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Needle in a haystack: Protein complex purification from *Thermoplasma acidophilum* with a phage display library

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Vollständiger Abdruck der Fakultät für Chemie der Teschnischen Universität München zur Erlagung des akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat.) genehmigten Dissertation.

Vorsitzender: Univ. - Prof. Dr. J. Buchner

Prüfer der Dissertation:

1. Hon. - Prof. Dr. W. Baumeister
2. Univ. - Prof. Dr. S. Weinkauf

Die Dissertation wurde am 10.01.2013 bei der Technischen Universität München eingereicht und durch die Fakultät für Chemie am 22.04.2013 angenommen.

„A tudomány nem képes megoldani a természet végső rejtélyeit. Azért nem képes, mert mi is a természet részei vagyunk, s ezzel részei vagyunk annak a rejtélynek is, amelyet megoldunk.“

“Science cannot solve the ultimate mystery of nature. And that is because, in the last analysis, we ourselves are a part of the mystery that we are trying to solve.”

Max Planck (1858-1947)

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ZUSAMMENFASSUNG

Thermoplasma acidophilum ist ein thermoazidophiles Archaeon der *Euryarchaeota*, das bei 59°C und pH 2 wächst. Es stellt einen attraktiven Modellorganismus für die visuelle Proteomik dar, deren Ziel es ist, mithilfe von Kryo-Elektronenmikroskopie und Mustererkennungsverfahren, eine umfassende zelluläre Kartierung makromolekularer Komplexe zu erreichen. *T. acidophilum* weist einen vielfältigen Bestand an Proteinkomplexen auf, in dem Gruppierungen zu finden sind, die wie vereinfachte Variationen ihrer eukaryontischen Pendants aussehen und die in Faltung, Abbau und Umsatz von Proteinen involviert sind. Viele dieser sehr großen Proteinkomplexe sind strukturell charakterisiert, es lassen sich jedoch viele hypothetische und partiell charakterisierte Komplexe finden, deren molekulare Architektur und biochemische Funktion noch nicht erforscht wurde.

Um einen Einblick in die molekulare Architektur von Proteinkomplexen zu bekommen, ist die Isolierung intakter Strukturen unabdingbar. Obwohl heutzutage eine große Anzahl an Proteinaufreinigungstechniken zur Verfügung steht, existiert aufgrund jeweils bestimmter Nachteile keine universell einsetzbare Methode, die es erlaubte, alle möglichen Komplexe intakt und in aktivem Zustand zu erhalten. Niedrige Expressionsraten von nativem / rekombinantem Protein, Aggregation, Dissoziation oder Entfaltung können aufgrund unzureichender Mengen an nativ gefaltetem Protein strukturelle Untersuchungen ebenfalls begrenzen. Falls genetische Werkzeuge nicht zur Verfügung stehen, kann eine antikörperbasierende Methode zur Aufreinigung entwickelt werden, die eine schnelle und präzise Isolierung von Zielproteinen direkt aus ihrer nativen Umgebung möglich macht und eine ausreichende Menge an Protein für die EM-Analyse liefert. Desweiteren verlangt die Einzelpartikel-Elektronenmikroskopie, im Gegensatz zur Röntgenstrukturanalyse, nicht die Züchtung von Proteinkristallen als Voraussetzung für die Strukturanalyse, was die benötigte Zeit zur Probenvorbereitung drastisch senkt.

Im Zuge der vorliegenden Untersuchungen wurde eine scFv-basierte Phagen-Display-Bibliothek entwickelt, mit dem Ziel Proteinkomplexe aus *T. acidophilum* mit spezifischen Antikörpern schonend aufzureinigen. Während die antikörperbasierte Phagen-Display-Technologie ursprünglich für einzelne Zielantigene entwickelt wurde, war es in unserem Fall

nötig, sie auf eine komplexe Proteinmischung von mindestens dreihundert Proteinen anzupassen. Als Ausgangspunkt erstellten wir die Phagen-Display-Bibliothek, indem wir Mäuse mit hochmolekularen Proteinfraktionen (Sup12-HMWF) aus *T. acidophilum* immunisierten und scFvs aus Milz Gesamt-mRNA konstruierten.

Um die Antigenvielfalt zu verringern, wurde die Phagenselektion zunächst gegen unterschiedliche K_2HPO_4-KH_2PO_4-Elutionsfraktionen von Hydroxylapatit-getrennten Sup12-HMWF durchgeführt. Nach der Identifizierung spezifisch bindender Phagen versuchten wir die Aufreinigung von Proteinkomplexen mithilfe von auf Agarosekügelchen immobilisierten Phagen. Dieses Konzept war jedoch aufgrund der strukturellen Limitation der filamentösen Phagenpartikel nicht erfolgreich. Wir haben die Strategie daher in Richtung eines antikörperbasierenden Pull-down Assays in Lösung geändert. Da sich die Expression von löslichen E-Tag-markierten scFvs infolge der zeitaufwendigen Lokalisationsbestimmung (Zytosol, Periplasma und/oder Medium) nicht als sinnvoll erwies, entwickelten wir einen modifizierten pET28-Vektor (pET28-*Sfi*I), um die Expression von scFvs mit einem C-terminalen 6-His-Tag zu ermöglichen. Der Pull-down von Proteinkomplexen mithilfe von auf Agarose immobilisierten His-Tag-markierten scFvs resultierte in der erfolgreichen Aufreinigung des molekularen Chaperonins Thermosom (Ta0980 und Ta1276), es konnten jedoch infolge der rauhen Elutionsbedingungen keine intakten Partikel erhalten werden. Um Dissoziation und mögliche Denaturierung der Proteinkomplexe zu vermeiden, die aufgrund der Antikörper-Antigen-Interaktion während der Elution vorkommen können, wurden die Komplexe im nächsten Pull-down Assay mithilfe von Ni-Affinitätschromatographie aufgereinigt, der eine Größenausschlusschromatographie folgte. Dieser neue Aufreinigungsansatz ermöglichte es uns, strukturell intaktes Thermosom (Ta0980 and Ta1276) und 20S-Proteasom-Partikel (Ta0612 and Ta1288) zu erhalten, sowie ein Protein, bei dem es sich wahrscheinlich um Peroxiredoxin (Ta0152) handelt, dessen Komplex wir jedoch nicht durch EM visualisieren konnten.

Im nächsten Versuch setzten wir die ursprüngliche Antigenlösung (Sup12-HMWF) ein, was zu einer sehr viel größeren Zahl bindender Phagen führte als bei Selektion gegen höher aufgereinigte Hydroxylapatitfraktionen. Um die Effizienz der Phagenselektivität noch feiner abzustimmen, wurde ein Deselektions-Schritt gegen häufig vorkommende Proteinkomplexe (Thermosom, Proteasom) in das Biopanning-Protokoll eingeführt, und die Wirksamkeit dieses

Archiv-Reinigungsschritts mithilfe eines vergleichenden ELISA-Assays verfolgt. Von 373 getesteten Klonen wurden 217 spezifisch bindende identifiziert, von denen 62 (30%) potentiell neue Zielantigene zu erkennen schienen. Trotz der erfolgreichen Klonierung konnte allerdings keiner dieser scFv-Klone in phagenfreier Form sequenziert und exprimiert werden, was auf die Anreicherung einer ungewöhnlichen Form von scFv hindeutet.

Im nächsten Phagenselektionsversuch wechselten wir zu individuellen rekombinanten Proteinen, von denen angenommen wurde, dass sie mutmaßliche Bausteine möglicher Proteinkomplexe einer Größe von über 300 kDa darstellen. Die Biopanning-Assays wurden in verschiedenen Variationen durchgeführt (wechselnder Typ und Menge Sup12-HMWF-selektierter Phagen, Anzahl der Selektionszyklen, Inkubations/Waschbedingungen und Reinheit des Zielantigens). Unter Benutzung der gut funktionierenden Zwei-Schritt-Chromatographie gelang es uns, das mutmaßliche intermediäre Filament Ta1488 aufzureinigen. Die Aminosäuresequenz sagt vorher, dass Ta1488 einen multimeren Komplex bildet, allerdings konnte dies durch die EM-Analyse der aufgereinigten Fraktionen nicht nachgewiesen werden. Wir haben ebenfalls Antikörper gegen das archaeelle Homolog des fixABCX-Komplexes gefunden. Diese Antikörper konnten jedoch keine verwertbaren Mengen des vermeintlichen Komplexes einfangen. Stattdessen wurde aus dem Zellextrakt ein Formiat-Dehydrogenase-Homolog (Ta0425) zusammen mit einem Protein der kleinen Untereinheit (Ta0424) angereichert, der etwa einem Molekulargewicht von 450-500 kDa entspricht. Die Fähigkeit, einen Komplex auszubilden, wurde durch EM-Analyse bestätigt. Da die meisten der nativen und rekombinanten Zielproteine niedrige Expressionsraten aufwiesen und/oder sehr empfindlich gegenüber den Aufreinigungsbedingungen waren, wurden die Phagen im nächsten Versuch mit einer neuartigen Selektionstechnik, durch Nitrozellulose-immobilisiertes Antigen, isoliert, um diese Hürden zu überwinden. Die Durchführbarkeit dieser Methode wurde durch die Aufreinigung des 5 MDa großen 2-Oxo-Säure-Dehydrogenase-Multienzymkomplexes bestätigt, der erfolgreich von nitrozellulose-gebundenen antigen-isolierten scFv eingefangen werden konnte.

Zuletzt haben wir eine Archiv-Selektion gegen den SAMP-Kandidaten Ta0895 durchgeführt, mit dem Ziel, SAMP-markierte Proteine / Proteinkomplexe durch einen Pull-down-Assay einzufangen. Die Phagenselektion ergab eine bedeutende Anzahl an Phagen, die sowohl unter

ELISA- als auch unter Western Blot-Bedingungen rekombinantes Ta0895 spezifisch erkannten. Dies deutet auf eine erfolgreiche Anreicherung von SAMP-spezifischen Phagen hin.

Alles in allem diente dieses Projekt der Entdeckung von strukturell unbekannten mutmaßlichen Proteinkomplexen, die mit spezifischen Antikörpern detektiert und isoliert werden können, sowie der Bereitstellung einer geeigneten Aufreinigungsprozedur für Einzelpartikel-EM-Untersuchungen. Das Projekt umfasste auch die Ausarbeitung der Selektionstechniken und ihrer Screening-Systeme, sowie darüberhinaus die Entwicklung einer schonenden, antikörperbasierten Aufreinigungsstrategie, die dazu geeignet ist, hochmolekulare Proteinkomplexe für die strukturelle Untersuchung zu isolieren. Im Gegensatz zu anderen Antikörper-Archiven, bei denen der Antikörper-Satz gegen ein einziges Ziel-Antigen gerichtet ist, liefert das in dieser Arbeit erzeugte scFv-Archiv eine vielfältige Zusammenstellung von Antikörpern, und ermöglicht die Selektion spezifischer Bindungsparter für eine Reihe von Zielproteinen aus der hochmolekularen Proteinfraktion von *T. acidophilum*.

Zusammengefasst kann unsere archivbasierte Phagen-Display Strategie für die Proteinkomplexaufreinigung als Plattform für Untersuchungen in der visuellen Proteomik dienen, deren Ziel es ist, ein Template-Archiv für die Erstellung eines zellulären Atlases anzulegen.

ABSTRACT

Thermoplasma acidophilum is a thermoacidophilic archaeon that thrives at 59°C and pH 2 and belongs to the *Euryarchaeota* lineage. It has become an attractive model organism for visual proteomics approaches, which aims to provide a comprehensive cellular atlas of macromolecular complexes with the aid of cryo-electron tomography and pattern recognition procedures. *T. acidophilum* exhibits a colourful protein complex inventory, amongst which there are assemblies that look like simple variations of their eukaryotic counterparts and many of them are involved in protein folding, degradation and turnover. Most of these large protein complexes are structurally characterized, however, there are still many hypothetical and partially characterized assemblies whose molecular architecture and biochemical function is not yet explored.

To get an insight into the molecular architecture of protein complexes the isolation of intact structures is indispensable. Although a vast number of protein purification techniques are available, there is no universal method which would be effective to keep all the complexes in their intact and active state suitable for biochemical and structural analyses. One of the gentle and rapid purification methods is the antibody-based affinity purification, which allows the isolation of target complexes directly from their native environment, and serves sufficient amount of them for EM-analysis. Furthermore, in contrast to X-ray crystallography, single particle EM does not require the growth of protein crystals prior to the structural analysis, which radically reduces the time needed for sample preparation.

In this study, a scFv-based phage display library was developed, aiming the gentle purification of protein complexes from *T. acidophilum* with specific antibodies. While the antibody-based phage display technology was developed originally for single target antigens, we had to adapt it to a complex protein mixture containing at least 300 proteins. As a starting point, we created the phage display library by immunizing mice with the high molecular weight protein fractions (Sup12-HMWF) of *T. acidophilum* and constructing scFvs from the spleen total mRNA. To lessen antigen diversity, the phage selection and screening was first carried out against different K_2HPO_4-KH_2PO_4 elution fractions of OH-apatite separated Sup12-HMWF. After identifying positive binders we attempted the purification of protein

complexes with agarose bead immobilized phages, which conception failed due to the structural limitation of filamentous phage particles. Therefore, we had to change the strategy to a soluble antibody-based pull-down assay. Since the expression of soluble E-tagged scFvs did not prove to be reasonable as it required too much time and labor to monitor the location (cytosol, periplasm and/or medium) of soluble proteins, we developed a modified pET28 vector (pET28-*Sfi*I) to allow expression of scFvs with a C-terminal 6 Histidine fusion tag. The pull-down of protein complexes using the agarose-immobilized His-tagged scFvs resulted in the successful purification of the molecular chaperonin thermosome (Ta0980 and Ta1276), however, no intact particles could be obtained due to harsh elution conditions. To avoid dissociation and possible denaturation of protein complexes caused by antibody-antigen interaction abolishing eluents/agents, in the next pull-down approach complexes were purified by Ni-affinity chromatography followed by size exclusion chromatography. With the new purification approach we were able to isolate structurally intact thermosome (Ta0980 and Ta1276) and 20S proteasome particles (Ta0612 and Ta1288) and the probable peroxiredoxin (Ta0152), however, in the latter case the complex could not be visualized by EM.

As a next attempt, we applied the original antigen mixture (Sup12-HMWF), which resulted in many more phage binders than the selection against the further purified OH-apatite fraction. To further fine tune the efficiency of phage selection, de-selections against abundant protein complexes (thermosome, proteasome) were introduced in the biopanning, which was monitored with a comparative ELISA-assay to visualize the efficiency of library clean-up. Out of 373 tested clones 217 positive binders were identified, of which 62 (30%) appeared to recognize potential new target antigens. However, in spite of the successful cloning, none of the de-selected scFv clones could be sequenced and expressed in a phage-free form, indicating the enrichment of an unusual scFv form.

In the next phage-selection approach, we switched to individual recombinant proteins, which were assumed to be putative building blocks of probable protein complexes over the size of 300 kDa. The biopanning assays were carried out in different implementations (varying type and amount of Sup12-HMWF-selected phage inputs, number of selection cycles, incubation/washing conditions and purity of the target antigen). Using the well-working two-step chromatography method we were able to purify the putative intermedier filament Ta1488, however the EM analysis of purified fractions did not prove that Ta1488 form a

multimeric complex as it was predicted from its amino acid sequence. We also found antibodies against the archaeal homologue of the fixABCX complex, these antibodies, however, could not capture usable amounts of the putative complex. Instead, a formate dehydrogenase homologue protein (Ta0425) was enriched from the cell extract together with its putative small subunit constituent (Ta0424), corresponding to 450-500 kDa molecular weight, which complex forming ability was proved by EM-anaysis.

Since most of the native and recombinant target proteins exhibited low expression levels and/or were highly sensitive to the purification conditions, to overcome these hurdles, in the next approach phages were isolated with a novel selection technique using nitrocellulose membrane-immobilized antigens. The viability of this method was proved by the purification of the 5 MDa 2-oxo acid dehydrogenase multi-enzyme complex, which was successfully captured by a nitrocellulose-bound antigen-isolated scFv.

Finally, we carried out a library selection against the SAMP candidate Ta0895 protein aiming the capturing of SAMP-labelled proteins/protein complexes by pull down assay. Following phage selection, a significant number of phages proved to specifically recognize recombinant Ta0895 in both ELISA and Western blot conditions, which indicated the successful enrichment of SAMP specific phages from the system.

Taken together, the project aided the discovery of structurally unknown, putative protein complexes by finding and isolating them with specific antibodies to provide a feasible purification procedure for single particle EM studies. The project included the elaboration of selection techniques together with their screening systems, furthermore, the development of a gentle, antibody-based purification strategy suitable for isolating high molecular weight protein complexes for structural studies. In contrast to other antibody libraries, where the antibody set is targeted against a defined antigen, the scFv-library generated in this work provided a diverse antibody set allowing for the selection of specific binders for a variety of protein targets from the high molecular weight protein fraction of *T. acidophilum*. In conclusion, our phage display library-based protein complex purification strategy can serve as a platform for studies on visual proteomics approaches that aim the creation of a template library for the generation of a cellular atlas.

1. INTRODUCTION

1.1 *Thermoplasma acidophilum* as a model organism

Thermoplasma acidophilum is a thermoacidophilic archaeon that was first isolated from a self-heating coal refuse pile (1). The microorganism thrives at 59°C and pH 2 and belongs to the *Euryarchaeota* lineage, being a member of the genus *Thermoplasma*. *T. acidophilum* is a facultative anaerobe microbe which uses oxygen as terminal electron acceptor or S^0 if cultured anaerobically. The cell morphology is pleomorphous with the cell size varying between 0.2 and 2 µm. The common feature of the genus *Thermoplasma* is that the cells lack rigid cell wall and are delimited only by a plasma membrane. The genome size of *T. acidophilum* is around 1.5 Mbp and comprises 1507 open reading frames of which 1482 encodes for protein (2).

Proteomics studies based on 2DE-MALDI-TOF MS approach provided information on the expressed cytosolic proteins and macromolecular complexes of *T. acidophilum* (3). In the protein complement of *T. acidophilum* there are a number of macromolecular assemblies playing important role in protein folding, degradation and methabolic pathways. Based on database searches, many of those highly expressed proteins that form complexes are identified and structurally characterized, such as the archaeal chaperon thermosome, the VCP-like ATPase (VAT), which participates in numerous cellular activities (4-8), the "protein graveyard" 20S proteasome, (9) or the tricorn protease, the core of a modular proteolytic system (10). Molecular sieve chromatography in combination with LC-MS/MS helped to reveal less abundant cytosolic proteins on the basis of size distribution and bioinformatics analysis and thus, further enlarging the inventory of protein complexes (11). Using state-of-the-art mass spectrometric technologies 1025 proteins were identified, covering 88% of the cytosolic proteome of *T. acidophilum* (12). In the cytosolic proteome of *T. acidophilum* a large proportion of the proteins are organized into dimer, trimer, oligomer or multimer forms, amongst which 35 macromolecular assemblies have been identified with sizes over 300 kDa. These large protein structures serve as adequate candidates to create template libraries for visual proteomics studies and thereby promoting the generation of a comprehensive cellular atlas of macromolecular complexes (13). Visual proteomics approaches aim to determine

spatial relationships of macromolecular complexes inside an unperturbed cellular environment by using cryoelectron tomography and pattern recognition procedures. The lack of cell wall, relatively small genome and low cellular complexity make *T. acidophilum* a favourable model organism for visual proteomics approaches.

The question, how extremophiles can survive harsh environments, has remained in the focus of research for decades. The ability to respond to extreme environmental challenges requires not only particular physiological functions but also structural suitability. These often results in strange appearances and the presence of unusual biochemical pathways. *T. acidophilum* is an interesting organism representing a colorful protein complex inventory, amongst which there are assemblies that look like simple variations of their eukaryotic counterparts. With the help of single particle electron microscopy and X-ray crystallography many of these large complexes have been structurally characterized, however, there are still many hypothetical and partially characterized protein complexes whose molecular architecture and biochemical function is not yet explored. The characterization of these macromolecular assemblies could help to elucidate the structure and function of their similar but more complex eukaryotic homologues.

1.2 Protein complexes

1.2.1 Protein complexes and their structure determination

Protein complexes are assemblies of multiple proteins that play essential roles in living organisms in accomplishing and regulating cellular processes. To be able to perform their molecular functions macromolecules fold into specific three-dimensional shapes acquiring their functional, native state. The final architecture is determined by the primary structure of molecules (i.e their amino acid sequences). Within the long polypeptide chains there are regions which form ordered secondary structure elements, namely α-helices and β-sheets. To carry out biochemical processes, many single protein molecules are organized into multi-molecular complexes allowing the interaction partners to function as one entity, executing several multi-step reactions in an ordered way at a time. These multi-molecular assemblies

give the proteins the quaternary structure, in which the number and type of individual proteins (subunits) determines their functional characteristics and structural appearances. The quaternary structure of a protein complex depends on the oligomerization capacity of the protein subunits, which can form dimers, trimers, tetramers and other oligomers. The subunit content of a multimeric protein may be identical, similar or completely different concerning the type of proteins. Protein complexes being assembled with the same type of proteins are called homo-oligomers or homo-multimers, while complexes containing dissimilar subunits are known as hetero-oligomers or hetero-multimers. Many naturally occurring proteins and enzymes are arranged in oligo- or multi-protein complexes to carry out their biological functions in the intracellular environment or in the cell membrane. Such well-known protein complexes are for instance the homo-tetramer hemoglobin, the bacterial chaperone GroEL, the multi-enzyme complex 50S and 70S ribosomes, the PSI and PSII photosystems, the 26S proteasome, the pyruvate-dehydrogenases, and include furthermore several ion-channels, microtubules and other cytoskeletal proteins (14).

To understand molecular functions and interactions taking place in different locations of the cell - like cytoplasm, nucleus or membrane - the structural and functional investigation of protein complexes is essential. Molecular structural biology studies the 3-dimensional structure of biological macromolecules, how they acquire their structure, and how alterations in their structures affect their function. The methods that structural biology uses for determining 3D structures generally involve measurements on vast numbers of identical molecules at the same time. The most widely applied methods for structural determination include X-ray christallography, electron microscopy, NMR and CD-spectroscopy. Single particle electron microscopy is an increasingly important tool for the structural analysis of macromolecular complexes, whose main advantage is over other methods that it does not require the growth of protein crystals prior to structural analysis. Cryoelectron microscopy (CEM) is particularly important for membrane proteins and large protein complexes where generating crystals is often the main barrier to structural determination. Furthermore, (CEM) allows the observation of specimens that have not been stained or fixed in any way, showing them in their native environment in contrast to X-ray crystallography, which generally requires placing the samples in non-physiological environments that occasionally leads to functionally irrelevant conformational changes. However, by combining single particle EM

maps with high-resolution models of subunits or sub-complexes from X-ray crystallography and NMR spectroscopy, it is possible to build up an atomic model of a macromolecular assembly (15).

Computational data acquisition is used for data processing, image analysis, averaging and aligning different datasets thereby, contributing to visualizing complete molecular architectures. Bioinformatics approaches help to acquire complicated datasets and look for patterns among the diverse sequences that give rise to particular shapes. A number of computer programs developed for protein structure prediction, modelling and structural alignment are now available and used to deduce information on protein structure (HNN, PSA, Swiss model, PyTOM, PQS, PyMOL).

1.2.2 Purification strategies of protein complexes

Most purification schemes involve some form of chromatography, which are based on the separation of protein components. Classical protein purification procedures are based on the combination of chromatography techniques allowing the optimal purification yield required for further studies. Since there is no universal method which would be effective to keep all complexes in their intact and active state, the purification strategy of an unknown structure should always be determined experimentally on an individual basis, which often requires a number of time-consuming polishing steps. Different chromatography techniques with different selectivities like size exclusion chromatography, glycerole and/or sucrose gradient ultra centrifugation, ion-exchange chromatography, hydrophobic interaction chromatography or hydroxyl-apatite chromatography can form powerful combinations for the purification of any biomolecule. Although multi-step purification procedures often yield excellent purity of proteins, those, which may be fairly easy to obtain in a pure state, are not always stable at the applied conditions. Some proteins tend to aggregate, dissociate or unfold over a period of time, which may lead to a significant loss of actively folded protein assemblies. The time of purification procedure should therefore be always reduced to a minimum in order to avoid unwanted changes in protein structure prior to the structural analysis.

To accelerate the purification procedure novel methods have been developed. The development of recombinant DNA techniques has revolutionized the production of affinity tagged proteins in large quantities, facilitating their solubility and their purification by the appropriate affinity chromatography (SUMO-tag (16), FLAG-tag (17), Strep-tag (18) His-tag (19), GST-tag (20)). Although heterologous expression systems may enable the production of high quantity of target proteins, the host contaminants are still present and problems related to solubility, structural integrity and biological activity can still exist. Therefore, chromosomally tagged protein purification technologies are becoming more and more popular if genetic tools are available for the investigated host. Free-flow electrophoresis (FFE) is used for the analytical and preparative separation of intracellular components from the native cellular environment (21, 22). It separates charged particles ranging in size from molecular to cellular dimensions according to their electrophoretic mobilities (EPMs) or isoelectric points (pIs). However, it is not always an applicable method, since it may cause the disassembly of instable protein structures in the electric field. Native polyacrylamide gel electrophoresis combined with electron microscopy grid blotting is a fast and efficient method to transfer high molecular weight protein complexes from the acrylamide gel matrix directly to electron microscopy grids, which promote a quick structural analysis (23). This technique can be applied if the proper separation of certain protein complexes from each other is not feasible by conventional chromatography methods. However, it can only be used for protein bands containing high quantities of protein samples.

Single particle electron microscopy approaches does not require high protein quantities for the structural determination in contrast to X-ray crystallography. Therefore, the antibody-based small-scale pull-down assays may alone supply sufficient amount of protein samples for image analysis and data acquisition. This can be crucial, if the protein target is expressed at extremely low level and cannot be traced by conventional chromatography methods. The capturing of natively folded proteins with highly specific monoclonal antibodies directly from cell extracts is a mild and fast purification method, which usually does not require additional purification steps and can also be used to study specific protein-protein interactions. Large sets of combinatorial antibody libraries have nowadays been constructed and selected for target specificities using the phage display technology (24), which has revolutionized

antibody engineering by extending the antibody repertoire with potential new binders. By combining the epitope recognizing variable regions of IgG (Immunoglobulin G) molecules, small molecular weight, recombinant antibody libraries can be generated (scFv, Fab, dsFv) that allow a wider utility of antibodies by means of their increased stability (25-29). Conventional protein pull-down assays can be used to enrich high molecular weight protein complexes by highly specific antibodies and are frequently used in proteomics studies to investigate the structural constitution and interaction partners of protein assemblies (30-32).

1.3 Phage display technology

1.3.1 Overview

Phage display technology is a powerful method to study protein–protein, protein–peptide, and protein–DNA interactions. The technology was first described in 1985 by George Smith, who introduced bacteriophage as an expression vector that displays peptides on the surface of a virion (24). Since then it has become a widely used technique in cell biology, immunology, pharmacology and drog discovery. In phage display technology the DNA encoding the protein of interest is fused to a gene that encodes for one of the coat proteins, thus allowing the presentation of large peptides and proteins on the surface of a filamentous phage. The bacteriophage - displaying the target protein - delivers its genetic material into *E. coli* via an expressed F-pilus displayed on the surface of the cell envelop. This way, the connection between genotype and phenotype, allows screening and amplification of large protein libraries in a process called “in vitro selection”, which is analogous to natural selection. Phage display libraries containing up to 10^{10} variants can be constructed simultaneously, allowing high-throughput screening of protein interactions, instead of low-throughput individual engineering of proteins or peptides to express, purify, and analyze each variant of them one at a time. In the past decades a large number of phage display libraries have been constructed, leading to various approaches and techniques for library screening procedures (25, 33-38).

1.3.2 Bacteriophages and their role in phage display technology

Bacteriophages (or simply phages) are viruses that infect bacteria by injecting genetic material into the host cell. Infection occurs by attachment to specific receptors on the surface of bacteria, including lipo-polysaccharides, teichoic acids, proteins or flagella. The host range of phage is determined by its receptor specificity and only those bacteria can be infected which possess the phage specific receptor. The phage genome can be single- or double-stranded DNA or RNA arranged in either circular or linear forms. The protective coating of the genome is composed of phage genome encoded proteins. Phages use bacterial ribosomes to translate their mRNAs to proteins required for replication and virion assembly. There are two versions of viral reproduction cycles: the lytic and the lysogen types. Some phages, like T4 are only capable of lytic growth, in which the phage genome replicates independently from the host chromosome and the assembled virions cause immediate cell lysis when exiting the cell. In contrast, lysogen or temperate phages, like lambda phages, can maintain their genome as a "prophage" in a dormant state, in most cases within the chromosome of a bacterial host, which allows the phage genome to replicate together with the host chromosome. The release of phage genome will occur at certain environmental conditions followed by proliferation of new virions via the lytic cycle. From this point of view filamentous phages are exceptional, because they do not kill the host cell while exiting it, thus they are considered to be non-lytic phages making them the most frequently used helper phages in phage display technology.

Phages mostly used in phage display technology are filamentous, however, there are also examples of studies based on T4 (39), T7 (40) or lambda phage (41) aided library screening methods. Filamentous phages have filament- or rod-like shape and possess a circular, single-stranded DNA genome. They belong to the family Inoviridae, including a number of Enterobacteria, Pseudomonas, Xanthomonas and Vibrio phage species (http://www.ictvonline.org). Filamentous phages which infect F' episome bearing Gram negative bacteria are known as Ff phages. Their infection procedure starts with the attachment of protein pIII to the F-pilus, leading to a fusion of the phage and the bacterial membrane. Finally, the viral genome is released into the cytoplasm. The most commonly used filamentous phages in phage display technology are the closely related M13, f1 and fd phages which have highly similar structural and functional characteristics.

The genomes of M13, f1 and fd phages are more than 98% identical and their gene products are interchangeable. Ff phage particles are approx. 930 nm long and 6.5 nm in diameter exhibiting a worm-like shape (Figure 1.1). Its genome comprises ~6400 nucleotides, encoding for 11 genes. The viral mass is approximately 16.3 MDa, and composed of varying copy numbers of five capsid proteins (Figure 1.2). The most abundant protein component is the major coat protein pVIII (gene VIII), which contributes with ~2700 copies to the structure of virion. The positively charged C-terminal part of pVIII is located inside the phage particle close to the DNA, while the N-terminal part is exposed to the surface. The blunt end of the phage contains 3 to 5 copies of both pVII and pIX proteins (genes VII and IX) and on the other end there are about 5 copies of proteins pIII and pVI (42). Most display applications exploit pIII as a fusion tag of recombinant peptides or proteins. The surface exposed N-terminal domain of pIII is responsible for binding to the F-pilus of bacteria, while the C-terminal domain is buried in the particle contributing to the integrity of the capsid.

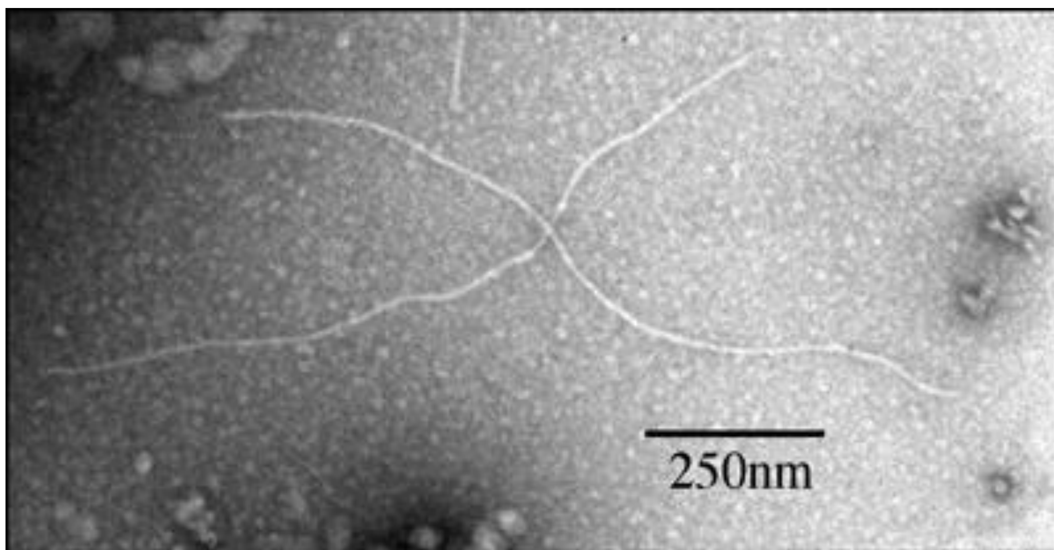


Figure 1.1: Negatively stained EM image of M13KO7 Helper phage (www.haverford.edu/biology/Johnson)

Phages can tolerate the presence of large DNA sequences encoding for additional genetic information within the viral genome. Since the coat dimensions are flexible, the number of pVIII copies is adjusted to the size of genome being packed. This feature enables the

application of these phages as cloning vectors in phage display applications, in which a large number of different clones are constructed simultaneously during cDNA library construction.

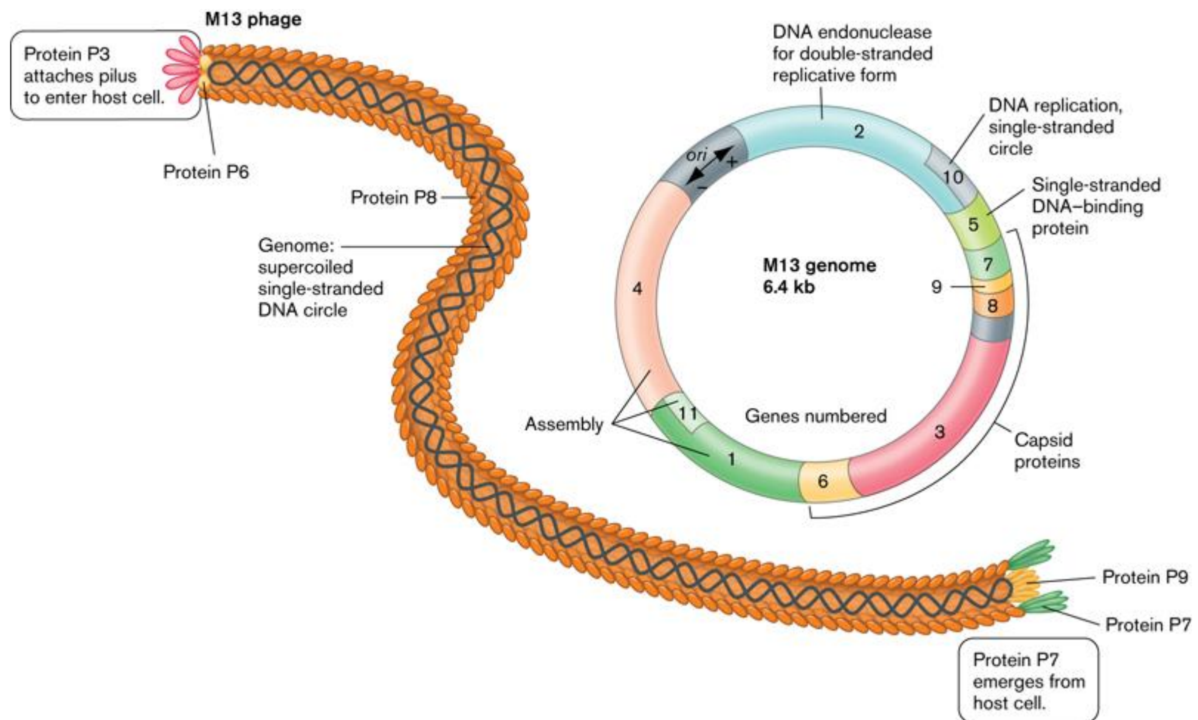


Figure 1.2: Structrure of Ff phages and the genome of M13 phage (34).

1.3.3 Phagemid vectors

Phagemid is a type of cloning vectors developed as a hybrid of M13 phage and plasmids. It can replicate independently as a plasmid in bacteria, but it also can be packaged as single-stranded DNA in viral particles. The production of double-stranded DNA is allowed by the prokaryotic ori region (*ColE1* ori) that derives from the naturally occurring *E. coli* plasmid ColE1. Besides that, the vector contains the Ff origin of replication (M13, f1 or fd type) to enable single-stranded replication by rolling circle mechanism and packaging into phage particles. Along with this, phagemids also encode an antibiotic resistance marker for maintenance as a plasmid in *E. coli* and a fusion gene under the control of a regulated promoter. Initially, phagemid vectors were used to generate single-stranded DNA for dydeoxy sequencing (43). Recently, most of the phagemid vectors are used for general DNA cloning,

transcription or in vitro mutagenesis, such as the pUC19 derivative pTZ19R/U (Fermentas), pBS (Stratagene), pCDNA3 (Invitrogene), pCF20 (USB) and there are a number of expression constructs as well, like the commonly used pBluescript II (Stratagene), pAMP1 (BRL), pCDM8 (Invitrogene) and pEX1 (Clontech). These vectors are applicable in *E. coli* hosts but there are also available phagemids as *E. coli/Saccharomyces* shuttle vectors, like pFL64 (ATCC) or the pGhost shuttle vectors (Applichem), which have broad host range.

Phage display vectors

The common feature of phage display vectors is that they possess an expression/display cassette beside the phagemid backbone. The display cassette contains an inducible promoter (e.g. lac) followed by a leader sequence (or signal sequence) that directs the synthesized peptide/protein to the bacterial periplasm where the phage particle is assembled. The most frequently used signal sequences are pelB (44-46) and the M13-derived gIII leader peptide (47-49). In the periplasm, the signal sequence is cleaved by a signal peptidase before the fusion protein is incorporated into the phage coat. There are restriction sites for cloning purposes and a fusion peptide tag (e.g. poly-histidie-tag, myc-tag, Flag-tag) that allows detection of soluble expression products downstream of the leader sequence. The expression/display cassette ends with the phage coat protein gene (usually pIII or pVIII), which allows the expressed protein/peptide to be incorporated into the phage particle and to be displayed. Another substantial property of phage display vectors is that they lack necessary viral components for phage assembly consequently, they are not infectious. For the conversion into infective phages the phagemid-bearing cells must be super-infected with helper phage (VCSM13 or M13KO7) that supplies all the necessary genes required for single-stranded DNA replication and packaging into phage particles.

1.3.4 Theory of phage displaying

The process starts with the transformation of appropriate *E. coli* cells with the phagemids carrying the DNA fragments of a given library (e.g. Fab, scFv, cDNA, peptide library) thus, generating a phage display library. Commonly used *E. coli* strains for phage display purposes

are TG1, JM110, SS320, DH5αF’, XL1-Blue or ER2738. These strains carry the F' episome, needed for the growth of the F-pilus. Figure 1.3 demonstrates the process of phage displaying with the substantial elements. The first step is the infection of *E. coli* cells with helper phages and the introduction of helper phage genome into the host cell. The virus genome starts replicating and virus components will be expressed from the double-stranded DNA. In the mean time, fusion protein expression and single-stranded DNA replication occurs from the phagemid vector with the assistance of helper phage proteins. During virion assembly, single stranded phagemids are packaged into the phage capsid, whilst the recombinant protein fused to the coat protein also incorporates into the virion. The assembled phage particles then continuously exit the host cell by budding and accumulate in the medium. Helper phages are usually engineered to be packed less efficiently than phagemids due to their defective replication origin. However, they provide all the necessary structural protein components to pack the phagemid DNA in a way that the resultant phage particles contain predominantly phagemid DNA. This feature enhances the efficiency of library screening procedure, since the protein displaying helper phage background is dramatically reduced. The expressed peptides/proteins are displayed in an intact form available for other macromolecular targets for interaction studies. In this way, not only 5-20 amino acid long peptides but also larger proteins, like antibody Fab fragment, scFvs or any other protein can be displayed.

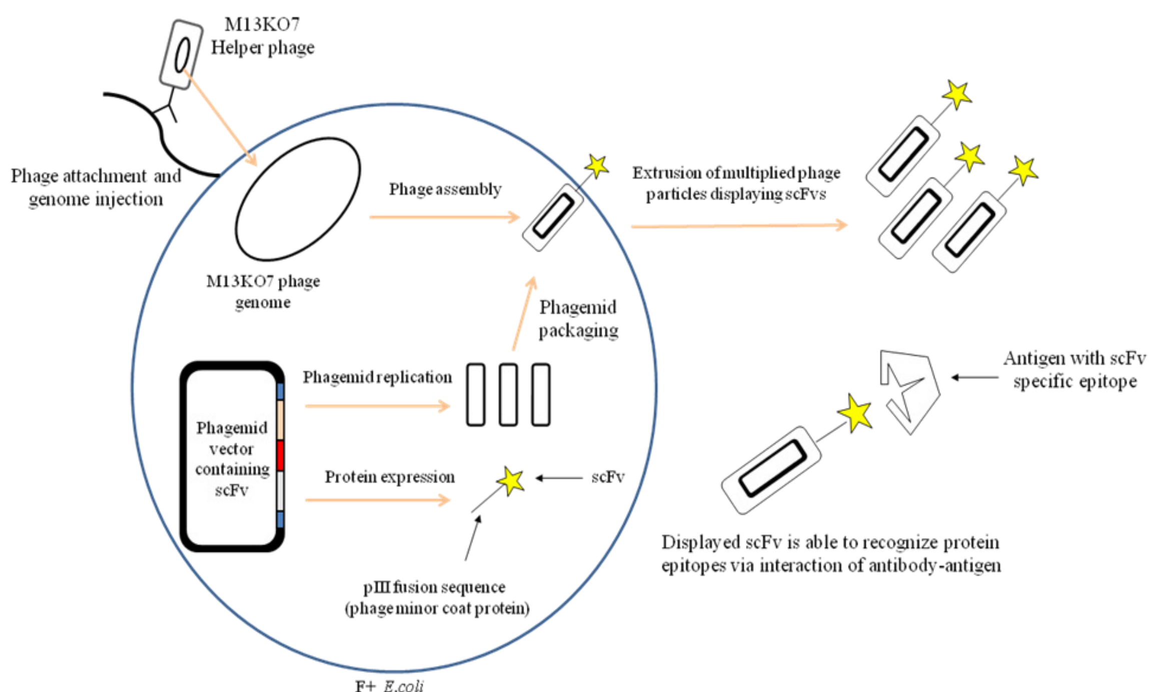


Figure 1.3: Theory of phage displaying demonstrated with the example of scFv-displaying system

1.3.5 Phage display libraries and their applications

The first phage display application was incarnated in the enrichment of only one expressed and displayed protein against the wild type phage population (24). In the past decades the technique has become a widely used high-throughput method for library screening procedures in molecular interaction studies, since a vast number of peptides, proteins, protein domains and antibodies have been routinely displayed on phages (50-52). Phage display-based libraries serve as robust tools for selecting proteins or peptides with specific binding properties from huge numbers of variants and, as such, considerably reduce the time and simplify the work required for time-consuming screening procedures.

In the aspect of fragment sizes being displayed, peptide or protein displaying libraries can be distinguished. Both libraries bear the common feature that the information of displayed fragments is encoded on DNA inserts, which are cloned in the phage display vector. Biological display systems apply natural libraries, in which the genomic information coding for a given protein repertoire derives from a biological host and its biological reactions, while non-biological libraries provide synthetically produced peptides or semi-synthetic derivatives. Natural peptide libraries are constructed by ligating randomly fragmented genomic DNA with phagemids (53-55). Synthetic peptide libraries contain randomly synthesized DNA fragments encoding for artificial peptide sequences (56-60). In the last few years a number of natural cDNA libraries have also been constructed and developed for phage display purposes (61-63). By the display of cDNA libraries we can get insights into a portion of the transcriptome of a variety of organisms. Antibody phage display libraries are also considered to be cDNA libraries however, they only represent certain domains of full length protein sequences. The isolation of monoclonal antibodies using large antibody fragment libraries has been one of the most successful applications of phage display (25, 50, 64) and become a powerful alternative to hybridoma technology (65).

Antibody libraries and display formats

Efficient techniques have been developed to design and build large libraries of antibody fragments, and several ingenious selection procedures have been established to derive

antibodies with the desired characteristics. IgG (Immunoglobuline G) is an isotype of immunoglobulines that is found most abundantly in the circulation. IgGs are 150 kDa molecules consisting of two identical heavy chains and two identical light chains, forming a fork-like structure. They are secreted by the plasma B cells into the circulation. The effective display formats for antibodies are scFvs (single chain variable fragment) (25, 66, 67), Fab fragments (fragment antigen binding) (26, 27, 34, 68), dsFvs (disulfide-stabilized variable fragment) (28, 29, 69) and diabody fragments (70, 71). The common feature of these fragments is that they all contain the epitope recognizing variable region of IgGs in different formats (Figure 1.4).

Fab-displaying libraries essentially comprise both heavy and light chain constituent of the whole Fab fragment. To display Fab fragments on phage, one of the chains is fused to pIII, and the partner chain is expressed un-fused. The two chains are separately secreted into the periplasmic space, where they associate to form an intact 50 kDa Fab fragment. ScFv libraries encode only for the variable region of IgGs and are thus, the truncated versions of Fab, lacking its non-variable regions. The variable fragments of the heavy and light chains are connected via a linker peptide, which stabilizes the formation of scFv. The smaller size of the scFv format makes these libraries genetically more stable than Fab libraries. Another form of antibody libraries is the dsFv-displaying library, in which an intermolecular disulphide bond stabilizes the variable fragments, engineered between structurally conserved framework positions of the two chains (28, 29, 69). Antibody libraries may also be constructed for displaying diabodies, which are the dimerized versions of scFvs (70, 71). Diabodies can be designed for bivalent or bispecific interactions from a bicistronic cassette containing two VH-VL fusion products (72). By reducing the length of the linker peptide of scFvs, the pairing between the two domains on the similar chain will be hindered, thus, they are forced to pair with the complementary domains of another chain. One of the two VH-VL fusion products is fused to the phage coat protein by which the diabodies can be incorporated into the capsid and displayed (71).

While most of the known IgGs consist of two identical heavy and two identical light chains, the serum of *Camelidae* contains also a considerable fraction of heavy chain antibodies (HcIgG). Camelid “heavy chain” IgGs consist of two heavy chains and completely lack light chains, although they are fully functional (73). Another characteristic feature is the lack of

CH1 non-variable region in the full heavy chain, as it is spliced out during mRNA processing due to loss of a signal sequence. Besides *Camelidae*, the serum of cartilaginous fish (sharks, rays, chimaeras) also contains heavy chain antibodies but in a different format (74-76). They possess five constant regions in their heavy chains which end with variable domains called IgNARs (Immunoglobulin new antigen receptor). Single domain antibodies (nanobodies) consist of a single monomeric variable antibody domain and are derived from heavy chain antibodies. Nanobody phage display libraries are made from the variable domain of camelid (dromedaries, alpacas, lamas, camels) antibodies (VHH fragments-display) and from IgNAR antibody isotypes of cartilaginous fish species (VNAR-display). The most commonly used camelid type nanobodies are highly soluble and very stable under challenging conditions as high salts, detergents or at elevated temperatures, which advantage can be exploited in numerous medical and biotechnological applications (77-82).

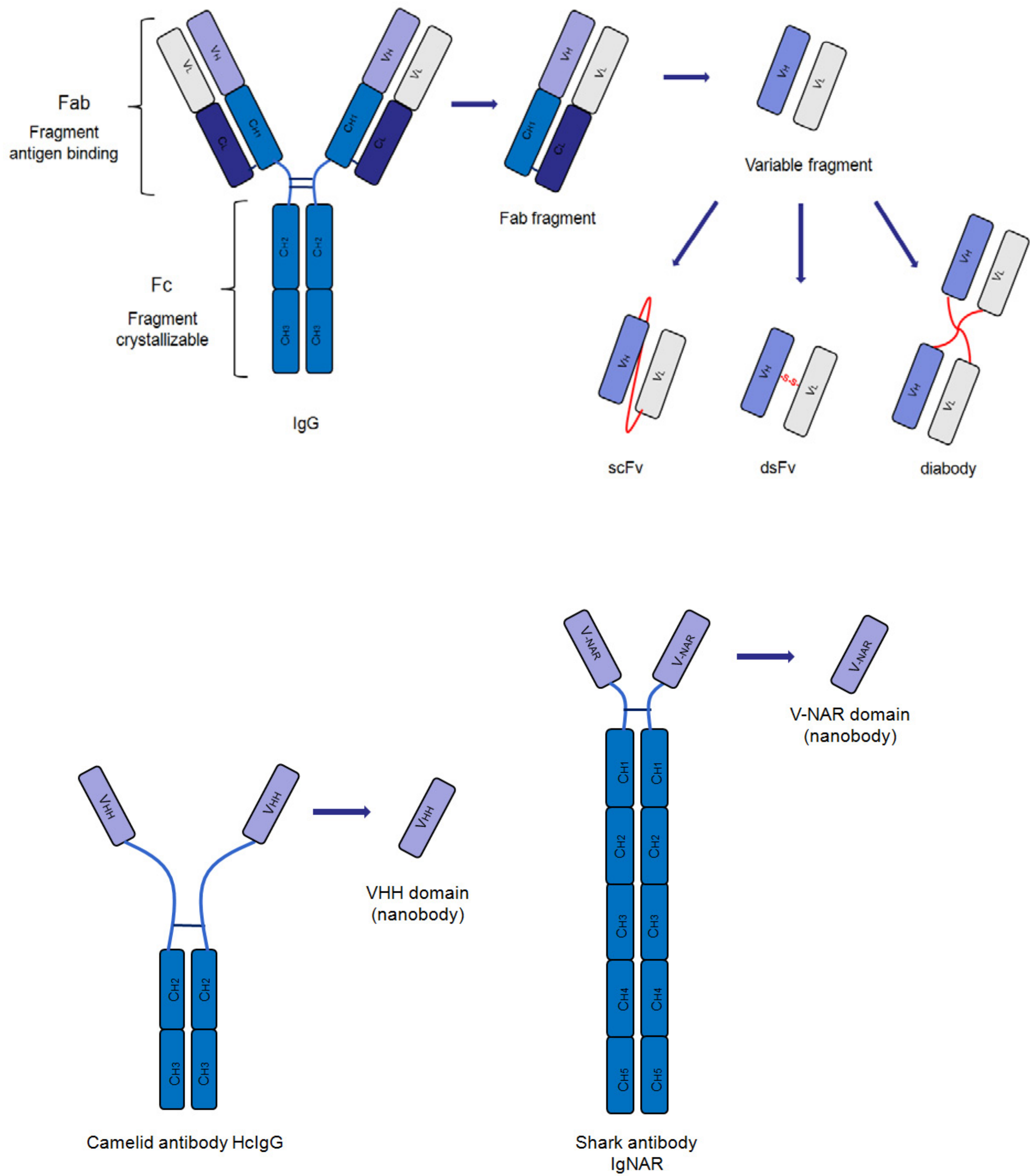


Figure 1.4: Structure of IgG and its derivatives for phage display purposes IgG: Immunoglobuline G, VH: Varible fragment of heavy chain, VL: Variable fragment of light chain, scFv: Single chain variable fragment, dsFv: disulphide-stabilized varible fragment, CH1-5: Constant regions of the heavy chain, CL: Constant region of the light chain, HcIgG: Heavy chain IgG, VHH: Heavy chain variable domain of the heavy chain IgG, IgNAR: Immunoglobulin new antigen receptor, V-NAR: Heavy chain variable domain of IgNAR.

2. AIM OF THE STUDY

In this study we aimed the isolation of native protein complexes from the cytosolic extract of *T. acidophilum* using an antibody library, thereby providing homogenous samples for EM-based structural studies. For this purpose we adapted the mono-antigen targeted phage display technology to a multi-antigen targeted version and generated a combinatorial scFv-library against the high molecular weight fraction of *T. acidophilum* which contained more than 300 proteins as subunits of protein complexes over the size of 300 kDa. Since protein complexes are sensitive to harsh purification conditions, our goal was to develop an antibody-based mild and fast protein isolation technique, which can keep the molecular assemblies in their intact and active form for subsequent structural and biochemical analyses. The project involved the implementation of sophisticated selection and screening assays that aided the identification of positive binders from a huge antibody repertoire against low abundant complexes, analogue to finding needles in a haystack with “magnetic” needles found in another haystack.

3. MATERIALS AND METHODS

3.1 Applied materials

3.1.1 Chemicals

Chemical	Chemical formula	Company
Acetic-acid	$C_2H_4O_2$	Merck
Acrylamide/bisacrylamid mix (30%, mixing ratio 37.5:1)	C_3H_5NO /$C_7H_{10}N_2O_2$	Roth
5-aminosalicylic acid (5-ASA)	$C_7H_7NO_3$	Sigma-Aldrich
Ammonium persulfate (APS)	$(NH_4)_2S_2O_8$	Roth
Ammonium sulphate	$(NH_4)_2SO_4$	Merck
Ampicilin	$C_{16}H_{19}N_3O_4S$	Sigma-Aldrich
Adenosine tryphosphate (ATP)	$C_{10}H_{16}N_5O_{13}P_3$	Sigma-Aldrich
β-mercaptoethanol	$HOCH_2CH_2SH$	Bio-Rad
Bromophenolblue	$C_{19}H_{10}Br_4O_5S$	Merck
Calcium chloride	$CaCl_2$	Merck
Carbenicilin	$C_{17}H_{18}N_2O_6S$	Sigma-Aldrich
Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$	ApplyChem
Cobalt (II) sulphate	$CoSO_4 \cdot 7H_2O$	Merck
Coomassie brillant blue R250	$C_{45}H_{44}N_3O_7S_2 \cdot Na$	Serva
Copper (II) chloride	$CuCl_2 \cdot 2H_2O$	Merck
Dimethyl sulphoxide (DMSO)	C_2H_6OS	Serva
Dithiothreitol (DTT)	$C_4H_{10}O_2S_2$	Merck
Dipotassium phosphate	K_2HPO_4	Merck
Disodium phosphate	Na_2HPO_4	Merck
Disodium tetraborate	$Na_2B_4O_7 \cdot 10H_2O$	Merck
Ethanol (absolute)	C_2H_6O	Merck
Ethylenediaminetetraacetic acid (EDTA)	$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$	Merck
Glucose	C6H12O6	Sigma-Aldrich
Glycerol (~ 87%)	C3H8O3	Merck
Glycine	C2H5NO2	Sigma-Aldrich
Hydrogen chloride	HCl	Merck
Hydrogen peroxide	H2O2	Merck

Imidazole	$C_3H_4N_2$	Merck
Iron (III) chloride	$FeCl_3 \cdot 6H_2O$	Merck
Isopropyl alcohol	$(CH_3)_2CHOH$	Sigma-Aldrich
Isopropyl β-D-1-thiogalactopyranoside	$C_9H_{18}O_5S$	Sigma-Aldrich
Kanamycin-sulphate	$C_{18}H_{36}N_4O_{11}$	Sigma-Aldrich
Magnesium chloride	$MgCl_2 \cdot 6H_2O$	Merck
Magnesium sulfate	$MgSO_4 \cdot 7H_2O$	Merck
Manganese (II) chloride	$MnCl_2 \cdot 4H_2O$	Sigma-Aldrich
Methanol	CH_4O	Sigma-Aldrich
Monopotassium phosphate	KH_2PO_4	Merck
Monosodium phosphate	NaH_2PO_4	Merck
Polyethyleneglycol (PEG 8000)	$C_{2n}H_{4n+2}O_{n+1}$	Merck
Potassium chloride	KCl	Merck
Sodium azide	NaN_3	Sigma-Aldrich
Sodium carbonate	Na_2CO_3	Merck
Sodium chloride	NaCl	Sigma-Aldrich
Sodium bicarbonate	$NaHCO_3$	Merck
Sodium hydroxide	NaOH	Merck
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	Merck
Rubidium chloride	RbCl	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	$NaC_{12}H_{25}SO_4$	Roth
Sulfuric acid	H_2SO_4	Merck
Tetramethylethylenediamine (TEMED)	$(CH_3)_2NCH_2CH_2N(CH_3)_2$	Sigma-Aldrich
Thiamine hydrochloride	$C_{12}H_{17}ClN_4OS \cdot HCl$	Sigma-Aldrich
Thiamine pyrophosphate (TPP)	$C_{12}H_{19}N_4O_7P_2S^+$	Sigma-Aldrich
Triethylamine	$C_6H_{15}N$	Sigma-Aldrich
Tris-(hydroxymethyl)-aminomethane	$(HOCH_2)_3CNH_2$	Merck
Uranyl acetate	$UO_2(CH_3COO)_2 \cdot 2\,H_2O$	Sigma-Aldrich
Vanadyl sulfate	$VOSO_4 \cdot 5H_2O$	Merck
Zinc sulfate	$ZnSO_4 \cdot 7H_2O$	Sigma-Aldrich

Other ingredients:

Agarose	A9539	Sigma-Aldrich
Bacto Agar	214030	BD
Bacto Tryptone	211705	BD
Bacto Yeast extract	210934	BD
Skim milk powder	70166	Sigma-Aldrich
Tween20™	170-6531	Bio Rad

3.1.2 Kits

EzWay™PAG Protein Elution Kit	K33010	Koma Biotech
Pure Yield Plasmid Miniprep System	A1222	Promega
Pierce® Co-immunoprecipitation Kit	26149	Thermo Scientific
QIAprep Spin Miniprep Kit	27104	Qiagen
QIAquick Gel Extraction Kit	28704	Qiagen
QIAquick PCR purification Kit	28104	Qiagen
QuikChange® Site-Directed Mutagenesis Kit	200518	Agilent Technologies
Rapid DNA ligation Kit	K1422	Fermentas
Ready-To-Go RT-PCR beads	27-9266-01	GE Healthcare
RNeasy Midi Kit	75144	Qiagen
Wizard® SV Gel and PCR Clean-up system	A9281	Promega

3.1.3 Standards

GeneRuler™ 1 kb DNA Ladder (250-10000 kb)	SM0311	Fermentas
HMW Native Marker Kit (66-669 kDa)	17044501	GE Helthcare
PageRuler™ Prestained Protein Ladder (10-250 kDa)	SM0671	Fermentas
Prestained Protein Molecular Weight Marker (20-120 kDa)	SM0441	Fermentas

3.1.4 Dyes and staining solutions

DNA loading dye	R0611	Fermentas
GelRed^{TM} DNA dye	41001	Biotium
Instant Blue protein dye	07-300-150	Fisher Scientific

6 x SDS protein sample buffer:
375 mM Tris-Cl, pH 6.8
6 % (w/v) SDS
48 % (v/v) glycerol
9 % (v/v) β-mercaptoethanol
0.03 % (w/v) bromophenol blue

3.1.5 Antibodies

Goat anti-rabbit IgG-AP conjugate	sc-2034	Santa Cruz Biotechnology
His-probe (6-18) rabbit polyclonal IgG	sc-803	Santa Cruz Biotechnology
Mouse anti-M13KO7-HRP conjugate	45-001-419	Amersham Pharmacia Biotech
Goat anti-mouse IgG-AP conjugate	sc-2047	Santa Cruz Biotechnology
Rabbit anti-E-tag monoclonal antibody	A190-133P	Bethyl Laboratories

3.1.6 Bacterial strains

E. coli BL21(DE3)	C6000-03	Invitrogene
E. coli HB2151	27-9401-01	Amersham Biosciences
E. coli Rosetta 2 (DE3)	71400-4	Merck Chemicals Ltd
E. coli TG1	27-9401-01	Amersham Biosciences
E. coli TOP10	C4040-10	Invitrogen
E. coli Tuner (DE3) pLysS		Biochemistry core facility of MPI
E. coli XL10 Gold	200-314	Agilent technologies
Thermoplasma acidophilum DSM 1728		

3.1.7 Enzymes

Restriction enzymes:

*Ecl*136II	ER0251	5’...GAG$^{\downarrow}$CTC...3’ 3’...CTC$_{\uparrow}$GAG...5’	Fermentas
Fast Digest *Nco*I	FD0573	5’...C$^{\downarrow}$CATGG...3’ 3’...GGTAC$_{\uparrow}$C...5’	Fermentas
Fast Digest *Not*I	FD0593	5’...GC$^{\downarrow}$GGCCGC...3’ 3’...CGCCGG$_{\uparrow}$CG...5’	Fermentas
Fast Diges *Sfi*I	FD1824	5’...GGCC$^{\downarrow}$CAGCCGGCC...3’ 3’...CCGGGTCGG$_{\uparrow}$CCGG...5’	Fermentas
Fast Digest *Xho*I	FD0694	5’...C$^{\downarrow}$TCGAG...3’ 3’...GAGCT$_{\uparrow}$C...5’	Fermentas
Fast Digest *Nde*I	FD0584	5’...CA$^{\downarrow}$TATG...3’ 3’...GTAT$_{\uparrow}$AC...5’	Fermentas
*Not*I	R0189M	5’...GC$^{\downarrow}$GGCCGC...3’ 3’...CGCCGG$_{\uparrow}$CG...5’	New England Biolabs
*Sfi*I	R0123L	5’...GGCC$^{\downarrow}$CAGCCGGCC...3’ 3’...CCGGGTCGG$_{\uparrow}$CCGG...5’	New England Biolabs
*Sph*I-HF	R3182S	5’...GCATG$^{\downarrow}$C...3’ 3’...C$_{\uparrow}$GTACG...5’	New England Biolabs

Other enzymes:

Bensonase® Nuclease	E1014	Sigma-Aldrich
DNase I from bovine pancreas	10104159001	Roche
Lysozyme from chicken egg white	L7651	Sigma-Aldrich
Pfu DNA polymerase	EP0571	Fermentas
T4 Ligase	M0202L	New England Biolabs

3.1.8 Plasmids and phagemids

M13KO7 Helper phage	N0315S	New England Biolabs
pCANTAB 5E	27-9401-01	Amersham Biosciences
pET28a	69864-3	Novagen

3.1.9 Primers

The applied primers were HPLC-purified and synthesized by Metabion GmbH.

Primer No.	Primer name	Primer sequence
1	pET28SphI for pET28(*Sfi*I) construct	CCGCAAGGAATGGTGCATGCAAGGAGATGG
2	pET28*Sfi*I for pET28(*Sfi*I) construct	GGTACGATGGCCGGCTGGGCCATGGTATATCTCCTTCTTAAAGTTAAA
3	pET28*Not*I for pET28(*Sfi*I) construct	CTCCGTCGACAAGCTTGCGGCCGCACTCGAG
4	T7 promoter sequencing primer	TAATACGACTCACTATAGGG
5	T7 terminator sequencing primer	GCTAGTTATTGCTCAGCGG
6	pCANTAB 5E sequencing primer 1	GGAATTGTGAGCGGATAACAAT
7	pCANTAB 5E sequencing primer 2	CCAGTACAAACCACCAACGCCTGT

3.1.10 Media

Unless otherwise stated additives were added to medium after autoclaving and after medium has cooled to 50-60°C. Heat-sensitive additives were sterilized through 0.22 µm pore size sterile filters.

Liquid media:

T. acidophilum medium:	Solution A:	0.1 ml	$CoSO_4 * 7H_2O$ (1 w%)
		0.5 ml	$CuCl_2 * 2H_2O$ (1 w%)
		1.93 g	$FeCl_3 * 6H_2O$
		0.18 g	$MnCl_2 * 4H_2O$
		0.45 g	$Na_2B_4O_7 * 10H_2O$
		0.3 ml	$Na_2MoO_4 * 2H_2O$ (1% (w/w))
		0.38 ml	$VOSO_4 * 5H_2O$ (1% (w/w))
		2.2 ml	$ZnSO_4 * 7H_2O$ (1% (w/w))

Adjusted to 1000 ml with $_{dd}H_2O$

Autoclaved at 121°C for 20 min

Solution B:	0.74 g	$CaCl_2 * 2H_2O$
	3.72 g	KH_2PO_4
	2.47 g	$MgSO_4 * 7H_2O$
	13.2 g	$(NH_4)_2SO_4$

Adjusted to 1000 ml with $_{dd}H_2O$

Autoclaved at 120°C for 20 min

The final medium contained:
10 ml Solution A
100 ml Solution B
20 ml yeast extract (10% (w/v), autoclaved)
2 ml ccH_2SO_4 (98%)
50 ml glucose (20% (w/v), filter sterilized)
Adjusted to 1000 ml with autoclaved $_{dd}H_2O$

LB-medium: 10 g Bacto tryptone
5 g Yeast extract
10 g NaCl
Dissolved in 1000 ml $_{dd}H_2O$

Autoclaved at 121°C for 20 min

LB-Amp: LB-medium containing 100 µg/ml ampicillin
LB-Kan: LB-medium containing 50 µg/ml kanamycin

2 x YT-medium: 16 g Tryptone
10 g Yeast extract
5 g NaCl
Dissolved in 1000 ml $_{dd}H_2O$

Autoclaved at 121°C for 20 min

YTCbG: 2 x YT containing 2% (w/v) glucose and 100 µg/ml carbenicillin
YTCbK: 2 x YT containing 100 µg/ml carbenicillin and 50 µg/ml kanamycin
YTCbN: 2 x YT containing 100 µg/ml carbenicillin and 100 µg/ml nalidixic acid

Solid media:

Bacto-agar (1.5 % (w/v) was added to media prior to autoclave as solidifying agent. Additives and antibiotics were added to the agar-medium after cooling to 50-60°C and immediately poured into Petri dishes.

Solid LB-agar media: LB-Kan
LB-Amp

Solid YT-media: YTCbG
YTCbN

Minimal medium for *E. coli* TG1 and HB2151: Prepared from the following solutions:

1M $MgCl_2 \cdot 6H_2O$:	20.33 g $MgCl_2 \cdot 6H_2O$ dissolved in 100 ml H_2O_{MilliQ} then autoclaved
1M $CaCl_2 \cdot 2H_2O$:	14.7 g $CaCl_2 \cdot 2H_2O$ dissolved in 100 ml H_2O_{MilliQ} then autoclaved
20% (w/v) glucose:	20 g of glucose dissolved in 100 ml H_2O_{MilliQ} then filter sterilized
1M thiamine hydrochloride:	33.73 g thiamine hydrochloride dissolved in 100 ml H_2O_{MilliQ} then sterilized by filtration.
Agar-solution:	15 g Bacto-agar dissolved in 500 ml H_2O_{MilliQ} then autoclaved
Buffer solution:	20 g Na2HPO4 3 g KH2PO4 1g NH4Cl

Dissolve in 500 ml 100 ml H_2O_{MilliQ} and adjust pH to 7.4 with NaOH

Autoclaved at 121°C for 20 min

The buffer and agar solutions were combined after t cooling (50-60 °C) and supplemented with 1 ml of 1M $MgCl_2 \cdot 6H_2O$, 1 ml of 1M $CaCl_2 \cdot 2H_2O$, 1 ml of thiamine hydrochloride and 5 ml of 20% (w/v) glucose. The medium was mixed well and poured into plates.

3.1.11 Buffers and stock solutions

The applied buffers and stock solutions were prepared with H_2O_{MilliQ} , autoclaved or filter sterilized by using Millex®-GS syringe filter (0.22 µm). The clarification and degassing of chromatography buffers was carried out by a Nalgene reusable filter system using Pall membrane filters (0.45 µm or 0.22 µm).

10 x PBS:	80 g	NaCl
	2g	KCl
	14.4g	Na_2HPO_4
	2.4g	KH_2PO_4

Dissolved in 1000 ml H_2O_{MilliQ} and pH was adjusted to 7.4 with NaOH

Autoclaved at 121°C for 20 min

1 x PBST:	100 ml	10 x PBS
	0.5 ml	Tween 20

Diluted to1000 ml with H_2O_{MilliQ}

2 % MPBS: 20 g skim milk powder dissolved in 1000 ml 1 x PBS
Always freshly prepared

3 % MPBS: 30 g skim milk powder dissolved in 1000 ml 1 x PBS
Always freshly prepared

MPBST: 3 g skim milk powder diluted in 1000 ml 1 x PBST
Always freshly prepared

1M Tris-Cl, pH 7.5: 121.1 g Tris base adjusted to 1000 ml with H_2O_{MilliQ}
pH was adjusted with HCl to 7.5

Autoclaved at 121°C for 20 min

10 x TBS: 1.5 M NaCl
200 mM Tris-Cl pH 7.5

Autoclaved at 121°C for 20 min

1 x TBST: 1 x TBS containing 0.05% Tween 20

MTBS: 1 x TBS containing 3% (w/v) Skim milk powder
Always freshly prepared

4 x separating gel buffer: 1.5 M Tris-Cl, pH 8.8
0.4 % (w/v) SDS
Filter sterilized and stored at 4°C

4 x stacking gel buffer: 0.5 M Tris-Cl, pH 6.8
0.4% (w/v) SDS
Filter sterilized and stored at 4°C

10 x Tris-Glycine buffer: 30.3 g Tris base
144 g Glycine
Adjusted to 1000 ml with H_2O_{MilliQ} and filtered

Native running buffer: 1 x Tris-Glycine buffer

SDS running buffer: 1 x Tris-Gycine buffer containing 0.1 % SDS
(Laemmli buffer)

Transfer buffer: 1 x Tris-Glycine buffer containing 10% (v/v) methanol and 0.05% (w/v) SDS

10 x Coupling buffer: 0.08 M Na_2HPO_4
(Modified PBS) 0.02 M KH_2PO_4
1.4 M NaCl
0.1 M KCl

pH adjusted to 7.4

Autoclaved at 121°C for 20 min

IMAC-buffers for His-tagged scFv purification:

Ni-NTA-equilibration buffer: 1 x Coupling buffer supplemented with 10 mM imidazole
Ni-NTA-wash buffer: 1 x Coupling buffer supplemented with 20 mM imidazole
Ni-NTA-elution buffer: 1 x Coupling buffer supplemented with 250 mM imidazole

-pH adjusted to 7.4 with NaOH

IMAC-buffers for purifying recombinant proteins of *T. acidophilum*:

Ni-NTA-equilibration buffer:	50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole
Ni-NTA-wash buffer:	50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole
Ni-NTA-elution buffer:	50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole

-pH adjusted to 8.0 with NaOH

RTBF I solution: 30 mM KOAc (potassium acetate)
100 mM RbCl
10 mM $CaCl_2$
50 mM $MnCl_2$
15% glycerol

pH adjusted to 5.8 with acetic acid

Sterilized with 0.2 µm filter

RTBF II solution: 10 mM MOPS
75 mM CaCl2
10 mM RbCl
15% glycerol

pH adjusted to 6.5 with KOH

Sterilized with 0.2 µm filter

50 x TAE-buffer: 242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA
Dilute to 1000 ml with H_2O_{MilliQ} and adjust pH to 8.0

1 x TES-buffer:	0.2 M Tris-Cl, pH 8.0
	0.5 mM EDTA
	0.5 M sucrose

Sterilized with 0.2 µm filter

3.1.12 Antibiotic stock solutions

100 mg/ml ampicillin:	1 g dissolved in 10 ml H_2O_{MilliQ} and filter sterilized
100 mg/ml carbenicilin:	1 g dissolved in 10 H_2O_{MilliQ} and filter sterilized
50 mg/ml kanamycin:	0.5 g dissolved in 10 ml H_2O_{MilliQ} and filter sterilized
34 mg/ml chloramphenicol:	0.34 g dissolved in 10 ml ethanol and filter sterilized
100 mg/ml nalidixic acid:	1 g dissolved in 10 ml 1M NaOH and filter sterilized

3.1.13 Chromogenic substrate solutions

Sigma*Fast* BCIP®/NBT tablet (dissolved in 10 ml H_2O_{MilliQ})	B5655	Sigma-Aldrich
1-Step Ultra TMB-ELISA	34028	Thermo Scientific
1-Step TMB-Blotting	34018	Thermo Scientific
One Step NBT/BCIP	34042	Thermo Scientific

3.1.14 Chromatography columns

HiTrap FF crude	GE Healthcare
Superose 6	GE Healthcare
Superose 12	GE Healthcare
Hydroxyapatite (Bio-Gel HTP)	Bio-Rad Laboratories

3.1.15 Laboratory equipment

Tools:

Agarose gel chamber	MPI workshop
Blotting filter papers	Extra thick Western Blotting Filter Paper, 88605, Invitrogene
Concentrator tubes (Amicon Ultra and Microcon)	Millipore
Cryotubes (1.8 ml)	Nunc 375418
ELISA plates (Microlon flat bottom, high binding)	Greiner Bio One 655061
Immuno-tubes (MaxiSorp) 4 ml	Nunc 444474
Inoculation loops (Sterile, plastic)	Greiner Bio One 731165
Microcentrifuge tubes (1.5 ml, 2 ml)	Safe Lock tubes, Eppendorf
Microtest Flat Bottom 96-well plates	BD-Falcon, 353936
Needles	Terumo
Nitrocellulose membrane (Reinforced NC)	Whatman, Optitran BA-S85
PAGE chambers	2050 Midget Electrophoresis Unit, LKB Bromma, XCell SureLock Mini-Cell Electrophoresis System, Life Technologies
Parafilm	Bemis Flexible Packaging
Pipette tips (Sterile filter tips)	Greiner Bio One
Pipettes	Pipetman Classic, Gilson
Plastic Petri dishes	Greiner Bio One
Sterile centrifuge tubes (50 ml and 15 ml)	BD-Falcon, TPP
Sterile filter system	500 ml Filter System, 431097, Corning
Sterile pipettes (50 ml, 25 ml, 10 ml, 5 ml)	Greiner Bio One
Sterile scalpels	Bayha
Syringes	BD, Braun Omnifix, Norm-Ject
Western blot incubation chambers	Genscript

Instruments:

Autoclaves	Tecnomara Deutschland GmbH, LTA 32/25, Zirbus GmbH
Blotting apparatus	Trans-Blot SD, Semi-Dry Electrophoretic Transfer Cell, 170-3940, Bio-Rad
Centrifuges	Sorvall RC-5B refridgerated superspeed centrifuge, DuPont Multifuge 3L-R, Heraeus, Thermo Scientific
Microplate-washer	Biotek ELx 50/16
FPLC	ÄKTA purifier 10/100, GE Healthcare ÄKTA Explorer GE Healthcare
Fume hood	Waldner
Incubator	Function Line Microbiological Incubator, Heraeus
Power supply	Consort E443
Shakers	Infors HT Minitron Innova 300 water bath shaker New Brunswick
Spectrophotometer	Implen NanoPhotometer (UV/Vis) Novaspec II, Pharmacia Biotech
Sterile bench	Heraeus LaminAir, HA 2448 GS
Tabletop centrifuges	Eppendorf 5415C, 5417 R Univapo 150 H, Montreal Biotech Inc
Thermomixer	Eppendorf, Thermomixer comfort
UV transilluminator	Herolab
Vortex	Vortex Genie 2, Scientific Industries Cell disruptor Genie, Scientific Industries

3.2 Methods

3.2.1 Creation of the scFv-library

The procedure of mouse immunization and detailed protocol of the scFv library construction is described in Supplementary data 7.1.

3.2.2 Affinity selection of scFv-displaying phages on plastic-immobilized antigen

Unless otherwise stated, the selection of phages was carried out by using plastic-immobilized antigens as described in Supplementary data 7.2.

3.2.3 Affinity selection of scFv-displaying phages on nitrocellulose-immobilized antigen

The isolation procedure of scFv-displaying phages from a nitrocellulose membrane is described as a novel affinity selection method in 4.3.2.

3.2.4 Molecular biology methods

3.2.4.1 Preparation of chemically competent *E. coli*

Competent cells of *E. coli* TOP10 and BL21(DE3) strains were prepared by the $RbCl_2$ method (83). For this, 10 µl of a frozen cryo culture was inoculated into 3 ml sterile LB medium in a polypropylene tube and shaken at 37°C, 160 rpm overnight (ON). The ON culture was transferred into 200 ml fresh LB medium and the incubation was continued at the same conditions until OD_{600} reached 0.5-0.7, at which value the culture was immediately placed on ice and cooled down for 15 minutes. After this point each step was done strictly on ice and at sterile conditions. Cells were collected by centrifugation at 2000 rpm, 4°C for 10 minutes then gently resuspended in 200 ml ice-cold RBTF I buffer and incubated on ice for 15 min. Incubation was followed by an another centrifugation step at 1000 rpm, 4°C for 5 min, after

which the supernatant was removed. Cells were carefully resuspended in 6 ml ice-cold RBTF II buffer, and aliquots of 50, 100 and 150 µl were distributed in 1.5 ml Eppendorf tubes and stored at -80°C.

3.2.4.2 Heat shock transformation of chemically competent *E. coli*

To transform *E. coli* strains ($RbCl_2$-competent or “Chemically” competent cells from Invitrogen and Agilent Technologies) with plasmid/phagemid DNA the heat shock transformation protocol was carried out as follows. 50-100 µl competent cells were thawed on ice for 15 minutes than mixed with 1-2 µl purified plasmid DNA or 2-10 µl ligation mixture. Cells were incubated with the plasmid DNA on ice for 20 minutes then heated at 42°C for 30 seconds. After this the cells were placed immediately on ice for 2 minutes then mixed with 500-1000 µl sterile LB or SOC medium and gently shaken at 37°C for 45 minutes. Cells were plated out on sterile, antibiotic-containing LB-agar media and incubated at 37°C ON.

3.2.4.3 Amplification and isolation of plasmid DNA from *E. coli*

For plasmid amplification a few µl of the maintained cryo stocks were spread on appropriate agar-plates and grown at 37°C ON. Next day, a single colony was used to inoculate 3-5 ml antibiotic-containing liquid medium and cultured at 37°C, 180 rpm ON. The purification of plasmid DNA from *E. coli* was implemented with the help of two alternative plasmid isolation kits (QIAprep Spin Miniprep Kit or Pure Yield Plasmid Miniprep System Kit) according to the manufacturer’s protocol. The concentration of purified plasmid DNA was measured and appropriate amounts were used for sequence analysis, PCR-reaction, transformation of *E. coli* or cloning procedures. The remaining plasmid DNA was stored at -20°C

3.2.4.4 Agarose gel electrophoresis

The quality of DNA samples were assayed with agarose gel electrophoresis using a horizontal gel electrophoresis apparatus. 1% or 1.5% agarose gels were prepared in 1 x TAE-buffer and poured into the gel chamber. After solidification the gel was covered with 1 x TAE-running

buffer, and the comb was removed. DNA samples were mixed with 6 x DNA loading dye in the ratio of 5 : 1 and loaded in the sample pockets in a maximum volume of 25 µl. The approximate lengths of DNA fragments were determined with the help of 1 kb DNA ladder (Fermentas). Separated DNA molecules in the gel were post-stained with 3 x GelRed™ fluorescent nucleic acid dye for 20 min and exposed to 302 nm UV light and images were recorded.

3.2.4.5 DNA-extraction from agarose gel

DNA fragments of interest were excised and purified with the QIAquick Gel Extraction Kit according to the manufacturer's protocol.

3.2.4.6 Restriction digestion of DNA

Plasmid and phagemid DNA were digested with the corresponding restriction endonucleases from Fermentas and New England Biolabs (NeB). Unless otherwise stated, reaction mixtures contained 2 µl reaction buffers, 0.5-1 µg purified plasmid/phagemid DNA and 1 µl enzyme for single or 1-1 µl enzymes for double digestion purposes. The digestion mixture was adjusted to 20 µl with nuclease free water. The reaction temperatures and reaction times were set according to the manufacturer's protocol. In case of buffer-incompatibility, plasmids were digested with one enzyme then purified prior to the next digestion procedure. The *Sfi*I-*Not*I digestion procedures were carried out as follows.

Digestion with NeB enzymes:

Solution (NeB)	Volume (µl)
10 x NeB buffer 4	2
*Sfi*I enzyme (NeB)	1
Plasmid/phagemid DNA	5-8 µl (1 µg)
$_{dd}H_2O$	9-12 µl
Incubation at 50°C for 1 hr	
Addition of 1 µl *Not*I enzyme (NeB) and digestion for 1 h at 37°C	

Digestion with Fast Digest enzymes:

Solution (Fast Digest, Fermentas)	Volume (µl)
10 x Fast Digest Green buffer	2
Fast Digest *Sfi*I enzyme	1
Plasmid/phagemid DNA	5-8 µl (1 µg)
$_{dd}H_2O$	9-12 µl
Incubation at 50°C for 15 min	
Addition of 1 µl Fast Digest *Not*I enzyme and digestion for 0.5 h at 37°C	

3.2.4.7 Clean-up procedure of digested DNA

To remove protein and reaction buffer components from the digested DNA or PCR products samples were purified with QIAquick PCR Purification Kit (Qiagen) or Wizard® SV Gel and PCR Clean-up system Kit (Promega) according to the manufacturer's protocol. After this clean up step the DNA concentration was measured at 260 nm then the digested or amplified DNA was used immediately or stored at -20°C.

3.2.4.8 DNA-ligation assay

The digested scFv inserts and pET28(*Sfi*I) vector were ligated with T4 DNA ligase (New England Biolabs) as follows:

*Sfi*I-*Not*I digested scFv insert 6 µl

*Sfi*I-*Not*I digested pET28(*Sfi*I) 2 µl

10 x T4-ligase buffer 1 µl

T4-ligase 1µl

Incubation at 16°C for 16 h

2-10 µl of the ligation mixture was directly used to transform *E. coli* cells or, if *Not*I used immediately, stored at -20°C.

3.2.4.9 Plasmid maintenance and amplification

Sequenced plasmid DNAs were maintained in *E. coli* TOP10 or XL10 Gold strains. From the freshly transformed bacterial lawn, 2-4 single colonies were picked and transferred into 3-3 ml of LB medium containing the corresponding antibiotics and grown overnight at 37°C and 180 rpm. Sub-cultures were supplemented with 30% sterile glycerol and distributed in 3-4 cryo-tubes and stored at -80°C.

3.2.4.10 DNA-sequencing

DNA-sequencing of the cloned inserts was carried out by the Biochemistry Core facility of the institute using a modified Sanger dideoxy terminator cycle sequencing method. Sequencing was performed with the help of ABI BigDye kit version 3.1, on an ABI 3730 48-capillary sequencer and 36 cm capillaries. The evaluation of DNA sequences was carried out by using DNA STAR sequence analyzer software. Primer No.4 and 5 was used for sequencing pET28-scFvs and primer No.6 and 7 for sequencing pCANTAB 5E-scFvs (3.1.9).

3.2.4.11 Determination of DNA concentration

The determination of DNA concentration was carried out by measuring the optical absorbance of DNA solution at 260 nm. The measurements were performed with 3 parallel samples using a spectrophotometer (Implen NanoPhotometer).

3.2.5 Cultivation of *E. coli* for heterologous protein expression and preparation of cell extracts

3.2.5.1 Expression of *T. acidophilum* structural genes

To express recombinant *T. acidophilum* proteins the pET28 vector (Novagene) or its derivatives were used (3.1.8). His-tagged recombinant proteins were expressed at varying temperature, induction time and inducer (IPTG) concentration in a set of expression strains. Table 7.2 of 7.9 describes the expression conditions. If otherwise not stated, an aliquot of the

bacterial cryo stock was inoculated into 3-100 ml of antibiotic(s)-containing medium and grown ON at 37°C. Bacteria were then transferred into fresh LB-medium and further incubated at 37 °C in the presence of the appropriate antibiotic(s). In the exponential growth phase (OD_{600}=0.8), protein expression was induced by adding IPTG to the cell culture. Expressions, using the Novagen autoinduction medium were carried out according to the manufacturer's protocol. Small-scale expressions were carried out in 10 ml LB-medium while the large-scale expression of proteins was performed in 2-10 L medium with proper aeration, applying the best expression conditions. After the required induction time bacteria were collected by centrifugation at 4°C, 4000 x g for 20 minutes. The pellet was resuspended in $_{dd}H_2O$ and centrifuged again for 20 minutes after which the supernatant was discarded and the pellet was stored at -80°C.

3.2.5.2 Expression of mouse scFvs

The cloning of scFvs in pCANTAB 5E and their expression on phage in *E. coli* TG1 was performed as described in 7.1.2 and 7.2, respectively. The expression of soluble, E-tagged scFvs was carried out in the amber suppressor *E. coli* HB2151 strain similarly to His-tagged proteins (3.2.5.1). Alternatively, scFvs were cloned into a modified pET28 vector and expressed as C-terminally His-tagged proteins in small and large scale, as described (3.2.5.1). Detailed description of applied recombinant strains with the necessary information is listed in supplementary data (7.8).

3.2.5.3 Whole cell extract preparation

10-15 g frozen biomass was thawed on ice then resuspended in 20 ml $_{dd}H_2O$ containing 1/2 tablet of EDTA-free protease inhibitor cocktail (Roche). The cell suspension was supplemented with 1 mg/ml lysozyme and with the appropriate volume of 10 x buffer (IMAC or Coupling buffer, 3.1.11) to obtain 1x buffer concentration and incubated on ice for 1 h. To decrease the viscosity of the lysate 50 µg/ml DNAse (Sigma-Aldrich) or 2-3 µl of Bensonase® was added. For proper cell wall degradation the lysate was sonicated on ice 5-8 times for 1 minute (duty cycle: 30 %, output control: 6-8, Sonifier 250, Branson). Crude cell extract was

centrifuged at 4°C, 30 000 x g, 40 minutes and the clear supernatant containing the soluble protein fraction was immediately used.
For small scale preparations the pellet of 1.5 ml cell culture was resuspended in 150 µl buffer containing protease inhibitor cocktail and lysed by mechanic cell wall disruption. 1/3 volume of zirconium beads were added and the cell suspension was vortexed for 8-10 minutes at 4°C (Cell Disruptor Genie, Scientific Industries). The crude cell lysate was then centrifuged at 13 000 x g, 4°C for 10 minutes and the supernatant was immediately used or stored at -20°C in the presence of 15% glycerol.

3.2.5.4 Periplasmic extract preparation

Periplasmic extracts were prepared by mild osmotic shock. Pellets of 25 ml cell cultures were resuspended in 0.5 ml ice-cold 1 x TES buffer after which 0.75 ml of ice-cold 1/5 TES was added and the cell suspensions were incubated on ice for 30 minutes then centrifuged at 13 000 x g, 4°C for 10 minutes. Supernatants containing the soluble periplasmic fraction were transferred into a new tube and used immediately or stored at -20°C in the presence of 15% glycerol.

3.2.6 Culturing and cell extract preparation of *Thermoplasma acidophilum*

3.2.6.1 Growth and harvest of *T. acidophilum*

T. acidophilum cells were cultured as described earlier (84) with minor modifications (3). Briefly: 4 ml *T. acidophilum* cryo stock or 1 ml fresh culture was added to 50 ml medium and grown at 59°C and 120 rpm in oil bath until OD_{600} reached 1.0 - 1.2 (2-3 days). Cells were centrifuged at 4000 g for 10 minutes at RT, washed with $_{dd}H_2O$ andstored at -80°C.

3.2.6.2 Preparation of *T. acidophilum* cytosolic extract

Frozen cell pellet was thawed on ice and resuspended in $_{dd}H_2O$ (2 ml/1 g cell pellet) containing EDTA-free protease inhibitor cocktail (Roche). Cell lysis was triggered by

elevating the pH of the suspension to 7.5 with non-buffered 1M Tris. After cell lysis DNase I or bensonase enzymes were added to digest released DNA and RNA polymers and the cell lysate was incubated on ice for 1 h. Crude extract was centrifuged at 30 000 x g, 4°C for 20 min to remove cell debris and the supernatant was further centrifuged for 15 min to sediment the membrane fraction. Clear cytosolic extract was immediately used or frozen with 15% glycerol and stored at -20°C.

3.2.6.3 Long-term storage of *T. acidophilum*

100 ml of a *T. acidophilum* culture was cooled down to room temperature then the pH was adjusted to 3 using 7% NH_4OH. As cryoprotectant, 15 g sucrose was dissolved in the cell suspension and 1.5 ml aliquots were flash frozen in liquid nitrogen and stored at -80°C.

3.2.7 Protein purification

3.2.7.1 Ni-affinity chromatography of recombinant His-tagged proteins

Large scale affinity purification of His-tagged proteins was carried out by IMAC (Immobilized metal ion affinity chromatography) technique (85) using a 1 ml HisTrap™ FF crude column (Ni-Sepharose™ 6 Fast Flow, GE Healthcare) that was connected to a fast protein liquid chromatography (FPLC) system (ÄKTA Purifier 10, GE Healthcare). The purification was carried out in a cold cabinet at 10°C with a flow rate of 0.8 ml/min Elution profile was monitored with a UV-detector operating at 230, 254 and 280 nm. The applied buffers and their preparation are described in unit 3.1.11. Prior to loading protein sample (cell extract) the column was first equilibrated with the appropriate running buffer supplemented with 10 mM imidazol. Unbound proteins were washed with 20 mM imidazole-containing running buffer until A_{280} reached a constant base line. The elution of proteins was implemented with a 20-250 mM imidazole gradient which was expanded to 40-45 column volume. Fractions of 0.8 ml volumes were collected during the gradient elution then analyzed on SDS-PAGE. Fractions of interest were pooled and concentrated by using Amicon Ultra concentrator tubes with appropriate MWCO (molecular weight cut off) or directly used for size exclusion chromatography (3.2.7.2).

The small scale and batch purification of His-tagged proteins was performed on Ni-NTA Agarose matrix (Ni-NTA coupled to Sepharose® CL 6B, Qiagen) applying the same buffer conditions as for the large scale chromatography. To exchange the storing buffer of Ni-NTA beads and to purify the target proteins the reactions were set up in 50 ml Falcon tubes. Each centrifugation step was carried out at 4°C, 1000 x g for 1 minute and supernatants were gently removed by pipetting. The volume of settled resin was 50 or 250 µl, respectively which was first washed 3 times with 1 ml H_2O_{MilliQ} to remove ethanol then equilibrated 3 times with 1 ml of the appropriate equilibration buffer. The cytosolic extract (100-200 µl or 20-40 ml) was added to the equilibrated resin and incubated at 4°C for 1 h by continuous rotation. The resin was then collected by centrifugation and washed 3 times with 1040 ml of washing buffer depending on the volume of applied Ni-NTA resin. The elution of bound proteins was carried out in 3-5 steps using 0.1-1 ml of elution buffer. Elution procedure was continued until no protein was detected in the last fraction. Elution fractions were collected in separate tubes and their protein concentration was analyzed as described (3.2.8.2). Samples of flow-through, washing and elution fractions were analyzed by SDS-PAGE. Elution fractions containing proteins of interest were pooled, concentrated and loaded on a gel filtration column or dialyzed against the appropriate buffer and stored/used according to the experimental set up.

3.2.7.2 Size exclusion chromatography

Superose 12 chromatography

The enrichment of macromolecular complexes from *T. acidophilum* cytosolic extract was carried out with size exclusion chromatography on a Superose 12 semi-preparative column (GE-Healthcare). The column was connected to an FPLCsystem (ÄKTA Purifier 10, GE Healthcare). 500 µl (max. 4 mg protein) of the cytosolic extract was loaded on the column that was previously equilibrated with running buffer (25 mM K_2HPO_4-KH_2PO_4 buffer, pH 7.5 containing 1mM ATP, 1mM DTT and 5 mM $MgCl_2$). During the separation the injected samples were running at 10°C with 0.4 ml/min flow rate and the protein elution profile was monitored with a UV-detector operating at 280 nm. 35 protein-containing fractions, each of 0.6 ml volume, were collected. Fractions 8 and 9 from each run, containing the high molecular weight protein complexes (over the size of 300 kDa) were pooled and concentrated to 2.5 ml volume. The protein solution was mixed with 15% glycerol, aliquoted to 100-200 µl

volumes, frozen in liquid nitrogen and stored at -80°C until further use. This protein solution served as antigen mixture for mouse immunization and as target protein mixture for the phage-biopanning and scFv-screening procedure. Additionally, the high molecular weight protein fraction was used as intput protein sample in further fractionation procedure by hydroxy-apatite chromatography (3.2.7.3).

Superose 6 chromatography

After Ni-NTA separation scFv-captured macromolecular complexes were separated from smaller contaminants on a semi-preparative Superose 6 chromatography column (GE-Healthcare). The column was connected to an FPLC system (ÄKTA Purifier 10, GE Healthcare) and operated at 0.4 ml/min flow rate and 10 °C. The protein elution profile was monitored with a UV-detector operating at 280 nm. A sample volume of 300-500 µl was loaded and 0.4 ml fractions were collected. Aliquots of them were loaded on SDS-gels and evaluated.

3.2.7.3 Hydroxy-apatite chromatography

Hydroxyapatite chromatography was used to further fractionate Superose 12-separated high molecular weight protein fractions of *T. acidophilum*. The sample was loaded on a 20 ml hydroxyapatite column (Bio-Gel HTP, Bio-Rad Laboratories) which was operated at 4°C and equilibrated with the loading buffer (10 mM K_2HPO_4-KH_2PO_4, pH 8.0). Step elution of bound proteins was carried out sequentially with buffers containing 150, 300 and 500 mM K_2HPO_4-KH_2PO_4, pH 8.0, at 0.5 ml/min flow rate. Protein elution profiles were detected and the 3 protein-containing fractions were collected. Protein fractions were dialyzed ON against 20 mM Na_2HPO_4-NaH_2PO_4 buffer, pH 8.0 at 4°C and concentrated with Amicon Ultra concentrator tubes, 5 kDa MWCO.

3.2.8 Protein analysis

3.2.8.1 Polyacrylamid gel electrophoresis (PAGE)

The protein content and quality of the protein samples were checked with PAGE under denaturing or non-denaturing conditions, depending on the information of interest.

PAGE under denaturing conditions (SDS-PAGE)

The separation of proteins under denaturing and reducing conditions was carried out at room temperature according to Laemmli's protocol (86). Materials and buffer solutions used for the gel preparation and running procedure are described in 3.1. The separation of protein samples was performed on 15 % SDS polyacrylamid gels, which were prepared as follows:

15% separating gel:	$_{dd}H_2O$	15 ml
	4 x separating gel buffer	15 ml
	30% acrylamid/bis-acrylamid mix (37.5 : 1)	30 ml
	10% APS (w/v)	0.24 ml
	TEMED	0.03 ml
6% stacking gel:	$_{dd}H_2O$	33 ml
	4 x stacking gel buffer	15 ml
	30% acrylamid/bis-acrylamid mix (37.5 : 1)	12 ml
	10% APS (w/v)	0.48 ml
	TEMED	0.06 ml

SDS-PAGE protocol:

Gels were placed in the electrophoretic chamber (Mighty Small II-System (8 x 7 cm, GE Healthcare) and covered with 1 x Running buffer. For total protein separation 10 µl of pellet was resuspended in 90 µl of $_{dd}H_2O$ then 20 µl of 6 x SDS-sample buffer was added to the suspension and heated at 95°C for 10 minutes. 5 µl of this mixture was loaded. Other protein samples were diluted in $_{dd}H_20$, if it was necessary and supplemented with the sample buffer in the ratio of 5 : 1 then heated at 75°C for 10 minutes. 10-15 µg protein was loaded in a maximum volume of 12 µl. 5 µl pre-stained molecular marker was loaded in one of the wells to be able to monitor protein separation. The applied electric field in the initial phase was 135 V and increased to 160-165 V when the bromophenolblue dye reached the border of stacking and resolving gel. The gel was run until the blue dye reached the end of the gel, after which the gels were rinsed with $_{dd}H_2O$ and stained with Instant Blue protein staining solution for 1 h then rinsed with water and scanned/stored.

Native-PAGE

NuPAGE® Novex 3-8% Tris-acetate precast gels, (Invitrogen) were used to separate proteins at native conditions. 5 µl HMW Native Marker (GE Healthcare) and protein samples mixed with 6x native sample buffer were loaded (max. volume 20 µl) in the wells of a gel which had been placed into an electrophoretic chamber (XCell Sure Lock Mini Cell, Invitrogene) and covered with native running bufferl. The chamber was placed to 4°C and connected to a power supply. The applied electric field was 100 V during the separation (until the bromophenolblue dye reached the bottom of gel). After this the gel was removed and rinsed with $_{dd}H_2O$ and stained with Instant Blue protein staining solution for 1 h then rinsed with water and scanned/stored.

3.2.8.2 Determination of protein concentration

Protein concentration was determined by measuring the absorbance of protein solutions at 280 nm. The measurements were performed with 3 parallel samples using a spectrophotometer (Implen NanoPhotometer).

3.2.8.3 Mass spectrometry

The MS/MS analysis of protein samples was carried out by the Biochemistry Core Facility of the institute. The protein samples were first digested with trypsin either in solution or in gel (87). Digested peptide mixtures were separated by on-line nanoLC and analyzed by electronspray tandem mass spectrometry. The experiments were performed on an Agilent 1200 nanoflow system connected to an LTQ Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark). The data analysis was performed with the MaxQuant software as described (88) supported by Mascot or Andromeda as the database search engine for peptide identifications. Peaks in MS scans were determined as three-dimensional hills in the mass-retention time plane. MS/MS peak lists were filtered to contain at most six peaks per 100 Da interval and searched against a concatenated forward and reversed version of the *Thermoplasma acidophilum* and *Escherichia coli* databases (extracted from NCBInr) and Mouse database (International Protein Index database: ftp://ftp.ebi.ac.uk/pub/databases/IPI/old/MOUSE) and contained frequently observed contaminants like proteases and human keratins. The initial mass tolerance in MS mode was set to 7 p.p.m. and MS/MS mass tolerance was 0.5 Da. Cysteine carbamidomethylation was searched as a fixed modification, whereas N-acetyl protein and oxidized methionine were searched as variable modifications.

3.2.8.4 Electron microscopy

Sample preparation

For EM-analysis, protein samples were prepared by negative staining on copper grids (100 x 400 meshes coated with a continuous carbon film, PLANO GmbH). The grids were first glow discharged for 30 seconds in a plasma cleaner. For negative staining, the grid was placed C-side down on a 5 µl drop of the protein sample and incubated for 60 seconds. After the incubation the grid was blotted with the help of a filter paper (Whatman, Grade No.1) In the same way, grids were washed and blotted quickly 2 times with 5 µl $_{dd}H_2O$ and incubated for 60 seconds on a 2 µl droplet of 2 % uranyl-acetate before drying by blotting with filter paper. The grids were then analysed or stored at room temperature.

Data acquisition

The electron micrographs were acquired with a transmission electron microscope (CM 200 FEG TEM, 160 kV, Philips) equipped with a TVIPS CCD camera (CCD size: 4096 x 4096 pixel, CCD physical pixel size: 15 mue, conversion factor: 67 counts per incident electron). The applied defocus and magnification of the images varied between -2 and -3 µm and 539600 and 93126, respectively.

3.2.9 Immunology methods

3.2.9.1 Monoclonal ELISA assay

ELISA assay was used to determine antigen specificity of monoclonal scFv-displaying phages derived from the scFv library after the phage biopanning procedure. The growth of monoclonal phages was carried out as described in supplementary data (7.2.1). ELISA-assay was carried out with Nunc MaxiSorp® flat-bottom 96 well plates. The plate was first coated with 100 µl of the appropriate antigen solution (10-100 µg/ml) diluted in PBS (1-10 µg protein/100 µl PBS/well) at 4°C, ON with gentle horizontal shaking. Additionally, a negative control plate was prepared for each antigen plate containing 100 µl of 2 % MPBS/well and incubated on the same way. Next day, plates were washed 3 times with 350 µl PBS using an automated microtiter plate-washer (Biotek ELx50/16). To block free binding surfaces, wells were filled with 250 µl of 2 % MPBS and incubated with shaking at room temperature for 2 h. After blocking, plates were washed 3 times with PBS in the same way as described. After washing step, 100-100 µl of PBST was added to each well then mixed with 90-90 µl of the corresponding phage supernatants (see 7.3.1) or 90-90 µl of soluble scFv-containing supernatants (see 7.3.2). Plates were covered with Parafilm and incubated for 1.5 h at room temperature. Wells were washed 3 times with PBST then 3 times with PBS. After washing steps the following antibody solutions were added:

- Phage detection: 100-100 µl of 5000 x diluted HRP-conjugated anti-M13KO7 antibody.
- Soluble scFv detection: 100-100 µl of 5000 x diluted HRP-conjugated anti-E-tag antibody.

Plates were incubated with the antibody solution for 1 h at room temperature then washed 3 times with PBST and 3 times with PBS as already described. To develop ELISA-signals either of the HRP substrate solutions was added, as follows:

5-aminosalicylic acid substrate solution:

- 1 tablet of 5-aminosalicylic acid was dissolved in 50 ml of 20 mM Na_2HPO_4-NaH_2PO_4, pH 6.0. Prior to development, 4 µl of H_2O_2 was added to the substrate solution then plates were incubated with 100 µl of this mixture for 20-30 minutes at RT. Reaction was stopped with 50 µl of 1M HCl.

TMB substrate solution:

- 50-100 µl of 1-Step Ultra TMB-ELISA substrate solution (Thermo Scientific) was added to the plates and incubated for 10-20 minutes at RT after which reaction was stopped with 2M H_2SO_4.

3.2.9.2 Western blot assay

Western blot assay was carried out using a horizontal, semi-dry electro-blotting system. Nitrocellulose membrane was placed on a thick blotting filter paper sheet, which was previously soaked in transfer buffer. SDS-gel containing the separated protein samples was placed on the nitrocellulose membrane immediately after run and covered with a pre-soaked thick filter paper sheet after which the lid was attached to the blotting chamber. The protein transfer was carried out applying 15V electric field for 48 min. After the transfer, the membrane was placed into 3 % MPBS solution and incubated at RT for 2 h or at 4°C ON to block protein-free binding spaces on the nitrocellulose. Membrane was After the blocking the membrane was placed into the target primary antibody solution. The following antibodies were used in our experiments:

A. 5 X diluted polyclonal phage solution in PBST
B. 15 X diluted monoclonal phage solution in PBST
C. 1000 x diluted Rabbit anti-poly-histidine antibody in TBST
D. 100-1000 x diluted monoclonal His-tagged scFv in TBST

The membrane was incubated with the primary antibody solution for 1 h at RT, then washed 3 x 5 min with the corresponding wash buffer (PBST or TBST). This step was followed by the incubation with the secondary antibody for 30 min, then washed 1 x 10 and 2 x 5 min with the appropriate wash buffer (PBST or TBST). The following secondary antibodies were used:

1. 5000 x diluted Mouse anti-M13-HRP conjugate in PBST for primary antibodies A and B
2. 5000 x diluted Goat anti-rabbit-AP conjugate in TBST for primary antibody C
3. 1000 x diluted Rabbit anti-poly-histidine antibody in TBST for primary antibody D

When a combination of A.1 and B.1 and D.3 was probed a tertier antibody solution was applied:

- 5000 x diluted Goat anti-mouse-AP in PBST for combinations A.1 and B.1
- 5000 x diluted Goat anti-rabbit-AP in TBST for combination D.3

The membrane was incubated with the tertier antibody for 30 min then washed with the corresponding wash buffer (PBST or TBST).
The signal was developed with the appropriate substrate solution after the last washing step. The used substrates were:

- One Step Western blot TMB solution for HRP-conjugated antibodies and
- One Step Western blot BCIP/NBT solution or Sigma Fast BCIP/NBT tablet dissolved in 10 ml ddH_2O for AP-conjugated antibodies.

The membrane was incubated in the substrate solution for 5-10 min or until the desired color developed after which the membrane was rinsed with water and conserved between cellophane sheets.

4. RESULTS AND DISCUSSION

4.1 Creation of the scFv library

Superose12-separated high molecular weight protein fraction (>300 kDa) of *T. acidophilum* cytosolic cell extract was used to immunize mice. The preparation of *T. acidophilum* cell extract and its subsequent Superose 12 chromatography purification was performed as described in 3.2.6.2 and 3.2.7.2, respectively. Detailed work-flow of the mouse immunization procedure and spleen harvest is described in 7.1.1. The extraction of total RNA and the scFv-library construction was carried out according to a basic protocol described in 7.1.2 using pCANTAB 5E vector. The scFv-library contained 1.4×10^8 independent clones of which six clones were randomly selected and sequenced. All the sequences were different (Figure 7.1.3.) indicating a diverse scFv library.

4.2 Selection of scFv-library against complex antigen mixtures

In the first approaches the original phage display library was selected and screened against mixtures of protein complexes that were separated by two different chromatography techniques. References, based on proteomics analyses and protein complex purification studies of *T. acidophilum* served as starting points for the selection of target antigen mixtures. Previous studies described that OH-apatite chromatography, glycerol gradient ultracentrifugation and molecular sieve chromatography are applicable methods to separate high molecular weight protein complexes from smaller constituents (3, 11, 89, 90). In the first scFv selection experiments we applied the Superose 12 separated high molecular weight protein fraction of *T. acidophilum* (Sup12-HMWF) and its further separated OH-apatite elution fractions. The applied protein mixtures contained considerable amounts of protein complexes over the size of 300 kDa. The preparation of target protein samples was carried out as described in protein purification methods 3.2.7.2 and 3.2.7.3.

4.2.1 ScFv selection using the OH-apatite separated Sup12-HMWF of *T. acidophilum*

4.2.1.1 Phage selection against the OH-apatite separated Sup12-HMWF

ScFv-displaying phages were selected through 3 biopanning cycles using 500 mM and 300 mM K_2HPO_4 (OHA-500 mM and OHA-300 mM) OH-apatite elution fractions. Phage propagation and selection procedures were carried out as described in 7.2 with minor modifications as follows: For amplification of the scFv library 100 ul of the original library (~1 X 10^8 clones) was inoculated into 100 ml of YTAG. The antigen mixture was incubated in 50 mM $NaHCO_3$ buffer, pH 9.6 ON. The elimination of unbound phages from the immuno-tubes included 5x PBST and 5x PBS washing steps, respectively. The protein concentration of the target mixture was 100 mg/ml, which was decreased by a factor of 2 at each subsequent biopanning cycle. After the third selection cycle, an average of 1 x 10^3 fold increase of CFU/ml was obtained compared to the first round using OHA-500 mM, yielding 2 X 10^7 CFU/ml of third generation TG1. In case of OHA-300 mM the CFU did not increase during the three selection cycles and yielded only 1 x 10^3 CFU/ml output colonies.

Antigen-binding specificities of third generation polyclonal phages

Western blot assay was performed to analyze binding properties of polyclonal phages to denatured subunits of probable complexes present in OHA-500 mM and OHA-300 mM fractions. Phages of the third generation library were amplified and purified as described in 7.2. The Western blot assay of polyclonal phages selected for OHA-500 mM resulted in two major protein bands in the range of 60 and 25 kDa (Figure 4.1). In contrast, no positive binders were obtained for OHA-300 mM.

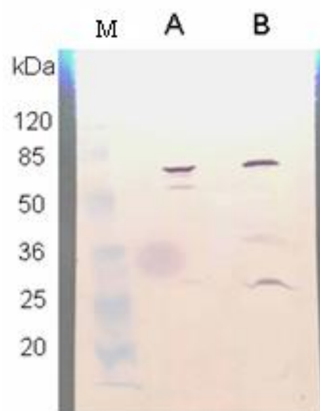
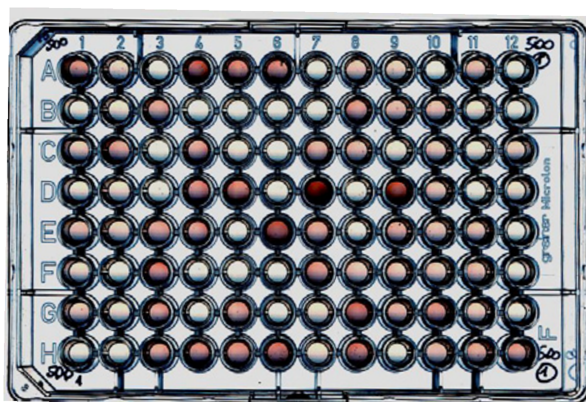


Figure 4.1: Western blot analysis of output 3 phages selected against OHA-500 mM as antigen mixture. (A) *T. acidophilum* cytosolic cell extract as test antigen, (B) OHA-500 mM of *T. acidophilum* as test antigen, M: Molecular weight marker.

Screening of individual phages for native protein complex binding capacity

Monoclonal TG1 cell lines were established from output 3 colonies to propagate monoclonal phages as described in 7.3.1. 96-96 individual colonies were picked from both of the third generation output plates and screened for the corresponding target fractions, according to protocol 3.2.9.1. OHA-500 mM used as target antigen yielded more than 50% positive signals (Figure 4.2), whereas, the selection for 300 mM elution fraction did not result in any positive phage binder (data not shown).



Figur 4.2: ELISA-assay of OHA-500 mM-selected monoclonal output 3 phages against the respective selection mixture. ELISA signals were developed with a HRP substrate system (5-aminosalicylic acid and H_2O_2).

Detection of protein complexes recognized by OHA-500 mM-selected phages with Western blot assay

Phages derived from monoclonal cell-lines were further screened by Western blot assay. The analyzed protein complex-containing fractions included the cytosolic cell extract of *T. acidophilum* and the selection target mixture OHA-500 mM. 24 ELISA-positive monoclonal phages were tested for protein binding properties, of which 7 gave positive signal at 60 kDa and 3 at 25 kDa, corresponding to the recognition pattern of polyclonal phages. One weak positive clone was obtained that recognized a protein at 20 kDa. No signal pattern was obtained in case of remaining phage clones, which however, does not necessarily mean that the phages were not specific to any of the target antigens. Signal intensities may vary due to unequal protein ratio in the separated protein mixture, which results in inadequate target quantity suppressed by abundant proteins. Low concentration (originating from poor expression level of certain scFv-displaying phage clones) of purified, specific phages can also result weak signal intensity. Besides, the 3D structure of target complexes and their subunit composition can be dramatically influenced by assay conditions, which can strongly affect the scFv-antigen binding by means of structural changes in the target antigen. Therefore, epitopes for specific scFvs often remain hidden at native conditions. Good examples are for this ELISA and Western blot assays, where the target antigens of interest are accessible either in their native structure or in denatured forms, respectively. This should also be considered while evaluating results arising from both screening techniques. Since our studies are focusing on intact protein complex isolation, ELISA-assay alone can serve as satisfying screening method for identifying specific scFv-binders, however, combining with Western blot analysis the evaluation and verification is more precise.

4.2.1.2 Affinity purification of protein complexes using agarose bead-immobilized scFv-displaying phages

Two ELISA and Western blot positive phages A2 and D1 from OHA-500 mM-selected monoclonal output 3 TG1 were selected and coupled to activated agarose beads to capture protein complexes from *T. acidophilum* cytosolic extract. In the elution fractions no protein was detectable, indicating that no protein binding occurred or it remained under detection level.

This might be explained with the fact that the quality and quantity of the bait protein strongly influences the purity and yield of the prey molecule. The maximum saturation of beads with the desired antibody results in higher prey binding capacity, which leads to a better purification yield. Filamentous phage particles are constituted of thousands of the major coat protein (pVIII) whose amino terminal end forms the outer hydrophilic surface of the virion. Therefore the primery amines ($-NH_2$) on the N-terminus are exposed to the reactive aldehyde groups in the cross-linking reaction. Consequently, the great mass of potential coupling sites is occupied by the phage minor coat protein, which dramatically reduces the number of potentially attachable scFvs in a surface unit. Thus, the low copy number scFv cannot capture usable amount of prey. Additionally, these huge filamentous phages can clog the pores of the resin causing poor protein distribution. With this end in view we concluded that the use of phage based pull-down assays have their limits and not applicable for protein purification purposes.

4.2.1.3 Affinity purification of protein complexes using surface-immobilized scFvs

Since scFv-displaying phage particles did not prove to be applicable for protein pull-down assays we targeted the expression of soluble scFvs. The expression of phage-free, affinity tagged scFvs (~27-28 kDa) was carried out in two different ways, using pCANTAB 5E and pET28(*Sfi*I) expression systems, respectively.

Expression of free E-tagged scFvs derived from positive phagemid clones

In the first approach we applied *E. coli* HB2151 non-suppressor strain, which allows free, E-tagged protein expression from the same phagemid without the need to re-clone scFvs into a new expression vector. To carry out soluble protein expression phagemids HB2151 cells were infected with third generation OHA-500 mM selected polyclonal phages. The growth of HB2151 and the propagation of phages were performed similarly as described in 7.2 using YTCbGN and YTCbKN media instead of YTCbG and YTCbK, respectively. Individual colonies harboring pCANTAB 5E-scFvs were harvested from plates and used to establish monoclonal cell-lines. The expression of monoclonal scFvs was carried out via IPTG induction as described in 7.3.2. The specificity of soluble scFvs expressed into the medium

was tested with monoclonal ELISA-assay using anti-E-tag antibodies (3.2.9.1). OHA-500 mM was used as test antigen mixture for testing antibody specificities.

The monoclonal ELISA test of soluble E-tagged scFvs resulted in slight positive signal intensities compared to those expressed on phages, indicating that the concentration of soluble free scFvs in the medium is significantly lower than their displayed versions. Since the concentration of expressed soluble scFvs may strongly differ in their three possible locations (cytosol, periplasm, medium) no conclusions can be drawn from the real expression rate of a certain clone. Therefore expression tests of two randomly selected clones were performed to monitor secreted, periplasmic and cytosolic expression levels. Periplasmic and whole cell extracts were prepared and SDS-PAGE and Western blot analyses were performed to detect scFvs in the three different cellular locations. These assays did not provide unambiguous results regarding the location of scFvs, only MS/MS analysis detected traces of scFvs in the periplasms and whole cell extracts, while almost nothing in the media.

The expression of eukaryotic antibodies and their derivatives in prokaryotic systems is a challenging task, which in many cases requires time-consuming testing procedures for obtaining the desired yield and quality of recombinant protein. The HB2151-pCANTAB 5E expression system did not prove to be appropriate either for high-throughput screening or for expression optimization of soluble scFvs due to more limitations. HB2151 needs to be infected with phages via the F-pilus, however, it infects quite poorly, which lessens transfection efficiency. pCANTAB 5E enables only E-tagged scFv expression with an N-terminal leader sequence which directs the protein into three different locations. The testing of clones for soluble scFv expression requires time-consuming expression tests, moreover, the undigested leader sequence on scFvs may influence proper antibody folding and epitope recognizing ability if the protein is enriched in the cytoplasm.

Expression of His-tagged scFvs derived from positive phagemid clones

For the faster and easier implementation of expression tests, positive scFv inserts were re-cloned into a modified pET28 vector (pET28-*Sfi*I) to allow expression of scFvs with a C-terminal 6 Histidine fusion tag. The 6-His fusion tag allows fast and reproducible affinity

purification using considerably cheaper IMAC resins instead of costly, low binding capacity antibody-affinity columns. pET28 may be introduced in a variety of expression strains to optimize recombinant expression level and protein solubility. To be able to clone scFvs directly into pET28, the insertion of a *Sfi*I restriction site was required. The schematic picture of construction procedure is shown in 7.4. The detailed cloning protocol of scFvs in pET28(*Sfi*I) is described in molecular biology methods (3.2.4). After sequence analysis of re-cloned scFvs, plasmids were used to transform *E. coli* BL21(DE3) to perform expression. The small scale expression of His-tagged scFvs was tested at 37°C in the presence of 1mM IPTG as an induction agent for 2.5 and 5 h, respectively. The presence of scFvs was examined from the total cell extract of hosts and assayed by Western blot using His-probe antibody. The expression tests of 10 randomly selected scFvs (corresponding ELISA-assay of clones see Figure 4.2) are demonstrated with SDS-PAGE and their corresponding Western blot assay (Figures 4.3 and 4.4). A minor increase in scFv rate was observed with extending the induction time. The presence of scFvs separated along with the host proteins is not unambiguous on the SDS-gels, however, they show slight differences in their protein pattern if un-induced control samples are compared to the induced ones.

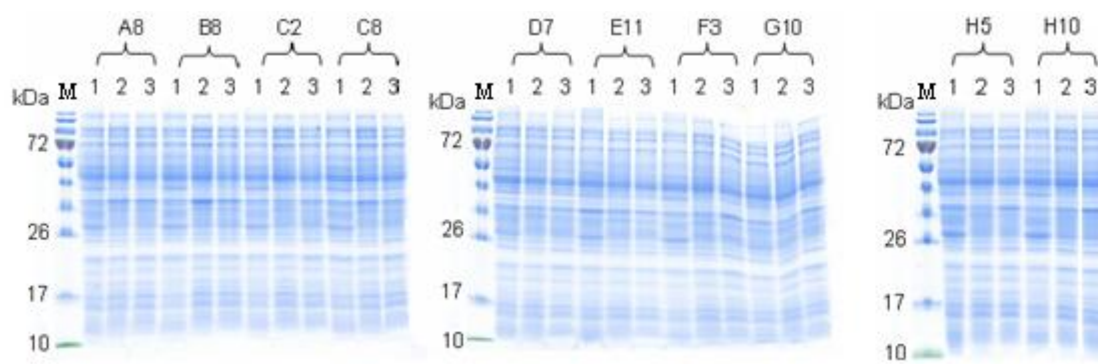


Figure: 4.3: Expression tests of OHA-500 mM-selected His-tagged scFvs separated by 15% SDS-PAGE. A8-H10 represent total cell extracts of BL21(DE3) expressing individual scFv clones. 1: Un-induced control (5 h), 2: 1 mM IPTG induction, 2.5 h induction time, 3: 1 mM IPTG induction, 5 h induction time. M: Molecular weight marker.

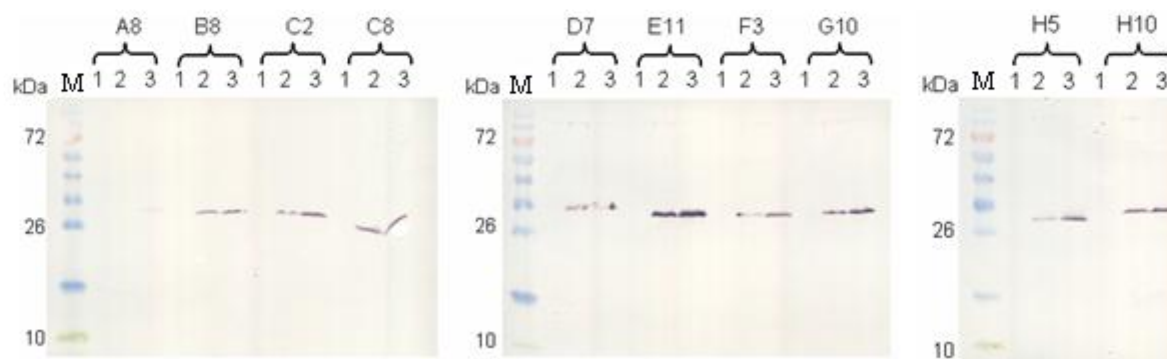


Figure 4.4: Expression tests of OHA-500 mM-selected His-tagged scFvs analysed by Western blot assay. A8-H10 represent total cell extracts of BL21(DE3) strains expressing scFv antibodies (the corresponding SDS-PAGE is shown in Figure 4.3). 1: Un-induced control (5 h), 2: 1 mM IPTG induction, 2.5 h induction time, 3: 1 mM IPTG induction, 5 h induction time. M: Molecular weight marker.

<u>Small scale purification of His-tagged scFvs derived from positive phagemid clones</u>

Small-scale affinity-purification of 8 scFvs from the soluble cytosolic fraction was performed using Ni-NTA beads, as described in 3.2.7.1. Protein purity was tested with SDS-PAGE, which detected ~26 kDa protein bands in the elution fractions (Figure 4.5). Western blot assays of the elution fractions provided evidence of soluble scFv expression with varying intensities (Figure 4.6). The Western blot assay of further scFv solubility tests is shown in Figure 7.6 of 7.7. The size of His-tagged scFvs varies between 27-29 kDa due to sequence length differences in the variable segments of heavy and light chains.

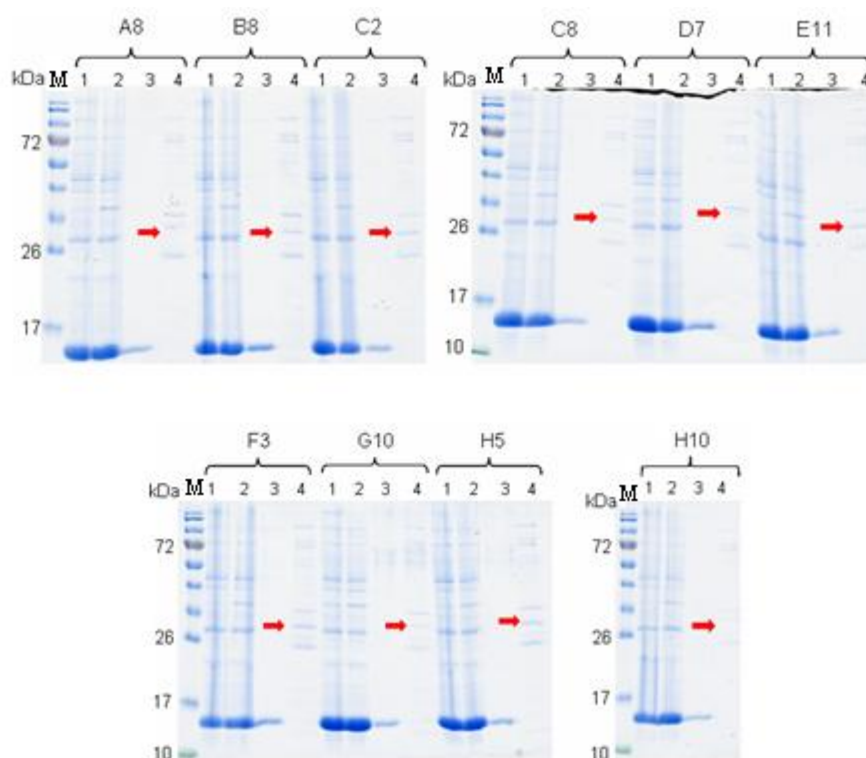


Figure 4.5: 15% SDS-PAGE analysis of Ni-NTA-purified soluble His-tagged scFvs. Small-scale expression and Ni-NTA purification of scFvs from the soluble cell fraction were carried out as described in 3.2.5.3 and 3.2.7.1, applying 1 x Coupling buffer. Bands of A8-H10 represent individual scFv clones. 1: Cytosolic extract, 2: Flow through, 3: Washing step, 4: 250 mM imidazole elution fraction. Red arrows show His-tagged scFvs. M: Molecular weight marker.

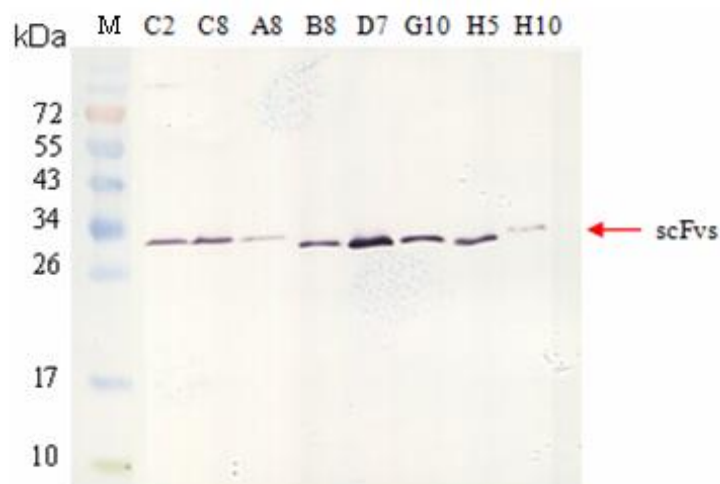


Figure 4.6: Western blot assay of Ni-NTA-purified soluble His-tagged scFvs (corresponding to SDS-PAGE in Figure 4.5). Bands of C2-H10 represent affinity-purified His-tagged scFvs. M: Molecular weight marker.

Identification of protein complexes using Ni-NTA-purified His-tagged scFvs

The antigen recognizing ability of purified scFvs was verified by Western blot assay, applying OHA-500 mM of *T. acidophilum* cytosolic extract as target mixture. Ni-NTA-purified His-tagged scFvs were used as primary antibody, while His-probe antibody and alkaline phosphatase conjugated anti-rabbit were used as secondary and tertier antibodies, respectively (Figure 4.7).

Three of the tested scFv clones (corresponding ELISA-assay of clones see in Figure 4.2 of 4.2.1.1) showed reactivity in the size range of 58 kDa (B8, C8, G10) and three clones in the size range of 35 kDa (A11, D7, F3). No scFv binding was obtained in case of four clones, which might be indicative of low scFv concentration applied for the incubation or the low concentration of specific target protein in the cytosolic extract.

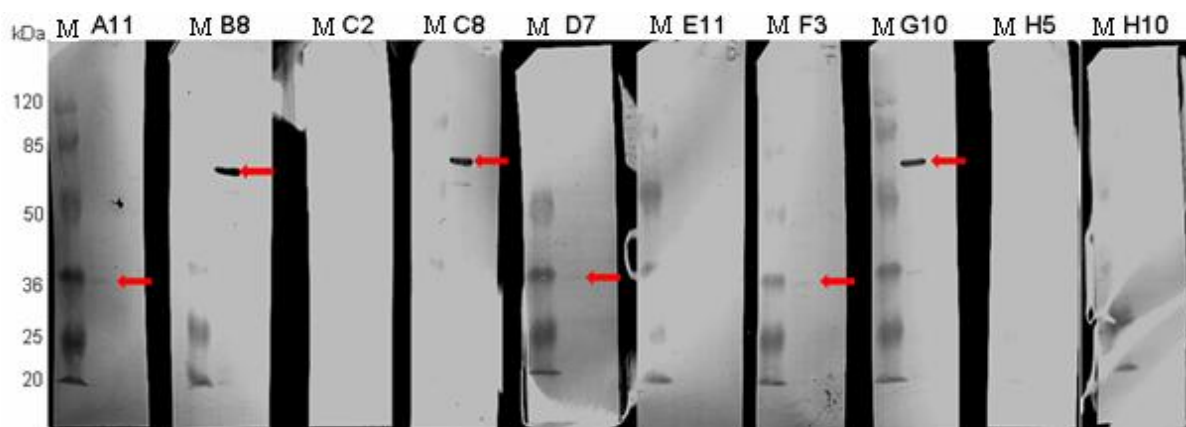


Figure 4.7: Identification of protein bands by affinity-purified His-tagged scFvs selected for OHA-500 mM proteins. Bands of A8-H10 represent affinity-purified scFvs tested for protein specificity against OHA-500 mM as test antigen mixture. Red arrows show proteins recognized by the specific scFv clones. M: Molecular weight marker.

Affinity purification of protein complexes on agarose bead-immobilized scFvs

Our first attempts showed that scFv-displaying phages coupled to agarose beads were not applicable for protein pull-down assays. Therefore, in the second approach we used agarose-

immobilized purified scFvs. To prove the viability of this approach the C8-scFv was chosen and purified from 2 L *E. coli* BL21(DE3) culture that was induced for 5 h with 1mM IPTG, at 37°C. Elution fractions containing soluble His-tagged scFvs were collected and concentrated Figure 4.8 shows the 15% SDS-PAGE result of IMAC purification.

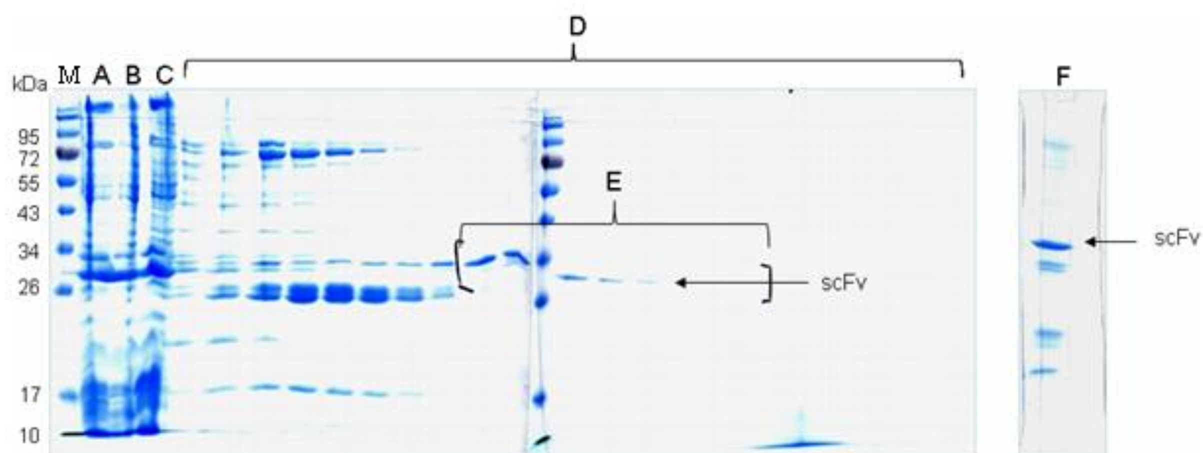


Figure 4.8: 15% SDS-PAGE-separated IMAC-chromatography fractions of the C8-scFv. Purification was carried out according to protocol 3.2.7.1 using 1 x Coupling buffer. (A) BL21(DE3) cytosolic extract, (B) Flow through, (C) Washing step, (D) 20-250 mM imidazole gradient elution fractions, (E) Pure fractions of C8-scFvs were pooled and concentrated (F) for pull-down assays. M: Molecular weight marker.

Purified C8 scFvs were coupled to activated agarose beads and used to pull down specifically bound complexes from *T. acidophilum* cytosolic extract. Coupling procedure and pull-down assay was performed according to the manufacturer's protocol (Pierce® Co-Immunoprecipitation Kit, Thermo Scientific). Samples of each purification step were loaded on 15% SDS-PAGE to analyze purity (Figure 4.9). Two major protein bands at 58 kDa were detected corresponding perfectly to the Western blot analysis (Figure 4.7). The first elution fraction (Figure 4.9, fraction No. 8) was analyzed by MS/MS, which identified Ta0980 (α) and Ta1276 (ß) subunits of the molecular chaperonin thermosome. To verify that the protein structure remained intact during the elution procedure an aliquot of the same fraction was analyzed by single particle electron microscopy in negative stain. (Figure 4.10)

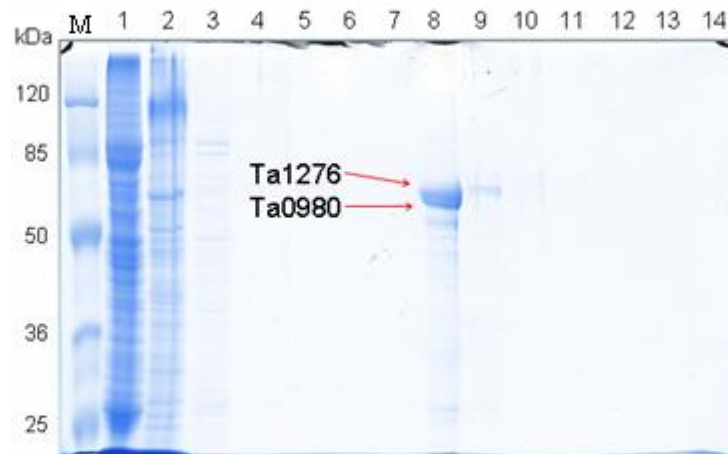


Figure 4.9: Affinity purification of thermosomes using agarose-immobilized scFvs. 1-10: Purification fractions of pull-down assay separated on 15% SDS-PAGE, 1: *T. acidophilum* cytosolic extract, 2: Flow through, 3-7: Washing steps, 8-11: Eluates, 12-14: Negative control, M: Molecular weight marker.

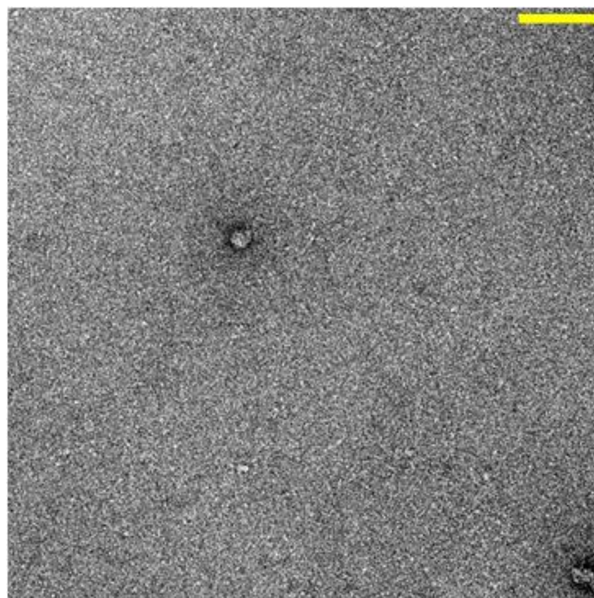


Figure 4.10: Negatively stained electron micrograph of thermosomes purified with agarose-immobilized C8-scFv. This method did not support the purification of intact thermosomes as no defined structures could be detected. The yellow scale bar corresponds to 100 nm.

Thermosome complexes fell apart during the elution procedure losing their native structure due to the harsh elution (low pH) condition. The subunits of protein complexes are held together by non-covalent bindings, which include ionic bonds, hydrogen bonds, Van der Waals and hydrophobic interactions which also dominate in antibody-antigen interactions. The disruption of antibody-antigen complex demands relatively harsh elution conditions, such as extreme pH (pH 2.2, pH 10), chaotropic ions (3 M KSCN, 3.5 M NaSCN) or denaturing agents (6-8 M urea, 3-4 M guanidine-HCL) which can also affect the native structures of target protein complexes. Extreme pH values might denature both bait (antibody) and prey proteins. The elution of antigen from the antibody at neutral pH or other gentle conditions is also possible in certain cases. However, the main disadvantage of using mild conditions is that they are not strong enough to abolish intermolecular interactions, which often results in inefficient elution rate. Therefore, elution conditions have to be tested experimentally and established individually for each antigen-antibody pair. Although thermosome was thought to be stable it lost its integrity at low pH. Based on this experiment we concluded that the purification of complexes in their native state by this antibody-based pull-down assay does not fulfill the expectations.

4.2.1.4 Purification of protein complexes using a two-step chromatography method

To avoid dissociation and possible denaturation of protein complexes caused by antibody-antigen interaction abolishing eluents/agents, target complexes were purified together with His-tagged scFvs in a two-step chromatography method. As imidazole-based elution buffers generally do not affect intra and intermolecular interactions and they can be adjusted to suitable pH and salt conditions IMAC-based affinity chromatography was used as the first purification step. An additional purification step based on size exclusion chromatography was used to separate high molecular weight scFv-captured complexes from smaller contaminants. This method does not require purified scFvs and enables the same buffer conditions (pH, salt components and salt concentration) during both purification procedures.

Purification of scFv-captured thermosome from *T. acidophilum* cytosolic extract

IMAC-purification of scFv-captured complexes:

E. coli and *T. acidophilum* cell lysates were prepared in Coupling buffer according to protocol 3.2.5.3 and 3.2.6.2, respectively. The two cell lysates were mixed and sedimented together at 30 000 x g and 4°C for 40 min. Clear supernatant, containing the soluble cytosolic fractions was transferred into a new polypropylene tube and incubated at RT for 1 h. The sample was amended with 10 mM imidazol and loaded on a 1 ml volume HisTrap FF crude IMAC column which was connected to an ÄKTA purifier FPLC system. Aliquotes of the eluted fractions were loaded on 15% SDS-PAGE and analyzed (Figure 4.11). The presence of both bait and prey was observed in fractions 18 to 34 and their stoichiometric elution pattern indicated the efficiency of this purification method. To make sure that the thermosome remains intact during the purification, thermosome-containing fractions were pooled and subjected to size exclusion chromatography.

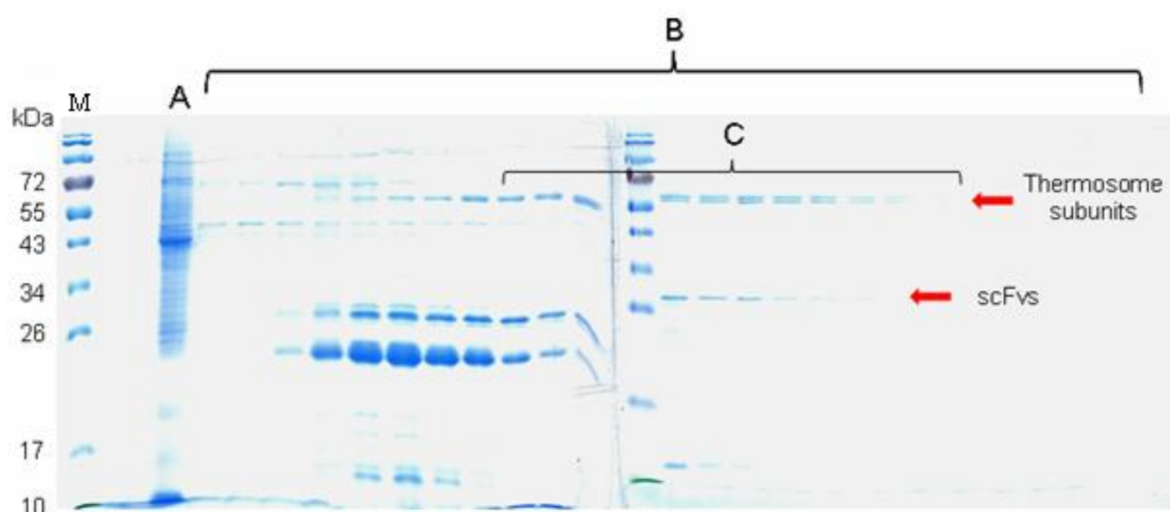


Figure 4.11: 15% SDS-PAGE-separated IMAC-purified fractions of scFv-captured thermosomes. (A) Flow through of combined *E. coli* and *T. acidophilum* cell extracts, (B) 20-250 mM imidazole gradient elution fractions, (C) Elution fractions containing scFv-captured thermosomes. M: Molecular weight marker.

Superose 6 chromatography of IMAC-purified scFv-captured thermosomes

Fractions of Superose 6 chromatography were analyzed by 15% SDS-PAGE. (Figure 4.12) Thermosome subunits were detected in fractions 11-18 representing a Gaussian distribution. The peak maximum corresponds to 11.5 ml retention volume, where ~900-1000 kDa molecular weight complexes are eluted which is in good agreement with the size of the thermosome complex. The stoichiometry of thermosome subunits and the scFvs along the two-step chromatography purification indicates that the termosomes were captured by their highly specific scFvs. The purification assay yielded 80 µg, 98% purity of thermosomes from 2 g *T. acidophilum* biomass.

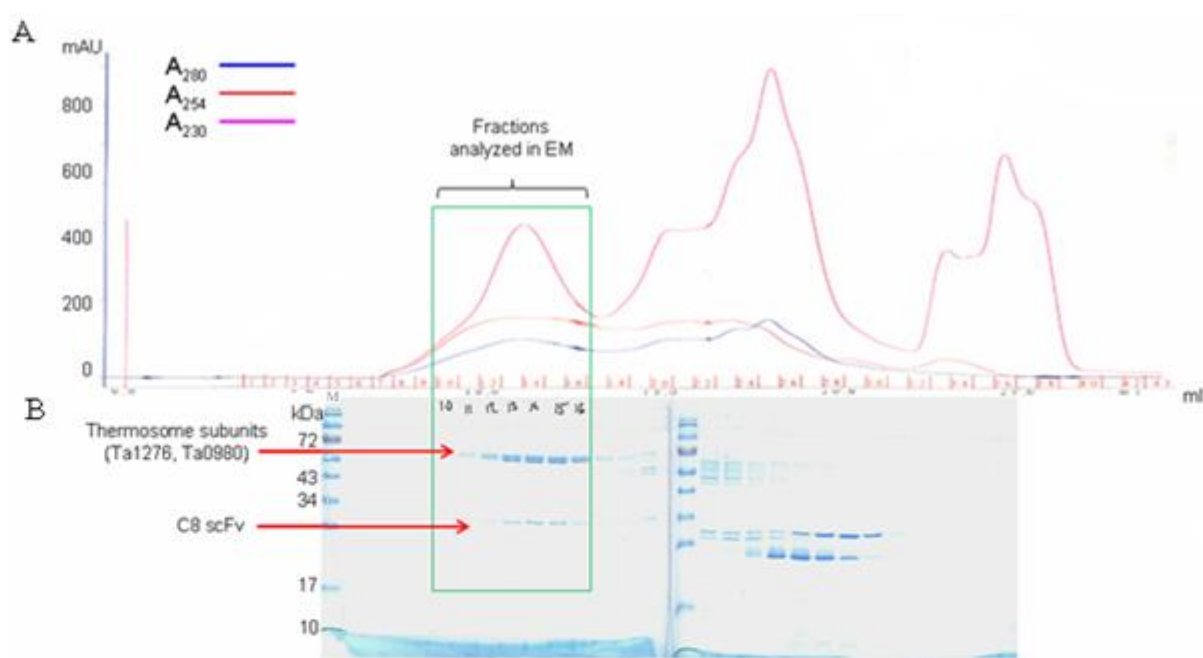


Figure 4.12: Chromatogram of Superose 6-purified scFv-captured thermosomes (A) and the 15% SDS-PAGE analysis of the corresponding chromatography fractions (B). Fractions 11-18 contain the captured thermosome particles. M: Molecular weight marker.

To monitor the structural integrity of thermosome complexes fraction 15 was analyzed by single particle electron microscopy in negative stain. Thermosomes are seen in their intact structure (Figure 4.13), which can serve as a perfect, homogenous particle set for data collection, class averaging and 3D reconstruction. The attachment of thermosome particles to EM-grid enabled class averaging from two different views. Side view demonstrates two ring-constituents of the complex, while top view indicates the well known eight member ring architecture.

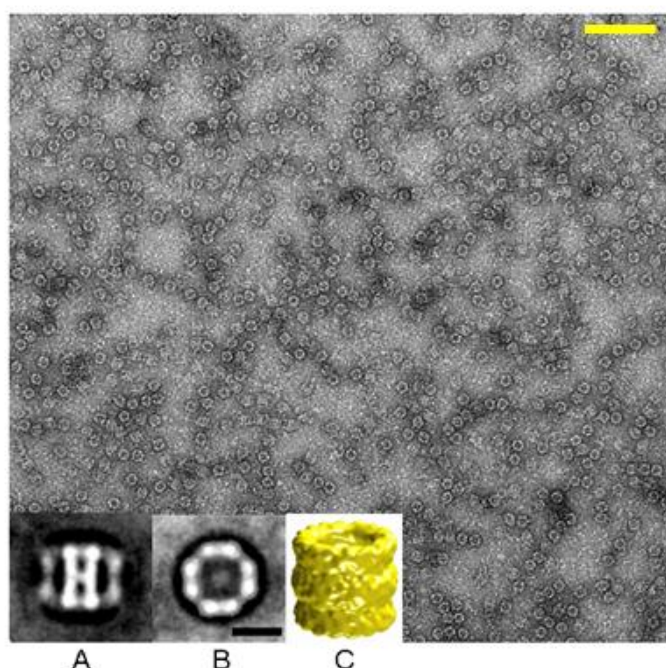


Figure 4.13: Electron micrograph of scFv-captured, Superose 6-purified and negatively stained thermosome particles. The yellow scale bar corresponds to 100 nm, the black scale bar corresponds to 10 nm. (A) Class average of side view, (B) Class average of top view, (C) 3D-reconstruction from the data.

The thermosome is a 940.8 kDa molecular weight complex, forming two stacked, eight-membered rings, which comprise the alternating α and ß subunits (91). Our antibody-based purification technique combined with the two-step chromatography separation method allowed the isolation the intact complex. With the purification of this well-known complex we demonstrated the applicability of the scFv-based purification technique for other high molecular weight protein complexes from the native cell extract.

Purification of scFv-captured proteasome complexes from *T. acidophilum* cytosolic extract

The next scFv candidate for purification purposes was A11-scFv (corresponding ELISA-assay is shown in Figure 4.2). The purification of complexes was performed in the same way as described (4.2.1.4). ScFv-containing protein fractions were analyzed by single particle EM in negative stain and the major proteins of the fractions were identified by MS/MS. Figure 4.14 demonstrates the results of the second purification step.

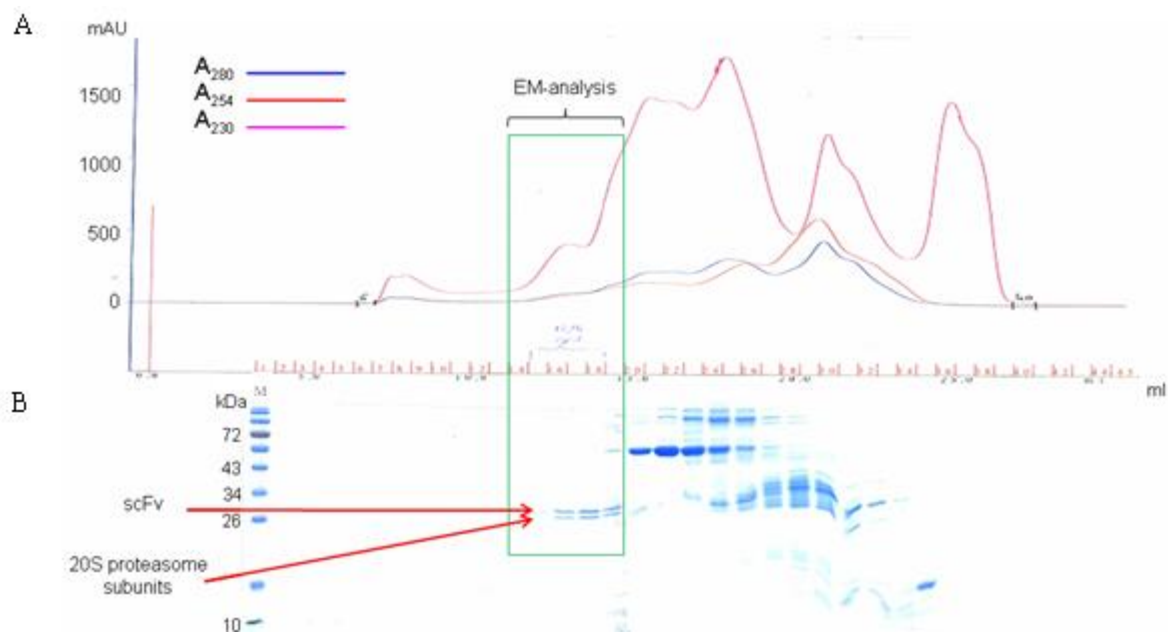


Figure 4.14: Chromatogram of Superose 6-purified scFv-captured proteasomes (A) combined with the 15% SDS-PAGE of respective chromatography fractions (B). Fractions 14-19 - indicating pure proteasome bands - were analyzed by MS/MS and EM. M: Molecular weight marker.

A11-scFv was identified as recognizing a ~26 kDa protein target (data not shown). Accordingly, a protein band of 26 kDa was detected in fractions 14-19. The protein band showed stoichiometric conformity with a slightly bigger protein in the same retention volumes. The MS/MS analysis identified α and ß subunits of *T. acidophilum* proteasome, Ta1288 and Ta0612, respectively. Furthermore, the analysis identified significant amounts of antibody in the same protein samples. The real molecular weight of α-subunit is 25.8 kDa, while ß-subunit is 23.2 kDa, but our SDS-PAGE running conditions did not allow the proper

separation of the two subunits. This resulted in one major protein band at ~26 kDa, representing the two overlapping subunits of proteasome and a band at 27 kDa, representing A11-scFvs. The first elution peak of the size exclusion chromatography corresponds to 12.5 ml retention volume where 600-800 kDa molecular weight complexes like the 686 kDa proteasome particles are eluted (Figure 4.14, green rectangle). Single particle EM-analysis of fractions 14-19 revealed the characteristic shape of side and top views of proteasome particles (Figure 4.15).

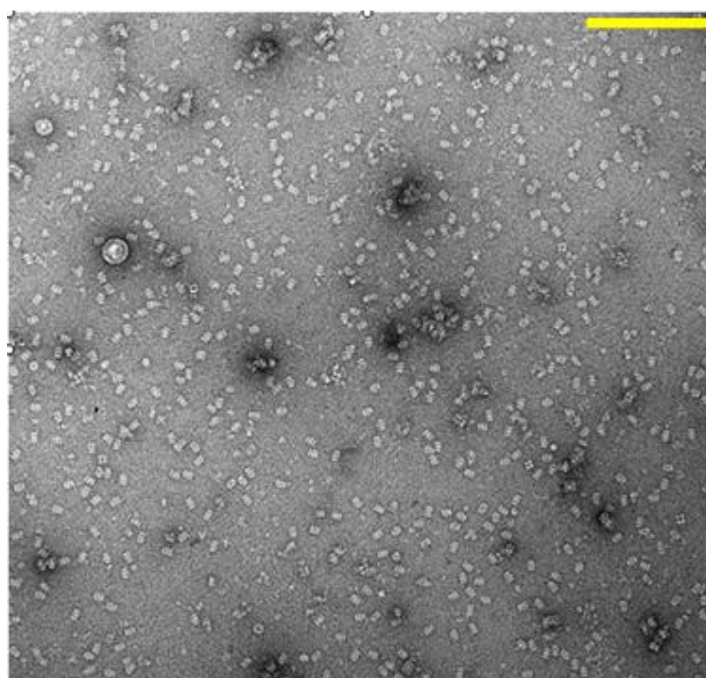


Figure 4.15: Negatively stained electron micrograph of Superose 6-purified and scFv-captured proteasomes. The yellow scale bar corresponds to 200 nm. Sample was prepared from Superose 6 elution fraction No.16.

The 20S proteasome of *T. acidophilum* is interacting with the CDC48 homolog ATPase (Ta0840) (92, 93). This interaction was demonstrated in pull-down and biochemical assays with heterologously expressed proteins, however in our pull-down assays with two 20S proteasome specific antibodies no traces of Ta0840 could be detected. This might be due to the longer purification procedure in which the two interacting partners dissociate.

Purification of scFv-captured peroxoredoxin from *T. acidophilum* cytosolic extract

The next antibody candidate for the complex purification was G11-scFv (Figure 4.2 of 4.2.1.1). The cloning and expression of His-tagged scFvs and the protein purification assay was carried out as described in 4.2.1.3. The protein content of the Superose 6 chromatoghraphy fractions was analyzed with SDS-PAGE and MS/MS and the scFv-containing fractions were analyzed with EM in negative stain.

Two major protein bands were detected by the SDS-PAGE analysis in fractions 16-20 (Figure 4.16), whose sizes corresponded to the molecular weight of scFv (26 kDa, upper band) and the expected molecular weight of the target protein recognized by the anibody (~20 kDa, lower band) based on previous Western blot assays (data not shown). MS/MS analysis identified the 20 kDa band as Ta0152 (probable peroxiredoxin) and confirmed the presence of the scFv as the other major protein component in the same fractions, proving that the scFv specifically recognized Ta0152. The chromatography peak maximum of fractions 16-20 was obtained at 15.4 ml retention volume, corresponding to a molecular weight of 150-250 kDa. Previous proteomics studies showed that Ta0152 possess complex forming ability suggesting a ~500 kDa molecular weight, which size was in good agreement with its hexadecameric homologue of *Aeropyrum pernix* (94). Our result might indicate that the antibody purified Ta0152 ring was prevented from ordered aggregation which was detected in earlier studies (89), therefore the shape of the complex could not be visualized by EM (data not shown).

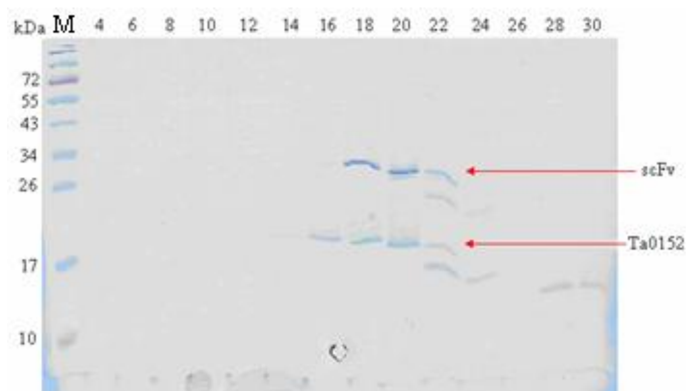


Figure 4.16: 15 % SDS-PAGE analysis of Superose 6-purified scFv-captured peroxiredoxin. Lane 4-30: Elution fractions of Superose 6 size exclusion chromatography. Fractions 16-20 were analyzed by MS/MS and EM. M: Molecular weight marker.

4.2.2 Superose 12-enriched high molecular weight protein fraction (Sup12-HMWF) of *T. acidophilum* cytosolic extract as target mixture

As previous results showed, the specificity of scFv-displaying phages in the third generation scFv-library is limited and disproportional to proteins that are present in the cytosol at high concentration (3) and are good antigens (thermosome and proteasome). The use of OH-apatite chromatography as a second target fractionation step further increases the enrichment of these abundant protein complexes in the OHA-500 mM target mixture. The abundance of thermosomes and proteasomes in the applied antigen mixture favors their corresponding scFvs to be selected during the selection procedure. Thus, those scFv-displaying phages, which are specific for low expression level protein complexes are not exposed to high selection drift due to unequal protein ratios. This hinders the diversity of phage binders during the biopanning cycles.

To avoid segregation of highly abundant target complexes by further fractionation, the next selection procedure was carried out against the crude Superose12-separated cytosolic extract of *T. acidophilum* (this protein mixture was used for mouse immunization).

4.2.2.1 Phage selection and screening against Sup12-HMWF

ScFv-displaying phages were selected through 3 biopanning cycles using Sup12-HMWF as target protein mixture. The phage propagation and selection procedure was carried out according to basic protocol 7.2 with minor modifications as follows: to amplify scFv library 100 ul (~1 x 10^8 clones) of the original library was inoculated into 100 ml of YTAG. Target antigens in the immuno-tubes were incubated in 1 x PBS buffer, ON. In each cycle, the elimination of unbound phages included 5 PBST and 5 PBS washing steps, respectively.

After the third selection cycle an 8 x 10^3 fold-increase was obtained compared to the first round, yielding 4 X 10^8 CFU/ml. Third generation phages were checked by Western blot assays to test diversity of the polyclonal phage mixture.

The Western blot assay of polyclonal phages selected for Sup12-HMWF resulted in 15 clearly visible protein bands, of which 11 bands were detected over the size of 43 kDa. Figure 4.17 shows the diversity of third generation phages arising from two different phage selection procedures.

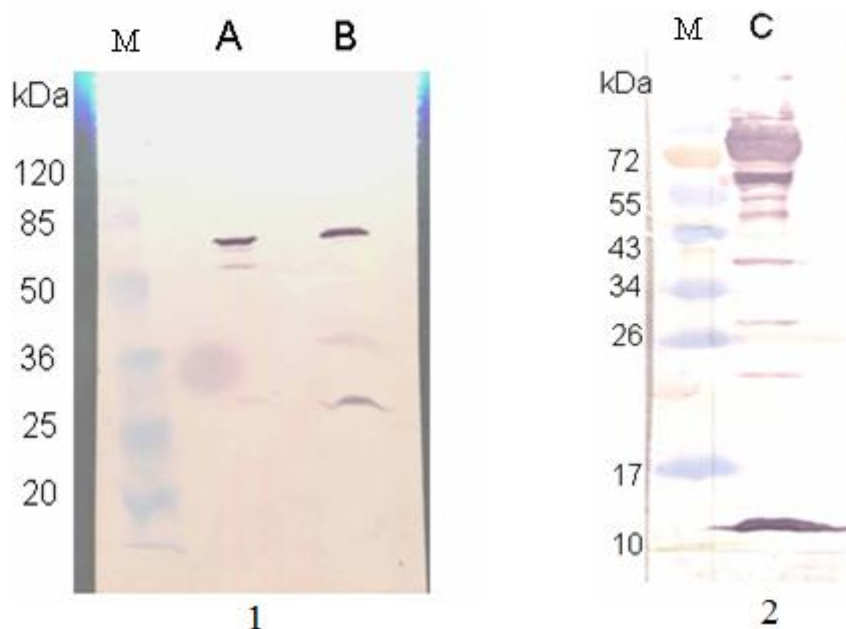


Figure 4.17: Comparison of polyclonal phage diversity arising from two different phage selection strategies using Western blot assays. The first Western blot (1) demonstrates amplified output 3 phages selected against OHA-500 mM proteins, the second Western blot (2) shows amplified output 3 phages selected against Sup12-HMWF proteins. Applied test antigens (A) *T. acidophilum* cytosolic extract, (B) OHA-500 mM, (C) Sup12-HMWF. Western blot signals were developed with Sigma*FAST* BCIP/NBT substrate system. M: Molecular weight marker.

The use of Sup12-HMWF as target antigen mixture resulted in many more detectable phage binders than the selection against its further purified OH-apatite fraction. The selection of appropriate target mixture to be used during the phage biopanning is therefore a prime importance that strongly influences the enrichment of low copy number or low affinity phage binders in the next generation libraries.

4.2.2.2 Phage selection against low abundant complexes combined with de-selections against abundant complexes

To increase the diversity of our scFv libraries, de-selection steps against abundant protein complexes were introduced. The de-selection approach is a library clean-up approach, in which the third phage selection cycle is followed by further biopanning steps using purified antigens to remove their binders in order to facilitate the discovery of scFvs against other complexes.

De-selection of phages against recombinant thermosome and proteasome

Sup12-HMWF-selected and amplified output 3 phages were used as phage input for the de-selection assay (corresponding Western blot assay of phages see in Figure 4.17 of 4.2.2.1). Immunotubes were coated with 4 ml of 100 µg/ml protein mixture diluted in PBS. The protein mixture contained 40 % α-proteasome, 30 % α-thermosome and 30 % ß-thermosome. Tubes were incubated, blocked and washed according to the standard protocol and filled with 4 ml PBS containing 100 µl purified phage solution. Tubes were incubated with the phages for 1.5 h at RT. Output phages (output phage 4) were added to 11 ml of exponentially growing TG1 cells and incubated at 37°C for 30 minutes. The infected cells were grown ON at 30°C, counted, and collected. The de-selection process was repeated again as described with minor modifications: after incubation with target antigens, 2 ml of the output phage solution (output phage 5) was added to 10 ml exponentially growing TG1 cells. The rest of phages was mixed with 15% glycerol and stored at -80°C. The infected cells were grown ON at 30°C, counted and collected.

The de-selections yielded 7.5 x 10^{10} CFU/ml of output phage 4 and 1.4 x 10^{10} CFU/ml of output phage 5. Since the input phage mixture contained 8 x 10^{12} CFU/ml of phages, this meant a ~10^2 fold decrease of phage number in each de-selection cycle.

ELISA screen of de-selected phages for protein complex binding specificity

Output 5 TG1 colonies were picked and used to establish monoclonal cell lines for ELISA-assay. Monoclonal ELISA was performed as described in protocol 3.2.9.1 using two different test antigens. Phages were tested against Sup12-HMWF as real target antigen mixture and against thermosome-proteasome mixture as negative target antigen mixture (Figure 4.18).

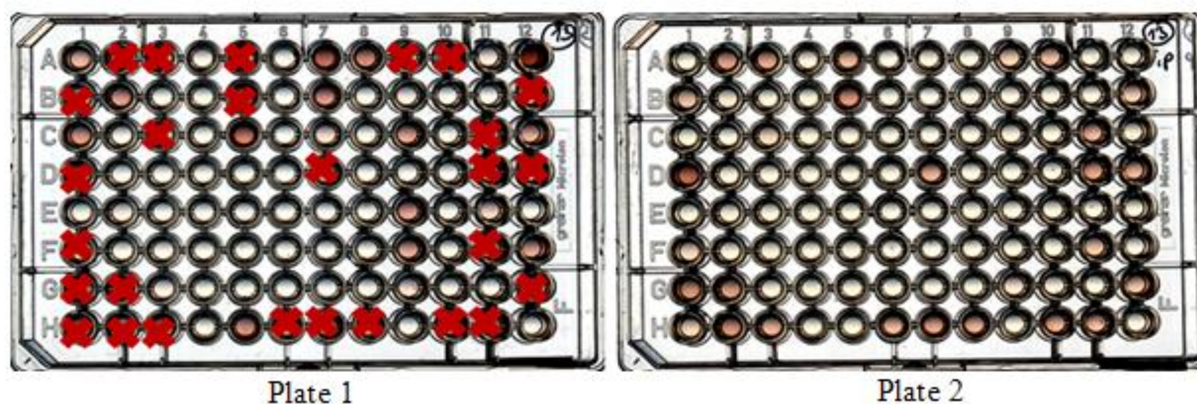


Figure 4.18: ELISA-assays of de-selected monoclonal output 5 phages tested against two different protein mixtures. Plate 1: ELISA-plates coated with the real target antigen mixture Sup12-HMWF, Plate 2: ELISA-plates coated with the de-selection target mixture (purified, recombinant α- and β-thermosomes and α-proteasome. ELISA signals were developed with HRP substrate system (5-aminosalicylic acid and H_2O_2). Red X-s depict those positive phage clones which gave positive signal on both plates and were excluded from further screenings.

All together, 373 monoclonal phages were screened by ELISA, of which 217 showed positive signals against Sup12-HMWF. Among these 155 (70%) indicated specificity on the negative target plate as well, while 62 (30%) showed specificity only on the real target plate. For further testing, these 62 clones were screened for protein binding specificities with Western blot assay using Sup12-HMWF. Monoclonal phage propagation and Western blot assay were carried out as described in 7.2 and 3.2.9.2, respectively. Figure 4.19 shows the Western blot tests of 7 randomly selected phages.

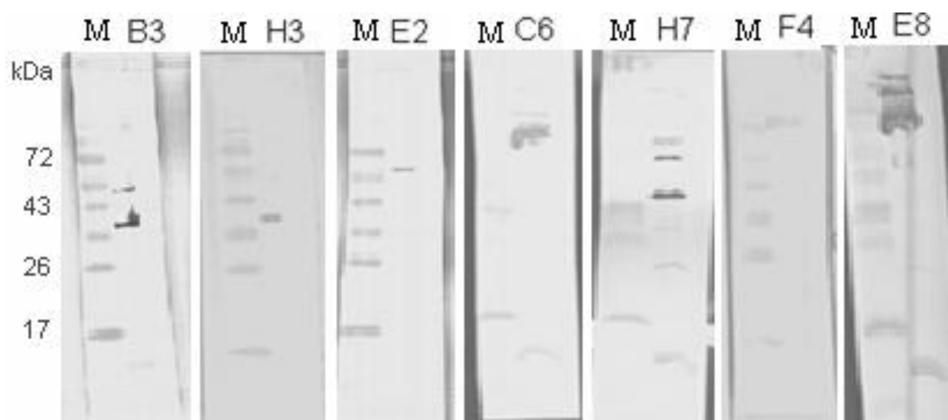


Figure 4.19: Western blot assays of de-selected output 5 monoclonal phages against Sup12-HMWF antigen mixture. B3-E8 represent output 5 phage clones arising from the last de-selection cycle. M: Molecular weight marker.

From the 62 selected ELISA-positive phage clones 52 proved to be specific against protein components of Sup12-HMWF. The Western blot assay of positive ELISA-clones revealed specific phage binders against new target antigens. Furthermore, the lack of binders against the 72 kDa and 26 kDa protein (characteristic for the thermosome and proteasome subunits) was also indicative for the efficiency of the de-selection/screening procedure.

This experiment revealed that a vast number of interfering phages can be removed from the scFv-library via introducing negative de-selections against recombinant thermosome and proteasome subunits which are abundantly present in the Sup12-HMWF target antigen-mixture. However, the removal of unwanted phages from the library is never 100% due to the presence of multi-specific and low-affinity phages. These phages can, however, be easily distinguished in the system with a comparative ELISA-assay by using the negative target antigens as an additional control. The phage de-selection approach is therefore a usable and efficient method if used in combination with a comparative ELISA-assay.

Cloning and heterologous expression of de-selected scFvs

In the next steps, 26 scFv clones were selected to be tested for soluble His-scFv expression in *E. coli* BL21(DE3) cells. The cloning of scFv inserts was carried out as already described (4.2.1.3). Surprisingly, the digestion of pCANTAB 5E-scFv DNAs produced an additional DNA fragment at 500 bp besides the 750 bp scFv fragment in 70% of the clones. These constructs were sequenced to ascertain proper scFv coding frames. However, the sequence analysis of each clone failed due to unidentified reasons. It could originate from mixed sequences in the DNA template, which can occur due to multiple priming sites, frame-shift mutations or slippage due to homo-polymer or repeat regions in the template. In spite of this, *Sfi*I-*Not*I-digested 750 bp long fragments were excised from the agarose gel and cloned into pET28(*Sfi*I), but despite of successful cloning, the sequence analyses failed again. As small scale expressions did not result any His-tagged scFvs these kinds of experiments were terminated.

4.3 Protein complex purification with scFvs selected against recombinant *T. acidophilum* proteins

A set of target proteins - putative building blocks of probable protein complexes like Ta0202 (iron ABC transporter, ATP-binding protein), Ta0316 (putative ATP-ase, twitching mobility releated protein), Ta0326-27-28-29 (subunits of a putative, cytosolic fixABCX complex) Ta1073 (5'-diphosphate-sulfoquinovose synthase (SQD1) homolog protein), Ta1194 (phosphopantetheine adenylyltransferase), Ta1315 (ribulose-phosphate 3-epimerase), Ta1435-36-37-38 (subunits of the 2-oxoacid dehydrogenase complex) and Ta1488 (hypothetical protein COG coiled coil) (3, 11)- were heterologously expressed. The applied vectors, expression strains and the corresponding expression conditions of the respective recombinant targets are listed in table 7.2 of 7.9. The 15% SDS-PAGE analysis of protein expression profiles is shown in Figure 7.5 of 7.6.

4.3.1 Phage selection against recombinant targets

The conventional phage selection procedure (7.2) was used to select scFv-displaying phages for different implementations. The biopanning assays were carried out on plastic-immobilized surface and the type and amount of phage inputs, the number of selection cycles, the incubation/washing conditions and the target antigens were varied.

4.3.1.1 Phage selection against Ta0316, Ta1194, Ta1315 and Ta1435-38

Biopanning assays were carried out using Ta0316, Ta1194, Ta1315 and the co-expressed subunits of the Ta1435-36-37-38 complex as antigens. The recombinant proteins were expressed as described (Table 7.2 of 7.9), and purified by large-scale IMAC affinity chromatography according to protocol 3.2.7.1.

Phage biopanning and screening against Ta0316, Ta1194 and Ta1435-38

Output 3 phages (4.2.2.1) were used as phage input in biopanning experiments. IMAC-purified recombinant protein fractions were pooled and diluted to 100 µg/µl concentration in PBS and used to coat immuno-tubes (1 tube/protein). The biopanning procedure was carried out as described in 7.2 with minor modifications, as follows: 400 µl amplified output 3 phages was used for the phage selection and 5 washing steps were applied with PBST and PBS, respectively. Only one selection cycle was performed on the plastic-immobilized recombinant antigens to test panning output. Colonies were obtained from each output which were collected and stored as output stocks. The obtained output phages (8 x 10^4 CFU/ml) were immediately used for Western blot analysis to check protein specificity. Western blot assays were performed as described (3.2.9.2) using the corresponding recombinant proteins as test antigens.

The Western blot assay did not indicate any positive phage binder probably due to the low phage concentration in the incubation mixture (1.6 X 10^4 CFU/ml). The number of phages in the panning output can be influenced by different factors like the purity and concentration of the target antigen, the binding affinity to the target and the number of phages applied in the panning procedure. In our case the reason of the radical decrease in the phage output number could also be that the applied input phage solution was previously selected against a number of different protein targets and not against a defined one. If those phages, which are specific for the corresponding target proteins are under-represented in the input mixture, further biopanning cycles must be considered. On the other hand, the sensitivity of Western blot assay has also to be taken into account while evaluating signal intensities. Since the number of phage particles in the output mixture is always less then in the input phage solution, output phages must be amplified prior to Western blot assays in order to maximize the number of potential phage binders. The fact that the panning output yielded 4 x 10^4 fold less CFU/ml then the output CFU obtained after the previous selection cycle (4 x 10^8, see 4.2.2.1) is also an explanation for the poor signal intensities.

To screen for positive binders among the output phages, colonies from the panning output plates obtained after Ta1194 and Ta1315 selection were picked and used to create monoclonal cell lines. Furthermore, phages were also tested against Sup12-HMWF as a second test antigen. Surprisingly, no positive signal occurred on either of the test plates containing the

recombinant proteins, however, a certain number of positive binders were obtained on both plates coated with Sup12-HMWF (see supplementary data 7.4, ELISA plate 1-2). Although some phages proved to be specific for either of the target proteins in this target mixture, the lack of signals on the recombinant target plates indicated an unsatisfactory selection procedure against these targets.

Another critical point of this selection approach was the use of low expression level and/or unstable recombinant proteins. The selected recombinant candidates (Ta1315, Ta1194) showed a strong tendency to precipitate shortly after purification. This is especially crucial, if the protein is expressed recombinantly at an extremely low level (Ta1073). The proteins were therefore used in a fresh, affinity purified form for the coating of immuno-tubes to reduce storage time. However, proteins already started to precipitate prior to the ELISA assay, which resulted in less properly folded target antigen. Taking into account that the three biopanning steps and the additional ELISA screening requires a minimum of 7 days and the application of freshly purified protein sample for the coating procedure is not always feasible, we focused on more stable targets.

4.3.1.2 Phage selection against Ta1488

The next recombinant antigen used for the affinity selection of phages was the 35 kDa putative intermedier filament Ta1488. This protein proved to be stable in a wide temperature range and is expressed recombinantly at extremely high level. The 15% SDS-PAGE analysis of Ta1488 heat treatment is shown in Figure 7.4 of 7.6. The protein purity obtained after IMAC-chromatography was more than 90% and the concentrated protein stock showed no precipitation tendency even after several week storage at 4°C.

Phage biopanning and screening against Ta1488

The biopanning of scFv-displaying phages was carried out as described in 7.2 with minor modifications, as follows: 100 µl of output 5 phages (4.2.2.2) and 100 µl of amplified output 3 phages (4.2.2.1) were used as input phage mixtures. Two immuno-tubes were coated with 50 µg/ml affinity purified Ta1488 and used as panning target for both phage inputs. After one

selection cycle, the resulting panning output colonies were picked and used to propagate monoclonal phages as described in 7.3.1. Monoclonal ELISA-assay was performed: 2 plates were coated with the Sup12-HMWF and 2 plates with the affinity purified Ta1488. Both monoclonal phage plates were tested on both protein target antigens.

The ELISA assay resulted in 1-1 positive signal on the test plates coated with the recombinant Ta1488 target protein (Figure 4.20) and many more signals on the plates coated with Sup12-HMWF (ELISA plates 3-4 in Figure 7.3 of 7.5).

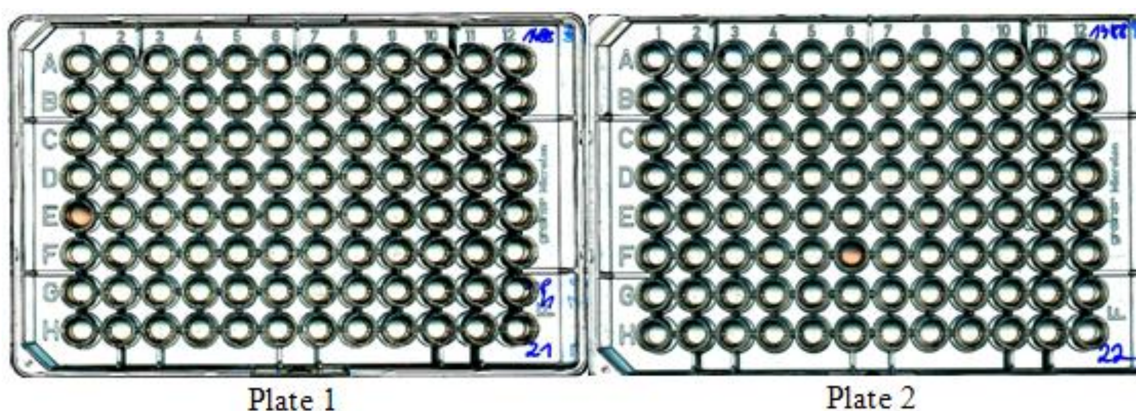


Figure 4.20: ELISA-assays of Ta1488-selected phages tested on affinity-purified Ta1488. Plate 1: Sup12-HMWF-selected, amplified output 3 phages (4.2.2.1) further selected against Ta1488. Plate 2: De-selected output 5 phages (4.2.2.2) further selected against Ta1488. ELISA signals were developed with HRP substrate system (5-aminosalicylic acid and H_2O_2).

ScFv clone E1 from plate 1 and scFv clone F6 from plate 2 (Figure 4.20) were further tested by Western blot to assure Ta1488 selectivity of phages on antigens separated under denaturing conditions. A few further clones that proved to be reactive only against Sup-HMWF (plates 3-4 in Figure 7.3 of 7.5) were also tested. The results of Western blot assays are shown in Figure 4.21 and 7.7 of 7.7. The tested clones were E1, C11, A2, A5, B2, C4 (ELISA plate 1 in Figure 4.20 and ELISA plate 3 in Figure 7.3 of 7.5) and F6, B3, A6, C1, C12, D8, G4 (ELISA plate 2 in Figure 4.20 and ELISA plate 4 in Figure 7.3 of 7.5).

ScFv clone E1 and F6 showed clear reactivity against recombinant Ta1488 in Western blot assay as well, serving unambiguous evidence of being specific for Ta1488. However, their reactivity against the Sup12-HMWF antigen mixture was observed only in ELISA conditions. Clones B3 and C11 appeared to specifically recognize a 35 kDa molecular weight protein target in the Sup12-HMWF antigen mixture by Western blot assay, of which only clone B3 proved to be reactive against the recombinant Ta1488. A few other phage clones proved to recognize a 60 kDa protein (probably thermosome) in the Sup12-HMWF antigen mixture (see clones A2, A5, B2, D8 in Figure 7.7 of 7.7), which indicated the presence of aspecific or multispecific phages in the system. Some clones exhibited slight reactivity against the recombinant Ta1488, which, however, is often observed in case of overloaded protein.

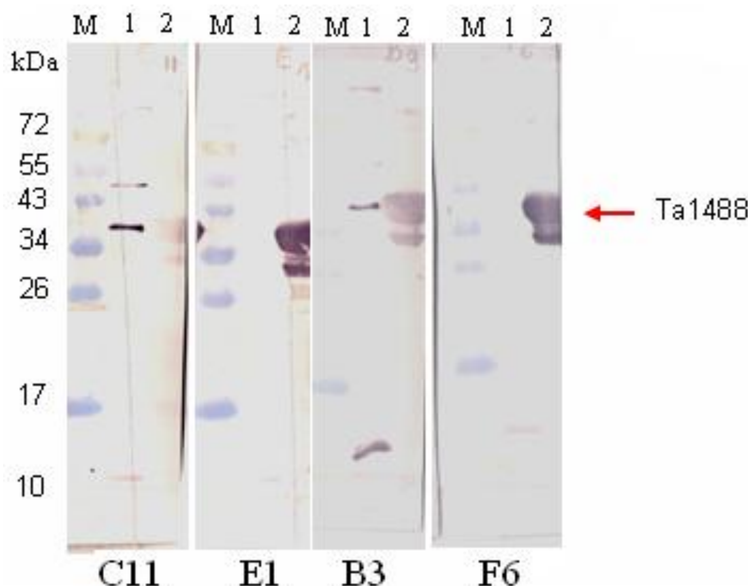


Figure 4.21: Western blot assay of Ta1488-selected monoclonal phages examined against two different test antigens. C11 and E1 phage clones correspond to ELISA plate 1 and plate 3 of Figure 4.20 and 7.3, respectively. B3 and F6 represent phage clones corresponding to ELISA plate 2 and plate 4 of Figure 4.20 and 7.3, respectively. 1: Sup12-HMWF test antigen, 2: Affinity purified recombinant Ta1488 test antigen. M: Molecular weight marker.

Since the binding affinity of respective scFvs to the natively folded target antigen was indispensable, only E1 and F6 phages suited the screening requirements by means of being specific for the recombinant Ta1488 not only in Western blot but also at ELISA conditions.

Cloning and heterologous expression of Ta1488 specific scFvs

Since the DNA-sequence of clones E1 and F6 turned out to be identical, clone E1 was selected to express His-tagged scFv in *E. coli* BL21(DE3). Unfortunately, no protein expression was obtained at any of the investigated conditions either in *E. coli* BL21(DE3) or other *E. coli* hosts (Rosetta, Tuner). Since mammalian genes frequently use codons which are rare in bacteria, the DNA-coding sequence of scFv was optimized for *E. coli* (Eurofins MWG Operon) and the synthetic scFv was cloned into pET28(*Sfi*I) and expressed in BL21(DE3) strain. The original and codon optimized E1-scFv sequences are shown in Figure 7.13 of 7.8. The synthetic E1-scFv expressed well in *E. coli*, however, in form of inclusion body. To avoid inclusion body formation, scFv expression conditions were further optimized using *E. coli* Rosetta cells (Table 7.3 of 7.9).

Purification of scFv-captured Ta1488 from the cytosolic extract of *T. acidophilum*

6-His E1 scFv was used to capture native Ta1488 from *T. acidophilum* by incubating and purifying the two cytosolic cell extracts as described previously (4.2.1.4). Fractions of Superose 6 chromatography were analyzed by SDS-PAGE (Figure 4.22) and proteins were identified by MS/MS. The 27 and a 35 kDa protein bands detected in fractions 19-21 were identified as the scFv and Ta1488, respectively. The third dominant band at 85 Da was identified as Ta1475, the ribonucleoside-diphosphate reductase, which is an abundantly expressed cytosolic protein of *T. acidophilum* (3) and it was probably purified together with the scFvs being bound to them as an extra protein prey or just a contaminant.

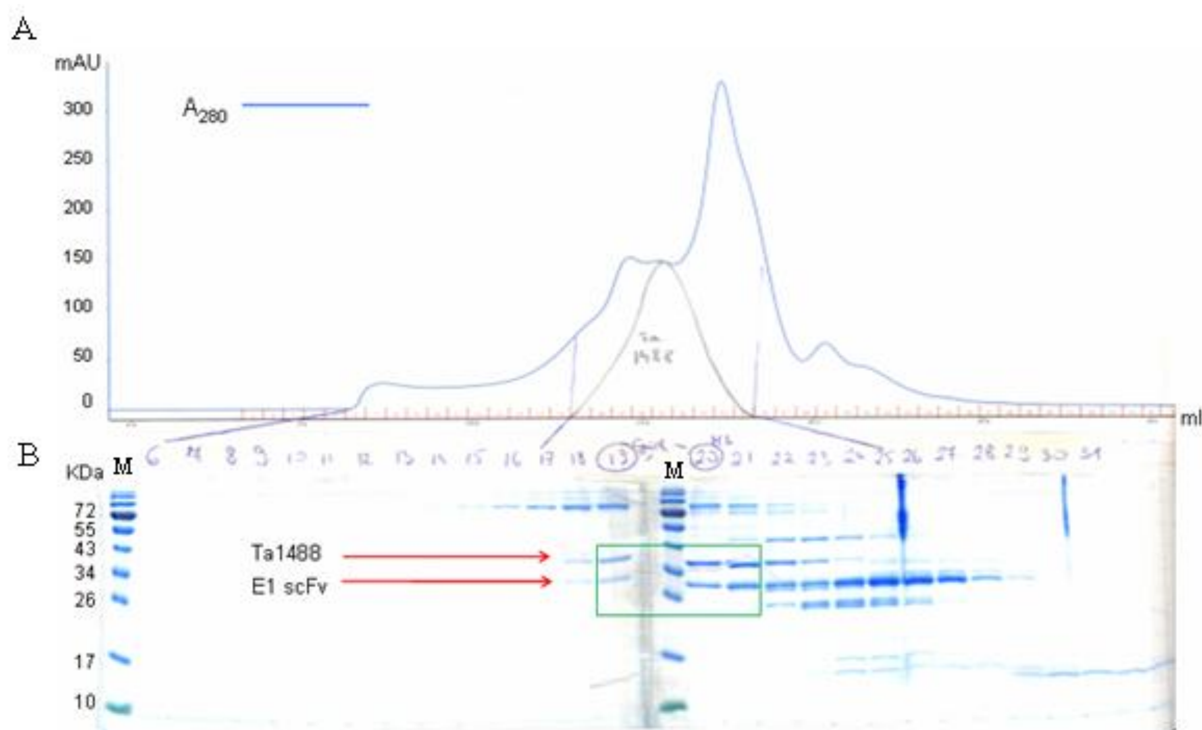


Figure 4.22: Chromatogram of Superose 6-purified scFv-captured Ta1488 (A) combined with 15% SDS-PAGE of respective chromatography fraction (B). Fractions 19-21 were analyzed by MS/MS and by EM. Green rectangle indicates the scFv and Ta1488 bands. M: Molecular weight marker.

The chromatography peak maximum of Ta1488 was at approximately 15.5 ml volume, which corresponded to 150-250 kDa which size did not allow a proper EM analysis. This size indicates that Ta1488 does not form multimeric complex as it was predicted from its amino acid sequence (2) or it disassembles during the purification and still undefined chemical supplements are needed in the purification buffers to keep the structure intact. Since only one scFv clone was tested in this assay and the sterical inhibition of multimerization by the antibody is also not excluded, the testing of other potential binders recognizing other epitopes is considered.

4.3.1.3 Phage selection against Ta0328

To purify the archaeal homologue of the fixABCX complex of *Rhodospirillum rubrum* (95) we targeted to isolate scFvs against Ta0328. In *T. acidophilum* Ta0326, Ta0327, Ta0328 and Ta0329 display high similarity to the corresponding subunits of this membrane complex, which participates in an electron transfer chain by shuttling electrons to the nitrogenase (95). There was proteomics evidence that this complex is not membrane-bound in *T. acidophilum* therefore its purification from the cytosolic extract with an appropriate antibody seemed realistic (11). Since the recombinant Ta0328 showed a fair protein expression level (Figure 7.5 of 7.6) and was relatively stable at short-term storage conditions, it was expressed in large-scale according to protocol 3.2.5.1 then purified for scFv selection/screening applications by Ni-affinity chromatography followed by Superose 6 size exclusion chromatography, as described in 3.2.7.1 and 3.2.7.2, respectively.

Phage biopanning and screening against Ta0328

Concerning the amount and purity of recombinant Ta0328, the selection of scFv-displaying phages was carried out on a special way, to avoid enrichment of non-specific and unwanted phage binders. For this, the conventional biopanning procedure was complemented with sequential de-selection steps applying two different negative targets. The first negative selection was performed by coating the immunotubes with the crude cell extract of *E. coli* TG1. The second de-selection step was carried out against the mixture of recombinant thermosome and proteasome complexes. The first de-selection step was introduced to eliminate disturbing *E. coli* protein binders, while the other one was to reduce bispecific or multispecific binders.

Three panning cycles were implemented as described in 7.2 with the following modifications (Figure 4.23). The first biopanning cycle started with inoculating 400 ml of medium with 1-1 aliquot of 5 different glycerol stock tubes containing the original scFv library. After ON amplification the phages were precipitated and resuspended in 6 ml of PBS then 500 µl of the suspension was used as phage input for the biopanning. In the first biopanning assay 2 tubes were coated with 400 µg/ml of *E. coli* cell extract, 2 tubes with 400 µg/ml of the thermosome-proteasome mixture (200-200 µg) and one tube with 400 µg/ml of Ta0328. The phage input

was first incubated for 30 minutes in the first tube containing the *E. coli* proteins then poured into the second tube and incubated for an additional 30 minutes. The consecutive steps were carried out similarly to the previous ones with the thermosome-proteasome-containing tubes. After the final de-selection step phages were poured into the Ta0328 protein-containing tube and incubated for 1 h. Tubes were washed 10 times with PBST then 10 times with PBS, after which phages were eluted and used for cell infection as described. For the second selection cycle the half volume of the TG1 output stock was inoculated into 100 ml of medium and used as starting culture for the next biopanning procedure. The second biopanning was performed similarly to the first one with the difference that 200 µg/ml of Ta0328 target antigen and 15-15 washing steps were applied with PBST and PBS, respectively. In the third selection cycle phages were incubated only once for 1 h with the de-selecting antigens and finally for 1 h with the real target tube, containing 100 µg/ml recombinant Ta0328.

The first modified biopanning procedure yielded 1.5×10^5 CFU/ml after the first phage selection cycle, while the second and third selection cycles resulted in 1.5×10^6 and 1.8×10^8 CFU/ml, respectively. The increase of CFU in the phage output (at constant phage input number) indicated the enrichment of certain scFv-displaying phages.

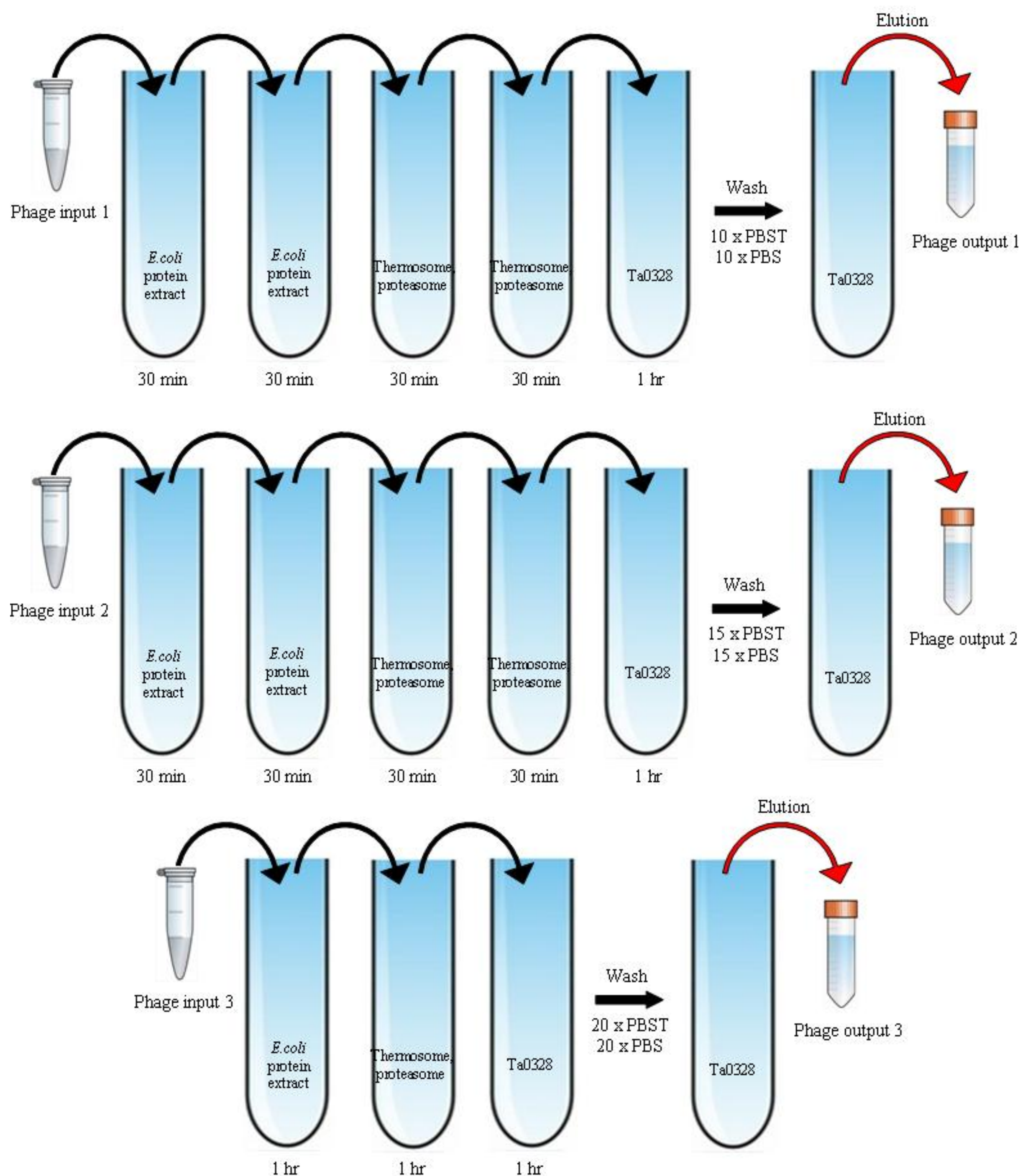


Figure 4.23: Work-flow of the modified phage-biopanning using recombinant Ta0328 as target protein. The schematic picture shows the sequential incubation steps along the three biopanning cycles with the appropriate antigens being bound to the immuno-tubes and the corresponding washing and elution steps. Phage input 1 represents the starting point of the first cycle, phage input 2 and 3 the second and the third one, respectively.

ELISA-screening of monoclonal output phages was implemented as described in 3.2.9.1. One plate of each was prepared from output 2 and output 3 colonies and tested for target specificity using Ta0328 as test protein (Figure 4.24). The negative control plates were coated with the mixture of recombinant thermosome, proteasome and the diluted cell extract of *E. coli*. Since the purity of Ta0328 was less then 70% and the complete elimination of aspecific phages is never accomplishable, the screening against protein contaminants coming from the recombinant host was also necessary.

Figure 4.24: ELISA-assays of Ta0328-selected monoclonal phages examined on purified Ta0328. Plate 1: Ta0328-selected output 2 phages. Plate 2: Ta0328-selected output 3 phages. Phages were selected by a modified biopanning method as described in 4.3.1.3. ELISA signals were developed with 1-Step Ultra TMB ELISA substrate system.

Eight binders were chosen to be screened with Western blot assay, as well (Figure 4.25). Only 4 out of the 8 candidates proved to specifically recognize recombinant Ta0328 as target antigen with various signal intensities, however, none of these appeared to detect the native protein in the Sup12-HMWF antigen mixture, probably due to its low expression level (12). In spite of this the 4 clones were cloned and expressed in *E. coli* for further purification purposes.

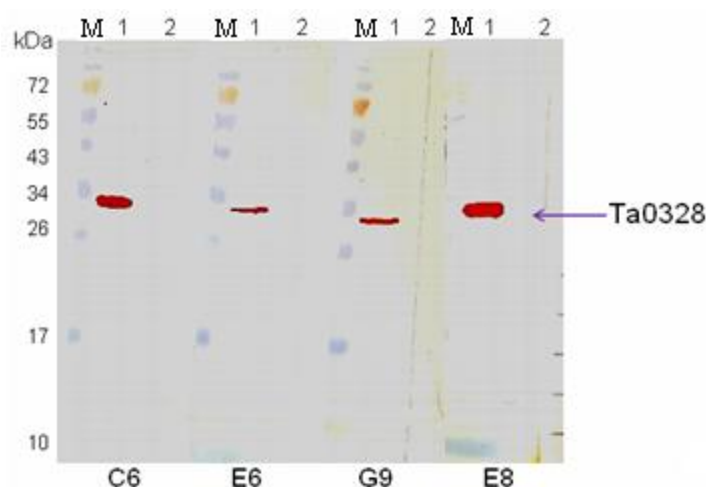


Figure 4.25: Western blot assays of Ta0328-selected monoclonal phages examined against two different test antigens (corresponding ELISA-assays of scFv clones are shown in Figure 4.24). C6, E6 and E9 represent output 3 phages (see ELISA plate 2 in Figure 4.24) E8 represents an output 2 phage (see ELISA plate 1 in Figure 4.24). 1: Purified recombinant Ta0328 as test antigen, 2: Sup12-HMWF as test antigen mixture. Western blot signals were developed with 1-Step TMB-Blotting substrate system (Thermo Scientific). M: Molecular weight marker.

Cloning and heterologous expression of Ta0328 specific scFvs

His-tagged scFvs were expressed in *E. coli* BL21(DE3) at 30°C for 5 h in the presence of 1 mM IPTG. To reduce the number of unwanted proteins in the complex purification assay, soluble scFvs were first pre-purified on a HisTrap^{TM} column as described (3.2.7.1) using 1 x Coupling buffer. His-tagged scFv containing fractions were collected, concentrated and 0.4 mg (~ 400 µl) was used for the complex purification assay.

Purification of scFv-captured Ta0328 from the cytosolic extract of *T. acidophilum*

The purification of the scFv-captured complexes from *T. acidophilum* cytosolic extract was carried out as described (4.2.1.4) with minor modifications, as follows: 400 µl of affinity purified scFv was added to the *T. acidophilum* cytosolic extract in 1 x Coupling buffer amended with 10% glycerol. The mixture was incubated with continuos rotation at 4°C for 1 h then 250 µl of binding buffer equilibrated Ni-NTA resin was added and incubated for an additional hour in the presence of 10 mM imidazole. Batch purification of scFv-captured complexes was carried out using 1 x Coupling buffer amended with 10 % glycerol then

elution fractions were further purified on a Superose 6 column. Fractions were analyzed on SDS-PAGE and the proteins of fractions of interest were identified by MS/MS. Figure 4.26 represents the data of this experiment.

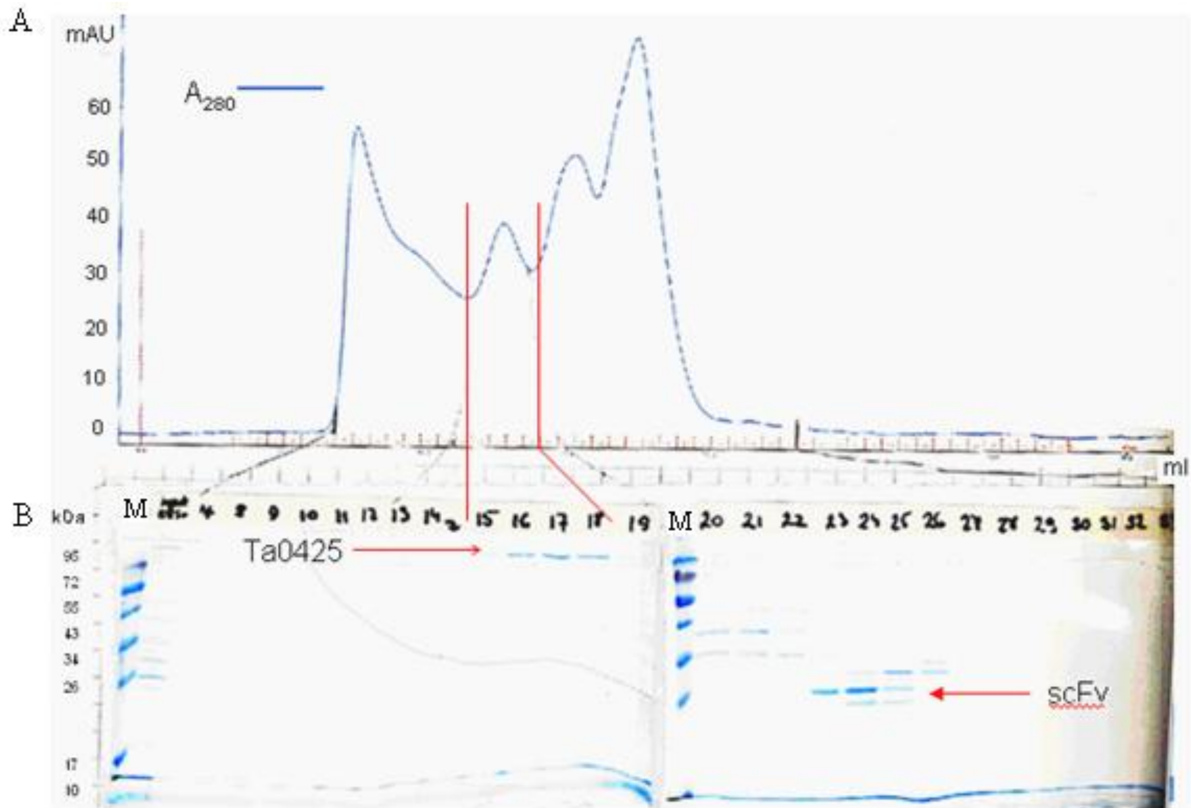


Figure 4.26: Superose 6 chromatogram of Ta0328 selected scFv-captured protein complexes (A) combined with the 15% SDS-PAGE of respective chromatography fraction (B). Fractions 15-21 were analyzed by MS/MS and by EM. M: Molecular weight marker.

In fractions 20-22 two dominant protein bands and many other less abundant proteins were detected, in the molecular range over 34 kDa. Ta0328 was detected by MS/MS in fractions 20-22 at very low level together with Ta0326. Ta0329 was detected only in fraction 20, while Ta0327 could not be traced. ScFvs were detected along fractions 19-26 with a significant increase in fractions 23 and 24. This result indicated that the fixABCX complex was captured specifically but its amount was very low and its native size (approx. 300 kDa) is lower as it was expected. Surprisingly, the 110 kDa protein in the high molecular weight protein fractions (16-18) was identified as Ta0425, the α-subunit of the putative cytosolic formate

dehydrogenase complex. The retention volume of its chromatography peak maximum corresponded to a size of ~450 kDa, which indicated the presence of a large protein complex. Ta0424 (16 kDa), a probable subunit of the complex was also identified by MS in the same fractions as a major protein component, although it was not apparent on SDS-PAGE. To clarify antigen specificity of Ta0328-selected scFv clones, the purification procedure was carried out in the same way as described above, testing the other three scFv clones for protein binding properties. Interestingly, each scFv clone exhibited the same purification pattern similarly to clone E8, although their amino acid sequence was not identical (Figure 7.10 of 7.8). Worthy to note, that the sedimented Ni-NTA resin showed a dark red color at the end of incubation with the antibody-cell extract mixture, indicating the enrichment of a metalloprotein. After eluting the proteins from the resin, the eluted protein solution also showed a slight pink color. However, the color was continuously paling during the storage and completely disappeared within a short period of time. This suggested that the bound protein is sensitive to aerobic conditions and probably reacted with molecular oxygen which shifted the optical absorption spectrum of the protein solution. In the control experiment, the Ni-NTA resin did not exhibit the red color after being incubated with the *T. acidophilum* cell extract and no protein was detected in the elution fractions by SDS-PAGE. Since the control experiment was carried out without adding of scFvs in the system, these results suggested, that Ta0425 purified together with the scFvs by means of antibody-antigen binding and not by means of the Ni-chelating ability of the protein.

The reason why the Ta0425 complex is captured not clear yet. It well might be that the complex is weakly associated to one of the proteins bound by the antibody or to the antibody itself and it was released upon separation on the Superose 6 column. This kind of complex dissociation on size exclusion columns is common as it was experienced in case of 26S proteasomes, *Streptomyces* 20S proteasome, *Thermoplasma* ribosome and many other complexes (I. Nagy personal communication). The fact that no scFv was found in fractions containing the Ta0425 complex supports this speculation. Since the purity of Ta0425 and Ta0424-containing protein fractions was over 90%, fraction 17 was analyzed in negative stain by EM to check protein complex integrity (Figure 4.27).

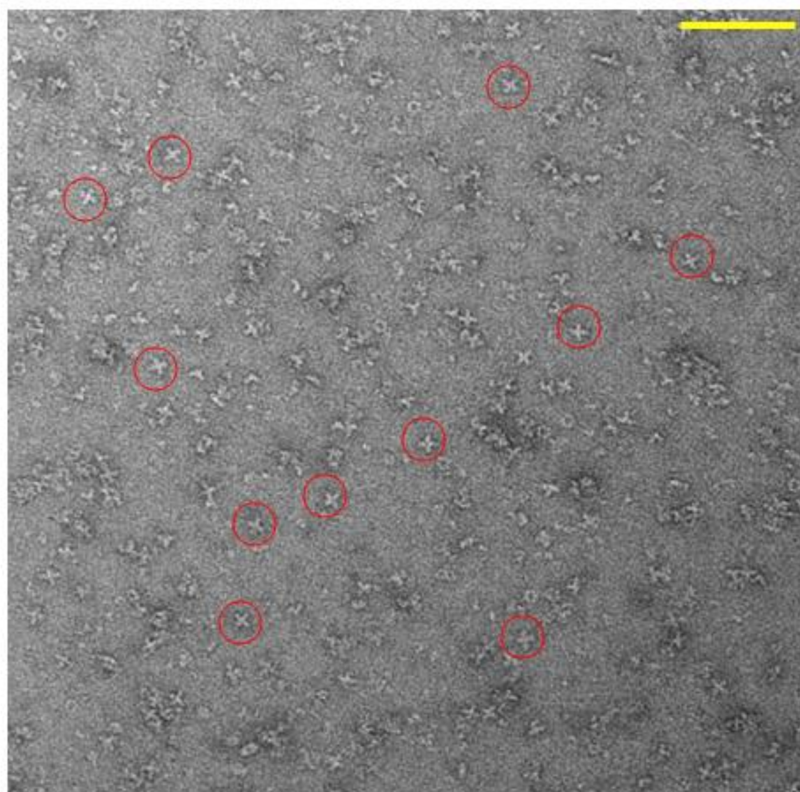


Figure 4.27: Negatively stained electron micrograph of the Superose 6-purified putative formate-dehydrogenase protein complex from fraction 17. Yellow scale bar corresponds to 100 nm. Red circles depict highly similar X-like structures which are probable formate dehydrogenase particles.

Ta0425 is a structurally not characterized protein complex exhibiting high homology to formate dehydrogenase H. Furthermore, Ta0425 showed significant homology to succinate dehydrogenases and uncharacterized anaerobic dehydrogenases, as well (COG3383) (96). On the gene level, the N-terminus of Ta0424 and the C-terminus of Ta0425 overlap by four bases, indicating an operon structure. Since both proteins were detected in the same Superose 6 fractions it is very likely that Ta0424 and Ta0425 form a complex. The estimated molecular mass of the protein is 450-500 kDa, which can be analyzed by cryo-EM. To substantiate the roles and stoichiometry of subunits within the respective complex, detailed structural and functional analyses are needed. Since recombinant expression of Ta0425 was not successful, the purification of its native form from *T. acidophilum* with scFv will help to elucidate the structure and function of this protein complex.

4.3.2 A novel biopanning method: Isolation of phages from nitrocellulose membrane-bound target proteins with the help of Western blot technique

As discussed in 4.3, recombinant target proteins are crucial players in biopanning since they facilitate the screening procedure of protein complex recognizing antibodies. However, most of the target proteins exhibit low expression levels and are highly sensitive to the purification conditions. To overcome these hurdles a novel phage biopanning method was developed, in which the phages were panned on nitrocellulose membrane-immobilized antigens instead of the conventionally used plastic surfaces. By this technique we aimed to purify proteins which due to different reasons like low expression level (Ta0202, Ta0326-27, Ta0825, Ta1073, Ta1435) or aggregation tendency during the purification (Ta0437-38, Ta0547, Ta1194, Ta1315) could not be captured. The protein expression pattern of these recombinant proteins is demonstrated in Figure 7.5 of 7.6. Detailed description of proteins is listed in Table 7.2 of 7.9.

4.3.2.1 Isolation of target specific phages from nitrocellulose membrane

Total protein extracts were prepared from *E. coli* cells expressing target antigens and 2 - 2 aliquotes of each samples were loaded on a 15% SDS-gel next to each other by leaving 1 empty well between the different extracts. The gels were subjected to Western blotting after gel electrophoresis, after which the isolation of phages from the membrane was carried out as follows: After ON blocking in 3% MPBS the membrane was washed 3 times for 5 minutes in PBST then placed in 12 ml incubation mixture that contained 2-2 ml purified, Sup12-HMWF-separated input 4 phages and de-selected input 6 phages (4.2.2.1 and 4.2.2.2, respectively). The membrane was washed 3 times for 5 minutes with PBST after 2 h incubation then the first protein lanes were subjected to detection with the appropriate secondary and tertier antibodies. The second lanes were placed in PBST and stored at RT to maintain native conditions. The developed Western signals served as references in the process in which the corresponding membrane pieces were matched and the target protein together with bound phages were excised from the native nitrocellulose membrane at the corresponding protein size (Figure 4.28).

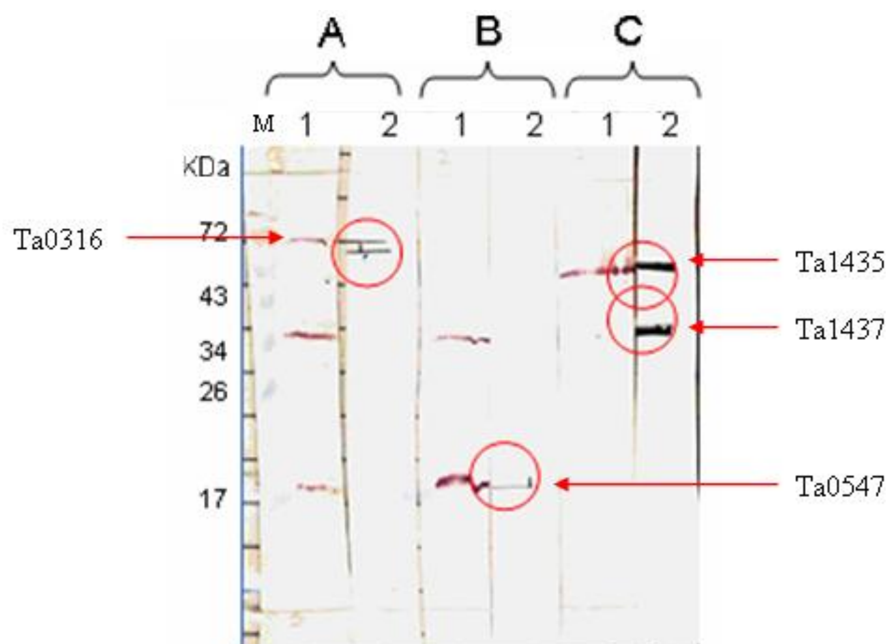


Figure 4.28: Excision of specific phage-containing bands from the nitrocellulose membrane. 1: membrane parts developed with Sigma*FAST* BCIP/NBT substrate system, 2: non-developed/native part of the membrane. A-C demonstrate the applied whole cell extracts containing the expressed target proteins, (A) Ta0316, (B) Ta0547, (C) Ta1435-38 in co-expressed form. Red circles depict excised membrane parts, which correspond to the molecular weight of target proteins. M: Molecular weight marker.

Membrane pieces carrying the putative target specific phages were placed in an Eppendorf tube containing 200 µl of 100 mM TEA buffer and incubated at RT for 30 minutes with gentle shaking. Incubation was followed by adding 100 µl of 1M Tris-Cl pH 7.5 to the tubes to neutralize the eluted phage solution then 150 µl of this mixture was added to 450 µl exponentially growing TG1 culture. Cells were incubated at 37°C for 30 minutes then centrifuged at 10 000 x g for 1 minute. The pellet was resuspended in fresh YT-medium and spread on YTCbG plates. Cells were grown at 30°C, ON and the grown bacterial lawn was scraped thoroughly in YT medium. The cells were centrifuged, washed and resuspended in YT medium containing 15 % glycerol for long-term storage at -80°C.

Western blot signals were obtained only in case of Ta0316, Ta0547 and Ta1435 target proteins (Figure 4.28), therefore only these proteins remained in focus. The growth of output colonies on YTCbG plates indicated that viable phages were eluted from the nitrocellulose membrane. This fact was also confirmed by the TG1 colonies coming from the Ta1437-containing membrane piece (for which no Western signal was detected).

The Western blot assay of polyclonal phages serves not only as a visual reference revealing the diversity of scFvs in the respective phage solution but also as an antigen surface for the phage biopanning. Moreover, the elution of specific phages from an isolated area of the membrane could help to filter out unwanted phages through the biopanning cycles. As it manifested from previous experiments (4.2.1) the lack of signal intensity against certain antigens does not necessarily mean the lack of specific phages in the polyclonal mixture. Therefore, with the help of nitrocellulose excision technique rare phage binders may be enriched from the diverse scFv-library by means of separating them from the abundant repressor phages being attached to their specific targets.

4.3.2.2 Selection of nitrocellulose-isolated phages on plastic-immobilized antigen

Since the membrane-bound proteins represented the denatured versions of target antigens the nitrocellulose-isolation approach was further refined with 1 cycle general biopanning in case of Ta0316 and Ta0547 selected phages using the plastic-immobilized recombinant cytosolic cell extracts. The resulting output colonies were used to establish monoclonal cell lines then screened by ELISA for specific phages. The Sup12-HMWF and the freshly prepared cytosolic extract of the recombinant protein expressing strains were used as screening targets.

The phage biopanning did not result considerable number of output colonies which was indicative for poor phage elution yield. A few colonies exhibited reactivity against the target proteins, but similar reactivities were detected on the negative control plates. Therefore, they did not pass the quality control criteria. Additional biopanning cycles combined with de-selection steps against *E. coli* protein components might result positive phages (4.3.1.3).

4.3.2.3 Direct screening of nitrocellulose-isolated phages

Output colonies harboring the putative Ta1435 specific nitrocellulose-eluted scFvs were used to establish monoclonal cell lines and screened by ELISA-assay (Figure 4.29). The ELISA assay revealed more then 10 positive signals, of which 8 were chosen to be propagated and tested by Western blot assay. Western blot assays were performed against the corresponding recombinant cell extract (Figure 4.30) and against the cytosolic extract of *T. acidophilum* (Figure 4.31).

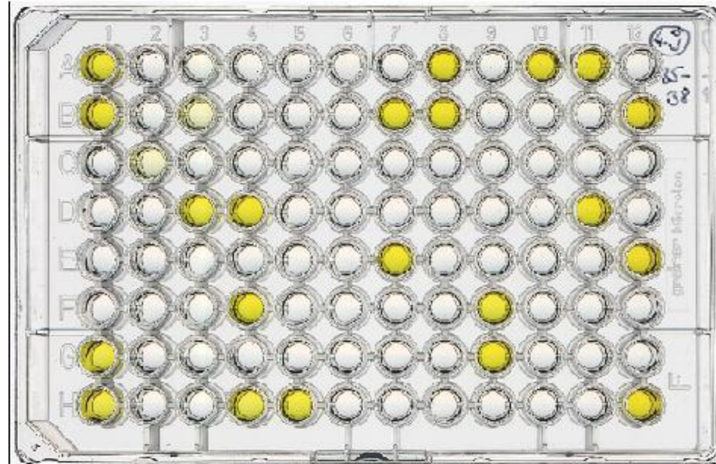


Figure 4.29: ELISA-assay of putative Ta1435 specific monoclonal phages arising from the nitrocellulose-isolation procedure, tested on the Ta1435-38-harboring recombinant cell extract. Signals were developed with 1-Step Ultra TMB ELISA substrate system.

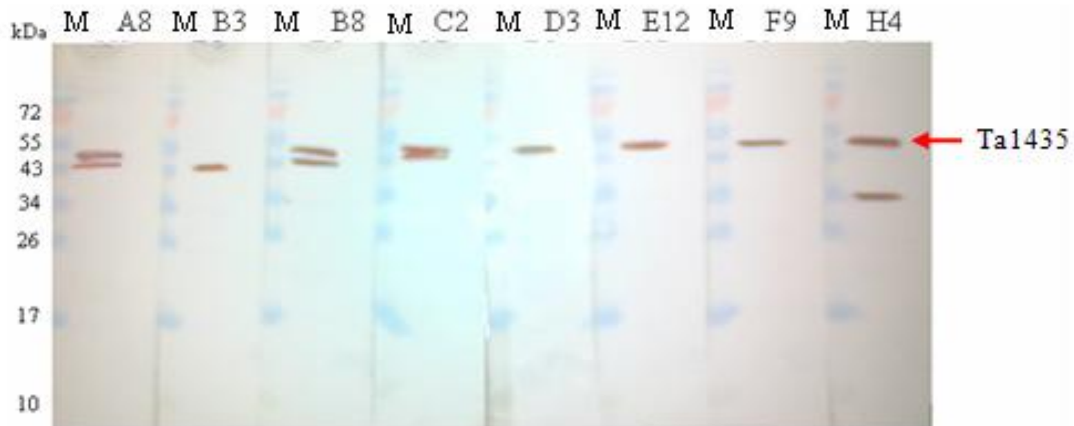


Figure 4.30: Western blot analysis of nitrocellulose membrane-isolated Ta1435 specific monoclonal phages (corresponding ELISA-assay of scFv clones is shown in Figure 4.29). A8-H4 represent membrane-eluted output phage clones tested on the recombinant total cell extract of Ta1435-38. Western blot signals were developed with 1-Step TMB-Blotting HRP substrate system (Thermo Scientific). M: Molecular weight marker.

Figure 4.31: Western blot analysis of nitrocellulose membrane-isolated Ta1435 specific monoclonal phages. A8-H4 represent membrane-eluted output phage clones tested on *T. acidophilum* cytosolic extract as target antigen. Western blot signals developed with 1-Step TMB-Blotting HRP substrate system. M: Molecular weight marker.

In case of both target antigens the same signal pattern was obtained at ~48 kDa, similarly to the corresponding polyclonal phage test (Figure 4.28), which was indicative of the successful nitrocellulose-based biopanning method. Since both Western blot assays confirmed the specificity of phages for Ta1435, it further strengthened the applicability of this selection method. Worthy of note, that one phage isolation step already yielded usable number of specific clones without the need of a further biopanning step on plastic surfaces. The previous experiment (4.3.2.2) suggested that an additional biopanning step could lead to the loss of previously isolated rare phages due to the usage of impure protein target and the overgrowth of abundant phages. The nitrocellulose-isolation assay and additional biopanning steps should be tested experimentally in case of the individual recombinant proteins.

4.3.2.4 Purification of scFv-captured 2-oxo acid dehydrogenase complex from *T. acidophilum*

In the next steps, scFvs were cloned in the modified pET28(*Sfi*I) vector and expressed in BL21(DE3) cells. ScFv clone B3 was expressed, purified and used to purify the Ta1435-1438 protein complex from *T. acidophilum* cell extract. Interestingly, the Superose 6 chromatography separation exhibited similar elution pattern, as those arising from the Ta0328 selection procedure (Figure 4.26), with three additional weak protein bands in fractions 7-8

(column void) which indicated the presence of a complex over 1 MDa. The expected molecular weight of intact Ta1435-38 is 5 MDa (97) which value was in good agreement with our chromatography results. The MS/MS analysis revealed the presence of the four subunits Ta1435, Ta1436, Ta1437 and Ta1438 of the 2-oxo acid dehydrogenase complex in fractions 7 and 8. The presence of a significant amount of the scFv in these fractions proved that the complex was purified by means of antibody affinity. The success of scFv-selection was further proved by the fact that Ta1435 was present in the expected stoichiometric amount with the other three subunits: ($Ta1437_2$/$Ta1438_2$) : (Ta1436) : ($Ta1435_2$) stoichiometry of 1 : 1 : 0.1. Fraction 8 was analyzed with EM, which revealed less intact particles with a diameter of 50 nm and more disassembled complexes (Figure 4.32).

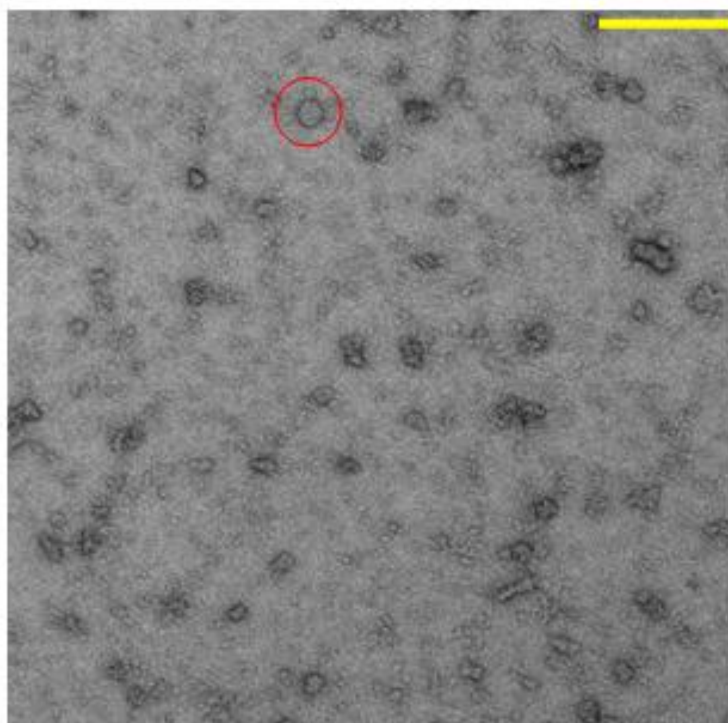


Figure 4.32: Negatively stained electron micrograph of scFv-captured 2-oxo acid dehydrogenase complexes of Superose 6 elution fraction 8. Yellow scale bar corresponds to 200 nm. The red circle depicts a structurally intact 2-oxo acid dehydrogenase particle. The other particles are probably high molecular weight remnants of the respective complex that fell apart during the purification.

Ta1435-Ta1438 are gene products of four, closely spaced, adjacent genes showing significant sequence identities with the bacterial and eukaryotic components of 2-oxo acid dehydrogenase complexes (OADHCs) that perform the oxidative decarboxylation of 2-oxo acids to corresponding acyl-CoAs. The 2-oxo acid dehydrogenase complex is composed of multiple copies of four subunits. Ta1436 is a dihydrolipoamide acetyltransferase component of the complex, which forms the E2 core by the assembly of 42 subunits. Ta1437 and Ta1438 are the α- and β-chains of a probable 3-methyl-2-oxobutanoate dehydrogenase component that forms the E1 tetramer of the complex. Ta1435 is the dihydrolipoamide dehydrogenase component that forms the mobile E3 dimer of the respective complex. Reportedly, the recombinantly expressed subunits are active and assemble into a large multienzyme complex constituting the proof that this complex is functional in *T. acidophilum* (97, 98). However, the whole complex comprising each four subunits has not been detected in *Thermoplasma*, because the motile subunit Ta1435 was never connected to the E1-E2 complex (89).

4.4 Protein purification with scFvs selected for small archaeal modifier proteins (SAMPs)

Small archaeal modifier proteins (SAMPs) are low molecular weight proteins possessing β-grasp fold and C-terminal diglycine motif, which, similarly to ubiquitin are conjugated to protein targets in *Haloferax volcanii* (99). Studies on *Haloferax volcanii* revealed the diversity of pathways modified by SAMPylation suggesting that this kind of conjugation plays a central role in Archaea. Comparative genomic analysis identified four SAMP candidates from *T. acidophilum* (100) of which only one, Ta0895 was confirmed by MS/MS-analysis to have a modifier role. Although the intracellular level of Ta0895-conjugated proteins is extremely low, a variety of proteins have been proved to be conjugated to it (unpublished data).

4.4.1 Phage selection against Ta0895

Since MS/MS-analyses revealed the existence of Ta0895-conjugated proteins in the cytosolic fraction of *T. acidophilum*, we aimed to capture Ta0895-labelled proteins/protein complexes by pull down assay. Since Ta0895 is expressed in *E. coli* in a highly soluble form and requires only an IMAC purification step to achieve excellent protein purity (Figure 4.33), it proved to be an ideal target for the scFv selection procedure.

<u>Phage biopanning and screening against Ta0895</u>

The selection of scFv-displaying phages was carried out through 3 biopanning cycles according to protocol 7.2, with minor modifications, as follows: The first selection cycle was performed in 4 immuno-tubes, which were coated with 100 μg/ml of affinity purified recombinant Ta0895. A loopful of each phage library glycerol stock was used to inoculate medium for the first starting culture and phages were grown in 400 ml YTCbK ON. 3 ml of the purified phages was mixed with 13 ml of 2% MPBS and 4 ml of this mixture was added to each immuno-tubes. In the second and third selection cycles only 2-2 immuno-tubes were coated with 50 μg/ml and 25 μg/ml of antigen, respectively. The subsequent selection cycles started with inoculating the half of the previous, amplified output TG1 stock into 100 ml medium. The output phages, arising from the respective biopanning steps, were always

combined and mixed before being added to the cells. The immuno-tubes were washed 5, 10 and 15 times with PBST then PBS, respectively.

The first phage biopanning resulted in 2 x 10^4 CFU/ml, the second 5 x 10^4 CFU/ml and the third 2 x10^6 CFU/ml output colonies, respectively. Input 3 phages (amplified from the output 2 colonies) were tested with Western blot assay for Ta0895 specificity. The assay resulted in a strong positive signal appeared at the corresponding protein size (~11 kDa) which provided evidence of the presence of Ta0895 specific phages in the polyclonal mixture (Figure 4.33).

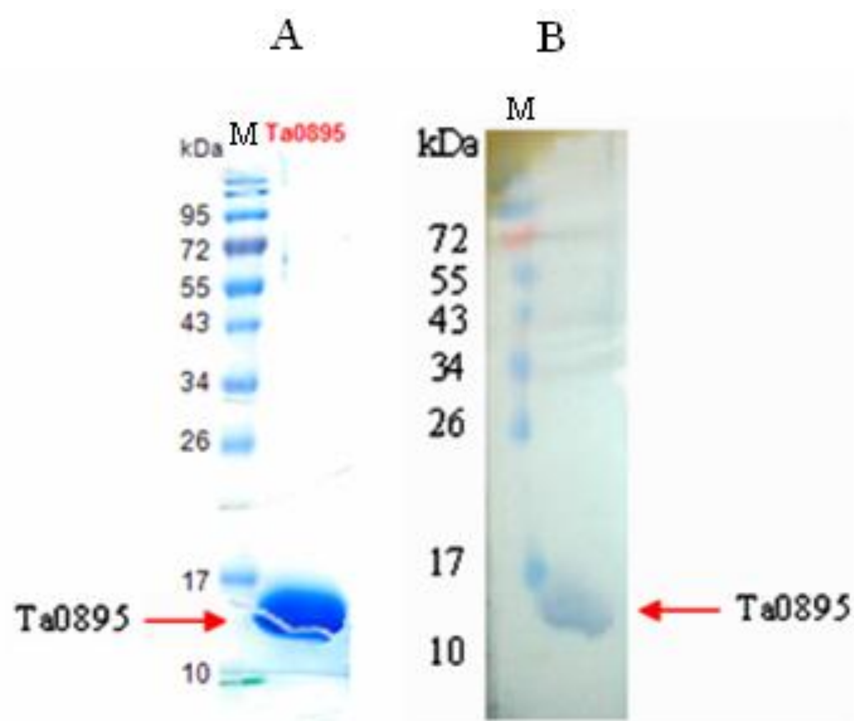


Figure 4.33: 15% SDS-PAGE of affinity purified, recombinant Ta0895 (A) and the Western blot assay of Ta0895-selected, polyclonal input 3 phages tested against the respective antigen (B). Western blot signals were developed with 1-Step TMB-Blotting HRP substrate system (Thermo Scientific). M: Molecular weight marker.

Output TG1 colonies were picked from each output plate and tested for Ta0895 specificity by monoclonal ELISA. 4-4 colonies were picked from output plate 1 and 2, while the rest of the clones were picked from output plate 3. The result of monoclonal phage ELISA-assay tested against the affinity purified Ta0895 as target antigen is shown in Figure 4.34.

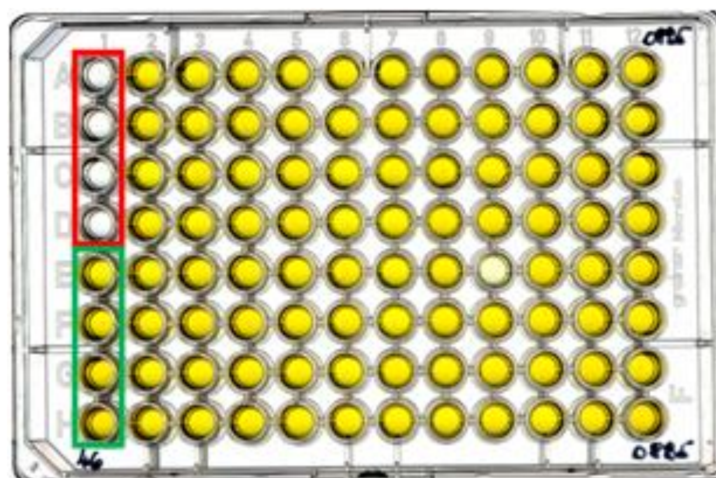


Figure 4.34: ELISA-assay of Ta0895-selected monoclonal phages tested against affinity-purified Ta0895. Red rectangle demonstrates output 1 phages, green rectangle represents output 2 phages. The rest of the clones demonstrate output 3 phages. ELISA signals were developed with 1-Step Ultra TMB ELISA substrate system (Thermo Scientific).

While the 4 examined output 1 phages did not yield any positive phage binders, the second and third selection cycles resulted in 98% of specific clones, which was highly above an average selection yield. This example of phage selection clearly shows that in certain cases specific binders may also be properly enriched by only two biopanning cycles, while other instances require three or more cycles to filter out those few candidates. 6 ELISA-positive phage clones were tested by Western blot assay according to protocol 3.2.9.2 using the affinity purified Ta0895 and *T. acidophilum* cytosolic extract as test antigens. The Western blot assay of the respective monoclonal phages is shown in Figure 4.35.

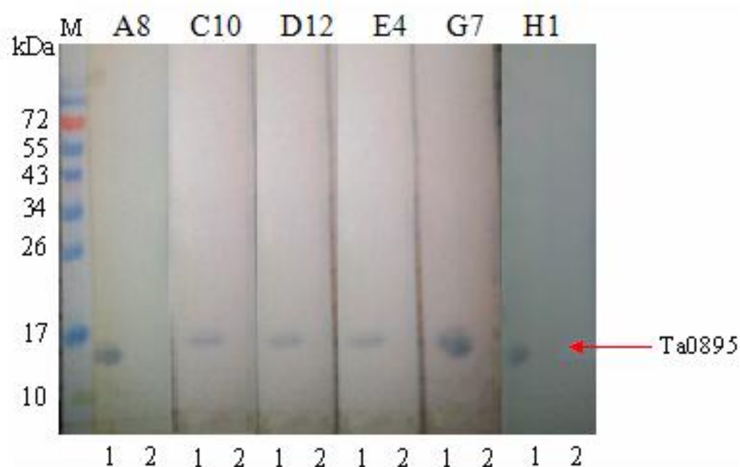


Figure 4.35: Western blot assays of Ta0895-selected monoclonal phages against two different test antigens. A8-H1 represent output 3 phages. 1: Affinity-purified Ta0895 as test antigen., 2: *T. acidophilum* cytosolic extract as test antigen mixture. Western blot signals were developed with 1-Step TMB-Blotting substrate system (Thermo Scientific). M: Molecular weight marker.

Each of the randomly selected phage clones proved to specifically recognize the nitrocellulose-bound recombinant Ta0895. Similarly to the polyclonal phage assay (Figure 4.33), a positive signal at the corresponding protein size of recombinant Ta0895 (~11 kDa) indicated the reactivity of phages to the respective antigen. In spite of this, no positive protein band was obtained on the *T. acidophilum* cytosolic extract, which indicated the extremely low level of Ta0895-conjugated proteins in the cytosol. To overcome this hurdle, the Ta0895-labelled proteins must be enriched from the cell extract by a pull down assay, using soluble scFvs coupled to agarose beads.

Cloning and heterologous expression of Ta0895 specific scFvs

ScFv clones derived from the monoclonal phage test (Figure 4.35) were cloned into the modified pET28(*Sfi*I) and used to express soluble His-tagged scFvs. Since none of the scFv clones were expressed in any of the applied expression strains, codon optimization was required. Clone A8 and G7-scFv were selected for the gene optimization procedure, of which only G7-scFv could be synthesized (Eurofins MWG Operon). Optimized G7-scFv was expressed in soluble form in Rosetta(DE3) cells. Expression conditions are shown in Table 7.3 of 7.9.

Purification of scFv-captured Ta0895-labelled proteins from *T. acidophilum*

Although the recombinant expression of G7-scFv yielded usable amount of soluble protein, the vast amount was detected in inclusion bodies. The purification of scFv from the soluble fraction was carried out by affinity chromatography and an additional ion-exchange chromatography, yielding 60% protein purity. Additionally, the purified protein exhibited a strong tendency to precipitate during storage. Therefore, the expression and purification of the scFv need to be further optimized to achieve eligible purity and stability for pull down studies. Thus, the purification of Ta0895-labelled proteins with the specific G7-scFv remains for future studies.

5. CONCLUSIONS AND PERSPECTIVES

Protein purification methods have to overcome many hurdles working against obtaining high amount of pure and soluble proteins to satisfy the criteria of biochemical assays and/or structural analyses. Native protein purification from the natural host often fails due to low expression levels, while recombinant protein expression technologies have to face inefficient expression levels or formation of inclusion bodies. The combination of the advantageous properties of different techniques may aim either homologous expression of affinity tagged proteins or in cases where there are no genetic tools available antibody based purification methods can be developed. In the frame of this study we aimed to create an scFv-based phage display library for the gentle purification of protein complexes of *T. acidophilum* with specific antibodies. The phage display technology was developed originally for single protein targets that we had to adapt to our needs to raise antibodies against a protein mixture containing at least 300 proteins. The project included the elaboration on selection techniques together with their accurate screening systems, furthermore, the development of an antibody-based purification strategy suitable for isolating high molecular weight protein complexes for EM studies. The high molecular weight protein fractions of Superose 12-separated cell extract (Sup12-HMWF) were used to immunize mice and induce specific antibodies. A combinatorial scFv-library was created in pCANTAB 5E phagemid vector by recombination of heavy and light chain encoding sequences of IgGs using mouse spleen total mRNA as template.

In the first attempt we tried to identify potential phage binders from the antibody repertoire against high molecular weight protein complexes. The first antigen cocktail used for phage biopanning was the Sup12-HMWF which was further fractionated by OH-apatite chromatography to lessen the antigen diversity. The 500 mM K_2HPO_4-KH_2PO_4 elution fraction (OHA-500 mM) yielded more than 50% positive binders of output 3 phages, while the selection for the 300 mM K_2HPO_4-KH_2PO_4 elution fraction (OHA-300 mM) did not result in any reactive phage binders. The remarkable difference in the selection outputs could be due to the segregation of antigenic targets by the OH-apatite fractionation. ELISA-positive phages (24) were tested for binding specificities by Western blotting, which revealed 7 binders against a 60 kDa, 3 binders against a 25 kDa and 1 binder against a 20 kDa protein target. After identifying positive phage binders we attempted the purification of protein complexes

with agarose bead immobilized phages using a conventional pull-down technology. However, this conception failed due to the structural limitation of filamentous phage particles as the ratio of the major coat proteins and the scFv fused to the minor coat protein is ~2700 : 5. Therefore, a pull-down assay using soluble antibodies as bait was developed. The expression of soluble E-tagged scFvs was carried out in a non-suppressor *E. coli* strain (HB2151) which could not be used directly to express phage-free form of affinity-tagged antibodies. However, this expression system did not prove to be reasonable for scFv expression as it required too much time and labor to monitor the location (cytosol, periplasm and/or medium) of soluble proteins. For the faster and easier implementation of expression tests, we developed a modified pET28 vector (pET28(*Sfi*I)) to allow expression of scFvs with a C-terminal 6 Histidine fusion tag. The viability of the new expression system was validated by Western assays that proved the expression of soluble scFvs in most of the cases. This pull-down approach with agarose-immobilized scFvs resulted in successful purification of the molecular chaperonin thermosome (Ta0980 and Ta1276), however, no intact particles could be obtained due to harsh elution conditions. To avoid dissociation and possible denaturation of protein complexes caused by antibody-antigen interaction abolishing eluents/agents, in the next pull-down approach thermosome complexes were purified by a two-step chromatography method. Cell extracts from *T. acidophilum* and the *E. coli* host expressing the specific scFv were mixed, incubated and purified by His-tag affinity chromatography and subsequent size exclusion chromatography. This resulted in pure and intact thermosome particles ideal for EM data collection, class averaging and 3D reconstruction. With the example of the thermosome we demonstrated the viability of this scFv-based purification technique for isolating high molecular weight protein complexes directly from cell extracts. We were able to isolate intact proteasome particles (Ta0612 and Ta1288) and probable peroxiredoxin (Ta0152) components with the same method, however, in the latter case the complex could not be visualized by EM (94).

Since the use of OHA-500 mM as selection target mixture revealed only thermosome, peroxiredoxin and proteasome specific phage binders from the library, in the next selection approach we applied the original antigen solution (Sup12-HMWF) to capture binders against other targets. This resulted in many more phage binders than the selection against OH-apatite fraction, indicating that the appropriate selection target has prime importance and can strongly influence the enrichment of hidden, low copy number or low affinity phage binders in the

next generation libraries. To further fine tune the efficiency of phage selection, de-selections against abundant protein complexes (thermosome, proteasome) were introduced in the biopanning. The library clean-up was monitored with a comparative ELISA-assay that aided to visualize the efficiency of de-selection method. Out of 373 tested clones 217 were identified as positive binder, of which 62 (30%) appeared to recognize potential new target antigens. Subsequent Western blots revealed 6-8 new characteristic protein bands recognized by the de-selected phages, providing evidence that the exclusion of thermosome and proteasome binders from the third generation phage library facilitates the discovery of new scFvs against other target complexes. Interestingly, in spite of the successful cloning, none of the de-selected scFv clones could be sequenced (the reason is misty) and in contrast to their phage-displayed versions they were not expressed at all, indicating the enrichment of an unusual scFv form.

In the next phage-selection approach, we used individual recombinant proteins as selection targets, which were assumed to be putative building blocks of probable protein complexes. The biopanning assays were first carried out on plastic-immobilized antigens in different implementations (varying type and amount of Sup12-HMWF-selected phage inputs, number of selection cycles, incubation/washing conditions and purity of the target antigen). The critical attendant of the recombinant target approach was that many of the targeted recombinant antigens possessed low expression levels (Ta0202, Ta0326-27, Ta0825, Ta1073, Ta1435) and/or were highly instable and/or sensitive to the purification conditions (Ta0437-38, Ta0547, Ta1194, Ta1315). Since many of the recombinant candidates showed a strong tendency to precipitate shortly after the purification we focused on more stable targets, such as the putative intermedier filament Ta1488. Only one biopanning cycle was carried out using equal amounts of two different phage input solutions (second generation de-selected phages (output 5) and amplified third generation output 3 phages), which resulted in one specific, identical scFv out of 96 tested clones in both cases. Since the soluble expression of scFv could not be achieved using the common optimization methods, the DNA-coding sequence of the scFv was optimized for *E. coli* which yielded usable amount of soluble antibody. Using the well-working two-step chromatography method we were able to purify Ta1488 from the cytosolic extract of *T. acidophilum*, however the EM analysis of purified fractions did not prove that Ta1488 form a multimeric complex as it was predicted from its amino acid sequence (2).

We aimed to purify the archaeal homologue of the fixABCX complex of *Rhodospirillum rubrum (95)* by isolating scFvs against one of the putative subunit component, Ta0328. Concerning the low amount and purity of recombinant Ta0328 we introduced library clean up steps against *E. coli* host proteins, thermosomes and proteasomes to avoid enrichment of non-specific and unwanted phage binders. We identified 4 different scFvs which passed the screening criteria and they were used to capture the fixABCX complex by the two-step chromatography method. Interestingly, both of them exhibited the same purification pattern, however, none of them could capture usable amount of the complex. Instead, a formate dehydrogenase homologue protein (Ta0425) was enriched from the cell extract together with its putative small subunit constituent (Ta0424), corresponding to 450-500 kDa molecular weight and the EM-anaysis revealed an X-like shape. Since the formate dehydrogenase releated protein is structurally not characterized, the purification of its native form with specific scFvs would contribute to EM studies and further biochemical investigations.

Since most of the native and recombinant target proteins exhibited low expression levels and/or were highly sensitive to the purification conditions, in the next approach a novel phage biopanning method was developed to overcome these hurdles. In this conception, the phages were isolated from nitrocellulose membrane-immobilized denatured antigens instead of the conventionally used plastic surfaces. The Western blot assay of polyclonal phages served as a visual reference revealing the diversity of scFvs in the respective phage solution. Furthermore, the elution of specific phages from an isolated area of the membrane could help to filter out unwanted phages through the biopanning cycles, thereby promoting the enrichment of rare phage binders. The viability of this method was proved by the purification of the 5 MDa 2-oxo acid dehydrogenase multi-enzyme complex. Interestingly, the enzyme complex was purified together with Ta0425 exhibiting a similar purification pattern as those arisen from the Ta0328 selection procedure.

The last library selection approach aimed the capturing of SAMP-labelled proteins/protein complexes by pull down assay (99). The SAMP candidate used as target antigen for the phage biopanning was Ta0895, which was proved to be conjugated to other proteins in the cytosolic fraction of *T. acidophilum*. Due to the high expression level and highly soluble form of recombinant Ta0895, a conventional phage biopanning could be carried out using plastic-immobilized antigen. Following phage selection, each of the randomly selected phage clones

proved to specifically recognize recombinant Ta0895 in both ELISA and Western blot conditions, which indicated the successful enrichment of SAMP specific phages from the system. ScFv sequences were optimized for *E. coli* to attempt soluble form protein expression, however, the expression and purification of scFv need to be further optimized to achieve eligible purity and stability. The pull-down of Ta0895-conjugated proteins therefore remains for future studies, in which the role of this kind of conjugation will be examined.

Interstingly, no phage binder could be found against several abundant proteins like the subunits of the 70S ribosome and VAT ATPase (Ta0840). The reason of this might be diverse. These proteins might be weakly antigenic or the library was too diverse or the expressed binders are incompatible with propagation, while others can be susceptible to proteolysis during propagation. The underrepresentation of these phages can be further enhanced by the overgrowth of “background” phages. Furthermore, in spite of their relatively high genetic stability, scFvs are prone to dimer-and trimerisation which can further hamper selection and enrichment of such scFvs. Since the scFv selection and screening is implemented at phage level, the enrichment of many potential binders is hindered by the defective phage display. These disadvantages all affect the diversity, affinity and specificity of output phages arising from the selection cycles, leading to the segregation of most persistent phages.

In conclusion, our results show that the construction of a scFv-library against a targeted antigen cocktail is a feasible approach to develop specific antibodies recognizing high molecular weight protein complexes and thereby can contribute to identify/purify structurally non-characterized proteins/protein complexes. The novelty in our phage display approach was that the antibody production was stimulated against a complex antigen mixture instead of a defined epitope, allowing the generation of a diverse scFv library possessing binding specificities against diverse protein targets. The selection and screening using complex target mixtures helped to reveal specific antibodies against three different proteins (thermosome, proteasome and peroxiredoxin), while the use of individual recombinant targets promoted the antibody selection against four proteins (Ta1488, Ta0425/0424, Ta1435/1436/1437/1438 and Ta0895). The combination of de-selection and selection steps in the biopanning assays helped to filter out frequent and unwanted phages from the system and enhanced the selection of potential new binders. Our novel nitrocellulose-based phage biopanning technique enabled a

fast and accurate antibody selection, which may be used as a powerful alternative to the conventional plastic surface-based method if the proper phage selection is limited by inadequate amount, purity and/or stability of the targeted antigen. Furthermore, using specific scFvs we were able to carry out protein complex purifications without the need of harsh, interaction abolishing eluents, which is a crucial prerequisite for single particle electron microscopy studies. Finally, our phage display approach permitted the generation of antibodies against the SAMP-candidate Ta0895 protein, which opens avenues for elucidating the function of this posttranslational labeling in *T. acidophilum*.

Taken together, the creation of a scFv-library against the high molecular weight protein fraction of *T. acidophilum* provided a diverse antibody set, which, in contrast to other libraries could be selected against a variety of protein targets and resulted in a set of specific binders which recognize specific targets. The project aids the discovery of structurally unknown, putative protein complexes by finding then isolating them with specific antibodies to provide a feasible purification procedure for single particle EM studies. Our phage display library-based protein complex purification strategy can serve as a platform for studies on visual proteomics approaches that aim the creation of a template library for the generation of a cellular atlas.

6. ABBREVIATIONS

A:	Adenine
ATP:	Adenosine triphosphate
AP:	Alkaline phosphatase
B:	Cytosine or guanine or thymine
Bp:	Base pair
C:	Cytosine
°C:	Degree Celsius
CEM:	Cryo-electron microscopy
CFU:	Colony forming unit
D:	Adenine or guanine or thymine
$_{dd}H_2O$:	Double distilled water
DNA:	Deoxyribonucleic acid
E. coli:	*Escherichia coli*
EDTA:	Etylene diamine tetra-acetic acid
ELISA:	Enzyme-linked immunosorbent assay
G:	Guanine
g:	Gramm
x g:	Times gravity
h:	Hour
H:	Adenine or cytosine or thymine
HRP:	Horseradish peroxidase
IgG:	Immunoglobuline G

IMAC: Immobilized metal affinity chromatography

IPTG: Isopropyl-ß-D-thiogalactopyranoside

K: Guanine or thymine

kb: Kilobase

kDa: Kilodalton

LB: Luria-Bertani medium

M: Adenine or cytosine

MS: Mass spectrometry

Mw: Molecular weight

MWCO: Molecular weight cut-off

N: Any nucleotide base

Ni-NTA: Nickel-nitriloacetic acid

nm: Nanometer

OD_{600}: Optical density measured at a wavelength of 600 nm

OH-apatite: Hydroxy-apatite

OHA-500 mM: 500 mM K_2HPO_4-KH_2PO_4 elution fraction of *T. acidophilum* cytosolic extract

OHA-300 mM: 300 mM K_2HPO_4-KH_2PO_4 elution fraction of *T. acidophilum* cytosolic extract

ON: Overnight

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PEG: Polyethylene glycol

pH:	Pondus hidrogenii – equal to the negative logarithmic value of the Hydrogen ion (H^{+}) concentration
R:	Adenine or guanine
RNA:	Ribonucleic acid
rpm:	Revolutions per minute
RT:	Room temperature
S:	Guanine or cytosine
scFv:	Single chain variable fragment
SDS:	Sodium dodecyl sulphate
Sup12-HMWF:	Superose 12-separated high molecular weight protein fraction
T:	Thymine
T. acidophilum:	*Thermoplasma acidophilum*
TBS:	Tris-buffered saline
V:	Adenine or cytosine or guanine
W:	Adenine or thymine
Y:	Cytosine or thymine

7. SUPPLEMENTARY DATA

7.1 Creation of the scFv library

7.1.1 Immunization of mice and harvest of spleens

Superose12-separated high molecular weight protein fractions of *T. acidophilum* cytosolic cell extract (Sup12-HMWF) was used for the immunization of eight Balb/c type, 5-6 weeks old female mice. Mice were immunized with 45 μg of Sup12-HMWF 3 times with 2 weeks intervals. The immunization procedure was carried out using Freund's adjuvant as previously described (101). Spleens of mice were removed three days after the last injection and 70 mg pieces of them were subjected to RNA extraction procedure.

7.1.2 Construction of the scFv repertoire in a phagemid vector

The next steps were carried out as described in Short protocols in immunology, Unit 14.3, Phage display of single-chain antibody constructs, basic protocol 1: Construction of a scFv repertoire in a phagemid vector, page 15-21 (102). The detailed protocol is described below:

1. Total RNA from the spleen was extracted using a total RNA extraction kit according to the manufacturer's protocol (RNeasy Midi kit, Qiagen) and recovered in 0.5 ml of DEPC-treated water then used immediately as template for RT-PCR. The remaining RNA was stored in 50-μl aliquots at -80°C.

2. Four reverse-transcription reactions (RT reaction) were set up for two-step RT-PCR (reverse transcription PCR) according to the instructions of the RT-PCR kit (Ready-To-Go-RT-PCR beads, Amersham Pharmacia Biotech). Each reaction had a final volume of 48 μl and contained 20 μg of total RNA and 0.5 μg of oligo-$dT_{12\text{-}18}$. The RT reaction was terminated by incubating 10 min at 95°C then tubes were placed on ice.

3. PCR-amplification: The following primer mixes were added to each tube (see table 1) in 2 ml that resulted in a final primer concentration of 50 pmol/50 µl reaction.

   Tube 1: Primers MuVH1-6-BACK (primer no.1 to 6 mixed according to percentages in Table 1. and primer Mu-JH1-FOR(S) (primer no.10)

   Tube 2: Primers MuVH1-6-BACK (primer no.1 to 6 mixed according to percentages in Table 1. and primer Mu-JH2-FOR(S) (primer no.11)

   Tube 3: Primers MuV_K1-7-BACK(S) (primer no. 16 to 22 mixed according to percentages in Table 1.) and primer Mu-J_K1-FOR (primer no 32)

   Tube 4: Primers MuV_K1-7-BACK(S) (primer no. 16 to 22 mixed according to percentages in Table 1.) and primer Mu-J_K2-FOR (primer no 33)

   Using the RT-PCR kit, the reaction included a final concentration of 1 x PCR buffer, 0.2 mM dNTPs, and 1 to 2 U of a thermo-stable DNA polymerase.

4. PCR amplification was carried out according to the following parameters:

1 cycle:	5 min	95°C	initial denaturation
35 cycles:	30 sec	95°C	denaturation
	1 min	55°C	annealing
	1 min	72°C	extension
1 cycle:	5 min	72°C	final product extension

   The PCR product was kept at 4°C

5. 5-µl aliquot of the PCR reactions was analyzed on a 1.5 % agarose gel against the 1 kb ladder DNA size marker (Fermentas).

6. Remaining PCR products were separated on a 1.5 % preparative agarose gel, stained and visualized as described in protocol 3.2.4.4. The 350-bp V_H fragments (reactions 1 and 2) and the 330-bp V_K fragments (reactions 3 and 4) were excised.

7. DNA fragments were extracted as described in method 3.2.4.5 and recovered in 50 µl of 10 mM Tris-Cl, pH 8.5.

8. DNA-concentration was measured according to protocol 3.2.4.11.

9. For re-amplification of the gel-purified V_H and V_K fragments four, 50 µl PCR reactions were prepared, containing:
   - 50 ng of template DNA
   - 50 pmol/reaction of primers mixed according to table 1.
   - 1 to 2 U high-fidelity thermostable DNA polymerase
   - 0.2 mM dNTP mix
   - 1 x PCR buffer

For V_H:

Tube 1: 50 ng of product 1 as template with primers MuVH1-6-BACK (primer no.1 to 6) and Mu-JH1-FOR(L) (primer no.13)

Tube 2: 50 ng of product 2 as template with primers MuVH1-6-BACK (primer no.1 to 6) and Mu-JH1-FOR(L) (primer no.14)

For V_K:

Tube 3: 50 ng of product 3 as template with primers MuVH1-7-BACK(L) (primer no.24 to 30) and Mu-JK1-FOR (primer no.32)

Tube 4: 50 ng of product 4 as template with primers MuVH1-7-BACK(L) (primer no.24 to 30) and Mu-JK2-FOR (primer no.33)

10. PCR amplification was carried out according to the following parameters:

1 cycle:	5 min	95°C	initial denaturation
30 cycles:	30 sec	95°C	denaturation
	1 min	55°C	annealing
	1 min	72°C	extension
1 cycle:	5 min	72°C	final product extension

The PCR products were kept at 4°C

11. PCR products were recovered from the preparative agarose gel then quantified as in steps 6 to 8.

12. 5 assembly PCR reactions were set up as follows:

    Template: 75 ng of product 1 was combined with 25 ng of product 2, 75 ng of product 3 an 25 ng of product 4.

    Primers: 50 pmol of Mu-*Sfi*-BACK (primer no.9), 37.5 pmol of Mu-JK1-FOR (primer no.32) and 12.5 pmol of Mu-VK2-FOR (primer no.33).

    50-µl PCR reaction contained 1 to 2 U of a thermostable DNA polymerase, 1 x PCR buffer and 0.2 mM dNTP mix.

13. PCR amplification was carried out according to the following parameters:

1 cycle:	5 min	95°C	initial denaturation
30 cycles:	30 sec	95°C	denaturation
	1 min	55°C	annealing
	1 min	72°C	extension
1 cycle:	5 min	72°C	final product extension

PCR products were kept at 4°C.

14. 5 µl of the PCR products was analyzed on a 1 % agarose gel (expecting an average length of 750 bp).

15. The assembled PCR products were recovered from a 1 % preparative agarose gel and quantified as described in steps 6 to 8.

16. 5 µg of pCANTAB 5E phagemid DNA was digested with 5 U of *Sfi*I for 3 h at 50°C then with 5 U of *Not*I for 2 h at 37°C.

17. The digested vector was loaded in a 4 cm wide well of a preparative, 1% agarose gel. The upper (vector) band was recovered from the gel and purified, quantified as described in steps 6 to 8.

18. The assembled scFv repertoire (step 15) was digested with *Sfi*I and *Not*I as described in step 16 and purified using a Microcon PCR filter and recovered in 22 µl $_{dd}H_2O$.

19. Five identical DNA ligation reactions were set up that contained the following ingredients:

 100 ng *Sfi*I + *Not*I digested, purified pCANTAB 5E vector DNA
 80 ng *Sfi*I + *Not*I digested, purified scFvs inserts
 2 µl 10 x ligation buffer
 1 µl T4 DNA ligase (NeB)
 $_{dd}H_2O$ to a final volume of 20 µl

 Mixture was incubated for 16 h at 16°C.

20. Ligations were combined and loaded on a Microcon-PCR filter to exchange the buffer to $_{dd}H_2O$. 500 µl volume of water was added to the cartridge and centrifuged again. DNA was recovered in a final volume of 50 µl $_{dd}H_2O$.

21. Electroporation-competent *E. coli* TG-1 cells were dispensed in 100 µl aliquots into prechilled 1.5 ml microcentrifuge tubes and kept on ice. Electroporation device was set up for *E. coli* electroporation protocol.

22. 20 individual electroporations were performed by adding 2.5 µl of the purified ligation product to 100 µl electroporation-competent cells. Cells were briefly mixed by gentle pipetting and placed back on ice for 40 to 50 sec.

23. DNA/cell mixture was transferred into a chilled 0.2 cm cuvette then immediately inserted into the electroporation chamber and treated with 4- to 5 -msec electric pulse. The cuvette was removed from the electroporation device and 900 µl pre-

warmed SOC medium was added to the mixture. Cells were pipetted up and down then transferred to a 13 ml sterile polypropylene tube. The tubes (20) were placed for 1 h in a 37°C incubator and shaken at 250 rpm.

24. 10 µl aliquots of the regenerated cells were combined into a single tube and library size was determined by plating ten-fold serial dilutions of the transformed cells on selective YTCbG agar plates. Plates were incubated for 16 h at 30°C.

25. The remaining cells were centrifuged for 10 min at 5000 x g at RT. Pellets were resuspended in 100 µl of fresh SOC medium then spread on one YTCbG plate/tube. Plates were incubated for 16 h at 25°C.

26. Using a cell scraper, cells were collected from each plate into 10 ml of YTCbG medium. After this, the suspension were combined and collected as in step 25. Cells were resuspended in 10 ml of YTCbG medium containing 15 % glycerol. Serial dilutions were plated onto YTCbG plates to determine library amplification. Glycerol stocks of the library were stored in 1 ml aliquots at -80°C.

Primer set for cloning scFvs

Primer No.	Primer name	Primer sequence	% in mix
First PCR V_H5' end primers:			
1	MuVH1-BACK	agccggccatggcc CAGGTYCARCTGCAGCAGYCTGG	33
2	MuVH2-BACK	agccggccatggcc GAGGTYCAGCTGCARCARTCTGG	28
3	MuVH3-BACK	agccggccatggcc CAGGTGCAGCTGAAGGAGTCAGG	8
4	MuVH4-BACK	agccggccatggcc GARGTGAAGCTGGTGGARTCTGG	21
5	MuVH5-BACK	agccggccatggcc GAGATCCAGCTGCAGCAGTCTGG	6
6	MuVH6-BACK	agccggccatggcc CAGATCCAGTTGGTGCAGTCTGG	4
7	MuVH7-BACK	agccggccatggcc SAGGTKMMKCTKVARGAGTCWGG	
8	MuVH8-BACK	agccggccatggcc SARGTNMWVCTGVWGSARHCHGG	
For 5' *Sfi*I second and assembly PCR (*Sfi*I site underlined):			
9	Mu*Sfi*-BACK	ATCTATGCGGCCCAGCCGGCCATGGCCSARRT	
First PCR V_H3' end primers:			
10	MuJH1-FOR-S	CGAGGAGACKGTGASHGWGGT	75
11	MuJH2-FOR-S	CGAAGAGACAGTRACCAGAGT	25
12	MuJH3-FOR-S	CGAWCYHYDVTCACHGTYTCY	
Second PCR V_H3' end long primers (BamHI site in linker underlined):			
13	MuJH1-FOR-L	ccagaaccgccaccgccggatccaccacctcc CGAGGAGACKGTGASHGWGGT	75
14	MuJH2-FOR-L	ccagaaccgccaccgccggatccaccacctcc CGAAGAGACAGTRACCAGAGT	25
15	MuJH3-FOR-L	ccagaaccgccaccgccggatccaccacctcc CGAWCYHYDVTCACHGTYTCY	
First PCR Vκ5' end short primers			
16	MuVK1-BACK-S	SAMATTGTKCTSACHCARTC	30
17	MuVK2-BACK-S	GAYATCCAGATGACHCARWC	18
18	MuVK3-BACK-S	GATGTTGTGATGACCCARAC	12
19	MuVK4-BACK-S	GAYATTGTGATGACNCAGKC	25
20	MuVK5-BACK-S	GATGTTTTGATGACCCAAAC	6
21	MuVK6-BACK-S	GACATCAAGATGACCCAGTC	5
22	MuVK7-BACK-S	GACATTGTGATGTCACAGTC	4
23	MuVK8-BACK-S	RRHRYYBWDMTVACHCARWC	
Second PCR Vκ5' end long primers			
24	MuVK1-BACK-L	ggcggtggcggttctggtggaggtggatct SAMATTGTKCTSACHCARTC	30
25	MuVK2-BACK-L	ggcggtggcggttctggtggaggtggatct GAYATCCAGATGACHCARWC	18
26	MuVK3-BACK-L	ggcggtggcggttctggtggaggtggatct GATGTTGTGATGACCCARAC	12
27	MuVK4-BACK-L	ggcggtggcggttctggtggaggtggatct GAYATTGTGATGACNCAGKC	25
28	MuVK5-BACK-L	ggcggtggcggttctggtggaggtggatct GATGTTTTGATGACCCAAAC	6
29	MuVK6-BACK-L	ggcggtggcggttctggtggaggtggatct GACATCAAGATGACCCAGTC	5
30	MuVK7-BACK-L	ggcggtggcggttctggtggaggtggatct GACATTGTGATGTCACAGTC	4
31	MuVK8-BACK-L	ggcggtggcggttctggtggaggtggatct RRHRYYBWDMTVACHCARWC	
First, second and assembly PCR Vκ3' end primers (*Not*I site underlined)			
32	MuJK1-FOR	gccacctgcggccgc CCGTTTKATYTCCARYTTKGTSCC	75
33	MuJK2-FOR	gccacctgcggccgc CCGTTTCAGYTCCAGCTTGGTCCC	75
34	MuJK3-FOR	gccacctgcggccgc CCGYTTKAKYTCCARYTTKGTNCC	25

Table 7.1: Primer set for cloning scFv constructs for phage display

7.1.3 Sequence analysis/alignment of scFv clones obtained after the library construction

```
Clone 1  ---F-AAQPA-AEVQLQQSGAELVKPGASVKISCKASGYTFTDHAIHWVKQR
Clone 2  ---FYAAQPAMAQVQLQQPGAELVKPGASVKLSCTASGFNIKDTYMHWVKQR
Clone 3  ---FYAA-PA-A-VQL-QSGAELVKPGASVKISCKASGYSFTGYYMHWVKQS
Clone 4  ---FYAA-PAMAQVQLQQSGAELMKPGASVKISCKATGYTFSSYWIEWVKQR
Clone 5  ---FYAA-PA-AQVQLQQSGAELVKPGASVKLSCKASGYTFTSYYMFWVKQR
Clone 6  ---F-AA-PA-AEVQLQQSGAELMKPGASVKISCKATGYTFSSYWIEWVKQR
            * ** ** * *** *.****:*******:**.*:*:.:..  : ****

PEQGLEWIGRIDPANGNTKYDPKFQGRATITADTSSITAYLQLSSLTSEDTAVYYCATIT
PEQGLEWIGRIDPANGNTKYDPKFQGKATMTADTSSNTAYLQLSSLTSEDSAVYYCARLY
HGKSLEWIGYISCYNAATSYNQKFKGKATFTVDTSSSTAYMQFNSLTSEDSAVYYCARNY
PGHGLEWIGKILPGSGGTNYNEKFKDKATLTADKSSSTAYMQLSSLTSDDSAVYFCARAG
PGQGLEWIGEINPTNGGTNFNEKFRNKATLTVDTSSSTAYMQLSSLTSEDSAVYYCTISG
PGHGLEWIGEILPGSGSTNYNEKFKGKATFTADTSSNTAYMQLSSLTSEDSAVYYCARLY
  :.***** *    .. *.:: **:.:**:*.*.** ***:*:.****:*:***:*:

TVVGGHAMDYWGQGTTLTVSSGGGGSGGGGSGGGGSDVLMTQTPLTLSVTIGQPASISCK
YGSNYWYFDVWGAGTTLTVSSGGGGSGGGGSGGGGSDVLMTQTPLTLSVTIGQPASISCK
RYDEGYYFDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIVMT-TPLTLSVTIGQPASISCK
------ANAYWGQGTLVTVSSGGGGSGGGGSGGGGSDVVMTQTPLTLSVTIGQPASISCK
YGT---YFAYWGQGTTVTVSSGGGGSGGGGSGGGGSDVLMTQTPKFLLVSAGDRVSITCK
YGSNYWYFDVWGAGTSLTVSSGGGGSGGGGSGGGGSDIQMTQTPLSLPVSLGDQASISCR
          ** ** :*********************: ** **  * *: *: .**:*:

SSQSLLNSDGKTYLNWLLQRPGQSPKRLIYLVSKLDSGVPDRFTGSGYGTDFTFTISTVQ
SSQSLLDSDGKTYLNWLLQRPGQSPKRLIYLVSKLDSGVPDRFTGSGSGTDFTLKISRV-
SSQSLLDSDGKTYLNWLLQRPGQSPKRLIYLVSKLDSGVPDRFTGSGSGTDFTLKIS-V-
SSQRLLESDGKTYLSWLLQRPGQSPKRLIYLVSKLDSGVPDRFTGSGSGTDFTLKISRV-
ASQSVSNA-----VAWYQQKPGQSPKLLIYYASNRYTGVPDRFTGSGYGTDFTFTISTVQ
SSQSLVHSNGNTYLHWYLQKPGQSPKLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRV-
:** : .:      : *  *:****** *** .*:  :******:*** *****:.** *

AE-LAVYFCQQDYSSPW-FG-GTKLEIKRAG-------------RCAGAVSGSAG----I
A-DLGVYYCWQGTHFPT-FGGG-KLELKRAAA------------GAPGAVS---------
A-DLGVYYCWQGTHFTW-VRWRDQA-TETG-P--------------A--VSGSAGTACR-
AEDLGVYYCWQGTHFPYTFG-G-KL-L-GRP------I-IRW--RA--TVES-LA-----
AEDLAVYFCQQDYSPPYTFGAGTKLELKRAA------------RR-RIRIRWNRVPH-LL
A-DLGVYFCSQSTHVP--SV-GPSWSNGRP---------------CRIRIRWNRV---L-
*  *.**:* *.    .        .                      :
```

Figure 7.1: Sequence analysis of 6 randomly selected scFv clones obtained after library construction. Sequences were aligned with the ClustalW multiple sequence alignment program (103) " * " indicates positions which have a single, fully conserved residue, "**:**" indicates that one of the following 'strong' groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW " **.** " indicates that one of the following 'weaker' groups is conserved: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY, " **-** " indicates gaps in the alignment.

7.2 Affinity selection of scFv-displaying phages on plastic-immobilized antigens

The affinity selection of scFv-displaying phages was carried out as described in Short protocols in immunology, Unit 14.3, Phage display of single-chain antibody constructs, basic protocol 2: Affinity selection of scFv-displaying phage on plastic-immobilized antigen, page 22-25 (102).

Growth of *E. coli* cells:

1. Day 1: A TG1 bacterial colony was transferred from a minimal medium plate into 5 ml of 2 x YT medium and grown ON at 37°C with shaking.

2. Day 2: The cell suspension was diluted 1:100 into 20 ml of fresh 2 x YT medium and grown with shaking at 37°C until OD_{600} reached a value of 0.4 to 0.6 after which it was infected with phages.

Growth and rescue of phage library:

3. Day 3: An aliquot of the bacterial library glycerol stock (~ 1 x 10^{10} clones) was inoculated into 100 ml of 2 x YTCbG medium and grown with shaking at 37°C until the OD_{600} reached 0.5.

4. Cells were infected with M13KO7 helper phage at ratio of 1 : 20 (number of bacterial cells/helper phage particles, taking into account that 1 OD_{600} = ~ 2 x 10^8 bacteria/ml). Cells were incubated for 30 min at 37°C without shaking then transferred to a 37°C shaker/incubator and grown for 30 min with shaking at 200 rpm.

5. The infected cells were centrifuged for 10 min at 3300 x g, at RT and the pellet was resuspended in 100 ml of YTCbK medium. Cells were incubated ON with shaking at 220 rpm, at 30°C.

6. Day 2: ON-grown cell culture was centrifuged for 10 min at 8000 x g (or 30 min at 3300 x g), 4°C. Supernatant was transferred into a new polypropylene tube and 1/5 volume PEG/NaCl was added. After mixing, tubes were left on ice for 1-2 h. Phage precipitate was centrifuged for 30 min at 10.800 x g, 4°C then the white pellet was resuspended in 40 ml of $_{dd}H_2O$. 8 ml PEG/NaCl was added to the water then tube was mixed and left on ice for further 20-40 min. Phage precipitate was centrifuged for 10 min at 10.800 x g, 4°C then supernatant was carefully aspirated. Phages were centrifuged briefly again and the remaining PEG/NaCl was aspirated entirely.

7. Phage pellet was resuspended in 5 ml PBS and centrifuged for 10 min at 11.600 x g, RT. The supernatant was filtered through a 0.45 μm sterile filter and stored at 4°C until usage or stored at -80°C in the presence of 15% glycerol for later purposes.

8. Ten-fold serial dilutions were made in sterile PBS to titer of the phage stock. Early log phase TG1 cells were infected with phage dilutions by mixing and incubating them for 30 min at 37°C. Infected cells were spread on YTCbG plates and grown ON at 37°C.

Biopanning: First selection cycle:

9. Day 2: Immuno-tubes were coated ON at 4°C with 4 ml of the required antigen. 10 to 100 μg/ml antigen was diluted in PBS or in 50 mM $NaHCO_3$, pH 9.6.

10. Day 3: Next day, tubes were washed 3 times with PBS by pouring PBS into the tube and immediately pouring it out. Tubes were filled with 2% MPBS, covered with Parafilm and incubated for 2 h at room temperature to block the tube. Tubes were washed 3 times with PBS.

11. For panning, 10^{11} to 10^{12} rescued phages (from step 7) were added into the tube in 4 ml of 2% MPBS and incubated for 1 to 2 h at RT with continuous rotation. For each wash, tubes were filled with buffer then emptied. Immunotubes were washed 10 times with PBST for the first selection cycle, then 10 times with PBS to remove detergent. For the second and subsequent selection cycles tubes were washed 20 times with PBST, then 20 times with PBS.

12. The remaining PBS was removed from the tubes and phages were eluted in 1 ml of 100 mM triethylamine (TEA) with continuous rotation for 30 min at RT.

13. During the incubation period, 13-ml polypropylene culture tubes with 0.5 ml of 1 M Tris-Cl, pH 7.4 were prepared to neutralize eluted phages from step 12.

14. After elution, another 200 µl of 1 M Tris-Cl, pH 7.4 was added to the immuno-tubes to neutralize the remaining phages. Tubes were rotated such that every part of it came in contact with the neutralizing solution.

15. 750 µl of the eluted, neutralized phage solution was added to 9.25 ml exponentially growing TG1 culture (step 2). Parallel with it, 4 ml of TG1 culture was added to the immunotube. Both cultures were incubated for 30 min at 37°C without shaking to allow infection of the cells.

16. The 10 and 4 ml infected TG1 suspensions were pooled and 100 µl of it was saved to make 4 or 5 ten-fold serial dilutions. Aliquots of the dilutions were plated on YTCbG plates and incubated ON at 37°C to determine the panning output.

17. The remaining infected cells were centrifuged for 10 min at 3300 x g, RT. The bacterial pellet was resuspended in 1 ml of 2 x YT medium and spread entirely on four YTCbG plates. Cells were grown at 30°C ON, or until colonies were visible.

Additional cycles:

18. Day 4: To collect output colonies the plates were overlayed with 5 to 6 ml of 2 x YT medium supplemented with 15% glycerol and cells were loosened with a cell scraper. 50 to 100 µl of the scraped bacteria was inoculated into 50 to 100 ml of YTCbG medium and incubated at 37°C with shaking at 180 rpm until the OD_{600} reached 0.5. The remaining bacteria were stored as output 1 glycerol stocks at -80°C.

19. 10 ml of the exponentially growing culture from step 18 was infected with M13KO7 helper phage in the ration of 1:20. Cells were incubated for 30 min at 37°C without shaking and an additional 30 min at 37°C with shaking. Infected cells were centrifuged for 10 min at 3300 x g, RT then pellet and the pellet was resuspended in 50 ml of YTCbK medium. Cells were incubated ON at 30°C with shaking.

20. Day 5: 40 ml of the ON-grown cell culture was centrifuged for 10 min at 8000 x g (or 30 min at 3300 x g), 4°C. Supernatant was transferred into a new polypropylene tube then 1/5 volume PEG/NaCl was added. The liquids were mixed and left for 1 to 2 h on ice. Phage precipitate was centrifuged at 10.800 x g for 10 min then supernatant was aspirated off. Tubes were centrifuged again briefly then the remaining PEG/NaCl was aspirated off entirely. White pellet was resuspended in 2 ml PBS and centrifuged for 10 min at 11.600 x g, 4°C to remove most of the remaining bacterial debris.

21. The resulting phage preparation was titered and 1 ml of it was used as input for the next round of affinity selection. The remaining phages were stored at 4°C for short-term or at -80°C for long-term in the presence of 15% glycerol.

22. The phage selection was repeated for another 2 or 3 cycles as described above. In each cycle, the concentration of antigen in the immunotube was decreased by a factor of 2.

The ratio between panning input and panning output was monitored in each cycle. Phages from single infected bacterial colonies were screened by monoclonal ELISA as described in protocol 3.2.9.1 and tested for binding properties on different antigen test plates. The diversity of resulting positive scFv-clones was assessed by DNA sequence analysis as described in methods (3.2.4.10).

7.3 Establishment of monoclonal cell lines for ELISA-assay

7.3.1 Establishment of TG1 cell lines and growth of monoclonal phages

To be able to grow monoclonal scFv-displaying phages for ELISA-assay, isolated TG1 colonies were picked from the library output plates and inoculated into 100 µl YTCbG medium in a 96-well flat bottom microtiter plate (Corning). The plate was covered with the lid and cells were grown ON at 37°C with shaking at 600 rpm. Next day, 10 µl of the cell suspensions was inoculated into 100 µl resh YTCbG with a help of a multi channel pipette and grown at 37°C, 600 rpm for 1 h (at which time the OD_{600} reached ~0.5-0.7). At this point 25 µl of M13KO7 helper phage mixture was added to each well (10^9 PFU phage mixed with YTCbG medium) and the plate was incubated at 37°C for 30 min without shaking then for 1 h with shaking at 600 rpm. After this, the plate was centrifuged at 1300 x g for 15 min, RT. The supernatant was gently removed from the pellets and cells were resuspended in 200 µl of YTCbK medium then grown at 30°C, 700 rpm, ON. The plate was centrifuged at RT, 1300 x g for 30 min on the next day and the resulting supernatant containing the phages were transferred to the well of ELISA-plates coated with the appropriate antigens being tested (3.2.9.1).

7.3.2 Establishment of HB2151 cell lines and expression of E-tagged scFvs

The growth of monoclonal HB2151 cells and the expression of E-tagged scFvs were carried out as described (7.3.1) with minor differencies, as follows: After 2-3 h of growth (at which point OD_{600} reached 0.8-0.9), cells were induced by adding 1mM IPTG then incubated at 30°C, 700 rpm, ON. Cells were grown in YTCbGN and YTCbKN media instead of YTCbG and YTAK media, respectively.

7.4 Construction of a modified pET28 (pET28(*Sfi*I))

To clone scFvs from pCANTAB 5E phagemid directly into pET28 a *Sfi*I restriction site was introduced into the vector. The work-flow of vector construction is shown in Figure 7.2. The purification of PCR products, restriction digestions and ligation procedures were carried out as described in molecular biology methods (3.2.4). The PCR-amplification of DNA fragment carrying the *Sfi*I site was carried out as described (7.1.2, step 13). The reaction mixture contained the following ingredients:

20 ng of template DNA (pET28a)
100 ng primer 1
100 ng primer 2
10 µl 2 mM dNTP mix
10 µl 10 x Pfu buffer
1 µl Pfu enzyme
Nuclease-free water to 100 µl

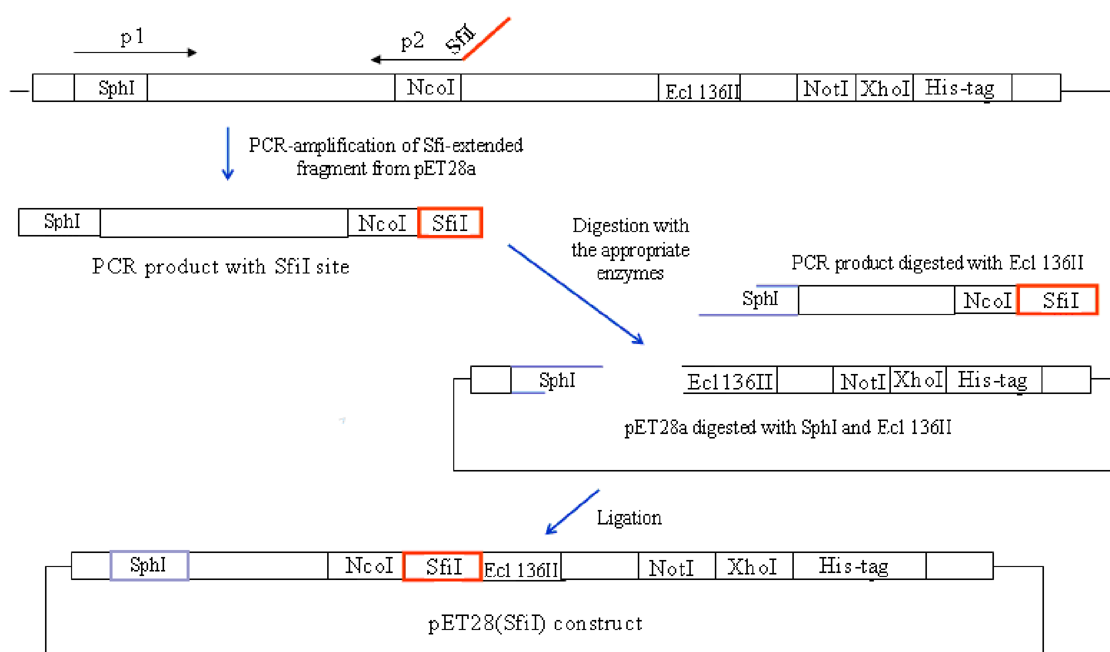


Figure 7.2: Work-flow of the modified pET28 vector construction (pET28-*Sfi*I). Arrow p1 and p2 represent forward and reverse primers applied for PCR amplification (see primer No. 1 and 2, in 3.1.9). SphI, NcoI, Ecl136II, NotI, XhoI and *Sfi*I represent positions of restriction sites for the respective enzymes.

7.5 ELISA-assays of monoclonal phages

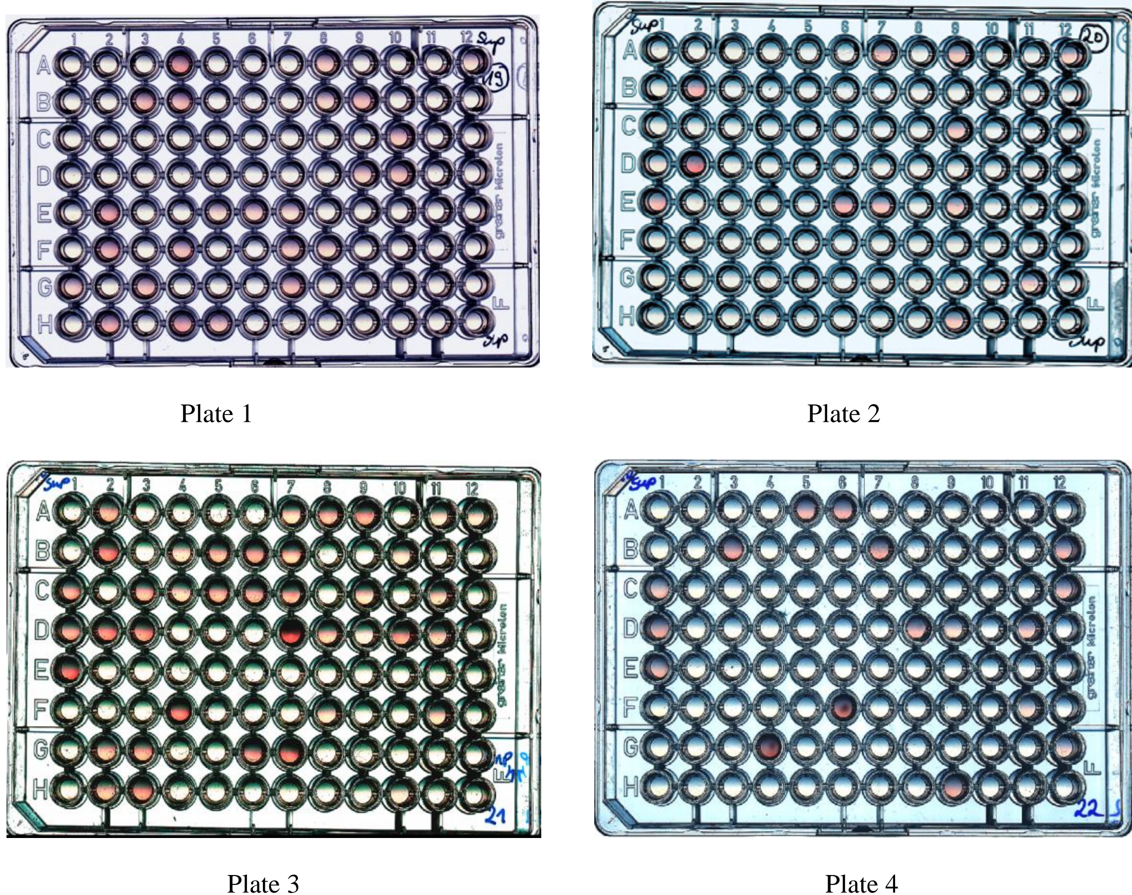


Figure 7.3: Plate 1: ELISA-assay of Ta1315-selected monoclonal phages using Sup12-HMWF as test antigen mixture. Phage selection was carried out as described in 4.3.1.1 Plate 2: ELISA-assay of Ta1194-selected monoclonal phages using Sup12-HMWF as test antigen mixture. Phage selection was carried out as described in 4.3.1.1. Plate 3: ELISA-assay of Ta1488-selected output 3 phages using Sup12-HMWF as test antigen mixture. Phage selection was carried out as described in 4.3.1.2. Plate 4: ELISA-assay of Ta1488-selected output 5 phages using Sup12-HMWF as test antigen mixture. Phage selection was carried out as described in 4.3.1.2. ELISA signals were developed with a HRP substrate system (5-aminosalicylic acid and H_2O_2).

7.6 SDS-PAGE analysis of proteins

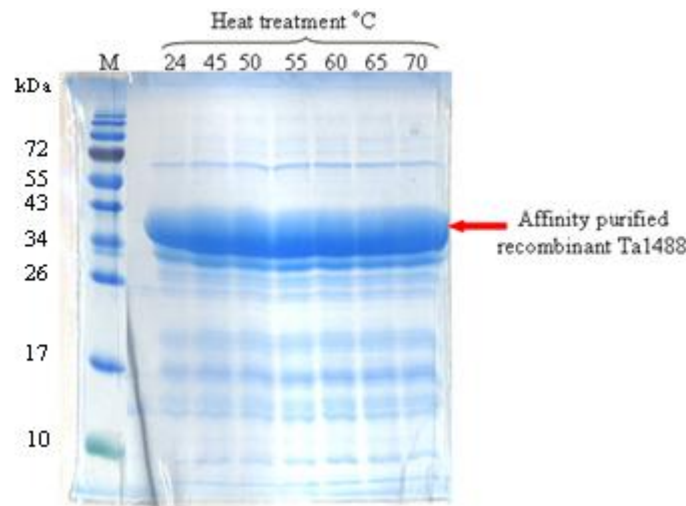


Figure 7.4: 15% SDS-PAGE of a heat treated and affinity purified recombinant Ta1488. Protein samples were heated for 10 minutes at 24, 45, 50, 55, 60, 65 and 70°C then centrifuged for 1 minute at 13 000 x g and the supernatant was loaded on 15% SDS-PAGE. M: Molecular marker.

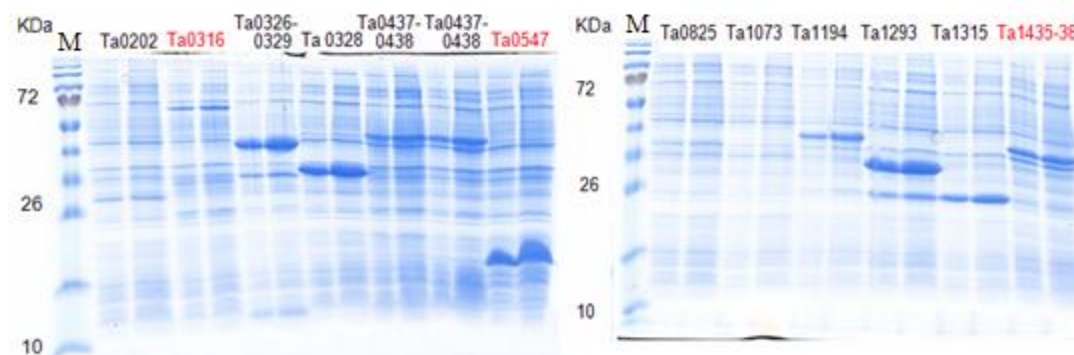


Figure 7.5: 15% SDS-PAGE of total *E. coli* cell extracts containing recombinant *T. acidophilum* proteins. The recombinant expression of respective proteins was carried out as described in table 7.2 of 7.9. Proteins highlited in red color demonstrate antigen targets used for the nitrocellulose-selection method of phages. M: Molecular marker.

7.7 Western blot assays

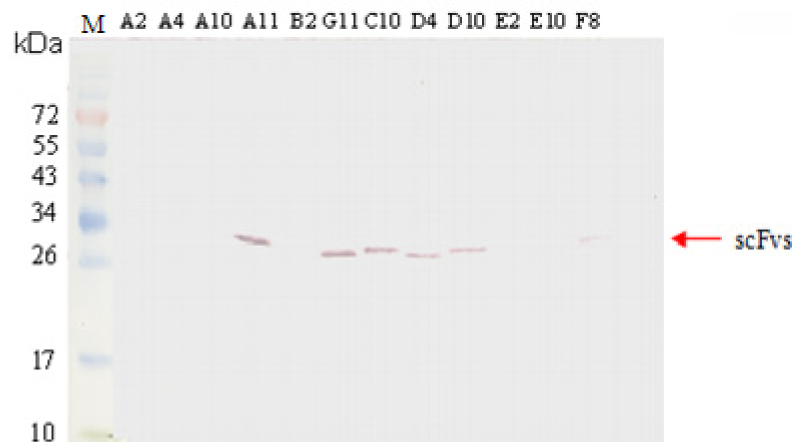


Figure 7.6: Western blot assay of Ni-NTA-purified soluble His-tagged scFvs selected for OHA-500 mM (corresponding ELISA-assay of clones see in Figure 4.2 of 4.2.1.1). Small-scale expression and Ni-NTA purification of scFvs from the soluble cell fraction were carried out as described in 3.2.5.3 and 3.2.7.1, applying 1 x Coupling buffer. A2-F8 represent elution fractions of Ni-NTA-purification. Western blot signals were developed with Sigma*FAST* BCIP/NBT substrate system. M: Molecular marker.

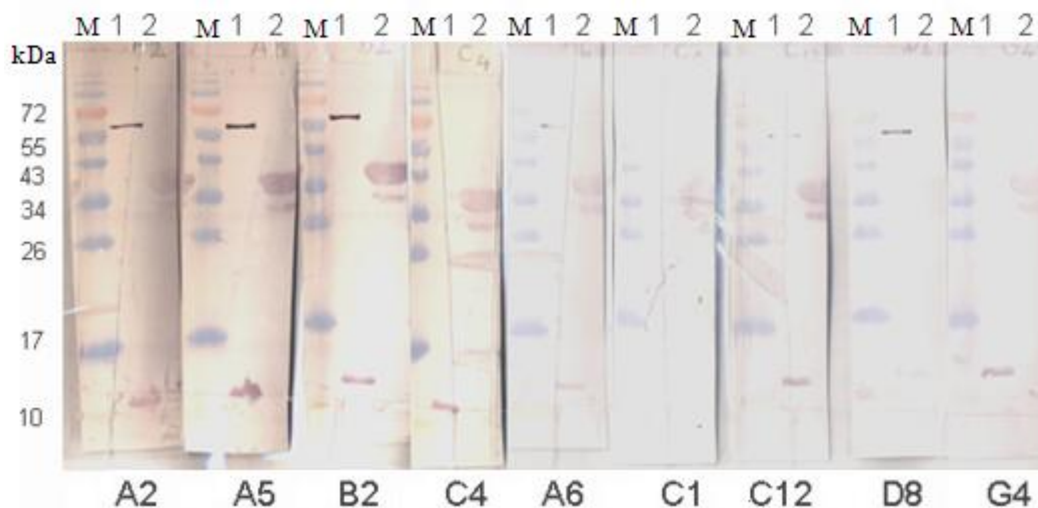


Figure 7.7: Western blot assay of Ta1488-selected monoclonal phages examined against two different test antigens. A2-C4 represent phage clones correspond to ELISA plate 1 and plate 3 in Figure 4.20 and 7.3, respectively. A6-G4 represent phage clones correspond to ELISA plate 2 and plate 4 in Figure 4.20 and 7.3, respectively. 1: Sup12-HMWF as test antigen mixture, 2: Affinity purified recombinant Ta1488 as test antigen. Western blot signals were developed with Sigma*FAST* BCIP/NBT substrate system. M: Molecular marker.

7.8 Amino acid sequences of scFv clones used for protein purification

2-oxo acid dehydrogenase specific scFvs (specific for Ta1435-38 complex)

```
D3-scFv  MAQVQLKESGPSLVKPSQTLSLTCSVTGDSITSGYWNWIRKFPGNKLEYMGYITFSGSTY
F9-scFv  MAQVQLKESGPSLVKPSQTLSLTCSVTGDSITSGYWNWIRKFPGNKLEYMGYITFSGSTY
B3-scFv  MAQVQLKESGPSLVTPSQTLSLTCSVTGDSITSGYWNWIRKFPGNKLEYMGYITFSGSTY
C2-scFv  MAXVQLKESGPSLVTPSQTLSLTCSVTGDSITSGYWNWIRKFPGNKLEYMGYITFSGSTY
         ** ***********.*********************************************

D3-scFv  YNPSLKSRISITRDTSKNQYYLQLNSVTTEDTATYYCARARDYGMDYWGQGTSLTVSSGG
F9-scFv  YNPSLKSRISITRDTSKNQYYLQLNSVTTEDTATYYCARARDYGMDYWGQGTSLTVSSGG
B3-scFv  YNPSLKSRISITRDTSKNQYYLQLNSVTTEDTATYYCARARDYGMDYWGQGTSLTVSSGG
C2-scFv  YNPSLKSRISITRDTSKNQYYLQLNSVTTEDTATYYCARARDYGMDYWGQGTSLTVSSGG
         ************************************************************

D3-scFv  GGSGGGGSGGGGSDIVMTQTPLTLSVTIGQPASISCKSSQSLLDSDGKTYLNWLLQRPGQ
F9-scFv  GGSGGGGSGGGGSDIVMTQTPLTLSVTIGQPASISCKSSQSLLDSDGKTYLNWLLQRPGQ
B3-scFv  GGSGGGGSGGGGSDIVMTQTPLTLSVTIGQPASISCKSSQSLLDSDGKTYLNWLLQRPGQ
C2-scFv  GGSGGGGSGGGGSDVVMTQTPLTLSVTIGQPASISCKSSQSLLDSDGKTYLNWLLQRPGQ
         **************:*********************************************

D3-scFv  SPKRLIYLVSKLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPQTFGGGT
F9-scFv  SPKRLIYLVSKLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPQTFGGGT
B3-scFv  SPKRLIYLVSKLDSGVPDRFIGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPQTFGGGT
C2-scFv  SPKRLIYLVSKLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPYTFGGGT
         ******************** ******************************** ******

D3-scFv  KLELKR
F9-scFv  KLELKR
B3-scFv  KLEIKR
C2-scFv  KLEIKR
         ***:**
```

Figure 7.9: Sequence analysis of Ta1435-38 complex specific scFv clones derived from phage selection procedure 4.3.2.1. Corresponding ELISA- and Western blot-assays of scFv clones used for the complex purification see in Figure 4.29-4.31, respectively. Sequences were aligned with the ClustalW multiple sequence alignment program (103). " * " indicates positions which have a single, fully conserved residue, "**:**" indicates that one of the following 'strong' groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW " **.** " indicates that one of the following 'weaker' groups is conserved: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY.

<u>Formate-dehydrogenase specific scFvs (specific for Ta0425-0424 complex)</u>

```
E6-scFv  MAQVQLQQSGDDLVKPGASVKLSCKASGYTFTHYWINWIKQRPGQGLEWIGEINPSNGGT
G9-scFv  MAQVQLQQPGXELVKPGASVKLSCTGSGFNIKHTYMHWVKQRPEQGLEWIGRIDPANGYT
C6-scFv  MAQVQLQQPGAELMKPGASVKISCKATGYTFSSYWIEWVKQRPGHGLEWIGEILPGSGST
E8-scFv  MAQVQLQQSGPELVKPGTSVKLSCKASGYTFTSYWMHWVKQRPGQGLEWIGEIDPSDSYT
         ********.* :*:***:***:**..:*:.:.  ::.*:**** :******.* *... *

E6-scFv  NFNEKFKKKATLTVDRSSRTAYMQLSSLTSEDSAVYYCTRSGYG---TYFDNWGQGTSLT
G9-scFv  KYDPKFQGKATITADTSSNTAYLQLSSLTSEDSAVYYCARLYYGSPYWYFDVWGAGTTLT
C6-scFv  NYNEKFKGKATFTADTSSNTAYMQLSSLTSEDSAVYYCAR----GGHWAMDYWGQGTSLT
E8-scFv  NYNQKFKGKATLTVDKSSSTAYMQLSSLTSEDSAVYFCARHG----NYAMDYWGQGTSVT
         ::: **: ***:*.* ** ***:*************:*:*         :* ** **::*

E6-scFv  VSSGGGGSGGGGSGGGGSDVLMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYL
G9-scFv  VSSGGGGSGGGGSGGGGSDIVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYL
C6-scFv  VSSGGGGSGGGGSGGGGSDVVMTQTPLSLPVSLGDQASISCRSSQSIVHSNGNTYLEWYL
E8-scFv  VSSGGGGSGGGGSGGGGSDIVMTQTPLTLSVTIGQPASISCKSSQSLLDSDGKTYLNWLL
         *******************::******:*.*::*: *****:****::.*:*:***.* *

E6-scFv  QKPGQSPKLLIYKVSNRFSGVPDRFSXSGSGTDFTLKISRVXAEDLGVYFCSQSTHVPYT
G9-scFv  QKPGQSPKLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPYT
C6-scFv  QKPGQSPKLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPYT
E8-scFv  QRPGQSPKRLIYLVSKLDSGVPDRFTGSGSGTDFTLKISRVEAXXLGVYYCWQGTHFPWT
         *:****** *** **:  *******: ************** *  ****:* *.**.*:*

E6-scFv  FGGGTKLELKR
G9-scFv  FGGGTKLEIKR
C6-scFv  FGGGTKLEIKR
E8-scFv  FGGGTKLEIKR
         ********:**
```

Figure 7.10: Sequence analysis of Ta0328-selected scFv clones derived from phage selection procedure 4.3.1.3. Corresponding ELISA- and Western blot-assays of scFv clones used for the complex purification see in Figure 4.24 and 4.25 of 4.3.1.3, respectively. Sequences were aligned with the ClustalW multiple sequence alignment program (103). " * " indicates positions which have a single, fully conserved residue, "**:**" indicates that one of the following 'strong' groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW " **.** " indicates that one of the following 'weaker' groups is conserved: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY, " **-** " indicates gaps in the alignment.

Small archaeal modifier protein specific scFvs (specific for Ta0895)

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A8-scFv     SCFVMAQVQLQQSGSELVRPGASVKLSCKASGYTFTSYWMHWVKQRPEQGLEWIGWIDPE
C10-scFv    ----MAQVQLQQPGAELVRPGASVKLSCKASGYTFTSYWMNWVKQRPEQGLEWIGRIDPV
G7-scFv     ----MAQVQLQQSGPELEKPGASVKISCKASGYSFTGYNMNWVKQSHGKSLEWIGDINPN
                ********.*.** :******:*******:**.* *:****   :.***** *:*

A8-scFv     NGNTIYDPKFQGKASITADTSSNTAYLQLSSLTSEDTAVYYCAR-MDYWGQGTTLTVSSG
C10-scFv    NGNTNYDPKFQGKATITADTSSNTAYLQLSSLTSEDTAVYYCARSMDYWGQGTSLTVSSG
G7-scFv     NGGTIYNQKFKGKATLTVDKSSSTAYMHLNSLTSEDSAVYYCAR-GAYWGQGTTLTVSSG
            **.* *: **:***::*.*.**.***::*.******:*******   ******:******

A8-scFv     GGGSGGGGSGGGGSDIVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYLQKPG
C10-scFv    GGGSGGGGSGGGGSDVVMTQTPLSLPVSLGDQASISCRSSQSLAHSNGNTYLHWYLQKPG
G7-scFv     GGGSGGGGSGGGGSDIVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYLQKSG
            ***************:***************************.**************.*

A8-scFv     QSPKLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVXAEDLGVYFCSQSTHVPYTFGGG
C10-scFv    QSPKLLIYKVSNRFSGVPDRFSGSGSXTDFTLKISRVEAEDLGVYYCFXGSHAPYTFGGG
G7-scFv     QSPKLLIYKVSNRFSGVPDRFSGSGSGTDFTLQISRVEAEDLGVYYCFQGSHVPYTFGGG
            ************************** *****:**** *******:*  .:*.*******

A8-scFv     TKLELKR
C10-scFv    TKLELKR
G7-scFv     TKLELKR
            *******
```

Figure 7.11: Sequence analysis of Ta0895-specific scFv clones derived from phage selection procedure 4.41. Corresponding ELISA- and Western blot assay of scFv-clones are shown in Sequences were aligned with the ClustalW multiple sequence alignment program (103). " * " indicates positions which have a single, fully conserved residue, "**:**" indicates that one of the following ’strong’ groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW " **.** " indicates that one of the following ’weaker’ groups is conserved: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY.

Ta1488 specific scFvs

```
E1-scFv  MAQVQLQQPGAEPVKPGASVKLSCTVSGFNIKDTYMHWVKQRPEQGLEWIGRIDPANGNI
F6-scFv  MAQVQLQQPGAEPVKPGASVKLSCTVSGFNIKDTYMHWVKQRPEQGLEWIGRIDPANGNI
         ************************************************************

E1-scFv  KYDPKFQGKATITADTSSNTAYLQLSSLTSEDTAVYYCARWAMITGFDYWGQGTSLTVSS
F6-scFv  KYDPKFQGKATITADTSSNTAYLQLSSLTSEDTAVYYCARWAMITGFDYWGQGTSLTVSS
         ************************************************************

E1-scFv  GGGGSGGGGSGGGGSDIQMTQSPSSMFASLGERVTITCKASQDINSYLSWFQQKPGKSPK
F6-scFv  GGGGSGGGGSGGGGSDIQMTQSPSSMFASLGERVTITCKASQDINSYLSWFQQKPGKSPK
         ************************************************************

E1-scFv  TLIYRANRLVDGVPSRFSGSGSGQDYSLTISSLEYEDMGIYYCLQYDEFPYTFGGGTKLE
F6-scFv  TLIYRANRLVDGVPSRFSGSGSGQDYSLTISSLEYEDMGIYYCLQYDEFPYTFGGGTKLE
         ************************************************************

E1-scFv  IKR
F6-scFv  IKR
         ***
```

Figure 7.12: Sequence alignment of Ta1488-specific scFv clones derived from phage selection procedure 4.3.1.2. Corresponding ELISA- and Western blot-assays of scFv clones used for the protein purification see in Figure 4.20 and 4.21 of 4.3.1.2, respectively. Sequences were aligned with the ClustalW multiple sequence alignment program (103). " * " indicates positions which have a single, fully conserved residue.

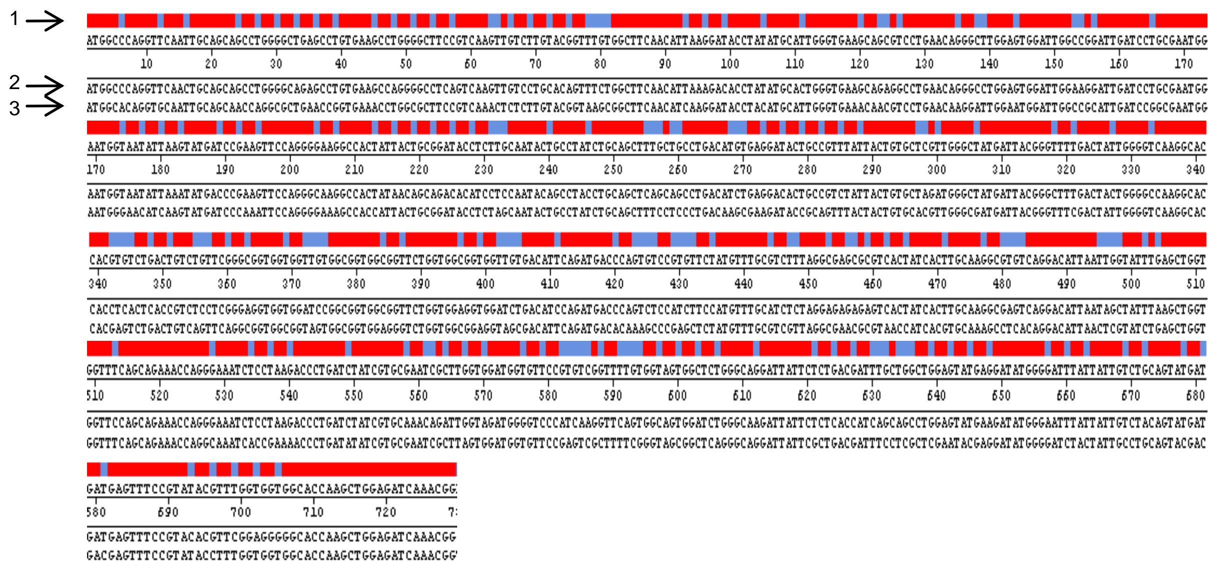

Figure 7.13: Sequence alignment of the original and the *E. coli*-optimized versions of Ta1488 specific scFv clones derived from phage selection procedure 4.3.1.2. Corresponding ELISA- and Western blot-assay of E1-scFv clone used for the protein purification are shown in Figure 4.20 and 4.21 of 4.3.1.2, respectively.

7.9 Recombinant proteins applied for scFv-selections

Protein	Vector	Origin of insert	Host	Fermentation
Ta0202	pET28	*T. acidophilum*	*E. coli* BL21(DE3)	30°C, 16 h, 1 mM IPTG
Ta0316	pET28	*T. acidophilum*	*E. coli* Rosetta(DE3)	37°C, 3 h, 1 mM IPTG
Ta0328	pET28	*T. acidophilum*	*E. coli* Tuner (pLysS)	30°C, 3 h, 1 mM IPTG
Ta1073	pET28	*T. acidophilum*	*E. coli* BL21(DE3)	30°C, 12 h, 1 mM IPTG
Ta1194	pEXP5-CT/TOPO	*T. acidophilum*	*E. coli* Rosetta(DE3)	ON, 37°C, autoinduction medium (Novagen)
Ta1315	pEXP5-CT/TOPO	*T. acidophilum*	*E. coli* Rosetta(DE3)	ON, 37°C, autoinduction medium (Novagen)
Ta1435-38	pET-Duet/pACYC-Duet	*T. acidophilum*	*E. coli* BL21(DE3)	ON, 37°C, autoinduction medium (Novagen)
Ta1488	pET28	*T. acidophilum*	*E. coli* BL21(DE3)	30°C, 12 h, 1 mM IPTG

Table 7.2: Recombinant *T. acidophilum* proteins applied for phage selection and screening assays.

Clone ID	Vector	*E. coli* codon optimization	Expression strain	Fermentation	Specificity
C8-scFv	pET28	-	*E. coli* BL21(DE3)	30°C, 5 h, 1 mM IPTG	Thermosome
A11-scFv	pET28	-	*E. coli* BL21(DE3)	30°C, 5 h, 1 mM IPTG	Proteasome
G10-scFv	pET28	-	*E. coli* BL21(DE3)	30°C, 5 h, 1 mM IPTG	Peroxiredoxin
E1-scFv	pET28	+	*E. coli* Rosetta(DE3)	30°C, 5 h, 1 mM IPTG	Ta1488
E6-scFv	pET28	-	*E. coli* BL21(DE3)	30°C, 5 h, 1 mM IPTG	Ta0425
E8-scFv	pET28	-	*E. coli* BL21(DE3)	30°C, 5 h, 1 mM IPTG	Ta0425
B3-scFv	pET28	-	*E. coli* BL21(DE3)	30°C, 5 h, 1 mM IPTG	2-oxo acid dehydrogenase
G7-scFv	pET28	+	*E. coli* Arctic express	18°C, 24 h, 0.8 g/L $MgSO_4$, 0.2% L-arabinose, 1 mM IPTG	Ta0895

Table 7.3: Specific scFv-clones applied for protein complex purification. Amino acid sequences of the scFv clones are shown in Figure 7.8-7.13.

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9. ACKNOWLEDGEMENTS

This work was performed in the Department of Molecular Structural Biology, Max Planck Institute of Biochemistry, Martinsried.

I would like to express profound gratitude to Professor Dr. Wolfgang Baumeister, head of the Department of Molecular Structural Biology, for giving me the opportunity to pursue my Ph.D thesis in this department, and for his generous support and supervision.

I would like to express my deepest gratitude to Dr. István Nagy not only for his patient, continuous guidance and supervision of my project, but also for his optimism, enthusiasm and his encouragement in the crucial parts of the project. His wide range of knowledge and his professional thinking helped me to deepen my scientific learning and facilitated to implement complex scientific experiments in a reasonable way. I am also thankful to him for his critical reading and correction of my thesis among his busiest time of work and for his valuable recommendations.

I would like to thank Prof. Dr. Tomohiro Tamura for the experimental design of phage display library. I am highly grateful to Dr.Yasuo Mitani for constructing the scFv-library and introducing me into the phage display project. I am thankful to him for his patient, precise and devoted training of the technique during the initial stage of this work and for giving me the opportunity to acquire the essential experimental work. His continuous helpfulness has been very much appreciated.

My grateful thanks are also extended to Dr. Stephan Nickell, whose professionalism and inspiring attitude had a good influence on me. His critical suggestions and ideas at giving scientific talks have been highly appreciated by me.

I would like to thank all my colleagues for creating a pleasant working atmosphere and for their contribution to this project. Thanks to Marius Boicu for performing the FPLC-purification and EM-analysis of protein samples and for providing me with hundreds of perfectly prepared SDS-gels. I am also thankful to Ganesh Pathare, Andreas Schweitzer,

Daniel Bollschweiler, Oana Michalache, Marga Schüler, Marion Jasnin, Inga Wolf, Amy Schönegge, Stefan Bohn, Shoh Asano and Roland Knispel for their kindness and for giving me helping hands whenever it was necessary.

I would like to take the opportunity to thank the Microchemistry Core Facility, especially Dr. Sabine Suppman for her cooperation in the recombinant protein expressions, Snezan Marinkovic for the DNA-sequencing and Dr. Cyril Boulegue and Beatrix Scheffer for performing the MS/MS-analyses of protein samples.

I am also thankful to our secretaries, Birgit Book and Sabina von Polenz, for their always patient and kind help with all the official and personal things.

Special thanks to Dr. Sarah Breuer not only for her invaluable support and encouragement, but also for her friendship and the nice time spent together in the lab.

My special thanks are extended to Dr. József Kukolya for his kindness, cordiality and placing reliance on me towards the Ph.D study.

Last but not least, I would like to thank my beloved family for their support- my husband, Péter, for his deep understanding, constant supports and good soul, my mother for her endless unselfishness, favour and efforts that she in me invested, my brother for his always encouraging and optimistic attitude and my father for trusting me. Without these, I would not have been able to achieve so much! Thank you!