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One-step purification of recombinant glutamate carboxypeptidase II and its homolog

by

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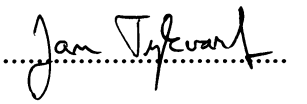

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1. PREFACE

We live in the post-genomic era. As the genomes of almost all model and pharmaceutically relevant organisms have been sequenced, attention of scientists has been slowly moving from DNA towards proteins.

Since information within DNA is coded in its primary sequence, simple and high-throughput methods, such as chain-termination sequencing method [1], could have been established to obtain that information. On the other hand, primary sequence of protein molecules bears substantially less information about a function of the protein. Secondary and tertiary structure, different cofactors, possible binding partners and many other factors can influence the protein's function. Therefore, it is necessary to purify and characterize every protein individually to obtain reliable information about its function [2].

Fortunately, establishment of DNA recombinant technology approach [3], discovery of type II restriction endonucleases [4,5] and utilization of PCR (polymerase chain reaction) techniques [6,7] has enabled the growth of a whole new branch of biochemistry and molecular biology. The availability of these technologies made enabled us to perform large scale production of recombinant proteins, both for industrial and research purposes.

For more efficient recombinant protein manipulation, it is often genetically fused to the peptide or protein sequence - a tag. The primary role of a tag is to simplify and unify purification and subsequent characterization of the target protein. Purification via a tag is based on a specific and sufficiently strong affinity of tag peptide/protein sequence and its binding partner. Such tag is therefore called an affinity tag.

Except for this utilization, the tags have been used for more diverse purposes, such as increasing yield of recombinant proteins production, enabling their detection or investigating their potential protein partners [8].

2. OBJECTIVES

- summarize the current scientific literature about different tags, focusing on their diverse features and use
- prepare plasmids for heterologous expression of Avi-tagged forms of glutamate carboxypeptidase II (GCPII) and NAALADaseL in *Drosophila* S2 Schneider cells
- create a stable transfectant of *Drosophila* S2 Schneider cells which would enable the large-scale production of Avi-tagged GCPII and NAALADaseL proteins
- optimize conditions for one-step purification of these recombinant proteins on the Streptavidin Mutein Matrix
- purify the above mentioned two recombinant proteins on Streptavidin Mutein Matrix and determine yields of purification and purity of target proteins
- investigate the influence of Avi-tag on the function of the purified proteins

3. INTRODUCTION

Many authors compared standard and affinity protein purification methods [8-12]. From their results it could be concluded that the yield and efficiency of any purification procedure depend mainly on the level of optimization which has been performed by the researcher and also on the nature of the purified protein.

Nevertheless, there are several reasons why to prefer affinity purification approach. Firstly, despite the above mentioned conclusion, an affinity purification protocol is more versatile than a standard purification protocol which is mostly worked out just for a particular protein. Secondly, the effort which has to be invested into establishing standard purification protocol is considerably higher than in the case of affinity purification. Thirdly, affinity purification procedure mostly consists of only one or two purification steps in contrast to multiple-step purification which has to be commonly performed in case of standard purification. Finally, affinity purification generally tends to have higher yields than standard purification [8].

3.1 Classification of the affinity tags

Affinity tag can be defined as an exogenous amino acid (AA) sequence with a high affinity for a specific biological or chemical ligand. For example, His-tag is represented by six histidine residues which specifically bind to the nickel ions (see 3.11.1.1) [8]. Most of the available affinity tags have been developed in the last 20 years [13]. Generally, they can be divided into two different groups depending on the nature of the affinity tag and its binding partner [12]:

- affinity tag (peptide or protein) which binds to small molecular ligands linked to a chromatography resin
- affinity tag (peptide) which binds to its protein-binding partner immobilized on a chromatography resin

Homogeneity of purified protein, cost-effectiveness, and regeneration ability of the affinity matrix are three major attributes in which the affinity tags differ.

For the first group of affinity tags a small molecular ligand is linked to the resin. Therefore the production of such a matrix is considerably less expensive than if a whole protein has to be attached. Another advantage of a small molecular ligand matrix is its possible

repeated use. Since the capability of protein coupled matrixes to bind an affinity tag is mostly dependent on the tertiary structure of the binding protein, usually higher loss of binding capacity and specificity occur after each purification than with small molecule ligand matrixes [2,8].

On the other hand, the purity of elution fraction is generally higher in case of protein coupled matrixes [12]. Since protein attached to the matrix binds its ligand through many non-covalent bonds, such an interaction tends to be highly specific. In case of small ligand matrixes, there are fewer non-covalent bonds which have to be stronger and thus enable easier partial non-specific binding.

Another attribute which might influence the purity of elution fraction is a possible protein/peptide-protein interaction between the tag itself and other proteins from the purified solution. Large protein affinity tags tend to contain such kind of impurities [12].

3.3 Influence of tags on expression yield

Since tags are designed and prepared via molecular cloning methods, precise DNA sequences which would be the most suitable for initiation of translation at the N-terminal methionine residue of the tag can be chosen. Additionally, as majority of proteins are now produced by heterologous expression systems, DNA sequence of the tag can be designed to follow the codon preferences in the host organism. When a problem with translation efficiency arises, it is often caused by interfering of the mRNA secondary structure with ribosome binding site [14].

It is also a generally accepted fact that the N-terminal amino acid of the protein determines the rate of protein degradation within the cell [15]. Hence, by choosing the right N-terminal tag the life-time of the fusion protein within the cell can be substantially prolonged. Interestingly, recent results show that even positioning of the tag to the C-terminus of purified protein can considerably increase its stability against intracellular proteolysis [16].

Currently, *Escherichia coli* is the most widely used heterologous expression system. According to the recent data from several large structural genomic centres, more than a half of all recombinant proteins produced in *E. coli* accumulate in the form of insoluble aggregates [17]. This represents a great problem mainly for high-throughput protein production and purification. The common method for increasing solubility is expression of recombinant protein at low temperature [18,19]. Unfortunately, this approach might not always be effective, yielding low amounts of the target protein if successful at all.

However, it has been shown that some affinity tags possess the ability to increase solubility of their fusion partners in *E. coli* [19,20]. The mechanism by which the protein/peptide tag enhances solubility is still not completely understood. Solubility-increasing affinity tags tend to be proteins rather than peptides. Highly soluble proteins are considered to be good solubility-enhancers but several works showed that certain soluble proteins are more effective than others [21]. Recently, protein tags which enhance solubility significantly but do not function as affinity tags have also been described [22,23].

Perhaps the best studied and most validated solubility-increasing proteins are *E. coli* maltose-binding protein (MBP, see 3.11.4.1) [24] and N-utilization substance A (NusA, see 3.11.4.2) [22]. For more information about these and several other tags see section 3.11.4.

3.4 Design of the fusion protein

The appropriate architecture of the fusion protein is as important as the right choice of the tag. Tag may be placed either within or at the end (N- or C-terminal) of the target protein. The latter possibility is much more frequent. If structure or model of the target protein is available, the tag should be placed at the more flexible part of the molecule (loops within the sequence or that end of the molecule which is more distant from prospective functional site of the protein) [25]. In order to influence the translation initiation and resistance against intracellular proteolysis, the tag has to be placed at the N-terminus of fusion protein (see 3.3).

Once the purification is finished the tag might need to be removed. Several approaches are available but the most common one involves cleaving off the tag by specific endoprotease (see 3.9). For such a cleavage a special peptide sequence has to be designed within the tag. The recognition sequence has to be easily accessible for the endoprotease and therefore some extra residues (linkers) are typically added to its both ends. The examples of possible design of fusion protein are shown in Figure 1 (p. 10). These examples do not cover all possible combinations but should serve just for illustration.

During molecular cloning a special attention has to be paid also to the preservation of the correct open reading frame.

3.5 Detection of fusion protein

At present, every major biotechnology company offers a list of monoclonal and polyclonal antibodies against diverse affinity tags, such as His-tag (see 3.11.1.1), FLAG-tag (see 3.11.1.2) or glutathione-S-transferase (GST) (see 3.11.2.2) [26]. Other tools for affinity tags detection involve avidin-biotin interaction or spectroscopy. For more details see 3.11.5.

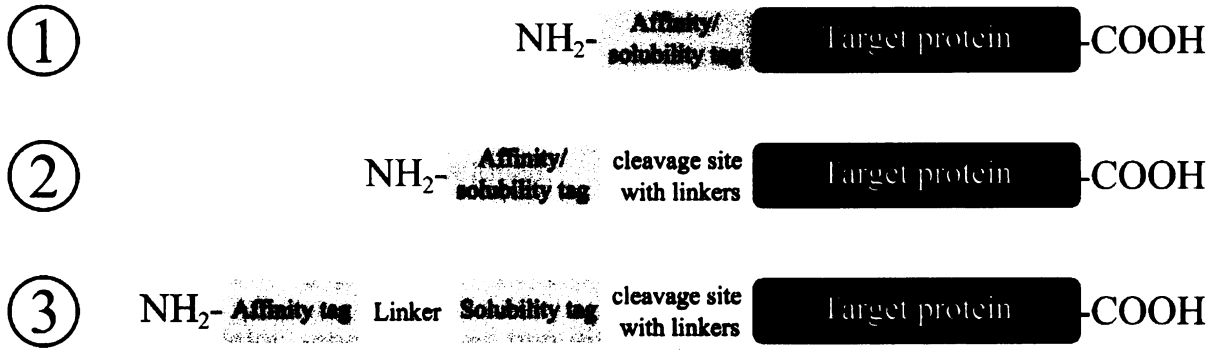


Figure 1: Different strategies for the design of a fusion protein. For clarity, only N-terminal fusions are shown. (1) Affinity tag serves also as a solubility enhancer and is added directly to the N-terminus of target protein. (2) Affinity tag which possesses also solubility-increasing feature is separate from target protein by peptide sequence which contains specific cleavage site for endoprotease. (3) Fused protein contains two tags, one for affinity purification and second for enhancement of the solubility. There is also cleavage site for endoprotease to enable release of the whole combinatorial tag after purification. Additionally, a linker is designed between both tags to prevent their interaction and subsequent dysfunction. Modified from [8].

3.6 Selection of expression host

The choice of expression system for production of recombinant fusion protein can significantly influence the process of purification. The predominant system used for heterologous protein expression is *E. coli* [2]. Unfortunately, as a prokaryotic expression system it is not suitable for all eukaryotic proteins and hence heterologous expression in other hosts (e.g. *Saccharomyces cerevisiae*, *Pichia pastoris*, insect and mammalian cell lines) was developed [27-31]. The right choice of expression host enables the researcher to obtain sufficient amount of properly folded and post-translationally processed protein which is a necessary prerequisite for successful purification. Furthermore, some tags are specific only for certain expression host (see 3.11.3.2 and 3.11.4.3).

3.7 Tags and protein crystallization

Tags might have negative effect on crystallization of fusion protein. Generally, it is assumed that large tags (proteins such as GST or MBP) have a negative impact on the ability of protein to crystallize. On the other hand, it is believed that small tags (peptides such as His-tag, FLAG-tag or Arg-tag) do not influence the crystallization of the fusion protein significantly. A direct comparison of several small affinity tags has been published recently by Bucher *et al.* [32]. The results have shown that even small peptide might influence the formation and quality of the protein crystals.

Until more work will be done and some consistent results will be obtained in this area of research, the cleavage of the tag prior the crystallization will probably be the most often used approach.

3.8 Combinatorial tagging

In the past several years, a number of approaches that utilize two different tags fused to a single target protein have been developed.

3.8.1 Tandem affinity purification (TAP)

This purification setup involves two different affinity tags placed in sequence at the C- or N-terminus of target protein. Usually, a cleavage site for specific endoprotease is also designed between tag and its fusion partner. By applying two consequent affinity purification steps, a highly pure protein is obtained. Such purified sample can be used for crystallization trials [31]. The other widely applied use of TAP is isolation of protein complexes and subsequent identification of potential protein partners of a target protein. One of the most used experimental setup involves combination of calmodulin-binding peptide with protein A (see 3.11.1.5) [33].

3.8.2 Combination of tags with diverse functions

The other approach to combinatorial tagging is creation of a fusion protein which will have one affinity tag and one tag with different function. In recent years, MBP fused with His-tag has been shown as one of the most favourite combination (see 3.11.4.1) [34]. Another interesting use of this technique is the combination of affinity tag with a „rainbow tag" which presents a simple and continuous way how to observe the localization and proper folding of fusion protein during purification (see 3.11.5.2) [35,36].

3.9 Removal of the tags

The tag may influence the behaviour of target protein in undesired fashion. Hence, it is often cleaved off after the purification. In some cases, e.g. production of recombinant proteins for pharmaceutical purposes, it is necessary to produce the protein in its wild-type form to avoid unexpected reaction of the patient during administration [8].

Several approaches exist for tag cleavage. However, none of them works unconditionally. There is always ambiguity whether the designed cleavage site will be cleaved off efficiently or not.

Even if efficient cleavage is achieved, another round of purification has to be performed to remove cleaved tag and eventually the protease. That makes the purification more laborous

and can also decrease the yield of whole procedure. Moreover, the tagged fraction of fusion protein should be also removed to ensure homogeneity of the protein preparation.

3.9.1 Chemical removal of the tags

Chemical removal of the tag is not a frequent method of choice. It can be performed by the treatment with cyanogen bromide or hydroxylamine if the unique methionine residue is placed between the fused protein and the tag [37]. The chemical method is rather unspecific and might lead to protein denaturation and side chain modification of amino acids in the target protein.

3.9.2 Enzymatic removal of the tags

In comparison with the chemical one, the enzymatic removal of the tag has two obvious advantages. Firstly, the cleavage performed by enzymes is much more accurate and the danger of the fused protein amino acid side chain modification is minimal. Secondly, the cleavage conditions are much milder.

Either exopeptidases or endopeptidases can be used to cleave the tag off.

3.9.2.1 Application of endopeptidases

This is the most common way to cleave the tag off. This approach involves endopeptidases with highly specific cleavage sites. List of the most utilized endopeptidases together with their cleavage sites and references of their use is given in Table 1 (p. 13).

To easily describe substrate specificity of peptidases, a nomenclature for description of amino acids (AAs) in the protease cleavage sequence was established by Schechter and Berger in 1967 [38]. The AAs forming cleaved peptide bond in a substrate are designed P_1-P_1'; the residues N-terminal to the cleavage point are labelled P_1, P_2, P_3, counting from the cleavage site, while residues in the substrate C-terminal to the cleavage site are labelled P_1', P_2', P_3', etc.

All endopeptidases have more stringent requirements on the AA sequence on their P than P' site (see Tab. 1, p. 13). That represents an additional problem if the tag is located at the C-terminus of target protein. There are two endopeptidases (enterokinase and Factor Xa) which cleave the recognition sequence with no specificity on the P_1' position and thus do not leave any part of the tag attached to the target protein. Even though it was assumed that the TEV peptidase requires certain AA on the P_1' position a recent study shows that the peptidase sufficiently cleaves substrates with almost any AA in the P_1' position [39].

Table 1: List of most often used endopeptidases for the tag cleavage. The position of endopeptidase cleavage site is indicated with an asterisk (*). Residues in bold remain in the protein after cleavage (if the tag is placed at N-terminus of target protein). Modified from [8].

Enzyme	Cleavage site	Comments	References°
Enterokinase	DDDDK*	Possible secondary cleavage sites at other basic AA FLAG-tag contains whole cleavage site	[40-42]
Factor Xa	IDGR*	Possible secondary cleavage sites at GR* Require an extra affinity tag for on column tag removal	[41-43]
Thrombin	LVPR***GS**	Possible secondary cleavage sites Biotin labelled for removal of the endopeptidase	[41,42]
PreScission	LEVLFQ***GP**	GST tag for removal of the endopeptidase	[44,45]
TEV peptidase	EQLYFQ***G**	Broad substrate specificity in the P_1' position His-tag for removal of the endopeptidase	[39,46-48]
3C peptidase	ETLFQ***GP**	GST tag for removal of the endopeptidase	[42]
Sortase A	LPET***G**	Ca^{2+}-induction of cleavage Requires an extra affinity tag for on column tag removal	[49]
Granzyme B	D***X**, N***X**, M*N, S*X	Risk of non-specific cleavage Require an extra affinity tag for on column tag removal	[50]
Intein	self-cleavable	Artificial AA left after cleavage in some applications On column (chitin-beads) cleavage	[51,52]

° Only a few relevant references are included

To simplify subsequent isolation of target protein from endopeptidase and cleaved tag, variants of endopeptidases bearing an affinity tag have been developed. One of the most favourite approaches is to use endopeptidase which is tagged with the same affinity tag as the target protein. After the tag is cleaved off the whole reaction mixture is applied again on the same affinity matrix which was used for the purification. The flow-through fraction which should contain only the target protein without the affinity tag is collected [46]. In this experimental setup it is also assumed that possible impurities bound to the affinity matrix during the first purification would now bind similarly and would not appear in the flow-through fraction. That should increase the homogeneity of purified protein. The example of this approach can be seen in Figure 9 (p. 28).

3.9.2.2 Application of exopeptidases

New method for cleavage of the tag has been developed recently. It involves utilization of exopeptidases which have generally a higher scissile activity than endoproteases. Either aminopeptidases or carboxypeptidases, with respect to the design of the fusion protein, could be

chosen. The special caution has to be taken if the exopeptidases belonging to the metallopeptidase family are used for removal of His-tag since His-tag could chelate ions in the enzyme active site and thus disrupt its activity [53].

The list of currently used exopeptidases together with some comments and references is given in Table 2.

Table 2: List of the most common used endoproteases for tag cleavage. Standard abbreviations for AAs are used. X refers to any AA. Modified from [8].

Enzyme	Comments	References°
Aeromonas aminopeptidase	Cleaves N-terminus, effective on M, L. Requires Zn^{2+}	[54]
Aminopeptidase M	Cleaves N-terminus, does not cleave X-P	[55]
Carboxypeptidase A	Cleaves C-terminus, no cleavage at X-R, P	[55]
Carboxypeptidase B	Cleaves C-terminal R, K	[55]

° Only a few relevant references are included

One common feature of all exopeptidases is their ability to cleave different dipeptide bonds with various efficiencies. This feature is used during the design of fusion tag in the way that the majority of it will be cleaved off during treatment with appropriate exopeptidase [8]. Due to exopeptidase's broad substrate specificity it is sometimes impossible to design tag of our choice for exopeptidase cleavage. In addition, the broad substrate specificity can also lead to incorrect tag cleavage.

3.9.2.2.1 TAGzyme system

The key element of this system is a recombinant rat dipeptidyl aminopeptidase I (DPPI or DAPase). Together with two additional recombinant proteins, plant glutamine cyclotransferase (Qcyclase) and bacterial pyroglutamyl aminopeptidase (pGAPase), the system facilitates cleavage of a short tag (less than 25 AA) at a very high rate and specificity [56-59]. Until now, TAGzyme system has been used mainly for cleavage of the His-tag [60]. Only N-terminal tags can be cut off via this approach.

DAPase is a cysteine dipeptidase which has quite a broad substrate specificity but there are several stop positions in which it is unable to continue the cleavage, such as Gln, Lys and Arg residue in the P_2 position. If a stop position occurs at the N-terminus of purified protein simple treatment with DAPase can be performed to cut off the tag [56]. However, usually the stop position has to be designed into the sequence. If a glutamine is the last residue of the tag (placed at the uneven position) adjacent to the desired N-terminal AA of the target protein, the DAPase will cleave off the whole tag in front of that glutamine residue. Qcyclase, which is specific to the N-terminal glutamine, will subsequently form pyroglutamyl at the N-terminal of

the fusion protein to prevent further DAPase cleavage. Afterwards, removal of DAPase and Qcyclase (both proteins are currently available in their tagged form) and treatment with pGAPase, which removes the pyroglutamyl residue, will lead to the complete removal of the tag [8,60]. For a schematic depiction of TAGzyme system sees Figure 2.

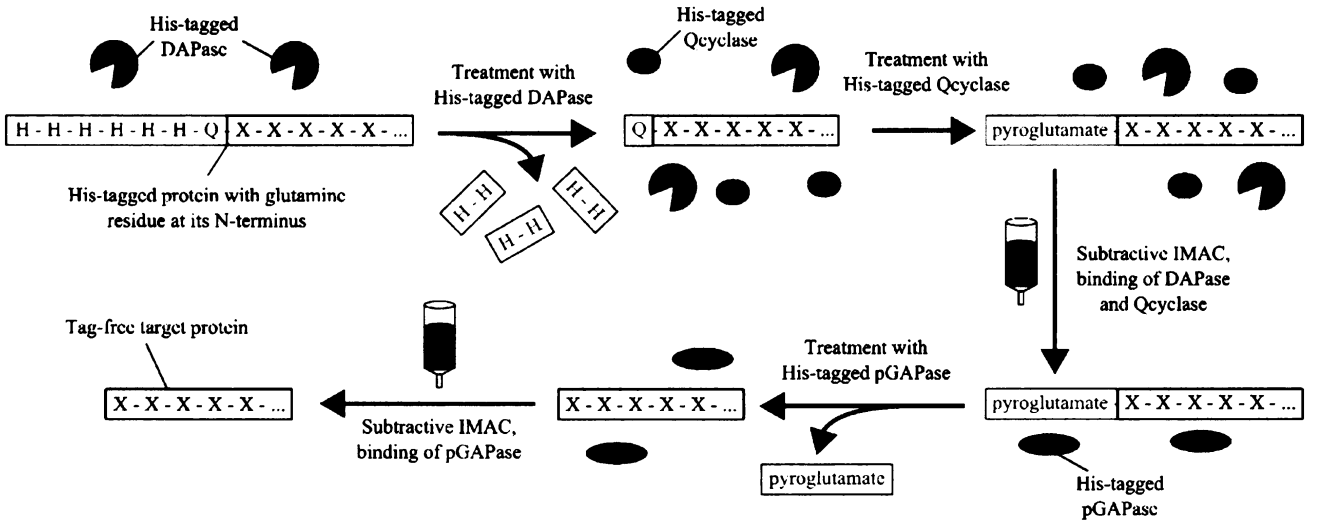


Figure 2: Schematic summary of the TAGzyme cleavage strategy utilizing glutamine as a cleavage stop point. Standard one-letter abbreviations for AAs are used. DAPase-dipeptidyl aminopeptidase I, Qcyclase-glutamine cyclotransferase, pGAPase- pyroglutamyl aminopeptidase, IMAC-immobilized metal-affinity chromatography.

DAPase has its active site located at the surface of the molecule which explains the ability of a rapid processing even if a low ratio of DAPase to the tagged protein is used (1:5000 or 0,2 μg of DAPase per 1 mg of cleaved protein).

These features together with minimal unspecific cleavage of DAPase at least balances the necessity of additional reactions and purifications that have to be performed during tag removal and make the TAGzyme system an interesting tool for efficient tag removal.

3.10 Possible negative effects of the tags

To consider expression yield, the fusion protein might be expressed in much less amount or not at all in comparison with its wild type form [61-63]. An explanation for this might be that large protein tags are believed to represent high metabolic burden for the host expression system and thus might also decrease the expression of fusion protein [2].

The tag could also prevent the proper binding of monoclonal antibody to the target protein and thus disallow the detection of fusion protein.

The negative effect on crystallization was already mentioned (see 3.7) [32]. There is also a possibility that the tag will interfere with the biological function of the target protein

[64]. Hence, proper comparison studies of wild type form of the target protein and its fusion form have to be performed.

Unfortunately, since negative results are not commonly published, it is difficult to judge how many unsuccessful purification protocols were developed, how many experimental setups were shown to be ineffective and how many binding or elution conditions were found to be unsatisfactory. Moreover, very few reports on the comparison of different tags can be found in the scientific literature. Thanks to the above mentioned facts, the correct choice of tag still remains a laborous operation.

Finally, a very interesting study was published recently by Lichty *et al.* [12]. This work compares several dual-affinity tags by purifying two different proteins (one highly and the other slightly expressed) on both affinity matrixes. The results suggest that no matter how perfect the affinity tag and purification conditions are the original amount of purified protein is most determining for the overall yield of purification. Hence, it is recommended to invest at least as much effort in establishing appropriate host expression system as in the subsequent optimization of the purification itself.

3.11 Examples of the tags

On the following pages, the list of the tags which are used throughout scientific research is presented. The registry is divided into several categories which gather the tags with some important common feature. The list is not complete but the author believes that it covers the most important representatives from all fields in which the tags might be utilized.

3.11.1 Small peptide affinity tags

These tags function primarily as the affinity tags. Their possible influence on expression level can be mediated by facilitation of translation or prevention of intracellular degradation but they are generally thought to lack the ability to enhance solubility of the fusion protein (see 3.3). These tags represent a small metabolic burden for host expression system which might also positively influence the yield of expression.

3.11.1.1 His-tag (polyhistidine tag)

His-tag is by far the most used affinity tag. More than 60% of the proteins produced for structure studies include His-tag [65]. It may be composed of 2-10 histidine residues, while the most common form of His-tag is 6xHis-tag which consists of six histidine residues. These residues mediate the non-covalent interaction (chelation) of this tag to the transition metal

cations (Ni^{2+}, Co^{2+}, Zn^{2+}, or Cu^{2+}) [66,67]. The idea of immobilized metal-affinity chromatography (IMAC) dates back to 1975 [68]. However, it fully advances just after development of His-tag. In this experimental setup the transition metal ions are immobilized on a matrix through special chemical groups. Ni^{2+}-NTA, nickel ions adsorbed through nitrilotriacetic acid (NTA), and Co^{2+}-CMA (Talon), cobalt ions adsorbed through carboxylmethyl aspartate (CMA), are currently the most used metal-chelated affinity chromatography resins [69,70].

The metal-chelated matrixes can be prepared easily in high amounts and therefore they are much less expensive than the matrixes with immobilized proteins. In addition, the matrixes can endure much severe handling and hence can be used repeatedly without significant loss of binding capacity and specificity.

Purification via His-tag can be performed in both native and denaturing conditions. Bound protein can be eluted in two ways - either by chelating competitors (imidazole) or by low pH (under 5.0), which causes protonation of histidine imidazole rings and abandons their chelation to the transition metal ions [71]. The first approach tends to be milder for purified protein and hence it is often used. During the loading and washing steps of purification it is recommended to add a low amount of imidazole to the solution to prevent unspecific binding to the matrix. The concentration of imidazole in the wash and elution solutions is crucial for successful purification and should be optimized as a first parameter if the procedure does not function appropriately [72,73]. Unfortunately, the imidazole might negatively influence NMR experiments, competition studies, crystallographic trials, and can lead to the formation of protein aggregates [73].

His-tag is commonly located at either N- or C-terminus of target protein. It works in both of the setups but it can have a different influence on target protein depending on its position [64].

The purification via His-tag has been carried out in a number of expression systems including bacteria [74], yeast [75], baculovirus-infected insect cells [76], S2 Schneider insect cells [77], and mammalian cells [78].

More than 100 structures of His-tagged proteins have been deposited in the Protein Data Bank [71]. The tag is believed to have minor effect on the crystallization of target protein as supported by the recent comparative study [32]. The influence on protein biological activity cannot be excluded completely but due to the size of tag the probability of such intervention is quite low. Nevertheless, if this is the case, it can be often solved by moving the tag to the opposite terminus of the target protein [64].

On the other hand, the proteins with metal centre (e.g. metalloproteases) are not suitable for this purification system since the metals could be chelated by the NTA, CMA or His-tag of another fusion protein which would lead to the inactivation of the protein [8].

However, the advantages of His-tag purification clearly outweigh its disadvantages.

3.11.1.2 Arg-tag (polyarginine tag)

The Arg-tag was originally described in 1984 [79] and consists of 5-9 consecutive arginine residues. Arginine, as the most basic amino acid, keeps positive charge even in high pH conditions. Hence, Arg-tagged fusion proteins are simply purified on cation exchange resin in high pH. In such conditions almost all other proteins will flow through the resin without binding. The tagged protein is eluted with high ionic strength (commonly by linear NaCl gradient) [79]. Since a regular ion exchange resin is used the cost of the method is low.

Highly positively charged Arg-tag can have a severe influence on the tertiary structure of the fused protein. It was shown that the Arg-tag does not prevent crystallization of one particular protein but it significantly affects the mosaicity of crystals and their final resolution [32].

One very interesting feature of Arg-tag is its ability of binding on a flat surface. That can be used for studying interaction of target protein with ligands [80]. For example, GFP with Arg_6-tag, which is able to reversibly and specifically bind onto a mica surface, was established as a standard substrate for electron and scanning probe microscopy applications [81].

If the Arg-tag is placed at C-terminus of the target protein, it can be cleaved off by carboxypeptidase B (see 3.9.2.2). However, contradictory reports were published about the effectiveness and specificity of that cleavage [80,82].

3.11.1.3 FLAG-tag

FLAG-tag is a short, hydrophilic 8 amino acids long peptide (see Tab. 3, p. 32). This peptide is specifically recognized and bound to the antibody M1 [83]. Until now, several more antibodies (e.g. M2 and M5) have been raised against FLAG-tag with different recognition and binding characteristics [71]. The matrix for this purification contains immobilized antibody and therefore is sensitive to outer conditions and expensive.

FLAG-tag can be located at C- or N-terminus of the protein. The system can be utilized in variety of host expression systems such as bacteria [84], yeast [85], S2 Schneider insect cells [86], and mammalian cells [87].

The elution of the fusion protein can be performed by EDTA-containing buffer since the binding of FLAG-tag to M1 antibody was shown to be calcium-dependent [40]. The purity of elution fraction from this purification system is quite high (above 90%) even in a direct comparison with other affinity purification methods [12].

The 3xFLAG-tag has been developed to improve a detection of FLAG-tag. Its three-tandem FLAG epitope enables a detection of up to 10 fmol of the expressed fusion protein [88].

The FLAG-tag can be removed by treatment with enterokinase, which specifically recognizes the five AA long C-terminal part of the FLAG-tag sequence (see Tab. 1, p. 13).

3.11.1.4 Softags

Softags are small peptides, 6-13 AA long, with various primary sequences, recognized by polyol-responsive monoclonal antibodies (PR-mAbs). PR-mAbs antibodies are obtained during selection of hybridome clones on the basis of their ability to elute the bound antigen in the presence of high ionic strength and low molecular weight polyhydroxylated compounds (e.g. solution of 1 M NaCl and 40% propylene glycol) [89]. Such antibodies were raised against small peptides (Softags) and were additionally used for their affinity purification [90]. Because of the mild elution features of PR-mAbs this purification setup is especially appropriate for purification of labile, multisubunit enzyme complexes and for facilitation of the study of protein interactions [91]. The features of the antibody-coupled matrix are quite the same as in the case of the FLAG-tag matrix (see 3.11.1.3).

3.11.1.5 CBP (Calmodulin-binding peptide)

This purification system was developed in 1992 [92] and employs a 26 amino acids long peptide derived from C-terminus of skeletal-muscle myosin light-kinase chain, which binds calmodulin with nanomolar affinity in the presence of 0.2 mM $CaCl_2$ [93]. In this experimental setup the calmodulin protein is immobilized on the chromatography matrix.

The calmodulin-binding peptide can be placed at either C- or N-terminus of the target protein but the N-terminal location may lead to the reduction of the efficiency of translation. Moreover, C- terminal location may conversely result in high expression yield [94].

Since no endogenous proteins that would interact with calmodulin are present in *E. coli*, this system is highly specific for purification of the fusion protein from that expression host. [95]. The tight binding of the tag enables severe washing conditions. On the other hand, the elution is very mild, performed by adding chelating agent (e.g. EDTA, ethylenediaminetetraacetic acid). That, unfortunately, makes this purification protocol

unsuitable for proteins with metal in their active site (e.g. metallopeptidase). The purification in eukaryotic host cells is not recommended since the protein mixture will contain many endogenous proteins which may interact with calmodulin in a calcium-dependent manner [96].

The CBP is a part of well characterized tandem affinity purification (TAP) protocol for studying protein interaction (see 3.8.1). This tag is used together with protein A, bacterial protein which is able to bind specifically to immunoglobulin [97], and a cleavage site for TEV protease (see 3.9.2.1) is additionally designed between these tags. The fusion protein is expressed in its physiological conditions which enables binding of its natural protein partner. Consequently, the protein is sequentially purified via protein A and then via CBP. After the purification the elution fraction is resolved by SDS-PAGE and its components are further characterized (see Fig. 3, p. 32) [98,99].

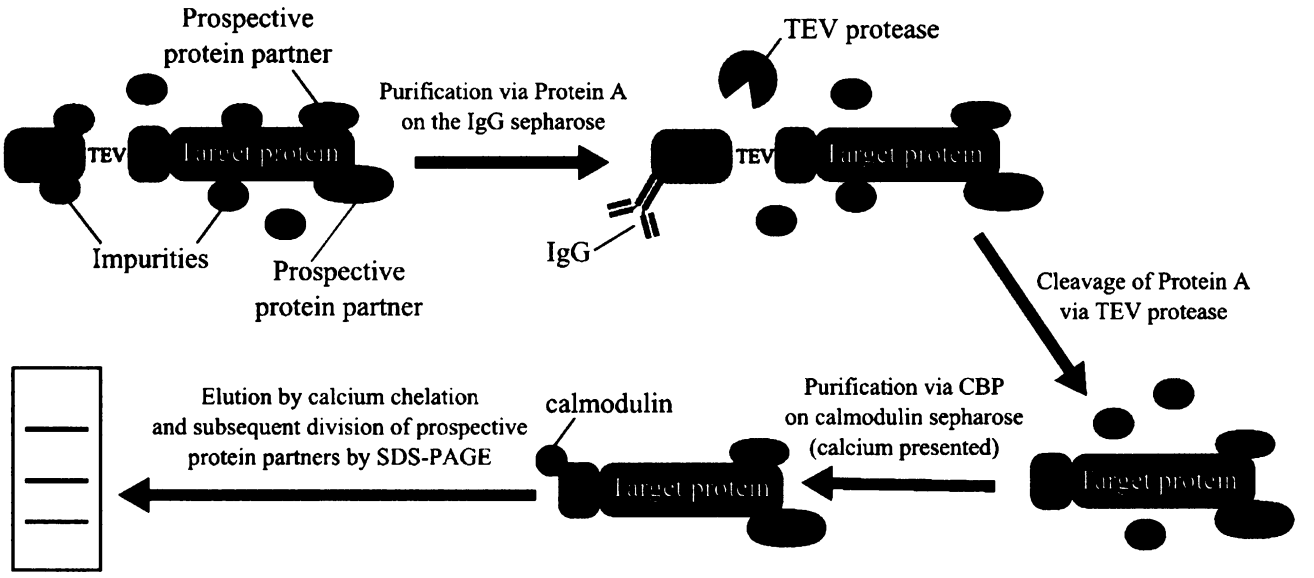


Figure 3: Schematic representation of TAP strategy utilizing Protein A and CBP. ProtA-protein A, TEV-recognition peptide sequence for TEV protease, CBP-calmodulin-binding peptide, IgG-immunoglobulin G.

3.11.2 Large protein affinity tags

This group of tags works primarily as affinity mediating tags but might also have a positive influence on rate of translation and the solubility of fusion protein (see 3.3). Corresponding to their size, these tags represent a high metabolic burden for the host expression system and thus can negatively influence the yield of expression.

3.11.2.1 CBD (Cellulose-binding domain)

There are more than 13 different families of proteins with cellulose-binding domain. Their size ranges from less than 30 to more than 150 amino acids [100]. A model for the interaction of families I, II, and III CBDs with cellulose is shown in Figure 4 (p. 21). Cellulose

represents a very advantageous affinity matrix since it is inert, has low non-specific binding, is available in many different forms, and has been approved for pharmaceutical purposes [71].

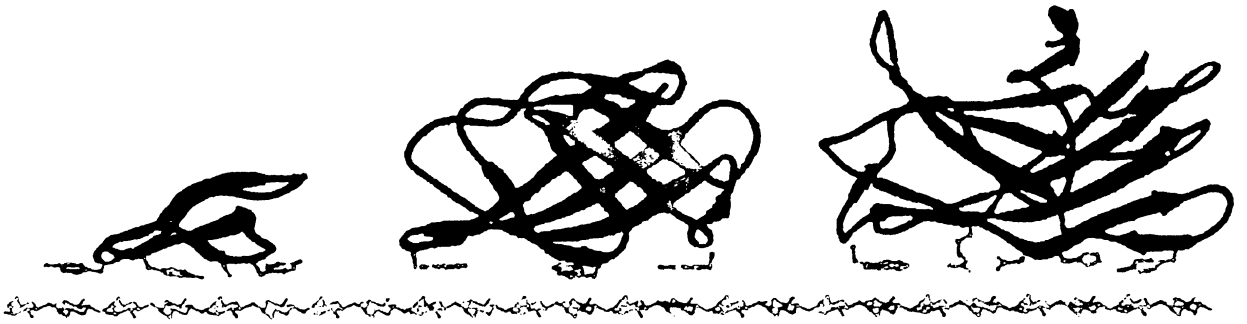


Figure 4: Model for the interaction of families I, II and III CBDs with cellulose. The panel shows the three CBDs aligned along a single cellulose chain. The family I fungal CBD is shown in blue, the family II bacterial CBD is shown in orange, and the family III bacterial CBD is shown in green. Amino acid side chains, which interact with the cellulose, are shown in yellow. Cellulose chains are shown in white. Modified from [101].

The binding of CBD is mediated mainly by hydrogen bonds and van der Waals interactions [100,101]. Some of the CBDs bind irreversibly to cellulose and thus can be used as an immobilized tool for target enzyme [102]. On the other hand, CBDs of families I, II and III interact reversibly with crystalline cellulose and are therefore suitable for affinity purification. CBDs from family I bind really strongly and thus only buffers containing urea or guadinium chloride can sufficiently elute bound protein. On the other hand, CBDs from family II and III can be eluted gently with ethylene glycol [103].

Recombinant CBD tagged proteins have been produced in bacteria, yeast, baculovirus-infected insect cells, and mammalian cells [100].

3.11.2.2 GST (gluthathione-S-transferase)

GST is a 26 kDa protein which was first described as an affinity tag in 1988 [104]. Fusion protein can be purified by affinity chromatography on immobilized gluthathione matrix. Gluthathione, γ-Glu-Cys-Gly tripeptide, can be prepared without too much difficulty and thus the price of the affinity matrix is affordable.

The binding kinetics of GST to gluthathione is slow which, together with formation of dimers by GST in the solution, represents a major drawback in the use of this affinity tag in large-scale purification. Additionally, it has been published that GST might have unpredictable impact on the solubility of fusion protein [71]. The elution is commonly performed with 10 mM gluthathione. Recent comparison study of different affinity tags showed that elution fraction from GST affinity purification contains considerable amount of impurities [12].

GST can be placed at N- or C-terminus of the fused protein. Successful expression in bacteria [104], yeast [105], baculovirus-infected insect cells [106], and mammalian cells [107] has been described.

3.11.2.3 ChBD (Chitin-binding domain)

ChBD from *Bacillus circulans* composes of 51 amino acids and is commonly available in combination with self-splicing intein (see 3.9.2.1) [108]. The most often used intein originates from *Saccharomyces cerevisiae* VMA1 gene and consists of 451 amino acids [109]. Polysaccharide chitin serves as an affinity matrix. It shares similar advantages as cellulose in terms of cost and endurance (see 3.11.2.1).

In this purification setup the elution is provided by self-cleavage of the intein. This cleavage can be induced by thiol reagents, such as 1,4-dithiothreitol or 2-mercaptoethanol. However, the intein biochemistry is quite a new and complex field and proper explanation of the mechanism of action and inducement of the self-cleavage are beyond the scope of this thesis; for more information see [8]. The high salt concentration and the use of non-ionic detergents can lead to reduction of non-specific binding during loading the protein preparation [71].

A protein with C-terminal chitin-binding domain fused with intein has been successfully expressed in bacterial system [111,112].

3.11.2.4 ELP (Elastin-like polypeptide)

ELP consists of several-to-numerous repeats of a pentapeptide motive. This sequence enables a fusion protein to undergo a reversible, from soluble to insoluble phase, transition upon temperature up-shifts [113]. Even more interesting is the fact that the length of the ELP has been shown to influence not only temperature of phase transition but also yield of the whole purification [114]. The robustness and simple experimental setup makes this method suitable for large-scale purification of commercially relevant proteins [71].

The question remains, how much the phase transition might influence the biologic activity of purified protein. Additionally, for further use of fused protein, the ELP has to be cleaved off. The method involving intein (see 3.11.2.3) has been developed recently [115].

3.11.2.5 PHB-binding-tag (polyhydroxybutyrate-binding tag)

This system represents a non-conventional but very astute method for purification of target protein. Some bacteria and archae organisms are able to synthesize polyhydroxyalkanoic

acids (mainly polyhroxybutyrate, PHB) in the form of polymers as carbon and energy resources [116]. A group of proteins called phasins which showed specific affinity towards the PHB, were discovered within these organisms [117]. Naturally, phasins probably influence the size of PHB bodies and the rate of their accumulation [118].

Through simple genetic modifications the creation of PHB granules was also achieved in the wide variety of common expression systems (e.g. *E. coli, Saccharomyces cerevisiae*) [119,120]. In this purification setup phasins are used as affinity tags (PHB-binding tag) and are fused together with the target protein.

One particular experimental setup which utilized also self-cleavable intein (see 3.11.2.3) designed between PHB-binding-tag and target protein was described. This fusion protein was expressed in the engineered strain of *E. coli* which produced PHB polymer granules. By virtue of that, no additional chromatography resin was necessary. Cell lysate was centrifuged after expression. Since PHB polymer is insoluble it was in a pellet. Then the pellet was washed out from impurities and placed to the intein cleavage buffer. Cleavage was performed and purified protein was released from PHB polymer granules. After another centrifugation only purified, tagless target protein was present in the solution (see Fig. 5) [121].

Figure 5: Schematic representation of PHB strategy utilized by specially engineered strain of *E. coli* and self-cleavage intein. In the Figure is shown total cleavage of the intein molecule which is not always the case.

This approach is extremely simple and cost-effective but since not many references on this purification system can be found it will probably bear also some disadvantages.

3.11.3 Tags based on biotin-avidin interaction or its analogy

Avidin is a tetrameric 69 kDa protein produced in the oviducts of birds, reptiles and amphibians which is deposited in the whites of their eggs. The tetrameric protein contains four identical subunits (homotetramer) each of which can bind biotin (vitamin H) with a high degree of affinity and specificity. The biotin-avidin interaction is the strongest non-covalent interaction known, with $K_D \sim 10^{-15}$M [122]. Streptavidin, 55 kDa prokaryotic ortholog of avidin, shares the

same binding ability as avidin but does not contain glycosylations. Hence, it can be easily large-scale expressed in *E. coli* and subsequently commercially utilized. Streptavidin or its analogue (see below) is immobilized to the chromatography resin, therefore this purification method requires careful handling with matrix and the cost for the resin is high. Chemical formula of biotin and the avidin-biotin complex X-ray structure are shown in Figure 6.

All these tags belong to the group of small peptide affinity tags. Hence their influence on expression yield is not significant (see 3.11.1).

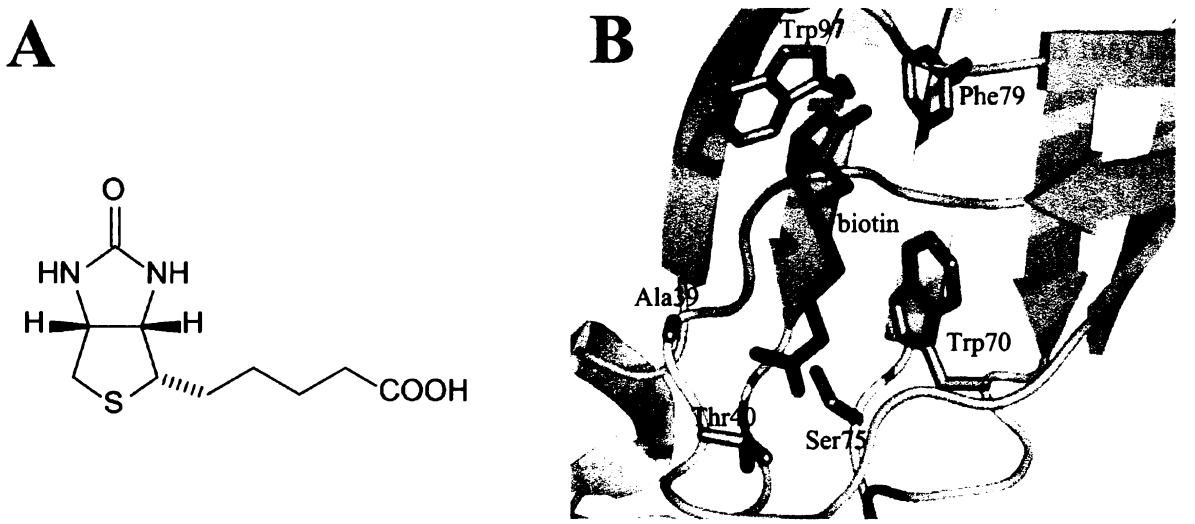


Figure 6: The D-biotin-avidin interaction. Panel A: Chemical formula of D-biotin (vitamin B_7) is shown. **Panel B:** Picture of the X-ray structure of avidin-biotin complex. Just biotin-binding part of the one monomer unit of avidin is depicted. Residues which contribute to the binding of biotin are coloured orange. Biotin is coloured magenta. The PDB (1avd) file was taken from [123] and adjusted by The PyMOL Molecular Graphics System (http://www.pymol.org).

3.11.3.1 Strep-tag II

Strep-tag II is an oktapeptide which specifically binds to streptavidin (see Tab. 3, p. 32) [124]. To tighten this interaction, a modified streptavidin was developed, under commercial name Strep-tactin, with 100 times higher affinity to Strep-tag II (K_D~10^{-6}M) [125,126].

Endogenously presented proteins which are able to bind to Strep-tactin are blocked by avidin prior to the purification. This approach increases homogeneity of purified protein since in contrast with other proteins Strep-tag II is unable to bind to avidin. Moreover, mild conditions of elution could be used in this purification system. Physiological buffer can be used with addition of 2.5 mM desthiobiotin. This chemical compound is an analogue of D-biotin and is capable of reversible binding to the Strep-Tactin matrix (see Fig. 7, p. 25) [127].

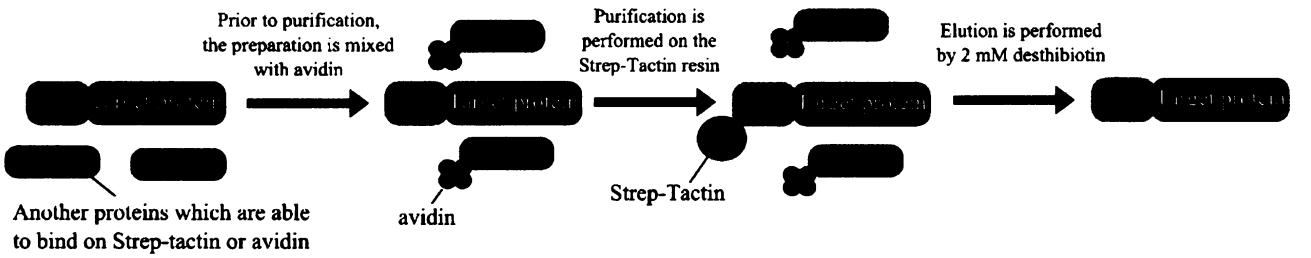


Figure 7: Schematic representation of Strep-tag II purification procedure. For better lucidity the possible cleavage site for endoprotease is not included in the scheme.

This purification method is recommended for purifying proteins under anaerobic conditions and also for metal-containing enzymes [128,129]. Strep-tagged proteins may also be used for NMR and crystallization experiments [130], even though a recent comparison crystallographic study has shown that from five small affinity tags utilized only Strep-tag II prevents the formation of fusion protein crystals [32].

The tag can be placed at either C- or N-terminus of the target protein and has been produced in bacteria [131], yeast [132], plant [133], and mammalian cells [134].

Detection of such fused protein is possible via streptavidin which is fused with some visualisation agent. Even if the strength of binding to streptavidin is not too high (K_D~10^{-4}M) it suffices for majority of common utilization.

3.11.3.2 Nano-tag

Nano-tag represents an evolutionary successor of Strep-Tag II (see 3.11.3.1). It was obtained via high-throughput testing of $2x10^{13}$ 15-mer peptides. The aim of the test was to get short peptide tag which would bind to streptavidin more tightly than Strep-tag II (K_D~10^{-4}M) but preserve all its positive features [135]. Two tags were gained from this study, Nano-tag_9 (K_D~10^{-8}M) and Nano-tag_{15} (K_D~10^{-9}M) (see Tab. 3, p. 32).

One major drawback can be found in this purification protocol. It was shown from a crystallographic study of a Streptavidin-Nano-tag complex that for the proper function of this tag a formyl-methionine residue has to be present at the N-terminus of the tag. That makes this approach usable only in prokaryotes expression systems and *in vitro* translation systems [136]. Logically, the Nano-tag is supposed to be placed at the N-terminus of the fusion protein.

3.11.3.3 Avi-tag

Several highly conserved protein sequences in *E. coli* are specifically biotinylated by the *E. coli* biotin-(acetyl-CoA-carboxytransferase) ligase (*Bir*A, EC 6.3.4.15). Using molecular engineering techniques a minimal substrate (Avi-tag) for *Bir*A was found, consisting of

14 AAs. It contains one lysine residue which is specifically biotinylated on its ε-amino group (see Tab. 3, p. 32) [137].

Ironically, the interaction between biotin and streptavidin is too strong for use in protein purification. The elution conditions would be so harsh that the purified protein would be almost certainly destroyed. It has been determined that the most favourable dissociation constant for purification purposes ranges in nanomolar values [138]. Hence, modified streptavidin analogues, monomeric streptavidin muteins, which embody nanomolar dissociation constants toward biotin were developed through molecular engineering [138,139]. Purification on the mutein matrixes can be performed under physiological conditions and elution is done with 2 mM D-biotin. Matrix can be easily regenerated with minimal loss of binding capacity [138] and the elution can be sufficiently performed just with two column volumes of elution buffer which provides considerable concentration of purified protein (see Fig. 8) [139].

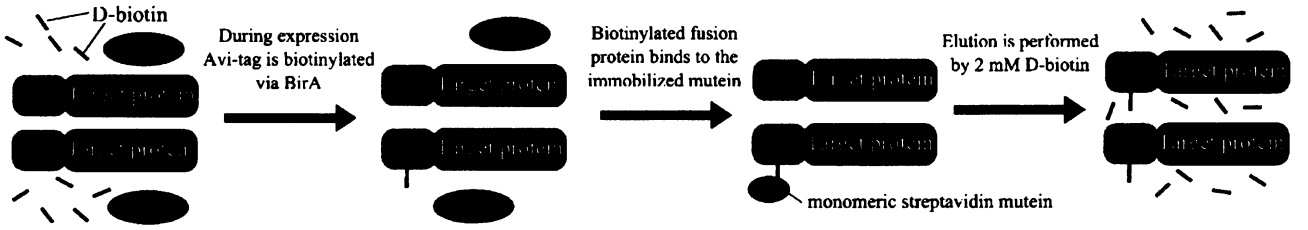


Figure 8: Schematic representation of Avi-tag purification system. For clarity the possible cleavage site for endoprotease is not included in the scheme.

If the fusion protein is supposed to be expressed in different expression host than in *E. coli*, an additional transfection with plasmid coding for *Bir*A has to be done. In that case various signal peptide sequences can be added to the *BirA* molecule in order to target the protein into a particular cellular compartment. Some of these sequences are diverse among different organisms (e.g. transport to the ER; 5-20mer peptide with majority of hydrophobic AA residues) while others are conserved (e.g. retention to the ER; Lys-Asp-Glu-Leu sequence at the C-terminus of the target protein called KDEL). The localization of *BirA* could be crucial for the final yield of Avi-tagged protein biotinylation. Even if it seems to be an additional work, expression of the target protein in the host that does not contain endogenous *BirA* ensures specific biotinylation of target protein and thus increases overall homogeneity of the subsequently obtained purified protein [140-143].

The biotinylated Avi-tagged protein can be utilized for various purposes. It is possible to simply and sensitively detect fusion protein via streptavidin conjugated with a visualisation marker. The protein can be also easily immunoprecipitated together with prospective protein

partner or immobilized to streptavidin coated chips [141,144]. Moreover, a recent report has shown that Avi-tag has a little effect on crystallization of its fusion partner [32].

Proteins fused with Avi-tag were successfully expressed in bacteria [142], baculovirus-infected insect cells [145], *Drosophila* S2 Schneider cells [143], and mammalian cells [140].

The necessity of prior biotinylation via *Bir*A represents a major drawback for this method. Since the biotinylation is never complete and non-biotinylated Avi-tagged protein is unable to bind to streptavidin mutein matrix, this non-biotinylated portion of fusion protein is lost even before the purification itself starts.

To conclude, this purification setup requires more labour for optimization but once the expression system is established it represents a very interesting tool for purification and handling of fusion proteins.

3.11.4 Solubility-enhancing tags

Peptides or proteins which are able to increase solubility of their fusion partners belong to this group. Many proteins have been shown to posses this feature. For lucidity, just several of them will be named and described. However, in theory, every protein which is able to increase the solubility of its fusion partner would belong to this group of tags.

Solubility-enhancing tags are needed almost exclusively for *E. coli* host expression system since mainly this system tends to produce proteins in the form of insoluble inclusion bodies. In these days, problems with solubility of heterologously expressed recombinant proteins seem to be the bottle neck of high-throughput systems utilized by structural genomic centres. Probably in response to that several papers describing and comparing features and abilities of different solubility-enhancing tags have been published [23,62,63,146].

3.11.4.1 MBP (Maltose-binding protein)

A vector that facilitates the expression and purification of fusion protein with MBP in *E. coli* was developed in 1988 [147]. MBP itself is a 40 kDa protein encoded by the *mal*E gene in *E. coli* [148]. Originally, the MBP system was supposed to be used for one-tag affinity purification. It binds specifically to cross-linked amylose and can be eluted by the 10 mM maltose under physiological conditions [71].

Both N-terminal and C-terminal MBP was successfully expressed in bacteria [149].

Currently, a highly utilized approach is to use combinatorial tagging and add some small peptide affinity tag (His-tag) to the MBP. In this kind of experimental setup MBP plays a role of solubility-enhancer and His-tag serves as an affinity purification tag [34]. Together with

utilizing His-tagged endoprotease (e.g. TEV protease), the pure target protein can be obtained in just two purification steps which makes this purification protocol very popular (see Fig. 9).

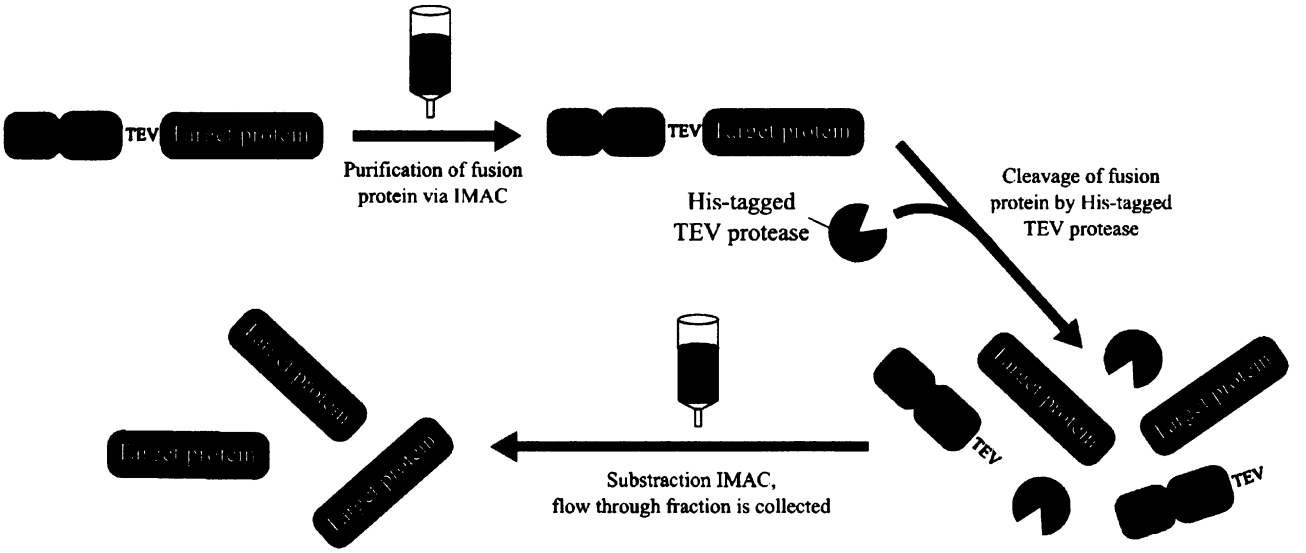


Figure 9: Schematic representation of combinatorial tagging, utilized His-tag and MBP. IMAC – immobilized metal-affinity chromatography.

On the other hand, it was published that MBP fusion could result in the protein degradation and its N-terminal localization in the reduction of the efficiency of translation [61]. Moreover, a 40 kDa protein such as MBP represents quite a high metabolic burden for host expression system during the large-scale expression.

3.11.4.2 NusA (N-utilization substance A)

NusA is a 55 kDa protein which serves as a pause-enhancement transcription factor in *E. coli* [150]. NusA has proven to be, together with MBP, one of the most potent solubility-enhancing protein discovered so far [151,152]. Beyond its natural good solubility there is one additional feature which might help NusA to increase solubility of its fusion partner. Its presence makes RNA-polymerase pause more often during transcription. Since in prokaryotes the transcription and translation are closely connected, the pausing of RNA-polymerase would provide more time for the translated protein to fold properly and thus would prevent its precipitation [151].

NusA does not work as an affinity tag. Therefore, combinatorial tagging approach has to be used to perform purification. Small tags such as His-tag or FLAG-tag are mostly chosen [153]. Similarly to MBP, NusA represents a high metabolic burden for expression host system.

3.11.4.3 SUMO (small ubiquitin-like modifier)

SUMO proteins are a family of small proteins (around 12 kDa) which are able to post-translationally modify diverse proteins within the eukaryotic cell and thus be involved in the various cellular processes, such as nuclear-cytosolic transport, transcriptional regulation, apoptosis, protein stability, response to stress, and progression through the cell cycle [154].

SUMO serves only as an expression-enhancing tag and thus it has to be also combined with other affinity tag in the same fashion as NusA (see 3.4.11.2). SUMO was shown to both increase solubility of its fusion partner and aid in its proper folding [146,153].

The use of SUMO is mostly constrained to *E. coli* since highly conserved SUMO proteases that may cleave off the fusion protein during its production are present in eukaryotes [8]. On the other hand, these specific proteases, such as yeast SUMO protease-1 Ulp1, can be used to cleave off almost whole SUMO tag after the purification [155]. Interestingly, the SUMO proteases do not recognize the primary sequence but the tertiary structure of SUMO which ensures almost zero possibility of unspecific cleavage [146].

3.11.4.4 SET-tag

SET-tag is an artificially created polypeptide tag, less than 40 amino acids long, which was described recently [2,23]. It also does not possess features to be utilized as an affinity tag and therefore has to be combined with another affinity tag (see 3.8.2).

In contrast to MBP and NusA, SET-tag increases the solubility of its fusion partner by enhancement of the electrostatic repulsions. The tag contains several acidic residues which prevent formation of aggregates during large-scale expression of fusion protein [23].

3.11.5 Detection tags

Monoclonal antibodies against the tags can be used for a simple detection of the fusion protein. Despite of that, under certain circumstances it can be convenient to add some other tag with a special visualisation feature. Combinatorial tagging approach is used for affinity purification of the corresponding fusion protein (see 3.8).

3.11.5.1 c-myc-tag

Myc codes for the protein which serves as a transcriptional factor and its mutation often leads to cancer development [156]. The first antibodies against c-myc protein were developed in 1985 (see Tab. 3, p. 32) [157]. c-myc tag is a 10 AA sequence derived from the C-terminus of the human c-myc protein which is specifically and strongly recognized by some of the raised

antibodies. In contrast to FLAG-tag (see 3.11.1.3), these antibodies are mainly used for the detection of c-myc-tagged fusion protein by Western-blotting, immunoprecipitation, and flow-cytometry methods [158]. Even if it is theoretically possible, myc-tag is not commonly used as an affinity tag.

The c-myc-tag can be expressed in different protein contexts and diverse expression systems but it is still efficiently recognized by the antibodies [159-163].

3.11.5.2 Rainbow tags

This name associates several proteins with one common feature - the simple and continuous visualisation of the tag protein by naked eye. This ability is tightly connected with the tertiary structure of the rainbow tag and since it is assumed that tag's and its fusion partner's tertiary structure mutually influence each other, the rainbow tag serves as an indicator of the proper folding of its fusion partner [35].

Rainbow tags are represented by proteins such as flavin mononucleotide (FMN)-binding domain of cytochrome P450 reductase (displaying a blue-green or yellow colour depending on the oxidation state of the FMN cofactor) or the red heme-binding cytochrome b5 [35,164].

3.11.5.3 Visualization tags

Visualization tags differ from the rainbow tags only in their requirement for an external energy source for visualisation. The typical representative of this group is green fluorescent protein (GFP) originally isolated from the jellyfish *Aequorea victoria* [36]. In recent years, many engineered forms of GFP or other fluorescent proteins with modified features, such as emission and/or absorption spectra or intensity of radiation, were developed [165,166].

3.11.5.4 S-tag

S-tag is a 15-mer peptide tag which strongly interacts with the S-protein (see Tab. 3, p. 32). Both S-tag and S-protein are derived from ribonuclease A (RNaseA, EC 3.1.27.5) [167]. The interaction between them depends on pH, temperature, ionic strength and leads to the restoration of ribonucleolytic activity of the complex [168]. This activity is eventually utilized for the detection of fusion protein. The discovery of a hypersensitive fluorogenic substrate for RNaseA has made this system an interesting tool for detection in combination with high-throughput screening [169].

It is also possible to use this system for affinity purification but since the elution conditions are really harsh (pH ~ 2) it is not recommended [71].

Table 3: List of the most used affinity tags and some of their characteristics. Standard abbreviations for AAs are used. Modified from [71].

Name	Residues	Sequence	Matrix	Elution conditions	Section
His-tag	2-10 (usually 6)	HHHHHH	Ni^{2+}-NTA, Co^{2+}-CMA (Talon)	20–250 mM imidazole or low pH < 5.0	3.11.1.1
Arg-tag	5-6 (usually 5)	RRRRR	cation-exchange resin	NaCl linear gradient from 0 to 400 mM at the alkaline pH > 8.0	3.11.1.2
FLAG-tag	8	DYKDDDDK	monoclonal antibody	2–5 mM EDTA or pH < 3.0	3.11.1.3
Softags	6-13	e.g. Softag1 SLAELLNAGLGGS	monoclonal antibody	nonchaotropic salt and a low molecular weight polyhydroxylated compound (polyol)	3.11.1.4
CBP	26	KRRWKKNFIAVS-AANRFKKISSSGAL	calmodulin	EGTA or EGTA with 1 M NaCl	3.11.1.5
CBD	27–189	domain	cellulose	family I: guanidine-HCl or urea > 4 M family II/III: ethylene glycol	3.11.2.1
GST	271	protein	glutathione	5–10 mM reduced glutathione	3.11.2.2
ChBD	51	domain	chitin	fused with intein: 30–50 mM dithiothreitol, 2-mercaptoethanol or cysteine	3.11.2.3
ELP	18-320	polypeptide	none	adjusting precise temperature	3.11.2.4
PHB-binding-tag	app. 20 kDa	protein	PHB granules	fused with intein: 30–50 mM dithiothreitol, 2-mercaptoethanol or cysteine	3.11.2.5
Strep-tag II	8	WSHPQFEK	Strep-Tactin (modified streptavidin)	2.5 mM desthiobiotin	3.11.3.1
Nano-tag	9-15 (usually 15)	DVEAWLGER(VPLVET)	Strep-Tactin (modified streptavidin)	2.5 mM desthiobiotin	3.11.3.2
Avi-tag	15	GLNDIFEAQKIEWHE	streptavidin monomer mutein	2 mM D-biotin	3.11.3.3
MBP-tag	396	protein	cross-linked amylose	10 mM maltose	3.11.4.1
c-myc-tag	10	EQKLISEEDL	monoclonal antibody	Low pH	3.11.5.1
S-tag	15	KETAAAKFERQHMDS	S-fragment of RNaseA	3 M guanidine thiocyanate, 0.2 M citrate pH 2.0, 3 M magnesium chloride	3.11.5.4

Table 4: Advantages and disadvantages of some commonly used tags. Modified from [2]. The table continues on page 34.

Name	Advantages	Disadvantages
His-tag	Low metabolic burden	Moderate specificity of an affinity resin
	Inexpensive affinity resin	Unsuitable for metalloenzymes
	Work in both native and denaturing setups	
	Detection via monoclonal antibody	
Arg-tag	Low metabolic burden	Low specificity of an affinity resin
	Inexpensive affinity resin	Might influence tertiary structure
	Immobilizing in a directed orientation	
FLAG-tag	Low metabolic burden	Expensive affinity resin
	High specificity of an affinity resin	Unsuitable for metalloenzymes
	Detection via monoclonal antibody	
	Contain cleavage site within sequence	
Softags	Low metabolic burden	Expensive affinity resin
	Mild elution conditions	
	Detection via monoclonal antibody	
CBP	Low metabolic burden	Expensive affinity resin
		Unsuitable for metalloenzymes
		Addressed for expression in prokaryotes
CBD	Inexpensive affinity resin	Harsh elution conditions
GST	Inexpensive affinity resin	High metabolic burden
	Mild elution conditions	Low specificity of an affinity resin
	Detection via monoclonal antibody	Homodimeric protein
ChBD	Inexpensive affinity resin	High metabolic burden
	Combination with self-cleavage intein	
ELP	Affinity resin is not necessary	Phase transition during purification
PHB-binding-tag	Affinity resin produced during expression	unknown
	Simple experimental setup	
Strep II-tag	Low metabolic burden	Expensive affinity resin
	Mild elution conditions	Might prevent formation of crystals
	High specificity of an affinity resin	
	Detection via modified streptavidin	
Nano-tag	Low metabolic burden	Expensive affinity resin
	Mild elution conditions	Addressed for expression in prokaryotes
	High specificity of an affinity resin	
	Detection via modified streptavidin	
Avi-tag	Low metabolic burden	Expensive affinity resin
	Mild elution conditions	Cotransfection by *E. coli Bir*A
	High specificity of an affinity resin	Variable efficiency of biotinylation
	Immobilizing in a directed orientation	
	Detection via modified streptavidin	
MBP	Inexpensive affinity resin	High metabolic burden
	Enhances solubility	Low specificity of an affinity resin
	Mild elution conditions	
	Detection via monoclonal antibody	

NusA	Enhances solubility	High metabolic burden
	Pause-enhancement transcription factor	Not an affinity tag
SUMO	Enhances solubility	Moderate metabolic burden
	Aid proper folding of fusion protein	Not an affinity tag
	Specific SUMO proteases are available	Addressed for expression in prokaryotes
SET-tag	Enhances solubility	Might influence tertiary structure
	Low metabolic burden	
myc-tag	Enables detection via antibody	Expensive affinity resin
		Harsh elution conditions
rainbow tags	Continuous visualisation by naked eye	High metabolic burden
	Checking of target protein's proper folding	Not an affinity tag
visualisation	Visualisation via external energy source	High metabolic burden
tags	Checking of target protein's proper folding	Not an affinity tag
S-tag	Detection via colorimetric assay	Expensive affinity resin
		Harsh elution conditions

3.13 Affinity purification put into practice

The aim of this thesis was to establish an efficient affinity purification protocol for the recombinant proteins using one of the tags described above. As target proteins, human recombinant protein GCPII and its paralog NAALADaseL were selected. In the following pages, the current knowledge about these proteins is reviewed briefly.

3.13.1 Glutamate carboxypeptidase II (GCPII)

The gene for glutamate carboxypeptidase II (EC 3.4.17.21) spans over 62 kbp region of 11p11.2 in the human genome [170]. In that region 18 exons are present and translated into a 750 AAs long protein.

GCPII is a type II transmembrane protein which belongs to the M28 di-zinc metallopeptidase family on the basis of sequence homology to the aminopeptidases of this family. It consists of a short N-terminal intracellular part (AAs 1-19), single transmembrane segment (AAs 20-43) and bulky extracellular region (AAs 44-750) [171]. It was shown that just the extracellular part of the protein (designed EXST) is sufficient for proper folding and enzymatic activity of the peptidase [172]. There are 10 potential N-glycosylation sites within the extracellular part of GCPII which are necessary for correct protein activity [173]. Since GCPII requires complex post-translational modifications, eukaryotic expression system for large-scale expression of EXST had to be established and subsequently purification protocol for EXST was also worked out [30].

Very pure EXST was obtained via this purification protocol and consequently the crystal structure of extracellular part of GCPII was solved [171]. Three domains, each involved in formation of the enzyme's active site, were identified in the structure. Additionally, EXST crystallizes as a symmetric homodimer with the dimer interface of total 2457 $Å^2$. This information supports a previously published data which indicated that GCPII forms dimer in solution [174].

3.13.1.1 Enzymatic activity of GCPII

Until now, two endogenous substrates for GCPII have been described, N-acetyl-L-aspartyl-L-glutamate (NAAG) and folylpoly-γ-glutamate [175,176]. The first compound is the most abundant peptide neurotransmitter in our nervous system and GCPII is involved in its inactivation (hydrolytic cleavage) within the neural synapses [178]. The latter compound is a precursor of folic acid (vitamin B_9) and GCPII enables its absorption through brush border membrane of small intestine [177]. In both reactions, GCPII cleaves off C-terminal L-glutamate (see Fig. 10). Biochemical characterization of NAAG cleavage by EXST was performed and kinetic parameters were obtained [30].

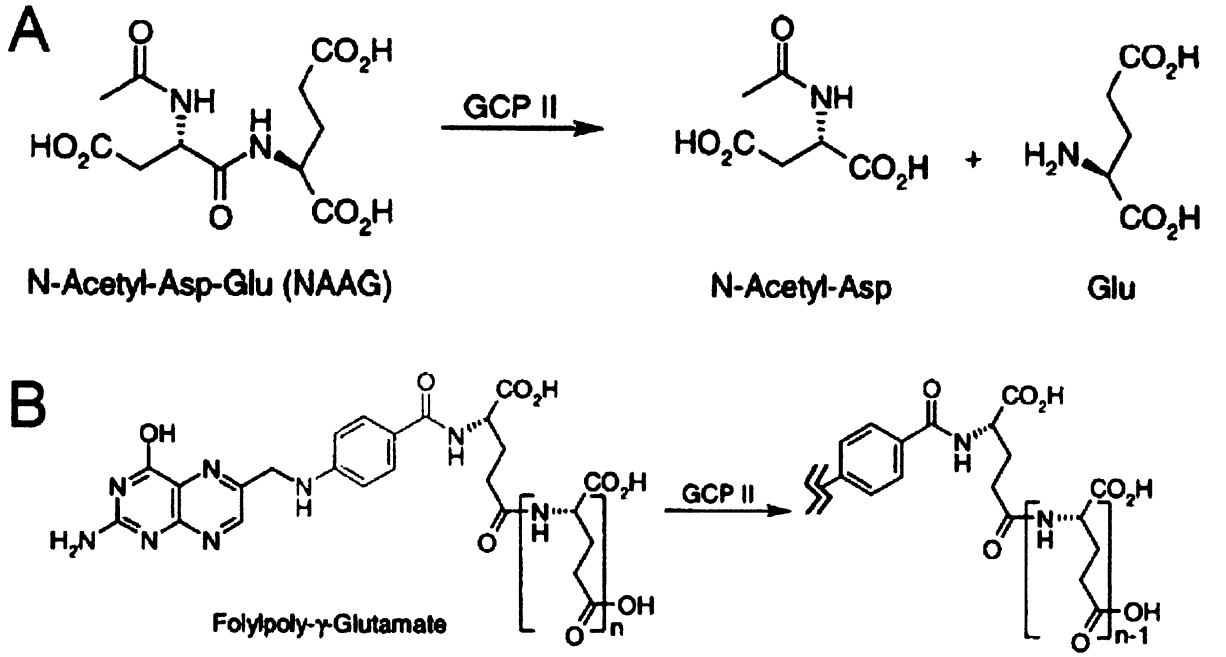


Figure 10: Hydrolysis of the natural substrates of GCPII. Panel A: N-acetyl-aspartyl (NAA) and neurotransmitter glutamate (Glu) are produced during hydrolysis of N-acetyl-aspartyl-glutamate (NAAG). **Panel B:** Cleavage of folylpoly-γ-glutamate by GCPII.

3.13.1.2 Biological importance of GCPII

Glutamate carboxypeptidase II is a widely studied peptidase. It seems to participate in several important biological processes which are not directly interconnected. That indicates that GCPII may behave as a multi-functional protein [179].

One of the well documented functions of GCPII is the inactivation of NAAG neurotransmitter in the brain. Specific inhibition of GCPII activity (preserving cleavage of NAAG) leads to the neuroprotective effect in both the acute and chronic neurological disorders [180,181]. Attempts have been made to describe the complex mechanisms of NAAG neuroprotective effect in these neurological disorders [182].

As mentioned previously, GCPII may play an important role during the uptake of dietary folate [Halsted].

Finally, GCPII is used as a diagnostic and prognostic marker for prostate cancer [183]. In 1999, a radiographic test that used murine antibody against GCPII (7E11-C5.3) linked to ^{111}indium was approved by US Food and Drug Administration [184]. The antibody bears commercial mark ProstaScint. Additionally, radioimmunotherapy utilization of GCPII has also been investigated. Monoclonal antibody J591 stably bound to β-emitting radioisotope ^{111}indium is mostly used. Phase I radioimmunotherapy trials in patients with progression hormone-independent prostate cancer have begun recently [185].

3.13.2 N-acetylated-α-linked-acidic-dipeptidase-like protein (NAALADaseL)

NAALADaseL was first isolated from rat ileum by Shneider *et al.* in 1997 [186]. This protein, originally called I100, was shown to be type II transmembrane protein consisting of 764 AAs and comprising 10 potential N-glycosylation sites. Due to its close homology to the rat NAAG-peptidase (rat ortholog of human GCPII which is also known as NAALADase I), the protein was renamed as NAALADase-like protein [186]. Two years later, the cDNA of human NAALADaseL was obtained [187]. Gene coding NAALADaseL was located onto the 11 chromosome, at 11q12 arm, spreading over 14 kbp.

Human NAALADaseL is a 740 AAs long protein which shares 35% identity and 54% similarity to GCPII. On the basis of the sequence homology, NAALADaseL has been assigned to the M28 family of metallopeptidases. In comparison to GCPII, the residues which participate in the coordination of zinc ions and also the potentially catalytic glutamate residue are conserved in NAALADaseL but it substantially differs in the amino acids involved in the substrate binding [188]. That could indicate possible hydrolytic activity of NAALADaseL with different specificity than GCPII. Similarly to GCPII, NAALADaseL is predicted to

consist of three major domains; short intracellular N-terminal domain (AAs 1-5), single transmembrane segment (AAs 6-27) and large extracellular part (AAs 28-740). 7 potential N-glycosylation sites can be found within the extracellular region of the protein [187].

3.13.1.2 Enzymatic activity of NAALADaseL

Though it has not been confirmed it is assumed that just the extracellular part of the protein (designed EXSTL) will be sufficient for proper folding and possible enzymatic activity of the protein. Prospective enzymatic activity of mouse and rat NAALADaseL has been analyzed. The NAAG-hydrolyzing activity of NAALADaseL seems to be unlikely but DPPIV activity has been observed in both rat and mouse NAALADaseL [186,187]. Until now, no study investigating enzymatic activity of human NAALADaseL has been published.

3.13.1.3 Biological importance of NAALADaseL

The NAALADaseL expression profile on the protein level in human or other species has not been described yet. Without that and a proper biochemical characterization of NAALADaseL it is difficult to assume the physiological role of this protein.

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals, enzymes and other material

Avidity, Colorado (USA)

pBirAcm

Bio-Rad, Hercules (USA)

agarose, protein assay dye reagent

Biotum, California (USA)

GelRed

Gibco, California (USA)

SF900II media, 10% fetal bovine serum (FBS), 100x Defined Lipid Concentrate, 50x Yeastolate Ultrafiltrate

Finnzymes, Espoo (Finland)

Phusion DNA polymerase, *Phusion* DNA polymerase buffer

Hampton Research, California (USA)

50% (v/v) pentaerythritol propoxylate PO/OH 5/4

Hirschmann Gerätte, Eberstadt (Germany)

Pasteur pipettes

Invitrogen, California (USA)

TOPO TA Cloning Kit, TOP 10 cells, *Drosophila* S2 Schneider cells, pMTBip/V5/His A

Koh-i-noor Hardmuth, České Budějovice (CZ)

transparent 96-well microplate with flat bottom

Lach-Ner, Neratovice (CZ)

hydrochloric acid, sodium acetate, sodium carbonate, sodium hydroxide, sodium thiosulphate, sodium chloride, calcium chloride, silver nitrate, ammonium sulphate

Millipore, Massachusetts (USA)

0.5 ml Microcon Centrifugal Filter Unit (10 kDa c/o)

New England BioLabs (NEB), Ipswich (USA)

restriction endonucleases (*BclI, BglII, MscI, KpnI, XhoI*), NEBuffer 2, NEBuffer 3, BSA, T4-DNA ligase, T4 DNA-ligase buffer

Nextal Biotechnologies, Montreal (Canada)

crystallization plate

P-Lab, Prague (CZ)

black 96-well polypropylene microplate with flat bottom

Penta, Prague (CZ)

MOPS, methanol, acetic acid, acetone, formaldehyde, isopropanol, ethyleneglycol, ethanol, formic acid

Pierce, Illinois (USA)

D-biotin, Neu-HRP (1 mg/ml), Casein Blocker, SuperSignal West Dura/Pico Chemoluminescence substrate, anti-mouse-IgG antibody conjugated with HRP (0.8 mg/ml), plastic gravity-flow disposable columns

Promega, Madison (USA)

Pfu DNA polymerase, *Pfu* DNA buffer

Qiagen, California (USA)

QIAprep spin Miniprep Kit, QIAquick Gel extraction kit, 50bp ladder DNA marker, X-gal

ROCHE s.r.o., Prague (CZ)

Streptavidin Mutein Matrix

ROTH, Karlsruhe (Germany)

ROTISZINT eco cocktail

Sartorius Stedim Biotech S.A., Aubagne (France)

Vivaspin 6 (centricon unit for 2-6 ml of a sample)

Serva, Heidelberg (Germany)

Coomassie Brilliant Blue G250, bromphenol blue, BSA

Sigma-Aldrich, Buchs (Switzerland)

EDTA, PEG 3350, SDS, LB Broth, LB Agar, 2-mercaptoethanol, glycerol, glycine, Tris, acrylamide, RNaseA, TEMED, N,N'-bisacrylamide, Gly-Pro-AMC, boric acid, sucrose

Top-Bio, Prague (CZ)

PPP Master Mix, dNTP's, Coloured DNA marker 155-970

4.1.2 Instruments

MLS-3020U autoclave, Sanyo (Japan)

Innova 4300 rotate incubator, New Brunswick Scientific (Germany)

centrifuges: Megafuge 2.0R, Heraeus Instruments (Germany)
Biofuge pico, Heraeus Instrument (Germany)
Eppendorf 5414R, Eppendorf (Germany)

Biometra Tgradient Thermal Cycler, Labrepco (USA)

UV Transilluminator, UltraLum (USA)

Horizontal electrophoresis apparatus, Gibco (USA)

ThermoCellMixing block MB-102, Bioer (Germany)

Optima digital thermostat GD-100 P12, Grant (UK)

Bright-line hemacytometer-cell counting chamber, Sigma-Aldrich (USA)

Eppendorf 2510 electroporator, Eppendorf (Germany)

Soniprep 150 sonicator, Sanyo (USA)

KS 260 basic shaker, IKA (Germany)

Grant-Boekel BFR25 Platform Rocker, Grant (UK)

Unicam 9450 pH meter, Unicam (UK)

PowerPac HC power supply, Bio-Rad (USA)

Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad (USA)

Vertical Polyacrylamid Gel Eletrophoresis, Sigma-Aldrich (USA)

CCD camera LAS 3000, Fujifilm Corporation (Japan)

CanoScan 8400F scanner, Canon (Japan)

NanoDrop ND-1000 spectrometer, Thermo Scientific (USA)

Aminco-Bowman Series 2 Luminescence Spectrometer, Thermo Electro Corporation (USA)

GENios microplate reader, Tecan (Schwitzerland)

Tri-Carb 2900TR liquid scintillation analyzer, PerkinElmer (USA)

Nikon SMZ-660 Stereo Microscope, Nikon (Japan)

Nikon TMS Inverted Phase Contrast Microscope, Nikon (Japan)

Nikon CoolPIX 995 camera, Nikon (Japan)

Olympus SP500uz camera, Olympus (Japan)

Mar345 Image Plate System, Rayonix (Germany)

Nonius FR591 Rotating Anode Generator, Enraf Nonius (Netherland)

4.2 Methods

4.2.1 DNA manipulations

4.2.1.1 PCR (polymerase chain reaction)

All reaction components except *Phusion* polymerase (Finnzymes) were pippeted into the PCR eppendorf tube. The eppendorf tube was placed into the Biometra Tgradient thermal cycler (Labrepco) and the program was started. After increasing temperature above 95°C, for *Phusion* polymerase, or above 92°C, for *Pfu* polymerase, the corresponding polymerase was added into the reaction mixture and the PCR was performed. The composition of each PCR reaction together with times and temperatures of reaction cycles are shown in Table 5 (p. 41). For sequence of individual primers see Table 13 (p. 81).

Table 5: Components of individual PCR reaction. 1U is defined as the amount of enzyme that will incorporates 10 nmol of dNTPs into acid-precipitable material in total reaction volume of 50 µl in 30 min at 37°C.

reaction	1	2
template DNA	1 µl pMTEXSTL (plasmid, 7.6 ng/ul)	5 µl pBirAcm (plasmid)
primers (20 µM)	0.5 µl FNAL28 0.5 µl RNAL740ST	1 µl FBirABglII 1 µl RBirAXhoIXbaI
DNA polymerase	0.5 U *Phusion* polymerase	0.67 U *Pfu* polymerase
buffer	6 µl *Phusion* buffer	5 µl *Pfu* buffer
dNTPs (10 mM)	1 µl	1 µl
distilled water	21 µl	36 µl
cycles	98°C 30 sec; 98°C 10 sec; 65°C 20 sec; 72°C 40 sec; 72°C 5 min; 4°C ---; 30	94°C 30 sec; 94°C 30 sec; 50°C 1 min; 72°C 2 min; 72°C 5 min; 4°C ---; 30

reaction	3	4
template DNA	5 µl pBirAcm (plasmid)	5 µl pBirAcm (plasmid)
primers (20 µM)	1 µl FBirABglII 1 µl RBirANoKDEL	1 µl FBirAKpnI 1 µl RBirANoKDEL
DNA polymerase	0.67 U *Pfu* polymerase	0.67 U *Pfu* polymerase
buffer	5 µl *Pfu* buffer	5 µl *Pfu* buffer
dNTPs (10 mM)	1 µl	1 µl
distilled water	36 µl	36 µl
cycles	same as reaction 2	same as reaction 2

4.2.1.2 „Gene fusion" PCR

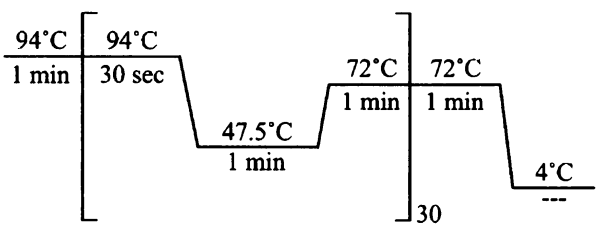
This experimental setup did not require the presence of template double-stranded DNA sequence. Instead of that, six primers (Avi0F, Avi1R, Avi2F, Avi3R, Uni3F and Uni4R) were designed in the fashion to form the amplified DNA sequence in the solution. For sequence of individual primers see Table 13 (p. 81). Subsequently, standard PCR reaction was performed with this primers using following conditions.

Components : 2 µl of each primer (10 µM) Avi0, Avi1, Avi2, Avi3, Uni3, Uni4

1.5 µl *Pfu* DNA polymerase, 3 µl *Pfu* DNA polymerase buffer, 1 µl dNTPs (10 mM)

distilled water to the final volume of 30 µl

Cycles:



4.2.1.3 DNA digestion with restriction endonucleases

DNA molecules (plasmids, PCR products) were digested with appropriate restriction endonucleases (NEB) under conditions recommended by the manufacturer. The DNA concentration, reaction volume, temperature and time of digestion depended on the nature of the experiment. The exact information about each reaction is given in Table 6.

Table 6: Components of individual restriction reaction. 1 U is defined as the amount of enzyme required to digest 1 μg of λ DNA in 1 hour at 37°C in a total reaction volume of 50 μl.

reaction	**1**	**2**	**3**
digested DNA	20.5 ul Avi-tag (PCR, reaction 1)	1 ul pMTBipEXST (plasmid, 8 mg/ml)	2 ul pMTBipAviEXST (plasmid, ~0,3 mg/ml)
restriction endonucleases	15 U *BclI* 10 U *BglII*	20 U *BglII* -	5 U *BglII* 4.5 U *MscI*
buffer	2.5 μl NEB2	2 μl NEB2	2 μl NEB3
distilled water	-	15 μl	14 μl
time and temperature	for 1 hour at 37°C	for 1 hour at 37°C	for 1 hour at 37°C

reaction	**4**	**5**	**6**
digested DNA	15 μl pCRII-TOPOEXSTL (plasmid, 0.45 mg/ml)	1 μl pMTBipAviEXST (plasmid, 5.6 mg/ml)	1 μl pMTBipAviEXSTL (plasmid, 0.55 mg/ml)
restriction endonucleases	30 U *BclI* 30 U *XhoI*	30 U *BglII* 30 U *XhoI*	3 U *MscI* 4 U *KpnI*
buffer	3 μl NEB3	3 μl NEB3	3 μl NEB3
BSA	0.3 μl BSA	0.3 μl BSA	0.3 μl BSA
distilled water	8 μl	21 μl	25 μl
time and temperature	overnight at 37°C	overnight at 37°C	overnight at 37°C

reaction	**7**	**8**	**9**
digested DNA	40 μl BirAKDEL (PCR, reaction 2)	2 μl pMTBip/V5/His A (plasmid)	15 μl BirA (PCR, reaction 3)
restriction endonucleases	10 U *BglII* 20 U *XhoI*	5 U *BglII* 10 U *XhoI*	10 U *KpnI* 20 U *XhoI*
buffer	5 μl NEB3	5 μl NEB3	5 ul NEB1
BSA	0.5 μl BSA	0.5 μl BSA	0.5 μl BSA
distilled water	2.5 μl	42 μl	27.5 μl
time and temperature	overnight at 37°C	overnight at 37°C	overnight at 37°C

reaction	**10**	**11**
digested DNA	15 ul BirA (PCR, reaction 4)	2 ul pMTBip/V5/His A (plasmid)
restriction endonucleases	10 U *BglII* 20 U *XhoI*	5 U *KpnI* 10 U *XhoI*
buffer	5 μl NEB3	5 μl NEB1
BSA	0.5 μl BSA	0.5 μl BSA
distilled water	27.5 μl	42 μl
time and temperature	overnight at 37°C	overnight at 37°C

Digested plasmids were subsequently dephosphorylated using Antarctic phosphatase (NEB). Into a restriction digest reaction of a total volume of 20 μl, 0.5 μl of Antarctic phosphatase was added. The mixture was incubated in 37°C for 10 min.

Digestion reactions were then loaded on an agarose gel and gel purified (see 4.2.1.4).

4.2.1.4 Agarose gel electrophoresis

10x TBE buffer : 890 mM Tris-HCl, 890 mM boric acid, 20 mM EDTA, pH 8.0

sample buffer (5x) : 40% (w/v) sucrose, 0.1% (w/v) bromphenol blue, 0.02% NaN_3

DNA molecules were analyzed using horizontal gel electrophoresis apparatus (Gibco) in agarose gel prepared in TBE buffer and supplemented with GelRed (stock solution diluted 1:10 000, Biotium). The percentage of agarose within the gel was chosen according to the nature of the experiment (from 0.8% to 2% (w/v)). To determine the relative molecular mass of tested DNA, DNA marker with appropriate size range was loaded on the agarose gel. DNA was resolved electrophoretically by 120 V for 20-30 min and visualized using UV transilluminator (UltraLum) at 302 nm.

If resoluted for preparative analysis, DNA was excised from the gel and subsequently isolated using the QIAquick Gel extraction kit (Qiagen) according to the manufacturer's protocol.

4.2.1.5 DNA ligation

The total volume of the ligation reaction was usually 10 µl. The ratio of plasmid:insert was set up on the basis of individual needs of each experiment (approximately 1:10) and the reaction was performed in a T4-ligase buffer with 1µl of T4-ligase (NEB). The mixture was incubated overnight at room temperature.

4.2.1.6 TOPO TA cloning

TOPO TA Cloning Kit (Invitrogen) containing plasmid pCRII-TOPO vector and TOP10 *E. coli* strain was used. This plasmid was already linearized and contained DNA topoisomerase I covalently attached via 3'-phosphate group to the thymidine which forms one-nucleobase cohesive ends of the plasmid. The molecule of insert DNA was specifically monoadenylated by treatment with PPP Master Mix (Top-Bio; 15 µl of the mix with 15 µl of DNA for 15 min at 72°C). After the mixing of the insert and vector DNAs the DNA topoisomerase I catalyzed the covalent connection of these two fragments and cleaved itself off. The whole procedure was performed according to the manufacturer's protocol.

4.2.1.7 Transformation of *E. coli* cells by heat shock

Competent DH5α *E. coli* cells were prepared using the calcium chloride protocol [189]. 50 µl suspensions of DH5α *E. coli* competent cells was mixed gently with 10 µl of the ligation reaction (see 4.2.1.4) or with 0.5-1 µl of plasmid DNA and incubated on ice for 30 min.

Subsequently, a heat shock of the mixture was performed in 42°C for 90 sec followed by 60 sec incubation on ice. 500 µl of sterile LB media (Sigma; prepared using distilled water and autoclaved) was then added to the cells and the mixture was incubated in 37°C for 60 min before spreading 30-200 µl of the mixture on agar plates pre-incubated in 37°C.

The agar plates were prepared using 15 ml of LB agar (Sigma) and appropriate antibiotics (ampicillin at a final concentration of 100 µg/ml). If the transformed plasmid enabled the Blue-White screening test, 40 µl of X-gal (40 µg/ml dissolved in dimethylformamide; Qiagen) was applied to the agar plates. The plates coated with the transformed cells were incubated overnight in 37°C.

4.2.1.8 Transformation of *E. coli* cells by electroporation

Competent ER2925 *E. coli* cells were thawed on ice. 40 µl of thawed cells were pipetted into the ice-cold electroporation cell and then 1 µl of plasmid DNA was added. Then the surface of the cell was wiped and the cell was placed into the electroporator (Eppendorf). One pulse (1250 V, 8 msec) was applied. Immediately afterwards, 100 µl of ice-cold sterile LB media (Sigma, St. Louis, USA; prepared using distilled water and autoclaved) was added to the cells and the whole mixture was kept at the room temperature for 10 min. Subsequently, the mixture was spread onto agar plates pre-incubated at 37°C.

The agar plates were prepared using 15 ml of LB agar (Sigma) and an appropriate antibiotic (ampicillin at a final concentration of 100 µg/ml). The plates coated with the transformed cells were incubated overnight at 37°C.

4.2.1.9 Preparation of plasmid DNA via QIAprep spin Miniprep Kit

2-12 ml of LB media (Sigma, with ampicillin at a final concentration 100 µg/ml) was inoculated by 1 colony of transformed *E. coli* cells (see 4.2.1.5) and shaken 220 rpm in 37°C overnight. The cell culture was then centrifuged at 3350 g for 10 min and the pellet was obtained. The plasmid DNA within the pellet was subsequently isolated using QIAprep spin Miniprep Kit according to the manufacture's protocol (Qiagen).

4.2.1.10 Measurement of DNA concentration and purity

DNA obtained from minipreparation (see 4.2.1.7) was analyzed on the Nanodrop ND-1000 spectrophotometer (Thermo Scientific). 1 µl of a sample was put into the measuring cell and the absorbance was measured at 230, 260, and 280 nm. DNA concentration of measured sample was calculated from the absorbance at 260 nm together with two ratios indicating the

purity of the sample. 260/280 ratio should have ranged around 1.8 and 260/230 ratio should be between 1.8 and 2.2 for pure double-stranded DNA.

4.2.1.11 DNA sequencing

Firstly, the sequencing mixture was prepared. It consisted of 0.33 µl of corresponding primer (10 µM), x µg of sequenced plasmid (x was counted as size of plasmid in kbp multiplied by 0.1) and water, added to a final volume of 14 µl.

Just MT primer was used for sequencing plasmid pMTBipAviEXST and primers MT, LSecNA1, LSecNA2, and LSecNA3 were used for sequencing plasmid pMTBipAviEXSTL. The vectors coding for differently localized BirA protein were sequenced by the MT and BGH primers which border the multiple cloning site of the pMTBip/V5/His 5 plasmid. For sequences of individual primers see Table 13 (p. 81).

Such prepared mixtures were sent to sequencing facility at the Charles University, Faculty of Natural Sciences, Department of Biology.

4.2.2 Recombinant protein expression in insect cells

4.2.2.1 Stable transfection of *Drosophila* S2 Schneider cells using calcium phosphate

Solution A: 36 µl of 2 M $CaCl_2$, 9 µg of recombinant DNA, 0.5 µg of pCoBLAST, tissue culture sterile water to the final volume of 300 µl

Solution B: 300 µl of 2x HEPES-Buffered Saline (HBS)

2x HBS: 50 mM HEPES, 1.5 mM Na_2HPO_4, 280 mM NaCl, pH 7.14

Drosophila Schneider S2 cells (Invitrogen) were cultured in a 35 mm plate in SF900II media (Gibco) supplemented with 10% new fetal bovine serum (Gibco) at 22-24°C until they reached a density of 2 to $4x10^6$ cells/ml. Solution A was added dropwise to Solution B with continuous vortexing and the final mixture was incubated at room temperature for 40 min. Subsequently, the solution was mixed and added dropwise to the cells. The cells were then incubated for 16-24 hours at 22-24°C. The transfected cells were then transferred into a 15 ml sterile falcon tube and centrifuged at 500 g for 2 min. The media was aspired, 3 ml of fresh SF900II media with 10% new fetal bovine serum (complete media) were added and the cells were replaced into the new plate. After one-day incubation at 22-24°C, the media was carefully aspired and 3 ml of fresh complete media containing either blasticidin (final concentration 5 µg/ml) or hygromycin (final concentration 300 µg/ml) were added. The

selective media was replaced every 4-5 days until resistant cells started growing out (usually 3-4 weeks).

4.2.2.2 Trypan Blue exclusion assay

Trypan Blue: 0.4% solution of Trypan Blue in PBS, pH 7.4, filtered through 0.22 μm filter

PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4

Trypan Blue Exclusion Assay is used for distinguishing between living and dead cells. Dead cells take up the Trypan Blue dye and stain blue, while living cells are capable of excluding the dye and stay transparent.

Typically, 20 μl of the cell suspension were mixed with 880 μl of PBS and 100 μl of the Trypan Blue solution were added. The mixture was left to stand for 5 min at room temperature then mixed thoroughly and 13 μl of the solution were pipetted under a hemacytometer (Sigma) cover-slip. The cells were observed under a contrast microscope (Nikon) at 100x magnification. The numbers of viable as well as dead cells were counted in 15 min from adding the dye and the cell concentration was calculated.

4.2.2.3 Influences on the *in vivo* biotinylation of Avi-tagged recombinant proteins

The plasmid pMTBipAviEXST was used for preparation of stable transfectant of *Drosophila* S2 Schneider cells (see 4.2.2.1). Afterwards, the transfectant was transfect with one of the three additional plasmids coding for biotin ligase (*BirA*; see section 5.1.3) with different signal sequences. The clones were let grown for 10 days in a 66 mm plate in SF900II media. Subsequently, the number of living cells was determined by Trypan blue exclusion assay (see 4.2.2.2). Each clone of transfectant cells was distributed into a 6-well plate to the final amount of cells $7x10^6$ in each well. D-biotin was added into each well to cover the concentration range from 0 to 160 μg/ml.

The cells were incubated for three days and afterwards the production of transfected protein was induced by addition of 100 mM $CuSO_4$ (final concentration 1 mM). The cells were harvested after three more days, centrifuged at 500 g for 2 min and the cell media was analyzed for the presence of biotinylated and non-biotinylated version of AviEXST.

4.2.2.4 Large-scale production of recombinant protein (AviEXST or AviEXSTL)

A 250 ml spinner flask was filled with 100 ml SF900II media and cells were seeded at approximately $2x10^6$ cells/ml. 1 ml of 100x Defined Lipid Concentrate (Gibco) and 2 ml of 50x Yeastolate Ultrafiltrate (Gibco) were added per 100 ml of the media. The spinner was

incubated at 22-24°C with a constant stirring rate of 90-125 rpm. The side arms were loosed approximately a quarter (1/4) turn.

When the cell density reached 10^7 cells/ml (2 to 4 days) the cell suspension was transferred into 3-liter spinner flask, 400 ml of SF900II media was added together with 4 ml of 100x Defined Lipid Concentrate (Gibco) and 8 ml of 50x Yeastolate Ultrafiltrate (Gibco). The spinner was incubated at the same conditions as above. When the cells reached density 10^7 cells/ml, they were induced with 100 mM $CuSO_4$ (final concentration 1 mM). On days 1 and 3 post-induction the media was supplemented with 5 ml of 200 mM L-glutamine and 5 ml of 20% D-glucose in sterile water. The cell density, viability and the recombinant protein production was checked on a daily basis and the cells were harvested once there was a decrease in cell viability or when protein production reached a plateau (typically fifth day post-induction).

4.2.3 Purification of Avi-tagged protein on the Streptavidin Mutein Matrix

4.2.3.1 Small-scale purification via Avi-tag

Equilibration buffer:	450mM NaCl, 300mM Tris-HCl (pH 7.2)
Wash buffer:	150mM NaCl, 100mM Tris-HCl (pH 7.2)
Elution buffer:	150mM NaCl, 2mM D-biotin, 100mM Tris-HCl (pH 7.2)
Resin:	Streptavidin Mutein Matrix (ROCHE s.r.o)

0.4 ml of resin slurry (50% suspension in ethanol) was pipetted into 15 ml falcon tube and 1 ml of Wash buffer was added. Then the mixture was centrifuged at 470 g for 3 min at 4°C on Megafuge 2.0R (Heraeus Instruments), the supernatant was removed and substituted with another 1 ml of Wash buffer. This procedure was repeated 3 times. If the regenerated resin slurry was used, only one washing step was applied.

In the meantime, 9 ml of conditioned media from S2 Schneider cells was thawed and centrifuged at 3360 g for 10 min at 4°C on Megafuge 2.0R (Heraeus Instruments) to get rid of possible debris. Both resin and media were placed to the cold room and from now on all procedure steps were performed at 4°C.

9 ml of media was mixed, while stirring, with 4.5 ml of Equilibration buffer and after that the sample was taken to future analysis (Load). Afterwards, the 13.5 ml of the prepared solution was transferred into the 15 ml falcon tube with washed resin and the tube was left on the rocker overnight.

Next day, the plastic disposable column (Pierce) was prepared and washed once with 1 ml of Wash buffer. Afterwards, the conditioned media together with the resin beads was poured into the column and by gravity-flow the resin was separated from the media (Flow-through). Then 0.2 ml of Wash buffer was applied onto the column to wash out the rest of media (Wash1) and another 2 times 1 ml of Wash buffer to wash out the non-specifically bound proteins (Wash2 and 3).

After that, approximately 0.2 ml of Elution buffer was applied onto the column in a way to soak into the whole resin column but still not let the upper part of it dry (Elution1). After the soaking, the stopper was placed into the bottom of the column and it was allowed to stand in the Elution buffer for 1 hour. Subsequently, the stopper was removed and the bounded proteins were eluted, without waiting, applying 2 times 0.4 ml of Elution buffer (Elution2 and 3). Finally, another 0.8 ml of Elution buffer was applied onto the column to elute possible strongly bound proteins (Elution4).

Afterwards, as a part of regeneration of Streptavidin Mutein Matrix (see 4.2.3.3) several millilitres of Wash buffer were applied onto the column. During that procedure, 50 µl of resin suspension (in the Wash buffer) was taken and boiled with 10 µl of sample buffer(6x). Then the beads were spinned down and the supernatant was analyzed (Resin).

All samples were then analyzed on the SDS-PAGE (see 4.2.4.1)

4.2.3.2 Large-scale purification via Avi-tag

Equilibration buffer:	450mM NaCl, 300mM Tris-HCl (pH 7.2)
Wash buffer:	150mM NaCl, 100mM Tris-HCl (pH 7.2)
Elution buffer:	150mM NaCl, 2mM D-biotin, 100mM Tris-HCl (pH 7.2)
Resin:	Streptavidin Mutein Matrix (ROCHE s.r.o.)

250 ml of conditioned media from S2 Schneider cells was thawed, centrifuged at 3360 g for 10 min at 4°C on Megafuge 2.0R (Heraeus Instruments) and then filtered through 0.22 µl membrane (Millipore) to get rid of possible debris. Both resin and media were placed to the cold room and from now on all procedure steps were performed at 4°C.

In the meantime, 2 ml of regenerated resin slurry (50% suspension in Wash buffer) was pipetted into 15 ml falcon tube and 4 ml of Wash buffer was added. Then the mixture was centrifuged at 470 g for 3 min at 4°C on Megafuge 2.0R (Heraeus Instruments) and the supernatant was removed.

250 ml of media was mixed, while stirring, with 125 ml of Equilibration buffer and after that the sample was taken to future analysis (Load). Afterwards, the washed resin beads were quantitatively transferred from the falcon tube into the 375 ml of equilibrated S2 media. The flask with the media was let on the rocker overnight.

Next day, the flask was put on the table for several minutes to let the resin settle down. Meanwhile, the plastic disposable column (Pierce) was prepared and washed once with 1 ml of Wash buffer and then put on the end of 150 ml funnel.

Subsequently, the conditioned media was carefully poured onto the column without the resin beads (the column of resin beads significantly decrease the flow rate through the column). After the majority of media was flown through, the rest of the media together with the resin beads was applied onto the column. The flask itself was rinsed out with several millilitres of flow-through fraction and transferred again onto the column (Flow-through).

Then 1 ml of Wash buffer was applied onto the column to wash out the rest of media (Wash1) and another 2 times 4 ml of Wash buffer to wash out the non-specifically bound proteins (Wash2 and 3).

After that, approximately 1 ml of Elution buffer was applied onto the column in a way to soak into the whole resin column but still not let the upper part of it run dry (Elution1). After the soaking, the stopper was placed into the bottom of the column and it was let stand in the Elution buffer for 1 hour. Subsequently, the stopper was removed and the bound proteins were eluted, without waiting, by the 2 times 1 ml of Elution buffer (Elution2 and 3). Finally, another 2 times 2 ml of Elution buffer was applied into the column to elute possible strongly bound proteins (Elution4 and 5).

Afterwards, as a part of regeneration of Streptavidin Mutein Matrix (see 4.2.3.3) several millilitres of Wash buffer was applied onto the column. During that procedure, 50 µl of resin suspension (in the Wash buffer) was taken and boiled with 10 µl of sample buffer(6x). Then the beads were spinned down and the supernatant was analyzed (Resin).

All samples were then analyzed on the SDS-PAGE (see 4.2.4.1)

4.2.3.3 Regeneration of Streptavidin Mutein Matrix

Wash buffer:	150mM NaCl, 100mM Tris-HCl (pH 7.2)
Elution buffer:	150mM NaCl, 2mM D-biotin, 100mM Tris-HCl (pH 7.2)
Regeneration buffer:	100mM glycine-HCl (pH 2.8)
Storage buffer:	0.2% (w/v) sodium azide, 20mM MOPS pH (7.0)

Volumes of used solutions were dependent on the volume of the beads (column volume). The regeneration of resin was performed in the disposable column (Pierce).

One after another 5 cv (column volume) of Wash buffer, 5 cv of Elution buffer, 6 cv of Regeneration buffer and 5 cv of Wash buffer were applied onto the column and let flow-through by gravity flow. Afterwards, if the resin was not meant to be used soon, it was transferred into the Storage buffer.

The resin was stored and all regeneration procedures were performed at 4°C.

4.2.4 Protein manipulations

4.2.4.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Resolving gel (10% acrylamide):

2.5 ml 1.5 M Tris-HCl (pH 8.8), 2.3 ml 44% acrylamide mixture (42.8 g acrylamide, 1.2 g N,N'-bisacrylamide, water to the final volume of 100 ml), 100 µl 10% (w/v) SDS, 100 µl 10% (w/v) ammonium persulfate (APS), 10 µl TEMED, water to the final volume of 10 ml

Stacking gel (5% acrylamide):

1.25 ml 1 M Tris-HCl (pH 6.8), 0.75 ml 44% acrylamide mixture, 50 µl 10% SDS, 50 µl 10% APS, 10 µl TEMED, water to the final volume of 5 ml

Running buffer (5x):

15.1 g Tris, 94 g glycine, 5 g SDS, 900 ml water, pH 8.8 (pH not adjusted)

Sample buffer (6x):

3.5 ml 1 M Tris (pH 6.8), 3 ml glycerol, 1 g SDS, 600 µl 2-mercaptoethanol, 1.2 mg bromphenol blue, water to the final volume of 10 ml

Gels were prepared, let to polymerize and then placed into a vertical electrophoresis apparatus (Sigma). The upper and lower reservoirs of the apparatus were filled with the 1x running buffer. Prior to the loading onto gel, the samples were mixed with the sample buffer (6x) and boiled for 2-3 min. Electrophoresis was run at 150 V (PowerPac HC, Bio-Rad) until the bromphenol blue dye reached the bottom of the resolving gel. Proteins in the gel were then either visualized by silver staining (see 4.2.4.2) or subjected to Western blotting (see 4.2.4.3).

4.2.4.2 Silver staining of polyacrylamide gel

Solution 1: 12% (v/v) acetic acid, 50% (v/v) methanol, 0.5 ml 37% formaldehyde, water to the final volume of 1 litre

Solution 2: 50% methanol

Solution 3: $Na_2S_2O_3.5H_2O$ (0.2 g/l)

Solution 4: 2 g $AgNO_3$, 0.5 ml 37% formaldehyde, water to the final volume of 1 litre

Solution 5: 60 g Na_2CO_3, 4 mg $Na_2S_2O_3$, 0.5 ml 37% formaldehyde, water to the final volume of 1 litre

Solution 6: 12% (v/v) acetic acid, 50% (v/v) methanol

Proteins resolved by SDS-PAGE (see 3.2.4.1) were visualized by silver staining. After the electrophoresis, the gel was incubated for at least 30 min in Solution 1 and then washed three times in Solution 2 for 8 min. Subsequently, the gel was incubated in Solution 3 for 1 min, washed with water three times, incubated in Solution 4 for 20 min and again rinsed with water three times. The silver staining was developed by addition of Solution 5. When the protein bands were clearly visible, the gel was washed with water three times and the developing process was stopped by addition of Solution 6 for 10 min. The gel was then stored in Solution 2.

Stained gels were scanned on the scanner (Canon).

4.2.4.3 Western-blotting using chemoluminescence substrates

Blotting concentrate: 72.1 g glycine, 15.1 g Tris, water to the final volume of 500 ml

Blotting buffer: 10 ml blotting concentrate, 1 ml 10% (w/v) SDS, water to the final volume of 90 ml and afterwards add 10 ml methanol

After electrophoretic separation of proteins (see 4.2.4.1), the gel and a nitrocellulose membrane (7.5x5 cm) were equilibrated in the blotting buffer for 5 min. Two sheets of filter paper, pre-wetted in the blotting buffer, were placed onto the anode of the transfer apparatus and the nitrocellulose membrane was placed on the top. The gel was then placed on top of the membrane and covered with two pre-wetted sheets of filter paper. A test tube was rolled over the area of the stack to eliminate air bubbles. The transfer apparatus was then assembled and connected to power supply (Bio-Rad). Proteins were electroblotted at constant voltage of 15 V for 15 min. After electroblotting, the membrane was rinsed in PBS + 0.05% Tween 20 and membrane was blocked with 5 ml of Casein Blocker (Pierce) for at least 1 hour at 4°C with agitating.

Subsequently, for visualisation of AviEXST 1 µl of murine monoclonal antibody GCP-04 (1 mg/ml) in 5 ml of Casein Blocker was used [190]. If just a biotinylated fraction of AviEXST should have been visualised, 1 µl of Neutravidin-HRP (1 mg/ml) (Pierce) in 5 ml of Casein Blocker was utilized.

The membrane was incubated either with the solution of the primary antibody overnight or with Neutravidin-HRP for 1 hour at 4°C with agitating. Sequentially, the membrane was washed three times in PBS + 0.05% Tween 20 for 5 min with agitating.

For visualising murine monoclonal antibodies GCP-04, solution with 5 ml of Casein Blocker and 0.5 µl of goat anti-mouse antibody conjugated with HRP (0.8 mg/ml, Pierce) in 5 ml Casein Blocker was prepared and the membrane was incubated with it at 4°C for at least 1 hour with agitating. In the case of utilization Neutravidin-HRP, it was not necessary to use any secondary antibody. To remove the unbound secondary antibody, the blot was washed three times in PBS + 0.05% Tween 20 for 5 min with agitating.

0.4 ml of the Stable Peroxide Solution was mixed with 0.4 ml of the Luminol/Enhancer Solution (SuperSignal West Dura Chemoluminescence Substrate, Pierce) and the blot was incubated with this mixture for 5 min. The membrane was then dried between two sheets of filter paper and placed in transparent plastic foil. The chemoluminescence signal was detected using CCD camera LAS-3000 (Fujifilm).

4.2.4.4 Protein quantification from Western-blot

For protein quantification, the chemoluminescence signal intensities were recorded by CCD camera LAS-3000 (Fujifilm) and the image was analyzed using ImageQuant TL software (version 2003.02; Amersham Bioscience).

Concentration of GCPII in a sample was determined by comparing the signal intensity of the sample with calibration curve constructed using purified recombinant AviEXST or AviEXSTL of known concentration as a standard. Except precise concentrations, the total densities of samples and their mutual ratios were also examined.

4.2.4.5 Biotin-binding assay

100 µl of coating buffer (0.1 M carbonate buffer, pH 9.8) was pipetted into the 96-well black polystyrene microplate with flat bottom (P-LAB) and 1 µl of the sample diluted 20 times with the coating buffer. Then the microplate was let on the rocker overnight.

Next day, the samples were washed out from the well with 3 times 250 µl of PBS + 0.05% Tween 20. Afterwards, the wells were incubated for 1 hour at 4°C with 250 ul of Casein blocker (Pierce) to block the rest of the wells surface. Subsequently, the Casein blocker was substituted with the 150 µl of Neutravidin-HRP (1:2500) solution in Casein blocker. The plate was placed to the rocker at 4°C again. In 2 hours the solution was poured out and the wells were washed 3 times with 250 µl of PBS + Tween 20. After that, 200 ul of SuperSignal

West Pico Chemoluminescence Substrate (premixed from the Stable Peroxide Solution and the Luminol/Enhancer Solution) was added to each well. Immediately after substrate addition the luminescence was measured on the GENios microplate reader (Tecan). The gain of the reader was manually set up to 150 and integration time to 2 sec.

Every sample was measured in triplicates and standard deviations for each sample were calculated.

4.2.4.6 Bio-Rad Bradford protein assay on the 96-well microplates

The protein concentration in a sample was determined using 5xBio-Rad protein assay (Bio-Rad). The measurement was performed in the 96-well transparent microplate (Koh-i-noor). A bovine serum albumin (BSA) was used as an inner standard for the procedure. Every sample was measured in duplicates.

180 µl of 1xBio-Rad protein assay was transferred into each well. Subsequently, 20 µl of BSA solution was pipetted into the upper line of microplate to form calibration curve of 0, 0.25, 0.5, 1, 2, and 4 µg BSA. The samples were pipetted into the rest of the wells and then water was added to the wells to the final volume of 200 µl. The solution in each well was mixed rigorously by pipette tip after addition of a sample. Following five-minute incubation at room temperature, the absorbance was measured at 595 nm on the GENios microplate reader (Tecan).

4.2.4.7 Concentration of the AviEXST protein

Several elution fractions with AviEXST from optimizing experiments (approximately 2.5 ml) were mixed and concentrated. Firstly, a Vivaspin 6 centricon (Sartorius) was used to concentrate the sample to approximately 0.5 ml and simultaneously transfer the protein from elution buffer to the 20 mM NaCl, 20 mM MOPS, pH 7.4 solution. Afterwards, the solution was pipetted into the 0.5 ml centricon unit (Millipore) with 10 kDa cut-off and the solution was concentrated to approximately 25 µl. The protein concentration was measured by Bio-Rad protein assay (see 4.2.4.6) and the solution was used for crystallization (see 4.2.6.1).

4.2.4.7 Dialysis for kinetic characterization of AviEXST

50 ul of the Load, Flow-through and Elution2 fractions from both large-scale purification of AviEXST, 5 fractions together (see 5.4.1) were dialyzed against 2 litres of 20 mM NaCl, 20 mM Tris-HCl, pH 7.4. The dialyzing buffer was changed three times after 8 hours. The volume of each fraction after dialysis was determined.

4.2.5 Enzymatic activity assays

4.2.5.1 Radiometric measurement of NAAG-hydrolysing activity

Reaction buffer:	20 mM Tris-HCl, 20 mM NaCl, pH 7.4
Resin :	AG 1-X8 Resin, formate form (Bio-Rad)
^{3}H-NAAG :	50 nM ^{3}H-NAAG + 950 nM NAAG in Tris buffer, 50 Ci/mmol
Stopping solution:	200 mM Na_2HPO_4, 2 mM 2-mercaptoethanol, pH 7.4

The reaction buffer and a sample were brought to a final volume of 90 µl in an eppendorf tube. The mixture was incubated for 5 min at 37°C. To start the reaction, 10 µl of the ^{3}H-NAAG substrate was added to the solution and the reaction was incubated typically at 37°C for 20 min. The reaction was stopped by adding 100 µl of ice cold stopping solution.

The columns were prepared by adding a single glass bead (ø 3 mm) into a Pasteur pipette (Hirschmann) and by filling the columns with 2 ml of the resin. The resin was allowed to settle and then it was washed twice with 1.3 ml of sonicated distilled water.

180 µl of each assay sample was loaded onto the columns and the samples were allowed to drain. The columns were then put over 20 ml scintillation vials and glutamate was eluted two times with 1 ml of 1 M formic acid. 6 ml of the Rotiszint scintillation cocktail (ROTH) was added and each vial was shaken vigorously. The scintillation in DPM (disintegration per minute) units was measured at the Department of Nuclear Chemistry, IOCB Prague (Dr. Elbert) on the Tri-Carb 2900TR liquid scintillation analyzer (PerkinElmer).

For determination of K_M and k_{cat} values, series of reactions was performed with different substrate concentration ranging from 20 nM to 20 µM final concentration of NAAG. All reactions were done in duplicates.

4.2.5.2 Fluorimetric measurement of DPP IV activity

The reaction buffer (150 mM glycine, pH 8.5) and a sample were brought to a final volume of 147 µl in an Eppendorf tube. Then a substrate Gly-Pro-AMC (Sigma) was added into the reaction mixture to the 100 µM final concentration. Afterwards, the reaction was incubated for 4 hours at 37°C. Then 140 µl of the reaction mixture was transfer to the cuvette and the fluorescence was measured at the Aminco Bowman spectrometer (Thermo Electro Corporation) with 350nm/440nm excitation/emission wavelengths. The voltage of photomultiplier was set up manually along the positive control sample.

4.2.6 Crystallization trials

4.2.6.1 Hanging drop vapour diffusion crystallization

Crystallization solution:

33% (v/v) pentaerythritol propoxylate PO/OH 5/4, 1 % (w/w) PEG 3350, 100mM Tris, pH 8.0

AviEXST was crystallized in conditions used for EXST in past studies [191]. Three different additive setups were performed with AviEXST - with natural substrate (NAAG), with potent inhibitor (2-PMPA), and without any additive. Crystallization solution was mixed with 10 mM stock solution of NAAG or 2-PMPA to the final concentration 1 mM of these compounds.

1 µl of the stock solution of recombinant protein AviEXST (5.3 mg/ml) was pipetted onto a hanging-drop support. Equal volume of the prior prepared crystallization solution was then added to the droplet and sucked in and out to the pipette three times. Subsequently, the hanging-drop support was screwed to the crystallization plate's well filled with 1 ml of the crystallization solution.

Crystallization plates (Nextal Biotechnologies) were stored at 19°C and the hanging drops were observed under a Nikon SMZ-660 microscope (Nikon). The crystal photos were taken by the Nikon CoolPIX 995 camera (Nikon) in 2 weeks from the drops seeding.

4.2.6.2 Freezing of AviEXST crystals

Two crystals from each condition were fished out and immediately cryo-cooled in the liquid nitrogen (without soaking with additive cryoprotectant). The crystals were stored in the Dewar flask with liquid nitrogen until further measurement.

4.2.6.3 Measuring the diffraction pattern of AviEXST crystals

Diffraction data from cryo-cooled crystals were collected at 120 K on a Mar345 Image plate system (Rayonix) using a Nonius FR591 rotating anode generator (Enraf Nonius). 3 diffraction pictures from each crystal were taken and processed by HKL 3000 software [192]. The space group and unit-cell parameters were obtained and compared with the values from wild type EXST crystal.

5. RESULTS

Through the whole Result section the non-tagged extracellular portion of GCPII (AAs 44-750) will be denoted EXST and its Avi-tagged partner AviEXST. Similarly, the non-tagged extracellular portion of NAALADaseL (AAs 28-740) will be indicated as EXSTL and its Avi-tagged form AviEXSTL.

5.1 Plasmid DNA preparation for heterologous expression of *in vivo* biotinylated protein in insect cells

5.1.1 Preparation of Avi-tagged EXST (AviEXST)

Firstly, the double-stranded DNA coding for Avi-tag protein sequence (14 AA), spacer sequence (6 AA), and TEV protease restriction site sequence (9 AA), was prepared by „Gene fusion" PCR technique (see 4.2.1.2). The restriction sites *BclI* and *BglII* were introduced to the DNA sequence. The 112 bp product of PCR reaction was visualized on an agarose gel, excised and isolated (see 4.2.1.4). Subsequently, the DNA was treated with *BclI* and *BglII* restriction endonucleases (see 4.2.1.3, reaction 1) and gel purified (see 4.2.1.4).

Secondly, plasmid pMTBipEXST (EXST refers to extracellular part of GCP II; AA 44-750), which was prepared by Cyril Bařinka in our laboratory, was digested by *BglII* restriction endonuclease and dephosphorylated (see 4.2.1.3, reaction 2), resolved on an agarose gel and subsequently isolated from the gel (see 4.2.1.4).

Ligation was performed with these DNA fragments (see 4.2.1.5). Then competent *E. coli* cells (strain DH5α) were transformed by the ligation mixture (see 4.2.1.7) and the plasmid DNA from several obtained clones was isolated by minipreparation protocol (see 4.2.1.9).

Both *BclI* and *BglII* restriction sites are compatible with *BglII* site, but the ligation of *BclI* with *BglII* restriction site leads to the abolishment of that site. That fact was used during the testing of obtained plasmid DNAs. The DNA was digested with *MscI* and *BglII* restriction endonucleases to determine the correct orientation of the ligated insert DNA (see 4.2.1.3, reaction 3). Plasmid DNA of one positive clone was sequenced (see 4.2.1.11).

The schematic representation of DNA fragment coding for Avi-tag together with whole cloning procedure is depicted in Figure 11 (p. 57).

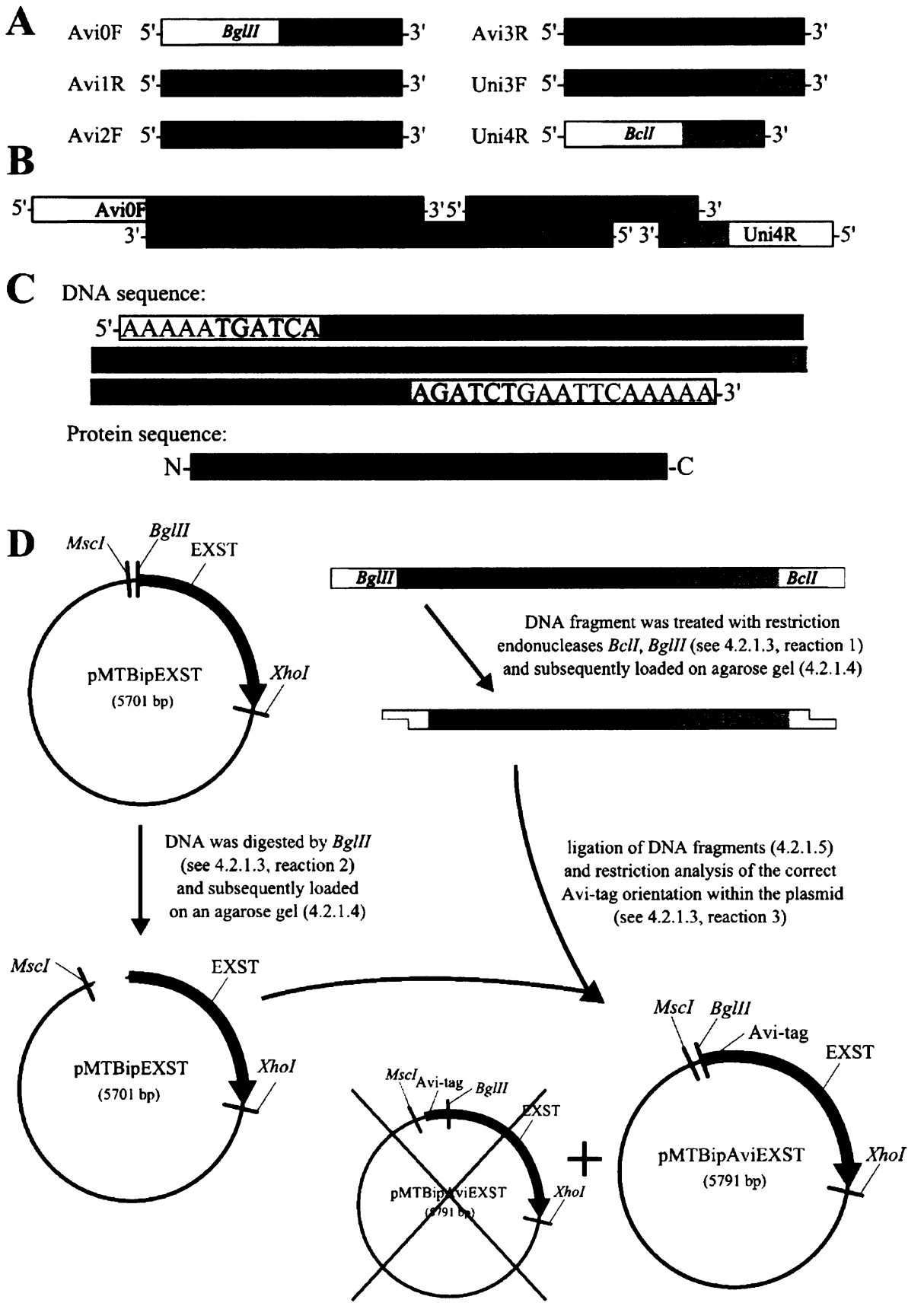


Figure 11: Schematic representation of construction of plasmid DNA coding for Avi-tagged GCPII. **Panel A:** The primers which were used for „Gene fusion" PCR and parts of the protein tags which they coded. **Panel B:** Mutual overlay of the primers. **Panel C:** DNA and protein sequence of the constructed Avi-tag. Standard one-letter abbreviations for nucleobases and AAs were used. The lysine residue within Avi-tag sequence which is specifically biotinylated by *BirA* is depicted in red. **Panel D:** Steps performed during preparation of plasmid coding for AviEXST. The false positive DNA plasmid of pMTBipAviEXST is crossed out. For the sequences of individual primers see Table 13 (p. 81).

5.1.2 Preparation of Avi-tagged EXSTL (AviEXSTL)

The plasmid DNA pMTBipEXSTL (EXSTL refers to extracellular part of NAALADaseL; AA 28-740), prepared by Pavel Šácha in our laboratory, was used as the template for PCR. The restriction sites were introduced upstream (*BclI*) and downstream (*XhoI*) of the amplified sequence (see 4.2.1.1, reaction 1). The restriction site *BglII* could not be used since it is present within the DNA sequence of EXSTL. Afterwards, the PCR product was treated with PPP Master Mix (Top-Bio) to add adenine to the 3-end of each DNA strand (see 4.2.1.6). The mixture was then resolved on an agarose gel and the band corresponding to EXSTL was excised and isolated (see 4.2.1.4).

The insert was ligated into the pCRII-Topo vector (see 4.2.1.6). The plasmid DNA was amplified by transformation of *E. coli* cells (strain DH5α) and a subsequent minipreparation was performed (see 4.2.1.7 and 4.2.1.9). Since *BclI* restriction site is sensitive to methylation, the final production of plasmid DNA was done in non-methylating *E. coli* strain ER2925 (see 4.2.1.8). Plasmid DNA was isolated from the positive clone (see 4.2.1.9) and sequenced (see 4.2.1.11). Subsequently, pCRII-TopoEXSTL plasmid was digested by *BclI* and *XhoI* restriction endonucleases (see 4.2.1.3, reaction 4), resolved on an agarose gel and the DNA band corresponding to EXSTL was gel purified (see 4.2.1.4).

In the meantime, plasmid pMTBipAviEXST was digested by *BglII* and *XhoI* restriction endonucleases (see 4.2.1.3, reaction 5), resolved on an agarose gel and the band corresponding to pMTBipAvi was isolated (see 4.2.1.4).

Afterwards the ligation was performed with these two DNA fragments, pMTBipAvi and EXSTL (see 4.2.1.5). *E. coli* cells (strain DH5α) were transformed (see 4.2.1.7) and plasmid DNA was isolated from several clones (see 4.2.1.9). Purity and concentration of isolated plasmid DNA was determined (see 4.2.1.10) and the successful ligation was verified by digestion with *KpnI* and *MscI* restriction endonucleases (see 4.2.1.3, reaction 6).

5.1.3 Preparation of *BirA* with different signal peptide sequences

In order to obtain the highest yield of *in vivo* biotinylation of Avi-tagged recombinant protein, three different cellular localizations of biotin ligase (*BirA*) - extracellular, cytoplasmic and within the ER - were tested. The secreted *BirA* was obtained by a fusion with the Bip signal peptide (sequence of 18 mainly hydrophobic AAs attached to the N-terminus of the target protein) which addresses the translated protein to the ER and subsequently out of the cell. The KDEL signal peptide (sequence of 4 AAs attached to the C-terminus of target

protein) together with the Bip signal peptide enabled the retention of *BirA* within ER and for targeting the *BirA* into the cytoplasm no signal peptide was added to the biotin ligase.

The *BirA* cDNA was amplified from a commercial plasmid pBirAcm (Avidity). Three different PCR reactions were performed in order to obtain *BirA* fused with Bip and KDEL signal sequence (targeting to ER; see 4.2.1.1, reaction 2), with Bip signal sequence (secretion of *BirA* into the media; see 4.2.1.1, reaction 3) or with no signal sequence (targeting *BirA* to cytoplasm; see 4.2.1.1, reaction 4). All three products of the PCR reactions were subsequently loaded on an agarose gel and isolated (see 4.2.1.4). Then the restriction reaction was performed with each DNA fragment using appropriate restriction enzymes. *BglII* and *XhoI* were used to cleave fragments coding for secreted *BirA* and *BirA* retained within ER (see 4.2.1.3, reactions 7 and 10) and *KpnI* together with *XhoI* to cleave fragment coding for cytoplasmic *BirA* (see 4.2.1.3, reaction 9) After cleavage the whole reaction mixtures were again loaded on an agarose gel and purified (see 4.2.1.4).

Meanwhile, the commercial plasmid pMTBip/V5/His A (Invitrogen) was cleaved by *BglII*, *XhoI* or *KpnI*, *XhoI* combination of restriction endonucleases (see 4.2.1.3, reactions 8 and 11). The reaction mixtures were subsequently resolved on an agarose gel and the bands corresponding to the linear vector DNA were isolated from both reactions (see 4.2.1.4).

The cleaved and isolated fragments from PCR reactions were ligated with the vector DNA cleaved with appropriate restriction enzymes (see 4.2.1.5). The competent *E. coli* cells (strain DH5α) were transformed with the ligation reactions (see 4.2.1.7) and one positive clone from each ligation was grown and its plasmid DNA isolated (see 4.2.1.9). The purity and concentration of such obtained DNA was determined (see 4.2.1.10) and the right sequence of the constructs was proven by sequencing (see 4.2.1.11).

Three plasmids were obtained by this approach: pMTBipBirA coding for secreted *BirA*, pMTBipBirAKDEL coding for *BirA* retained in ER, and pMTBirA coding for cytoplasmic *BirA*.

The preparation of plasmid coding for *BirA* retained within ER (i.e. construct containing both Bip and KDEL signal sequence) is schematically illustrated in Figure 12 (p. 60).

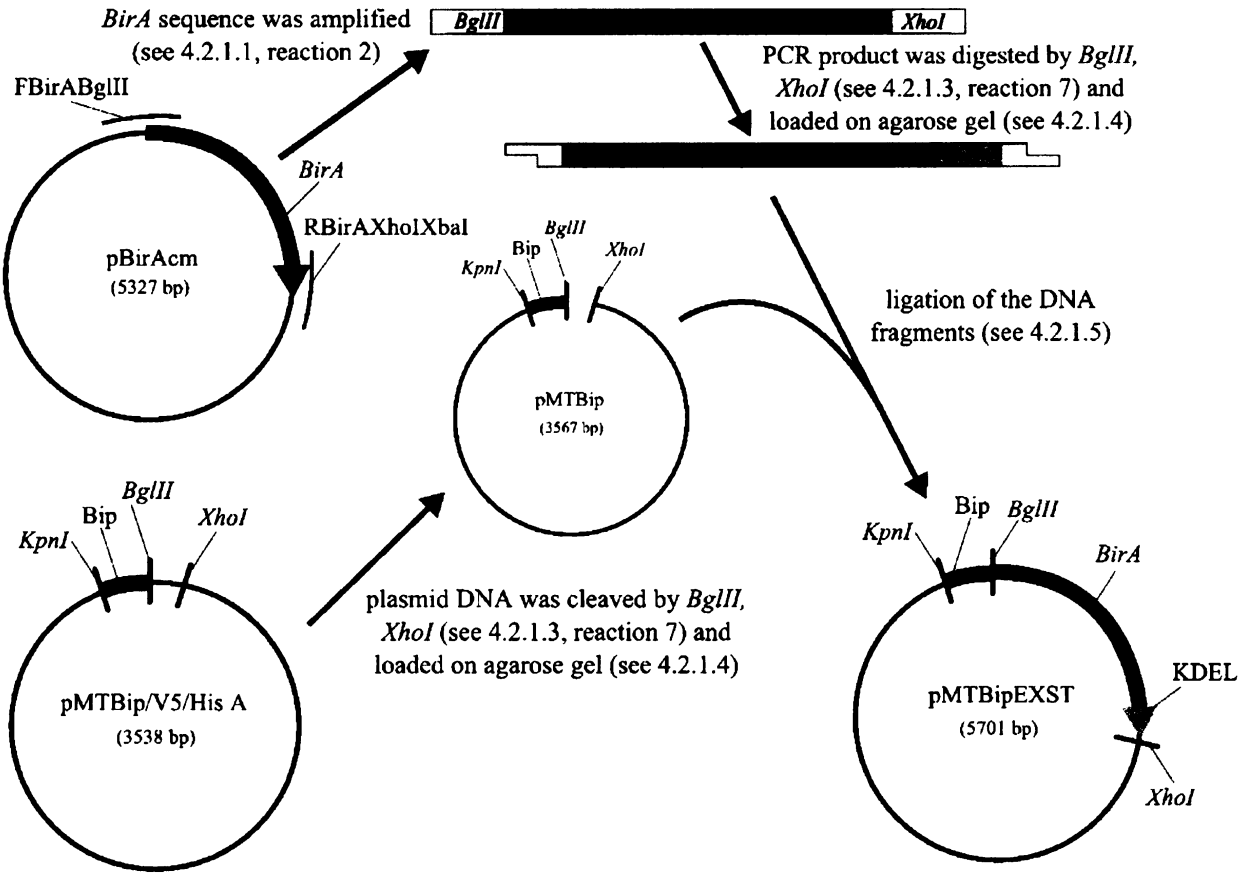


Figure 12: Schematic representation of the preparation of the plasmid DNA coding for BipBirAKDEL. For the sequence of the FbirABglII and RbirAXhoIXbaI primers see Table 13 (p. 81).

5.2 Preparation of stable *Drosophila* S2 Schneider cell transfectant

5.2.1 Influence of different factors on *in vivo* biotinylation yields

Drosophila S2 Schneider cells were stable transfected by the DNA plasmid coding for AviEXST (pMTBipAviEXST). This stable transfectant was additionally transfected by three different DNA plasmids coding for *BirA* protein (see 4.2.2.3). All three plasmids had the same metallothionein promoter (MT) but differed in the peptide signal sequence at the N-terminus of *BirA* (see 5.1.3). On the basis of previously published results [143], the influence of exogenously added D-biotin on the yield of biotinylation was also investigated

The conditioned media from different transfectants were analyzed for the presence of biotinylated and overall AviEXST by Western blotting (see 4.2.4.3). Neutravidin-HRP (Neu-HRP) was used for visualisation of AviEXST biotinylated fraction and the GCP-04 antibody for visualisation of both biotinylated and non-biotinylated AviEXST (see Fig. 13, p. 61).

The mutual densitometric comparison of the samples (see 4.2.4.4) showed that the highest production of AviEXST was in S2 cells with secreted *BirA*. On the other hand, the ratio of biotinylated to overall AviEXST was highest for the clone with the *BirA* protein

localized in the endoplasmic reticulum (ER). The results also suggested that the addition of exogenous D-biotin into the media has no or only a minor effect on the efficiency of *in vivo* biotinylation of the target protein by biotin ligase (*BirA*).

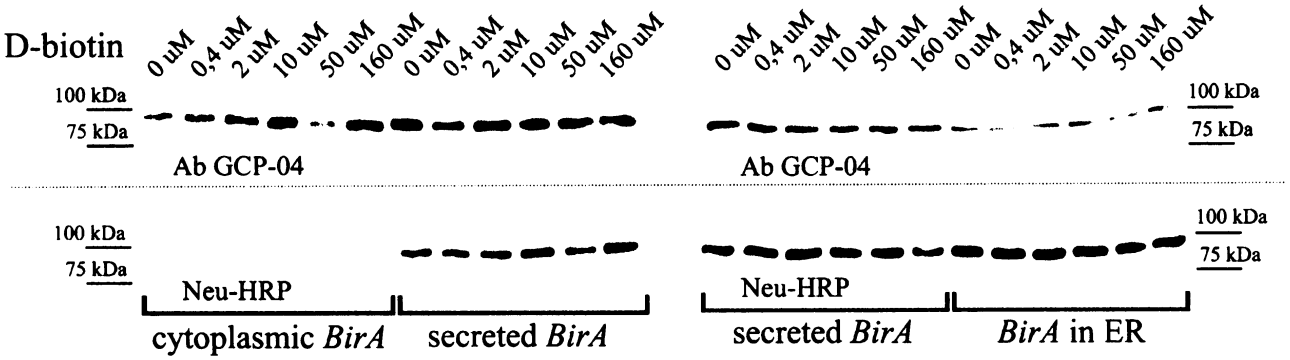


Figure 13: Western blot analysis of media from S2 cells. Four different blots are depicted here, two visualized using the GCP-04 antibody and two using the Neutravidin-HRP. The same volume of the samples was applied in each line. cytoplasmic *BirA*-series of transfectants with *BirA* localized in cytoplasm, secreted *BirA*-series of transfectants with *BirA* produced into the media, *BirA* in ER-series of transfectants with *BirA* retained in ER. Ab GCP-04-monoclonal antibody specifically recognizing EXST [190], Neu-HRP-Neutravidin conjugated with horseradish-peroxidase which specifically recognizes biotinylated molecules.

5.2.2 Large-scale expression of AviEXST and AviEXSTL

Large-scale expression of AviEXST recombinant protein was performed with the clone of *Drosophila* S2 Schneider cells which had localized the *BirA* in the ER and was co-transfected with the plasmid coding for AviEXST (see 4.2.2.4).

For production of recombinant AviEXSTL, the transfectant with *BirA* localized in ER was additionally transfected by plasmid pMTBipAviEXSTL (see 4.2.2.1) and large-scale expression was performed according to the protocol (see 4.2.2.4).

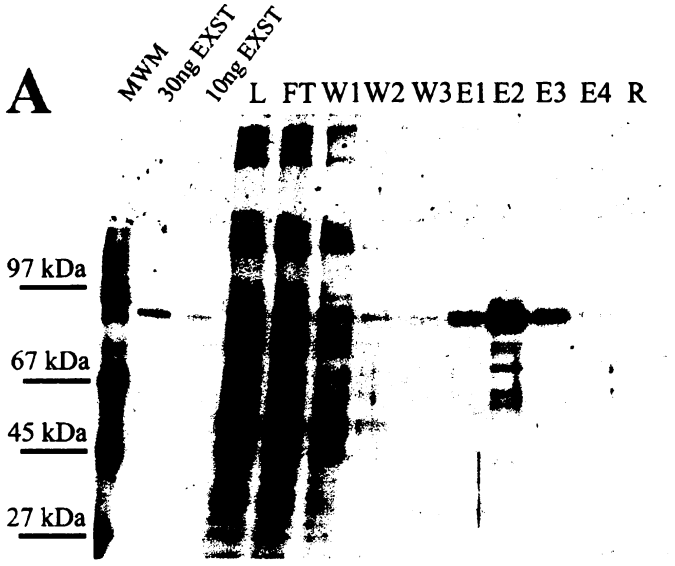
5.3 Optimization of purification via Avi-tag

The commercially available Streptavidin Mutein Matrix (ROCHE s.r.o.) was chosen as an affinity matrix for purification of recombinant Avi-tagged extracellular portions of GCPII and NAALADaseL, AviEXST and AviEXSTL respectively. Several small-scale purifications were performed with those non-dialyzed med. 0.2 ml of the resin matrix was incubated with 9 ml of the media overnight. Afterwards, the resin was separated from the media on the gravity-flow column and the purified protein was eluted. More than 85% of the bound target protein was present in the Elution2 (E2) fraction (for detailed protocol see 4.2.3.1).

During optimization experiments just a single parameter of the protocol was changed and the influence of that change on the amount of protein in E2 fraction, determined by Bio-

Rad protein assay (see 4.2.4.6), and purity of E2 fraction, determined by silver stained SDS-PAGE gels (see 4.2.4.1 and 4.2.4.2), was investigated.

The silver stained SDS-PAGE gels from one of these optimization experiments with non-dialyzed media containing AviEXST is shown in Figure 14. All fractions of the purification were loaded on the gel in the volumes which enabled easy determination of the effectivity of the purification and the purity of the E2 fraction (which contained more than 85% of the purified protein).



B

Fraction	Total amount of protein in the elution fractions [µg]
E1	< 2
E2	36
E3	< 4

Figure 14: Example of the small-scale purification of AviEXST. Panel A: Silver-stained gels with individual fractions from the small-scale purification. MWM (molecular weight marker) 1.5 µl; 30 ng EXST and 10 ng EXST refers to the amount of marker protein EXST; L(Load), FT(Flow-through), W1(Wash1) 5 µl; W2(Wash2), W3(Wash3) 15 µl; E1(Elution1), E2(Elution2) 10 µl; E3(Elution3), E4(Elution4), R(Resin) 15 µl. **Panel B:** Table with the total protein amounts in the elution fractions determined by Bio-Rad protein assay.

5.3.1 Influence of ionic strength and D-biotin on the purification

In order to standardize the purification protocol, the part of conditioned media from S2 cells containing AviEXST was dialyzed against 100 mM Tris-HCl (pH 7.2). Different equilibration buffers were used to achieve different ionic strengths during the incubation with the resin beads (see Fig.15, Panel A, Experiments 1-4, p. 63).

Surprisingly, none of the chosen conditions provided at least a similarly pure E2 fraction as was obtained from the purification of non-dialyzed media (compare with Fig. 14). The purity of the E2 fraction was highest in the conditions of the experiment 2 (100 mM Tris-HCl, 150 mM NaCl, pH 7.2) and both the decrease and increase of the ionic strength used in that experiment led to the less pure E2 fractions (see Fig. 15, Panel B, p. 63).

In a hope to improve the purity of elution fractions from purification of dialyzed media another series of experiments was performed. Similarly dialyzed media from S2 cells

containing AviEXST were mixed with equilibration buffer which was used in experiment 2, but additionally contained three different concentrations of D-biotin (see Fig. 15, Panel A, Experiments 5-7). The performed standard small-scale purification led to the more pure E2 fraction but also to the substantial less yield of the purified protein (both parameters were directly proportional to the concentration of D-biotin).

A

Experiment	Final composition of purified sample	Total protein in E2 fraction [µg]
1	100 mM Tris-HCl (pH 7.2)	**63**
2	150 mM NaCl, 100 mM Tris-HCl (pH 7.2)	**27**
3	200 mM $(NH_4)_2SO_4$, 150 mM NaCl, 100 mM Tris-HCl (pH 7.2)	**30**
4	400 mM $(NH_4)_2SO_4$, 150 mM NaCl, 100 mM Tris-HCl (pH 7.2)	**34**
5	2 µM D-biotin, 150 mM NaCl, 100 mM Tris-HCl (pH 7.2)	**20**
6	6 µM D-biotin, 150 mM NaCl, 100 mM Tris-HCl (pH 7.2)	**18**
7	10 µM D-biotin, 150 mM NaCl, 100 mM Tris-HCl (pH 7.2)	**14**

B

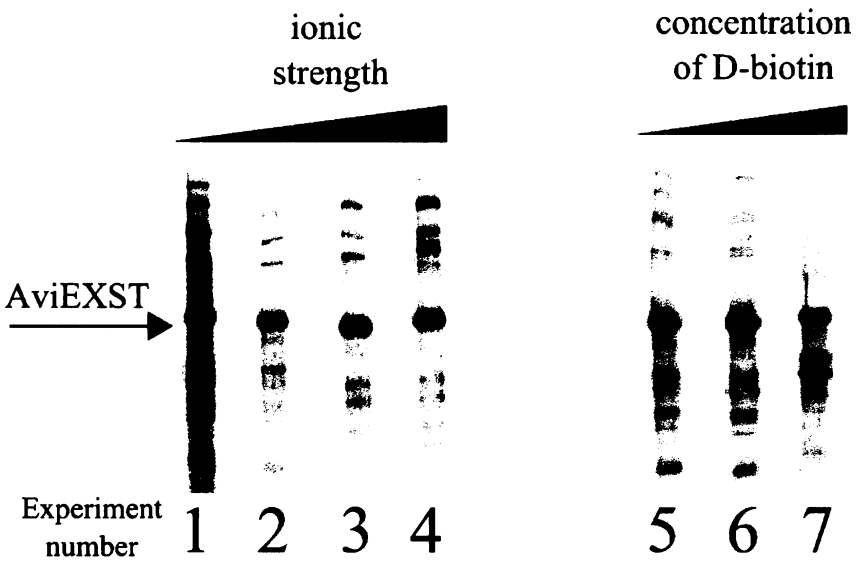


Figure 15: Summary of the optimization purification of AviEXST from dialyzed media. Panel A: The table contains the number of experiments, the final composition of the sample which was mixed with the Streptavidin Mutein Matrix resin and the total amount of protein measured by Bio-Rad protein assay in the E2 fractions from individual experiments. **Panel B:** Parts of the silver stained SDS-PAGE gels which show the protein composition of E2 fractions from different experiments. 10 µl of each sample was loaded on the gel. The picture was created by the Adobe Photoshop (v. 9.0).

Since purification from non-dialyzed media still showed the highest purity of elution fractions with comparable or better yields than any of the dialyzed version of purification further optimization experiments were performed using the non-dialyzed media from S2 Schneider cells.

5.3.2 Dependence of purification yield on the incubation times

To make the purification protocol timely effective, small-scale purification experiments which differed in the incubation time of non-dialyzed AviEXST media with regenerated Streptavidin Mutein Matrix (see Fig. 16, Panel A, Experiments 1-4) and experiments differing in the incubation time during which the regenerated resin is incubated with elution buffer before the elution itself (see Fig. 16, Panel A, Experiments 5-8), were performed (for detailed purification protocol see 4.3.2.1). Purity of all elution fractions analyzed by silver-stained SDS-PAGE was comparable to the previous experiments (see Fig. 16, Panel B).

A

Experiment	Incubation time of the purified sample with resin	Total amount of protein in E2 fraction [µg]	Experiment	Incubation time of the resin with elution buffer	Total amount of protein in E2 fraction [µg]
1	1 hour	**11**	**5**	none	**17**
2	4 hours	**20**	**6**	10 minutes	**16**
3	8 hours	**20**	**7**	30 minutes	**20**
4	16 hours	**25**	**8**	60 minutes	**22**

B

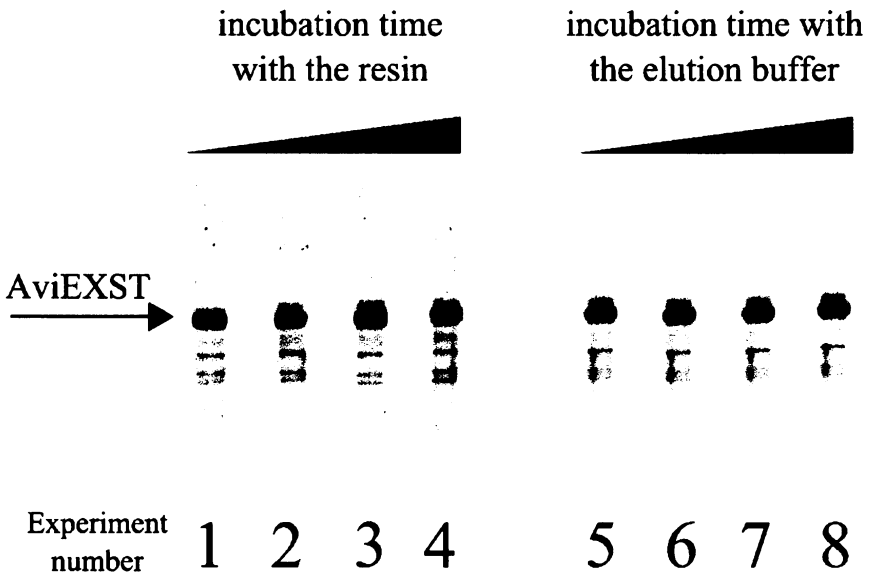


Figure 16: Purifications of AviEXST with different incubation times. Panel A: The table contains the number of experiments, the incubation times used during the experiments and the total amount of protein measured by Bio-Rad protein assay in the E2 fractions from individual experiments. **Panel B:** Parts of the silver stained SDS-PAGE gels which show the protein composition of E2 fractions from different experiments. 10 µl of each sample was loaded on the gel. The picture was created by the Adobe Photoshop (v. 9.0).

The obtained results indicated that the longer incubation of purified media with the resin led to approximately two times larger yield of the purified protein in the E2 fraction. On the other hand, the incubation of the resin with elution buffer before elution itself showed not to be so determining for the purification yield. However, this result was obtained for small-

scale purification setup and if larger amount of the resin would have been used the elution would not have had to be so quick. Therefore the 1-hour incubation of the resin with elution buffer before elution itself was used for the large-scale purification experiments.

5.3.3 Versatility of the purification method

Two different proteins, EXST (extracellular part of GCPII; AA 44-750) and EXSTL (extracellular part of NAALADaseL; AA 28-740), both fused with the same Avi-tag protein sequence were successfully purified into high homogeneity on the Streptavidin Mutein Matrix. Both purifications were performed in small-scale from non-dialyzed S2 cell media according to the protocol (see 4.2.3.1). For a brief summary of these two purifications see Figure 17. The purity of both E2 fractions was more than 90% (estimated from the SDS-PAGE).

A

Experiment	Purified protein	Total protein in E2 fraction [µg]
1	AviEXST	50
2	AviEXSTL	28

B

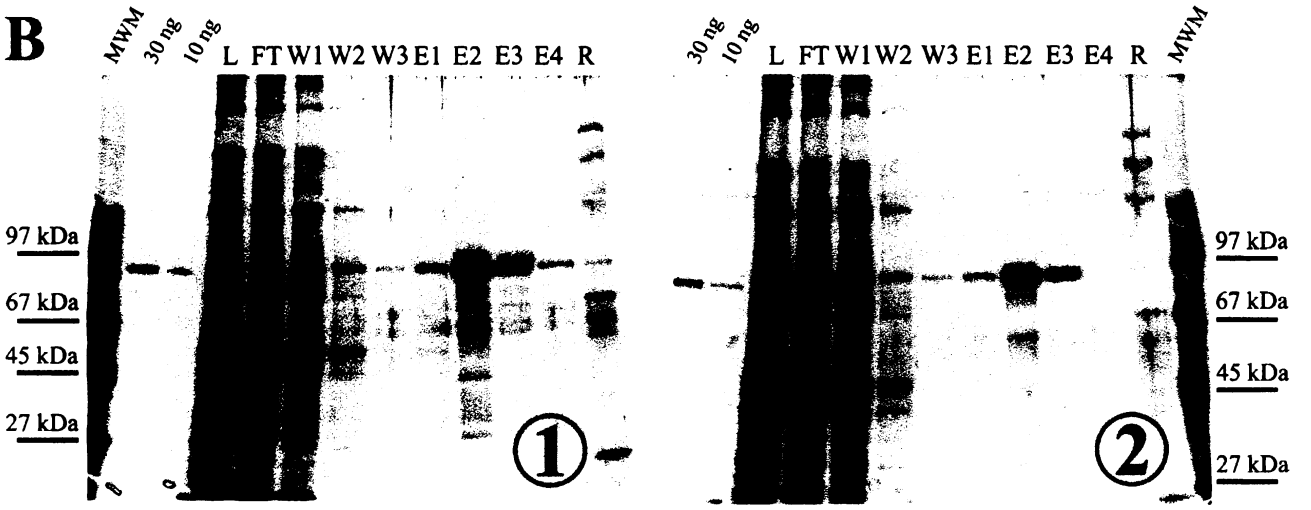


Figure 17: Purification of AviEXST and AviEXSTL. Panel A: Summary of purifications which highlights the differences among the individual experiments. If not stated otherwise, the conditions were the same as described in section 4.2.3.1. **Panel B:** Silver-stained gels with individual fractions from the purifications. MWM (molecular weight marker) 1.5 µl; 30 ng and 10 ng refers to the amount of marker protein EXST; L(Load), FT(Flow-through), W1(Wash1) 5 µl; W2(Wash2), W3(Wash3) 15 µl; E1(Elution1), E2(Elution2) 10 µl; E3(Elution3), E4(Elution4), R(Resin) 15 µl.

During these optimization procedures it was also found that the Streptavidin Mutein Matrix is easily regenerated (see 4.2.3.3) with retained capacity and specificity of the binding.

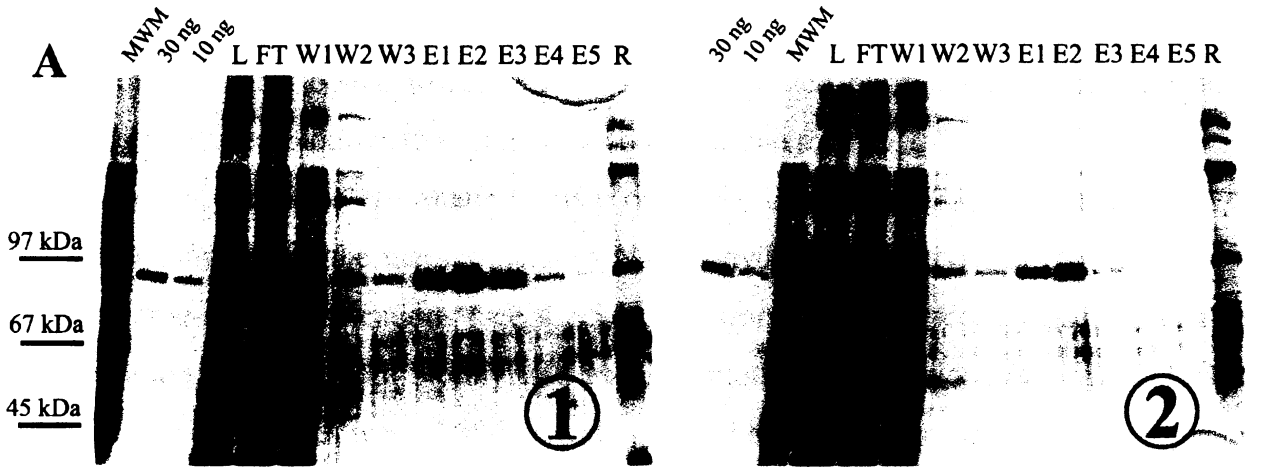
Another small-scale optimization experiment revealed that if the once purified media is loaded on the regenerated matrix additional amount of target protein will be obtained with yield equal to approximately one third of the originally purified protein.

After optimization of the purification procedure in the small-scale and determination that both Avi-tagged proteins were capable of efficient purification on Streptavidin Mutein Matrix, the large-scale purification of these proteins was performed.

5.4 Large-scale purification of AviEXST and AviEXSTL

5.4.1 Large-scale purification of AviEXST

On the basis of optimization experiments, the large-scale purification of recombinant protein AviEXST from 250 ml of S2 cell media was performed (see 4.2.3.2). After the first round of purification (Experiment 1) the same Streptavidin Mutein Matrix was regenerated (see 4.2.3.3) and mixed again with the flow-through fraction of previous purification (Experiment 2). The composition of the fractions collected during the purification was analyzed by the SDS-PAGE (see 4.2.4.1). The yield of the purification was estimated by Bio-Rad protein assay measured from the elution fractions 1, 2, and 3 (see 4.2.4.6). The results of this analysis are shown in Figure 18.



B

Experiment 1		Experiment 2	
Fraction	**total amount of protein in elution fractions [µg]**	**Fraction**	**total amount of protein in elution fractions [µg]**
E1	**17**	E1	**< 6**
E2	**326**	E2	**198**
E3	**18**	E3	**< 6**

Figure 18: Large-scale purification of AviEXST. Experiment 1 refers to first purification and Experiment 2 to the subsequent purification with the flow-through fraction from Experiment 1. **Panel A:** Silver-stained gels with individual fractions from the purifications. MWM (molecular weight marker) 1.5 µl; 30 ng and 10 ng refers to the amount of marker protein EXST; L(Load), FT(Flow-through), W1(Wash1) 5 µl; W2(Wash2), W3(Wash3) 15 µl; E1(Elution1) 5 µl; E2(Elution2) 1 µl; E3(Elution3) 5 µl; E4(Elution4), E5(Elution5) 10 µl; R(Resin) 15 µl. **Panel B:** Table with the concentrations and total amounts of protein in the Elution fractions.

The total yield after two rounds of the purification was determined to approximately 0.5 mg of the pure AviEXST protein from the 250 ml of conditioned media. The purity of the E2 fractions was estimated to be more than 95%. Additionally, the AviEXST protein was concentrated from 250 ml to 2 ml.

For the determination of the purification yield the biotin-binding assay was developed (see 4.2.4.5). This method enabled detection of the biotinylated portion of the AviEXST molecules via their binding to the surface of 96-well microplate and subsequent visualization by Neutravidin-HRP. The results from the measurements are summarized in Table 7.

Table 7: Results from the biotin-binding assay characterizing the AviEXST large-scale purification. L stands for the Load fraction from Experiment 1. FT stands for Flow-through fraction from Experiment 2 but simultaneously also for Load fraction from Experiment 2. FT^2 stands for Flow-through fraction from Experiment 2. X/L describes the ratios of mean luminescence between analyzed fraction and the Load fraction.

Sample	Mean luminescence	Volume of the sample in the well [ul]	Ratio of X/L luminescence
L	4 311 ± 172	0.05	1.00
FT	2 926 ± 288	0.05	0.68
FT^2	1 684 ± 211	0.05	0.39

The data from the measurement showed that approximately 30% of the biotinylated portion of AviEXST bound to the resin and after repeated purification another 30% of the originally presented biotinylated protein was bound to the matrix.

Finally, since the enzymatic activity of EXST is known [175], the purification yield was also quantified by the measurement of NAAG-hydrolysing activity of the fractions from the two large-scale purifications (see 4.2.5.1). Contrary to the previously used method, this quantification involved both biotinylated and non-biotinylated versions of AviEXST protein (see 5.5.1). The samples were first dialyzed against the same buffer (see 4.2.4.8) and then the activity was determined. The results are summarized in the Table 8.

Table 8: Results from the enzymatic activity characterization of AviEXST large-scale purification. L stands for the Load fraction from Experiment 1. FT stands for Flow-through fraction from Experiment 2 but simultaneously also for Load fraction from Experiment 2. FT^2 stands for Flow-through fraction from Experiment 2. E2 stands for Elution2 fraction from Experiment 1 while $E2^2$ stands for Elution2 fraction from Experiment 2. X/L describes the ratios of mean activity between analyzed fraction and the Load fraction. "act" is defined as a number of DPM (disintegrations per minute) in 1 µl of the sample. Total activity refers to the total amount of activity in each purification fraction while specific activity refers to the amount of activity in the 1 mg of the total protein in the purification fraction.

Sample	Activity [act]	Total activity [act.ml]	Ratio of X/L activities	Specific activity [(act.ml)/mg]
L	6 310	4 022 625	1.00	33 522
FT	2 970	2 561 625	0.64	21 347
FT^2	3 924	3 089 953	0.77	25 750
E2	169 500	406 800	0.10	1 232 727
$E2^2$	39 380	118 140	0.03	590 700

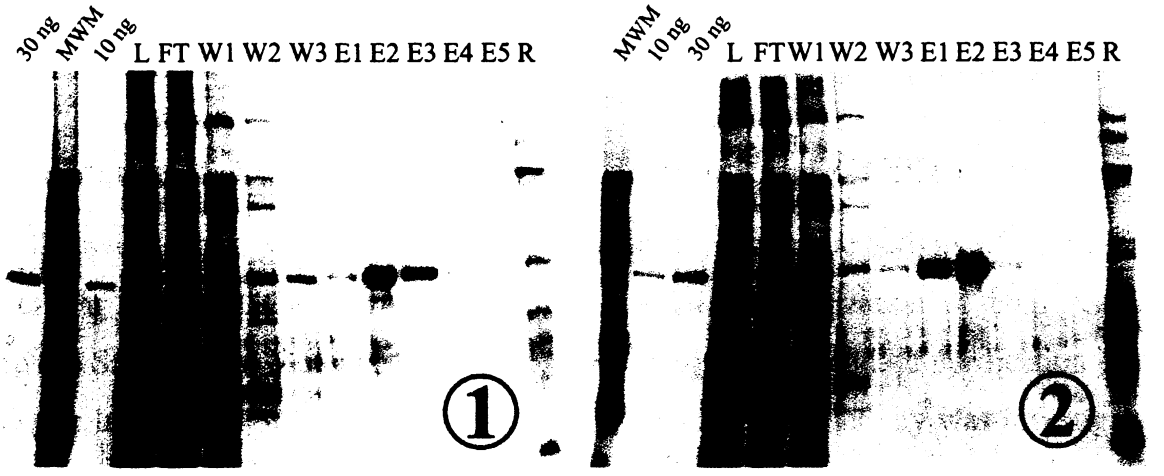
The results from the enzymatic activity detection suggested that the overall yield of the AviEXST affinity purification after two rounds was 13% (related to the conditioned media, Load) and approximately a 30 times increase in specific activity of the material was achieved.

5.4.2 Large-scale purification of AviEXSTL

The very same purification procedure as for AviEXST (see 5.4.1) was also applied to the AviEXSTL protein (see 4.2.3.3). Two subsequent purifications (Experiment 1 and 2) were performed and their yields determined.

The fractions from both purifications were analyzed by the SDS-PAGE (see 4.2.4.1). The yield of the purifications was estimated by Bio-Rad protein assay measured from the elution fractions 1, 2, and 3 (see 4.2.4.6). The results of this analysis are shown in Figure 19.

A



B

Experiment 1		Experiment 2	
Fraction	**Total amount of protein in elution fractions [µg]**	**Fraction**	**Total amount of protein in elution fractions [µg]**
E1	**< 6**	E1	**12**
E2	**370**	E2	**120**
E3	**9**	E3	**< 6**

Figure 19: Large-scale purification of AviEXSTL. Experiment 1 refers to first purification and Experiment 2 to the subsequent purification with the flow-through fraction from Experiment 1. **Panel A:** Silver-stained gels with individual fractions from the purifications. MWM (molecular weight marker) 1.5 µl; 30 ng and 10 ng refers to the amount of marker protein EXST; L(Load), FT(Flow-through), W1(Wash1) 5 µl; W2(Wash2), W3(Wash3) 15 µl; E1(Elution1) 5 µl; E2(Elution2) 1 µl; E3(Elution3) 5 µl; E4(Elution4), E5(Elution5) 10 µl; R(Resin) 15 µl. **Panel B:** Table with the concentrations and total amounts of protein in the Elution fractions.

Similarly to the AviEXSTL large-scale purification, the total yield after two rounds of the purification was determined to approximately 0.5 mg of the pure AviEXSTL protein from the 250 ml of conditioned media. The purity of the E2 fractions was estimated to be more than 95%. Additionally, the AviEXSTL protein was concentrated from 250 ml to 2 ml.

In order to determine the amount of biotinylated AviEXSTL in purification fractions, the biotin-binding assay was performed (see 4.2.4.5). Its results are summarized in Table 9.

Table 9: Results from the biotin-binding assay characterizing the AviEXSTL large-scale purification. L stands for the Load fraction from Experiment 1. FT stands for Flow-through fraction from Experiment 2 but simultaneously also for Load fraction from Experiment 2. FT^2 stands for Flow-through fraction from Experiment 2. X/L describes the ratios of mean luminescence between analyzed fraction and the Load fraction.

Sample	Mean luminescence	Volume in well [ul]	Ratio of X/L luminescence
L	5 255 ± 409	0.05	1.00
FT	2 898 ± 188	0.05	0.55
FT^2	2 127 ± 212	0.05	0.41

The results indicated that after two rounds of purification approximately 60% of biotinylated portion of AviEXSTL was bound to the Streptavidin Mutein Matrix.

5.5 Influence of Avi-tag on the properties of target proteins

5.5.1 Influence of Avi-tag on the enzymatic activity of GCPII

To investigate a possible effect of Avi-tag on the enzymatic activity of EXST protein (extracellular part of GCPII, AA 44-750) the kinetic characterization of both AviEXST, purified via Streptavidin Mutein Matrix, and EXST, purified by standard purification protocol used in our laboratory [30], was performed. NAAG-hydrolysing activity was investigated by radiometric measurements (see 4.2.5.1). The values of K_M, k_{cat} and k_{cat}/K_M for both proteins are shown in Table 10.

Table 10: Direct comparison of NAAG-hydrolysing activity of EXST and AviEXST proteins. EXST stands for extracellular part of GCPII (AAs 44-750). AviEXST stands for Avi-tagged extracellular portion of GCPII (AAs 44-750).

Sample	K_M [nmol/L]	k_{cat} [s^{-1}]	k_{cat}/K_M [10^5/s/(mol/L)]
EXST	1 100 ± 150	0.53 ± 0.017	4.8 ± 0.63
AviEXST	760 ± 90	0.67 ± 0.022	8.8 ± 1.08

The results from the NAAG-hydrolysing activity measurement suggested that the presence of a biotinylated Avi-tag at the N-terminus of the extracellular portion of GCPII (EXST) does not significantly influence its enzymatic activity.

5.5.2 Influence of Avi-tag on the enzymatic of EXSTL

As mentioned in section 3.13.2, the NAALADaseL was reported to possess dipeptidyl peptidase IV (DPPIV) activity [186,187] which enables the enzymes to cleave off dipeptides from the N-terminus of the substrate. For an efficient cleavage, the proline residue has to be

presented at the P1' site of the substrate. On the basis of those published results and the close homology of NAALADaseL with GCPII both NAAG-hydrolysing and DPPIV activity of purified AviEXSTL protein were investigated (see 4.2.5.1 and 4.2.5.2).

The results from the activity measurements of purified AviEXSTL protein showed that NAALADaseL possesses neither NAAG-hydrolysing nor DPPIV activity (summarized in Tables 11 and 12).

Table 11: Determination of AviEXSTL NAAG-hydrolysing activity. DPM - disintegrations per minute. E - the amount of purified protein added into the individual reaction. The final concentration of the substrate in each reaction was 100 μM. EXST stands for extracellular part of GCPII (AAs 44-750). AviEXSTL stands for Avi-tagged extracellular portion of NAALADaseL (Aas 28-740).

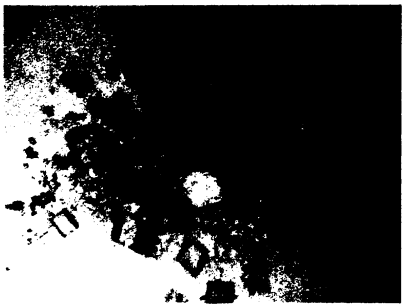
Sample	DPM	E [ng]
EXST	500	1.2
EXST	2 700	3.5
EXST	23 200	35
AviEXSTL	< 0	3.5
AviEXSTL	400	35
AviEXSTL	< 0	175

Table 12: Determination of AviEXSTL DPPIV activity. RFU - relative fluorescence unit. E - the amount of purified protein (in LNCaP lysate total amount of protein) added into the reaction. The final concentration of the substrate in each reaction was 100 μM and voltage put on the photomultiplier during measurement 640 V. EXST stands for extracellular part of GCPII (AAs 44-750). AviEXSTL stands for Avi-tagged extracellular portion of NAALADaseL (AAs 28-740).

Sample	RFU	E [ng]
EXST	0.03	175
AviEXSTL	0.14	35
AviEXSTL	< 0	175
LNCaP lysate	4.57	20 000

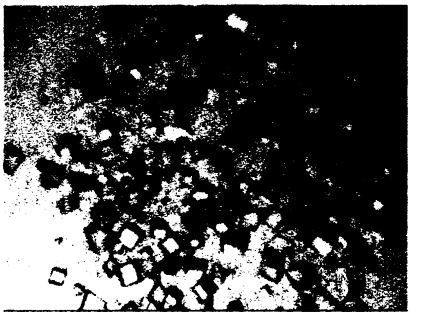
5.5.3 Crystallization of GCPII

In order to determine the Avi-tag influence on the crystallization ability of GCPII, the Avi-tagged extracellular portion of GCPII (AviEXST) was crystallized. The crystallization conditions were the same as used by Bařinka *et al.* for EXST (see 4.2.6.1) [191]. Crystals with three different substrates (with none, with the natural substrate NAAG, and with potent a inhibitor 2-PMPA) were obtained (see Fig. 20, p. 71) and their diffraction pattern was analyzed (see 4.2.6.3).



AviEXST (5.3 mg/ml)
no substrate
crystals ~ 0.2-0.3 mm

AviEXST (5.3 mg/ml)
1 mM NAAG
crystals ~ 0.1-0.2 mm



AviEXST (5.3 mg/ml)
1 mM 2-PMPA
crystals ~ 0.1 mm

Figure 20: Pictures of the AviEXST crystals. Three different setups (free enzyme, with natural substrate and with inhibitor) were performed. Size of the crystals is written under each picture. NAAG stands for N-acetyl-L-aspartyl-L-glutamate. 2-PMPA stands for 2-phosphonomethylpentandioic acid.

All three analyzed crystals diffracted in the size dependent manner and the biggest one (~ 0.3 mm) diffracted up to 2.3Å. An example of its diffraction image is given in Figure 21. Crystals from all three conditions belonged to the I222 space group and showed identical unit-cell parameters as the wild-type EXST protein [171].

The crystallographic study of AviEXST was performed in the laboratory of Structural Biology at IOCB under the kind supervision of Pavlína Řezáčová.

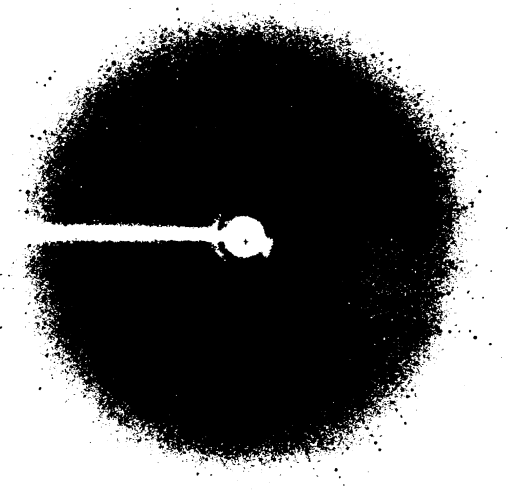


Figure 21: Diffraction image of the AviEXST crystal.

6. DISCUSSION

A working purification protocol has been established in our laboratory for purification of recombinant GCPII [30]. Unfortunately, the successful purification via this protocol is very sensitive to any modification. Just one amino acid substitution within the GCPII protein sequence might cause the need for further optimization of the established purification procedure [193]. Additionally, for protein homologs of GCPII, such as human GCPIII (87% similarity), the original purification protocol did not work sufficiently as well and had to be modified [194]. Therefore an alternative, standardized purification protocol utilizing an affinity purification approach was established as the subject of this thesis.

Currently, various different affinity tags are available (see section 3.11) and all of them have their pros and cons. The most commonly used His-tag might not be a suitable candidate since GCPII, and most likely also its close homologs, is a di-zinc metallopeptidase and the His-tag might be able to chelate the zinc ions and thus abolish the protein enzymatic activity. The Avi-tag was finally chosen for two main reasons. First, biotin-avidin interaction is one of the phenomena that is "relatively" reliable in science and works almost unconditionally. Second, the possible post-purification utilization of specifically biotinylated protein has been very tempting for us.

In order to attempt gaining a non-tagged product protein, several experiments were performed with the His-tagged TEV protease mutant (S219V), a kind gift from David S. Waugh, NCI Frederick, to cleave off the tag. The influence of different cleavage buffers, incubation times and temperatures on the effectivity of the cleavage was investigated. The results suggested that approximately 100 molar excess of the TEV protease efficiently cleaved off the tag while equimolar ratio of protease and tagged protein led to a partial cleavage. The investigated conditions had just a minor effect on the efficiency of the cleavage (data not shown). Since the consumption of the protease would be large and it would also require additional purification steps to get rid of TEV protease and the cleaved tag, we decided to keep the tag on the recombinant protein and study the whole fusion protein.

6.1 Creating an expression host for large-scale expression

During the establishment of an expression host (*Drosophila* S2 Schneider cells) a series of experiments with plasmids directing *E. coli* biotin ligase (*BirA*) to the different cellular compartments was performed (see 5.2.1).

The clones with *BirA* localized either in ER or in extracellular space showed production of biotinylated AviEXST (see Fig. 13, p. 61). Though the overall amount of AviEXST was higher in the clone with secreted *BirA*, the clone with *BirA* in ER showed higher biotinylation efficiency. Considering our previous experience with *Drosophila* S2 Schneider cells, which showed that the overall amount of produced recombinant protein varies highly after each single transfection, new transfectants with the ER localized *BirA* protein were prepared. One of these transfectants showed approximately 4 times higher production of overall AviEXST than the transfectant with secreted *BirA* (data not shown). This transfectant was subsequently used for large-scale expression of AviEXST.

For production of Avi-tagged NAALADaseL the clone with ER localized *BirA* was transfected by pMTBipAviEXSTL plasmid DNA. The transfection was performed just once showing a comparable yield with the best transfection performed with pMTBipAviEXST.

6.2 Optimization of Avi-tagged recombinant protein purification

The commercially available Streptavidin Mutein Matrix was used as an affinity resin. Together with resin the optimized protocol for both batch and column purification setup was also available (http://www.roche-applied-science.com/pack-insert/3708152a.pdf). However, our final optimized purification protocol differed from the recommended manufacturer's protocol in many parameters. The batch and column approaches were combined together, different composition of the buffers was chosen and all steps were performed at 4°C (for details see section 4.2.3).

The results of experiments with different incubation times of resin with elution buffer (see 5.3.2) indicated that the column purification setup might lead to the better overall yield than the batchwise large-scale purification method used in this thesis.

In an attempt to make the purification method more reproducible and to achieve the highest possible purity of the target protein, the conditioned media from S2 cells was dialyzed against defined buffer solution and the small-scale purifications with different ionic strengths or different concentrations of D-biotin within equilibration buffer were performed (see 5.3.1). Surprisingly, none of the conditions led to the protein with high or at least the same purity and with the same yield in comparison to the purification from non-dialyzed media (see Fig. 15. p 63). Since the SF900II media, which was used for cultivating S2 cells, is not chemically defined, we are not sure what might have caused the impaired purification after dialysis.

The optimization experiments were analyzed on the basis of its yield, determined by Bio-Rad protein assay. The inconsistent values of the overall protein amount in Elution2(E2)

fractions among the different experiments might have been caused by the manipulation with the conditioned media (Load). All experiments were performed within 2 months and during that time the media was thawed and re-frozen several times. Sometimes a precipitate formed and then the media was filtered through a 22 μm filter. That might be also an explanation of different yields during the small-scale experiment with AviEXST and AviEXSTL non-dialyzed conditioned media (see Fig. 17, p. 65). Therefore the amount of protein in Elution2 fraction should be compared just among the fractions within one particular series of experiments.

The second observed parameter was the purity of the elution fractions, determined by silver stained gels from SDS-PAGE. The elution fraction samples (mainly E2 fractions) were overloaded in order to visualize possible impurities. Two different amounts of GCPII standard (10 and 30 ng of purified EXST protein) were loaded on each gel and the gels were stained so that the standards have the same intensities. Despite of that the intensities of the bands corresponding to AviEXST/AviEXSTL in the E2 fractions should not be compared even among the gels from one experiment since the silver staining is not linear if the amount of the protein loaded in the line is too high.

Finally, during optimization experiments the versatility of the Avi-tag purification approach was demonstrated by the successful purification of GCPII close homolog, NAALADaseL (see 5.3.3). Extracellular portion of NAALADaseL (AAs 28-740, EXSTL) was prepared in its Avi-tagged form and subsequently purified into the similar degree of homogeneity as GCPII. Such a proven versatility of Avi-tag purification approach will hopefully enable an easier purification of different mutants and homologs of GCPII in the future.

6.3 Large-scale purification experiments

0.5 mg of purified AviEXST and AviEXSTL proteins was obtained by large-scale purification. That amount of protein is definitely sufficient for enzymatic analysis. However, there are other methods, such as crystallography, which demand higher amount of the pure protein. If the crystallization conditions are known, the above mentioned amount of purified protein for seeding dozens of drops is sufficient (see 5.5.3), but this amount of protein might not be satisfactory for searching of possible crystallization conditions. On the other hand, the purification method is relatively simple, fast and the Streptavidin Mutein Matrix can be easily regenerated so it is just a matter of time, money, and experimenter's will how many purifications he or she will perform. Moreover, using advanced liquid handling

instrumentation in crystallization trials ("robots") decreases the demand for the protein quantities needed for these experiments.

The quantification of large-scale purification was performed just with the three fractions from each purification (Load, Flow-through and Elution2 fractions) because more than 98% of total purified protein was present in these fractions (estimated from the silver-stained SDS-gels and measurements of total protein amounts in elution fractions). The biotin-binding assay, which detected just biotinylated portion of target proteins, indicated the 60% yield of both large-scale purifications after two rounds of purification (see Table 7, p. 67 and Table 9, p. 69). The quantification was performed semi-quantitatively since the binding of the biotinylated protein to the well surface is quite dependent on the composition of the sample (mainly on the protein concentration and presence of detergents) and thus the calibration curve was difficult to use (data not shown).

Additional quantification, based on the enzymatic activity, was used in the case of AviEXST large-scale purification. Results from that measurement indicated approximately 80% of the total AviEXST protein (biotinylated or not) present in the flow-through fraction after second round of purification (FT^2) and 13% of total AviEXST in the elution fractions (see Table 8, p. 67). The unexpected lower NAAG-hydrolysing activity of the flow-through fraction after first round of purification (FT) than the activity of the flow-through fraction after second round of purification (FT^2) might have been caused by the storage of the samples during the purification and subsequent dialysis at 4°C. That handling might have influenced individual samples differently and the FT fraction most severely. Another explanation might be the presence of some GCPII inhibitor which would be able to bind to the Streptavidin Mutein Matrix and after second purification its concentration would be so decreased that it would be no longer able to influence the GCPII activity. We also have to point out that the elution fractions contained much less protein than the Load and Flow-through fractions which could have altered the GCPII enzymatic activity as well and thus influence the results.

Considering the total amount of purified proteins during large-scale purifications (0.5 mg from 250 ml of conditioned media in both cases) together with the yields obtained from the quantification via biotin-binding assay (60% in both cases) we can estimate the total production of biotinylated AviEXST and AviEXSTL proteins by S2 Schneider cells as 3.3 mg/L. The total protein expression can be also determined for AviEXST from the quantification via its enzymatic activity. Since 13% of total protein was present in Elution2 fractions from both purifications, the overall production of AviEXST can be calculated to 15 mg/L. That would suggest approximately 20% effectivity of biotinylation of AviEXST

protein by biotin ligase (*BirA*). However, for the reasons discussed above these numbers should be taken more as an estimation rather than determination.

6.4 Influence of Avi-tag on protein function

The kinetic characterization of AviEXST versus EXST (extracellular part of GCPII, AAs 44-750) indicated that the Avi-tag protein sequence did not influence the NAAG-hydrolysing activity of GCPII (see section 5.5.1).

On the other hand, no enzymatic activity of AviEXSTL (Avi-tagged extracellular part of NAALADaseL, AAs 28-740) was determined (see section 5.5.2). No NAAG-hydrolysing activity was expected for this protein, but DPPIV activity for NAALADaseL (the full-length form immunoprecipitated from rat ileum) was reported by Shneider *et al.* [186]. The DPPIV activity is a relatively common activity and any cell lysate might be used as a positive control for such activity measurements. Therefore, we presume that Shneider *et al.* could work with a contaminated sample. Similar contamination was probably the reason for earlier false-positive report on DPPIV activity of GCPII [187].

On the other hand, AviEXSTL differs in many features from the protein which was shown to posses DPPIV activity. First of all, it is a human version of the protein, not rat. Second, the analysis was performed with just an extracellular portion of the whole protein, AAs 28-740. Third, the protein was produced in the insect cells which might have caused different post-translation modification in comparison with mammalian cells (e.g. different N-glycosylations). And finally, the Avi-tag was added to the extracellular portion of NAALADaseL protein (EXSTL). Each of these factors might have abolished DPPIV activity of AviEXSTL. However, DPPIV activity of the non-tagged extracellular part of NAALADaseL (EXSTL) from conditioned media was also analyzed and not found (data not shown). Therefore, we can speculate that the addition of the Avi-tag protein sequence was not the cause of the lack of DPPIV activity of the AviEXSTL protein.

As the final test of the Avi-tag influence on the tagged proteins, an attempt to crystallize GCPII fused with this tag was performed. Since the crystallization condition for GCPII (more precisely for its extracellular portion EXST) is known [191], only small amount of the purified protein was needed. The fused protein formed relatively large 3D crystal (see Fig. 20, p. 71). The size of the crystals corresponded to the kinetics of their formation which was partially influenced by the addition of either natural substrate (NAAG) or potent inhibitor (2-PMPA). With the potent inhibitor many crystal nuclei were formed after one day and therefore the protein in the solution soon ran out and the crystals stopped growing. On the

other hand, without any additive the growth of the crystals was slow and just several crystals for each drop were obtained. These crystals were 2 to 3 times bigger than those with 2-PMPA. Hopefully, the size of the crystals might be influenced by the change of the ratio among crystallized protein, precipitate and co-crystallizing agents. However, all crystals diffracted the x-ray radiation while the biggest one diffracted at the resolution of 2.3Å. That was close to the resolution limit of the instrument which was used for the measurement and probably with the more powerful X-ray source the resolution limit may be even lower.

The whole structure of AviEXST is hopefully going to be solved soon. There is just one reference in the scientific literature about the crystallization of Avi-tagged protein [32] and it is reported that the structure is identical to the non-tagged version of that protein and the Avi-tag is not visible. The same space group and parameters of the cell-unit of the obtained crystals in comparison with the crystal of wild-type protein may indicate that it might also be the case for AviEXST protein.

Even if there might be still space for further improvements of this purification approach, large-scale purification protocol that we present here affords highly pure recombinant protein in sufficient yields. The versatility of this affinity purification approach seems to enable the simple and fast purification of great variety of proteins (at least proteins closely related to the GCPII). The true generality of this purification approach will be hopefully proven in the near future.

7. CONCLUSIONS

- the plasmids for heterologous expression in the *Drosophila* S2 Schneider cells coding for extracellular domains of GCPII and NAALADaseL with fused Avi-tag protein sequence were prepared.
- *Drosophila* S2 Schneider cells transfectants with biotin ligase (*BirA*) localized in three different cellular compartments were prepared, co-transfected with plasmids coding for the recombinant proteins, analyzed and the co-transfectant with *BirA* located in ER of the host cell was selected for further use in protein expression.
- the one-step purification of two recombinant proteins using Avi-tag on the Streptavidin Mutein Matrix was optimized.
- large-scale purifications of GCPII and NAALADaseL proteins using protein engineered Avi-tag were achieved with yields of 0.5 mg of pure recombinant protein from 250 ml of conditioned media for both proteins.
- NAALADaseL with Avi-tag was purified and enzymatically characterized. Contrary to the previous reports no enzymatic activity of this purified protein was detected.
- GCPII containing Avi-tag was purified, enzymatically characterized, and crystallized yielding crystals diffracting to 2.3Å resolution.

8. LIST OF ABBREVIATIONS

AAs	amino acids
AMC	amino methyl coumarin
AviEXST	Avi-tagged extracellular part of GCPII containing AAs 44-750
AviEXSTL	Avi-tagged extracellular part of NAALADaseL containing AAs 28-750
BirA	biotin-(acetyl-CoA-carboxytransferase) ligase
CBD	cellulose binding domain
CBP	calmodulin binding peptide
ChBD	chitin binding domain
CMA	carboxylmethyl aspartate
DAPase	dipeptidyl aminopeptidase I
DNA	deoxyribonucleic acid
DPM	disintegrations per minute
DPPIV	dipeptidyl peptidase IV
EDTA	ethylenediaminetetraacetic acid
ELP	elastin-like polypeptide
ER	endoplasmic reticulum
EXST	extracellular part of GCPII containing AAs 44-750
EXSTL	extracellular part of NAALADaseL containing AAs 28-740
FBS	fetal bovine serum
FMN	flavin mononucleotide
GCPII	glutamate carboxypeptidase II
GCPIII	glutamate carboxypeptidase III
GFP	green fluorescence protein
GST	glutathione-S-transferase protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IMAC	immobilized metal-affinity chromatography
IOCB	Institute of Organic Chemistry and Biochemistry
MBP	maltose-binding protein
MOPS	4-morpholinepropanesulfonic acid
NAAG	N-acetyl-L-aspartyl-L-glutamate
NAALADase	N-acetylated-α-linked-acidic dipeptidase protein

NAALADaseL	N-acetylated-α-linked-acidic dipeptidase-like protein
NMR	nuclear magnetic resonance
NTA	nitrilotriacetic acid
NusA	N-utilization substance A
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pGAPase	pyroglutamyl aminopeptidase
PHB	polyhydroxybutyrate
2-PMPA	2-phosphonomethylpentandioic acid
Qcyclase	glutamine cyclotransferase
RFU	relative fluorescence unit
RNA	ribonucleic acid
RNaseA	ribonuclease A
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SUMO	small ubiquitin-related modifier
TAP	tandem affinity purification
TEMED	N,N,N',N'-tetramethylethylenediamine
TEV	tobacco etch virus
Tris	tris(hydroxymethyl)aminomethane

9. APPENDIX

Table 13: Names, sequences, and sizes of used primers.

Name	5'-3' Sequence	size
FNAL28	aaa tga tca atc ccc aaa aaa gcc aac tca ctg gc	35 bp
RNAL740ST	ttt ctc gag tca tca gag gtc agc cac agg cc	32 bp
FBirABglII	ctc ggg aga tct atg aag gat aac acc gtg cc	32 bp
RBirAXhoIXbaI	tct aga ctc gag tta cag ctc atc ttt ttc tgc act acg cag gg	44 bp
FBirAKpnI	atc ggg gta cca tga agg ata aca ccg tgc c	31 bp
RBirANoKDELXhoI	tct aga ctc gag tta ttc tgc act acg cag gg	32 bp
Avi0F	aaa aat gat cag gcc tga acg aca tc	26 bp
Avi1R	atc ttc tgg gcc tcg aag atg tcg ttc agg cc	32 bp
Avi2F	ttc gag gcc cag aag atc gag tgg cac g	28 bp
Avi3R	ggt tct cgc tgc cgc tgc cgc tgc cct cgt gcc act cg	38 bp
Uni3F	gca gcg gca gcg aga acc tgt act tcc agg gca gat ctg aat tc	43 bp
Uni4R	ttt ttg aat tca gat ctg c	19 bp
MT	cat ctc agt gca ata aa	17 bp
BGH	tag aag gca cag tcg agg	18 bp
LSecNA1	ctc ctg cca ccg gac tga gg	20 bp
LSecNA2	cgg aac ttt ggc ccc agc c	19 bp
LSecNA3	ggg tgc agg gga cgc ccc c	19 bp

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Svoluji k zapůjčení této práce pro studijní účely a prosím, aby byla řádně vedena evidence vypůjčovatelů.

Jméno a příjmení s adresou	Číslo OP	Datum vypůjčení	Poznámka