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The cell-free protein biosynthesis — applications and analysis of the system★✪

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The *in vitro* protein biosynthesis has the potentials to become a powerful technology for biochemical research. Beside the determination of structure and function the *in vitro* evolution of proteins is also of great interest.

The system described was used to produce bovine heart fatty acid binding protein (FABP) and bacterial chloramphenicol acetyltransferase (CAT) with and without fusion of the Strep-tag II affinity peptide. The proteins were purified after and during protein biosynthesis by using a StrepTactin Sepharose matrix. No significant influence of the Strep-tag and the conditions during the affinity chromatography on maturation or activity of the protein was observed.

The *in vitro* evolution of proteins is feasible by means of ribosome display. The selection of a specific mRNA coding for a shortened FABP with a N-terminal His-tag *via* the accompanying protein property was shown. Goal of the selection was to bind the FABP *via* the His-tag on Ni(II)-IDA-agarose. After nine cycles of transcription, translation, affinity selection and RT-PCR the protein with the His-tag could be enriched 10^8-fold.

In order to correlate a possible relationship between changes in protein population and biological function studies were initiated in which 2-dimensional protein patterns of the total *in vitro* system were compared after 0 and 2 h reaction time. The very interesting findings are that a number of proteins disappear, while others are newly formed during protein synthesis.

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Abbreviations: CAT, chloramphenicol acetyltransferase; DHFR, dihydrofolate reductase; FABP, fatty acid binding protein; GFP, green fluorescence protein; PRM, protein ribosome and encoding mRNA; TCA, trichloroacetic acid; 2-DE, two-dimensional gel electrophoresis.

The potentials of the *in vitro* protein biosynthesis system include not only the production of proteins, but especially the synthesis of cytotoxic, regulatory or unstable proteins which cannot be expressed in living cells (Stiege & Erdmann, 1995). Another advantage is the labeling with isotopes at specific positions which facilitates their detection or allows to study their structure and function by NMR spectroscopy. Further advantages are the purity of the synthesized proteins, it is easier to isolate them (Hauakanes & Kvam, 1993) and in general their superior biological activities. The system can also be used for the *in vitro* evolution of proteins with selected biological properties and it is possible to create proteins with improved or even new biological activities by introducing unnaturally modified amino acids into specific positions of a protein.

The use of a specific *in vivo* or *in vitro* synthesized protein for example for crystallization or NMR studies is to a great extent dependent upon its purity. For this purpose the recombinant production and purification of proteins with short affinity tails have gained widespread application in biotechnology (Nygren *et al*., 1994). In most examples investigated so far it was found that these short peptide extensions, between three and twelve amino acids in length, did not interfere with the biological function of the protein and therefore need not be removed *via* proteolysis. The Strep-tag II is an eight-amino-acid peptide that displays intrinsic binding affinity towards recombinant core streptavidin (Schmidt *et al*., 1996), named StrepTactin (Voss & Skerra, 1997). A specific advantage for protein purification is that competitive elution of the bound Strep-tag fusion protein from the StrepTactin matrix can be accomplished in the native state under very mild buffer conditions by competition with the biotin analog desthiobiotin. We have tested if the Strep-tag affinity purification is practicable and compatible with our cell-free protein biosynthesis system.

In vitro selection experiments using DNA or RNA, where the molecules are simultaneously genotype and phenotype, have shown that nucleic acid molecules with specific molecular recognition and catalytic properties can be isolated from complex pools of random sequences (Osborne & Ellington, 1997). Because proteins carry out a wider range of structural and catalytic roles in biology and are much more extensively used in diagnostics, therapeutic, and industrial applications, the selection and directed evolution of proteins is of great interest. Most of the methods used for the selection of proteins as carrier of the phenotype have been based directly or indirectly on living cells. Examples of such approaches include phage display (Smith & Petrenko, 1997), plasmid display (Schatz *et al*., 1996), and completely *in vivo* genetic approaches (Zhang *et al*., 1997; Moore & Arnold, 1996; Peled-Zehavi *et al*., 2000). However, *in vivo* approaches are limited by transformation efficiency to 1–10 $\times$ 10^9 different molecules. This limitation can be overcome by using *in vitro* systems based on cell-free translation. Proteins unfortunately are not genetic molecules and cannot be copied by any known enzymatic activity. Therefore, to construct an *in vitro* protein selection cycle, the amplifiable genomic information (mRNA) must be physically linked to the selectable information (protein). Two different approaches have been published which make such a coupling possible. The mRNA-protein fusion technique is a covalent linkage between the 3′ end of the mRNA and the carboxyl terminus of the protein *via* a puromycin (Roberts & Szostak, 1997). The ribosome display is based on the possibility of expressing proteins from mRNA lacking a stop codon and the direct use of the ternary complex, consisting of a protein, a ribosome and an encoding mRNA (PRM-complex), for affinity enrichment (Hanes & Plückthun, 1997). One limitation of the ribosome display is that libraries must be screened under conditions in which the PRM-complex is stable

(high Mg^{2+} and low temperature). Here we describe the establishment of the ribosome display technique in our lab.

Concerning the cell-free protein biosynthesis there are still a number of questions to be answered, in order to explain the fact that the *in vitro* system is much less efficient than the *in vivo* system (Spirin *et al*., 1988; Ryabova *et al*., 1989; Stiege & Erdmann, 1995). One interesting observation with the closed *in vitro* system, called the batch system, is that after an initial very active phase the reaction stops after 1 to 2 h. This has been observed in our *E. coli* translation system with the synthesis of FABP (Stiege & Erdmann, 1995). Similar results are obtained for the dihydrofolate reductase (DHFR) and CAT (our unpublished data) and green fluorescence protein (GFP) (Siemann *et al*., 2000). Thus questions arise: Which causes have been leading to the termination of protein synthesis in the batch system? So far several reasons could be excluded: mRNA hydrolysis cannot be the reason because in the coupled transcription/translation system T7-RNA polymerase permanently synthesizes new mRNAs. This system is stable as demonstrated by Siemann *et al.* (2000) and they were also able to eliminate energy shortages as a problem. Therefore we set up the hypothesis that the degradation and modification of enzymes and factors required for protein biosynthesis are the reason in loss of activity. Thus we decided on the basis of a batch synthesis of FABP to monitor the proteins of the reaction mixture after 0 and 120 min reaction time by high resolution two-dimensional gel electrophoresis (2-DE).

MATERIAL AND METHODS

Construction of plasmids

For the construction of plasmids, which served as templates for the *in vitro* transcription, we followed standard protocols (Sambrook *et al.,* 1989).

In vitro transcription

The mRNA transcripts were obtained by *in vitro* runoff transcription from linearized plasmids or from PCR-products with T7 RNA polymerase (Stratagene) following the protocol of Triana-Alonso *et al.* (1995) with minor modifications. The synthesized mRNA can be labeled with [α-^{35}S]CTP (Amersham). Transcripts were analyzed by agarose gel electrophoresis and in some cases by autoradiography after separation on 6% denaturing polyacrylamide gel.

In vitro translation

We used an optimized procaryotic lysate which was prepared by the method of Cronenberger & Erdmann (1975) with some modifications and composed of components described by Merk *et al.* (1999). The synthesized proteins were labeled with L-[U-^{14}C]leucine with a specific activity of 304 mCi/mmol (Amersham). After translation protein quantification was done by measuring the incorporated radioactivity present in trichloroacetic acid-precipitated aliquots of the reaction mixtures. The proteins were analyzed by autoradiography after separation on 15% polyacrylamide gels (Laemmli, 1970).

Strep-tag affinity purification

After* in vitro *protein biosynthesis. Purification of the Strep-tag fusion proteins was done by affinity chromatography according to the manufacturers protocol (IBA Göttingen, Germany) except that the volume of the affinity column (StrepTactin Sepharose) was reduced to 230 μl to purify 150 μl reaction mixture. The wash and elution volumes were 230 μl and 130 μl, respectively. Reaction mixtures from coupled transcription/translation were shortly centrifuged and subjected to the column. The isolated fractions were analyzed by TCA-precipitation and by an autoradiography after SDS/PAGE as described.

During* in vitro *protein biosynthesis. The StrepTactin Sepharose matrix (50 μl) was equilibrated with translation buffer and then the reaction mixture (100 μl) for the coupled transcription/translation was added. The translation reaction was carried out with constant shaking in order to keep the matrix in suspension. The matrix was collected by centrifugation for 1 min at 220 × ***g*** after protein synthesis and between the purification steps. After removing the supernatant the matrix was washed three times with 100 μl washing buffer followed by elution of the Strep-tag fusion protein with four times 100 μl elution buffer.

CAT-assay

The activity from *in vitro* synthesized CAT was detected with the FAST CAT® (deoxy) chloramphenicol acetyltransferase assay kit according to the manufacturers protocol (Molecular Probes, U.S.A.) with some modifications. The supernatant of a translation reaction after centrifugation at 15000 × ***g*** for 5 min was diluted 500-fold with buffer (50 mM Tris/HCl, pH 7.8, 2 mM dithiothreitol/ 0.03% bovine serum albumin) and between 1 μl and 17 μl were used in a total volume (same buffer) of 24 μl. From each of the FAST CAT substrate solution and the 9 mM acetyl CoA 4 μl were used. The reaction was stopped by adding 400 μl of ice-cold ethyl acetate. After a short centrifugation the top 300 μl ethyl acetate were transferred to a clean tube. The solvent was then evaporated and the dry sample was taken up in 20 μl ethyl acetate. Three μl of this solution were used for thin-layer chromatography.

Affinityselection of PRM-complexes and isolation of mRNA

The translation was stopped by adding $Mg(OAc)_2$ to a final concentration of 50 mM and cooling on ice (Holschuh & Gassen, 1982). The samples were centrifuged for 5 min at 4 °C at 15000 × ***g*** to remove insoluble components. The supernatant was applied on a Ni(II)-IDA-agarose column. After six washes with ice cold washing buffer (Tris/HOAc, pH 7.5, 150 mM NaCl, 50 mM $Mg(OAc)_2$, 5–10 mM imidazole), the retained PRM-complexes were eluted with ice-cold elution buffer (washing buffer with 300 mM imidazole). The released PRM-complexes were treated with EDTA and the mRNA was recovered by precipitation with isopropanol and glycogen.

Reversetranscription-PCR

Reverse trancription was performed using Superscript II reverse transcriptase (GIBCO/BRL) according to the supplier's recommendation. Thirty cycles of PCR were performed using Taq DNA polymerase (GIBCO/BRL) according to the supplier's recommendation. PCR products were analyzed by agarose gel electrophoresis and purified from the gel and directly used for transcription.

Sample preparation for 2-DE from cell-free protein biosynthesis reaction

Protein concentration in S30-lysate was determined as described by Bradford (1976). Plasmid pHMFA containing the structural gene for FABP and all elements for *in vitro* transcription/translation was constructed as described and added to obtain a final concentration of 2 nM. The biosynthesis reaction was carried out as described above without any labeling in a volume of 200 μl. After 0 and 2 h incubation 100 μl of the samples were removed. The first sample was immediately frozen in liquid nitrogen. The 2-h sample was centrifuged (5 min, 15000 × ***g***, room temperature) in order to remove a precipitate. The supernatant was then frozen in liquid nitrogen until the 2-DE was performed. For use in 2-DE the samples were thawed on ice and prepared as described by Klose & Kobaltz (1995). Equal volumes of 25 μl were loaded on to first-di-

mensional gels. The protein amount loaded on each gel was equivalent to 80 μg.

High resolution 2-DE

We performed the high resolution 2-DE as described by Klose & Kobaltz (1995) with minor modifications. The first dimension was run with carrier ampholytes in a pH-range from 4–11. The glass tubes contained gels of 23.5 cm length and with a 1.5 mm diameter (first dimension equipment from WITA, Teltow, Germany). Rod gels where incubated for 10 min in solution (WITA) before they were applied on the tops of the second dimension gels. The homogenuous 15% SDS/polyacrylamide gels were 27 × 35 cm in size, with 0.9 mm spacers. The gels were silver stained according to Heukeshoven & Dernick (1985).

RESULTS AND DISCUSSION

Affinity purification of cell-free synthesized Strep-tag II fusion proteins

The necessity to establish a one-step purification system for *in vitro* synthesized proteins is obvious. In general short affinity peptides do not interfere with the biological function of the protein and therefore need not be removed by proteolysis. This is a very important observation, because the conditions during proteolysis and the proteases itself have in many cases a negative influence to the activity and the stability of the purified protein. In addition, the affinity chromatography should be under physiological conditions so that the fusion protein can be obtained in the native state. The Strep-tag affinity peptide was tested with regard to these requirements to investigate if it is a useful tool for protein purification in an *in vitro* translation system.

First of all we have examined the quality of the Strep-tag purification. Therefore we fused the Strep-tag II to the C-terminus of the FABP- and the CAT-gene and cloned them into plasmids containing all elements for an efficient *in vitro* transcription and translation. These plasmids showed in a coupled transcription/translation reaction no difference in activity when compared to the constructs without Strep-tag. The recombinant proteins were subjected to affinity purification. About 87% of the FABP+StII and 79% of the CAT+StII were recovered from the column. 82% and 72%, respectively, could be isolated as pure products in the elution fractions as calculated by TCA precipitation of fractions of the affinity chromatography. The progress of the chromatography from FABP+StII is shown in the Coomassie stain (Fig. 1A) and in the autoradiogram of the protein gel (Fig. 1B). The purified product was isolated mainly within one elution fraction visible in the Coomassie stained gel as one band. These results indicate the quality of the Strep-tag purification.

The next question was if the Strep-tag interferes with the biological function of the fused protein. To get an answer to this question we tested the activity of the *in vitro* synthesized CAT with and without Strep-tag, before and after affinity chromatography. In comparison to the commercial available CAT (Sigma) the activity of the cell-free translated proteins were usually significantly higher. Neither the Strep-tag nor the conditions of the chromatography lead to decrease in activity (not shown).

After the purification system met our satisfaction we tried to separate the Strep-tag fusion proteins during synthesis. For that reason the influence of the StrepTactin sepharose matrix to the coupled transcription/translation reaction was examined. To one out of two identical 60μl coupled reaction mixtures were added 20 μl StrepTactin Sepharose and we used a plasmid coding for FABP without Strep-tag to determine the total amount of synthesized protein after translation. The products were analyzed by TCA precipitation and SDS/PAGE followed by autoradiography in a phosphorimager (Molecular Dynamics). The amount of the synthesized

protein in the presence of the matrix was reduced to 94 ± 2% compared with the unchanged reaction but also the by-products were decreased. The rest of the reaction mixture with matrix was treated with 0.5 % SDS (30 min, 50°C) and an identical volume was analyzed by SDS/PAGE to find out if some product was bound to the matrix. The autoradiogram revealed that the by-products in this sample were increased but not the main product (Fig. 2). The FABP itself seem to have no affinity for the matrix and the slightly reduced performance is a consequence of the matrix present in the system. The by-products are probably unfolded, insoluble proteins with some unspecific affinity for the matrix.

The insignificant influence of the StrepTactin Sepharose upon the translation system gave us the chance to separate a protein with Strep-tag II during a coupled transcritpion/translation reaction. CAT with Strep-tag II was synthesized in a 150 μl reaction in the presence of 50 μl StrepTactin Sepharose. The translation reaction was stopped after 90 min and the supernatant was removed. An affinity purification in a batch manner was carried out. About 71% of the synthesized product could be isolated at reasonable purity in the elution fractions as calculated by TCA precipitation of the different fractions. The progress of the chromatography is shown in the Coomassie stain (Fig. 3A) and in the autoradiogram of the protein gel (Fig. 3B).

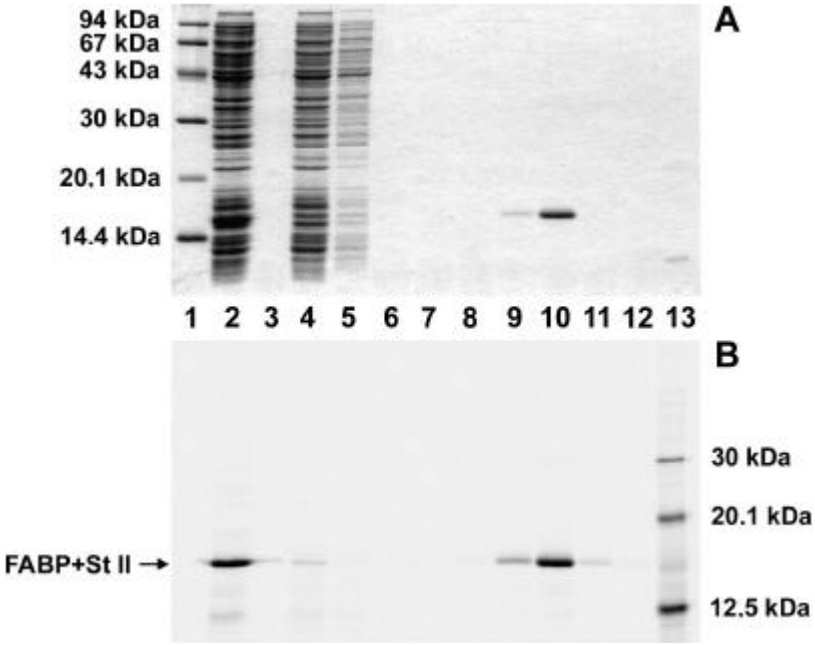

Figure 1. Purification of FABP with Strep-tag II using a StrepTactin affinity column.

The same percentage of every isolated fraction was analyzed by SDS/PAGE. (A) Coomassie stain and (B) autoradiography of the radioactively labeled products. The samples in the numbered lanes are as follows: (1) molecular mass standards; (2) reaction mixture; (3) flow-through of the sample loading; (4–6) wash fractions 1–3; (7–12) elution fractions 1–6 and (13) ^{14}C-labeled molecular mass standards.

Selection of a desired protein property by means of ribosome display

The ribosome display technique (Fig. 4) was first described by Mattheakis *et al.* (1994). Since we have a well working *in vitro* translation system we decided to test rather the ribosome display system than the mRNA-protein fusion technique. One reason is that the puromycin-mRNA fusion, which has to be repeatedly constructed for each selection cycle, is not only a time consuming factor but also a system in which the products are produced at lower yields.

For the reason to do *in vitro* evolution of proteins in the future we tried to establish the ribosome display technique on the basis of a model system. The used mRNA bears the untranslated region of phage T7 gene 10, which encodes a stem-loop structure directly at the beginning of the mRNA, followed by the coding sequence for FABP. The sequence coding

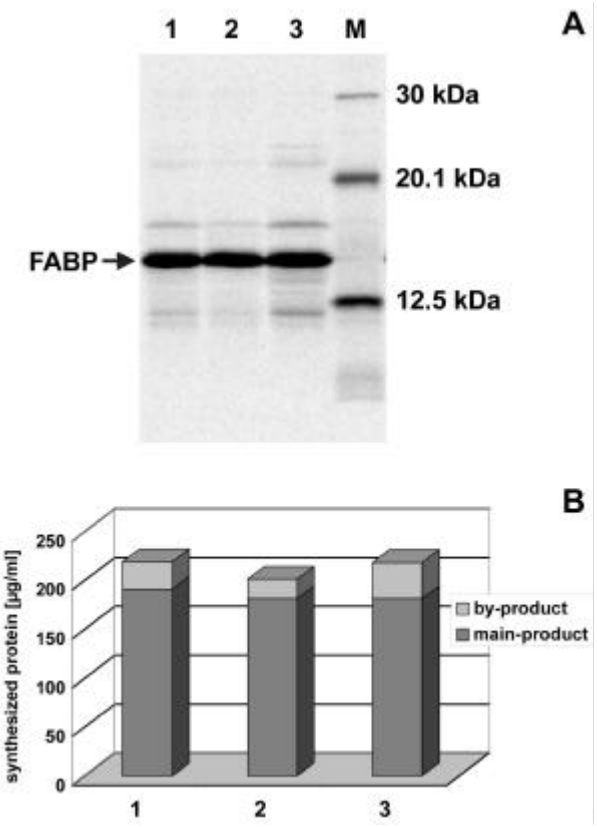

Fig ure 2. In flu ence of StrepTactin sepharose to a coupled transcription/translation reaction of FABP.

One out of two iden ti cal 60 μl re ac tions was car ried out in the pres ence of 20 μl af fin ity ma trix. The prod ucts of both re ac tions were an a lyzed by TCA pre cip i ta tion and SDS/PAGE. The rest of the re ac tion mix ture with ma trix was treated with 0.5% SDS and iden ti cal vol umes were an a lyzed. (A) Autoradiogram of the SDS/PAGE and (B) amount and dis tri bu tion of the prod ucts. Lanes 1, re ac tion in the ab sence of ma trix; 2, in the pres ence of ma trix; 3, rest of the re ac tion mix ture.

for the last ten amino acids of FABP and the stop codon were replaced by the lipoprotein terminator of *E. coli*, which encodes a 3′ stem-loop at the RNA level. This first mRNA (designated mFA) acts on the one hand as a con trol and was the ba sis for a sec ond mRNA (des ig nated mFAHis) cod ing for a N-terminal His-tag (six con sec u tive histidine res i dues) on the other hand. A mixture of these two mRNAs served as start ing pool and fea ture of the test selection was to bind on Ni(II)-IDA-agarose. Using ribosome display the mRNA coding for the FABP with His-tag should be enriched.

The mRNAs showed only a low level of un-spe cific bind ing to the NI(II)-IDA-agarose and neither of them was fa voured in the RT-PCR (not shown). Sev eral steps of op ti mi za tion in-creased the yield of mFAHis after one round of affinity selection up to 8% of input mRNA calculated by TCA precipitation of radio-labelled mRNA. The integrity of the mRNA was an a lyzed by denaturating poly acrylamide gel electrophoresis followed by autoradio-graphy in the phosphorimager system. As a re sult the amount of full length mRNA could be de tected and came to 5% (Fig. 5). The back-ground of the sys tem could be de tected on the same way with mFA as template and was about 0.3%.

A fi nal ex per i ment should re veal the qual ity of the system. Therefore mFA and mFAHis were mixed in dif fer ent ra tios and used for ri-bo some dis play (Ta ble 1). Their PCR prod ucts differ slightly in length (21 basepairs), be-cause of the His-tag with a linker codon and can thus be distinguished after agarose gel elec tro pho re sis. De pending on the ra tio of di-lution different numbers of cycles according to Fig. 4, undergoingselection on Ni(II)-IDA-agarose, were necessary to enrich the PCR product coding for FABP with His-tag (Ta-ble 1). Even out of a ratio of $1:10^8$ (one mFAHis- be tween 10 mil lion mFA-molecules) “one mol e cule” could be se lected af ter nine cy-cles (Fig. 6). The PCR products which went through nine cycles of selection were cloned and analyzed. Of 10 clones sequenced, 8 had the full His-tag se quence and the other two a short ened His-tag se quences cod ing for 5 and 4 his ti dines, re spec tively. The se quence anal y-sis showed that the clones con tained be tween 5 and 13 base changes. At the protein level, the se lected clones car ried be tween 0 and 5 ex-changed amino acids.

The results demonstrate that even the 10^8-fold en rich ment was suc cess ful. With our model sys tem we have shown that it is possi-

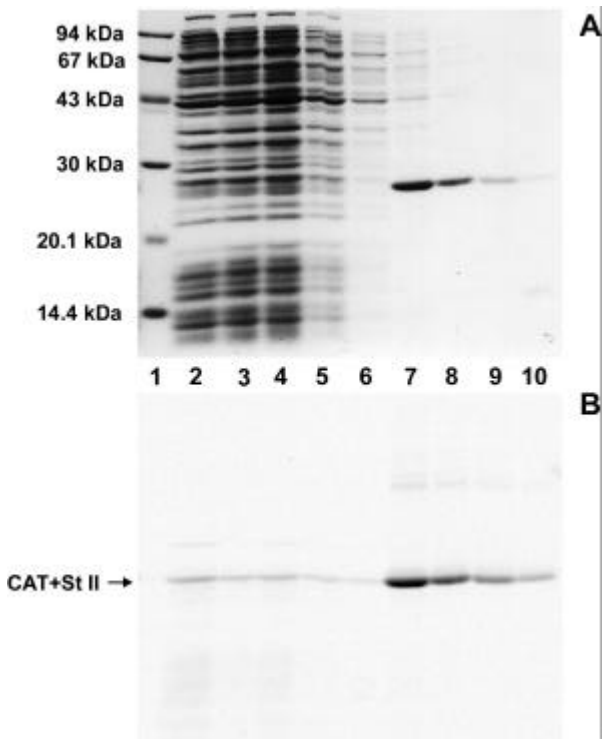

Figure 3. Removal of CAT with Strep-tag II during *in vitro* protein synthesis *via* StrepTactin Sepharose.

The same percentage of every isolated fraction except of the elution fractions was analyzed by SDS/PAGE. From the elution fractions the fourfold amount was separated in order to check the purity. The samples in the numbered lanes are as follows: (1) molecular mass marker; (2–3) supernatant of the reaction before and after centrifugation (5 min, 15000 × ***g***); (4–6) wash fractions; (7–10) elution fractions.

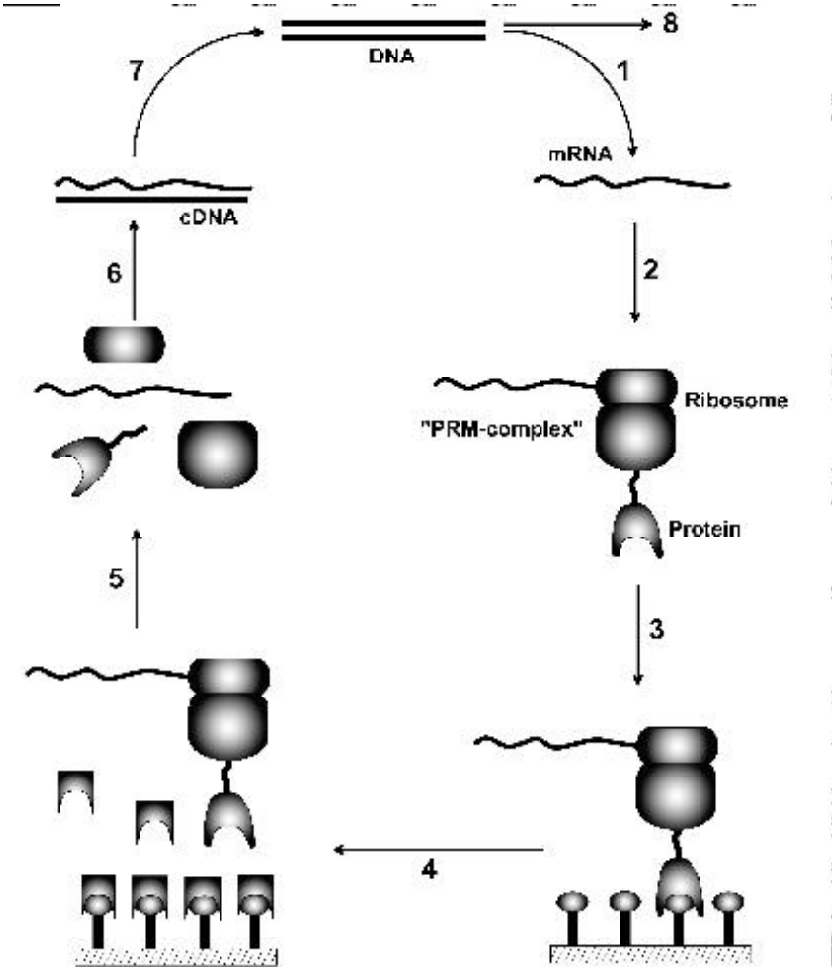

Figure 4. Principle of *in vitro* ribosome display for screening protein libraries for ligand binding.

(1) A DNA library containing a T7 promoter, ribosome binding site and stem-loops is first transcribed into RNA. (2) After purification, mRNA is translated *in vitro* in an *E. coli* S-30 system. Translation is stopped by cooling on ice, and the "PRM-complexes" are stabilized by increasing the magnesium concentration. (3) The desired "PRM-complexes" are affinity selected from the translation mixture by binding to the immobilized ligand. (4) The "PRM-complexes" can be eluted with a specific competitor. (5) Dissociation of the complexes by adding EDTA. (6) Reverse transcription of the isolated mRNA. (7) Amplification of the cDNA by PCR. This DNA is then used for the next cycle of enrichment and (8) can be analyzed by cloning and sequencing.

ble to carry out phenotypic selection for ligand binding with a protein molecule *in vitro* by using ribosome display.

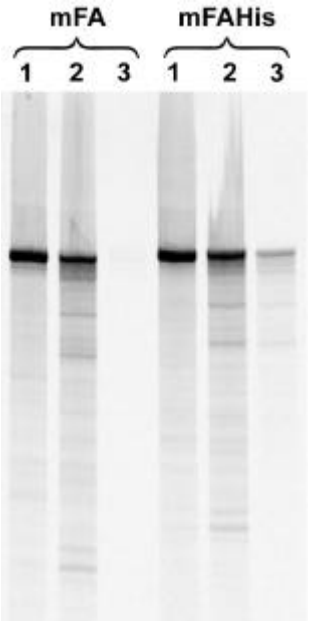

Figure 5. Analysis of mRNA during one round of affinity selection.

The radiolabeled mRNAs were isolated from the reaction mixtures and the "PRM-complex", respectively before and after Ni^{2+}-IMAC. (1) Reaction mixture, (2) reaction mixture after *in vitro* translation, (3) eluted "PRM-complexes". The mRNAs were separated on a 5% denaturing polyacrylamide gel. Shown is the autoradiogram of the PAGE.

Two-dimensional gel electrophoresis

Although we are at the beginning of our investigation of the dynamics of the protein bioreactor, we would like to present here our first results. The following data illustrated in histograms have been obtained from 11 gels: 5 gels (a–e) represent the group '0 h' (one example is shown in Fig. 7A) and 6 gels (f–k) represent the '2 h' (one example is shown in Fig. 7B) group at the end point of reaction.

The gels were analyzed with the MELANIE software (Appel *et al*., 1991). A synthetic gel was prepared as a reference on the basis of both groups i.e. the 0 h and 2 h experiments. Only spots observed at least in 3 gels were matched against each other. The spots from '0 h' group resulted in averaged 1743 (±6.8%) spots against 1943 (±10.8%) in '2 h' group. This fact requires further validation. The spot number is potentially increased due to proteolytical activity and/or modification of proteins. Investigations from Schindler *et al*. (1999) with a different type of protein bioreactor (dialysis system) were opposite to our findings, in their experiments the spot number was reduced after 2 h.

Table 1. Number of selection cycles necessary for enrichment of the diluted mRNA.

mFA and mFAHis were mixed at different ratios and used for ribosome display.

Ratio of mFAHis : mFA	Number of cycles necessary for selection
$1:10^2$	3
$1:10^4$	4
$1:10^5$	5
$1:10^8$	9

Spot intensities were measured as volume percent. We decided to consider only spots with a volume percent value greater than 0.01, because it is very difficult to determine small spots after Coomassie blue staining in order to identify them later. We tested

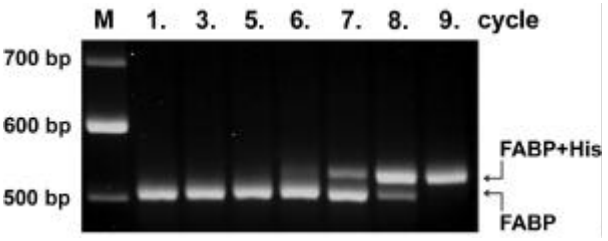

Figure 6. Enrichment of FABP+His from a mixture with FABP by ribosome display.

The mRNA of FABP+His was mixed with the mRNA of FABP at a ratio of $1:10^8$ and used for ribosome display. After affinity selection of "PRM-complexes" carrying a His-tag, the mRNA was amplified by RT-PCR and analyzed by agarose gel electrophoresis. Lane M is a 100 bp DNA ladder. The other lanes show the PCR products after different selection cycles.

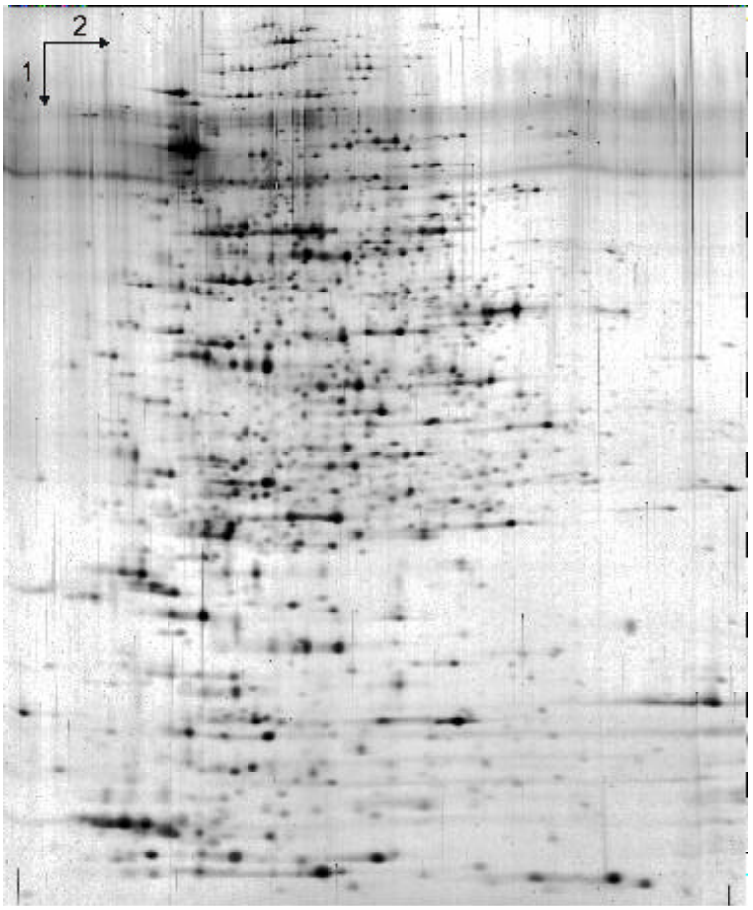

Figure 7A. Two dimensional polyacrylamide gel from the cell-free reaction mixture at the start point of protein biosynthesis.

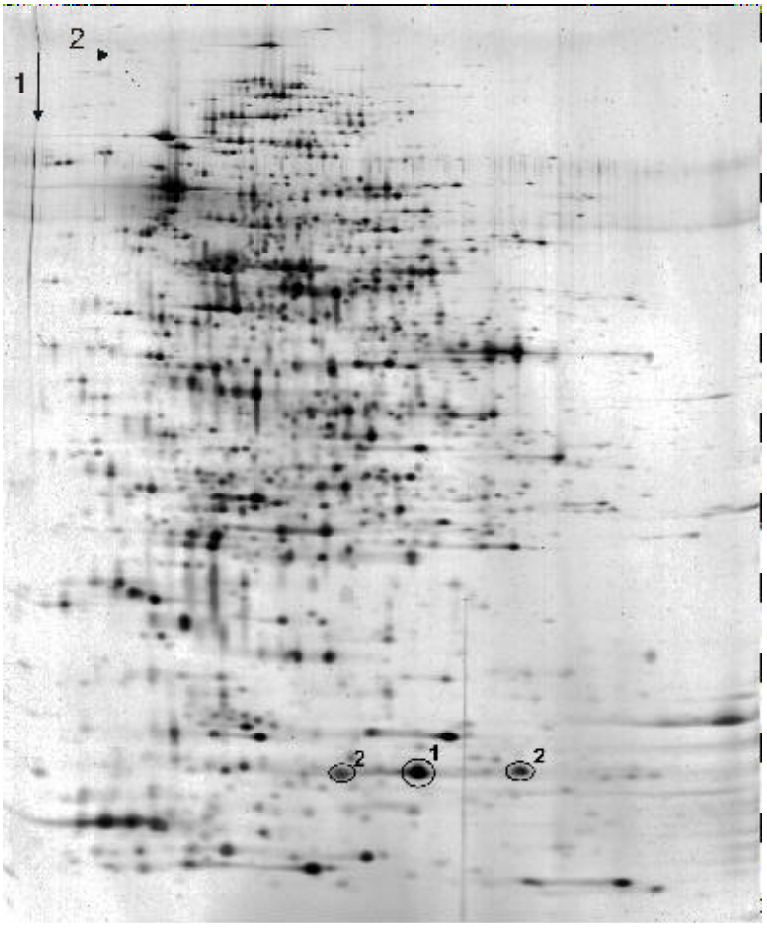

Figure 7B. Two dimensional polyacrylamide gel from the cell-free reaction mixture after 2 h of protein biosynthesis. The prod uct FABP is la beled as 1, the by-products as 2.

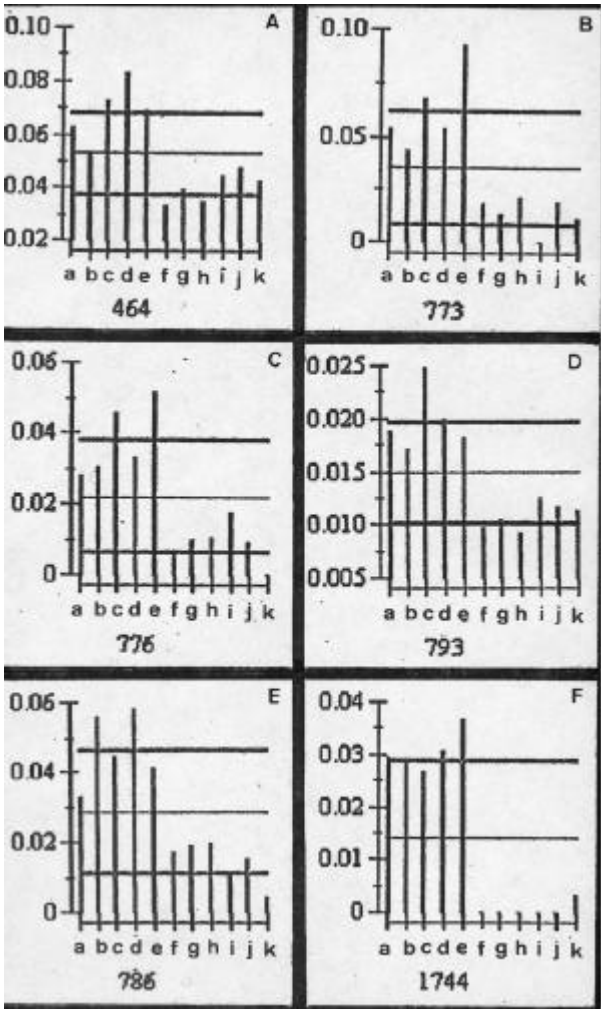

Fig ure 8. Five ex am ples for spots, which in ten si ties are re duced af ter 2 h, are shown in A–E in his tograms. One spot disappeared after 2 h and is shown in F. 0 h sam ples are pre sented in a–e and 2 h sam ples in f–k.

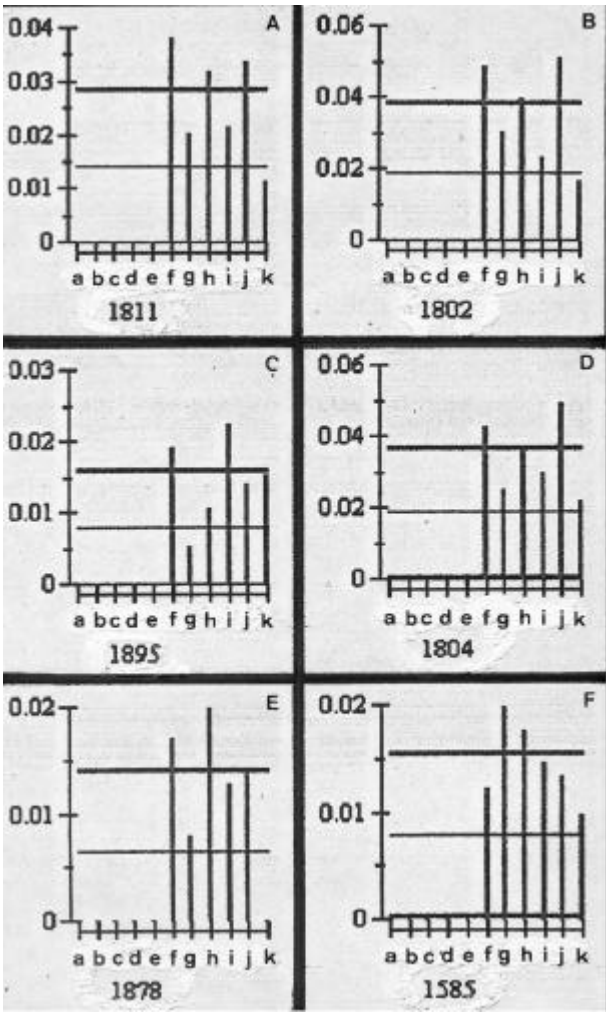

Fig ure 9. Six ex am ples for spots, which does not ex ist at the start of the pro tein biosynthesis re ac tion, but are formed af ter 2 h are shown in A–F in his to grams. 0 h sam ples are pre sented in a–e and 2 h sam ples in f–k.

the significance by which spots from one group against the other were reduced (examples are given in Fig. 8) or increased (examples are given in Fig. 9) with the Student's *t*-test and se lected spots about 0.975 with the volume percent change by a factor 2.

The results obtained can be summarized as follows and are documanted (Fig. 8A–E).

Spots dis ap pear af ter 2 h:	1
The in ten si ties of spots are re duced af ter 2 h:	9
New spots are formed after 2 h:	12
Spots are more intense af ter 2 h:	5

Currently we are working on the identification of these 27 pro teins and their pos si ble involvement in the *in vi tro* pro tein biosynthesis system.

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