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Two General Methods for the Isolation of Enzyme Activities by Colony Filter Screening

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Summary

We describe two general methodologies, based on filter-sandwich assays, for isolating enzymatic activities from a large repertoire of protein variants expressed in the cytoplasm of *E. coli* cells. The enzymes are released by the freezing and thawing of bacterial colonies grown on a porous master filter and diffuse to a second "reaction" filter that closely contacts the master filter. Reaction substrates can be immobilized either on the filter or on the enzyme itself (which is then, in turn, captured on the reaction filter). The resulting products are detected with suitable affinity reagents. We used biotin ligase as a model enzyme to assess the performance of the two methodologies. Active enzymes were released by the bacteria, locally biotinylated the immobilized target substrate peptide, and allowed the sensitive and specific detection of individual catalytically active colonies.

Introduction

Having implications of both scientific and industrial nature, the isolation of improved enzymes is an important challenge in protein engineering. Directed evolution has proven to be a powerful route to enhancing the stability, catalytic efficiency, or substrate specificity of enzymes [1]. Construction of libraries of mutant enzymes by various mutagenesis techniques [2–4] followed by screening has led to the isolation of remarkable enzyme variants [5]. In most of these screening strategies, the enzyme is assayed intracellularly by linking of the enzymatic reaction to a growth or survival advantage to the host organism [6–8]. These in vivo screening methods have the disadvantage that the enzymatic reaction of interest can interfere with the cellular metabolism and that the choices of parameters for solvent or substrate are limited. Assays performed in microtiter plates allow in vitro screening of enzyme activities. However, these assays are limited by the number of wells used to measure the catalytic performance with chromogenic or fluorogenic substrates [9]. Tawfik et al. [10] have devised a sensitive method, catELISA, based on the catalytic conversion of substrates into products, to be detected immunochemically at the end of the reaction. Other in vitro screening methods were proposed that use phage display [11–13] or cell display technology [14], which allows $>10^6$ clones to be assayed simultaneously. The usefulness of these methods at library level yet has to be demonstrated [12, 15].

In this article, we present a novel in vitro screening method for the isolation of enzymatic activities from a large repertoire of protein variants based on a filter-sandwich assay. Filter screening techniques have proven to be a valuable tool for isolating good-quality monoclonal antibodies from a large synthetic antibody repertoire expressed in bacterial cells [16–20]. We proposed that the filter sandwich screening technique could also be applied to enzymes. The principle of the methodology is depicted in Figure 1. *Escherichia coli* cells expressing enzyme are grown on a porous master filter receiving nutrients from an agar plate by diffusion (Figure 1A). The enzyme of interest is expressed in the cytoplasm of bacterial cells and does not diffuse in the master filter. This porous filter, loaded with bacterial colonies, is then laid on top of a second filter (termed "reaction filter"). Enzymes are released from the bacterial cells by freezing and thawing and can then convert suitable substrates on the reaction filter (Figures 1B and 1C). We used a biotin ligase (BirA) as a model enzyme. BirA catalyzes the formation of biotinyl-5′-adenylate from biotin and ATP. The first reaction is followed by a covalent attachment of biotin onto a specific lysine residue of a peptide [21, 22]. However, the method is applicable to all enzymatic reactions in which the enzyme can be expressed in bacteria and in which the substrate can be chemically modified with a linker arm [11]. The enzymatic activity of the secreted enzyme is assayed by two different approaches, shown in Figure 2. In the first approach (Figures 1B and 2A), the reaction filter is coated with the reaction substrate (in this case the biotin acceptor peptide [BAP]). The peptide is biotinylated if it comes in contact with active biotin ligase secreted from cells on the master filter. In the second approach (Figures 1C and 2B), the reaction filter is coated with human serum albumin (HSA). The enzyme is expressed as a fusion protein of BirA, calmodulin, and the albumin binding domain (ABD) and is immobilized noncovalently but in a stable fashion ($k_{off} < 10^{-3}$ s^{-1}) on the reaction filter by binding of the ABD to human serum albumin (HSA) [23–26]. The biotin acceptor peptide (which represents the substrate) linked to a high-affinity calmodulin binding peptide ($k_d < 2$ pM; [27]) is then immobilized by binding to calmodulin and can be biotinylated by the enzyme [11]. The product of the reaction, a biotinylated peptide, is detected with a streptavidin-horseradish peroxidase conjugate.

Results

catELISA with a Biotin Ligase

Before assessing the colony filter methodologies for the screening of enzymatic activities, we performed model experiments in microtiter plates by using the reaction schemes of Figure 2 in a catELISA (catalytic enzyme-

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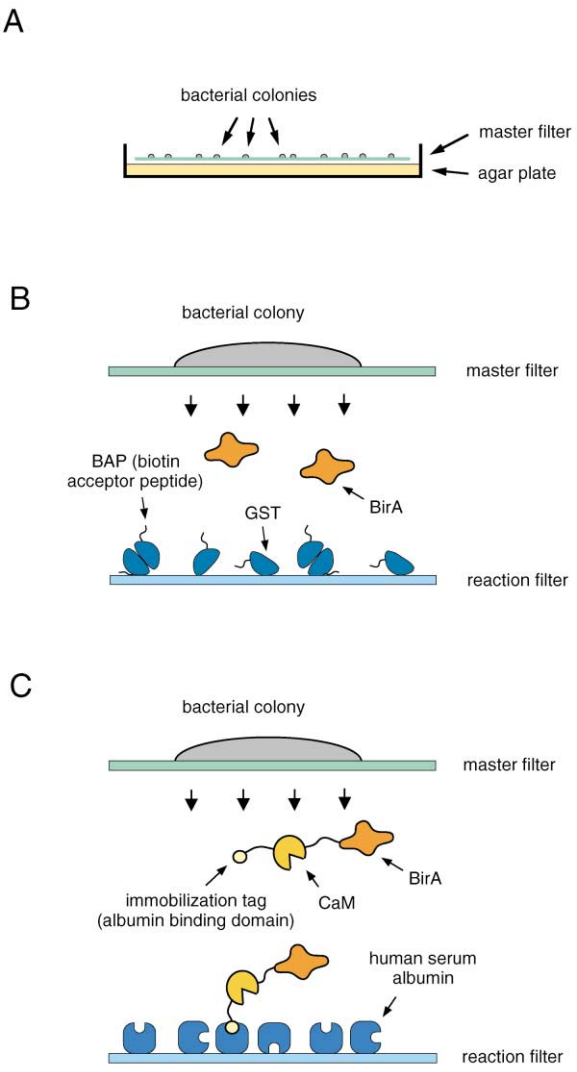

Figure 1. Schematic Representation of the Filter Sandwich Assay for a Biotin Ligase

(A) Bacterial colonies expressing enzyme on a hydrophilic filter on top of a solid medium.

(B) Bacteria on the master filter release biotin ligase (BirA) to the reaction filter, which is coated with a fusion protein of GST, and the biotin acceptor peptide (BAP), which represents the substrate of the reaction.

(C) Fusion protein of BirA-calmodulin-ABD-His_6 is released from the bacteria and immobilized on the human serum albumin (HSA)-coated reaction filter by the noncovalent interaction of the albumin binding domain (ABD) and HSA.

linked immunosorbent assay) format [10]. The catELISA experiment is a simplification of the enzyme filter colony screening method; the reaction is performed in physically separated reaction compartments, and purified enzyme or enzyme-fusion protein is used. This experiment allows the assessment of the integrity and functionality of the components of the reaction schemes of Figures 1 and 2, namely the integrity of enzymes, reaction substrates, and binding molecules.

We purified to homogeneity BirA-His_6 (Figure 3) and the fusion proteins consisting of BirA, calmodulin, and

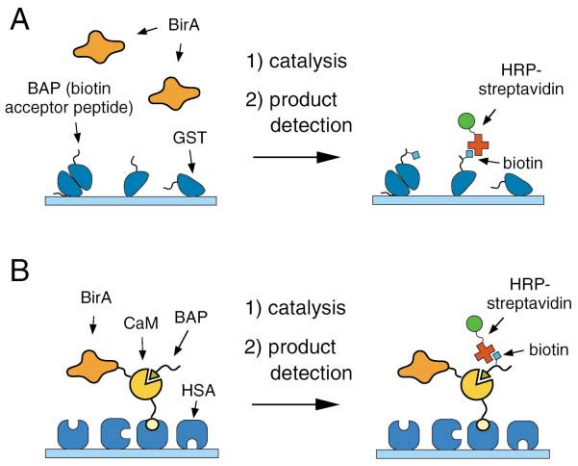

Figure 2. Schematic Representation of the Biotin Ligase (BirA) Activity Assay

(A) BirA biotinylates the immobilized biotin acceptor peptide (BAP) in a buffer supplied with biotin and ATP. The biotinylated peptide representing the product is detected by a streptavidin-HRP (horseradish peroxidase) conjugate.

(B) The fusion protein BirA-CaM-ABD-His_6 is immobilized on the reaction filter, which is coated with human serum albumin (HSA). The substrate peptide, linked to a high-affinity calmodulin binding peptidic moiety, is then added to BirA-CaM-ABD-His_6 and binds to calmodulin. BirA biotinylates the peptide, and the product is detected with streptavidin-HRP.

ABD (BirA-CaM-ABD-His_6), produced in the bacterial cytoplasm (Figure 4A). Also, the biotin acceptor peptide, fused to glutathione S-transferase (GST-BAP), was expressed in *E. coli* and purified on a glutathione resin (Figure 4B).

We used two different approaches to assay the activity of the biotin ligase (Figures 2A and 2B). In the first approach, the substrate of the reaction (GST-BAP) was immobilized on a microtiter plate. An ELISA experiment confirmed that GST-BAP expressed in *Escherichia coli* was not biotinylated by endogenous biotin ligase (Figure 5A; negative control). Mass spectrometry analysis of the fusion protein indicated that a portion of the BAP was proteolyzed (data not shown). The GST-BAP-coated wells were blocked with milk and incubated with 5 μg of BirA-His_6 in a reaction buffer supplied with ATP and biotin. Biotinylated GST-BAP, representing the product of the reaction, was detected with a streptavidin-HRP conjugate. The results of the catELISA experiment are shown in Figure 5A. A strong signal was obtained when either purified BirA-His_6 (from clone pCHH20/TG1) or BirA-CaM-ABD-His_6 (from clone pCHH19/TG1) was added to the reaction. Negative controls in which no enzyme was added, ATP or biotin was omitted in the reaction buffer, or the wells were coated with GST instead of GST-BAP were performed. All negative controls showed significantly lower signals, confirming that the BirA-mediated biotinylation reaction is specific.

In the second approach (Figure 2B), BirA-CaM-ABD-His_6 (5 μg per well) was immobilized on a microtiter plate coated with human serum albumin (HSA).

The peptidic reaction substrate GAAA**RWKKAFIAV SAANRFKKIS**TSGGGPGGLVSIFEAQKIEWH containing

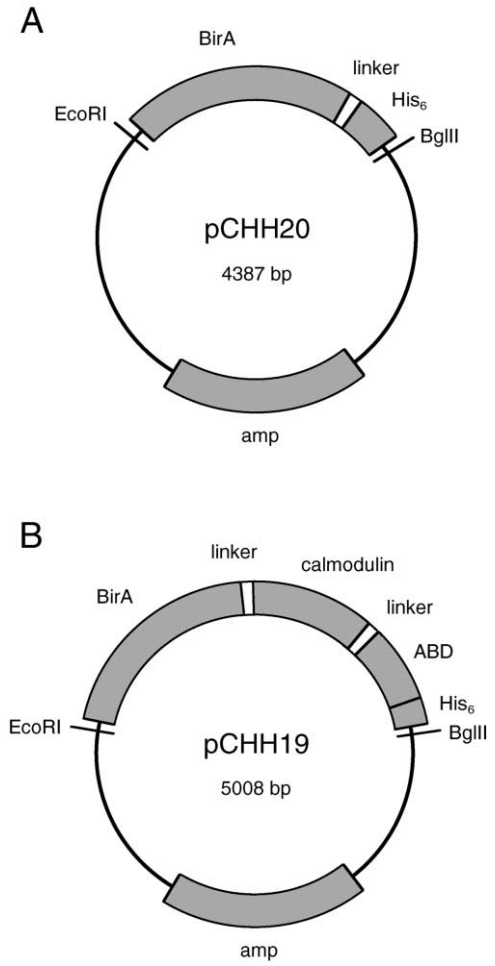

Figure 3. Vectors for the Cytoplasmic Expression of BirA

(A) Hexa-histidine-tagged BirA.

(B) Hexa-histidine-tagged BirA-calmodulin-ABD fusion protein. ABD stands for albumin binding domain; amp stands for ampicillin resistance gene; and BirA stands for biotin ligase gene.

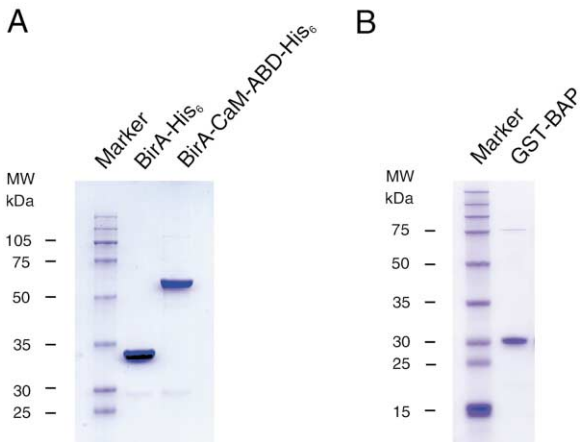

Figure 4. SDS-PAGE Analysis of Proteins Expressed in the Cytoplasm of *E. coli*

(A) Lane 1: molecular-weight marker. Lane 2: hexa-histidine-tagged BirA (from pCHH20/TG1) purified by nickel affinity chromatography. Lane 3: hexa-histidine-tagged fusion protein of BirA, calmodulin, and albumin binding domain (from pCHH19/TG1) purified by nickel affinity chromatography.

(B) Lane 1: molecular-weight marker. Lane 2: biotin acceptor protein (BAP) fused to the C terminus of GST via an 8 amino acid linker (from pCHH21/TG1) purified by glutathione sepharose affinity chromatography.

the BAP sequence (underlined) was anchored on calmodulin by means of its calmodulin binding moiety (boldface). The enzyme was allowed to catalyze the reaction in a buffer supplied with biotin and ATP, before the formation of reaction product was detected with streptavidin-HRP conjugate. Control reactions were performed as follows. A biotinylated calmodulin binding peptide [biotin-CAAARWKKAFIAVSAANRFKKIS; (27)] was used as a positive control, simulating the reaction product of the biotinylation reaction. In negative controls, either biotin or the substrate peptide was not added to the reaction mixture. The ELISA signals of the individual reactions are shown in Figure 5B. The positive control with a biotinylated peptide showed a strong signal indicating that the binding partners HSA/ABD, calmodulin/calmodulin binding peptide, and biotin/streptavidin-HRP are functional and that product can be detected. However, biotinylation of the substrate peptide could only be detected when additional biotin ligase (5 μg per well) was added to the reaction mixture. These data indicate that the intramolecular biotinylation reaction does not take place with the BirA-CaM-ABD-His_6 fusion protein, in spite of the fact that this enzyme is catalytically active (Figure 5A). This may be due to the relatively short linkers between calmodulin/BirA (13 amino acids; GGSGGGGSGGGGS) and calmodulin/BAP (8 amino acids; TSGGGPGG). However, the intramolecular reaction of Figure 5B (bar 3) gave a weak ELISA signal when the reaction was allowed to proceed for 3 hr at 37°C, yet the signal was significantly lower than the positive control. The ELISA signal does not increase when a fusion protein with a 40 amino acid linker between calmodulin and BirA is used [J. Bertschinger and D.N., unpublished data].

Biotin Ligase Activity Detected by Colony Filter Screening

We then investigated whether the two strategies depicted in Figure 2 could be performed by a modified screening methodology based on the two-membrane colony filter screening system of Skerra [18]. Bacterial cells harboring a plasmid for the cytoplasmic expression of BirA-His_6 (pCHH20/TG1) or BirA-CaM-ABD-His_6 fusion protein (pCHH19/TG1) were grown on a porous master filter, in contact with an agar plate providing nutrients to the filter by diffusion. As soon as small colonies were visible, the master filter was laid on a second agar plate, containing IPTG for the induction of protein expression. The activity of the biotin ligase enclosed in the bacterial cells was assayed via the strategies shown in Figure 2. In the first approach (Figures 1B and 2A), a second filter ("reaction filter") was coated with GST-BAP, incubated in reaction buffer supplied with biotin and ATP, and dried carefully with blotting paper. A filter sandwich was then formed with the semidry reaction

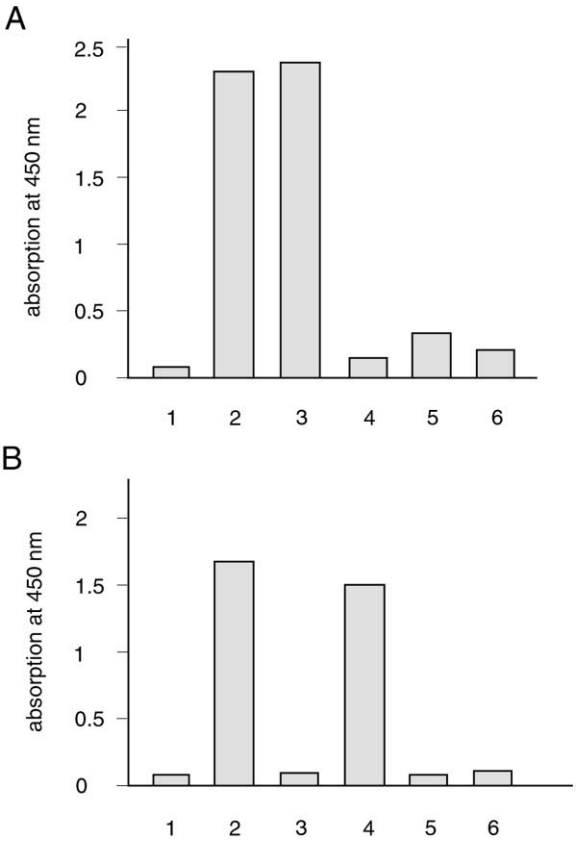

Figure 5. catELISA Experiments in which the Two Different Assay Formats from Figure 2 Were Used

(A) lane 1, no enzyme was added to the reaction (negative control 1); lane 2, BirA-His_6 was added to the reaction mixture; lane 3, BirA-calmodulin-ABD-His_6 fusion protein was added to the reaction mixture; lane 4, BirA-His_6 was added to the reaction mixture, devoid of biotin (negative control 2); lane 5, BirA-His_6 was added to the reaction mixture, devoid of ATP (negative control 3); lane 6, BirA-His_6 was added to the reaction mixture, but the microtiter well was coated with GST (negative control 4).

(B) lane 1, no BirA-calmodulin-ABD-His_6 fusion protein was added to the reaction mixture (negative control 1); lane 2, BirA-calmodulin-ABD-His_6 fusion protein and biotinylated peptide were added to the reaction mixture (positive control); lane 3, BirA-calmodulin-ABD-His_6 fusion protein and substrate peptide were added to the reaction mixture; lane 4, BirA-calmodulin-ABD-His_6 fusion protein and substrate peptide were added to the reaction mixture, and soluble BirA-His_6 was also added; lane 5, BirA-calmodulin-ABD-His_6 fusion protein and substrate peptide were added to the reaction mixture, devoid of biotin (negative control 2); lane 6, BirA-calmodulin-ABD-His_6 fusion protein and substrate peptide were added to the reaction mixture, devoid of ATP (negative control 3). See also the Results and Experimental Procedures sections.

filter and the master filter on top. Freezing and thawing the filter sandwich three times released the biotin ligase from the cells, and the biotin ligase was allowed to biotinylate the biotin acceptor peptide on the reaction filter at 37°C. The filter was then washed, and product formation was detected with streptavidin-horseradish peroxidase and a chemiluminescent substrate. Figure 6A shows an image of the reaction filter (left) obtained according to the protocol outlined above. The biotin acceptor peptide coated on the filter is biotinylated in the region where it was covered by bacterial colonies expressing the enzyme. Importantly, the biotin ligase released by the bacterial colonies did not diffuse, allowing the identification of individual colonies carrying the desired enzymatic activity. As a negative control, the filter was either coated with GST without the BAP, or TG1 *E. coli* cells that harbor the ampicillin resistance gene but that do not overexpress BirA were used (Figure 6A, middle and right filters). Biotinylation of the substrate by endogenous BirA protein was not detectable because of its low abundance and the competitive inhibition of the biotin carboxyl carrier protein.

In the second approach (Figures 1C and 2B), a filter sandwich was formed with the master filter and a reaction filter coated with human serum albumin. The master filter was covered with bacterial colonies, expressing BirA-CaM-ABD-His_6 in the cytoplasm. The fusion protein was released from the cells by freezing and thawing of the filter sandwich, allowing their noncovalent immobilization on the reaction filter by interaction with HSA. The reaction filter was then washed, and the substrate peptide GAAA**RWKKAFIAVSAANRFKKIS**TSGGGPGG LVSIFEAQKIEWH was added to the reaction filter, allowing its binding to the calmodulin moiety of BirA-CaM-ABD-His_6 by means of the calmodulin binding peptidic sequence (boldface) [11]. The reaction filter was incubated in reaction buffer supplied with biotin and ATP, and the resulting product was detected as in the first approach (see above). Control reactions were performed with biotinylated peptide (biotin-CAAARWK KAFIAVSAANRFKKIS as positive control), no peptide (first negative control), an irrelevant peptide (GGHRDYK DEGGGAAA**RWKKAFIAVSAANRFKKIS**; second negative control) or no biotin (third negative control). The results are shown in Figure 6B; the presence of a biotinylated peptide is detected in the positive control (left filter) and in the enzyme-catalyzed reaction (middle filter), but not in the negative controls (right filter; first negative control only is shown; similar pictures were obtained with the other two negative controls). The reaction filters of the positive control gave slightly stronger signals, in spite of the X-ray film being exposed for a 40-fold shorter time. This corresponds to an approximately 100-fold lower degree of biotinylation in the enzyme-catalyzed reaction, which was, however, clearly positive compared to the negative controls, in which no signal was detectable.

The fact that the intramolecular enzyme-catalyzed reaction gives a weaker signal, compared to that of the positive control, is in line with the results of model selection experiments performed with phage-displayed BirA-CaM fusion proteins [15]. In comparison to the catELISA performed on microtiter plates (Figure 5B), the reaction with BirA-CaM-ABD-His_6 can comfortably be detected in the filter screening assay (Figure 6B). This could be due to the more sensitive detection method used (ECL) or to a higher surface density of immobilized BirA-CaM-ABD-His_6/peptide complex, allowing an intermolecular reaction.

Discussion

We describe two novel methodologies for the isolation of enzymatic activities from a large repertoire of enzyme

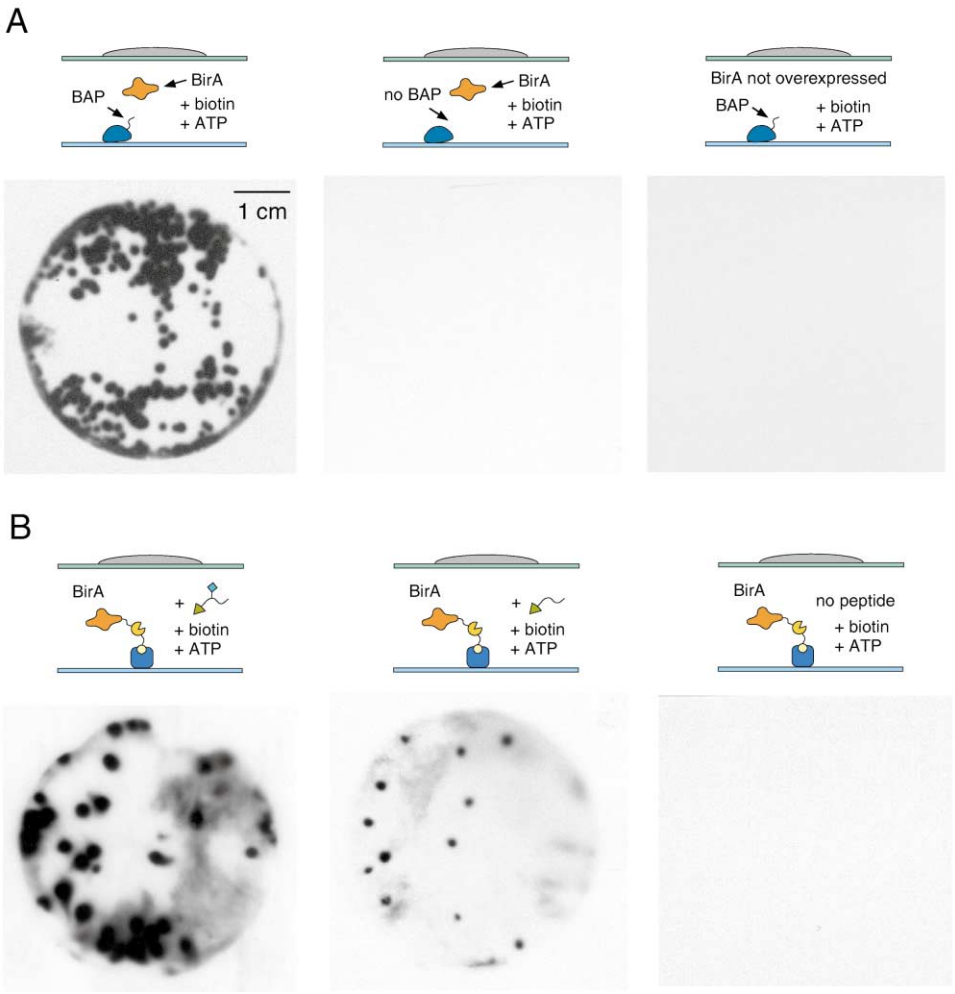

Figure 6. Two Different Filter Sandwich Activity Assays for the Biotin Ligase

The figure shows X-ray films, which were exposed to the reaction filters treated with streptavidin-HRP and the chemiluminescence ECL detection kit. The schematic drawing above each film illustrates the reaction conditions.

(A) Left filter, BirA-His_6 released from bacterial cells biotinylates the BAP in the presence of biotin and ATP (1 min exposure time); middle filter, the reaction filter is coated with GST that does not have a BAP fused to it (negative control 1; 1 min exposure time [the same filter was still negative for exposures as long as 30 min]); right filter, bacteria do not express biotin ligase (negative control 2; 1 min exposure time).

(B) Left filter, BirA-calmodulin-ABD-His_6 is released from the bacterial cells, and biotinylated peptide is added to the reaction (positive control; 30 s exposure time); middle filter, substrate peptide is added to the reaction (exposure time: 20 min); right filter, no peptide is added to the reaction (exposure time: 20 min). For further details, see the Results section.

mutants based on a filter-sandwich assay. The two-membrane colony screening system was initially developed for screening antibody Fab fragments secreted by bacterial colonies for binding to an antigen [16, 18]. Giovannoni et al. have recently used it to isolate good-quality antibodies by screening a large ($>10^8$ clones) naïve library of human antibody fragments by iterative colony filter screening [19]. We reasoned that one could modify this technology to screen for enzyme activities rather than for antibody binding.

The first experimental strategy (Figures 1B and 2A), in which a soluble enzyme converts substrates into products, which are attached to the reaction filter, proceeds with minimal diffusion of the released enzyme from the bacterial colony, allowing the identification of catalytically active colonies. Diffusion of the enzyme in the filter sandwich was successfully prevented by semidry reaction conditions. Product detection in the filter sandwich assay turned out to be much more sensitive than in the catELISA activity assay performed in microtiter plates, with an excellent signal to background ratio.

In the second experimental approach (Figures 1C and 2B), in which a calmodulin-tagged enzyme is captured on the reaction filter and is allowed to react with a calmodulin binding substrate derivative, the reaction product could only be identified in the filter sandwich assay but not in the catELISA experiments in microtiter plates. This observation may be due to a steric hindrance of the intramolecular biotinylation or to a more favorable reaction in *trans* occurring in the reaction filter because of a very high-density protein coating. It is likely that a longer linker between the substrate peptide and the enzyme, or between enzyme and calmodulin, could fa-

vor the intramolecular reaction and further increase the scope of the methodology. This experimental approach (Figure 1C) is possibly more interesting than the first one (Figure 1B) for the following reasons. The noncovalent, but stable, immobilization of the enzyme on the reaction filter serves to separate it from other enzymes released by the *E. coli* cells, which could interfere with the reaction of interest. Furthermore, less stable substrate derivatives (e.g., proteolytically labile peptides) can be added to the reaction filter in the absence of bacterial colonies and interfering proteins and thereby reduce the background of unwanted reactions.

Significance

The isolation of novel enzymes with improved catalytic properties and/or improved substrate specificities is a major challenge in protein engineering and has profound implications for the understanding of how enzymes work and for advanced industrial applications. Representing just one example taken from the research activity of our group, human enzymes with novel substrate specificity, which maintain their catalytic activity in serum, are urgently needed for effective tumor targeting [28]. The antibody-mediated delivery of such enzymes to the tumor environment will allow the selective generation of drugs from nontoxic precursors ("pro-drugs") in situ and therefore spare normal tissues and improve the therapeutic index of available chemotherapeutic agents [29]. Recombinant DNA methodologies allow the facile generation of very large repertoires of clones, expressing different enzyme mutants. However, general methodologies for the efficient screening of millions of enzymatic activities are still missing. We now describe two novel methodologies for the isolation of enzymatic activities from a large repertoire of enzyme mutants based on a filter-sandwich assay. In brief, the enzyme of interest is expressed in the cytoplasm of *Escherichia coli* cells, growing on top of a porous master filter. The enzyme is released to a second filter (reaction filter) in close contact to the master filter, allowing the catalytic conversion of substrates into products and their consequent detection with product-specific affinity reagents. Biotin ligase (BirA) was used as a model to demonstrate the feasibility and usefulness of the methodology. However, this method should be generally applicable to all catalyzed reactions in which the enzyme can be expressed in bacteria and in which the substrate can be chemically modified with a linker arm [11]. Several classes of enzymes, for example proteases, esterases, ligases, transferases, polymerases, and aldolases [11–13, 15], should be compatible with the methodologies presented in this article. Eventually, the usefulness of the these methodologies will heavily rely on the availability of good-quality affinity reagents (e.g., monoclonal antibodies [30] that can discriminate between reaction products and substrates [11, 15].

Experimental Procedures

Chemicals and Reagents

Peptides were synthesized by solid phase chemistry and purified by HPLC as described [27]. The positive-control peptide was biotinylated via the N-terminal cysteine by the use of biotin-NHS (Sigma, Buchs, Switzerland) and purified by HPLC. Peptide identity was confirmed by amino acid analysis and by MALDI-TOF (Protein Service Labor, ETH Zürich).

Ampicillin and IPTG were purchased from Applichem (Darmstadt, Germany). All the restriction enzymes and polymerases were purchased from Qbiogene (Basel, Switzerland), Axon Lab (Baden, Switzerland), and Boehringer Mannheim (Rotkreuz, Switzerland). T4 DNA ligase was obtained from Qbiogene (Basel, Switzerland). DNA primers were purchased from Microsynth (Balgach, Switzerland).

Bacterial Strains and Expression Vector Construction

All expression work was performed in *E. coli* strain TG1 [K12, Δ(*lac-pro*), *sup*E, *thi*, *hsdD5*/F′*traD*36, *proA*$^+$B$^+$, *lacI*^{q}, *lacZ*ΔM15].

The plasmid vectors were constructed by standard techniques [31].

pVG1 is a vector for periplasmic expression of hexa-histidine-tagged BirA. The BirA gene was amplified from pGEX-BirA (a kind gift of Professor Petri Saviranta, University of Turku, Finland; [21]) with the primers birasfiba and birahisnotfo and inserted into the SfiI and NotI sites of the expression vector pDN255 [32].

pVG3 is a vector for periplasmic expression of the hexa-histidine-tagged fusion protein BirA-CaM-ABD. The gene for BirA was amplified from pGEX-BirA with the primers birasfiba and biralinkfo. The genes for CaM and ABD were amplified from pSD4 [11] and pSB423 (a kind gift of Professor Mark Suter, University of Zurich, Switzerland; [25]), with the primer pairs linkba/camfonot and camlinkabpba/abphisnotfo, respectively. The PCR products were assembled in a three-way PCR assembly reaction with birasfiba and abphisnotfo as pull-through primers and inserted into the SfiI and NotI sites of the expression vector pDN255.

pCHH20 (Figure 3A), a vector for cytoplasmic expression of hexa-histidine-tagged BirA, was made by insertion of *E. coli* BirA, which was PCR amplified with the primers birecoba and hisbglfo and ligated into the EcoRI and BglII sites of pFV46.3 [33], a pQE12-based vector.

pCHH19 (Figure 3B) is a plasmid for the cytoplasmic expression of the hexa-histidine-tagged fusion protein BirA-CaM-ABD. The fused genes of BirA, CaM, and ABD were amplified from pVG3 with the primer pair birecoba/hisbglfo and were inserted into the EcoRI and BglII restriction sites of pFV46.3.

pCHH21 is a vector for the production of GST-BAP. The 5′ end-phosphorylated primers gstpepbirba and gstpepbirfo were assembled and ligated into the BamHI and XbaI sites of pGEX-BirA.

DNA Primers

The following primers were used: birahisnotfo 5′-CCATTGCGG CCGCTATCAGTGATGGTGATGGTGATGGCCACCTTTTTCTGCAC TACGCAGGGATATTTC-3′; biralinkfo 5′-CACCGCCACCAGAACC ACCTTTTTCTGCACTACGCAGGGATATTTC-3′; camlinkabpba 5′-GTACAAATGATGACAGCAAAGGCCGCGttagctgaaGCTAAAGTCT TAgc-3′; abphisnotfo 5′-CATTGCGGCCGCTATCAGTGATGGTGAT GGTGATGGCCACCAGGTAATGCAGCTAAAATTTC-3′; hisbglfo 5′-ACTAGATCTCTATCAGTGATGGTGATGGTG-3′; cambabam 5′-GGA GCAGGCGCCGGATCCATGGCTGACCAACTGACAGAAGAG-3′; birecoba 5′-ATCGAATTCATTAAAGAGGAGAAATTAACTATGAAGGAT AACACCGTGCCAC-3′; gstpepbirba 5′-GATCCGGCCTGGTTTCTA TCTTCGAAGCTCAGAAAATCGAATGGCACTGATAGT-3′; gstpepbirfo 5′-CTAGACTATCAGTGCCATTCGATTTTCTGAGCTTCGAAGATAG AAACCAGGCCG-3′; birasfiba 5′-ATGCGGCCCAGCCGGCCATGG CCATGAAGGATAACACCGTGCCAC-3′; linkba, 5′-ACCGCCACTTC CACCGCCACCAGAACCACCGTTGGCAG-3′; camfonot, 5′-CCGAT TGCGGCCGCCTTTGCTGTCATCATTTGTACA-3′.

Expression and Purification of Protein

Five milliliter overnight cultures of *E. coli* strain TG1 containing the expression vectors were used for the inoculation of 400 ml of 2YT media containing 100 μg/ml ampicillin. Cells were grown at 37°C with vigorous shaking until an OD_{600} of 0.6 was reached. Protein expression was induced by the addition of IPTG to a final concentration of 1 mM. Cells were incubated for an additional 12 hr at 25°C and then harvested. Proteins that were directed to the periplasm by the PelB leader sequence (pVG1 and pVG3) were isolated by

extraction of the periplasmic fraction as described [34]. A modified extraction buffer (50 mM Tris-HCl [pH 8], 300 mM NaCl, 1 mM EDTA, 10 mM imidazole, and 1 mg/ml polymyxin), compatible with nickel affinity chromatography purification, was used. Proteins that had a hexa-histidine-tag and were expressed into the cytoplasm (pCHH19 and pCHH20) were lysed with a buffer containing 50 mM Tris-HCl (pH 8), 300 mM NaCl, 1 mM EDTA, 10 mM imidazole, and 0.2 mg/ml lysozyme. Cells were additionally sonicated. The native fraction of the expressed protein was purified by nickel affinity chromatography with 300 μl of Ni-NTA agarose (Qiagen, Hilden, Germany). The lysate-Ni-NTA mixture was loaded into a column (Biorad, Glattbrugg, Switzerland) and washed twice with 6 ml of a buffer containing 50 mM Tris-HCl (pH 8), 300 mM NaCl, and 15 mM imidazole by gravity flow. The enzyme was eluted in 600 μl buffer containing 50 mM Tris-HCl (pH 8), 300 mM NaCl, and 250 mM imidazole. Expression yields for cytoplasmic expression were 1 mg per liter culture for both the BirA-His_6 and the BirA-CaM-ABD-His_6 protein. Periplasmic expression (from pVG1/TG1 and pVG3/TG1) yielded very low amounts of protein ($<$0.1 mg/liter culture).

Cells expressing the protein GST-BAP into the cytoplasm (pCHH21) were lysed with PBS (20 mM NaH_2PO_4, 30 mM Na_2HPO_4, and 100 mM NaCl [pH 7.4]) containing 0.2 mg/ml lysozyme. Cells were additionally sonicated. The protein was purified by glutathione sepharose affinity chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden) with 200 μl of slurry per mg of protein. The protein was eluted with PBS containing 20 mM GSH (Sigma, Buchs, Switzerland).

All proteins were analyzed by SDS polyacrylamid gel electrophoresis with precast 10% Bis-Tris gels (Invitrogen, Basel, Switzerland) and Coomassie staining. The presence of the biotin acceptor peptide (BAP) fused to GST was analyzed by MALDI-TOF (Protein Service Labor, ETH Zürich, Switzerland).

catELISA Experiments

Falcon Flexible Assay plates with 96 wells (Becton Dickinson, Heidelberg, Germany) were coated with GST-BAP protein (50 μg per well) in PBS (pH 7.4; 100 μl) at 37°C overnight. The wells were washed four times with TBST (Tris-HCl [pH 7.4], 100 mM NaCl, and 0.1% v/v Tween-20) and blocked with 1% milk (Rapilait, Migros, Switzerland) in PBS for 1 hr at room temperature. BirA-His_6 (5 μg) was then incubated in 100 μl of the reaction buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 12 mM $MgCl_2$, 10 mM ATP, 100 μM D-biotin [Sigma, Buchs, Switzerland], 1 mg/ml pyrophosphatase [Sigma, Buchs, Switzerland], and 0.1% v/v Tween-20) for 2 hr at 37°C. In negative control reactions, either biotin or ATP was omitted in the reaction buffer. Apyrase (40 μg per well; Sigma, Buchs, Switzerland) was added to the negative control reaction when ATP was omitted. The wells were washed four times with TBST and blocked with 1% milk in TBS for 30 min at room temperature. Then 100 μl of preblocked HRP-streptavidin (Amersham Pharmacia Biotech) in TBS (1:500) was added and incubated for 30 min at room temperature. The wells were washed five times with TBST, and the bound HRP was quantified in a colorimetric reaction with 100 μl of BM blue POD substrate (Roche, Basel, Switzerland). The reaction was stopped with 50 μl 1 M H_2SO_4, and the absorption at 450 nm was measured in a SpectraMAX instrument (Molecular Devices, Sunnyvale, CA, USA).

In a second assay format the micro titer plates were coated with HSA (human serum albumin; Sigma, Buchs, Switzerland) in PBS (1 mg/ml) at 37°C overnight. After being washed three times with TBSCT (50 mM Tris-HCl, 100 mM NaCl, 1 mM $CaCl_2$, and 0.1% v/v Tween-20 [pH 7.4]), the wells were blocked for 30 min with 1% milk in TBSC. Preblocked BirA-CaM-ABD-His_6 (5 μg) was added to each well, and these were incubated for 30 min at room temperature. The wells were washed four times with TBSC and incubated with 100 μl of 10^{-8}M preblocked substrate peptide GAAARWKKAFIAVSAAN RFKKISTSGGGPGGLVSIFEAQKIEWH for 30 min at room temperature. Unbound peptide was removed by being washed four times with TBSCT before 100 μl of the reaction buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 12 mM $MgCl_2$, 2 mM ATP, 100 μM D-biotin (Sigma, Buchs, Switzerland), 1 mg/ml pyrophosphatase (Sigma), and 0.1% v/v Tween-20) was added to the wells. The wells were incubated for 2 hr at 37°C. Finally, the presence of immobilized biotin was detected with HRP-streptavidin as described above.

Colony Filter Assay

Bacteria were grown in liquid 2YT medium containing 100 μg/ml ampicillin until an OD_{600} of 0.6 was reached. The bacteria were spread on a 4.7 cm diameter PVDF Durapore membrane filter (type GVWP, 0.22 μm; Millipore, Bedford, MA, USA), which was placed on a Petri dish containing 2YT agar with 100 μg/ml ampicillin. The plate was incubated at room temperature overnight. The PVDF Durapore membrane filter (master filter) was then placed onto a new Petri dish containing 2YT agar with 100 μg/ml ampicillin and 1 mM IPTG and incubated at room temperature overnight.

A second membrane (reaction filter) was coated with either GST-BAP or HSA, depending on whether the approach shown in Figure 1B or 1C was followed. The 5 cm diameter PVDF membrane (Immobilon-P; Millipore, Bedford, MA, USA) was incubated in GST-BAP (0.5 mg/ml) or HSA (1 mg/ml) in PBS at room temperature overnight. The filter was washed three times with TBST and blocked in 1% milk/TBS for 1 hr at room temperature. The GST-BAP-coated filter was additionally incubated in reaction buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 12 mM $MgCl_2$, 10 mM ATP, 100 μM D-biotin, 1 mg/ml pyrophosphatase, and 0.1% v/v Tween-20) for 10 min at room temperature.

The coated reaction filters were then placed into an empty Petri dish and covered with the master filter, with the bacterial colonies on top. The resulting "sandwich" was frozen and thawed three times in order to lyse the cells and to release the expressed protein. The filter "sandwich" with the GST-BAP-coated reaction filter was incubated for 1 hr at 37°C to allow the released BirA enzyme to biotinylate the BAP. The HSA-coated reaction filter on which the released fusion protein BirA-CaM-ABD-His_6 was immobilized was washed, blocked in 1% milk/TBSC, and incubated with of 10^{-8} M preblocked substrate peptide for 30 min at room temperature. Unbound peptide was removed by being washed three times with TBSCT before the filter was incubated in reaction buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 12 mM $MgCl_2$, 2 mM ATP, 100 μM D-biotin, 1 mg/ml pyrophosphatase, and 0.1% v/v Tween-20) for 1 hr at 37°C. The biotinylated BAP, which represents the reaction product, was detected as follows. The reaction filter was washed four times with TBSCT and blocked for 30 min in 1% milk/TBSC. HRP-streptavidin conjugate in 1% milk/TBSC (1:1000) was added to the filter and incubated for 30 min at room temperature. The reaction filter was washed five times with TBSCT, and the bound HRP was identified in a chemiluminescent reaction with the ECL kit from Amersham Pharmacia Biotech.

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