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Cell-Free Expression of Two Single-Chain Monoclonal Antibodies against Lysozyme: Effect of Domain Arrangement on the Expression[1]

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Single-chain antibodies (scFv), which can be produced in *Escherichia coli* cells, have been shown in numerous cases to be active in antigen binding. In the case of the two anti-lysozyme single-chain antibodies, $scFv_{LH}$ and $scFv_{HL}$, which have the reversed arrangement of the variable domains of the heavy and light chains of the corresponding monoclonal antibodies, the expression level differs greatly when they are produced in *Escherichia coli* [Tsumoto *et al.* (1995) *Biochem. Biophys. Res. Commun.* 201, 546-551]. Although the expression level of $scFv_{LH}$ is high *in vivo*, the single chain antibody with the reversed orientation ($scFv_{HL}$) was synthesized in a very low yield and no active product could be obtained. We report here the synthesis of these two anti-lysozyme single-chain antibodies in high yields and with high biological activities in a cell-free *E. coli* expression system in the presence of reduced and oxidized glutathione, protein disulfide isomerase (PDI), and chaperones. In immunological blotting assays, the synthesized scFvs with both arrangements exhibit specific binding activity to the corresponding antigens, hen egg-white lysozyme, and in an activity assay both inhibited the action of lysozyme. $scFv_{LH}$ is synthesized mainly as a product with the expected molecular weight, whereas $scFv_{HL}$ is produced with additional shorter fragments, suggesting that the low yield isolation through the expression *in vivo* is due to mistranslation or ribonucleolytic cleavage of the transcript. In the cell-free expression of scFv a certain amount of the product is precipitated but in the presence of chaperones the amount of soluble protein increased from 25 to 90% (PDI and chaperones). The overall expression level and the specific biological activity, however, were hardly influenced. The system reported here can provide significant amounts of various scFv fragments regardless of the order of variable regions, including those which are hardly expressed *in vivo*.

Key words: protein activity, protein disulfide-isomerase, protein folding, protein synthesis *in vitro*, single-chain antibody.

Recent progress in antibody engineering has made it possible to produce various antibody fragments grafting desired specificities towards antigens (*1*). The usual methodology for obtaining novel antibody fragments comprises two steps: the library of phage-displayed antibody fragments is panned against target antigens (selection) (*2*), and those are prepared using bacterial expression systems (preparation) (*3*). However, since the use of bacterial cells cannot be avoided, limitation of the library size and toxicity to the host cells are often observed.

Extensive research for a selection system and a secretory expression system for antibody fragment production has been performed (*4*). However, some antibody fragments cannot be expressed in the *Escherichia coli* periplasm even as an insoluble fraction (*5*). An alternative approach for obtaining recombinant antibody fragments from *E. coli* is to produce proteins as cytoplasmic inclusion bodies, which is time-consuming and requires laborious refolding steps *in vitro* (*6*).

Because of their smaller size and useful properties, Fv fragments, which are composed of the variable regions of immunoglobulins, have often been used. For better physical stabilization single-chain Fv fragments (scFvs) have been constructed (*7, 8*), in which the C terminus of the V_H or V_L chain is linked to the N terminus of the other chain.

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Abbreviations: BSA, bovine serum albumin; dpm, disintegrations per minute; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Fv, fragment of variable region of immunoglobulin; GSH, glutathione reduced; GSSG, glutathione oxidized; HEL, hen egg-white lysozyme; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid; PDI, protein disulfide-isomerase; POD, peroxidase; PVDF, polyvinyldifluoride; SDS-PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate; scFv, single-chain antibody/single-chain Fv fragment; TCA, trichloroacetic acid; V_H, variable region of H chain; V_L, variable region of L chain.

However, in some cases, the V_H-linker-V_L (V_HV_L) and V_L-linker-V_H (V_LV_H) arrangements of single-chain antibodies (scFv) were observed to differ greatly in the expression level when produced in *E. coli* (*9, 10*). This seems to be one of the major obstacles for generalization of the production of novel antibody fragments by means of a phage-displayed library (*10*).

Here we report the synthesis of significant amounts of functional scFvs (V_HV_L and V_LV_H) in a cell-free *E. coli* expression system (*11*) for a stable supply of scFv. The addition of chaperones to the translation system led to a dramatic increase in the solubility of the products.

EXPERIMENTAL PROCEDURES

Reagents—Protein disulfide-isomerase (PDI) from bovine liver was obtained from Pierce. Chaperones GroEL, GroES, GrpE, DnaK, and DnaJ were from Biomol (Hamburg, Germany). L-[*U*-^{14}C]Leucine with a specific activity of 304 mCi/mmol was from Amersham. T7 RNA-polymerase was from Stratagene. Ribonuclease inhibitor was from Promegea. *Eco*RI restriction enzyme was from NEB. Hen egg-white lysozyme was obtained from Seikagaku-Kogyo (Tokyo). *Micrococcus lysodeikticus* was from Sigma. The Chemiluminescence Western Blotting Kit (rabbit) was from Boehringer Mannheim. All other chemicals were of reagent grade appropriate for biochemical use.

Plasmid Isolation—Isolation of plasmid DNA for coupled transcription/translation was carried out with a plasmid isolation kit from Genomed (Bad Oeynhausen, Germany) according to the manufacturer's protocol. The isolated plasmid DNA was assayed by electrophoresis on a 1% agarose gel, and by restriction enzyme digestion analysis. Prior to transcription/translation the plasmids were linearized by restriction enzyme digestion with *Eco*RI.

Coupled In Vitro Transcription/Translation—We used an optimized prokaryotic lysate, which was prepared by the method of Herrlich and Schweiger (*12*), and composed of the components described by Baranov and Spirin (*13*). The modifications will be described elsewhere. The composition of the reaction mixture was as follows: 20 mM HEPES buffer, pH 7.5, 100 mM KCl, 11 mM $MgCl_2$, 1 mM EDTA, 0.02% NaN_3, 1% polyethyleneglycol 6000, 1 mM adenosine triphosphate, 1 mM guanosine triphosphate, 0.5 mM cytidine triphosphate, 0.5 mM uridine triphosphate, 10 mM phosphoenolpyruvate, 10 mM acetyl phosphate, 40 μM folinic acid, 8 U/ml pyruvatekinase, 100 U/ml ribonuclease inhibitor, protease inhibitors 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, 2 μM pepstatin, 600 μg/ml bulk tRNA, 900 pmol/ml ribosomes, 1 mg/ml S100, 50 μM L-[*U*-^{14}C]-leucine (50 dpm/pmol), 200 μM each of the other 19 amino acids, and 0.1-10 pmol/ml plasmid pUTN2 and pUTN3, respectively. The reactions were performed for 60 min at 37°C. Control reactions were performed under identical conditions without plasmids.

Protein disulfide-isomerase was used at a final concentration of 0.5 μM, and glutathione in the reduced and oxidized forms at concentrations of 0.1 and 1 mM, respectively. The concentrations of the chaperones in the reaction mixture were as follows: GroEL, GroES, GrpE, and DnaJ 0.4 μM each, and DnaK 1.4 μM.

Quantification of the Synthesized Protein—Protein quantification was performed by measuring the incorporated radioactivity present in the TCA-precipitates, assuming 23 leucines per scFv molecule. Five-microliter aliquots of the reaction mixtures were mixed with 50 μl 0.5% BSA as a carrier and then precipitated with 3 ml 10% trichloroacetic acid containing 2% amino acids (peptone No. 5 from Gibco). Aminoacyl- and peptidyl-tRNAs were hydrolyzed by heating at 90°C for 15 min. After leaving the TCA precipitates for 30 min on ice they were collected on GFC filters (Whatman), washed 5 times with 5% TCA and two times with acetone, and finally dried by suction. The radioactivity was measured with a scintillation counter (LS 6000, Beckman) after the filter disks have been shaken with 6 ml scintillation cocktail (Ready Protein, Beckman). Non-specific TCA-precipitable radioactivity was determined in control reactions without a template and subtracted from the results.

Analysis of Soluble and Precipitated Protein—After transcription/translation the reaction mixtures were mixed until a homogenous suspension was obtained, which was then centrifuged for 5 min at 15,000 × *g*. The suspensions as well as the supernatants were TCA-precipitated as described above, and then the amounts of soluble and precipitated material were calculated.

Samples (20-60 μl aliquots) were analyzed on 15% SDS polyacrylamide gels (*14*) after they had been precipitated with ice-cold acetone, dissolved in 20 μl PAGE sample buffer and then heated for 5 min at 90°C.

In order to renature the precipitated protein the pellets were dissolved in dialysis buffer (50 mM sodium phosphate, pH 7.4, 200 mM NaCl) containing 6 M urea, and then refolded by dialysis for three days with three changes of the dialysis buffer.

Aminoterminal Sequencing of HyHEL10scFV$_{LH}$—An aliquot of the refolded urea soluble fraction of scFv$_{LH}$ was separated by SDS-PAGE and then blotted onto a PVDF membrane. The full-length product was cut out. Aminoterminal sequence analysis was performed with an Applied Biosystems 473A Protein Sequencer after the first formylated methionine had been hydrolysed with HCl.

Immunodetection of HyHEL10scFv—The transcription/translation mixtures were separated by SDS-PAGE (*14*), and then blotted onto a PVDF membrane according to the standard protocol. The membrane was incubated with polyclonal anti-HyHEL10Fv antibodies and then treated as described in the following section.

Determination of Antigen-Binding Activity by Immunodetection—HEL-binding activity of the synthesized proteins was recorded as dotblot signals. Verification of the antigen-binding activity was performed by spotting various amounts of HEL onto a polyvinyldifluoride membrane. The membrane was incubated with 0.5 nmol scFv in 10 ml buffer. The scFvs were obtained through cell-free coupled transcription/translation reactions, and the precipitated and refolded products were used. The concentrations of scFvs were calculated on the basis of incorporated L-[*U*-^{14}C]leucine. Detection of binding of scFv to HEL was carried out with subsequent addition of polyclonal anti-HyHEL10Fv antibodies from rabbit and anti-rabbit IgG coupled to POD (horseradish). POD allowed the visualization of the antibody complex *via* a light reaction monitored by X-ray film exposure according to the manufacturer's protocol.

Inhibition Assaying of HEL Enzymatic Activity—The

lytic activity of HEL was measured as the decrease in turbidity of a *Micrococcus lysodeikticus* cell suspension according to Ueda *et al.* (*15*) with slight modifications. Various concentrations of transcription/translation products were mixed with 125 nM HEL (100 μl reaction volume), and then incubated for 60 min at 25°C in the incubation buffer (30 mM potassium phosphate buffer, pH 7.4, 0.1% BSA). Each mixture was then added to 400 μl of a *M. lysodeikticus* cell suspension (0.6 A_{450}/ml) in the incubation buffer. The initial rate of the decrease in A_{450} was monitored at 25°C for 5 min. Nonspecific inhibition of cell lysis was assayed by means of control reactions and subtracted from the results.

RESULTS AND DISCUSSION

Synthesis of HyHEL10 Single-Chain Fragments in an E. coli Cell-Free Linked Transcription/Translation System—The plasmids, pUTN2 and pUTN3 (Fig. 1) (*16*), encoding the single-chain antibodies of HyHEL10 with the V_HV_L (variable domain of heavy chain-linker-variable domain of light chain) and V_LV_H (reversed arrangement) arrangements served as templates in a linked transcription/translation system in which the transcription is performed by T7 RNA polymerase. The translation system is based on isolated ribosomes and the enzymatic supernatant S100 prepared from *E. coli* (*12*). The total amount of the synthesized protein was calculated by quantification of the radioactivity present in the TCA-precipitated L-[*U*-^{14}C]-leucine containing protein, assuming 23 leucines per scFv molecule. The amount of TCA-precipitable radioactivity in reactions without the plasmids was subtracted from the results. Figure 2 shows the dependence of the amounts of the synthesized $scFv_{HL}$ and $scFv_{LH}$ on the plasmid concentration. The scFvs were normally synthesized in a yield of about 30 μg per ml reaction mixture in 60 min. A plateau as to the amount of synthesized protein was reached at a template concentration above 10 nM. Certain amounts of the synthesized proteins were found to be produced in insoluble forms and thus they could be pelleted by a short centrifugation step.

Effects of Redox Potential, PDI and Chaperones on the Solubility of scFv Fragments—In order to minimize the aggregation and misfolding of the scFvs synthesized in the cell-free transcription/translation system, various reaction conditions were examined. The plasmids, pUTN2 and pUTN3, were routinely assayed at 5 pmol/ml. After incubation for 60 min, aliquots of a homogenous suspension obtained on vigorous mixing and of a supernatant obtained on centrifugation were taken. In order to calculate the percentage of soluble protein, the amounts of synthesized proteins in suspensions and supernatants were determined after precipitation with TCA. Under our normal reaction conditions (5-10 mM DTT), usually 70 to 75% of the synthesized proteins were detected as insoluble pellets. Under redox-defined conditions (0.1 mM GSH and 1 mM GSSG) the percentage of pelleted protein was reduced to 60%, most likely due to stabilization of the protein tertiary structure by intramolecular disulfide bonds. The addition of PDI increased the solubility to 50%, indicating the importance of disulfide shuffling catalyzed by PDI. Under redox conditions in the presence of PDI and the five chaperones, GroEL, GroES, GrpE, DnaJ, and DnaK, at least 90% of scFv produced in the cell-free system was detected as a soluble protein. During the preparation of this manuscript, Ryabova *et al.* also reported the effects of chaperones on the solubility of cell-free synthesized single-chain antibodies (*17*). The yield of active proteins was reported to range up to 50% of the amount of the synthesized protein, which was estimated to be 8.3 μg/ml.

Western Blotting Analysis of Products Expressed in the Cell-Free Translation System—The molecular weights and homogeneity of the synthesized proteins in the soluble and insoluble fractions were determined by autoradiography

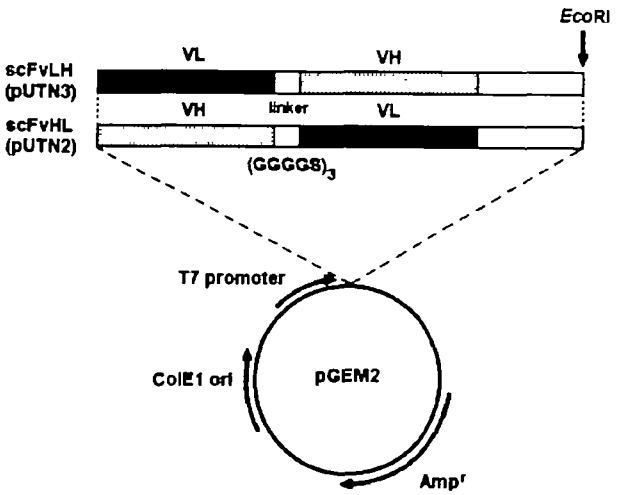

Fig. 1. **Plasmid construction for the expression of the HyHEL10-derived single chain antibodies, scFv, in a cell-free transcription/translation system.** The plasmids, pUTN3 and pUTN2, which served as templates for the synthesis of $scFv_{LH}$ and $scFv_{HL}$, respectively, are based on the pGEM2 vector. They differ in the orientation of the coding sequences of the V_L- and V_H-chains. Both chains are connected to each other by the linker, $(GGGGS)_3$. Prior to their use in the protein synthesis reaction, the plasmids were linearized by digestion with *Eco*RI.

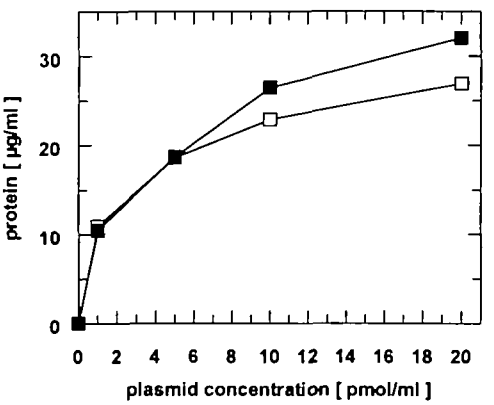

Fig. 2. **Expression of HyHEL10 scFv as a function of the plasmid concentration.** After 60 min incubation of the linked transcription/translation reaction mixture the amount of synthesized protein was calculated on the basis of [^{14}C]leucine incorporation into TCA-precipitates. A plateau of about 30 μg scFv per ml reaction mixture was reached when the plasmids were used at concentrations of greater than 10 pmol/ml. The examined templates were pUTN2 ■ ($scFv_{HL}$) and pUTN3 □ ($scFv_{LH}$).

(Fig. 3), and Western blot analysis (Fig. 5) following SDS-PAGE (*14*). On Western blotting and autoradiography the scFvs were found to each have an approximate molecular weight of 30,000 Da. The correct synthesis of $scFv_{LH}$ was verified by aminoterminal protein sequencing, where the first 8 amino acids could be detected (data not shown). In contrast to $scFv_{LH}$, $scFv_{HL}$ showed additional smaller fragments (Fig. 3). The patterns of the bands of the soluble and insoluble fractions of $scFv_{LH}$ are more or less the same and therefore independent of solubility. In the case of $scFv_{LH}$ approximately 97% of the incorporated radioactivity was in the full-length product, whereas only 25% was of the correct size in the case of the soluble fraction of $scFv_{HL}$ and 42% in the precipitated fraction. This was determined by scanning the radioactivity in the gel. The incorrect expression might be responsible for the inability to isolate this antibody by affinity chromatography when produced *in vivo*. Interestingly there are striking differences between the results of autoradiography and Western blotting for $scFv_{HL}$. Apart from the full length product two small fragments each gave a signal in the blot, whereas bigger ones, which are visible in the autoradiogram, do not bind the polyclonal antibodies (Figs. 4 and 5, lanes 4 and 5). Therefore we believe that premature termination of translation is not the reason for the fragmentation of $scFv_{HL}$. If premature termination does occur fragments with higher molecular weights, which are visible in the autoradiogram, should be detected on Western blotting as well. This should also be the case if ribonucleolytic cleavage leads to the fragmentation of $scFv_{HL}$, although that special folding, which will mask the relevant epitopes, cannot be excluded.

HyHEL10 scFv Synthesized In Vitro Has Specific Antigen-Binding Activity, as Determined by Immunodetection—The binding activities of the synthesized proteins as to hen egg-white lysozyme (HEL) were analyzed as dotblot signals (Fig. 5). For this assay the precipitated and refolded products were used. After synthesis under our standard conditions reaction mixtures were centrifuged for 5 min at $15,000 \times g$. In order to refold the proteins the pellets were completely dissolved in the dialysis buffer, which contained

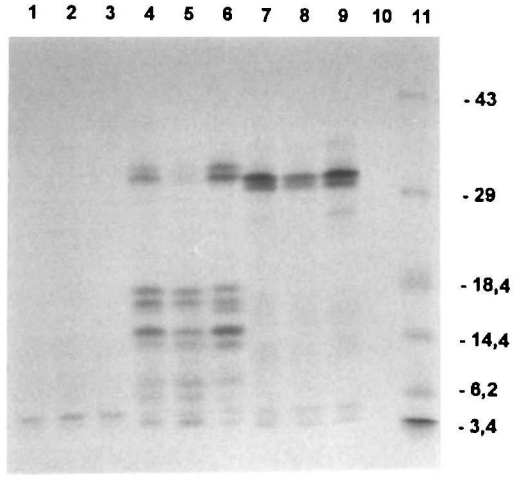

Fig. 3. **Autoradiography of the SDS-gel of the transcription/translation products of pUTN2 and pUTN3.** The samples in the numbered lanes in the autoradiogram are as follows: (1 and 2) reaction mixtures with the templates, pUTN2 and pUTN3, but without T7 RNA-polymerase; (3) the reaction mixture without the plasmids; (4) the reaction mixture with pUTN2 (V_HV_L) and chaperones; and (5) the soluble and (6) insoluble fractions of the reaction mixture with pUTN2 (without chaperones); (7) the reaction mixture with pUTN3 (V_LV_H) and chaperones; (8) the soluble and (9) insoluble fractions of pUTN3 (without chaperones); (10) molecular weight standards (not labeled); (11) ^{14}C-labeled molecular weight standards.

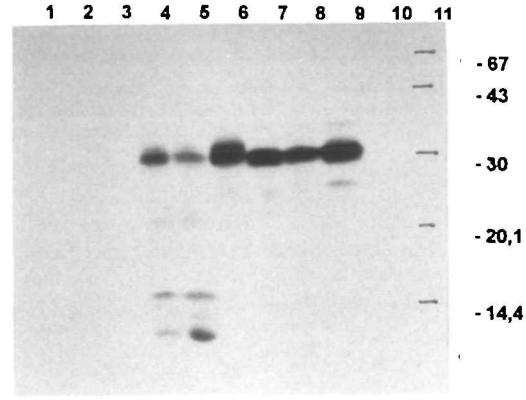

Fig. 4. **Western blot analysis of HyHEL10 scFv after the linked transcription/translation reaction.** The samples in lanes 1-9 are similar to in Fig. 3; (10) BSA; (11) a molecular weight standard. After polyacrylamide gel electrophoresis the samples were blotted onto a PVDF membrane using standard protocols. The scFvs were detected *via* a light emitting reaction using the specific binding of polyclonal anti-HyHEL10 Fv antibodies (rabbit) and anti-rabbit IgG coupled to POD, as described under "EXPERIMENTAL PROCEDURES."

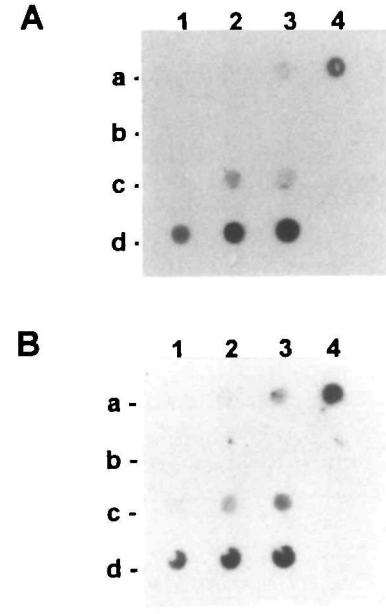

Fig. 5. **Antigen-binding activities of the synthesized single chain antibodies determined by dot blotting.** The spotting of dots on a PVDF was as follows: (line a, dots 1-4) 10, 50, 100, and 300 ng HEL; (line b, dots 1-4) 10, 50, 100, and 300 ng BSA; (line c, dots 1-3) 50, 100, and 200 fmol HyHEL10 $scFv_{HL}$; (line d, dots 1-3) 50, 100, and 200 fmol HyHEL10 $scFv_{LH}$. The membranes were incubated with 0.5 nmol HyHEL10 $scFv_{HL}$ (A) or 0.5 nmol HyHEL10 $scFv_{LH}$ (B) as the first antibodies. Further treatment was the same as described for the Western blotting in Fig. 4.

an additional 6 M urea, and dialyzed for three days with three changes of the dialysis buffer. Verification of the antigen-binding activity was performed by immunodetection. Various amounts of HEL were spotted onto a polyvinyldifluoride membrane, followed by incubation in 10 ml buffer containing 0.5 nmol of the urea-soluble and renatured fraction of $scFv_{HL}$ (Fig. 5A) or $scFv_{LH}$ (Fig. 5B) as the first antibody. Detection of the binding of scFv to HEL was carried out with subsequent addition of the second antibody, the polyclonal anti-HyHEL10 Fv antibody from rabbit, and finally binding of the anti rabbit IgG coupled to horseradish peroxidase (POD).

Both arrangements of the single chain antibodies (V_HV_L and V_LV_H) gave positive HEL binding signals, with intensities depending upon the amount of HEL spotted onto the membrane (Fig. 5). This indicates that both scFvs are bound specifically to the corresponding antigen HEL. As a control, both single chain antibodies were spotted directly in three amounts onto a membrane and, as expected, also gave positive signals with the polyclonal antibodies.

Although the described method does not allow precise quantitation, strong signals were observed from the $scFv_{LH}$-HEL complex, and weaker but also significant and specific signals for the $scFv_{HL}$-HEL complex. These results strongly suggest that both arrangements exhibit almost identical binding affinities to the antigen.

Quantification of Binding Activity and Effects of Chaperones—For the isolation of the single chain HyHEL10-antibodies produced in *E. coli* cultures an affinity chromatography step involving HEL-Sepharose has been used (*10*). Thus, only molecules with the ability to bind the antigen were assayed to quantify the binding activities and no data could be obtained as to percentage of nonactive antibodies expressed in *E. coli* cells. We, on the contrary, assayed reaction mixtures containing the whole *in vitro* synthesized protein to measure the antigen-binding activity. Quantitation was performed by measuring the inhibition of HEL-enzyme activity in the presence of increasing amounts of appropriate antibodies. The antibodies from the whole reaction mixture, from the soluble fraction and from the refolded urea-soluble fraction were used for lysis assaying of *M. lysodeikticus* cell suspensions according to Ueda *et al.* (*15*). The profile of inhibition of the lytic activity of HEL in the presence of increasing amounts of $scFv_{LH}$ from the different fractions is shown in Fig. 6. All fractions show similar inhibitory effects on HEL. A fourfold molar excess of $scFv_{LH}$ over HEL almost completely abolished the lytic activity of HEL. Comparing these data with the inhibitory effect of purified HyHEL10-$scFv_{LH}$ produced *in vivo* (*10*), we calculated the percentage of active single chain antibodies synthesized *in vitro* to be approximately more than 80%.

Although no activity of scFv with the V_HV_L orientation could be observed with the *in vivo* product, we were able to show that the *in vitro* synthesized $scFv_{HL}$ is active as well. In this case a fourfold molar excess of the synthesized product over HEL reduced the lytic activity to about 85%. However, this result is based on a rough approximation for determination of the $scFv_{HL}$ concentration, which in fact consists of a mixture of products. In the autoradiogram (Fig. 4) approximately only 25% of the synthesized $scFv_{HL}$ was detected as the full length product, as determined by scanning the autoradiogram. With this calculation 15%

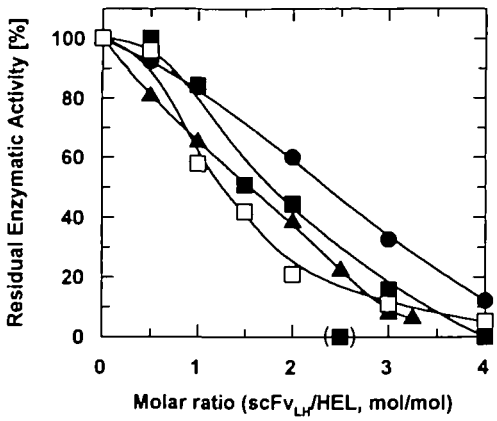

Fig. 6. **Profile of the inhibition of the enzymatic activity of lysozyme by the single chain antibody, HyHEL-$scFv_{LH}$.** Lysozyme (HEL) at 125 nM and the scFv fragments at various concentrations were incubated at 25°C for 60 min, and then mixed with a *M. lysodeikticus* cell suspension. The process of lysis of *M. lysodeikticus* cells was monitored on the basis of the decrease in turbidity by measuring the absorbance at 450 nm. The residual enzymatic activity observed with these measurements was plotted against the molar ratio of scFv to HEL. The $scFv_{LH}$ samples which were used are: the soluble fraction (▲), the precipitated and renatured fractions of a reaction without chaperones (●), as well as a reaction with chaperones (■). For comparison a sample of isolated $scFv_{LH}$ produced *in vivo* is shown as well (□).

inhibition of the lysozyme action is obtained with a 1:1 ratio of the single chain antibody to HEL, and this is the same activity as has been observed for $scFv_{LH}$ (Fig. 6).

As mentioned above, about 90% of scFv with the V_LV_H orientation is synthesized *in vitro* in a soluble form when chaperones and protein-disulfide isomerase are present. The specific binding affinity of this product is almost identical to that without chaperones, indicating that the yield of the soluble protein does not lead to an increase in binding affinity, but the absolute amount of the soluble and active product was increased by a factor of 3 when compared to with our standard conditions. The aggregated products obtained *in vivo* (*i.e.* inclusion bodies) can hardly be refolded without a redox potential or the addition of chaperones (Tsumoto, K. and Kumagai, I., unpublished results), suggesting that avoidance of aggregation in cell cultures must be critical for a high yield of active scFv. The expressed scFv *in vitro* was found to exhibit mainly full antigen binding activity.

Effect of the Order of Domains Linking the Antibody Variable Regions by Single-Polypeptide Chain on Expression In Vitro System—As reported previously (*16*), when HyHEL10 fragments where expressed *in vivo*, only scFv with the V_LV_H orientation was produced as intact molecules. In our cell-free protein synthesis system single chain antibodies with both orientations could be synthesized. $scFv_{HL}$ however was found to be expressed with additional shorter fragments (Fig. 3).

The translation products in the linked transcription/translation system reported here seem to be stable, since no additional degradation was observed with a longer incubation period (data not shown). When using the mRNA of the V_HV_L gene in the cell-free translation system, which was obtained by run off transcription, almost the same results

were obtained. When a mRNA lacking a stop codon was used, mainly shorter fragments resulted, which might be due to the peptide tagging by 10Sa RNA (*18, 19*) (data not shown), suggesting that the low yield isolation through expression *in vivo* is due to ribonucleolytic cleavage of the transcript leading to a mRNA devoid of a stop codon besides the possibility of mistranslation.

The possibility of mistranslation and certain instability of the transcript can hardly be predicted from the DNA sequence, and therefore must be taken into consideration when constructing an *in vivo* expression system involving various scFvs. However, significant amounts of scFvs with both arrangements were found to be obtained in the cell-free transcription/translation linked system, and the system reported here will overcome the problem of low expression of various other scFv fragments. Improvement of the system will be promising for the large scale synthesis of various scFvs, stable isotope labeled proteins (*20*), and unnatural amino acid incorporated ones (*21*), including continuous systems (*22, 23*).

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