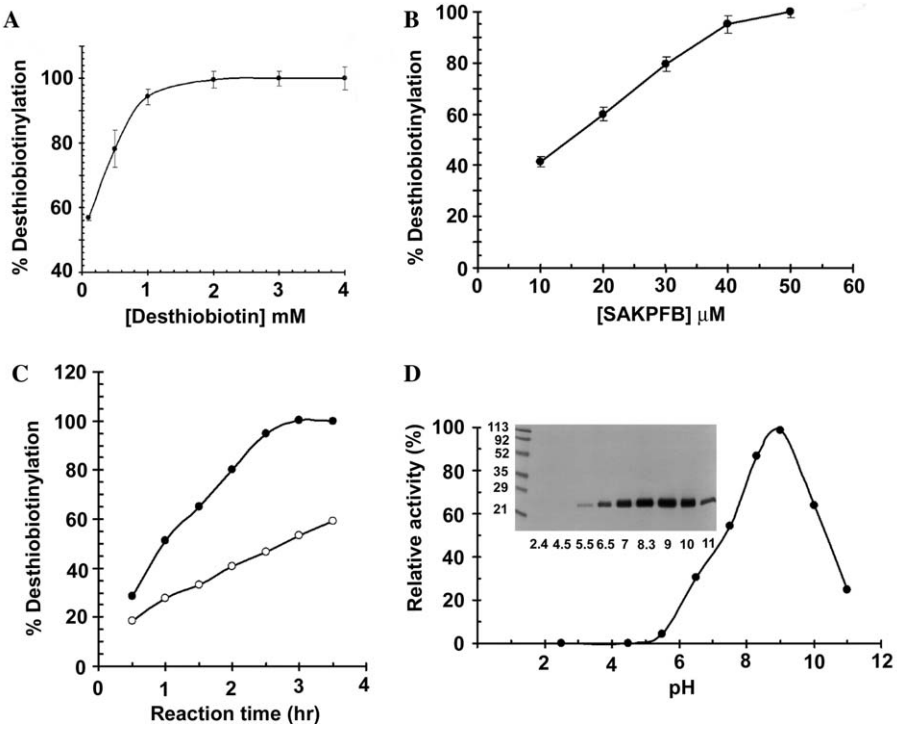

Fig. 3. Establishment of optimal conditions for enzymatic desthiobiotinylation. (A) Effect of desthiobiotin concentration. Reaction was carried out with 50 μM SAK-PFB and 0.45 μM BirA for 4 h. (B) Effect of SAK-PFB concentration. The reaction was carried out in a 4-h time period with 4 mM desthiobiotin and 0.45 μM BirA. The extent of desthiobiotinylation was estimated by an ELISA method. Data shown represent the mean values and standard deviations of the mean as determined from triplicate assays. (C) A typical time course of desthiobiotinylation reaction. SAK-PFB at 20 μM (open circle) and 50 μM (closed circle), desthiobiotin at 4 mM and BirA at 0.45 μM were used in this study. (D) pH activity profile of the BirA-mediated desthiobiotinylation. Buffers used to establish the pH range were as described in a previous paper [24]. Insert shows a Western blot of SAK-PFB desthiobiotinylated under the different pH conditions. The blot was probed with HRP-streptavidin.

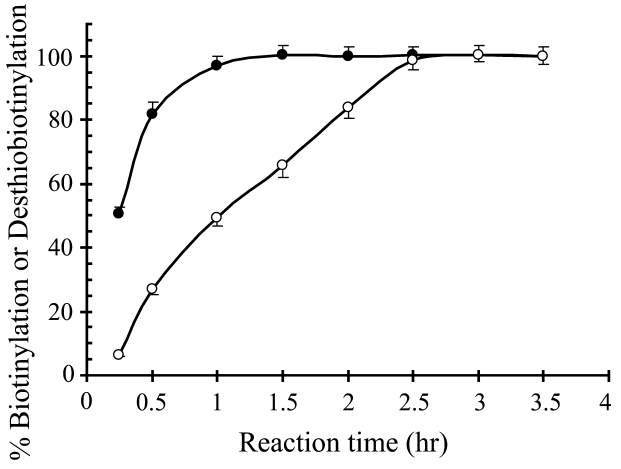

Fig. 4. Efficiency of BirA -mediated biotinylation versus desthiobiotinylation. A 50 μM SAK-PFB and 0.45 μM BirA were used for the experiments. Biotin and desthiobiotin were 100 μM and 4 mM, respectively. Closed circles represent biotinylation and open circles correspond to desthiobiotinylation. Data represent the mean values and standard deviations determined from triplicate assays.

BirA-mediated desthiobiotinylation took 3 h to complete using 4 mM desthiobiotin. This finding raises two major concerns. First, if desthiobiotin is contaminated with trace amounts of biotin, the high concentration of desthiobiotin required in the reaction may suggest the amount of biotin required to generate sufficient signal to be detected by the HRP-streptavidin probe. This means that the observed reaction may actually reflect biotinylation rather than desthiobiotinylation. Second, even if the observed result represents a true desthiobiotinylation reaction, it would be important to know what degree of biotinylation, if any, actually takes place in this desthiobiotinylation reaction. Any biotinylated SAK-PFB molecules are considered to be contaminants and are not suitable candidates for reversible affinity purification using the natural streptavidin- or avidin-agarose column.

Analyses of biotinylated/desthiobiotinylated SAK-PFB by surface-enhanced laser desorption/ionization-time of flight mass spectrometry

To address the above-noted concerns and to reduce possible ambiguity, a thrombin-specific cleavage site (LVPRGS) was introduced between the linker sequence and the PFB tag in SAK-PFB to generate an engineered fusion protein designated SAK-T-PFB (Fig. 1). Purified SAK-T-PFB was biotinylated or desthiobiotinylated (using 40 μM SAK-T-PFB and 3.5-h reaction time) and

then cleaved by thrombin. The molecular mass of the cleaved PFB tag was determined using the Ciphergen ProteinChip Biosystem II (also known as the SELDI-TOF mass spectrometry) which has a built-in MALDI-TOF mass spectrometer [29,30]. The smaller masses of these tags allow more accurate differentiation of the desthiobiotinylated tag from the biotinylated one. As a control, the molecular mass of the cleaved PFB tag from the nonmodified SAK-T-PFB was also determined. Based on internal calibration, the observed molecular mass of the nonmodified SAK-T-PFB was 2042.6 Da which matched closely with the expected value of 2042.3 (Fig. 5A). Interestingly, two minor peptide peaks (16 and 32 Da above the expected value) with the molecular masses of 2058.4 and 2074.8, respectively, were observed. These peaks likely represent the oxidized forms of the nonmodified PFB tag (a readily oxidized methionine residue and a less easily oxidized tryptophan residue are present in the PFB tag). Addition of biotin to PFB should increase the peptide molecular mass to 2268.6 Da (molecular mass of biotin is 244.31 Da. A water molecule is removed after linking biotin to the lysine residue in PFB). The actual observation matched very well with the prediction (Fig. 5B). Two peptide peaks were obtained with biotinylated PFB tag: a major peak with the molecular mass of 2268.5 and a minor one with a mass of 2284.6 which likely represents the oxidized biotinylated PFB tag. Desthiobiotinylated PFB had a similar spectrum except for the mass difference (Fig. 5C). The major peak of the desthiobiotinylated PFB tag showed a molecular mass of 2238.6 which matched exactly with the prediction. A minor peak corresponding to the oxidized desthiobiotinylated PFB tag (molecular mass of 2254.7) was also observed. Other than these, no peptide peak with the molecular mass matching exactly with that of the biotinylated PFB tag was observed with the desthiobiotinylated sample. Only a very tiny peak with a molecular mass slightly larger than that of biotinylated PFB tag was observed and its peak area was less than 2% of the total peak areas for the desthiobiotinylated PFB tag. These data unambiguously demonstrated that *E. coli* BirA ligase could use desthiobiotin as the substrate to mediate site-specific desthiobiotinylation of SAK-T-PFB. Furthermore, there is no evidence for any significant biotinylation taking place in the desthiobiotinylation reaction. The degree of completion of the biotinylation/desthiobiotinylation process could also be monitored by mass spectrometry. As shown in Fig. 5B, 100% biotinylation was essentially achieved. For the case of desthiobiotinylation (Fig. 5C), a small peak with the molecular mass of 2058.5 was also observed. This peak likely represents the oxidized PFB tag that was not desthiobiotinylated. An estimate of the area of this small peak suggested that 8% of the sample was not desthiobiotinylated, an observation which agreed closely with that determined by the ELISA method (Fig. 3C).

Purification of desthiobiotinylated SAK-PFB

To demonstrate the suitability of in vitro desthiobiotinylation for protein purification, SAK-PFB secreted from WB800[pSAK-PFB] was used as the starting

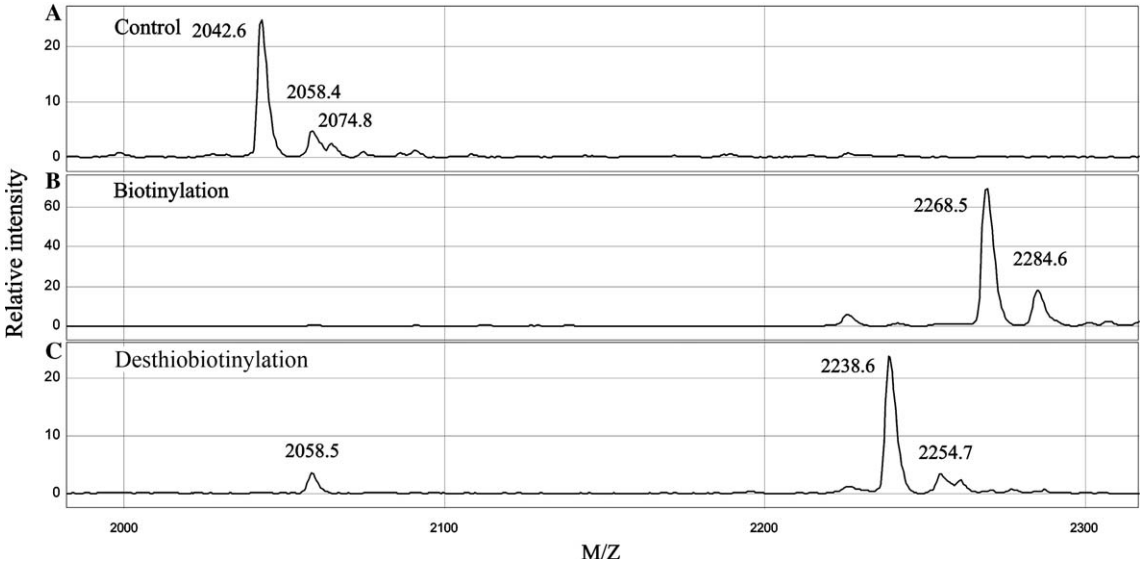

Fig. 5. SELDI-TOF mass spectra of thrombin-cleaved PFB tags. The ProteinChip Reader was calibrated both externally and internally. For clarity, mass spectra with external calibration are shown. The molecular mass indicated on the spectrum was obtained by internal calibration. (A) Nonmodified tag. The thrombin-cleaved tag and its mono- and dioxygenated derivatives have molecular masses of 2042.6, 2058.4, and 2074.8 Da, respectively. (B) Biotinylated tag. The biotinylated PFB tag and its mono-oxygenated derivative have the molecular masses of 2268.5 and 2284.6 Da, respectively. (C) Desthiobiotinylated tag. The molecular masses of the desthiobiotinylated PFB tag and its mono-oxygenated derivative are 2238.6 and 2254.7 Da, respectively. The tiny peak with molecular mass of 2058.5 represents oxidized PFB tag which was not desthiobiotinylated. Thrombin alone spotted on the chip in a parallel experiment did not yield any detectable signals (spectrum not shown).

material for purification. Proteins in the culture supernatant were concentrated by ammonium sulfate precipitation. After dialysis, samples were desthiobiotinylated and excess desthiobiotin was removed by extensive dialysis. Samples containing desthiobiotinylated SAK-PFB (~200 μg) and other contaminants from the culture supernatant were loaded to a 0.6-ml streptavidin-agarose column. Fig. 6A shows the recovery of pure SAK-PFB based on this purification scheme. Relative to SAK-PFB, desthiobiotinylated SAK-PFB showed a slower mobility in SDS-PAGE (Fig. 6A, lane 3 vs lane 2). That the protein was really desthiobiotinylated was demonstrated by a Western blot probed with HRP-streptavidin (Fig. 6B). No SAK-PFB could be detected in either the flowthrough or the wash fractions by Western blot probed with SAK specific antiserum (data not shown). Elution with 50 mM desthiobiotin consistently allows the recovery of 75–80% of desthiobiotinylated SAK-PFB within the first three fractions (1 column volume per fraction or 0.6 ml per fraction) based on three independent purifications. Elution profile from a typical run is shown in Fig. 6A (lanes 6–8). These fractions contained desthiobiotinylated SAK-PFB in high purity.

The streptavidin-agarose column could be easily regenerated. While regeneration worked well by washing the column with a PBS-based buffer, elution of HABA (a biotin analog which turns red on binding with streptavidin on the column) from the column suggested that a Tris-based buffer (100 mM Tris–HCl, 150 mM NaCl, pH 8.3) could regenerate the column even faster. Despite some losses in column performance with multiple reuses, we have been able to recover about 50% of the desthiobiotinylated SAK-PFB after eight rounds of column regeneration. This result came from the observation associated with two independently regenerated columns.

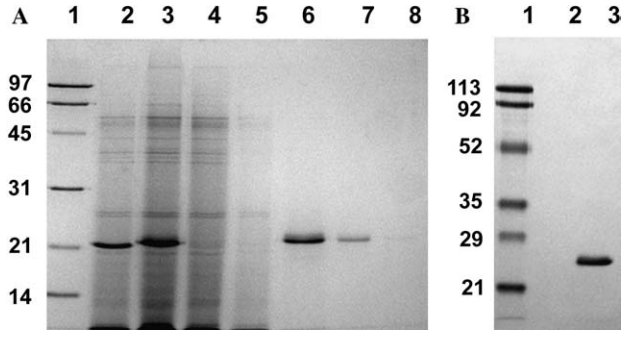

Fig. 6. Purification of SAK-PFB from the culture supernatant of *B.subtilis* WB800[pSAKPFB] by in vitro desthiobiotinylation and streptavidin-agarose chromatography. (A) Coomassie blue R250-stained SDS gel showing SAK-PFB, desthiobiotinylated SAK-PFB, and various fractions from the streptavidin-agarose column. Lane 1, molecular-weight markers. Lane 2, ammonium sulfate-precipitated culture supernatant containing SAK-PFB before desthiobiotinylation. Lane 3, ammonium sulfate precipitate after desthiobiotinylation. Lane 4, column flowthrough. Lane 5, 1-column volume wash. Lanes 6–8, the first three fractions of desthiobiotinylated SAK-PFB eluted off from the column using 50 mM desthiobiotin. (B) Western blot of desthiobiotinylated SAK-PFB probed with HRP-streptavidin. Lane 1, prestained molecular weight markers. Lane 2, ammonium sulfate precipitate containing SAK-PFB before desthiobiotinylation. Lane 3, ammonium sulfate precipitate after desthiobiotinylation. Samples in lanes 2 and 3 shown in panel B are identical to those shown in panel A.

Discussion

Escherichia coli BirA ligase has been applied successfully to biotinylate engineered proteins carrying biotinylation tags [28,32–34]. In this study, we demonstrate the feasibility to extend the application of *E. coli* BirA for site-specific desthiobiotinylation of recombinant proteins. In comparison with the biotin concentration normally used for biotinylation, the concentration of desthiobiotin used for desthiobiotinylation is at least 40 times more. Analysis of the structure of the BirA-biotin complex [35] revealed two major factors which could contribute to the lower affinity of BirA to desthiobiotin. First, BirA has several residues including Tyr-132, Gly-204, and Gly-206 that make close contact with the thiophene ring in biotin. Absence of the thiophene ring in desthiobiotin weakens these interactions. Second, the pentanoic acid in biotin is attached directly to the thiophene ring. The junction of this attachment makes close contacts with Gly-117, lys-183, Gly-186, and Leu-188 in BirA via van der Waals interactions and hydrogen bonding. In the case of desthiobiotin, a hexanoic acid is attached directly to the uredio ring. The interaction between the hexanoic acid and the BirA may not be optimized. Despite the lower affinity of BirA toward desthiobiotin, complete desthiobiotinylation could still be achieved within 3 h of reaction by using higher concentrations of desthiobiotin (4 mM) and SAK-PFB (50 μM). This reaction time frame is acceptable for most applications. By increasing the amount of BirA used (from 0.45 to 1 μM), we have been able to achieve complete desthiobiotinylation within 1 h as confirmed by both the ELISA method and SELDI-TOF mass spectrometry (data not shown).

Since molecular masses of biotinylated (19088.6 Da) and desthiobiotinylated (19058.6 Da) SAK-PFB molecules differ from each other by only 30 Da, SELDI-TOF MS analyses of these samples do not offer resolution high enough to differentiate unambiguously the mass difference between biotinylated and desthiobiotinylated SAK-PFB. This problem was solved by inserting a thrombin cleavage site before the PFB tag and determining the molecular masses of the thrombin cleaved biotinylated/desthiobiotinylated PFB tags instead. Since the peptides generated are relatively small and their chemical natures are reasonably similar, they should be ionized efficiently in a fairly quantitative manner.

This allows a more accurate quantitative analysis using the SELDI-TOF mass spectra. We demonstrated in this study that the degree of biotinylation/desthiobiotinylation observed from the SELDI-TOF spectra agreed well with the ELISA data. Moreover, the MS analyses confirmed real desthiobiotinylation of SAK-PFB and ruled out biotinylation as brought about by biotin contaminants which may be present in the commercial desthiobiotin.

Like BirA-mediated biotinylation, desthiobiotinylation efficiency is greatly dependent on the concentration of SAK-PFB. With a constant amount of BirA and desthiobiotin, desthiobiotinylation can proceed faster at a higher concentration of SAK-PFB. This special feature can be attributed to the bifunctional nature of BirA. Besides serving as a biotin ligase, BirA also functions as a repressor to shut off gene expression from the biotin biosynthetic operon at excess intracellular biotin [36]. To serve as a functional repressor, BirA forms a dimer to bind to the operator sequence in the biotin operon [37]. The dimerization surface is suggested to overlap with the substrate-binding site in BirA [38]. Higher concentrations of the PFB-tag containing protein will be more effective in competing against the BirA dimerization process and lead to better biotinylation/desthiobiotinylation.

Using SAK-PFB as a model in this study, we showed that desthiobiotinylated protein can be affinity purified with good recovery using streptavidin matrix. Elution with 50 mM desthiobiotin (instead of 10–20 mM as previously suggested [13]) could help minimize the tailing effect during elution and improve the recovery. This would be a desirable feature over the use of monomeric avidin for affinity purification of biotinylated proteins since tailing could be a problem with the latter elution scheme. An important attribute of using desthiobiotin instead of biotin for elution is that it allows the column to be regenerated. The flexibility of reusing the column matrix is in fact another great advantage of using desthiobiotinylation (in place of biotinylation) in protein purification. Here, the use of biotin-free desthiobiotin is critical since trace amounts of biotin contaminants could poison the streptavidin matrix.

An enzymatic approach not only offers a gentle method for desthiobiotinylation but also allows the modification to take place in a site-specific manner and enables a more precise control of the number of desthiobiotin introduced to each recombinant protein molecule. With MALDI-TOF mass spectrometric analysis of desthiobiotinylated SAK-PFB in a preliminary study, we found only a single desthiobiotin per recombinant protein molecule. If desired, the number of desthiobiotin per protein molecule can be increased to two by simply introducing desthiobiotinylation sites at both the N- and C-terminals. This precise control would be difficult with chemical modification.

Potential applications of site-specific enzymatic desthiobiotinylation are immense. Besides affinity purification of proteins as demonstrated in this study, it could also be applied to generate high-quality protein arrays, to recover high-affinity binders in a phage display system, and to create revitalizable enzyme bioreactors. While this study used desthiobiotin as the biotin analog, the same principle can potentially be extended to enzymatically introduce other biotin analogs (e.g., diaminobiotin and iminobiotin) to recombinant proteins to offer even more choices for potential applications.

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