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Evaluation of a New System for Developing Particulate Enzymes Based on the Surface (S)-Layer Protein (RsaA) of *Caulobacter crescentus*

Fusion With the β-1,4-Glycanase (Cex) From the Cellulolytic Bacterium Cellulomonas fimi *Yields a Robust, Catalytically Active Product*

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Abstract

Immobilized biocatalysts, including particulate enzymes, represent an attractive tool for research and industrial applications because they combine the specificity of native enzymes with the advantage that they can be readily separated from end product and reused. We demonstrated the use of the *Caulobacter crescentus* surface (S)-layer protein (RsaA) secretion apparatus for the generation of particulate enzymes. Specifically, a candidate protein made previously by fusion of the β-1,4-glycanase (Cex) from the cellulolytic bacterium *Cellulomonas fimi* with the C-terminus of RsaA was evaluated. Cex/RsaA cleaved the glycosidic linkage in the artificial substrate *p*-nitrophenyl-β-D-cellobioside with a K_M similar to that of native Cex (1.1 m*M* for Cex/RsaA vs 0.60 m*M* for Cex), indicating that the particulate Cex enzyme was able to bind substrate with wild-type affinity. By contrast, the k_{cat} value was significantly reduced (0.08 s^{-1} for Cex/RsaA vs 15.8 s^{-1} for Cex), likely owing to the fact that the RsaA C-terminus induces spontaneous unstructured aggregation of the recombinant protein. Here, we demonstrated that not only can an RsaA fusion protein be cheaply produced and purified to a

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high yield (76 mg/L of dry wt for Cex/RsaA), but it can also be efficiently recycled. The Caulobacter S-layer secretion system therefore offers an attractive new model system for the production of particulate biocatalysts.

Index Entries: Caulobacter surface (S)-layer protein RsaA; type I secretion; cellulomonas β-1,4-glycanase; cellulase; Cex; cellobiohydrolase; crosslinked enzyme crystals; particulate enzymes; immobilized biocatalysts.

Introduction

At the current time in industrial enzyme research, varied means are being implemented to immobilize both native and semisynthetic enzymes on or in solid supports, leading to greater stability and the possibility of biocatalyst recycling. To date, enzymes have been expressed on whole cells *(1,2)*; crystallized and chemically crosslinked to produce crosslinked enzyme crystals (CLECs) *(3–6)*; encapsulated in various supports including sol-gel *(7,8)* and synthetic polymer complements *(9)*; and adsorbed and crosslinked to various immobilization supports such as chitosan *(10)*, poly(vinyl alcohol) *(11)*, Sepabeads-epoxy *(12)*, agarose beads/gel *(13,14)*, and even a novel DNA-avidin biopolymer microgel *(15)*. The ultimate goal of all of these initiatives is twofold: first, to protect the enzyme such that it can function in increasingly harsh environments without adversely affecting its ability to bind substrate and efficiently catalyze a specific reaction; and, second, to facilitate efficient and economical separation of the biocatalyst from the endproduct. The latter consequence of biocatalyst immobilization offers the added advantage of potentially allowing recovery of the enzyme so that it can be reused, thus greatly reducing production costs.

In our laboratory, we are currently exploring the potential of using Caulobacter, a nonpathogenic, Gram-negative aquatic scavenger bacterium *(16,17)*, to produce functional particulate enzymes for use in biotechnology applications. Caulobacter is one of many genera of bacteria that are covered by a protein surface (S)-layer *(18,19)*. S-layers are two-dimensional arrays that are nearly always composed of a single protein that self-assembles into a paracrystalline lattice on the cell surface *(18,20–22)*. In the case of the best characterized *Caulobacter* species, *C. crescentus*, the S-layer is a hexagonal array composed of approx 40,000 copies of a 1026 amino acid/98-kDa protein, RsaA *(23)*. The RsaA monomers are secreted from the bacterium via a type I ABC transporter system *(24)* that recognizes an uncleaved C-terminal secretion signal located within the last 82 amino acids of RsaA *(25,26)*.

Exploiting the *C. crescentus* S-layer protein secretion apparatus represents an attractive cost-efficient method for the production of heterologous proteins for several reasons. First, Caulobacter can be grown to high density under straightforward and inexpensive growth conditions *(26,27)* and can be manipulated genetically because the *C. crescentus* genome has been sequenced *(28)*. Second, the Caulobacter type I secretion apparatus appears attuned to produce high yields of RsaA fusion proteins, because RsaA is the predominant protein species produced, accounting for 10–12% of the pro-

tein mass of the bacterium *(29)*. Third, fusion of the target proteins with only the C-terminal secretion signal of RsaA allows shedding of the RsaA fusion proteins into the culture medium. Interestingly, when cultures are shaken gently, both native RsaA and RsaA recombinant proteins spontaneously form visible aggregates in the culture medium by way of an unknown mechanism requiring at least the last 82 amino acids of the RsaA C-terminus *(26)*. Unlike the S-layer, these aggregates appear unstructured when viewed by electron microscopy and are unaffected by EGTA (which readily disrupts the calcium-mediated crystallization of the S-layer) (unpublished observations), suggesting that self-aggregation does not occur by a crystallization process comparable with that of the native S-layer. These aggregates can be collected simply and rapidly by coarse filtration of the culture medium through a fine nylon mesh. The aggregates can be washed free of cells and other debris by centrifuging and rinsing with water or buffers; typically, by using these simple methods, the fusion protein aggregates are 90–99% pure. Finally, C-terminal hybrid S-layer fusion proteins are not subject to proteolytic degradation by the Caulobacter host cell *(30)*.

We previously engineered recombinant RsaA proteins by fusion of genes of interest such as vaccine candidates to fragments of the *rsaA* gene encoding various RsaA C-termini all bearing the secretion signal sequence *(27)*. We have observed a positive correlation between the length of the RsaA C-terminal fragment and the yield of the heterologous fusion protein, with fusion to the last 336 amino acids of RsaA representing a good compromise between product size and yield *(26)*. To date, genes encoding foreign proteins of up to 600 amino acids have been successfully secreted and recovered from culture medium *(27)*. For the present study, we assessed the use of the RsaA secretion apparatus as a new model system for the production of functional particulate enzymes for potential use in an industrial setting.

It is not our intention to describe a fully developed product capable of immediate use in an industrial application but, rather, to demonstrate the general feasibility of using Caulobacter to bioengineer recombinant particulate enzymes. To do so, we chose a previously constructed recombinant protein that bears the catalytic domain of a β-1,4-glycanase (*p*NPCase) (Cex) from the cellulolytic bacterium *Cellulomonas fimi* fused to the RsaA C-terminus as our prototype particulate enzyme. Cex is one of several degradative enzymes required for the stepwise conversion of the insoluble plant biopolymers cellulose and xylan into soluble monosaccharides *(31)*. Cex represents a good candidate enzyme for the evaluation of our model system because extensive kinetic and structural data are available for this enzyme *(32–35)*. Furthermore, a simple functional assay for measuring the *p*NPCase activity of Cex using a readily available artificial substrate has already been designed and published *(34)*.

In the current study, we evaluated the utility of the Caulobacter secretory apparatus as a new system for the generation of recombinant particulate enzymes for use in biotechnology applications. Specifically, we

evaluated the recombinant fusion protein Cex/RsaA by asking four questions regarding its eventual use in an industrial setting:

1. What is the minimal length of the RsaA C-terminus necessary for consistently high yield and reasonable catalytic activity?
2. Is the particulate enzyme stable?
3. How do the Michaelis-Menten enzyme kinetics of the recombinant protein compare with that of native Cex?
4. Can the recombinant particulate enzyme be efficiently separated away from the endproduct and reused to catalyze additional reactions?

Materials and Methods

Bacterial Strains and Growth Conditions

The holdfast-reduced *C. crescentus* strain JS4011 *(29)* was used for all *Caulobacter* experiments. Growth of both *C. crescentus* and *Escherichia coli* was as previously described *(36)*.

Genes and Plasmids

The *C. fimi cex* gene encoding a 443 amino acid *p*NPCase enzyme, Cex (EC 3.2.1.8), was kindly provided by Drs. D. G. Kilburn and R. A. J. Warren, Department of Microbiology & Immunology, University of British Columbia. Cex has been classified as a member of the glycoside hydrolase family 10 (http://afmb.cnrs-mrs.fr/CAZY/). The construction of six pUC8-based plasmids encoding Cex/RsaA fusion proteins that we studied has already been described elsewhere *(26)*. Owing to the cloning strategy employed, these six Cex/RsaA constructs all have a seven amino acid LacZ N-terminal leader sequence fused to a *cex* gene fragment bearing a modified N-terminus, i.e., missing the first two amino acids of the mature Cex protein. A new Cex/RsaA336C construct, designated C_S/RsaA336C, for short Cex, bearing the complete native N-terminus of mature Cex, the catalytic domain, and the linker region (340 amino acids total) was therefore made. While the original Cex/RsaA336C (designated henceforth as C_P/RsaA336C, for published Cex) was made in the pUC8 background as previously described *(26)*, C_S/RsaA336C was made in a new vector, pUC8-N. This new pUC8-based vector, which has had the first seven amino acids of LacZ (including the translation start ATG) replaced with an *Nde*I site that bears an internal translation start site (CAT ATG), was generated using the oligonucleotide primers 8NDE1 5'-GGA ATT CCA TAT GTT CGC CTG TAA AAC CGC CAA TGG TAC C-3' and 8NDE2 5'-GGA ATT CCA TAT GGTG TTT CCT GTG TGA AAT TGT TAT CCG C-3' (*Nde*I site underlined) in an inverse polymerase chain reaction (PCR) using a pUC8 vector containing a 495-bp *E. coli fimH* gene (used for another project) as template DNA. The resulting PCR product was then digested with *Nde*I and self-ligated to produce pUC8-N *fimH* N14 T3. To generate pUC8-N, the *fimH* gene was

removed by digestion with *Nde*I and *Hin*dIII and then replaced with two annealed oligonucleotide primers (JN ESBH-1 5' T ATG ACG AAT TCC CGG GGA TCC CCA 3' and JN ESBH-2 5' A GCT TGG CCA TCC CCG GGA ATT CGT CA 3'), resulting in the generation of a small multiple cloning site containing *Nde*I, *Eco*RI, *Sma*I, *Bam*HI, and *Hin*dIII sites.

To generate the Cex-encoding fragment of the C_S/RsaA336C clone, PCR was performed on the full-length *cex* gene using the following oligonucleotides: CexFwd 5'-CG CAT ATG **GCG ACC ACG CTC AAG GAG GCC**-3' and C_SRev 5'-GC GGA **TCC CCC GGC CGG ACC GGA CGT CGG**-3'. The *Nde*I site bearing the translation start site is underlined, the *Bam*HI is double underlined, and the *cex* gene sequence is boldface. The resulting PCR fragment was then ligated into the *Nde*I and *Bam*HI sites of the pUC8-N vector.

Expression of Fusion Proteins, and Recovery, Preparation, and Quantification of Aggregates

Fusion proteins were all expressed in the holdfast-reduced *C. crescentus* strain JS4011 *(29)*. For aggregate production, cells were grown in M_{11} HiGG– defined medium in 2.8-L Fernbach flasks, and protein was collected and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as previously described *(26)*.

Prior to enzyme assays, aggregates were thawed, sonicated as described in the next section, and the protein concentrations of the sonicated aggregate solutions were determined using a Micro BCA™ protein assay reagent kit (Pierce). For the experiments used to calculate the Michaelis-Menten parameters and to study the recyclability of Cex/RsaA (described later), freshly washed aggregates were homogenized as described and dry pellet weights were determined. Aggregates were stored at 4°C and used within 1 wk. Further, for these catalytic assays, aliquots were routinely pelleted and washed and the supernatants tested to verify that there was no soluble enzyme activity prior to use.

Assay of Cex/RsaA Hybrid Fusion Proteins for p*NPCase Activity (Figs. 1–3)*

Prior to assaying for *p*NPCase activity, hydrated aggregates were thawed; mixed with an equal volume of 50 m*M* potassium phosphate buffer, pH 7.2; and sonicated on ice for 30 s on the low setting with a Braun-Sonic 2000 microprobe to disperse the aggregates evenly. *p*NPCase activity was measured as the release of the *p*-nitrophenolate anion from the synthetic substrate *p*-nitrophenyl-β-D-cellobioside (*p*NPC) as reported previously *(37)* using 1 m*M* substrate. Absorbance was measured at 400 nm using an UltroSpec II (LBK Biochrom) spectrophotometer, and the release of *p*-nitrophenol (*p*NP) was calculated using a standard curve established with free *p*NP.

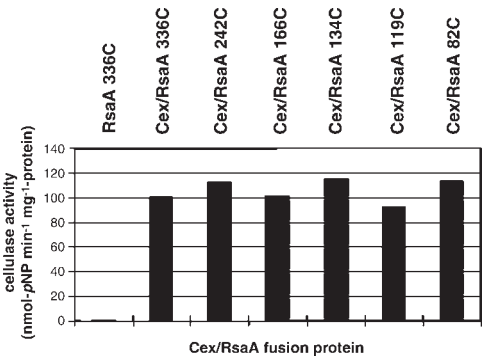

Fig. 1. *p*NPCase activity of hybrid Cex/RsaA fusion proteins. *p*NPCase activity was measured for each of the hybrid Cex/RsaA fusion proteins by determining spectroscopically the release of *p*NP from 1 m*M* *p*NPC at 400 nm. Cex/RsaA fusion proteins were recovered from culture medium, washed with distilled water, pelleted by centrifugation, and sonicated prior to the assay to disrupt the aggregates. RsaA336C without an N-terminal Cex fusion was included as a negative control. The results were normalized against a standard curve made with free *p*NP.

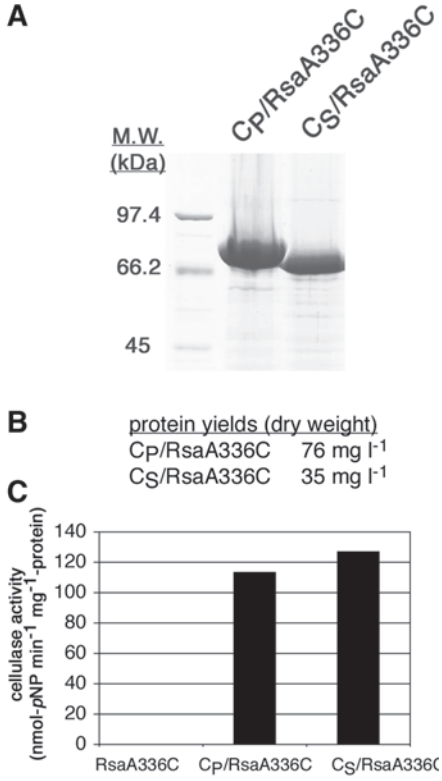

Fig. 2. Comparative purity, yield, and *p*NPCase activity of C_P/RsaA336C vs C_S/RsaA336C. **(A)** Cex/RsaA fusion protein aggregates were recovered from culture medium, solubilized with urea, and analyzed by SDS-PAGE on a 12% polyacrylamide gel. The molecular weight standards are as indicated. The predicted molecular weights of the fusion proteins are given in Materials and Methods. **(B)** Representative yields (milligrams/liter of dry weight) are given for each construct. **(C)** *p*NPCase activity was measured as described in the legend to Fig. 1. RsaA336C without an N-terminal Cex fusion was included as a negative control.

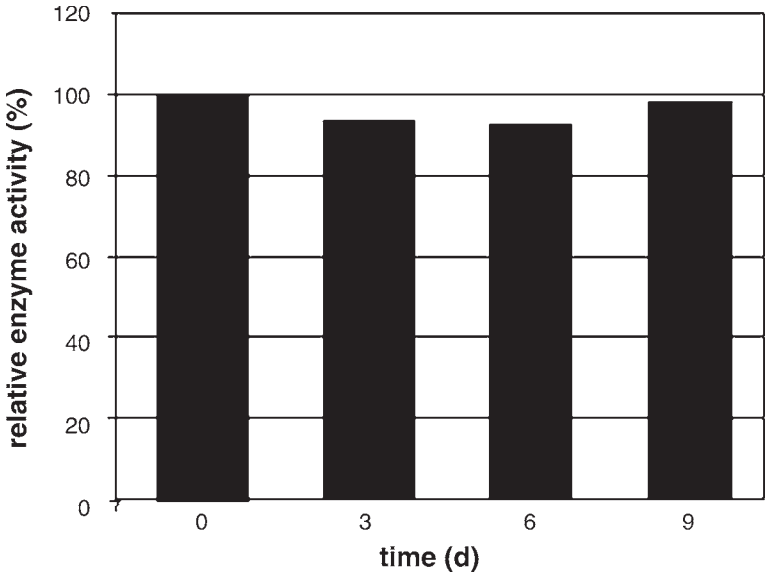

Fig. 3. Stability of *p*NPCase activity of Cex/RsaA particulate enzyme. The *p*NPCase activity of C_p/RsaA336C was measured as described in the legend to Fig. 1 after 0–9 d of storage at 4°C. The results are expressed as a percentage of the original activity.

Measurement of Stability of C_p/RsaA336C Fusion Protein

The stability of Cex/RsaA particulate enzymes was examined by measuring *p*NPCase activity after 0–9 d of storage at 4°C. Samples were assayed as just described, and the results are expressed as a percentage of the original activity.

Determination of Michaelis-Menten Parameters for C_p/RsaA336C

Fusion protein aggregates were homogenized with a Sorvall Omni-Mixer for 1 to 2 min at 25% of maximum speed to produce a suspension of approximately evenly sized particles, and dry pellet weights (milligrams) were used to calculate the amount of input protein for the enzyme assays. Homogenized pellets were used within 1 wk, and aggregates were washed once prior to use to eliminate soluble enzyme activity. To determine first the linear range of the initial rate at low substrate concentrations, a stopped assay based on the release of *p*NP from the substrate *p*NPC was used. Typically, 100 µL of C_p/RsaA336C particulate enzyme suspension was preincubated with 800 µL of 50 m*M* sodium phosphate buffer, pH 7.0, for 30 min. Reactions were then initiated by adding 10 µL of 18.9 m*M* *p*NPC and after different time intervals stopped with 500 µL of 1.0 *M* trisodium phosphate on ice. After centrifuging for 5 min at 19,000*g* at 4°C, a 1-mL aliquot was microfiltered (0.22 µm) and equilibrated to 37°C. Absorbance was measured at 400 nm using temperature-stabilized Cary AV-300 or Cary AV-4000 spectrophotometers. An extinction coefficient of 7.28 mM^{-1} cm^{-1} was used for 4-nitrophenol at 37°C as previously determined *(38)*.

To generate the Michaelis-Menten data, the initial rates of 11 substrate concentrations, varying from 0.2 to 5 times K_M, were used with a 3-min reaction time. The observed rates were corrected for background substrate hydrolysis, and the kinetic parameters k_{cat} and K_M were calculated by fitting the initial rate data to the Michaelis-Menten equation using Grafit version 4.0 *(39)*.

Active-Site Titration for C_P/RsaA336C

Active-site titration assays were performed for up to 48 h on aggregates prepared as already described using the mechanism-based inactivator 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-cellobioside. Turnover resulted in liberation of 2,4-dinitrophenol, which was measured optically at 400 nm, whereas 2-deoxy-2-fluoro-β-cellobioside remained irreversibly bound to the active site, thereby inactivating it *(34)*. Owing to the low absorbance reading obtained for the active-site titration (A_{400} = 0.01), we verified that the enzyme had been fully inactivated by performing an additional experiment as follows: The aggregates were thoroughly washed with sodium phosphate buffer after the titration assay, fresh *p*NPC was added, and the reaction was stopped and measured at 400 nm as described for the Michaelis-Menten kinetics.

Recycling of C_P/RsaA336C Fusion Protein

Assays were run as described previously for the determination of the Michaelis-Menten parameters, then samples were washed three times with sodium phosphate buffer, fresh substrate was added, and the assays were rerun. This process was repeated a total of 5–10 times per sample, and the results are expressed as a percentage of the original activity. After the last assay, the Cex/RsaA aggregates were pelleted and lyophilized, and the dry pellet weights were measured and used to calculate the average amount of enzyme lost per wash step. This value was then used to correct the catalytic activity values obtained after each assay run for enzyme loss.

Results

Determination of Optimal Fusion Protein Construct in Terms of Protein Yield and Catalytic Activity

Six previously engineered Cex/RsaA particulate enzymes were compared using a published quantitative assay for Cex activity *(34)*. Briefly, *p*NPCase activity was measured based on the release of the *p*-nitrophenolate anion (*p*NP) from the synthetic substrate *p*NPC. As can be seen in Fig. 1, all six Cex/RsaA fusion proteins exhibited relatively comparable levels of cellulolytic activity in this assay. When fusion protein length and protein yield *(26)* were also taken into account, the fusion construct bearing the last 336 amino acids of RsaA proved to offer the best compromise, and this C-terminal tail was adopted for all further study.

The original cloning strategy employed resulted in the addition of a gene segment encoding a seven amino acid LacZ N-terminal leader sequence fused to a *cex* gene fragment missing the first two amino acids of the mature Cex protein. Because the Cex catalytic domain is located near the N-terminus, it was possible that this N-terminal modification might be affecting enzyme activity. We therefore constructed an additional Cex/RsaA fusion protein, C_S/RsaA336C (short), which comprises the first 340 amino acids of mature Cex, i.e., including the complete native N-terminus of mature Cex, the catalytic domain, and the linker region. We then compared this new construct with the original Cex/RsaA336C construct, C_P/RsaA336C (published). The new Cex/RsaA fusion protein was of the predicted mol mass of 68.8 kDa and was isolated to high purity (Fig. 2A). Interestingly, however, whereas we obtained a dry weight yield of 76 mg/L for the original C_P/RsaA336C construct (Fig. 2B), consistent with our previous results *(26)*, the yield of the new construct, C_S/RsaA336C, was found to be only 35 mg/L (Fig. 2B), about 50% less than the yield obtained for C_P/RsaA336C. On the other hand, *p*NPCase activity (Fig. 2C) was found to be very comparable with that of all the other Cex/RsaA fusion constructs (Figs. 1 and 2C), suggesting that modifying the N-terminus did not have an adverse effect on *p*NPCase activity of the hybrid particulate enzymes. We chose to continue our studies with the highest-yielding C_P/RsaA336C construct.

p*NPCase Activity of Cex/RsaA Is Stable for 1 Wk at 4°C*

Altogether the results of our enzyme assay clearly demonstrated that Cex/RsaA particulate enzymes possessed the cellulolytic activity of the native *p*NPCase from *C. fimi*. We then assessed the stability of C_P/RsaA336C at 4°C by assaying the enzymatic activity of samples over time. The enzyme activity of C_P/RsaA336C remained relatively constant for more than 1 wk at 4°C (Fig. 3). Note, however, that although the supernatant of freshly pelleted and washed aggregates was consistently found to be devoid of enzyme activity (data not shown), we were able to measure low levels of enzyme activity in the supernatant surrounding the aggregates after a few days of storage at either 4°C or room temperature (data not shown), indicating that a small amount of hybrid particulate enzyme was solubilizing over time. Further, preliminary data suggested that storage for more than 2 wk at 4°C was associated with loss in enzyme activity (data not shown).

Cex/RsaA Hybrid Particulate Enzyme Binds Substrate With Native Cex-Like Affinity

To determine the Michaelis-Menten enzyme kinetics parameters for the Cex/RsaA hybrid particulate enzymes, we first defined the linear region of the initial reaction rate at low substrate concentration by measuring the release of *p*NP from *p*NPC using a stopped assay at pH 7.0, 37°C, as described in Materials and Methods. Because the initial rate was deter-

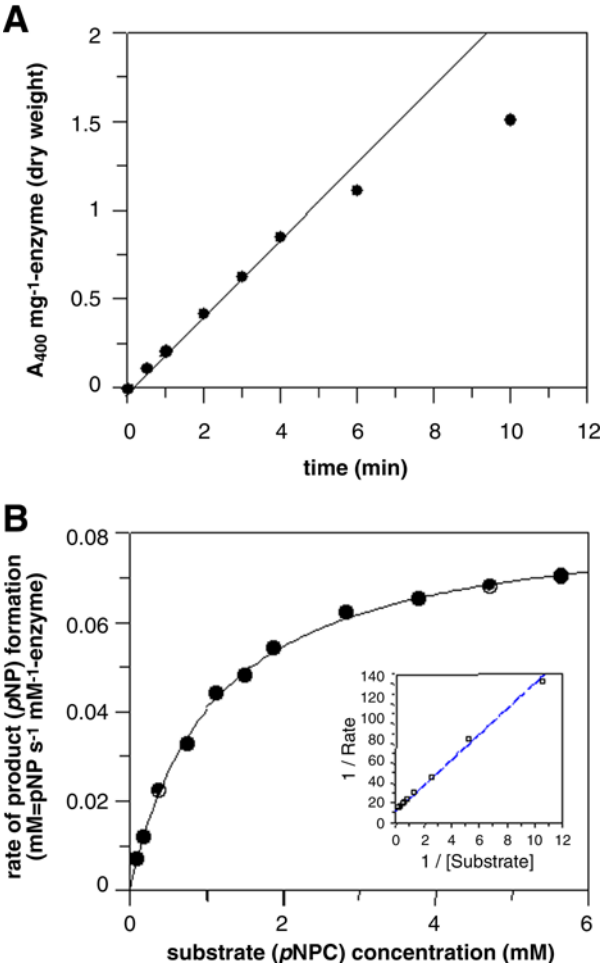

Fig. 4. Michaelis-Menten enzyme kinetics of Cex/RsaA336C particulate enzyme. **(A)** Determination of linear range of initial rate of enzymatic hydrolysis of *p*NPC by C_P/RsaA336C at low substrate concentration (0.2 m*M* *p*NPC). The release of *p*NP was calculated by measuring the absorbance at 400 nm and using an extinction coefficient of 7.28 mM^{-1} cm^{-1}. **(B)** Effect of increasing substrate (*p*NPC) concentration on initial velocity of C_P/RsaA336C-catalyzed release of *p*NP at pH 7.0, 37°C, using stopped assay time of 3 min. The kinetic parameters k_{cat} and K_M were calculated by fitting the initial rate data to the Michaelis-Menten equation using Grafit version 4.0 *(39)*.

mined to be linear over 0–4 min (Fig. 4A), the Michaelis-Menten data were collected with a reaction time of 3 min. The results showed that the binding constant (K_M) was similar to that of the native enzyme (Fig. 4B, Table 1). This suggested that the affinity of the hybrid particulate enzyme for its substrate was comparable with that of its native counterpart. However, we obtained a significantly reduced k_{cat} value for the Cex/RsaA fusion protein, indicating that the Cex/RsaA aggregates were only about 0.5% as efficient as native Cex under the same conditions (Table 1) *(34)*. This suggested either

Table 1
Michaelis-Menten Parameters
for Cex-Mediated Hydrolysis of *p*NPC at pH 7.0, 37°C

Cex enzyme	k_{cat} (s^{-1})	K_M (m*M*)	k_{cat}/K_M (s^{-1} mM^{-1})
C_P/RsaA336C[a]	0.084	1.1	0.073
Native (wild-type) Cex[b]	15.8	0.6	26.3

[a]Data obtained in this study (Fig. 4).
[b]Data obtained from ref. *34*.

that fewer of the active sites were accessible for substrate binding or that the majority of the active sites were available but had greatly reduced activity. To resolve these possibilities, an active-site titration analysis was performed and a value of about 0.7% (data not shown) was obtained, signifying that less than 1 in 100 of the active sites was functional and/or able to bind substrate. To confirm that all catalytically competent active sites had been labeled and inactivated, the same aggregates were then washed thoroughly and fresh substrate (*p*NPC) was added. No substrate hydrolysis was observed in this second assay, indicating that all catalytically competent active sites had been inactivated. Taken together these data indicate that <1% of the recombinant active sites were available and/or able to perform catalysis, as opposed to there being 100% of available active sites possessing only 1% residual activity.

Hybrid Cex/RsaA Aggregates Can Be Recycled Without Inactivation of Catalytic Activity

To determine whether the insoluble Caulobacter-based particulate enzymes could be purified away from the product and reused to catalyze the same reaction multiple times, C_P/RsaA336C enzyme activity was assayed, aggregates were collected by centrifugation and then washed three times, and the assay was repeated for a total of up to 10 consecutive runs. The relative activity of the enzyme decreased slowly over time (data not shown), with approx 50% of the original activity still detectable after the tenth cycle. Because this entire process represented a total of 40 processing steps (30 wash steps plus 10 substrate reactions), we sought to determine whether the loss in activity was owing to enzyme inactivation over time or loss of enzyme owing to processing (centrifugation and washing). The dry pellet weights were therefore determined after the last cycle and used to calculate the average amount of enzyme lost per wash step, which was then used to normalize the enzyme activity readings. As shown in Fig. 5, when enzyme loss is taken into account there is no significant decrease in enzyme activity, even after 10 runs, clearly demonstrating that Cex/RsaA particulate enzymes can be recycled without risk of inactivation.

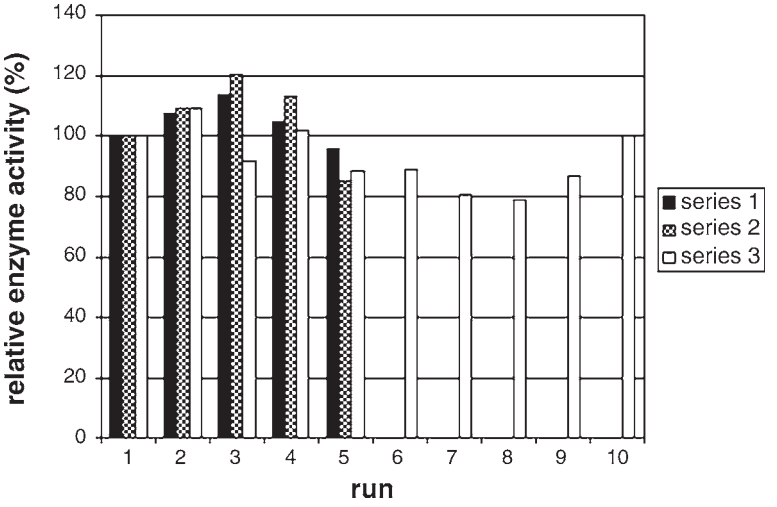

Fig. 5. Recyclability of Cex/RsaA336C particulate enzyme. Determination of relative enzyme activity remaining after up to 10 consecutive assay runs normalized for enzyme loss during washing and assaying steps. Enzyme assays were performed as described in Materials and Methods. Samples were then washed three times with sodium phosphate buffer, fresh substrate was added, and the aggregates were reassayed, for up to 10 cycles per sample. After the last assay, the Cex/RsaA aggregates were pelleted and lyophilized, and the dry pellet weights were measured and used to calculate the average amount of enzyme lost per cycle. This value was then used to correct the catalytic activity values obtained for enzyme loss after each assay run. The results are expressed as a percentage of the original activity.

Discussion

The Caulobacter S-layer secretion system has already proven to be a viable alternative to existing protein production systems. Indeed, the highly expressed *rsaA* gene of *C. crescentus* has been engineered to express genes of interest encoding proteins of up to 600 amino acids in fusion with the RsaA C-terminal secretion signal sequence *(25–27,29)*. Moreover, with this system Caulobacter cultures can be routinely grown to high density (OD_{600} of 1.5–2.5) in inexpensive chemically defined medium, resulting in the production of large quantities of hydrated aggregated fusion proteins in a matter of 2–4 d. Hybrid proteins can then be rapidly purified to 90–99% purity by simple coarse filtration of the culture medium *(26)*. Further, the foreign protein can be physically separated from the RsaA component of the fusion protein by the introduction of a protease cleavage site at the foreign protein-RsaA C-terminal junction and subsequent enzymatic digestion (unpublished results). In this report we demonstrate, for the first time, that RsaA hybrid fusion proteins produced using the Caulobacter secretion system retain the distinct biologic function of the native foreign protein being produced. Specifically, we assessed Cex/RsaA particulate enzyme (previously generated by fusion of the catalytic domain of the

*p*NPCase from *C. fimi* to the C-terminus of the Caulobacter S-layer protein RsaA) in terms of its catalytic activity, its tolerance to short-term storage, and the efficiency with which it could be recovered postcatalysis and reused.

Cex/RsaA particles bound substrate with an affinity comparable with that of the native Cex enzyme despite being fused to a 336 amino acid portion of RsaA. By contrast, our particulate *p*NPCase exhibited a 360-fold drop in catalytic efficiency (k_{cat}/K_M), suggesting either that very few of the active sites were accessible, most likely owing to occlusion caused by the aggregated nature of the hybrid fusion proteins themselves, or that the majority of the active sites were available but only weakly functional, owing to misfolding of the catalytic domain in Caulobacter. Using active-site titration analysis, we determined that, in fact, our Cex/RsaA hybrid particulate enzyme possessed a reduced number of accessible fully active catalytic sites as opposed to a large number of weakly active catalytic sites. Although it is probable that increased solubilization of the aggregates (e.g., by treatment with either urea or prolonged sonication) would lead to increased catalysis by freeing up more active sites, it must be noted that the ultimate goal of this research is to produce a particulate (i.e., insoluble) enzyme for industrial applications. Therefore, future improvements will need to focus on optimizing mechanical shearing of the enzyme particles to expose more active sites without dramatically increasing solubilization of the recombinant enzyme. It should also be taken into account that because of the ease and affordability of Cex/RsaA production, it may well be possible to compensate for lower specific activity simply by using a greater amount of particulate enzyme. Indeed, when considering the production of biocatalysts for use in large-scale processes, several factors of interest must be taken into consideration, of which perhaps the most influential are the initial cost of producing the enzyme and the efficiency with which it can be purified away from the product and recycled. As we have demonstrated here, the Cex/RsaA particulate enzymes can be easily recycled without risk of inactivation (Fig. 5), so the primary concern in this regard will be to optimize the purification and washing steps in order to minimize enzyme loss between reactions.

Often, one of the basic features of industrial bioprocessing enzymes is their stability, both in terms of storage and in terms of sample preparation and reaction conditions. It was therefore encouraging that not only could Cex/RsaA aggregates be frozen as hydrates at –20°C and then thawed and used, but they could also be stored for more than 1 wk at 4°C without significant loss of enzyme activity (Fig. 3). However, we were able to detect enzyme activity in the supernatant surrounding the aggregates after a few days of storage at either 4°C or room temperature (data not shown). Although this is in itself not a problem in terms of using the particulate enzyme in industrial applications, it could have a negative impact on enzyme recovery/recyclability, suggesting that it will be worthwhile to investigate ways of stabilizing the aggregates in their insoluble form.

Our first approach will be to explore various methods of chemical stabilization, such as the use of formalin crosslinking.

Biocatalyst immobilization technology has come a long way toward rendering enzymes more affordable, in particular by allowing them to function under optimal reaction conditions in which the native enzyme was significantly less active if not completely denatured, and by allowing them to be recovered and reused multiple times *(5,8,10,12,14,15,40,41)*. Among the enzyme-immobilizing methods currently in use, CLECs possess the distinct advantage of being extremely pure protein preparations, giving them a much higher specific activity per gram of catalyst, compared to enzymes that are expressed on whole cells and enzymes that are adsorbed to or encapsulated in solid supports *(6)*. There are, however, still a few drawbacks to most of these immobilization methods. For example, although entrapment and encapsulation generally yield immobilized enzymes with wild-type levels of activity, there can be significant contamination of the downstream product owing to enzyme leaching *(42)*. In addition, both encapsulation and CLECs suffer from limitations regarding the size of the substrate molecules able to diffuse through the matrix to the enzyme's active site *(6,42)*, and whole-cell methods require cell killing and chemical crosslinking, which is often concomitant with some denaturation of the surface-exposed enzyme, leading to reduced catalytic efficiency *(1,2)*. In terms of the Caulobacter model system for the production of hybrid particulate enzymes, our study indicates three basic goals for future research and development: to increase substrate access to the active sites and, thus, the specific activity of the hybrid particulate enzyme; to optimize the separation of enzyme and end product so as to facilitate maximum recovery of the enzyme for recycling in subsequent reactions; and to stabilize the insoluble aggregates to prevent enzyme loss during both storage and bioprocessing.

Conclusion

Using Cex/RsaA as a prototype, we demonstrated that the Caulobacter S-layer secretion apparatus offers a viable alternative to present methods of enzyme immobilization for generating affordable, recyclable biocatalysts for use in biotechnology applications. Indeed, we showed that recombinant Cex/RsaA meets or exceeds several of the desirable criteria of immobilized enzymes: namely, it is catalytically active, it can be simply and cheaply produced and purified in reasonable quantities (routinely 75 mg/L of dry wt), and it is reasonably stable during short-term storage at 4°C. Further, we showed that it can be separated away from the end product and reused. Finally, owing to the nature of the Caulobacter host organism, the synthesis of Cex/RsaA is amenable to economical, scaleable production methods. Future research will therefore concentrate not only on optimizing the stability and insolubility of RsaA-based fusion proteins as discussed, but also on targeting other catalytic proteins for fusion with RsaA to produce particulate enzymes with more immediate industrial potential.

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References

1. Freeman, A., Abramov, S., and Georgiou, G. (1999), *Biotechnol. Bioeng.* **62,** 155–159.
2. Strauss, A. and Gotz, F. (1996), *Mol. Microbiol.* **21,** 491–500.
3. Lee, T. S., Turner, M. K., and Lye, G. J. (2002), *Biotechnol. Prog.* **18,** 43–50.
4. Hoskin, F. C., Walker, J. E., and Stote, R. (1999), *Chem. Biol. Interact* **119–120,** 439–444.
5. Haring, D. and Schreier, P. (1999), *Curr. Opin. Chem. Biol.* **3,** 35–38.
6. Margolin, A. L. (1996), *Trends Biotechnol.* **14,** 223–230.
7. Ellerby, L. M., Nishida, C. R., Nishida, F., Yamanaka, S. A., Dunn, B., Valentine, J. S., and Zink, J. I. (1992), *Science* **255,** 1113–1115.
8. Shchipunov, Y. A., Karpenko, T. Y., Bakunina, I. Y., Burtseva, Y. V., and Zvyagintseva, T. N. (2004), *J. Biochem. Biophys. Methods* **58,** 25–38.
9. Trivedi, B. (1999), *Nat. Biotechnol.* **17,** 339–341.
10. Spagna, G., Barbagallo, R. N., Casarini, D., and Pifferi, P. G. (2001), *Enzyme Microb. Technol.* **28,** 427–438.
11. Ichijo, H., Nagasawa, J., and Yamauchi, A. (1990), *J. Biotechnol.* **14,** 169–178.
12. Pessela, B. C., Mateo, C., Fuentes, M., Vian, A., Garcia, J. L., Carrascosa, A. V., Guisan, J. M., and Fernandez-Lafuente, R. (2004), *Biotechnol. Prog.* **20,** 388–392.
13. Min, D. J., Andrade, J. D., and Stewart, R. J. (1999), *Anal. Biochem.* **270,** 133–139.
14. Alvaro, G., Fernandez-Lafuente, R., Blanco, R. M., and Guisan, J. M. (1990), *Appl. Biochem. Biotechnol.* **26,** 181–195.
15. Cao, R., Gu, Z., Patterson, G. D., and Armitage, B. A. (2004), *J. Am. Chem. Soc.* **126,** 726, 727.
16. Poindexter, J. S. (1981), *Microbiol. Rev.* **45,** 123–179.
17. Shapiro, L. (1976), *Annu. Rev. Microbiol.* **30,** 377–407.
18. Smit, J. (1986), in *Bacterial Outer Membranes as Model Systems* (Inouye, M., ed.), John Wiley & Sons, New York, pp. 343–376.
19. Sleytr, U. B. and Messner, P. (1983), *Annu. Rev. Microbiol.* **37,** 311–339.
20. Sara, M. and Sleytr, U. B. (2000), *J. Bacteriol.* **182,** 859–868.
21. Sara, M. and Sleytr, U. B. (1996), *Prog. Biophys. Mol. Biol.* **65,** 83–111.
22. Messner, P. and Sleytr, U. B. (1992), *Adv. Microb. Physiol.* **33,** 213–275.
23. Smit, J., Engelhardt, H., Volker, S., Smith, S. H., and Baumeister, W. (1992), *J. Bacteriol.* **174,** 6527–6538.
24. Awram, P. and Smit, J. (1998), *J. Bacteriol.* **180,** 3062–3069.
25. Bingle, W. H., Nomellini, J. F., and Smit, J. (1997), *J. Bacteriol.* **179,** 601–611.
26. Bingle, W. H., Nomellini, J. F., and Smit, J. (2000), *J. Bacteriol.* **182,** 3298–3301.
27. Nomellini, J., Toporowski, M. C., and Smit, J. (2004), in *Expression Technologies: Current Status and Future Trends* (Baneyx, F., ed.), Horizon Scientific Press, Norfolk, UK, pp. 477–524.
28. Nierman, W. C., Feldblyum, T. V., Laub, M. T., et al. (2001), *Proc. Natl. Acad. Sci. USA* **98,** 4136–4141.
29. Umelo-Njaka, E., Nomellini, J. F., Bingle, W. H., Glasier, L. G., Irvin, R. T., and Smit, J. (2001), *Vaccine* **19,** 1406–1415.
30. Umelo-Njaka, E., Bingle, W. H., Borchani, F., Le, K. D., Awram, P., Blake, T., Nomellini, J. F., and Smit, J. (2002), *J. Bacteriol.* **184,** 2709–2718.
31. Gilkes, N. R., Claeyssens, M., Aebersold, R., Henrissat, B., Meinke, A., Morrison, H. D., Kilburn, D. G., Warren, R. A., and Miller, R. C. Jr. (1991), *Eur. J. Biochem.* **202,** 367–377.

32. White, A., Withers, S. G., Gilkes, N. R., and Rose, D. R. (1994), *Biochemistry* **33,** 12,546–12,552.
33. Tull, D., Withers, S. G., Gilkes, N. R., Kilburn, D. G., Warren, R. A., and Aebersold, R. (1991), *J. Biol. Chem.* **266,** 15,621–15,625.
34. Tull, D. and Withers, S. G. (1994), *Biochemistry* **33,** 6363–6370.
35. Notenboom, V., Birsan, C., Nitz, M., Rose, D. R., Warren, R. A., and Withers, S. G. (1998), *Nat. Struct. Biol.* **5,** 812–818.
36. Bingle, W. H., Le, K. D., and Smit, J. (1996), *Can. J. Microbiol.* **42,** 672–684.
37. Langsford, M. L., Gilkes, N. R., Singh, B., Moser, B., Miller, R. C. Jr., Warren, R. A., and Kilburn, D. G. (1987), *FEBS Lett.* **225,** 163–167.
38. Kempton, J. B. and Withers, S. G. (1992), *Biochemistry* **31,** 9961–9969.
39. Leatherbarrow, R. J. (1990), Version 2.0 Ed., Erithacus Software, Staines, UK.
40. Kirk, O., Borchert, T. V., and Fuglsang, C. C. (2002), *Curr. Opin. Biotechnol.* **13,** 345–351.
41. van Beilen, J. B. and Li, Z. (2002), *Curr. Opin. Biotechnol.* **13,** 338–344.
42. Liang, J. F., Li, Y. T., and Yang, V. C. (2000), *J. Pharm. Sci.* **89,** 979–990.