An Improved Protein Bioreactor

EFFICIENT PRODUCT ISOLATION DURING IN VITRO PROTEIN BIOSYNTHESIS VIA AFFINITY TAG*

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In vitro protein biosynthesis became a powerful technology for biochemical research. Beside the determination of structure and function in vitro selection of proteins is also of great interest. In most cases the use of a synthesized protein for further applications depends on its purity. For this purpose the in vitro production and purification of proteins with short affinity tails was established. A cell-free protein synthesis system was employed to produce bovine heart fatty acid-binding protein and bacterial chloramphenicol acetyltransferase with and without fusion of the Strep-tag affinity peptide. The quantitative removal of fusion protein during cell-free synthesis from a batch reaction and a semicontinuous flow cell-free reactor were achieved. No significant influence of the Strep-tag and the conditions during the affinity chromatography on maturation or activity of the proteins were observed. The product removal from the continuous flow cell-free reactor is still an only partially solved problem, because the use of ultrafiltration membranes has some limitations. The results document that it should be possible to avoid these limitations by introducing an affinity system. Molecular & Cellular Proteomics 1:466–471, 2002.

The potentials of the in vitro protein biosynthesis system include not only the production of proteins but also the synthesis of cytotoxic, regulatory, or unstable proteins that cannot be expressed in living cells (1). Other advantages are the site-directed isotope labeling, as well as the incorporation of unnatural amino acids, and the direct expression of PCR products (reviewed in Ref. 2). The expression PCR can be used for revealing the products of the newly found genes or for producing and analyzing proteins carrying engineered or random mutations.

Different versions of cell-free protein synthesis systems derived from rabbit reticulocyte lysates, wheat germ, or Escherichia coli are used currently. For the production in a preparative scale the CFCF1 reactors (1, 3) and dialysis cells (4) have been established, because their yields are superior to those of a batch system. The continuous product removal from the CFCF reactor is still an only partially solved problem. The employment of ultrafiltration membranes has the limitations that the proteins may not pass through or might interact with the membrane. In addition it may well happen that some translation components during the reaction get lost. However, the use of a synthesized protein for further applications like crystallization or NMR studies depends to a great extent upon its purity. For this purpose, the recombinant production and purification of proteins with short affinity tails have gained widespread application in biotechnology (5). One of these short affinity tags termed Strep-tag (in this work Strep-tag I) is a nine-amino acid peptide (AWRHPQFGG) with intrinsic streptavidin binding activity ($K_{d} \approx 10^{-11}$ M) (6). It was shown that Strep-tag I allows single-step protein purification from bacterial expression systems (7), but its fusion to recombinant proteins is restricted to the C terminus. Another variant, designated Strep-tag II, was introduced (8) that did not show this limitation. This octapeptide (WSHPQFEK) possesses a binding affinity toward streptavidin and an even higher one toward a streptavidin mutant ($K_{d} \approx 10^{-6}$ M) named StrepTactin (9). One advantage of the Strep-tag system is the elution of the bound fusion protein from the affinity matrix in the native state under very mild buffer conditions with a specific competitor.

Here we report the feasibility and the compatibility of the Strep-tag affinity purification with our cell-free protein biosynthesis system, because in most cases, further analyses require a purified protein. In addition, the limitations caused by ultrafiltration membranes during product removal from a CFCF reactor should be overcome employing the affinity system described here.

EXPERIMENTAL PROCEDURES

Construction of Plasmids

Standard methods for molecular biology were used (10). The plasmids pHMFA, pFA+CStII, and pFA+NSStII (11) contain the sequence for the 5′ untranslated region of phage T7 gene 10 followed by the coding sequence for FABP and the T7 transcription terminator 150 bp downstream of the coding sequence. The pFA+CStII and the pFA+NSStII additionally have 30 bp encoding a two-amino acid linker and the Strep-tag II at the C terminus and the N terminus of the protein, respectively.

The plasmid pCAT coding for CAT and containing all elements necessary for efficient in vitro transcription/translation was constructed in two steps. First, the Ncol/BamHI fragment of pCAT3 (Promega) was inserted into pET-3d. Second, the Sphi/EcoRI fragment of this modified pET-3d was cloned into the pUC 19 vector.

The plasmid pCAT served as template for the construction of PCR products with a Strep-tag I and II at the end of the coding sequence. The forward primer was complementary to the T7 promoter and...
identical for both products. The reverse primers for introducing the Strep-tag I with linker and Xba restriction site were ST1 (5′-GTCGACTGATACACAAGCGTACGAGCTGGAAGGCGCTGCGCTCCTGGTGGCCTAGTACCTAG – 3′) and ST2 (5′-GTCGACTGATACACAAGCGTACGAGCTGGAAGGCGCTGCGCTCCTGGTGGCCTAGTACCTAG – 3′), which introduce the Strep-tag II with linker and Xba restriction site. PCR was performed using Phu DNA polymerase (Stratagene) according to the supplier’s recommendations. The PCR products were digested with XbaI, and the XbaI/XbaI fragments were subsequently cloned into the adequate digested pCAT vector. The two additional plasmids coding for CAT with C-terminal Strep-tag I and II were termed pCAT+Stl and pCAT+StII, respectively.

**Coupled in Vitro Transcription/Translation**

The coupled in vitro transcription/translation reaction is based on an *E. coli* S30 lysate (strain D10) and was performed as described (12) with several modifications. The coupled transcription/translation reaction was carried out for 90 min at 37 °C and contained the following components: 50 mM HEPES-KOH (pH 7.6), 70 mM Kacet, 30 mM NH4Cl, 14 mM MgCl2, 0.1 mM EDTA, 5 mM dithiothreitol, 0.02% NaN3, 0.2 mM L-[14C]leucine (25 dpm/pmol; Amersham Biosciences), 0.4 mM each amino acid (leucine omitted), 1 mM each of ATP and GTP, 0.5 mM each of CTP and UTP, 30 mM phosphoenolpyruvate, 10 mM acetyl phosphate (13), 8 µg/ml pyruvate kinase (Roche Molecular Biochemicals), 4% polyethylene glycol 2000, 20 mM Tris-HCl (pH 7.8), 20 µg/ml rifampicin, 0.1 mg/ml total *E. coli* rRNA, 0.1 mM folic acid, 100 units/ml RNase inhibitor (Promega), 2 µg/ml aprotinin (Roche Molecular Biochemicals), 1 µg/ml leupeptin (Roche Molecular Biochemicals), 1 µg/ml pepstatin (Roche Molecular Biochemicals), 30% (v/v) S30, 500 units/ml T7 phage RNA polymerase (Stratagene), 0.5–2 µM of a covalently closed plasmid.

**Analysis of the Synthesized Protein**

The incorporation of L-[14C]leucine into the synthesized proteins was determined by liquid scintillation counting of the trichloroacetic acid-insoluble material as described (14). The reaction products were also analyzed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) (15) followed by an autoradiography with a Storm 840 PhosphorImager (Amersham Biosciences).

**CAT Assay**

The activity from in vitro-synthesized CAT was detected with the FAST CAT® (deoxy) chloramphenicol acetyltransferase assay kit according to the manufacturer’s protocol (Molecular Probes) with some modifications. The supernatant of a coupled transcription/translation reaction after centrifugation at 15,000 × g for 5 min was diluted 500-fold with a buffer (50 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol, 0.03% bovine serum albumin), and between 1 and 17 µl of each solution, the FAST CAT® substrate and the 9 µM acetyl-CoA, were added. The reaction was stopped by extraction with 400 µl of ice-cold ethyl acetate. After a short centrifugation the top 300 µl of ethyl acetate was transferred to a clean tube, the solvent was evaporated, the dry sample was dissolved in 20 µl of ethyl acetate, and finally, 3 µl of this solution was analyzed after thin layer chromatography with a Storm 840 FluorImager (Amersham Biosciences).

**Strep-tag Affinity Purification**

Isolation after in Vitro Protein Biosynthesis—Purification of the Strep-tag fusion proteins was done by affinity chromatography according to the manufacturer’s protocol (Institut für Bioanalytik, Göttingen, Germany) except that the volume of the affinity column was reduced to 230 µl to purify 150 µl of the reaction mixture. The wash and elution volumes were 230 and 130 µl, respectively. Reaction mixtures were similarly centrifuged after coupled transcription/translation and subjected to the column. The isolated fractions were analyzed by trichloroacetic acid precipitation and an autoradiography after SDS-PAGE as described.

**Removal of Fusion Protein from a Batch System during in Vitro Protein Synthesis**—After the affinity matrix (50 µl) was equilibrated with translation buffer (50 mM HEPES-KOH (pH 7.6), 70 mM Kacet, 30 µM NH4Cl, 10 mM MgCl2, 0.1 mM EDTA (pH 8.0), 0.002% NaN3) the reaction mixture (150 µl) was coupled to the translation/transcript synthesis reaction mixture was pumped continuously from the reaction chamber onto the affinity column, which was filled with 530 µl of washing buffer (100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA) followed by elution of the fusion protein with 4 × 100 µl of elution buffer (washing buffer with 2× 25 mM desthiobiotin).

**Removal of Fusion Protein from an SFCF Reactor during Syntheses**—A coupled transcription/translation using an SFCF reactor (Fig. 1) was performed for 20 h at 30 °C. The volume of the reaction chamber was 750 µl, and in addition to the affinity column and the connecting hoses, the total volume of reaction mixture was 2150 µl. 6 ml of feeding buffer were used, consisting of translation buffer with 5 mM dithiothreitol, 4 mM MgCl2, 0.02% NaN3, 0.1 mM folic acid, 0.4 mM L-[14C]leucine (0.75 dpm/pmol; Amersham Biosciences), 0.4 mM each of the other 19 amino acids, 1 mM each of ATP and GTP, 0.5 mM each of CTP and UTP, 30 mM phosphoenolpyruvate (Roche Molecular Biochemicals), 10 mM acetyl phosphate (Sigma). During protein synthesis the reaction mixture was pumped continuously from the reaction chamber onto the affinity column, which was filled with 530 µl of StreptTactin-Sepharose, and sent back into the reactor. To isolate the product the column was washed three times with 800 µl of washing buffer followed by elution of the fusion protein with 6 × 400 µl of elution buffer. The isolated fractions were analyzed as described.
**RESULTS**

**Affinity Purification of Cell-free Synthesized Strep-tag Fusion Proteins**—The necessity to establish a one-step purification system for *in vitro*-synthesized proteins is quite apparent. In this study we have compared the quality and compatibility of the Strep-tag purification with our cell-free protein synthesis system. Therefore, we fused the two Strep-tag versions I and II to the C terminus of the CAT gene and Strep-tag II to the FABP gene by PCR methods and cloned them into plasmids containing all elements for an efficient *in vitro* transcription/translation. We have chosen these two genes, because FABP is a well-known standard in our laboratory, and CAT reveals the influence of the Strep-tag on the activity of the fused protein on the basis of its enzymatic activity (16). It was not known whether the additional 33 and 30 bp encoding the Strep-tag I and II, respectively, would influence the *in vitro* expression of the new genes, if the fused peptide would disturb the native structure of the proteins, and if the tag would be accessible for affinity chromatography.

The newly constructed and *in vitro*-synthesized fusion proteins showed, with regard to the amount of product, no significant difference when compared with the constructs without Strep-tag. The yields with CAT were 190 μg/ml, with the Strep-tag I 191 μg/ml and with the Strep-tag II 184 μg/ml. The amount of FABP was 228 μg/ml, with the C-terminal Strep-tag II 232 μg/ml and with the N-terminal Strep-tag II 201 μg/ml. The recombinant proteins were subjected to affinity chromatography. Between 70 and 87% of the fusion protein used for affinity purification were recovered from the column, and between 60 and 82% could be isolated as pure product in the elution fractions as calculated by trichloroacetic acid precipitation of the different fractions (summarized in Table I).

The quality of the chromatography products is shown in the precipitation of the different fractions (summarized in Table I). In the elution fractions as calculated by trichloroacetic acid used for affinity purification were recovered from the column, chromatography. Between 70 and 87% of the fusion protein.

![Fig. 2. Purification of FABP containing the Strep-tag II after the cell-free synthesis using a StrepTactin affinity column. Comparable amounts of every isolated fraction were analyzed by SDS-PAGE. A, Coomassie stain; B, autoradiography of the radioactively labeled products. The samples in the numbered lanes are as follows: 1, marker; 2, reaction mixture; 3, sample loading; 4–6, wash fractions 1–3; 7–12, elution fractions 1–6; 13, [14C]marker (only partly visible in the Coomassie stain).](image)

| Protein   | Amount of eluted protein μg/ml | % |
|-----------|--------------------------------|---|
| CAT       | 190                            | 2 |
| CAT II    | 191                            | 60|
| CAT II    | 184                            | 72|
| FABP I    | 228                            | 3 |
| FABP II   | 232                            | 82|
| FABP II   | 201                            | 75|

**Enzymatical activity of the in vitro-synthesized CAT with and without Strep-tag before and after affinity chromatography**

| Protein          | Assayed CAT activity Before affinity chromatography | After affinity chromatography |
|------------------|-----------------------------------------------------|-------------------------------|
| Commercial CAT   | 3                                                   | 4.8 ± 0.9*                    |
| CAT              | 3.9 ± 0.7                                           | 4.8 ± 0.9*                    |
| CAT-Strep-tag I  | 3.4 ± 0.7                                           | 3.2 ± 0.6                     |
| CAT-Strep-tag II | 2.9 ± 0.4                                           | 3.0 ± 0.3                     |

*The enzyme was subjected to a streptavidin-Sepharose column and recovered after treatment with washing buffer.

and after affinity chromatography, was assayed using a fluorescent deoxychloramphenicol substrate (17) (Molecular Probes). The observed activities were comparable with a commercially available CAT (Sigma) or even higher (Table II), so that neither the Strep-tag nor the conditions of the chromatography seem to affect the biological function of the fused protein. We did observe an influence of the Strep-tag on the solubility of CAT but not FABP. CAT is partly insoluble in our system, and that effect was increased by fusion with the Strep-tag I and II. It is known that hydrophobic interactions at the C terminus are essential for folding and stabilizing CAT (18). One possible reason for the increase in misfolding would be a disruption of these interactions caused by the presence of the Strep-tag sequences.

**Influence of Affinity Matrix to Coupled Transcription/Translation**—After the purification system performed according to our expectations we tried to separate the Strep-tag fusion proteins during the continued process of protein synthesis. For that reason the influence of the StrepTactin-Sepharose (IBA, Göttingen, Germany) on the coupled transcription/translations.

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**TABLE I**

Results of Strep-tag affinity chromatography with different *in vitro* synthesized proteins

| Protein | Position | Synthesized protein | Amount of eluted protein μg/ml | % |
|---------|----------|---------------------|--------------------------------|---|
| CAT     | I, C-terminal | 191 | 60 |
| CAT     | II, C-terminal | 184 | 72 |
| FABP    | II, C-terminal | 232 | 82 |
| FABP    | II, N-terminal | 201 | 75 |
A translation reaction was examined. 20 μl StrepTactin-Sepharose were added to one of two identical 60-μl coupled reaction mixtures, and a plasmid coding for FABP without Strep-tag was used to determine the total amount of synthesized protein after translation. The products were analyzed by trichloroacetic acid precipitation and SDS-PAGE followed by autoradiography (Fig. 3A). The amount of synthesized protein in the presence of the matrix was reduced by 6% compared with the unchanged reaction; unexpectedly, the by-products were also decreased (Fig. 3B). The rest of the reaction mixture with matrix was treated with 0.5% SDS, and comparable volumes were analyzed as described above. A, autoradiogram of the SDS-PAGE; B, amount and distribution of the products. 1, standard reaction; 2, reaction with matrix; 3, reaction with matrix treated with SDS.

Removal of CAT—The insignificant influence of the StrepTactin-Sepharose on the translation system gave us the opportunity to separate a protein with Strep-tag II during a coupled transcription/translation reaction. Thus, CAT with Strep-tag II was produced in the presence of StrepTactin-Sepharose and purified. About 82% of the synthesized product was bound to the matrix from which about 87% could be isolated in reasonable purity. The data of the chromatography results are shown in the Coomassie stain (Fig. 4A) and in the autoradiogram of the protein gel (Fig. 4B). The amount of eluted protein was comparable with a purification via the column method. Although the overall synthesis is generally decreased in such a batch system the amount of soluble CAT-Strep-tag II was increased slightly (data not shown).

Removal of FABP—The advantage of a dialysis system is the longer reaction time of cell-free expression and the consequently higher yields. After a reaction time of 20 h at 30 °C the yields of our SFCF reactor were between 700 and 1000 μg/ml for FABP and 600–800 μg/ml for CAT. The yields for the constructs with affinity tags were identical. The removal of fusion proteins via StrepTactin-Sepharose from an SFCF system (Fig. 1) was performed for 20 h followed by washing the affinity column and eluting of the bound fusion protein. 41% of the...
synthesized protein was found in the reaction mixture, 13% was found in the wash fractions, and 46% could be eluted from the affinity column. The Coomassie stain (Fig. 5A) of the protein gel and the corresponding autoradiogram (Fig. 5B) demonstrate the result of the chromatography. The fact that the elution fractions contained only 46% of the synthesized protein is because of the small volume of the affinity column (530 l) compared with the total volume of the reaction mixture (2150 l). In all likelihood an increased amount of affinity matrix will shift the contribution of the synthesized protein to the elution fractions. The amount of synthesized protein in the presence of the affinity column was reduced by 28 ± 6% compared with the unchanged reaction. Our results suggest that the reduced performance of the modified dialysis reactor is a technical problem that can be solved in the near future.

**DISCUSSION**

The introduction of an affinity system is a very useful addition to the development of an *in vitro* protein biosynthesis system, because the synthesis of the protein usually requires its isolation and purification. Because this step of the purification takes place during the process of protein biosynthesis it greatly reduces the time to isolate the protein product. Furthermore it is possible to avoid such negative effects during protein synthesis, by which the proteins are precipitated once a critical concentration is reached. The cell-free protein biosynthesis system described is a batch system in which the presence of StrepTactin-Sepharose allows the simultaneous removal of the protein product via a Strep-tag II affinity peptide.

Until now an important problem has been the continuous removal of the product from the reaction chamber of the protein bioreactor. Different solutions of this problem had been discussed by Stiege and Erdmann (1). Marszal and Scouten (19) synthesized dihydrofolate reductase in a coupled wheat germ batch system in the presence of the affinity ligand methotrexate (19). They could remove the synthesized protein from the reaction mixture, and on the basis of these results they also discussed a new type of continuous flow cell-free protein synthesis system. To the best of our knowledge such a novel type of protein bioreactor has never been developed or published.

In this paper we have demonstrated for the first time the continuous removal of the synthesized protein from an SFCF reactor, and now we are using the system reported here to solve the problem of product removal from the protein bioreactor (CFCF reactor). This step prevents the limitations observed by reactors based on an ultrafiltration membrane. Fig. 6 gives an example what our new type of protein bioreactor will look like. This reactor still has an ultrafiltration membrane, but in this case the membrane is only required to remove small molecular waste products and not the large molecular protein product synthesized. We think that our new type of protein biosynthesis reactor will be much more efficient for the synthesis of proteins with high molecular masses (>50 kDa) than the conventional membrane reactor, because the losses of components of the protein biosynthesizing machinery are avoided.

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