Dual Chain Avidins – Useful Tools for Fusion Protein Applications

MASTER'S THESIS
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Sivumäärä:	95 sivua
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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Tämän työn tavoitteena oli tutkia kaksiketjuhybridiavidiinien (dchAvd) ligandinsitomisominaisuuksia sekä rakennetta ja pohtia niiden käyttökelpoisuutta bioteknologisissa sovelluksissa. Muita tavoitteita oli optimoida näiden fuusioproteiinien tuottoa ja puhdistusta. Työn lähtökohtana toimivat dcAvd–rakenteet, joissa $cpAvd_{5\longrightarrow 4}$ on yhdistetty streptavidiinista, AVR2– tai AVR4–proteiinista valmistettuun $cpAvd_{6\longrightarrow 5}$ sirkulaarisesti permutoituun muotoon (dcASA, dcAA2, dcAA4). Näiden proteiinien sekvenssit sekä niiden biokemialliset ominaisuudet poikkeavat avidiinista eri tavoin, joten tämä joukko tarjoaa hyvän arviointiperustan kaksiketjuhybridiavidiinien toiminnallisuuden arvioinnille.

Tutkimusmenetelmät: rekombinanttiproteiineja tuotettiin *E. coli*:ssa pullokasvatus-menetelmällä sekä pienen skaalan fermentoinnilla ja Bac–to–Bac–hyönteis-solutuottosysteemillä. Proteiinit puhdistettiin affiniteettikromatografisella menetelmällä. Proteiinien puhdistumista, saantoa ja puhtautta seurattiin SDS–PAGE:n ja immunoblottauksen avulla. dcAA4:n biotiinin sitomisominaisuuksia tutkittiin fluoresenssispektroskopian avulla ja käyttäen Biacore optista biosensoria. dcAA4:n oligomeerisyyttä tutkittiin HPLC:n avulla ja sen heterogeenisyys pyrittiin osoittamaan immunologisilla kokeilla, joissa käytettiin monoklonaalisia vasta–aineita kullekin rakennuspalikalle.

Tutkimustulokset: dcAA4 tuottui paremmin kuin dcAA2 ja dcASA *E. coli* –pullokasvatuksessa. dcAA4 tuottui hyvin *E. coli* –fermentoinnilla. Hyönteissolutuotossa kaikki fuusio proteiinit tuottuivat heikosti, eikä niitä saatu eristettyä. dcAA4:n rakenne HPLC:n mukaan oli pseudotetrameeri. dcAA4 sitoi biotiinia vahvasti, mutta heikommin kuin avidiini ja AVR4. Sekä avidiinin että AVR4:n monoklonaaliset vasta–aineet tunnistivat dcAA4:n.

Johtopäätökset: Ligandinsitomistutkimusten perusteella dcAA4:ssä oli kaksi erilaista biotiinin sitomistaskua. Toinen taskuista sitoi biotiinia vahvemmin ja toinen heikommin. Vahva sitomistasku muistutti avidiinin sitomistaskua. dcAA4 on heterogeeninen, pseudotetrameerinen proteiini. dchAvd:t ovat lupaavia fuusioproteiineja bioteknologian erilaisiin sovelluksiin. Niiden käyttömahdollisuudet ovat laajoja, materiaalien ristisilloittajista diagnostisiin menetelmiin.

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Abstract

Background and aims: The aims of this study were to investigate the ligand binding properties and structure of dual chain hybrid avidins (dchAvd) as well as to discuss their applicability in biotechnological applications. Another aim was to optimize the production and purification of these fusion proteins. The study focused on dchAvd structures where a $cpAvd_{5\rightarrow4}$ is genetically combined with a $cpAvd_{6\rightarrow5}$ streptavidin, AVR2 or AVR4 protein (dcASA, dcAA2, dcAA4). The sequences and biochemical properties of these proteins differ from those of avidin thus serving as a good evaluation basis for the functionality of dual chain hybrid avidins.

Methods: dchAvd were produced in *E. coli* in bottle and in pilot–scale fermentor as well as in Bac–to–Bac insect cell production system. The proteins were purified with affinity chromatography and the purification, yields and purity were monitored by SDS–PAGE and Western Blot. The biotin binding properties of dcAA4 were studied with fluorescence spectroscopy and Biacore optical biosensor. The oligomerization state of dcAA4 was studied with HPLC and its heterogeneity was shown by immunological methods where monoclonal antibodies against avidin and AVR4 were used.

Results: dcAA4 was produced significantly better than dcAA2 and dcASA in *E. coli* bottle production. dcAA4 was produced very well in the fermentor. All three proteins were produced to certain extent in insect cell production system but none of them could be isolated. According to HPLC measurements dcAA4 is a pseudotetramer. dcAA4 bound biotin with high affinity but with lower affinity than avidin and AVR4. Both avidin and AVR4 monoclonal antibodies recognized dcAA4.

Conclusions: According to the ligand binding studies dcAA4 had two distinct biotin binding sites. The other one bound biotin with higher affinity and resembled wt avidin biotin binding site, and the other one bound biotin with weaker affinity. dcAA4 was a heterogenic, pseudotetrameric protein. The dchAvds are promising fusion proteins for a variety of biotechnological applications raging from material cross–bridging to diagnostics.

Preface

The lab work done in this Master of Science thesis was carried out in the Institute of Medical Technology (IMT), University of Tampere, in Molecular Biotechnology group led by Professor Markku Kulomaa, between August 2007 and May 2008. The writing was done in Helsinki between September 2008 and June 2009.

First I would like to thank my supervisor Professor Vesa Hytönen for the wonderful opportunity to do my thesis under his excellent guidance and knowledge, and for the superior scientific discussion that we had. I would like to thank the whole Molecular Biotechnology group for the wonderful working environment and all the fun we had. Especially I would like to than MSc Juha Määttä, MSc Soili Hiltunen, and MSc Tiina Paldanius for their friendship and support. For practical help with the laboratory work and especially with the insect cell culturing I would like to thank the group's technician Ulla Kiiskinen. For the help in the fermentation experiments I would like to thank technician Jukka Lehtonen.

Huge thanks goes to my dear friends Maria, Alise, Kaisa, Anmi, Anna Koskela, Kim, Arnevi, Iida, Johanna, Mari, Paula and Outi for making my life fun and interesting!

I would like to thank my loving parents Outi and Olli, and their spouses Tom and Jutta for the love and support they have given me during my studies. I want to thank my sister Saija for being the best sister in the whole world, and her family, especially my god son Elvis for all the fun we have had. I need to thank my grand parents Mani–pappa and Mili–mummi for their endless love and support.

Last but not least I want to thank my love Alari for standing next to me and believing in me, Nona for bringing light into my life, and Hugo for reminding me of how to live at this moment. I love you so much!

Content

Abbreviations

AMA–1	Apical Membrane Antigen 1
APS	ammoniumpersulfate
Avd	avidin
AVR2	avidin related protein 2
AVR4	avidin related protein 4
BBP	biotin binding protein
BCIP	5–bromo–4–chloro–3–indolyl phosphate
BFP	blue fluorescent protein
BSA	bovine serum albumin
cpAvd	circularly permutated avidin
CSCP	cellulose to starch cross–bridging protein
dcAA2	dual chain avidin–AVR2
dcAA4	dual chain avidin–AVR4
dcASA	dual chain avidin–streptavidin
dcAvd	dual chain avidin
dchAvd	dual chain hybrid avidin
DNA	deoxyribonucleic acid
dsDBP	double stranded DNA binding protein
dsDNA	double stranded DNA
ELP	elastin–like polypeptide
GFP	green fluorescent protein
GOx	glucose oxidase
GAM–AP	goat–anti–mouse–alkaline–phosphatase
HNTV	hantaan virus
HCMV	human cytomegalovirus
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
IPTG	isopropyl–β–Dthiogalactopyranoside
MDS	microsphere–based detoxification system
MSP1	Merozoite Surface Protein 1
NBT	nitro blue tetrazolium
OD	optical dencity
PCR	polymerase chain reaction
PfCP–2.9	*P. falciparum* chimeric protein 2.9
PBS	phosphate buffer saline
SA	streptavidin
SCWP	secondary cell wall polymerase
SDS–PAGE	sodiumdodecylsulphate–polyacrylamide gel
SIT	specific immunotherapy
SPR	surface plasmon resonance
TEM	transmission electron microscopy
TEMED	*N, N, N', N'* –tetramethylethyldiamine
wt Avd	wild type avidin

1 Introduction

Fusion proteins are a diverse group of engineered proteins. They are widely used for visualization of protein trafficking, folding and localization, in protein production and purification, in diagnostic applications, as drugs and drug candidates, as building blocks in nanobiotechnological applications and in conventional molecular biology amongst many other application in life sciences (Breitwieser et al. 2002, Levy et al. 2003, Walsh 2003, Bohle et al. 2004, Verkhusha and Lukyanov 2004, Wang et al. 2004, Heyman et al. 2007 and Conley et al. 2009). A protein that has been engineered to contain an additional oligopeptide (e.g. His–tag) is called a fusion protein as well as a protein containing two fully functional polypeptides (Heyman et al. 2007 and Zayakina et al. 2009). This means that the concept of fusion protein includes a wide variety of bi– or multifunctional proteins, regardless of the nature of the fusion partners; form peptide–protein fusions to protein–protein fusion and everything in between.

Probably the most used and best known protein fusions are those containing green fluorescent protein (GFP) originally found from jelly fish *Aequorea victoria* and its variants such as the blue fluorescent protein (BFP) (Prasher et al. 1992, Verkhusha and Lukyanov 2004 and Shaner et al. 2005). GFP is used as a fusion partner to visualize the localization, trafficking or folding of the protein of interest in a living cell (Verkhusha and Lukyanov 2004). It can be tagged to either end of the investigated protein and it has been shown that in numerous cases the GFP tag does not affect the function or targeting of the investigated protein (Gerdes & Kaether 1996).

Another widely used application of fusion proteins is the production and purification of recombinant proteins. The fusion partner of the desired recombinant protein serves to improve the production and/or facilitate the purification of the protein otherwise produced or purified in low quantities (Conley et al. 2009). In the case on improving the production of the desired recombinant protein the fusion partner can facilitate the expression of the fusion protein or facilitate the secretion of the fusion protein (Nyyssönen et al. 1993 and Paloheimo et al. 2003). Facilitation of the purification

step can be gained by using fusion partners with significant affinity towards a small molecule, polymers or metals (Glichuk and Volcov 2006, Govender et al. 2007 and Jiang et al. 2009). These affinities establish the basis for affinity chromatographic purification. Other specific and efficient purification methods like two phase extraction can be used for certain proteins with amphiphilic characteristics (Linder et al. 2004). These proteins and thus this method can be applied to fusion protein purification (Linder et al. 2004). A common nominator in above cases is that the function of the fusion partner is no longer needed after the protein of interest is purified. The fusion construct will usually have a small cleavage site in between the two fusion partners that allows the protein of interest to be cleaved from its partner.

The review of literature of this thesis will cover some examples of different kinds of fusion proteins from those used in every–day laboratory to fusion proteins used in nanobiotechnological applications and in medical and diagnostic applications. The experimental part of the thesis will describe methods of production and purification of three fusion proteins as well as the biophysical and biochemical analyses done to investigate the functional properties of one fusion protein. The three fusion proteins were different dual–chain hybrid avidins (dchAvd). These proteins are thought to have applicability in especially nanobiotechnology as building blocks in 3D structures since they pose interesting ligand binding properties and the binding properties could be modified quite easily.

2 Review of literature

2.1 Applicability of fusion proteins

As already discussed fusion proteins are widely used in almost all fields of cell and molecular biology. The benefit of fusion protein technology is the ability to add functions to proteins or enzymes without affecting their original function. This feature is the key and success of fusion proteins. The following chapter will discuss some widely used and commercially available fusion proteins and those that have been published and found useful but are not yet in the market for the big public.

2.1.1 Fusion proteins in conventional cell and molecular biology

GFP

The Nobel price winning fusion partner GFP and its derivatives are used in cell biological application in many different ways (Gerdes and Kaether 1996). This concept allows the monitoring of protein localisation, folding and trafficking in real time in living cells as well as the monitoring of protein production and production efficacy (Verkhusha and Lukyanov 2004 and Conley et al 2009). The GFP has been genetically modified to emit different wavelengths of visible light making the derivatives fluoresce different colours (Shaner et al. 2007). A wide spectrum of colours is commercially available ranging from green, cyan, blue, purple, yellow, and red to orange (Verkhusha amd Lukyanov 2004). This makes the simultaneous imaging of two or more proteins possible: Furuno and Nakanishi used GFP and its derivatives to visualize, track and quantify molecules and events in living mast cells. They investigated interactions between neurons and mast cells, the dynamic processes performed by intracellular signalling molecules and cellular structure. The aim was to provide basic yet valuable information for allergy and clinical immunological research (Furuno and Nakanishi 2005).

Protein production and purification

Another already mentioned and well known application of fusion proteins is affinity chromatographic purification. For example the (strept)avidin–biotin technology which will be discussed in detail later provides a good template for affinity chromatographic purification of the protein of interest (Laitinen et al. 2006). Avidin binds biotin and its derivatives such as 2–iminobiotin very tightly. This property can be used to specifically tag the fusion protein and separate it from a mixture of proteins. If the avidin part of the fusion protein is used only for the purification step, it can be coupled to biotinylated affinity matrix and proteolytically cleaved leading to highly purified recombinant protein. If the avidin–biotin binding is desired later on the fusion protein can be eluted as a whole and bound to biotinylated targets. An interesting purification method for recombinant proteins is the surfactant–based two–phase extraction system where the recombinant protein is fused to hydrofobin (Linder et al. 2004). Hydrofobins are proteins of filamentous fungi and they form layers, for example, on fungal cell walls or spores. They are moderately hydrophobic proteins but they are amphiohilic and extremely surface active. These characteristics are useful in the two–phase extraction system. The hydrophobin part of the fusion protein can be cleaved away or used to immobilize the recombinant protein onto hydrophobic surfaces. The two–phase extraction system is extremely good for recombinant proteins produced in large scale since it is cheaper than affinity chromatographic methods (Linder et al. 2004).

Some companies have established commercially available production systems where the recombinant protein is produced as a fusion partner with a specifically targeted protein or a peptide. The Canadian company Sembiosys Genetics Inc. (www.sembiosys.ca) offers a system in which the recombinant protein is fused to oleosin and produced on oilseed crops (Parmenter et al. 1995). These fusion proteins accumulate in oil bodies and can be extracted using a simple extraction method. The protein can be released from the fusion partner by protolytic cleavage. The Spanish company ERA Biotech (www.erabiotech.com) has established a production system where the protein is fused with a storage protein derived peptide, which accumulates host–independently in ER–derived protein bodies that can be separated by their high density. A similar idea works with elastin–like polypeptides (ELP) that simplify the

purification procedure of recombinant proteins. ELPs undergo reversible thermal denaturation and can be used for temperature based non–chromatographic separation of the fusion protein (Meyer and Chilkoti 1999). ELPs have been also reported to enhance the production levels of recombinant proteins in plants (Scheller et al. 2006, Patel et al. 2007 and Conley et al. 2009).

DNA polymerase and the polymerase chain reaction

The polymerase chain reaction (PCR) is a widely utilized every–day laboratory method in molecular biology. Wang and colleagues showed that fusion protein technology can be use to enhance the performance of both family A and family B DNA polymerases. The fusion protein contained a sequence non–specific dsDNA binding protein (dsDBP) fused to a DNA polymerase. The Sso7d dsDBP from *Sulfolobus solfataricus* contained point mutations to enhance its DNA binding properties. The fusion polymerase was reported to have increased processivity without compromising the enzymatic activity and since the processivity was concluded to be the limiting characteristic of the enzyme activity the fusion protein showed profound advantages compared to unmodified enzymes (Wang et al. 2004). The Finnish company Finnzymes (www.finnzymes.fi) offers a fusion DNA polymerase (Phusion® DNA polymerase) that has enhanced processivity and improved performance compared to other DNA polymerases on the market.

Phage display

Phage display is one of the earliest yet dominant display platforms for screening protein–protein interaction and affinities (Smith 1985). In phage display the gene of interest or a DNA fragment is inserted into the filamentous phage capsid protein gene creating a fusion protein that is incorporated into the virion. The virion retains infectivity and displays the recombinant protein in immunologically accessible form together with the gene sequence in its genome. These fusion phages can be subjected to binding selection rounds where a desired ligand (small molecule, antigen, protein) is immobilized on to a surface and the fusion phages are bound to them. After binding the non–bound phages are washed away. The bound phages are then pooled and amplified in bacterial host cells (figure 1) (Sachdev et al. 2007). The gene encoding the protein can be sequenced and subsequently expressed to produce the protein. The

collected phages can also be subjected to new selection rounds and screened for even better binding characteristics, so called molecular evolution. This is possible because each amplification step involves many errorprone PCR cycles, large library size and rapid panning cycles (Rothe et al. 2006).

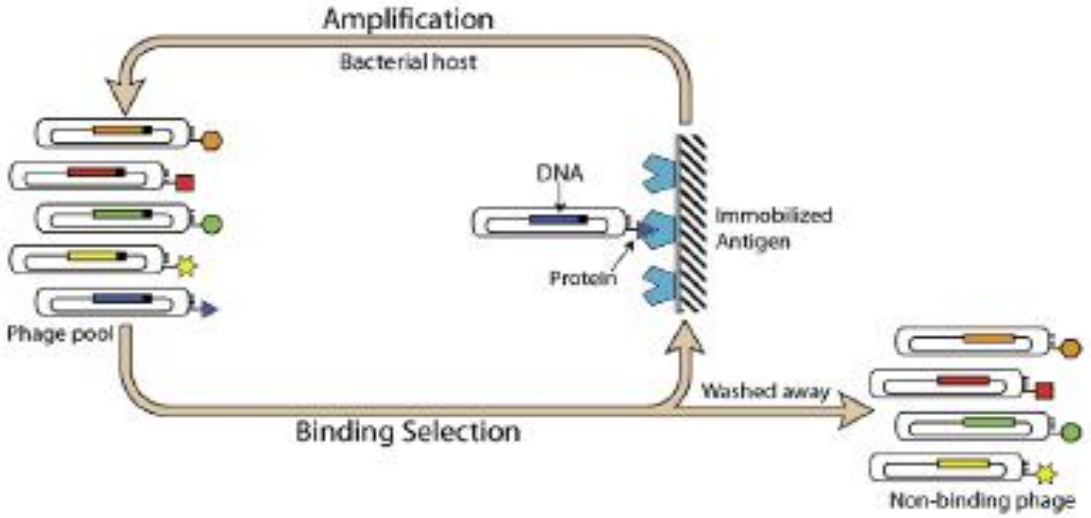


Figure 1. A schematic drawing of the selection of binding protein from phage display library. *The binding proteins are displayed on the surface of the phage particles as fusions to coat proteins (binding proteins as coloured, coat proteins as black). Ligand–specific proteins bind to immobilized ligand and non–binding phages are washed away. Retained phage can be amplified by a bacterial host, and the amplified pool can be cycled again to further enrich for ligand–binding clones. Individual binding clones can be subjected to DNA sequencing (Figure from Sachdev et al. 2007).*

2.1.2 Fusion proteins in medical applications

Fusion proteins have potential in medical applications for allergy treatment (Bohle et al. 2004), for treatment of autoimmune diseases (Völlenkel et al. 2004), as gene delivery vectors in gene therapy (Räty et al. 2004) and as vaccines (Hu et al. 2008, Hao et al. 2008 and Zhong et al. 2008). Another approach to utilize fusion proteins in the medical field is as tools to improve the performance of widely used diagnostic methods such as immunosorbent assays (Breitwieser et al. 2002, Ilk et al. 2002, Nomellini et al. 2007).

Diagnostics and immunoactive reagents

For many diagnostic approaches a combination of for example self–assembling module and immunological module could offer many advantages over the bare immunological module. The self–assembling fusion partner could be orientated onto

a specific surface in such a way that all of the immunological parts of the fusion proteins stand out in correct orientation and available for binding. Some examples of these fusion proteins are presented by Sára and colleges (Breitwieser et al. 2002, Ilk et al. 2002) and Nomellini et al. (Nomellini et al. 2007). In all the tree cases a recombinant bacterial cell surface layer (S–layer) protein was fused to an allergen or an immunoglobulin G (IgG) –binding protein G.

Crystalline bacterial cell surface layers termed as S–layers represent the outermost structure of many bacteria and archea (Sleytr and Beveridge 1999, Sára and Sleytr 2000). S–layers are two dimensional arrays of identical protein or glycoprotein subunits varying in size between 40 to 200 kDa (Sleytr and Beveridge 1999). The S–layer lattices are arranged in oblique, square or hexagonal symmetry (figure 2) and the S–layer subunits are linked to each other and to the underlying cell envelope layer by non–covalent interactions.

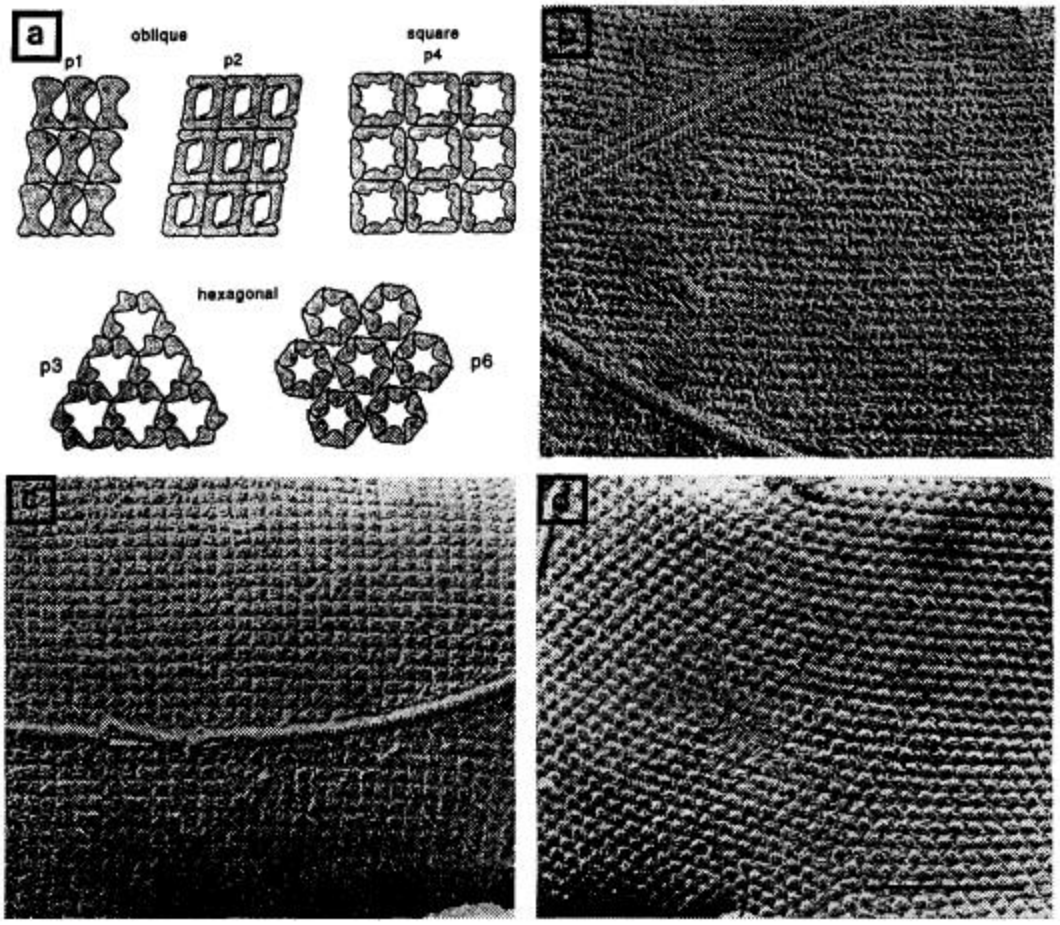


Figure 2. S–layer lattice types. *A schematic drawing of different S–layer lattice types. The number in the nomenclature of the lattices (for example p1) indicates how many proteins form the lattice unites (a). Electron micrographs of oblique S–layer lattice (b), square S–layer lattice (c) and hexagonal S–layer lattice (d) (Figure from Sleytr & Sára 1996).*

The N–terminal part of the S–layer subunit of members of the family *Bacillaceae* was found to play a role in anchoring the S–layer subunits to cell wall via a distinct secondary cell wall polymerase (SCWP) (Lemaire et al. 1998). Many isolated S–layer proteins have the ability to recrystallize into regular, two dimensional monolayer structures on solid surfaces such as silicon wafers, gold chips, silanized glass or plastic materials, as well as on Langmuir lipid films, liposomes and at the air–water interface (Lupas et al. 1994, figure 3). There are pores of identical size and morphology passing through the S–layer self–assembly products and functional groups show a regular and high density distribution trough out the whole lattice. These properties as well as the ability to bind SCWP make the S–layer proteins suitable and convenient tools for applications in biotechnology, molecular nanotechnology, biomimetics (Sleytr and Beveridge 1999) and solid–phase immunoassays (Breitweiser et al. 1996 and Breitweiser et al. 1998).

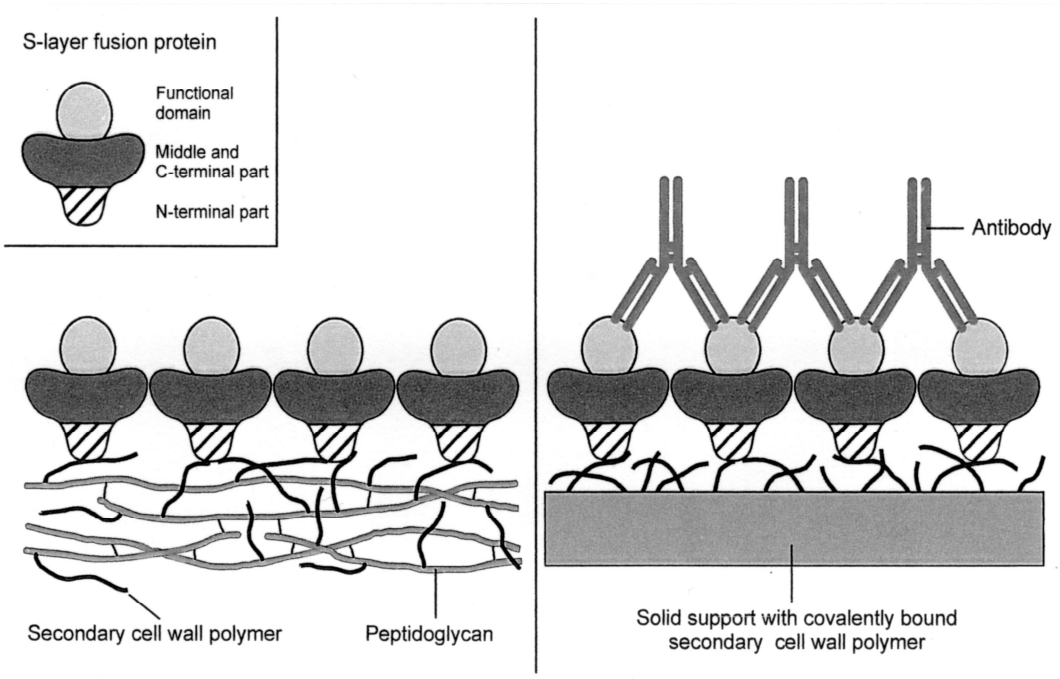


Figure 3. A schematic drawing of S–layer fusion protein attached to SCWP. *Drawings showing the formation of oriented functional S–layer lattices on peptidoglycan–containing sacculi on the left hand side and artificial supports coated with secondary cell wall polymer on the right hand side (Figure from Ilk et al. 2002).*

The fusion proteins presented by Nomellini et al., Breitweiser et al. and Ilk et al. were all demonstrated to assemble into regular structured protein lattices. The functional sequences were shown to be on the outer most part of the lattices aligned in predefined distances in the nanometer range available for further binding. (Breitwieser et al. 2002, Ilk et al. 2002, Nomellini et al. 2007) These properties can be thought as great advantages for example in immunobased–assays used in diagnostics because of the high density of correctly orientated active molecules on solid surface. They could broaden the use of diagnostic methods and cut down the coasts of health care by fastening the procedures and by making the reaction volumes and areas smaller (biochips) (Jain 2008).

Drugs and vaccinations

A fusion protein of *Geobacillus stearothermophilus* ATCC 12980 S–layer protein SbsC and Bet v1 was constructed by Breitweiser and colleagues (Breitwieser et al. 2002) and investigated in more closely by Bohle and colleagues as a specific allergen candidate for specific immunotherapy (SIT) of type I allergy (Bohle et al. 2004). It

was previously shown that IgE of birch pollen –allergy patients recognized the fusion protein (Breitwieser et al. 2002). The traditional SIT–treatment is limited in its effectiveness, which may in part be due to the nature of the current vaccines. The vaccines consist of crude allergen extracts adsorbed to aluminum hydroxide. The allergen is present in a complex mixture of allergenic and nonallergenic molecules, which makes it difficult to standardize. Aluminium hydroxide was shown to support the T_H2–like immune response rather than the more balanced TH1–like immune response (Wiedermann et al. 1998). These matters make the attempts to improve the allergenic molecules as well as the adjuvants of SIT–treatment justifiable. The fusion protein presented by Bohle et al. can be produced in recombinant form which facilitates the standardization and guarantees the invariable immunological characterization. Further more they have showed in their previous work that natural S–layers as adjuvants represent a promising tool to promote the desired T_H1–like response in SIT (Jahn–Schmid et al. 1997). In conclusion, a fusion protein of a major allergen and a bacterial S–layer protein resulted in a hypoallergen that was able to modulate the allergen–specific T_H2–dominated cellular response to a more balanced phenotype as well as enhanced the production of IL–10 (Bohle et al. 2004).

Völlenkle and co–workers designed a fusion protein for development of specific adsorbent for extracorporeal blood purification. The fusion protein comprised of a functional C–terminally truncated version of S–layer protein SbpA from *Bacillus sphearicus* CCM 2177 and two copies of the synthetic analogue of immunoglobulin G (IgG)–binding B–domain of protein A of *Staphylococcus aureus*, called the Z–domain (Völlenkle et al. 2004). The fusion protein (figure 4, panel c) was recrystallized on cellulose–based microbeads of diameter of 3 µm that were coated with SCWP (figure 4, panel b) to prepare biocompatible microparticles for microsphere–based detoxification system (MDS) which is a treatment that is used for removal of IgG from plasma of patients suffering from autoimmune disease. In MSD the plasma is recirculated into a filtrate compartment of the module and the addition of microparticles to the plasma circuit allows rapid removal of the pathogenic substance (figure 4, panel a) (Webber et al. 1994). The endotoxin content of the recrystallization products was very low and no cytotoxicity was detected. A 20 times higher IgG binding capacity of the DMP–treated S–layer fusion protein microbeads was observed compared to commercial adsorbents. In addition, a further advantage in

comparison to commercial adsorbents was noted in the fact that the ZZ–domains were exposed on the surface of the microbeads and diffusion–limited binding events could be excluded. Völlenkle and colleagues concluded that the use of biocompatible cellulose–based microbeads as carriers for the fusion protein, the possibility to cross–link the S–layer lattice, the high levels of IgG still bound, and the regeneration of the microbeads without losing the binding capacity were extremely promising for their use as immunosorbents in applications such as the MDS (Völlenkle et al. 2004).

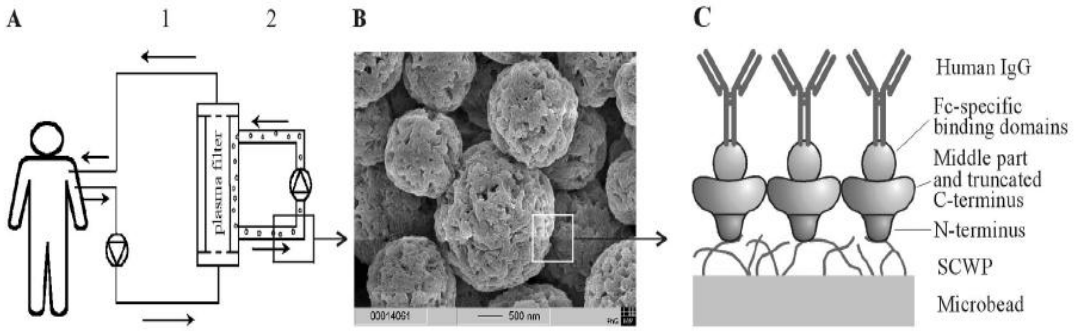


Figure 4. MSD. *A schematic drawing of the MDS system, showing the primary circuit (1) containing the whole blood of the patient and the secondary circuit (2), which contains the patients plasma and the fusion protein –coated microbeads, on which the IgG is bound (A). Scanning electron micrograph of the cellulose microbeads used for recrystallization of the fusion protein (B). Schematic drawing showing the S–layer fusion protein on microbeads precoated with SCWP and binding of IgG to the ZZ–domains (C) (Figure from Völlenkle et al. 2004).*

Räty et al. constructed an avidin–displaying baculovirus (Baavi) for enhanced, virusmediated gene delivery for gene therapy. The avidin sequence was incorporated into baculovirus envelope glycoprotein gp64 gene. It was shown to be fully functional and located on the surface of the baculovirus (Räty et al. 2004). This system exploits the avidin–biotin technology discussed later and brings it together with the viral gene delivery system. Baavi was shown to enhance transduction efficacy on its own. Further enhancement was gained by using biotinylated ligands as targeting molecules (Figure 5).

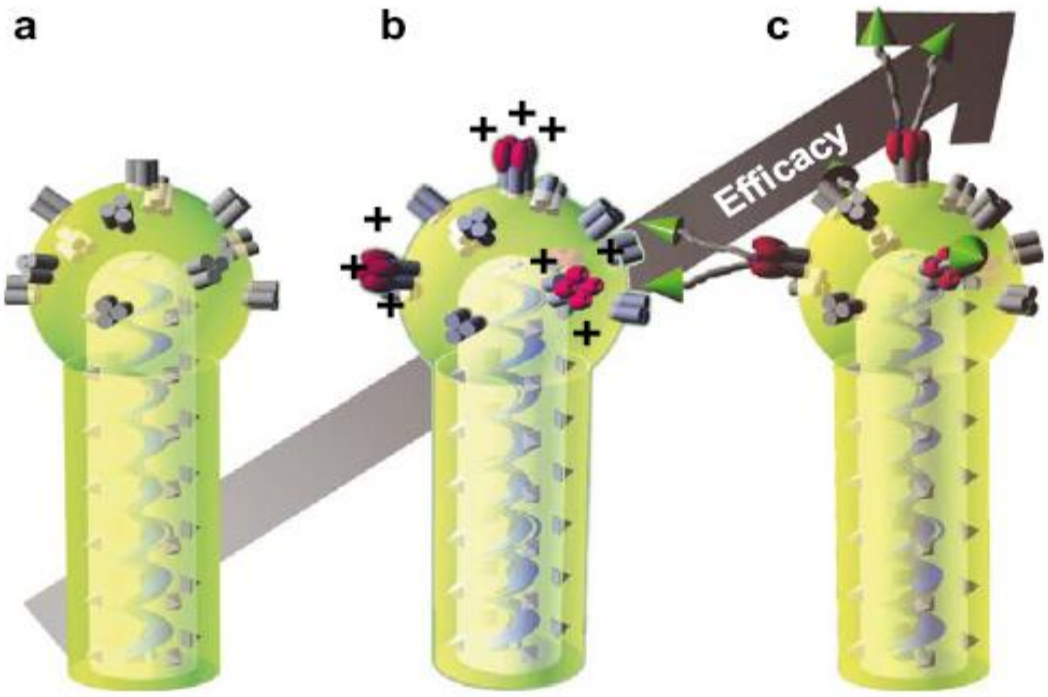


Figure 5. A schematic presentation of wild–type baculovirus and Baavi. *The wt baculovirus (a). Baavi with avidin (red) on the surface of the virus (b). Biotinylated ligands (green arrowed gray sticks) bound to the avidin moiety on the surface of the Baavi (c) (Figure from Räty et al. 2004).*

In their work Räty and colleagues referred that cationic molecules are able to cause adsorbtive endocytosis following the intake of the gene delivery vector. Thus, avidin having high net positive charge in buffers and media of pH 7.4 was concluded as an enhancer for transduction. In their work they tested the idea of versatile Baavi modification with biotinylated molecules to further enhance targeting and transduction by using biotinylated epidermal growth factor (EGF). An expected, specific targeting of Baavi to cells over expressing EGF receptor was detected. They concluded that the possibility of covering Baavi with a desired biotinylated molecule including peptides, proteins and other smaller molecules creates a broad range of opportunities to further improve Baavi as a gene delivery vector (Räty et al. 2004).

The characteristics of fusion proteins can also be exploited in vaccines. Hu and co–workers designed a vaccine against malaria that contained two small sized antigens of *Plasmonium falciparum* called the *P. falciparum* chimeric protein 2.9 (PfCP–2.9) (Hu et al. 2008). The fusion protein construct held a 19 kDa part of the 200 kDa Merozoite Surface Protein 1 (MSP1) called MSP1–19 and a three–domain substructure of the 83 kDa Apical Membrane Antigen 1 (AMA–1) called AMA–1

(III). MSP1 and AMA–1 are two leading asexual blood–stage vaccines candidates for malaria (Good et al. 1998). Both MSP1–19 and AMA–1 (III) can be recognized by the inhibitory antibodies but their small size may limit the ability of each alone to induce the high titer of antibodies required to be effective *in vivo*. PfCP–2.9 was found to induce the production of inhibitory antibodies in rabbits and monkeys and it was tested in healthy adults to evaluate its safety and immunogenicity in humans. The vaccine was found to be safe, well–tolerated and immunogenic. The study was the first clinical trial for PfCP–2.9 and showed promising results for a blood–stage malaria vaccine. The clinical trials were continued (Hu et al. 2008).

Other fusion proteins vaccines have been designed against human cytomegalovirus (HCMV) (Zhong et al. 2008) and against the Hantaan virus (HNTV) (Hao et al. 2008). Zhong and colleagues found out in their study that immunization with the fusion protein vaccine consistently generated strong HCMV–specific CD4+ and CD8+ T–cells which co–expressed INF–γ and TNF–α. They showed also that the vaccine induced a strong virus neutralizing capacity. They concluded that the chimeric HCMV vaccine provides as excellent platform for reconstituting protective immunity to prevent HCMV diseases (Zhong et al. 2008). Hao and co–workers developed a novel chimeric DNA vaccine plasmid against HNTV that carried a fusion protein gene linking IL–2 gene to G2 gene, a glycoprotein gene of HNTV which expressed a 72 kDa fusion protein. In their study they showed that the DNA vaccine induced both humoral and cellular immune response specific for HNTV G2 and that it could ne a candidate vaccine for HNTV infection (Hao et al. 2008).

2.1.3 Fusion proteins in nanobiotechnology

Nanotechnology is a branch of modern technology creating and utilizing materials, devices, and systems by control of matter on the nanometer scale (10^{-9} m). The characteristic feature in all nanotechnological applications is that at least one of the dimensions associated with the structure is measured in nanometers (nm). The diameter of double stranded DNA is approximately 2.5 nm, and the diameter of protein molecules is in range of 1–20 nm. Thus, the components of living cells are of ideal size to applications of nanoscience. The use of biomolecules in nanotechnology gave rise to the term nanobiotechnology. Nanobiotechnology is closely related to

other branches of science and gave rise to such fields of research as nanomedicine and nanobiopharmaceuticals. (Jain 2008, figure 6).

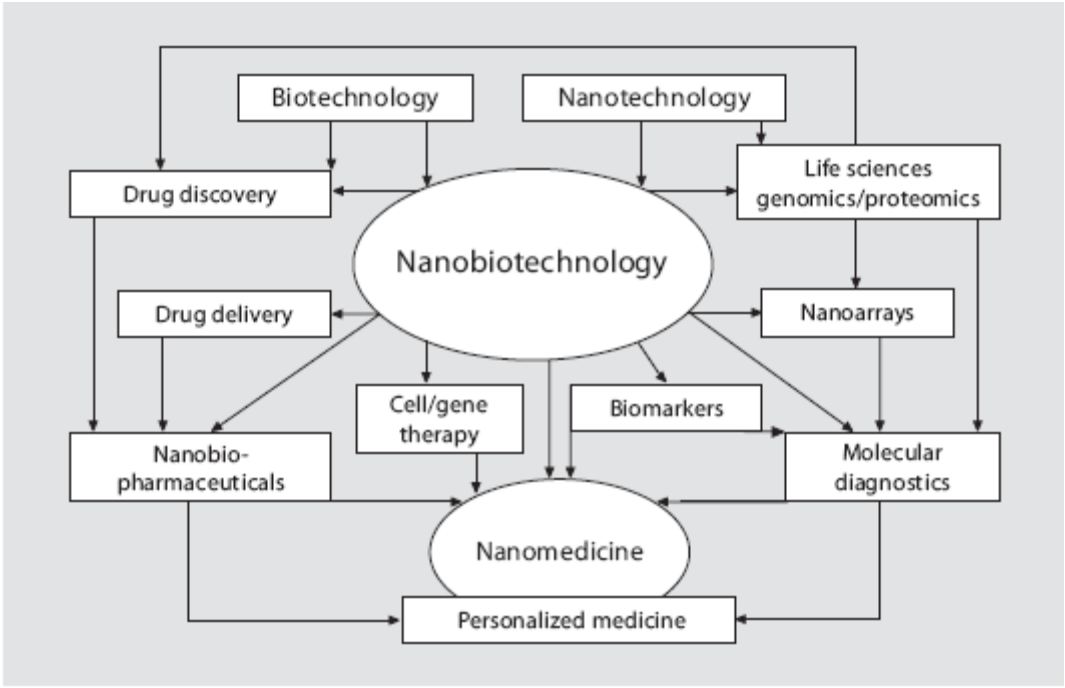


Figure 6. A schematic diagram representing the close relation ship of nanobiotechnology to other nanosciences and biotechnologies *(Figure from Jain 2008).*

Fusion proteins are of practical use in nanobiotecnology especially in composite materials bridging two different materials together (cross–bridging) or coupling nanoparticles, materials and other molecules to solid supports (Asuri et al. 2006 and Levy and Shoseyov 2004). Fusion proteins can be used in nanodiagnostic and other analytical methods for development of nanochips and nanoarrays that are sometimes referred as laboratory–on–a–chip systems (Jain 2008, Jain 2007 and Wu 2008). Biosensor technologies are also fields that can benefit from fusion proteins (Willner et al. 2007). An important feature in proteins used for nanobiotechnological applications is self–assembly. This gives a predictable structure and pattern for the formed nanomaterial and also the ability to control hierarchy in the formed structures. Another useful characteristic is (high) affinity towards a ligand that can be used for targeting of the protein onto the material or even onto a predetermined site on the material.

Some examples of fusion proteins that can be used in nanobiotechnology are a bifunctional starch–cellulose cross–bridge fusion protein by Levy et al., an SP1–

cohesin fusion protein by Heyman and colleagues, and an S–layer–streptavidin fusion protein constructed by Moll and co–workers.

Cellulose to starch cross–bridging protein (CSCP)

Levy and colleagues constructed a fusion protein containing a cellulose binding domain linked via an elastin linker to starch binding domain; cellulose to starch cross–bridging protein (CSCP). The cross–bridging capacity of the CSCP was demonstrated between granular or soluble starch and cellulose. The results showed that CSCP had the ability to cross–bridge granular starch and insoluble cellulose (Whatman filter paper). They showed also that CSCP was able to cross–bridge soluble starch and insoluble cellulose and the use of CSCP in combination with starch increased the tensile strength of paper over 250 % compared to paper treated with only starch (Levy et al. 2004). A variety of biomedical applications utilize starch and cellulose biodegradable polymers. Cellulose has been used as a component for material used for bone regeneration, artificial blood vessels, temporary skin substitutes, haemodialysis membranes and controlled drug release. Starch–based materials have been developed for orthopaedic implants, as bone replacements and for controlled drug delivery. These composites were shown to be biocompatible (Levy et al. 2004). The combination of cellulose and starch through the fusion protein may be extremely useful in various medical procedures when mild conditions and non–toxic chemicals must be used in order to avoid deactivation of accompanying active molecules. Levy et al. concluded that using different fusion proteins containing two different carbohydrate–binding domains will be used to design new class of chimeric polysaccharide–based biomaterials with potentially extended range of physical, chemical and biological properties (Levy et al. 2004).

SP1–GOx fusion protein; a scaffold for self–assemblage of molecules

Heyman and co–workers made an SP1 based fusion protein where a glucose oxidase (GOx) from *Aspergillus niger* was linked to SP1 via a native peptide linker from *A. niger* glycoamylase (GOx–SP1) (figure 7). SP1 is a homo–oligomerc protein complex (148.8 kDa) that was first isolated from aspen plants (*Populus tremula*). The protein complex is composed of 12 subunits (12.4 kDa) that are tightly bound to each other forming a ring structured homo–dodecamer (figure 7, panel a and b). The SP1 rings

tend to stack into nanotubes (figure 7, panel c) and they are resistant to extreme conditions like diverse pHs, high temperatures, organic solvents and various proteases (Wang et al. 2002 and Wang et al. 2006). GOx is a homodimeric glycoprotein (80 kDa per monomer) that catalyzes the oxidation of β–D–glucose. The GOx has considerable commercial importance as it is widely used in food processing, in production of gluconic acid, in quantitative determination of D–glucose in fermentation processes and medical diagnostics and as a pivotal enzyme in biofuel cells (Katz and Willner 2003 and Heyman et al. 2007).

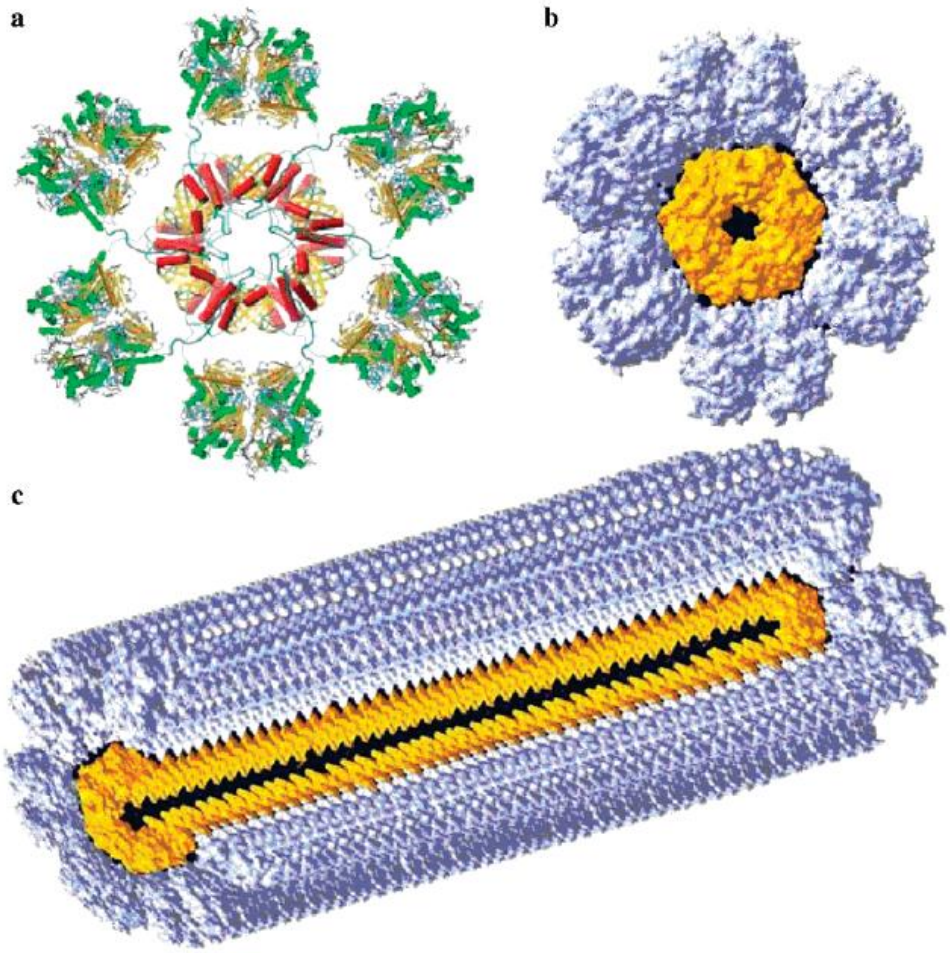


Figure 7. An illustration of GOx–SP1 fusion protein complex. *The dodecamer of SP1 in the centre of the complex and six GOx dimers surrounding it (a and b). An enzyme nanotube particle formed by the stacking of the dodecamers (c) (Figure from Heyman et al. 2007).*

In their results Heyman and co–workers showed that using SP1 as a molecular scaffold has two advantages: firstly the half–life ($T_{½}$) of GOx in GOx–SP1 was increased by a factor of 2 and 10 when compared to recombinant and native GOx, due to the stabilizing nature of SP1, and secondly the controlled stoichiometric ratio of

enzyme through SP1. They demonstrated a 1:12 scaffold–to–enzyme ratio (enzyme monomers) where 90% (w/w) of the total particle consists of active enzyme. In ordinary enzyme immobilization less than 10% of the total mass is immobilized enzyme and the rest constitutes of the substrate material (matrix). A high protein–to–matrix ratio was shown to be clearly advantageous. They also found multienzyme nanotube particles (figure 7, panel c) along with the dodecameric rings holding together 12 GOx monomers (figure 7, panel a and b). The nanotubes contained hundreds of enzymes per tube but still their nanometer range size retained the advantages of single molecular enzymes that remained in the solutions and favoured substrate and product mass–transfer. These complexes were also said to be large enough for removal from solution by standard filtration. In conclusion Heyman and co–workers reported that SP1 is an appealing molecular scaffold for self–assembling not only enzymes but also metal particles, peptides and protein domains creating a nanoplatform for chemical reactions especially reactor–type productions of biomolecules (Heyman et al. 2007).

S–layer–streptavidin fusion protein; self–assembly and high affinity

Moll et al. constructed fusion proteins of S–layer protein SbsB of *Geobacillus stearothermophilus* PV72/p2 and streptavidin of *Streptomyces avidinii*. The aim was to make proteins that could be used as templates for nanopatterned molecular arrays. In their study they designed six different fusion proteins of two different types. Four of the fusion proteins were so called N–terminal fusion proteins with streptavidin fused to the N–terminus of SbsB and two were so called C–terminal fusion proteins with streptavidin attached to the C–terminus of SbsB. The six fusion proteins were refolded to heterotetramers formed by one fusion protein and tree core streptavidins (figure 8).

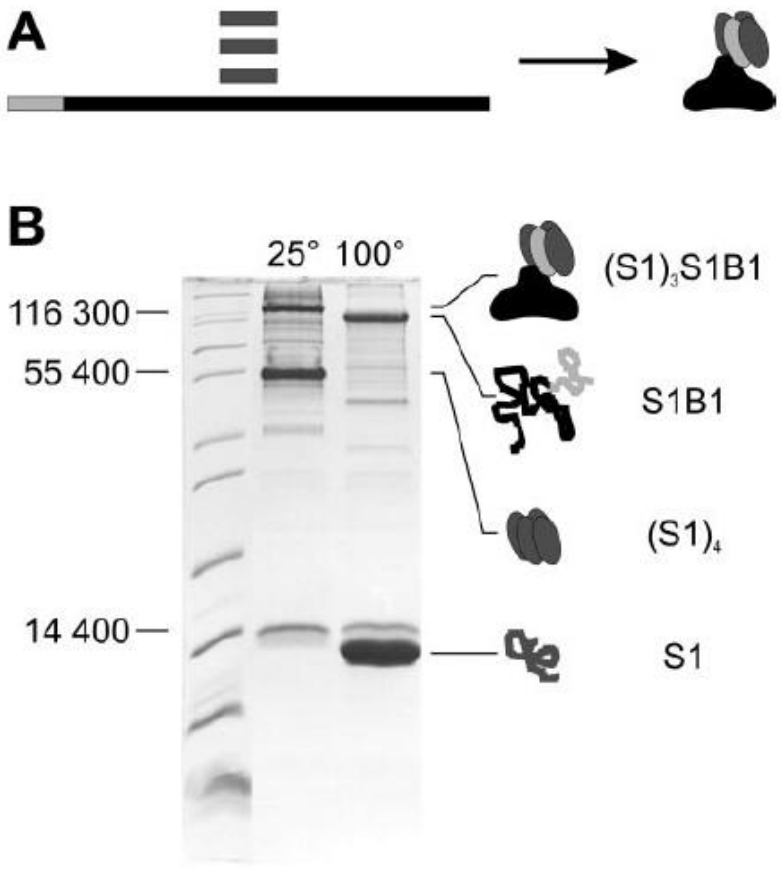


Figure 8. A schematic drawing of refolding of heterotetrameric fusion protein $(S1)_3$S1B1 (A) and analysis by SDS–PAGE (B). *Core streptavidiin (S1, dark grey) and fusion protein S1B1 (streptavidin S1, light gray; SbsB B1, black) (A). SDS–PAGE analysis: Without boiling of the sample (lane 25°C), streptavidin–SbsB heterotetramers (S1)3S1B1 (144.973 Da) and core streptavidin homotetramers $(S1)_4$ (49.840 Da) migrated at apparent molecular masses of 125.000 and 55.000 Da, respectively. After boiling (lane 100°C), the tetramers of both the fusion protein and core streptavidin were denatured to monomers (S1B1: 107.593 Da, S1: 12.460 Da) (B) (Figure from Moll et al. 2002).*

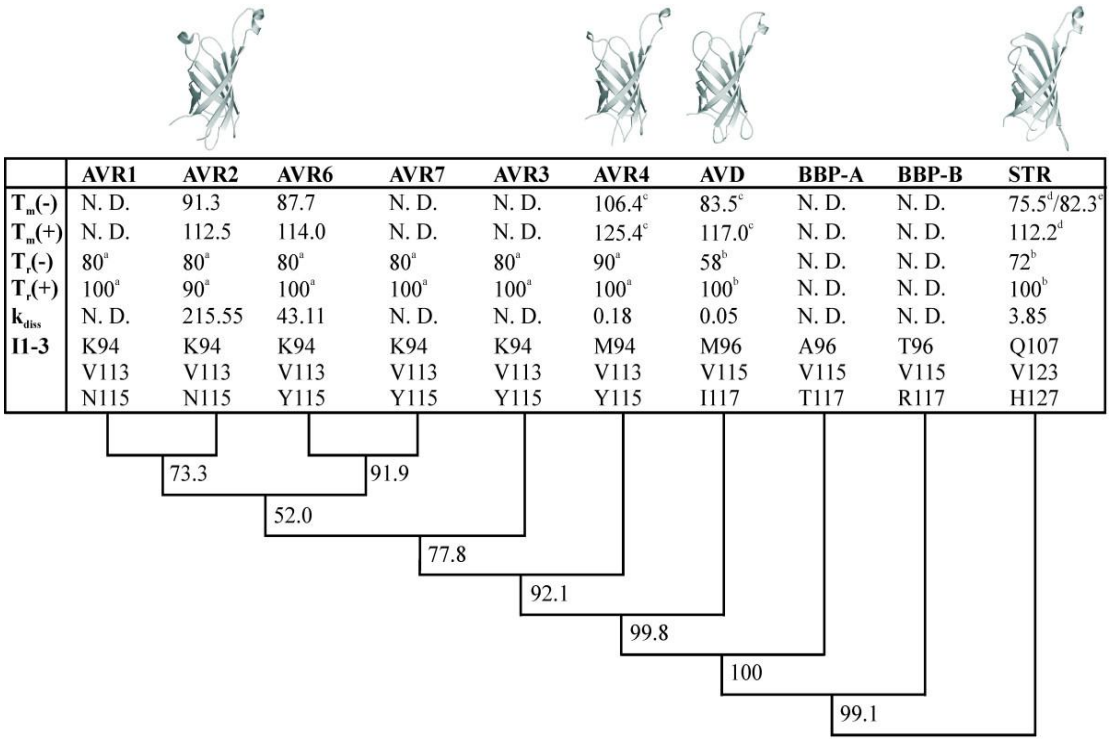
Following characteristics of the chimeric S–layer proteins were investigated: the capability to self–assemble in suspensions, on liposomes, on silicon wafers, and on cell wall fragments, their ability to bind *d*–biotin and two biotinylated marker proteins; ferritin and peroxidase. All four N–terminal heterotetrameric fusion proteins self–assembled into crystalline chimeric S–layer sheets in suspension similar to SbsB. The C–terminal fusion proteins did not self–assemble in suspensions but both of them formed monomolecular lattices on cell wall fragments of *G. stearothermophilus* PV72/p2 that contained SCWP. The lattice parameters were identical to those of SbsB. The biotin–binding properties of the fusion proteins and their self–assembly products were determined by fluorescent titration method. The fusion proteins and their self–assembly products showed an ability to bind ~3 ligands per protein tetramer whereas in the case of streptavidin homotetramer isolated from the same refolding

patch showed a binding ability of ~4 ligands per tetramer. To test whether the streptavidin moieties of the self–assembly products of the fusion proteins were accessible for biotinylated target molecules, biotinylated peroxidase was used as an enzyme marker and biotinylated ferritine was used as a marker that could be visualized by TEM. Over 95 % of the enzyme activity was found from the liposome fraction and less than 5 % of the enzyme activity was found from the supernatant. Liposomes coated with recombinant SbsB showed less than 3 % of the total enzyme activity. The electron micrographs showed dense binding of ferritine to both liposomes and cell wall fragments coated with the fusion protein but virtually no binding to liposomes coated with recombinant SbsB (Moll et al. 2002). Moll and co–workers concluded that the S–layer–streptavidin fusion proteins can be used for the engineering of defined functional structures in the nano sized range that cannot be routinely performed by other micro– and nanofabrication techniques.

Avidins and dual chain avidins; dual affinity molecules

Chicken avidin and its bacterial analog streptavidin (*Streptomyces avidinii*) bind a small vitamin, *d*–biotin, with great affinity ($K_d \approx 10^{-15}$ *M*) (Green 1975). Both avidin and streptavidin are antiparallel β–barrel proteins that assemble to form a tetramer. Each monomer binds one *d*–biotin molecule (Livnah et al. 1993 and Hendrickson et al. 1989). Despite the similarities of the tertiary and quaternary structures of avidin and streptavidin their primary structures are not conserved (Livnah et al. 1993 and Laitinen et al. 1999).

Avidin related proteins AVR2 and AVR4 resemble structurally very much avidin and streptavidin (Eisenberg–Domovich et al. 2005 and Hytönen et al. 2005a) and they form tetramers as well. AVR4 has almost as high affinity towards *d*–biotin as avidin ($K_d \approx 3.6 \times 10^{-14}$ *M*) and it is the closest relative to avidin in the avidin gene family (figure 9). On the other hand it is more thermostable than avidin or streptavidin (Hytönen et al. 2004). The greatest differences of AVR2 compared to avidin and AVR4 are functional; the biotin binding affinity of AVR2 is significantly lower than that of avidin or AVR4 and the isoelectric point (pI) of the protein is ~pH 5.0 compared to the pI of avidin and AVR4 of about pH 10 (Laitinen et al. 2002).



	AVR1	AVR2	AVR6	AVR7	AVR3	AVR4	AVD	BBP-A	BBP-B	STR
T_m(-)	N. D.	91.3	87.7	N. D.	N. D.	106.4[c]	83.5[c]	N. D.	N. D.	75.5[d]/82.3[e]
T_m(+)	N. D.	112.5	114.0	N. D.	N. D.	125.4[c]	117.0[c]	N. D.	N. D.	112.2[d]
T_r(-)	80[a]	80[a]	80[a]	80[a]	80[a]	90[a]	58[b]	N. D.	N. D.	72[b]
T_r(+)	100[a]	90[a]	100[a]	100[a]	100[a]	100[a]	100[b]	N. D.	N. D.	100[b]
k_{diss}	N. D.	215.55	43.11	N. D.	N. D.	0.18	0.05	N. D.	N. D.	3.85
I1-3	K94	K94	K94	K94	K94	M94	M96	A96	T96	Q107
	V113	V113	V113	V113	V113	V113	V115	V115	V115	V123
	N115	N115	Y115	Y115	Y115	Y115	I117	T117	R117	H127

Figure 9. The family tree of avidins'. *The conclusion of the phylogenetics and biochemistry of avidin (AVD) and avidin related proteins (AVR) including chicken biotin binding proteins BBP–A and BBP–B and streptavidin (STR). T_m(–): denaturizing temperature without d–biotin and T_m(+): with d–biotin (°C). T_r(–): transition temperature of oligomerisation without d–biotin and T_r(+): with d–biotin (°C). k_{diss}: dissociation rate constant of d–biotin (× 10^{-6} s^{-1}). I1–3: three important amino acid residues on the faces of the subunits 1 to 3. N.D.: not determined (Figure from Hytönen et al. 2005a). Please note that the structure of BBP–A is also determined by X-ray crystallography and it strongly resembles those of other family members (PDB: 2C1S, Hytönen et al. 2007).*

Dual chain avidin (dcAvd) has been constructed earlier by genetically connecting two circularly permutated avidin monomers (cpAvd) into one polypeptide chain (Nordlund et al. 2004). These pseudodimers form a dimer that is called the pseudotetramer, whish structurally corresponds to the wild type avidin (wt Avd) tetramer (figure 10). Each pseudodimer of dcAvd contains two biotin binding sites. By circular permutation the two termini of a protein's polypeptide chain are connected to each other via a linker and the new termini are usually made to loop areas (Uliel et al. 2001). The circular permutation was done to get the termini of the combinatorial wt Avd monomers close enough to be linked together via a tripeptide linker containing one serine and two glysines (figure 10, panel A). The dcAvd was made by combining $cpAvd_{5\rightarrow4}$ and $cpAvd_{6\rightarrow5}$, where the new termini were made to the loops between β–strand four and five in $cpAvd_{5\rightarrow4}$ and between β–strand five and six in $cpAvd_{6\rightarrow5}$. By investigating the biotin binding properties of cpAvds it was found that $cpAvd_{5\rightarrow4}$ resembled wt Avd. On the other hand the affinity of $cpAvd_{6\rightarrow}$

$_5$ towards biotin had reduced. Comparison of wt Avd and dcAvd did not reveal remarkable change on biotin binding (Nordlund et al. 2004).

In previous studies it has been observed that cpAvds form tetramers by themselves. These tetramers are less stabile than the pseudotetramers that are formed by dcAvds. This can be caused by the stiffness of the formed dcAvd pseudotetramer or by lower number of termini in dcAvd (Hytönen et al. 2005c). From these observations it can be concluded that the properties of the molecules are not fully understood although new information about the structure of dcAvd has been obtained by X–ray crystallography (Hytönen et al. 2006).

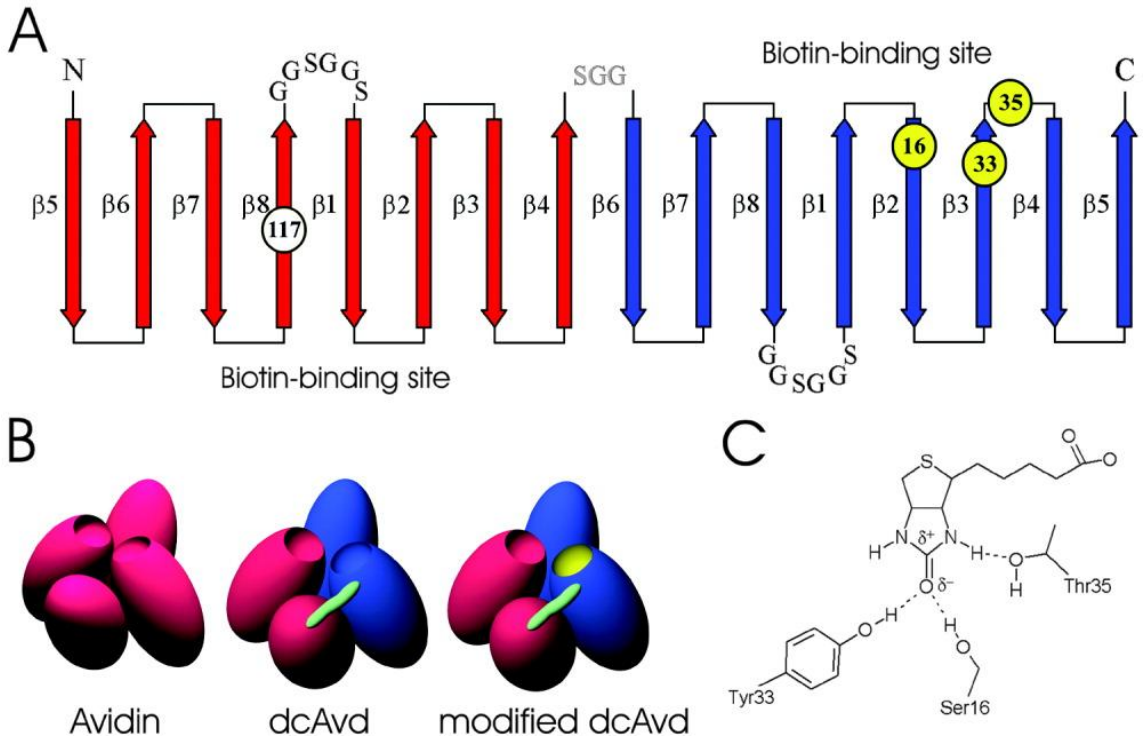


Figure 10. A schematic drawing of the construction of dual–affinity avidins. *Representative drawing of the secondary structure of dcAvd. The original circularly permuted monomers are colored red (cpAvd$_{5\rightarrow4}$) and blue (cpAvd$_{6\rightarrow5}$). The peptide linkers are shown in black and gray letters. Modifications are shown in circles. Yellow colour refers to mutations done to biotin–binding site. (A) Schematic representation of one possible quaternary structure of dcAvd and modified dcAvd. The peptide linker connecting the cpAvds is shown as a green tube. (B) Schematic representation of the biotin–binding residues mutagenised in this study and their interactions with biotin. (C)* ***(Figure from Hytönen et al. 2005c).***

The dcAvd pseudodimer has been further modified to contain two biotin–binding sites that differ in their biotin–binding properties (Hytönen et al. 2005c). The mutagenesis has been done separately to each cpAvd prior the combination of the

cpAvd building blocks. (Figure 10) The modifications were done in this fashion because the building blocks were too similar to be modified at the same time using PCR based methods (for example QuikChange PCR mutagenesis). By combining two structurally identical but sequentially distinct building blocks we could obtain a dual chain hybrid avidin (dchAvd) where the individual building blocks could be modified at the same time within the dcAvd structure. The modifications could be done quite easily and would save time and resources. This site directed mutagenesis could be performed by the use of sequentially distinct PCR primers available for the different building blocks.

Interesting fusion partners for avidin would be streptavidin, AVR2 and AVR4, for example, since they are close relatives and structurally similar but poses some what different biochemical properties (mentioned above). Constructs of $cpAvd_{5\rightarrow4}$ fused to 6→5 circularly permutated streptavidin, AVR2 and AVR4 ($cpSA/AVR2/AVR4_{6\rightarrow5}$) have been previously made (Hytönen, unpublished data). Preliminary results on protein size, biotin binding, thermostability and productivity in *E. coli* existed before the current study (Hytönen, unpublished data).

3 Aims of the study

1. Study the usefulness of dual chain hybrid avidins (dchAvds) as biotechnological tools.
2. Optimize the production and purification of dchAvds.
3. Study the ligand binding properties of dchAvds.
4. Verify the heterogeneity of the dchAvds.

4 Materials & Methods

4.1 Protein production in *E. coli*

Plasmid multiplication

DNA–constructs of 5→4 circularly permutated avidin cdAvd$_{5\rightarrow4}$ fused to 6→5 circularly permutated streptavidin, AVR2 and AVR4 (cpSA/AVR2/AVR4$_{6\rightarrow5}$) have been prepared in previous studies (Hytönen, unpublished data). To multiply the pBVboostFG plasmid constructs (figure 11) containing the desired dual chain avidin insert with region encoding OmpA signal sequence (figure 12), chemically competent *E. coli* TOP10 cells (Invitrogen) were transformed by standard heat shock method. Competent cells were taken from –80 °C freezer and thawed on ice. 5 to 10 ng of DNA in a volume of 1 to 5 µl was mixed to about 30 to 50 µl of competent cells and mixed gently by tapping. The mixtures were incubated on ice for 30 minutes. After the incubation the mixtures were heat shocked by incubating at 37 °C for exactly 2 minutes or at 42 °C for exactly 30 seconds. After heat shocking the mixtures were placed on ice and 250 µl of pre–warmed S.O.C. medium was added to them. After the addition of S.O.C. medium the mixtures were mixed well and placed in a 37 °C incubator for an hour. The mixtures were mixed every now and then during the incubation. 150 µl of the transformation mixture was plated onto LB medium plate containing gentamycin (7 mg/ml) and incubated overnight at 37 °C.

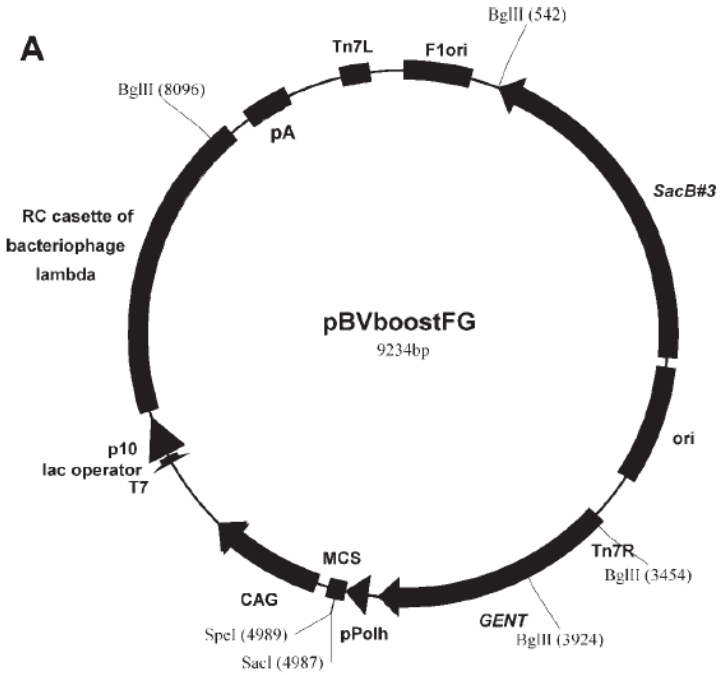


Figure 11. Map of the pBVboostFG vector. *(Figure modified from Laitinen et al. 2004)*

dcAA4 sequence

mnkpskfala lafaavtasg vasagtqptf gftvnwkfse
sttvftgqcf idrngkevlk tmwllrssvn digddwkatr
vginiftrlr tqkeggsggs arkcsltgkw tndlgsnmti
gavnsrgeft gtyitavtat sneikesplh gtqntinksg
gsttvftgqc fidrngkevl ktmwllrssv ndisydwkat
rvgynnftrl ctveeggark csltgkwtnn lgsimtirav
nsrgeftgty ltavadnpgn itlspllgiq hkrasqptfg
ftvhwnfse

dcAA2 sequence

mnkpskfala lafaavtasg vasagtqptf gftvnwkfse
sttvftgqcf idrngkevlk tmwllrssvn digddwkatr
vginiftrlr tqkeggsggs arkcsltgkw tndlgsnmti
gavnsrgeft gtyitavtat sneikesplh gtqntinksg
gstsvfvgqc fvdrsgkevl ktkwlqrlav ddisddwiat
rvgnndftrq htveeggark csltgewdnd lgsimtigav
ndngefdgty itavadnpgn itlspllgiq hkrasqptfg
ftvhwnfse

dcASA sequence

mnkpskfala lafaavtasg vasagtqptf gftvnwkfse
sttvftgqcf idrngkevlk tmwllrssvn digddwkatr
vginiftrlr tqkeggsggs arkcsltgkw tndlgsnmti
gavnsrgeft gtyitavtat sneikesplh gtqntinksg
gsattwsgqy vggaearint qwlltsgtte anawkstlvg
hdtftkvkps aasggsaaea gitgtwynql gstfivtaga
dgaltgtyes avgnaesryv ltgrydsapa
tdgsgtalgwt vawknnyrna h

Figure 12. The sequences of dcAA4, dcAA2 and dcASA. Prokaryotic signal peptide, OmpA, from *Bordatella avium in red, 5→4 linker in green, 6→5 linker in pink and linker between circularly permutated monomers in blue.*

Three liquid cultures of each construct were made using 5 ml of LB medium, gentamycin (7 µg/ml) and one colony (one clone). The liquid cultures were grown overnight in a 37 °C shaker (~225 rpm). The multiplied plasmids were harvested using Qiagen Plasmid Miniprep kit according manufacturers instructions.

Protein production

Chemically competent *E. coli* BL21–AI cells were transformed with pBVboostFG plasmid containing the desired insert, in this case the dcAA4 gene, dcAA2 gene or dcASA gene. The transformation was conducted by standard heat shock method, cells

were plated onto LB medium plates containing appropriate antibiotics and glucose; gentamycin (7 µg/ml), tetracycline (5 µg/ml), 0.1 % (w/v) glucose and grown over night in +37 °C. 10 ml liquid cultures were grown from the obtained transformed cell colonies. One colony was used for each culture containing 10 ml of LB medium, gentamycin (7 µg/ml), tetracycline (5 µg/ml) and 0.1 % (w/v) glucose. The cultures were grown over night in 26 °C ~200 rpm.

1000 ml of LB medium, gentamycin (5 mg/ml), 0.1 % (w/v) glucose and the previously made 10 ml liquid growth of *E. coli* BL21–AI cells were mixed and incubated in 26 °C ~200 rpm. The optical densities (OD) of the cultures were monitored by spectrometer using wave length 595 nm. As the OD of the cultures reached 0.2 to 0.4 the protein production was induced using 0.2 % (w/v) L–arabinose. The cultures were incubated in 26 °C ~200 rpm overnight. Cells were then collected by centrifugation (10 minutes, 5550 rpm, SLA–1500 rotor, 4 °C).

Protein purification with 2–iminobiotin affinity chromatography

The obtained cell mass was suspended to Set buffer (20 % sucrose, 2 mM EDTA pH 8, 30 mM Tris pH) using 4 ml of Set per 100 ml of culture. 50 µl of Lysozyme (5 mg/ml) per 100 ml of culture was added and the suspension was incubated on ice for 30 minutes. 10 ml of HilloI buffer (50 mM Tris pH 8, 2 mM EDTA pH 8, 150 mM NaCl, 1 % Triton X–100) per 100 ml of culture was added. After HilloI buffer addition the suspension was sonicated two times using amplitude 55 %, 2 minutes (5 seconds on and 1 second off) (Sonics, Vibra Cells, 500 VCX). A 100 µl sample, T, was taken from the sonicated cell mass. The lysate was centrifuged for 30 minutes, 9600 rpm, SLA–1500 rotor, 4 °C. During the centrifugation 2–iminobiotin resin (Sigma–Aldrich) was washed in an affinity chromatography column (BioRad, 10 ml) using 50 ml of pH 11 –buffer. After centrifugation the supernatant was carefully decanted into a beaker, a 100 µl sample, L1, was taken, an equal volume of pH 11 –buffer (50 mM Na_2CO_3, 1M NaCl) was added and the pH of the liquid was adjusted to pH 11 using 2M NaOH. The cell debris was suspended to 50 ml of PBS buffer and a 100 µl sample S was taken. The L1 liquid was divided to 50 ml Nunc bottles and the 2–iminobiotin resin was shared between the bottles. The L1–resin –mixture was incubated at 4 °C rolling shaker for at least an hour. After incubation the L1–resin –

mixture was centrifuged for 5 minutes, 500 g. The supernatant, L2, was carefully decanted away and stored. A 100 µl sample of L2 was taken. The resin was washed and centrifuged (5 min, 500 g) twice using pH 11 –buffer. The supernatants were discarded. The washed resin was transferred into the affinity chromatography column using pH 11 –buffer. The protein was eluted using 0.1M acetic acid taking 10x 1 ml fractions. A_{280} of each fraction was measured using NanoDrop™ spectrometer (NanoDrop™ 3300) and the protein concentration was calculated using equation 1.

$$A = \varepsilon cb \qquad (1),$$

where A is the absorbance (280 nm), ε is the molar extinction coefficient and b is the length of the light pathway (1 cm). To obtain the protein concentrations in mg/ml the molar extinction coefficient was divided by the molar mass, M, of the protein. For dcAA4; ε = 50 880 $M^{-1}cm^{-1}$ and M = 29 045 g/mol, for dcAA2; ε = 48 320 $M^{-1}cm^{-1}$ and M = 28 866 g/mol and for dcASA ε = 65 980 $M^{-1}cm^{-1}$ and M = 28 316 g/mol. (Protein Calculator v.3.3)

A 20 µl sample was taken from each elution fraction. An equal volume of 2x SDS–PAGE loading –buffer (1 M Tris pH 6.8, 2 % SDS, 10% glycerol, 0.2 % Coomassie Brilliant BlueR–250, 0.5 % β–mercaptoethanol) was added to all the samples that were taken; T, L1, S, L2 and the elution fractions. A SDS–PAGE –gel of the samples was run and the gel was either stained with Coomassie stain or Western–blotted (chapter 4.4 Protein analysis methods, sections *SDS-PAGE* and *Western Blot*).

The cell lysis was done by combining hypotonic shock method to either osmotic shock method or sonication to see if the lysis had an impact on the quality of the produced recombinant proteins. After lysozyme processing and Set & HilloI buffer addition the cells were centrifuged 15 000 g, 30 minutes, GSA rotor. The supernatant was decanted and stored and a 100 µl sample, L0, was taken from it. The pellet was suspended to 10 ml of ice cold 5 mM $MgCl_2$ per 100 ml of original culture volume. The suspension was centrifuged for 30 minutes, 15 000 g, GSA rotor. The L1 supernatant was decanted and stored. A 100 µl sample of L1 was taken. The remaining pellet was suspended to 50 ml of PBS buffer and a 100 µl sample S was

taken from it. The L0 and L1 supernatants were combined and the purification was continued as described above.

4.2 Protein production in *E. coli* by pilot–scale fermentation

Fermentation

Chemically competent *E. coli* BL21–AI cells were transformed with pBVboostFG plasmid containing the desired insert, in this case the dcAA4 gene. The transformation was conducted by standard heat shock method and cells were grown overnight in 37 °C followed by plating onto LB medium plates containing appropriate antibiotics and glucose, gentamycin (7 µg/ml), tetracycline (5 µg/ml), 0.1 % (w/v) glucose.

5 ml liquid cultures were grown from the obtained transformed cell colonies. One colony was used for each culture and special fermentation medium (appendix 1) with appropriate antibiotics was used for the growth. The cultures were grown overnight in 26 °C ~200 rpm. Antibiotics used were gentamycin (7 µg/ml) and tetracycline (5 µg/ml).

1 ml of the over night grown liquid culture was used to inoculate of 100 ml culture which was grown using the same parameters and ingredients as previously.

The device used for the fermentation was Infors Labfors 3. The initial volume of the fermentation reaction was 4 l. The antifoam agent used was sterilized struktol J 647. Fermentation medium used was the same used in previous liquid cultures except for containing only gentamycin (7 mg/l). The fermentation chamber containing the fermentation medium was sterilized before the fermentation by autoclaving. Cell culture, gentamycin, glucose, trace metals (appendix 2), $MgSO_4$, feed solution and induction substrates (appendix 3) were added using aseptic methods. 240 ml of liquid culture was used for inoculation. At the beginning of the fermentation the pO_2 of dissolved oxygen was 0 %, stirring speed was 500 rpm and OD was 0.288. Aeration was slowly raised manually to obtain 20 % dissolved oxygen (pO_2). A feed back loop to keep the oxygen level at 20 % was done by setting the stirring speed in range of

150 to 1100 rpm. Pumping of the feed solution was initiated at OD 0.8 – 1.2 and the flow speed was 5 %. Induction of protein production was done at OD 1.0 – 1.5 by adding 0.25mM IPTG (Fermentas) and 0.2% L–arabinose (w/v). The fermentation temperature was 28 °C. Fermentation was stopped after 24 h by harvesting the cell by centrifugation (5000 g, 15 min, 20 C).

Protein purification with 2–iminobiotin affinity chromatography

The purification procedure was done as described in section 8.1 (part: Protein purification with 2–iminobiotin affinity chromatography), except for the differences in the sonication step and the elution step. The cell suspension was sonicated twice using amplitude 50 %, 15 minutes (5 seconds on and 1 second off) and incubated in –20 °C for 30 minutes before each sonication. 20x 1 ml fractions were eluted instead of 10x 1 ml fractions. Same samples were taken during the purification process as described in section 8.1 (part: Protein purification with 2–iminobiotin affinity chromatography). The samples were ran into SDS–PAGE –gel and stained with Coomassie Brilliant Blue.

4.3 Protein production in insect cells

Transformation, targeted transposition and picking up the correct clones

Targeted transposition was done by transforming *E. coli* DH10BAC cells with pBVboostFG plasmid ($gent^r$) containing the desired insert, in this case the DNA encoding dcAA4, dcAA2 or dcASA proteins and the DNA encoding OmpA signal sequence. The DH10BAC cells contain the baculovirus genome as a bacmid (kan^r and lacZα positive) and a helper vector (tet^r) which produces (*in trans*) the proteins needed for the targeted transposition of the gene of interest into the bacmid.

A well visible amount of DH10BAC cells were taken from a pure culture LB plate grown over night at 37 °C and mixed with 100 µl of 100mM ice cold sterile $CaCl_2$. Cells were incubated on ice for 15 minutes and ~100 ng of plasmid DNA was added in a volume of 2 µl. The mixture was incubated on ice for further 30 minutes and after it given a heat shock by incubating 2 minutes in 37 °C. After heat shock the cells

immediately put on ice. 450 µl of S.O.C. medium was added and cells were incubated in 37 °C shaker for 3 hours. After incubation the 150 µl of the transformation mixture was plated onto BAC–REC –plates (5 µg/ml tetramycin, 7 µg/ml gentamycin and 50 µg/ml kanamycine) with 25 µl of X–gal reagent (40 mg/ml, Fermentas), 10 µl of 1M IPTG and 50 µl of sterile water to make the spreading easier. The plates were incubated in 37 °C incubator overnight.

For development of blue color to bacterial colonies, the plates were moved to 4 °C for 24 h. The clones containing the intact bacmid DNA develop blue colored colonies in the presence of IPTG and X–gal because the bacmid contains lacZα peptide coding sequence that compensates the *lac* mutation of DH10BAC cells. If the transformation and the targeted transposition have been successful the lacZα sequence will be disrupted and the colonies will be white. ~30 round and good looking colonies per construct were transferred to new BAC–REC –plates containing X–gal and IPTG (as previously) to confirm their white color. The plates were incubated in 37 °C incubator for 4 hours and then moved to 4 °C for 120 hours.

Extracting the viral DNA

5 ml liquid cultures were made using the confirmed white colonies. The liquid cultures contained 5 ml of LB medium, gentamycin (7 µl/ml), kanamycine (50 µg/ml) and a visible node of cells. The cultures were grown overnight at 37 °C.

1 ml of liquid culture was centrifuged for 1 minute 15 000 g. Supernatant was discarded thoroughly and cells were suspended to 300 µl of Bacmid I solution (15 mM Tris-HCl pH 8, 10 mM EDTA, 100 µg/ml RNase A). 300 µl of Bacmid II solution (0.2 M NaOH, 1 % SDS) was added and the mixture was mixed by inverting the tube. After mixing the mixture was incubater at RT for 5 minutes. 300 µl of Bacmid III solution (3 M KCH_3CO_2 pH 5) was added while vortexing slowly at the same time. The mixture was incubated on ice for 10 minutes. After incubation the mixture was centrifuged for 5 minutes 15 000 g, supernatant was moved to a new tube and centrifuged again for 5 minutes 15 000 g. The supernatant was moved to a 2 ml tube containing 800 µl of absolute isopropanol and centrifuged for 15 minutes 15 000 g. The supernatant was discarded. Little, barely visible pellets were washed

with 500 µl of 70 % ethanol and centrifuged for 15 minutes 16 000 g. The resulting, not visible pellets were dried for 30 minutes in a laminar flow hood and suspended to 40 µl of TE–buffer.

Transfection and primer stocks of recombinant baculoviruses

Transfection was conducted using lipofection method. Cells used for the transfection were Sf–9 insect cells and the medium used was Sf–900 II medium containing biotin. Two trasnfections were made from each bacmid construct using six–well plates. Each well contained ~1 000 000 cells attached to the bottom. For each well a solution 1 and solution 2 were prepared. Solution 1 contained 10 µl of previously extracted bacmid DNA and 100 µl of medium. Solution 2 contained 9 µl of CELLFECTIN™ reagent and 100 µl of medium. Solutions 1 and 2 were mixed together and incubated for 30 minutes at RT. After incubation 800 µl of medium was added to the transfection solution (solution 1 + solution 2). The culture medium was removed from the wells and the trasnfection mixture was added onto them. The cells were incubated at 28 °C for 4 hours. The transfection solution was removed and 2 ml of medium was added and incubation at 28 °C was continued for 72 hours.

The transfected insect cells were detached from the wells by pipetting and the obtained 2 ml primary virus stock was used to infect 15 ml of insect cells in liquid culture (~1 000 000 cell/ml). The mixture of insect cells and the virus containing transfection solution was incubated at 28 °C for ~96 hours.

After incubation 1 ml samples were taken from the liquid cultures and the wells. They were centrifuged for 5 minutes 1 000 g and supernatants and pellets were stored for further Western Blotting analysis. The liquid cultures were centrifuged for 5 minutes 1 000 g and the supernatants containing the baculoviruses were stored at 4 °C. These baculovirus stock solutions were used for further infections and protein productions.

Infection of insect cells and expression of recombinant protein

400 ml of Sf–9 insect cells were infected with 4 ml of previously obtained baculovirus stock solution. The growth medium was changes from biotin containing

Sf–900 to Sf–900 without biotin prior to infection. The infected culture was incubated at 28 °C, ~100 rpm for 72 hours.

Protein purification

The protein production was stopped by centrifuging the insect cells for 10 minutes, 500 g, GSA–rotor. 45 ml of the supernatant was stored and the rest was discarded. The cells were suspended to HilloI buffer using 15 ml of buffer per 50 ml of original culture volume. The cell suspension was sonicated three times 60 seconds using 40 % amplitude (1 second on, 1 second off). A 100 µl sample, T, was taken. The sonicated cells were centrifuged for 30 minutes, 15 000 g, GSA–rotor. The supernatant was carefully decanted away. The pellet was suspended to 50 ml of PBS and a 100 µl sample, S, was taken. A 100 µl sample, L1, was taken from the supernatant. 30 ml 5M NaCl was added and the pH was adjusted to pH ~10 to 11. L1 was shared to 50 ml Nunc bottles and the 2–iminobiotin resin was added. The L1–resin –mixture was incubated in 4 °C rolling shaker for ~2 hours. The L1–resin –mixture was centrifuged for 5 minutes, 2 500 g and the supernatant was stored. A 100 µl sample, L2, of the supernatant was taken. The resin was washed with pH 11 buffer and centrifuged 5 minutes, 2 500 g two times. The resin was transferred to a chromatography column and washed with 20 ml of pH 11 buffer. A 100 µl sample, R, of the resin was taken. The protein was eluted with 0.5M acetic acid taking 10x 1 ml fraction. A_{280} of each fraction was measured using NanoDrop™ spectrometer and the protein concentration was calculated using equation 1. A 20 µl sample was taken from each elution fraction. An equal volume of 2x SDS–PAGE loading –buffer was added to all the samples that were taken; T, L1, S, L2, R and the elution fractions. A SDS–PAGE –gel of the samples was run and the gels were Western blotted.

4.4 Protein analysis methods

SDS–PAGE

The proteins were separated and their purity, size and concentration was analyzed using SDS–PAGE gel having 5 % stacking gel and 15 % running gel. The stacking gel was made by mixing 680 µl of water, 170 µl of 30 % acrylamide mix (Sigma–

Aldrich), 130 µl of 0.5M Tris–HCl, pH 6.8, 10 µl of 10 % SDS, 10 µl of 10 % ammoniumpersulfate (APS) and 1 µl of *N, N, N', N'* –tetramethylethyldiamine (TEMED). The running gel was prepared by mixing 1.1 ml of water, 2.5 ml of 30 % acrylamide mix, 1.3 ml of 1.5M Tris–HCl, pH 8.8, 50 µl of 10 % SDS, 50 µl of 10 % APS and 5 µl of TEMED. Either 10 well combs or 15 well combs were used depending on the amount of samples. Samples were prepared by adding an equal volume of 2x SDS–PAGE sample buffer and boiled for 10 minutes. 2 to 20 µl of samples and 5 µl of MW marker (Fermentas PageRuler™ Prestained Protein Ladder or Ladder–plus, appendix 4 and 5) were loaded to each gel. Gels were run for 15 minutes under 80 V and for 60 minutes under 180 V current using Mini PROTEAN® II cell (Bio–Rad).

Western Blot

Proteins separated with SDS–PAGE –method were blotted onto a nitrocellulose membrane (BioRad) and recognized by antibodies to detect the identity and concentration of the proteins or the heterogeneity of the dual chain protein dcAA4. The blotting cassette was prepared at 4 °C under transferring buffer and the blotting was done using Mini Trans–Blot equipment (BioRad) with 100 V current for an hour at 4 °C. The mediating buffer for the blotting was transfer buffer.

After blotting the nitrocellulose membrane was washed with TBS–Tween (50 mM Tris, 150 mM NaCl, 0.5 % Tween) for ~5 min and blocked with 5 % milk in TBS–Tween. Blocking was done either at 4 °C, overnight or at RT, 30 minutes. After blocking the membrane was washed with TBS–Tween (3x 5 minutes). The membrane was incubated with primary antibody diluted 1:5000 in 5 % milk in TBS–Tween for an hour and then washed as previously. After the washings the membrane was incubated with secondary antibody diluted 1:2000 in 5 % milk in TBS–Tween for an hour and washed as previously. After TBS–Tween wash the membrane was washed with APA buffer (0.1 M $NaHCO_3$, 1 mM $MgCl_2 \cdot H_2O$, pH 9.8) (3x 10 minutes). The proteins were visualized with coloring solution containing 6 ml of APA buffer, 180 µl of NBT (10 mg/l) and 60 µl of BCIP (15 mg/l). The color development was stopped by washing the membrane with running water after the bands had appeared satisfactorily and dried. All the membranes were scanned for further inspection.

Coomassie staining

This staining was done so that all the proteins in the gels could be visualized for determination of the sample purity and homogenity. The gels were incubated in Coomassie solution for at least two hours and then treated with destaining solution. The gels were imaged and digitalized using a scanner.

HPLC gel filtration

High performance liquid chromatography (HPLC) gel filtration method was used to determine the exact size and degree of oligomerisation of dcAA4 protein and its complex with biotin (Äkta 900, Amerham Biosciences). The column used for the analysis was Tricorn™ High Performance Column Superdex™ 75 10/300 GL. The gel filtration standard used was provided by Bio–Rad and it contained lyophilized mixture of molecular weight markers ranging from 1 350 to 67 000 Da. The standard protein sample was used to make a standard curve where protein molecular weight could be read. The protein standard was suspended to 500 µl of HPLC –running buffer (50 mM $NaPO_4$ pH 7, 650 mM NaCl) and run through the column. The absorbances A_{280} were recorded as well as the elution times. 100 µl of dcAA4 (c ~0.5 mg/ml) without *d*–biotin and with excess *d*–biotin were run and A_{280} and elution times were detected. The standard curve was prepared using the known sizes of the standard proteins and their elution time. The molecular weights of the dcAA4 with and with out *d*–biotin were calculated using the equation describing the standard curve and their elution times.

Fluorescence spectroscopy

The dissociation of conjugated biotin was examined by fluorescence spectroscopy using fluorescent biotin (Bf560–BTN, Arcdia, Turku). The fluorescence of the Bf560–biotin is reversibly quenched when it is bound to a biotin binding protein and the binding is thus observed as a decrease in the intensity of the fluorescence. The reaction reaches equilibrium in a few minutes and it can be disturbed by the addition of a competitive ligand. As the fluorescent biotin is released from the binding site the fluorescence is recovered. This phenomenon can be used to measure ligand binding

properties of different biotin binding proteins such as dissociation rate constants. The proteins examined were bAVR4, dcAA4 and Avd. We prepared four different dcAA4 samples, one avidin and one AVR4 sample.

The measurements were conducted with QuantaMaster Spectrofluorometer System having following settings; temperature 25 °C, excitation wave length 560 nm, emitting wave length 576 nm, electric potential 1 000 V, lamp power 60 W and stirrer on. The software used was Felix32. The total volume of the samples was 3 000 µl and the buffer used was HPLC –running buffer.

All the measurements were done as follows; the fluorescence of 50 nM of free fluorescent biotin was measured for 100 seconds to obtain the scale of fluorescence in the reaction (measurement 1). The desired amount of protein was added and the fluorescence was measured for 500 seconds to observe the binding of Bf560–biotin to the protein (measurement 2). A hundred fold excess of non–fluorescent biotin (21.5 µl, 0.17 mg/ml) was added to the reaction. The fluorescence was measured for 3 600 seconds to observe the dissociation of Bf560–biotin (measurement 3). This was seen as an increase of fluorescence level. Graphs of all the measurements were obtained and they were of form RFU (relative fluorescent unit) vs. time (seconds).

In the measurements of avidin and AVR4 we used 50 nM of protein. In the measurements of dcAA4 we used either 25 nM, 50 nM, 200 nM of protein or 50 nM of protein presaturated with 25 nM of non–fluorescent *d*–biotin (Sigma–Aldrich). Table 1 summarizes the measured samples.

Table 1. The summary of the samples that were measured by fluorescence spectroscopy.

Sample name	Protein	c(protein)	c(d-biotin) used for presaturation	c(Bf560)
1	Avd	50 nM	none	50 nM
2	AVR4	50 nM	none	50 nM
3	AA4	50 nM	none	50 nM
4	AA4	25 nM	none	50 nM
5	AA4	200 nM	none	50 nM
6	AA4	50 nM	25 nM	50 nM

The dissociation reaction of Bf560–biotin and avidin is considered to be a one phase reaction since no co–operative binding of avidins is known up to date. This means that all the binding sites are equal and the reaction can be viewed as a single binding site reaction. The dissociation of the complex follows a simple exponential function;

$$B(t) = B_0 e^{-kt} \qquad (2),$$

where

B(t) = the amount of bound ligand at a moment of time t,
B_0 = the amount of bound ligand at a moment of time t = 0,
k = constant (dissociation rate constant) and
t = time.

To calculate the dissociation rate constants of the reaction the starting level of fluorescence was determined. It was calculated by averaging the last 100 seconds of the fluorescence signal of measurement 2. Averaging lowers the effect of instrument noise.

To determine the fluorescence scale of the reaction it was necessary to know how much the intensity of fluorescence was decreased by the addition of the protein. The fluorescence of free fluorochrome was obtained by averaging the last 100 seconds of the fluorescence signal of measurement 1.

The amount of bound ligand was determined by scaling the differences of each fluorescence intensity at a given time (measurement 3) and the starting level of the fluorescence (measurement 2) with the total decrease of the fluorescence signal (measurement 1 – measurement 2). Now it was possible to determine the amount of bound ligand as a function of time,

$$bound(t) = \frac{measuremetn,3(t) - measurement,2}{measurement,1 - mesurement,2} \qquad (3)$$

From equation 2 we can conclude that:

$$\frac{B(t)}{B_0} = e^{-kt} \| \ln$$
$$\Rightarrow \ln\left(\frac{B(t)}{B_0}\right) = -kt \qquad (4).$$

The dissociation rate constant was obtained from the data by plotting the relative amount of bound ligand against time and fitting a straight to the curve. The first 500 seconds of the data was not used for the analysis. The coefficient of the equation of the straight was the dissociation rate constant.

The amount of released ligand after an hour was determined using the equation 3 and time 60 minutes (3600 seconds).

Because dcAA4 was hypothesized to have two distinct binding sites for biotin; one very tight, avidin like, binding site and one weaker binding site, and because the data showed such binding properties, the data obtained from dcAA4 measurements was divided to two parts. The first part contained the whole data except for the first 500 seconds of the measurement 3 and it was handled as described above. The second part of the data contained the first 500 seconds of the measurement 3 and was handled as described above. The whole data set was also simulated to describe only the weaker binding site as follows; the relative amount of bound ligand from the whole avidin data was divided by two and subtracted from the relative amount of bound ligand from the whole dcAA4 data to describe only the weaker binding site. The resultant was then multiplied by two and handled as a measurement done for weak binding site sample only. The fitting was as described above.

Biacore® biosensor

The Biacore biosensor (BIAcore X optical biosensor, Biacore®) exploits the surface plasmon resonance (SRP) phenomenon and measures the mass of molecules on a surface in real time. The interaction is measured from the surface of a sensor chip where a ligand is immobilized. An analyte is delivered onto the surface using a microfluidic channels. A response is generated by the binding of the analyte to the sensor chip surface and it is proportional to the bound mass. The changes in mass can be detected down to a few picograms, and as small as pico– to nanomolar concentrations of starting material can be measured. (Biacore® Sensor Surphase Handbook). Kinetic data of analytes can be calculated from the obtained sensograms by mathematical calculations with the aid of the BIAevaluation® program.

The samples measured were dcAA4, bAvd, bAVR4. A dilution series of all the proteins were made; 1:1, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64. A_{280} of the 1:1 protein samples were measured using NanoDrop™ spectrometer and the concentrations were calculated using equation 1. The CM5 sensor chip used had 2–iminobiotin immobilized on its surface. The buffer used for the runs was pH 11 buffer and the protein dilutions were prepared into the same buffer. The surface of the sensor chip was regenerated using 0.1 M acetic acid and the regeneration was done after every run unless no response was detected from the previous run due to very low protein concentration.

Running buffer was injected onto the surface until the base line was reached (~300 seconds). The recording of the sensograph was started and after 20 seconds 40 µl of sample was injected onto the surface with pH 11 buffer. The sensogram was recorded for 350 to 450 seconds and the surface was regenerated.

The sensograms of different dilutions of the protein samples were fixed with the BIAevaluation® program so that the start of the run was set to y(0). The association was set to 20 seconds after the start. The highest peak of the sensogram was set to the stop of the injection in the analysis and the dissociation phase evaluation startpoint was set 60 seconds from the stop of the injection. After the evaluation of each sensogram the ones representing the different dilutions of the same protein were put

together. The kinetic data was obtained from these combined sensograms using the Langmuirian binding model implemented in BIAevaluation® program.

5 Results

5.1 Protein production

Protein production in E. coli
1000 ml of bacterial cells were cultured for the production of dchAvds. Two separate production trials were done for dcAA4 and dcASA and one for dcAA2 using the sonication lysis method. One production trial for each dchAvd was done using the osmotic shock lysis method. The purification samples and elution fractions were run into SDS–PAGE gel and the gels were either stained with Coomassie stain or blotted onto a nitrocellulose membrane and analyzed by Western blotting. The amount of protein was calculated from measured absorbance using equation 1.

The dcAA4 was produced in low quantity; the total amount of recombinant protein was ~260 µg when the cells were lysed by osmotic shock (table 4) and ~400 µg or ~26 µg when the cells were lysed by sonication (tables 2 and 3). Fragmentation of the protein was not seen after osmotic shock (figure 15). In contrast, fragmentation was detected after sonication (figures 13 and 14). This phenomenon was more visible in fractions with higher protein concentrations yielding to more avidin monomer sized fragmets (figure 13 and table 2) in contrast to more larger sized fragments that were present in fractions with lower protein concentration (figure 14 and table 3).

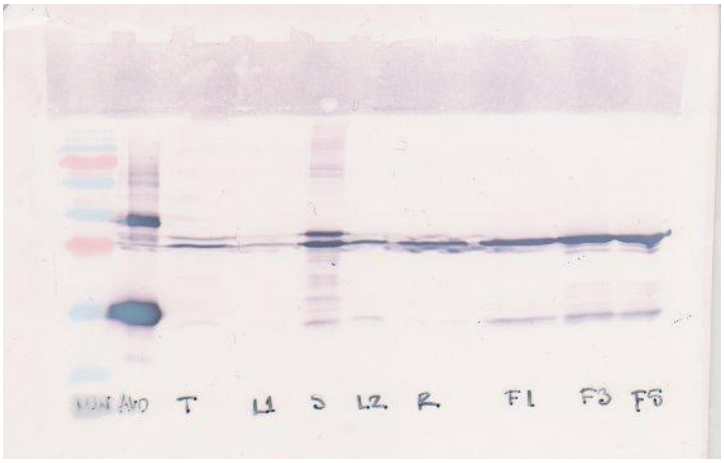


Figure 13. The Western blot of dcAA4 production and purification in E. coli; sonication lysis method. *The recombinant protein (~28 kDa) can be seen in all production and purification fractions. The fragmentation can also be seen in all samples. The fragments that are of higher mass than the recombinant protein are not seen in the elution fractions (F1, F3 and F5). The following fragments are present in the elution fractions F1: one ~28 kDa, one band just under 28 kDa and one ~11 kDa, F3: one band ~28 kDa, two bands just under 28 kDa and one ~11 kDa and F5: one band ~28 kDa, two bands just under 28 kDa and one ~11 kDa. Some protein is not eluted from the immobilization resin (R). The recombinant protein is concentrated during the purification procedure. T is the sample taken from the cell culture after sonicaiton. L1 is the supernatant after sonication and centrifugation. S is the sample taken from the pellet after sonication and centrifugation. L2 is the supernatant after binding the protein to 2–iminobiotin resin and centrifugation. MW is the molecule weight marker (Ladder-plus). Avd is the chicken avidin standard (1 mg/ml) and samples F1, F3 and F5 are elution fractions.*

Table 2. The A_{280} values and dcAA4 protein concentrations in elution fractions. *The amount of protein in the fractions was calculated by the formula $A = \varepsilon cl$ using $\varepsilon = 50\ 880\ M^{-1}cm^{-1}$. The fractions marked with orange were analyzed by Western blotting (figure 13).*

Fraction (1 ml)	A_{280}	protein concentration (mg/ml)	amount of protein (mg)
1	0.066	0.038	0.038
2	0.166	0.095	0.095
3	0.123	0.070	0.070
4	0.098	0.056	0.056
5	0.100	0.057	0.057
6	0.064	0.037	0.037
7	0.048	0.027	0.027
8	0.006	0.003	0.003
9	0.015	0.009	0.009
10	0.021	0.012	0.012
		Σ	**0.404**

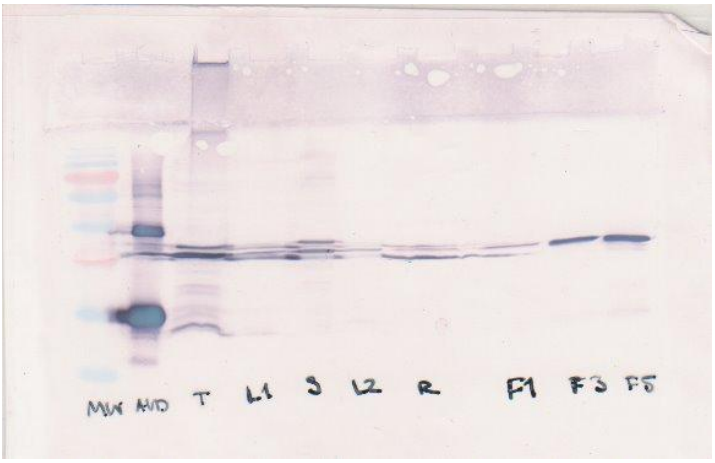
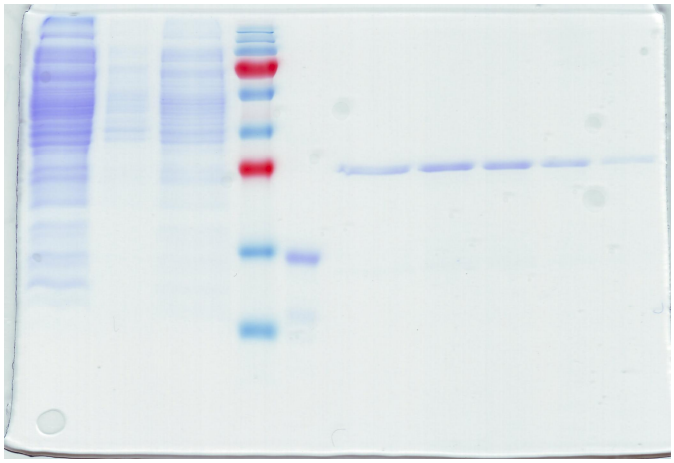


Figure 14. The Western blot of dcAA4 production and purification in E. coli; sonication lysis method. *The recombinant protein (~28 kDa) can be seen in all production and purification fractions. The fragmentation can also be seen in all samples. The fragmentation appears to yield many different sized pieces. Sample T: at least eight bands having a size of ~11 kDa to ~30 kDa, sample L1: at least four bands having a size of ~11 kDa to ~30 kDa, sample S: at least five bands around 28 to 30 kDa, sample L2: at least four bands from ~11 kDa to ~30 kDa, R: three bands around 28 to 30 kDa, F1: two bands around 28 to 30 kDa, F3 and F5: two band around 28 to 30 kDa and one ~11 kDa The recombinant protein is concentrated during the purification procedure. T is the sample taken from the cell culture after sonicaiton. L1 is the supernatant after sonication and centrifugation. S is the sample taken from the pellet after sonication and centrifugation. L2 is the supernatant after binding the protein to 2–iminobiotin resin and centrifugation. MW is the molecule weight marker (Ladder-plus). Avd is the chicken avidin standard (1 mg/ml) and samples F1, F3 and F5 are elution fractions.*

Table 3. The A_{280} values and dcAA4 protein concentrations in elution fractions. *The amount of protein in the fractions was calculated by the formula $A = \varepsilon cl$ using $\varepsilon = 50\ 880\ M^{-1}cm^{-1}$. The fractions marked with orange were analyzed by Western blotting (figure 14).*

Fraction (1 ml)	A_{280}	protein concentration (mg/ml)	amount of protein (mg)
1	0.006	0.003	0.003
2	-0.001	ND	ND
3	-0.011	ND	ND
4	0.018	0.010	0.010
5	0.020	0.011	0.011
6	0.002	0.001	0.001
7	-0.008	ND	ND
8	-0.022	ND	ND
9	-0.005	ND	ND
10	-0.012	ND	ND
		Σ	**0.026**



L0 L1 L2 mw Avd F1 F2 F3 F4 F5

Figure 15. The SDS–PAGE gel of dcAA4 production and purification in E. coli; osmotic shock lysis method. *The recombinant protein can be seen in fraction 1, 2, 3, 4 and 5 (F1–F5) in low amounts. The recombinant protein is very hard to detect from L0, L1 and L2 because of the other proteins present in greater amounts. Fragmentation of the protein is not seen. L0 is the supernatant after Lysozyme processing and centrifugation. L1 is the supernatant after osmotic shock and centrifugation. L2 is the supernatant after binding the protein to 2–iminobiotin resin and centrifugation. MW (Ladder–plus) is the molecule weight marker. Sample Avd is the chicken avidin standard (1 mg/ml) and samples F1 to F5 are the first five elution fractions.*

Table 4. The A_{280} values and dcAA4 protein concentrations in elution fractions. *The amount of protein in the fractions was calculated by the formula $A = \varepsilon cl$ using $\varepsilon = 50\,880\ M^{-1}cm^{-1}$. The concentrations were not determined (ND) for the fractions having negative A_{280} values. The fractions marked with orange were studied by SDS–PAGE (figure 15).*

Fraction (1 ml)	A_{280}	protein concentration (mg/ml)	amount of protein (mg)
1	0.094	0.054	0.054
2	0.078	0.045	0.045
3	0.122	0.070	0.070
4	0.090	0.051	0.051
5	0.046	0.026	0.026
6	0.020	0.011	0.011
7	-0.006	ND	ND
8	-0.012	ND	ND
9	-0.007	ND	ND
10	0.008	0.005	0.005
		Σ	**0.261**

The dcAA2 was not detected in the gel after the purification procedure where the cells were lysed by osmotic shock (figure 17). The purification yielded slightly negative A_{280} values except in elution fraction 2 (table 6). This was most probably due to problems in the spectrometer calibration. The total amount of dcAA2 recombinant protein in fraction 2 was 20 µg. The total amount of dcAA2 protein was ~48 µg when the cells were lysed by sonication and purified (table 5). The protein was detected in

samples T, L1, S and L2 and in very small amounts in samples R, F3 and F4 (figure 16). The protein was fragmented which is shown in figure 16.

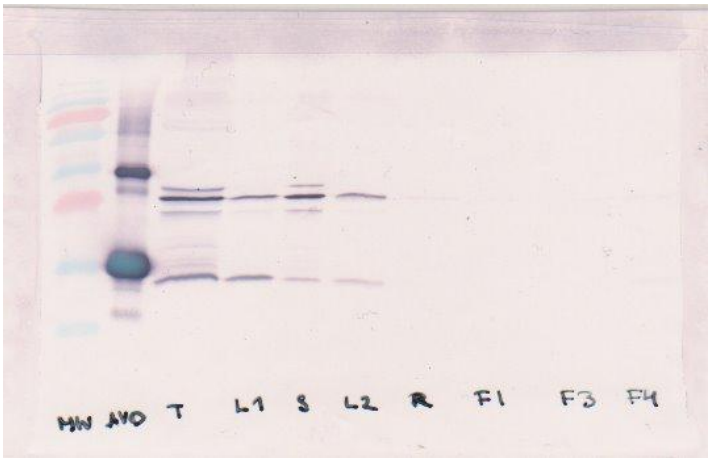
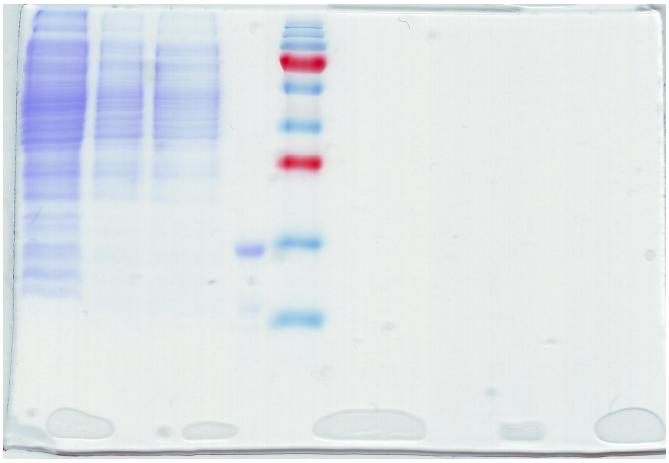


Figure 16. The Western blot of dcAA2 production and purification in E. coli; sonication lysis method. *The recombinant protein is seen in fractions T, L1, S, R, F3 and F4. In all of the fractions the protein is fragmented producing bands of sizes between ~30 and ~11 kDa Fraction T contains at least seven fragments, S at least four, L1 and L2 at least tree and R, F1, F3 and F4 at least 2 fragments. A lot of the recombinant protein seems to stay in the pellet containing the cell debris (S) after sonication and centrifugation. T is the sample taken from the cell culture after sonicaiton. L1 is the supernatant after sonication and centrifugation. S is the sample taken from the pellet after sonication and centrifugation. L2 is the supernatant after binding the protein to 2–iminobiotin resin and centrifugation. MW is the molecule weight marker (Ladder-plus). Avd is the chicken avidin standard (1 mg/ml) and samples F1, F3 and F5 are elution fractions.*

Table 5. The A_{280} values and dcAA2 protein concentrations in elution fractions. *The amount of protein in the fractions was calculated by the formula $A = \varepsilon cl$ using ε = 48 320 $M^{-1}cm^{-1}$. The fractions marked with orange were analyzed by Western blotting (figure 16).*

Fraction (1 ml)	A_{280}	protein concentration (mg/ml)	amount of protein (mg)
1	0.016	0.010	0.010
2	-0.008	ND	ND
3	0.033	0.020	0.020
4	0.009	0.005	0.005
5	-0.021	ND	ND
6	-0.005	ND	ND
7	0.002	0.001	0.001
8	0.007	0.004	0.004
9	0.004	0.002	0.002
10	0.009	0.005	0.005
		Σ	**0.048**



L0 L1 L2 Avd mw F1 F2 F3 F4 F5

Figure 17. The SDS–PAGE gel of dcAA2 production in E. coli; osmotic shock. *The recombinant dcAA2 was not detected in the eluted fraction 1 to 5 shown in the gel. The presence of dcAA2 in L0, L1 and L2 is impossible to determine. L0 is the supernatant after Lysozyme processing and centrifugation. L1 is the supernatant after osmotic shock and centrifugation. L2 is the supernatant after binding the protein to 2–iminobiotin resin and centrifugation. MW (Ladder–plus) is the molecule weight marker. Avd is the chicken avidin standard (1 mg/ml) and F1 toF5 are the first five elution fractions.*

Table 6. The A_{280} values and dcAA2 protein concentrations in elution fractions. *The amount of protein in the fractions 2 was calculated by the formula $A = \varepsilon cl$ using $\varepsilon = 48\ 320\ M^{-1}cm^{-1}$. The concentrations were not determined (ND) for the fractions having negative A_{280} values. The fractions marked with orange were studied by SDS–PAGE (figure 17).*

Fraction (1 ml)	A_{280}	protein concentration (mg/ml)	amount of protein (mg)
1	-0.013	ND	ND
2	0.033	0.020	0.020
3	-0.034	ND	ND
4	-0.007	ND	ND
5	-0.014	ND	ND
6	-0.004	ND	ND
7	-0.002	ND	ND
8	-0.040	ND	ND
9	-0.035	ND	ND
10	-0.011	ND	ND
		Σ	**0.020**

The dcASA was not seen in the coomassie stained gel after purification with osmotic shock (figure 20). The purification yielded low quantities of protein (table 9); in total 167 µg. The purification with sonication yielded even lower amounts of recombinant protein (tables 7 and 8); 23 µg and 15 µg respectively. The protein was fragmented greatly and it was present only in samples T, L1, S, L2 shown in figure 18 and in samples T, L1, S, L2 and R shown in figure 19.

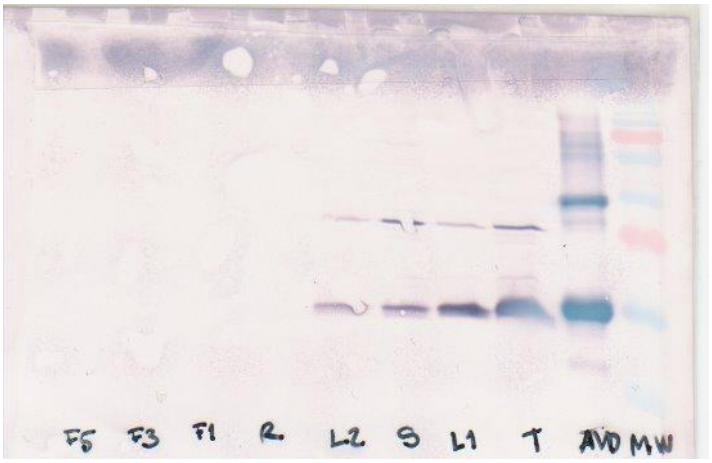


Figure 18. The Western blot of dcASA production and purification in E. coli; sonication lysis method. *The recombinant protein (~28 kDa) is produced by E. coli and fragmented into many different sized fragments (~28 to 11 kDa), the most abundant fragment being of size ~11 kDa. In fraction T there are at least six clearly visible band, in fractions L1 and S at least three, and in fraction L2 at least two clearly visible bands. The recombinant protein is not purified by the 2–iminobiotin resin markedly. T is the sample taken from the cell culture after sonicaiton. L1 is the supernatant after sonication and centrifugation. S is the sample taken from the pellet after sonication and centrifugation. L2 is the supernatant after binding the protein to 2–iminobiotin resin and centrifugation. MW is the molecule weight marker (Ladder-plus). Avd is the chicken avidin standard (1 mg/ml) and samples F1, F3 and F5 are elution fractions.*

Table 7. The A_{280} values and dcASA protein concentrations in elution fractions. *The amount of protein in the fractions was calculated by the formula $A = \varepsilon cl$ using ε = 65 980 $M^{-1}cm^{-1}$. The fractions marked with orange were analyzed by Western blotting (figure 18).*

Fraction (1 ml)	A_{280}	protein concentration (mg/ml)	amount of protein (mg)
1	-0.004	ND	ND
2	0.001	0.000	0.000
3	0.010	0.004	0.004
4	0.028	0.012	0.012
5	-0.017	ND	ND
6	-0.003	ND	ND
7	0.006	0.003	0.003
8	-0.023	ND	ND
9	-0.031	ND	ND
10	0.008	0.003	0.003
		Σ	**0.023**

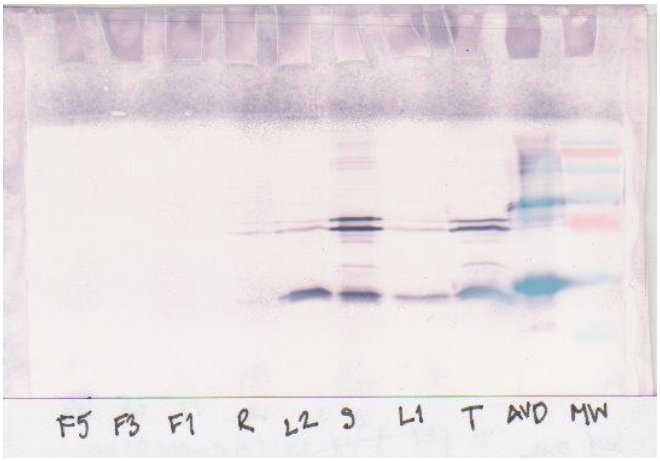
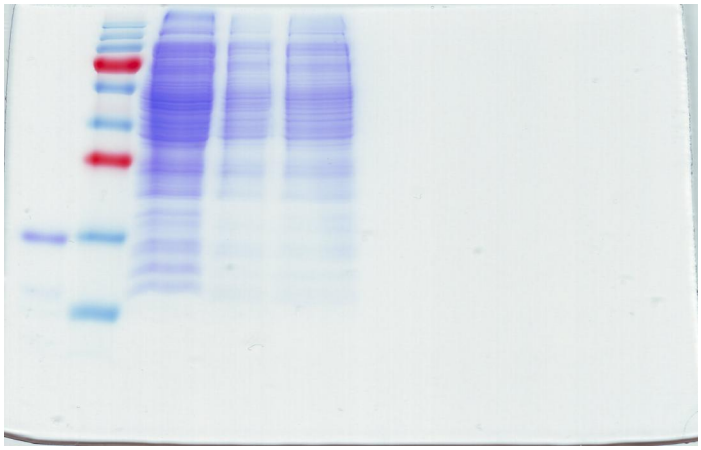


Figure 19. The Western blot of dcASA production and purification in E. coli; sonication lysis method. *The recombinant protein (~28 kDa) is produced by E. coli and fragmented into many different sized fragments (~30 to 11 kDa), the most abundant being of the size ~11 kDa. In fraction T there are at least six clearly visible band, in fraction L1 at least three, in fraction S at least seven and in fractions L2and R at least tree clearly visible bands. The recombinant protein is not purified by the 2–iminobiotin resin markedly. T is the sample taken from the cell culture after sonicaiton. L1 is the supernatant after sonication and centrifugation. S is the sample taken from the pellet after sonication and centrifugation. L2 is the supernatant after binding the protein to 2–iminobiotin resin and centrifugation. MW is the molecule weight marker (Ladder-plus). Avd is the chicken avidin standard (1 mg/ml) and samples F1, F3 and F5 are elution fractions.*

Table 8. The A_{280} values and dcASA protein concentrations in elution fractions. *The amount of protein in the fractions was calculated by the formula $A = \varepsilon cl$ using ε = 65 980 $M^{-1}cm^{-1}$. The fractions marked with orange were analyzed by Western blotting (figure 19).*

Fraction (1 ml)	A_{280}	protein concentration (mg/ml)	amount of protein (mg)
1	-0.008	ND	ND
2	0.000	0.000	0.000
3	-0.016	ND	ND
4	-0.051	ND	ND
5	0.006	0.003	0.003
6	-0.008	ND	ND
7	0.001	0.000	0.000
8	0.005	0.002	0.002
9	0.010	0.004	0.004
10	0.014	0.006	0.006
		Σ	**0.015**



Avd mw L0 L1 L2 F1 F2 F3 F4 F5

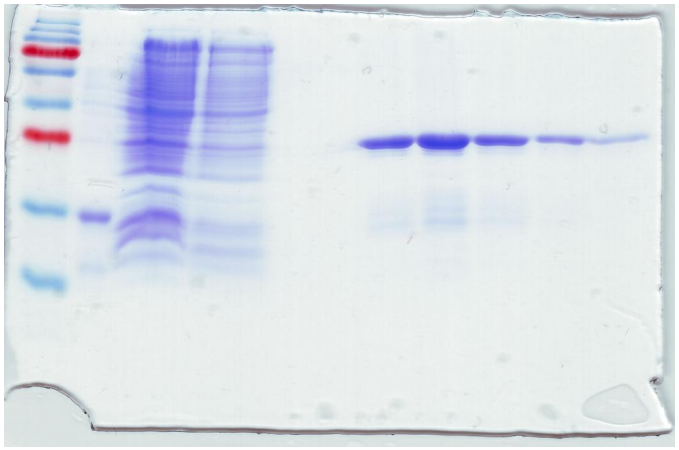
Figure 20. The SDS–PAGE gel of dcASA production in E. coli; osmotic shock. *The recombinant dcASA was not detected in the eluted fraction 1 to 5. The presence of dcASA in L0, L1 and L2 is impossible to determine. L0 is the supernatant after Lysozyme processing and centrifugation. L1 is the supernatant after heat shock and centrifugation. L2 is the supernatant after binding the protein to 2–iminobiotin resin and centrifugation. MW (Ladder–plus) is the molecule weight marker. Avd is the chicken avidin standard (1 mg/ml) and F1 toF5 are the first five elution fractions.*

Table 9. The A_{280} values and dcASA protein concentrations of the first five fractions eluted. *The amount of protein in the fractions was calculated by the formula $A = \varepsilon cl$ using ε = 65 980 $M^{-1}cm^{-1}$. The fractions marked with orange were studied by SDS–PAGE (figure 20).*

Fraction (1 ml)	A_{280}	protein concentration (mg/ml)	amount of protein (mg)
1	0.024	0.010	0.010
2	0.061	0.026	0.026
3	0.037	0.016	0.016
4	0.039	0.017	0,017
5	0.061	0.026	0.026
6	0.040	0.017	0.017
7	0.050	0.021	0.021
8	0.012	0.005	0.005
9	0.047	0.020	0.020
10	0.018	0.008	0.008
		Σ	**0.167**

Protein production in *E. coli* by pilot–scale fermentation

The purification of the fermentation was done in three separate steps because of the large amount of L1 obtained (1 l). In the first two purifications 400 ml of L1 was used as starting material for the purification and in the last purification 200 ml of L1 was used. The entire fermentation yielded over 25 mg of the recombinant dcAA4 protein (tables 10, 11 and 12). Samples from the first purification step were ran into SDS–PAGE gel and stained with Coomassie stain (figure 21).



mw Avd T L2 F2 F6 F7 F8 F9 F17

Figure 21. The SDS–PAGE gel of pilot–scale fermentation of dcAA4. *The recombinant protein was produced well by fermentation. The protein was seen very vaguely in elution fraction F2 but very strongly in all the other elution fractions in the gel. Only a little amount of fragmented protein was detected in F6 to F8. The purity of the protein was good. T is the sample total. L2 is the supernatant after binding the protein to 2–iminobiotin resin and centrifugation. MW (Ladder–plus) is the molecule weight marker. Avd is the chicken avidin standard (1 mg/ml) and F2, F6, F7, F8, F9 and F17 are elution fractions.*

Table 10. A_{280} values and protein concentrations of the first purification of the pilot scale fermentation of dcAA4. *The amount of protein in the fractions was calculated by the formula $A = \varepsilon cl$ using $\varepsilon = 50\,880\ M^{-1}cm^{-1}$. The fractions marked with orange were studied by SDS–PAGE (figure 21).*

Fraction (1 ml)	A_{280}	protein concentration (mg/ml)	amount of protein (mg)
1	0.081	0.046	0.046
2	0.109	0.062	0.062
3	0.037	0.021	0.021
4	0.097	0.055	0.055
5	0.777	0.444	0.444
6	6.357	3.629	3.629
7	7.376	4.211	4.211
8	4.033	2.302	2.302
9	1.497	0.855	0.855
10	0.594	0.339	0.339
11	0.311	0.178	0.178
12	0.228	0.130	0.130
13	0.190	0.108	0.108
14	0.083	0.047	0.047
15	0.067	0.038	0.038
16	0.077	0.044	0.044
17	0.085	0.049	0.049
18	0.049	0.028	0.028
19	0.052	0.030	0.030
20	0.014	0.008	0.008
		Σ	**12.624**

Table 11. A_{280} values and protein concentrations of the second purification of the pilot scale fermentation of dcAA4. *The amount of protein in the fractions was calculated by the formula $A = \varepsilon cl$ using $\varepsilon = 50\ 880\ M^{-1}cm^{-1}$.*

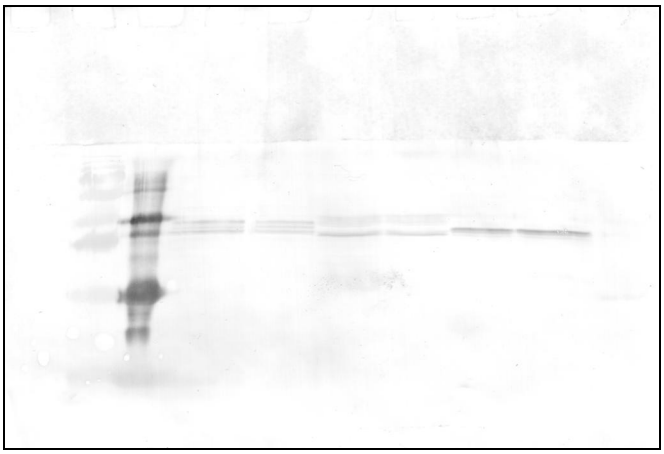
Fraction (1 ml)	A_{280}	protein concentration (mg/ml)	amount of protein (mg)
1	0.017	0.010	0.010
2	0.068	0.039	0.039
3	0.102	0.058	0.058
4	1.165	0.665	0,665
5	4.104	2.343	2.343
6	3.609	2.060	2.060
7	3.353	1.914	1.914
8	2.618	1.494	1.494
9	0.573	0.327	0.327
10	0.251	0.143	0.143
11	0.155	0.088	0.088
12	0.124	0.071	0.071
13	0.090	0.051	0.051
14	0.107	0.061	0.061
15	0.055	0.031	0.031
16	0.058	0.033	0.033
17	0.063	0.036	0.036
18	0.040	0.023	0.023
19	0.058	0.033	0.033
20	0.058	0.029	0.029
		Σ	**9.511**

Table 12. A_{280} values and protein concentrations of the third purification of the pilot scale fermentation of dcAA4. *The amount of protein in the fractions was calculated by the formula $A = \varepsilon cl$ using $\varepsilon = 50\,880\ M^{-1}cm^{-1}$.*

Fraction (1 ml)	A_{280}	protein concentratoin (mg/ml)	amoun of protein (mg)
1	0.003	0.002	0.002
2	0.031	0.018	0.018
3	0.173	0.099	0.099
4	1.135	0.648	0.648
5	1.728	0.986	0.986
6	1.657	0.946	0.946
7	1.666	0.951	0.951
8	1.264	0.722	0.722
9	0.657	0.375	0.375
10	0.377	0.215	0.215
11	0.277	0.158	0.158
12	0.109	0.062	0.062
13	0.063	0.036	0.036
14	0.039	0.022	0.022
15	0.050	0.029	0.029
16	0.050	0.029	0.029
17	0.065	0.037	0.037
18	0.004	0.002	0.002
19	0.001	0.001	0.001
20	0.011	0.006	0.006
		Σ	**5.343**

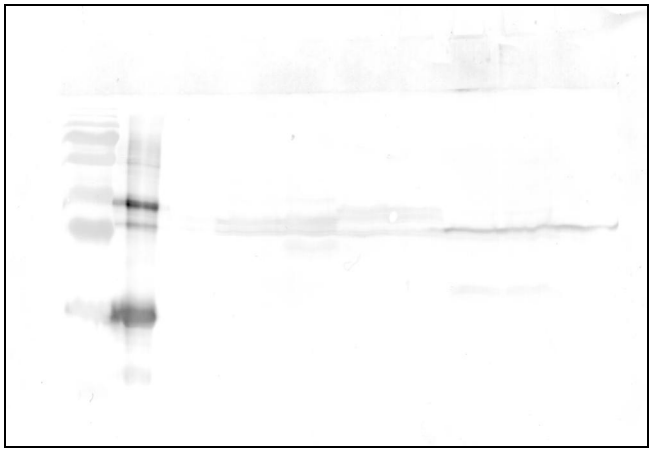
Protein production in insect cells

The samples drawn from the baculovirus stock solutions and the samples drawn from the wells where the transfection was done were run into SDS–PAGE gel and analyzed by Western blotting using polyclonal avidin antibody. All three recombinant proteins (dcAA4, dcAA2 and dcASA) were detected in both blots (figures 22 and 23). The proteins seemed to be glycosylated as expected; dcAA2 having three sites, dcAA4 having four sites and dcASA having one site per polypeptide (Laitinen et al. 2002). No fragmentation of the recombinant proteins was detected.



IS mw Avd $aa2_{(1)}$ $aa2_{(2)}$ $aa4_{(1)}$ $aa4_{(2)}$ $asa_{(1)}$ $asa_{(2)}$

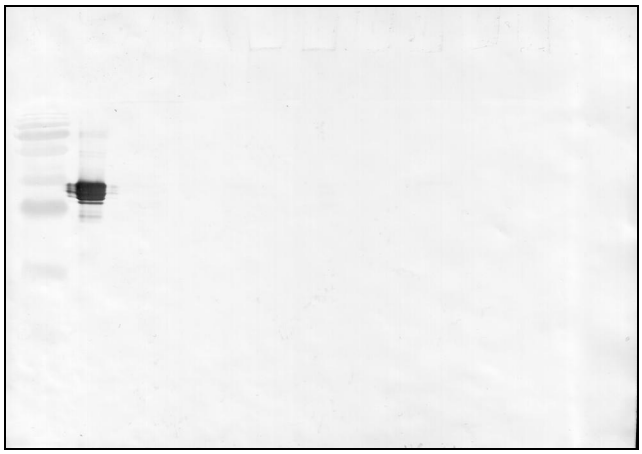
Figure 22. Western blot analysis of the transfection of the insect cells. *All of the recombinant proteins were detected. The proteins seem to be glycosylated as hypothesized. IS is negative control of insect cells, mw (Ladder–plus) is the molecule weight marker and avd is avidin standard (1 mg/ml). $Aa2_{(1)}$ and $aa2_{(2)}$, $aa4_{(1)}$ and $aa4_{(2)}$, $asa_{(1)}$ and $asa_{(2)}$ are the samples from two different wells containing the baculovirus vector carrying the DNA encoding for the same protein.*



mw Avd IS $aa2_{(1)}$ $aa2_{(2)}$ $aa4_{(1)}$ $aa4_{(2)}$ $asa_{(1)}$ $asa_{(2)}$

Figure 23. Western blot analysis of the baculovirus stock solutions. *All of the recombinant proteins were detected. The proteins seem to be glycosylated as hypothesized. IS is a sample of uninfected insect cells, mw (Ladder–plus) is the molecule weight marker and avd is avidin standard (1 mg/ml). $Aa2_{(1)}$ and $aa2_{(2)}$, $aa4_{(1)}$ and $aa4_{(2)}$, $asa_{(1)}$ and $asa_{(2)}$ are the samples from two different wells containing the baculovirus vector carrying the DNA encoding for the same protein.*

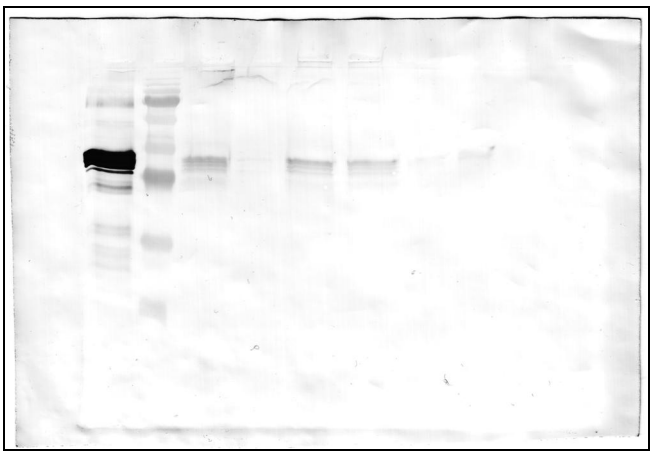
The samples from the protein production and purification in insect cells were run into SDS–PAGE gels and analyzed by Western blotting. dcAA4 was not detected in any purification or elution fraction (figure 24).



mw dcAvd T S L1 L2 F2 F4 F6 F8

Figure 24. Western blot analysis of the dcAA4 production and purification in insect cells. *No recombinant protein is detected. Mw (Ladder–plus) is the molecular weight marker, dcAvd is the dual chain avidin control sample (~0.3 mg/ml), T is sample total, S is the sample taken from the 2–iminobiotin resin after binding the protein to it, L1 is the supernatant after heat shock and centrifugation, L2 is the supernatant after binding the protein to 2–iminobiotin resin and centrifugation, F2, F4, F6 and F8 are elution fractions.*

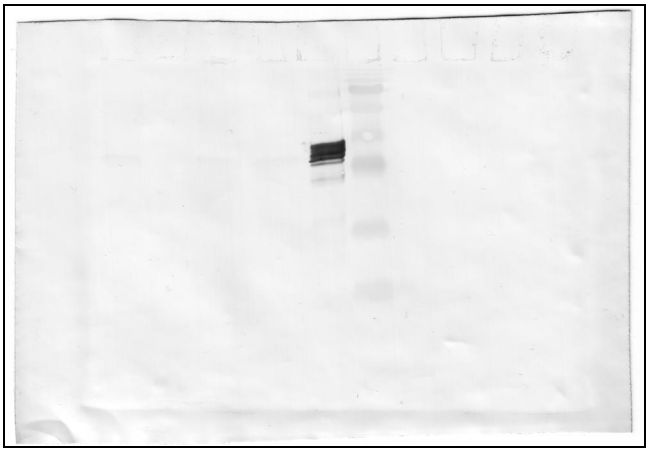
dcAA2 was detected in samples T, L1 and L2 and in barely visible amounts in elution fractions 2 and 4 but not in sample S (figure 25). The intensities of the bands in samples T, L1 and L2 seemed to be relatively close to each other.



dcAvd mw T S L1 L2 F2 F4 F6 F8

Figure 25. Western blot analysis of the dcAA2 production and purification in insect cells. *The recombinant protein was detected in samples T, L1, L2 and in elution fractions 2 and 4 in barely visible amounts. Mw (Ladder–plus) is the molecular weight marker, dcAvd is the dual chain avidin standard (~0.3 mg/ml), T is sample total, S is the sample taken from the 2–iminobiotin resin after binding the protein to it, L1 is the supernatant after heat shock and centrifugation, L2 is the supernatant after binding the protein to 2–iminobiotin resin and centrifugation, F2, F4, F6 and F8 are elution fractions.*

dcASA was detected in barely visible amounts in samples T, L1 and L2 but not in the elution fractions or the sample S (figure 26). The intensities of the bands in samples T, L1 and L2 seemed to be relatively close to each other.



T S L1 L2 dcAvd mw F2 F4 F6 F8

Figure 26. Western blot of the dcASA production and purification in insect cells. *The recombinant protein was detected in barely visible amounts in samples T, L1and L2. Mw (Ladder–plus) is the molecular weight marker, dcAvd is the dual chain avidin standard (~0.3 mg/ml), T is sample total, S is the sample taken from the 2–iminobiotin resin after binding the protein to it, L1 is the supernatant after heat shock and centrifugation, L2 is the supernatant after binding the protein to 2–iminobiotin resin and centrifugation, F2, F4, F6 and F8 are elution fractions.*

5.2 Protein analysis

HPLC gel filtration analysis

The elution time of dcAA4 without *d*–biotin was 28.83 minutes (figure 27) corresponding to molecular weight of 46.0 kDa calculated according to standard proteins (figure 29 and table 13). The elution time of dcAA4 with *d*–biotin was 28.77 minutes (figure 28) corresponding to molecular weight of 46.6 kDa (figure 29 and table 13). The theoretical molecular weight of the dcAA4 protein is 58 kDa. The result suggests that the state of oligomerisation of the dcAA4 with and with out biotin in pH 7.0 is pseudotetrameric. In gel filtration analysis, slightly lower molecular weight compared to the theoretical molecular weight is seen with other dcAvds as well (Hytönen et al. 2005c).

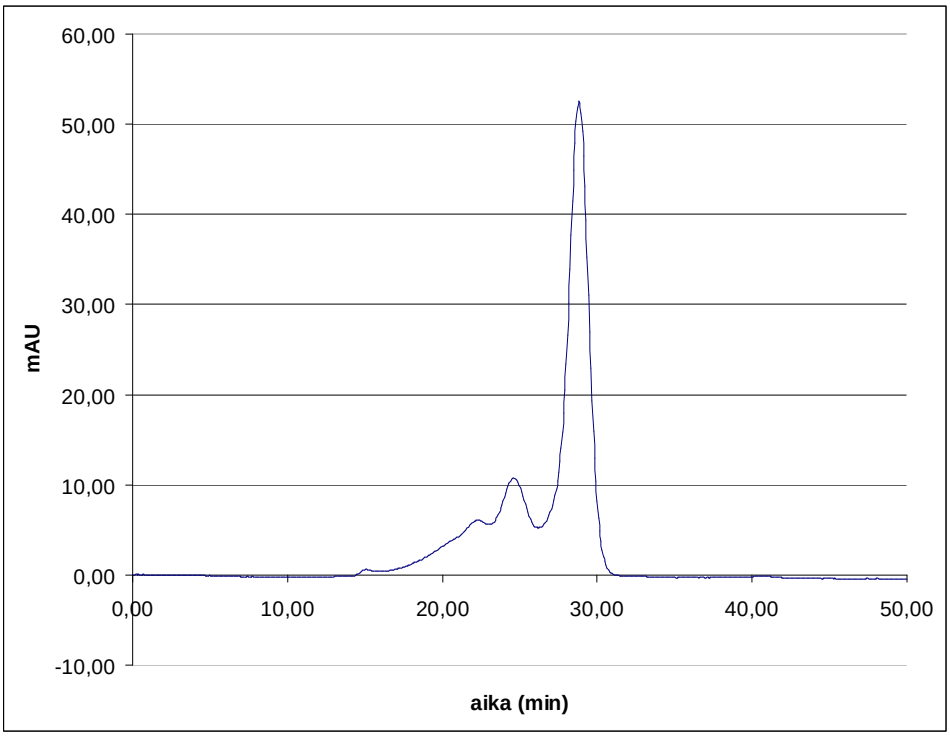


Figure 27. The elution diagram of dcAA4. *The elution time of dcAA4 was found to be 28.83 minutes.*

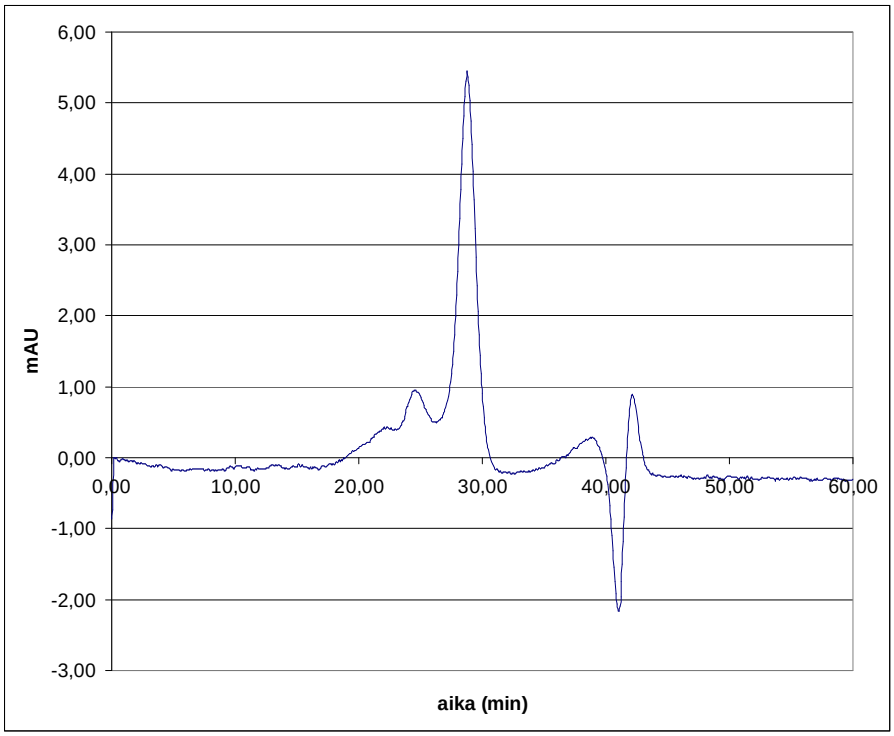


Figure 28. The elution diagram of dcAA4 in complex with d–biotin. *The elution time of dcAA4 in complex with d–biotin was found to be 28.77 minutes.*

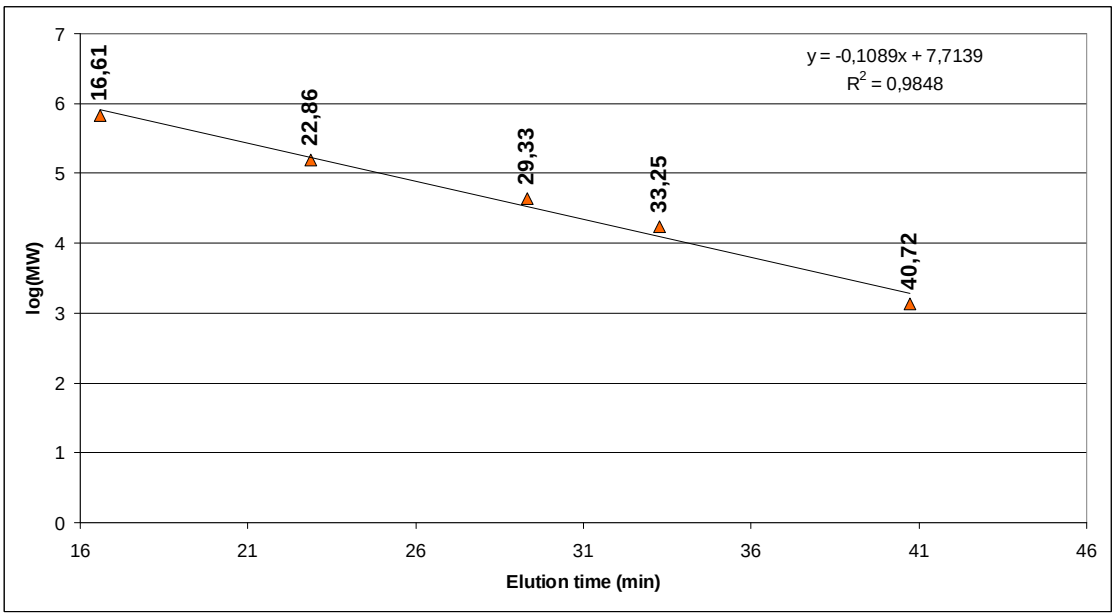


Figure 29. The logaritmic standard curve of HPLC. *The curve is of form log(MW) vs. elution time. The standard proteins are thyroglobulin (16.61 minutes, 670 000 Da), gamma–globulin (22.86 minutes, 158 000 Da), chicken ovalbumin (28.33 minutes, 44 000 Da), myoglobin (33.25 minutes, 17 000 Da) and vitamin B12 (40.72 minutes, 1 350 Da). The molecular weight of the protein can be calculated from the equation describing the standard curve if the elution time is known.*

Table 13. The molecular weight of dcAA4 and its complex with d–biotin calculated from the data obtained from the standard curve. *Theoretical values were calculated with the aid of protein calculator v.3.3 and the known size of biotin, M(biotin) ≈ 250 Da.*

Protein	Ligand	Elution time	log (MW)	MW (kDa)	MW; theoretical (kDa)
dcAA4	–	28.83	4.66	46.0	58
dcAA4	biotiini	28.77	4.67	46.6	59

Fluorometrical determination of Bf560–biotin dissociation rate constant

The samples were measured and the data obtained was handled as described in section 8.4 (part: *Fluorescence spectroscopy*). The dissociation rate constants calculated from the obtained data and the dissociation percentages of the protein–Bf560–BTN complex after an hour are shown in table 14. The dissociation rate constants were obtained by equation 4 by plotting $\ln(B/B_0)$ against time (figure 30 for dcAA4, appendix 6 for AVR4, appendix 7 for avidin, and appendix 8, 9 and 10 for dcAA4; samples 4, 5 and 6, see table 1, and appendix 11 for the simulated data of dcAA4). The dissociation of the protein–Bf560–BTN complex is shown in figure 31 for dcAA4 (appendix 12 for AVR4, appendix 13 for avidin, and appendix 14, 15 and 16 for dcAA4; samples 4, 5 and 6, see table 1, and appendix 17 for the simulated data of dcAA4). The sample names are used according to table 1 in section 8.4 (part: *Fluorescence spectroscopy*): The simulated data was obtained by subtracting the data obtained for avidin from the data obtained for dcAA4. The results suggest that there are two distinct binding sites for biotin in dcAA4.

Table 14. The dissociation rate constants of chicken avidin, AVR4 and dcAA4 and the dissociation percentages of protein–Bf560–BTN complexses after an hour. *The different rate constants obtained for dcAA4 describe the two different binding sites that are hypothesized to be present in the protein. The sample names are according to table 1: 1 for chicken avidin, 2 for AVR4 and 3, 4, 5 and 6 for AA4 (50 nM, 25 nM, 200 nM and 50 nM with 25 nM biotin presaturation, respectively). The data set indicates the time period used for the calculation of the dissociation rate constant.*

Sample name/data set	k_d (s^{-1})	Dissociation of the complex in 1 h (%)
1/last 3100 s	2.91E-05	17.19
2/last 3100 s	3.35E-05	15.98
3/whole data	1.26E-04	58.94
3/last 3100 s, tight binding	9.39E-05	
3/first 500 s, weak binding	7.02E-04	
3/simulated weak binding	7.68E-04	100
4/whole data	1.06E-04	54.79
4/last 3100 s, tight binding	7.24E-05	
5/whole data	7.67E-05	45.94
5/last 3100 s, tight binding	5.92E-05	
6/last 3100 s, tight binding	5.62E-04	92.97

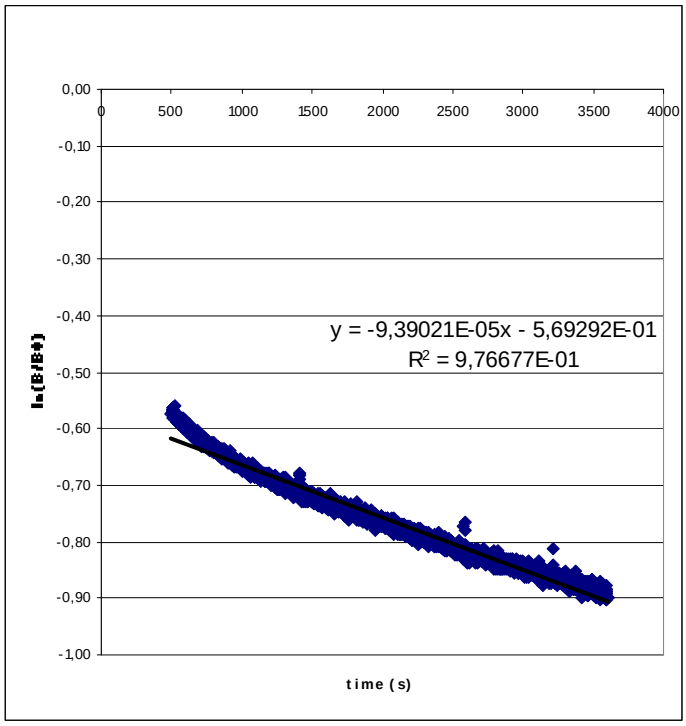


Figure 30. The dissociation rate constant of dcAA4. *$ln(B/B_0)$ vs. time gives the dissociation rate constant k_{diss} as the slope in the equation shown in the figure.*

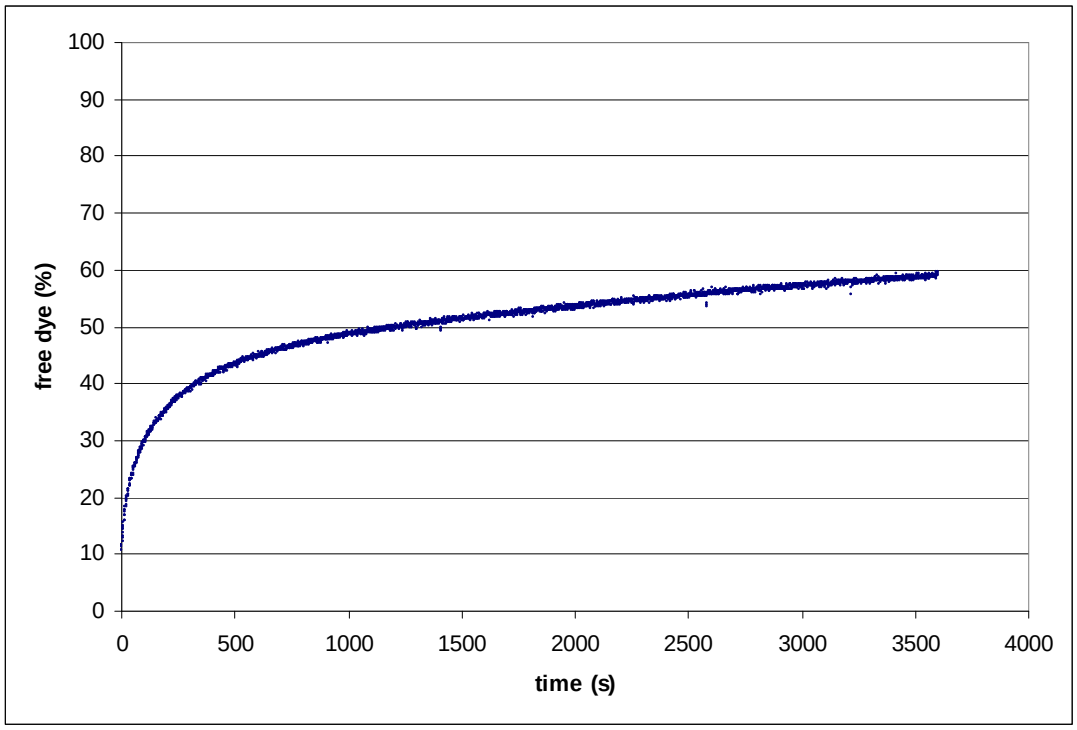


Figure 31. The relative amount of free Bf560–BTN released from the dcAA4–Bf560–BTN complex after the addition of free biotin. *A fast release of about 50 % of dye is visible during the first 500 seconds. During the last 3100 seconds a more slow release of the dye is seen resulting in the total release of approximately 59 % of the dye.*

Analysis of 2–iminobiotin–binding properties using BiaCore optical biosensor

The measurements and the data handling were done as described in section 8.4 (part: BiaCore biosensor). A series of sensograms (figure 32, avidin) were obtained for all the proteins (appendix 18 and 19, AVR4 and dcAA4) and the BiaCore program BIAevaluation was used for the rate constant and constant calculations (table 15). The data shows that k_d(dcAA4)>k_d(AVR4)>k_d(avidin). This is in line with previously described results from fluorescence analysis of the proteins. The data shows also that k_a(avidin)>k_a(AVR4)>k_a(dcAA4) and that K_d(dcAA4)>K_d(AVR4)>K_d(avidin).

Table 15. The kinetic data obtained for avidin, AVR4 and dcAA4 from BiaCore measurements and theoretical values measured in the same way found from literature. *The kinetic data obtained from the measurements show that avidin binds 2–iminobiotin stronger than AVR4 and dcAA4 and AVR4 is a stronger binder of 2–iminobiotin than dcAA4. This can be seen by comparing the dissociation rate constants of the proteins. The values found from literature and the values obtained in this study are not totally comparable since the measurements were done with different sensor chips. Values from literature from Hytönen et al. 2005b*

	Measured values			Theoretical values		
Protein	**k_a (1/M*s)**	**k_d (1/s)**	**K_d (M)**	**k_a (1/M*s)**	**k_d (1/s)**	**K_d (M)**
Avd	5.89E+04	6.84E-03	1.16E-07	5.50E+04	1.90E-03	3.40E-08
AVR4	1.06E+04	7.61E-03	7.17E-07	7.00E+03	1.60E-03	2.30E-07
dcAA4	1.01E+03	9.66E-03	9.61E-06			

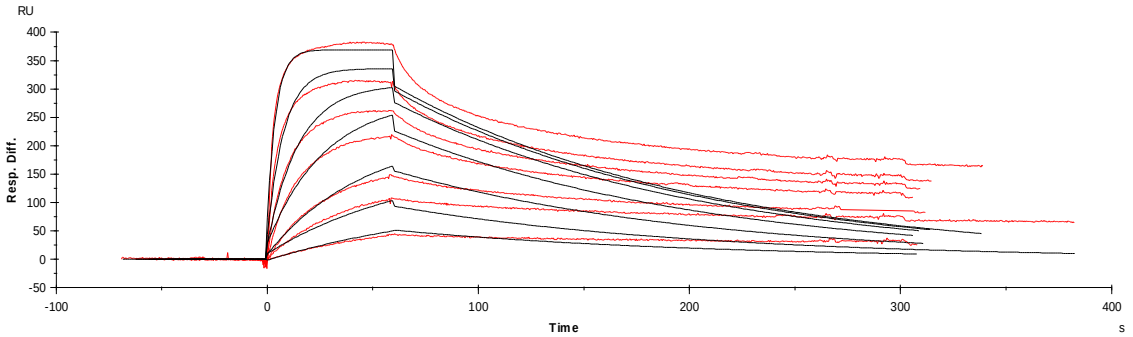
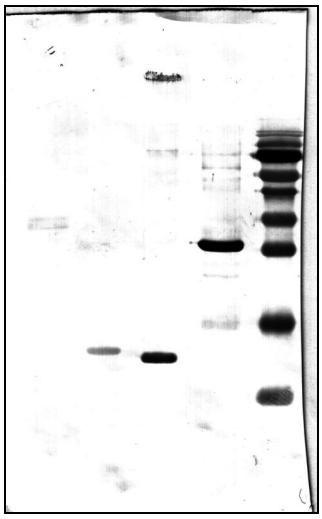


Figure 32. A BiaCore sensogram series obtained for avidin dilution series. *The response difference was measured in relative units and time was measured in seconds. A Langmuirian binding model is fitted to each sensogram and kinetic parameters are calculated by BIAevaluation.*

Monoclonal antibody recognition of dcAA4, dcAvd, bAvd and AVR4

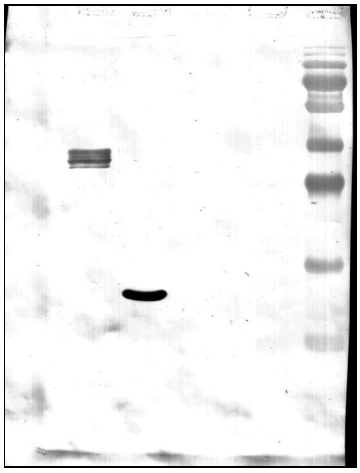
Samples were run to SDS–PAGE gel and blotted. Monoclonal antibodies against avidin and AVR4 were used as the primary antibodies. Two monoclonal antibodies against avidin were used; TDA2 and TDA6 which are mouse–anti–avidin antibodies. A monoclonal antibody against AVR4 was also used; mouse–anti–AVR4 (AVR4). The same secondary antibody was used for all blots; goat–anti–mouse–alkaline–phosphatase (GAM–AP). The samples were prepared so that each sample contained between 0.2 and 0.7 µg of protein.

AVR4 antibody recognized dcAA4 and AVR4 proteins the best but it also recognized avidin and dcAvd (figures 33). TDA2 recognized only dcAvd and avidin (figure 34). TDA6 recognized dcAvd, avidin and dcAA4 but not AVR4 (figure 35).



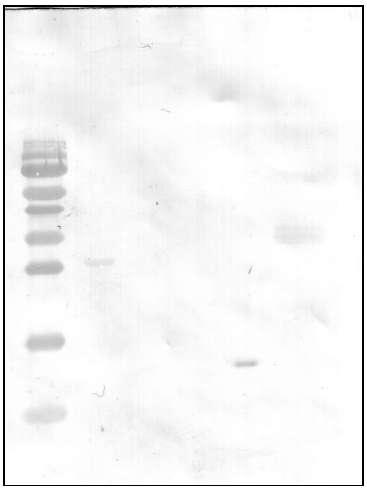
dcAvd bAvd AVR4 dcAA4 mw

Figure 33. A Western blot analysis for dcAA4, dcAvd, avidin and AVR4 by using monoclonal antibody against AVR4 protein. *The monoclonal antibody against AVR4 recognizes dcAA4 and AVR4 stronger than dcAvd and avidin. There were 0.7 µg of dcAA4, dcAvd and AVR4 proteins and 0.6 µg of avidin in the blot. Mw (Ladder) is the molecule weight marker.*



dcAvd bAvd AVR4 dcAA4 mw

Figure 34. A Western blot analysis for dcAA4, dcAvd, avidin and AVR4 by using TDA2 monoclonal antibody against avidin. *The monoclonal antibody TDA2 against avidin recognizes dcAvd and avidin but not AVR4 or dcAA4. There were 0.5 µg of dcAA4, avidin and AVR4 proteins and 0.6 µg of dcAvd in the blot. Mw (Ladder–plus) is the molecule weight marker.*



mw dcAA4 AVR4 bAvd dcAvd

Figure 35. A Western blot analysis for dcAA4, dcAvd, avidin and AVR4 by using TDA6 monoclonal antibody against avidin. *The monoclonal antibody TDA6 against avidin recognizes dcAvd, avidin and dcAA4 but not AVR4. There were 0.5 µg of AVR4, 0.4 µg of avidin, 0.2 µg of dcAA4 and 0.6 µg of dcAvd in the blot. Mw (Ladder) is the molecule weight marker.*

6 Discussion

The production of dchAvds was done in two different production systems, *E. coli* and Bac–to–Bac insect cell. dcAA4 was also produced in pilot–scale fermentation of *E. coli*. The proteins were purified by affinity chromatography using 2–iminobiotin resin. The production and purity of the proteins were recorded by SDS–PAGE and Western blot methods. The ligand binding studies were done by fluorescence spectroscopy and surface plasmon resonance (SPR) spectroscopy using BiaCore biosensor and the oligomerization state was studied by high performance liquid chromatography (HPLC).

dcAA4 was the only protein that was produced in yields enough to continue with the ligand binding studies and the oligomerization studies. It can be speculated that since AVR4 is more closely related to avidin than AVR2 and streptavidin (Laitinen 2002 and Hytönen et al. 2005a) the dcAA4 would be structurally more close to the dcAvd and thus more stabile than dcAA2 and dcASA. Also AVR4 is the most stable protein among this group, and it has been also successfully mixed with avidin in previous study resulting highly stable chimeric proteins (Hytönen et al. 2005b). A high resolution structure or even a simulated structural study of dchAvds would most probably provide answers to this question. The structure of dcAvd is available (PDB: 2c4i) and could be used as a template for the modelling. The structural analysis and modelling could also give explanations to why dcAA2 and dcASA are produced in significantly lower amounts than dcAA4. It would be interesting to see if production yields of dcAA2 and dcASA could be improved by pilot–scale fermentation production and if the fragmentations of both dcAA2 and dcASA could be lowered as was the case with dcAA4. Some 10 mg of dcAA4 was sent to Dr. Tomi Airenne, Åbo Akademi, for crystallization and X–ray studies, but no structure has been obtained up to date.

The fragmentation of the dchAvds seems to be a habit of *E. coli* growing in less controlled environment since almost no protein fragmentation was seen in pilot-scale fermentation whereas protein expressed in Erlenmeyer bottles showed significant amount of fragmented proteins. This can be due to the more stabile and controlled environment in

the fermentor (Gnoth et al. 2008). The fragmentation of recombinant protein in *E. coli* could also be overcome by stabilization of the recombinant protein by means of comparative mutagenesis (Gaves et al. 2008). For this approach more detailed structure of the protein is needed. The fragmentation pattern of the proteins is some what broad in size raging in size between ~11 kDa to over 30 kDa. This indicates that there is more than one site from which the proteins are cleaved. dcASA is fragmented mostly to ~11 kDa sized piece when dcAA2 and dcAA2 are fragmented to many pieces varying around 30 to 28 kDa. The ~11 kDa piece is visible in dcAA4 and dcAA2. As the ~11 kDa sized fragment is present in all the recombinant proteins it can be speculated that the linker between the two monomers combined to one polypeptide is quite prone to proteolytical cleavage. It can also be speculated that the fragments around size 30 kDa could be recombinant proteins containing the OmpA–signalling sequence.

In insect cells the glycosylation of dchAvds was seen as expected but the yields were very poor and the proteins could not be purified. This could have been due to the saturation of the biotin binding sites by biotin present in the production system. Although the growth media used was biotin–free, the cells grown in biotin–containing medium carry some biotin. The amounts of protein obtained from the productions were so low that even a small amount of biotin present could have been enough to saturate the proteins completely. The poor yields on the other hand were most probably due to the usage of an ompA signal peptide from *Bordatella avium* instead of eukaryotic signal peptide. In previous study, dcAvd was successfully expressed in baculovirus-insec cell expression system by using the signal peptide from chicken avidin (Nordlund et al 2004). The next approaches for the production of the dchAvds would be to try out the pilot-scale fermentation of dcAA2 and dcASA in *E. coli* and to optimize the insect cell production by replacing the prokaryotic secretion signal with eukaryotic secretion signal.

The HPLC studies showed that the dcAA4 molecule was a tetramer with and without biotin in pH 7. The size for the protein–ligand complex and the protein it self was approximately 46 kDa which is in line with previous results for avidin, dcAvd and their mutants (Hytönen et al. Proteins 2005).

The biotin binding studies of dcAA4 indicate that there could be two types of biotin binding sites within the protein; one that binds *d*–biotin more loosely, and one that binds *d*–biotin more tightly resembling wild type avidin. The experiments done by fluorescence spectroscopy showed that, during the first 500 seconds the release of biotin from dcAA4 was clearly faster than during the last 3100 seconds (table 1; sample 3: 50 nM protein, table 14: k_{diss}(first 500 s) = 7.02 * 10^{-4} s^{-1} vs. k_{diss}(last 3100 s) = 9.39 * 10^{-5} s^{-1}, figure 30). This was shown also in the experiment where dcAA4 was partially presaturated with *d*–biotin (table 1; sample 6: 25 nM of *d*–biotin with 50 nM protein). As hypothesized, the tight biotin binding pockets were saturated with biotin and the complex–forming–releasing reaction was mostly seen as the reaction of the weaker binding site, showing a dissociation rate constant (table 14: k_{diss} = 5.62 * 10^{-4} s^{-1}, appendix 10) very close to the dissociation rate constant obtained for the weak binding site from the experiments done with dcAA4 that was not presaturated. On the other hand, the tight binding pocket was more clearly observed in experiments done with excess dcAA4 compared to Bf560–BTN (table 1; sample 5: 200 nM protein, table 14: k_{diss}(last 3100 s) = 5.92 * 10^{-5} s^{-1}, appendix 9 and k_{diss}(whole data) = 7.67 * 10^{-5} s^{-1}). These experiments showed that with an excess of protein compared to the ligand, the tight binding dominates the reaction. In the experiments that were done with excess of Bf560–BTN compared to the amount of protein (table 1: sample 4) the results were comparable with the results obtained from dcAA4 and the tight binding pocket appeared only after excluding the first 500 s of the experiment (table 14: k_{diss}(whole data) = 1.06 * 10^{-4} s^{-1} vs. k_{diss}(last 3100 s) = 7.24 * 10^{-5} s^{-1}, appendix 8). The effect of the weak binding site on the dissociation rate constant is clear when the experiment are viewed as a whole (table 14: whole data), not as two separate data sets (table 14: first 500 s vs. last 3100 s). One can also easily see that fitting of single exponential does not yield a good fit, thus suggesting the presence of divergent binding sites. The rate constants decrease by order of magnitude when the whole data is compared to the data of the last 3100 s (table 14). The dissociation rate constant of dcAA4 (tight binding site) is greater than the ones obtained for avidin (k_{diss} = 2.91 * 10^{-5} s^{-1}, appendix 6) and AVR4 (k_{diss} = 3.35 * 10^{-5} s^{-1}, appendix 7) yet they are of same order of magnitude. On the other hand the dissociation rate constants obtained for the data of

the first 500 seconds of dcAA4 measurements, the pre saturated sample and the simulated data are close to each other and 10 fold greater than the ones for avidin, AVR4 and dcAA4. This implies that there are two different types of binding sites present in dcAA4.

The complexes of avidin and AVR4 with Bf560–BTN dissociate significantly slower (17.19% and 15.98% in an hour) than the complexes of dcAA4 with Bf560–BTN (58.94% in an hour) (table 14, appendix 12 and 13, and figure 31). The simulated data for the weak binding site of dcAA4 shows similar dissociation (100%) as the data obtained for the presaturated dcAA4 (92.97%) (table 14, appendix 17 and 16). The simulated data was obtained by subtracting the wt Avd data from the whole data obtained for dcAA4. The amount of free Bf–560–BTN after an hour of the addition of excess *d*–biotin is a little over 50 % in all the dcAA4 samples except for the pre saturated sample and the simulated weak binding (table 14). These results point further to the conclusion that there actually is a weak binding site present in the dcAA4 protein that releases the ligand rather quickly and a tight binding site that acts more like the binding site in avidin and AVR4.

The presence of two distinct binding sites in the protein can be observed by the fluorescence spectroscopy nicely since the measurement was done by free biotin in liquid; not by immobilised ligand as in the SPR measurements. The Biacore® measurements will give a somewhat suggestive result on how dcAA4 binds 2–iminobiotin that has been immobilized on a solid support. This method is not the most ideal way of measuring the rate constants for multimeric proteins since one or more binding sites might be occupied from the same oligomer. Whether one or more ligands are bound can not be distinguished, and distinction of two different binding sites will also be impossible. What the results from Biacore® showed was that avidin binds its ligand 2–iminobiotin with higher affinity when compared to AVR4 and dcAA4, and that AVR4 binds its ligand with greater affinity than dcAA4 (table 15). This was in line with the results from fluorescence spectroscopy and with values obtained from literature (Hytönen et al. 2005b). The values from literature cannot be directly compared to the values obtained in this work since they were done with different sensor chips. Each sensor chip is unique and might contain different densities of immobilized ligand on them. The

density of the ligand bound on the surface of the sensor chip has an affect on the protein binding. This is an issue especially when handling oligomeric proteins, such as avidins, since one protein can bind more than one ligand. If the ligand is small and the analyte is large, as 2–iminobiotin is compared to avidin, the steric crowding on the sensor chip can also prevent the analyte from binding to the surface (Biacore® Sensore Surface Handbook). This has an affect on the results and makes the comparison of absolute values impossible. On the other hand relative comparison of the results is possible.

The monoclonal antibodies against avidin recognized dcAvd, avidin and dcAA4 but not AVR4 as expected. The monoclonal antibody against AVR4 recognized dcAA4 and AVR4 very strongly and very faintly avidin and dcAvd. This could be partially due to background binding that could have been investigated by using a negative control for example bovine serum albumin (BSA). In earlier experiments, the monoclonal AVR4 antibody did not bind avidin (Ovod & Hytönen, unpublished data). The results suggest that dcAA4 is a heterodimer of avidin-like and AVR4-like subunits as hypothesized.

What makes streptavidin, AVR2 and AVR4 interesting fusion partners is that they are almost identical in their 3D structure and they bind the same ligand as avidin (Laitinen et al. 2002) but they have distinct biochemical properties compared to it (Hytönen et al. 2004). These similarities and differences provide a good basis for the evaluation of applicability and functionality of the dchAvds. The dchAvds could be made to bind two different molecules or the same molecule with two different affinities as shown in this thesis. The affinity of the two distinct binding sites binding the same ligand could be modified to be pH or temperature controlled. The applicability of these fusion proteins would be broad; they could be used as building blocks in nanostructures and immobilizers of substrates in biosensor technology as well as in diagnostics. The ability of having two distinct binding sites whose ligand binding could be controlled by the environment could function as a framework for controlled release of drugs. They could also be used in more conventional biochemistry as tags in affinity chromatography. Furthermore, these molecules could also be used to replace and broaden the already existing (strept)avidin-biotin technologies.

The current study showed that two closely related proteins can be incorporated into the same covalent dual chain molecule and that the resulting subunit fusion protein was functional. The compatibility problems resulting in poor yield of recombinant protein and its degradation could be encountered by means of molecular modelling and more detailed structural information. It has been shown that the stability of mRNA in *E. coli* can be significantly lowered if there are identical regions with in the mRNA (Deutscher 2006). It has been found that subunits of multimeric proteins often differ from one another in terms of primary sequence but resemble each other in 3D-structure. This is thought to be because protein folding can be disturbed by the existence of similar sequences within multimeric proteins (Wright et al. 2005). According to this model, similar sequences close to each other in the same polypeptide can interact with each other and therefore cause misfolding, leading to protein aggregation (Wright et al. 2005).

One possibility for extending this research could be the construction of dcAvds that combine new microbial avidins or biotin binding proteins (BBPs) from chicken together with members of the chicken avidin family. Because there is a vide variety of genetic material available, a library that displays all the different possibilities of combinations of circularly permutated biotin binding proteins could be constructed. The most interesting combinations could be chosen from these libraries and one could end up with quite surprising combinations of proteins. This kind of approach would be theoretically and practically a valuable tool.

Fusion proteins are widely exploited in broad range of biosciences. The fusion proteins studied in this thesis are of interest because they pose a useful characteristic of high affinity towards a small molecule, *d*–biotin. *D*–biotin can be coupled to other molecules, proteins, polymers and inorganic materials (Govender et al. 2007 and Guiliano 2008). The dchAvds can thus be used for cross–bridging molecules in variety of different applications where specific targeting or binding is needed. In advance, compared to previously constructed dcAvd and dual affinity dcAvd (Nordlund et al. 2004 and Hytönen et al. Protein 2005), these molecules could be genetically modified in some what

easier way in one step PCR–based mutagenesis. This makes the modification of binding properties of these proteins easier and faster compared to dcAvd. The one step genetic modification of dchAvds is possible because they contain two sequentially identical genes that can be modified separately at a same time without the problems that occur if repeated sequences are present. With dcAvd this is not possible because the two genes encountered in the pseudodimer are almost identical (Nordlund et al. 2004) and all mutations done to the other cpAvd will occur on the other as well. On the other hand we have shown that dcAA4 contains two distinct binding sites and thus it is comparable to the dual affinity avidin constructed earlier, yet potentially more convenient platform for further modification (Hytönen et al. Proteins 2005).

7 Conclusions

Production of dcAA2 and dcASA in *E. coli* was compromised due to partially cleaved end–product whereas dcAA4 produced more efficiently, especially in pilot–scale fermentor. The production of dchAvds was unsuccessful in baculovirus–insect cell expression system most probably due to wrong type of signal peptide used. dcAA4 was further characterized and more detailed information of its structure and ligand binding properties was obtained. The state of oligomerization of dcAA4 with and without *d*–biotin in pH 7 is pseudotetrameric. The dcAA4 pseudodimer holds two distinct biotin binding sites; one with weaker binding affinity and one stronger binding affinity. This information serves as an important framework for further studies and modifications of these unique fusion proteins. The molecules studied in this thesis could be potentially exploited in biotechnological applications varying from nanosciences and medical technologies to conventional biochemistry.

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Appendix

Appendix 1: *Fermentation medium*

KH_2PO_4	3.5	g/l
K_2HPO_4	5.0	g/l
$(NH_4)HPO_4$	3.5	g/l
$MgSO_4 \cdot 7H_2O$	0.5	g/l (*, **)
Glucose	5.0	g/l (*, +)
Yeast extract	5.0	g/l
Trace metals	1.0	ml/l (*, +)
Glycerol 87%	5.0	ml/l

* = applied after sterilization
** = sterilized separately
+ = sterile filtered Ø 0.22

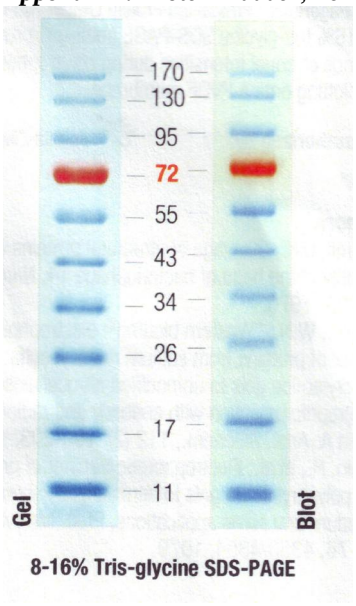
Appendix 2. *Trace metals 1000x stock*

$FeCl_3 \cdot 6H_2O$	1.6	g/l
$CoCl_2$	0.2	g/l
$CuCO_4$	0.1	g/l
$ZnCl \cdot 4H_2O$	0.2	g/l
$(NH_4)_6Mo7O_{24} \cdot 4H_2O$	0.2	g/l
H_3BO_3	0.05	g/l
HCl 37%	10	ml
H_2O	1000	ml

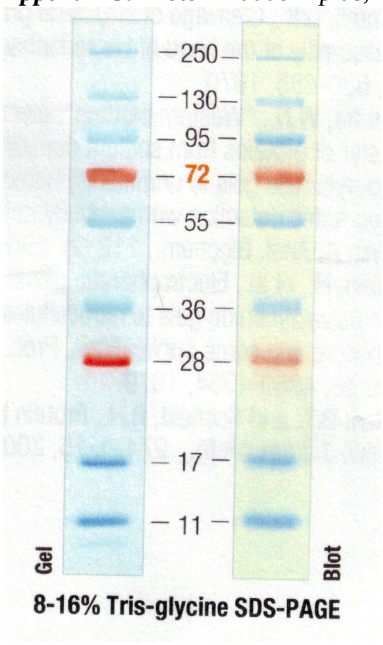
Appendix 3. *Feed solution*

Glycerol 87%	210	ml
H2O	190	ml
1000x trace metals	2	ml
$MgSO_4$ 0.25 g/ml	4	ml

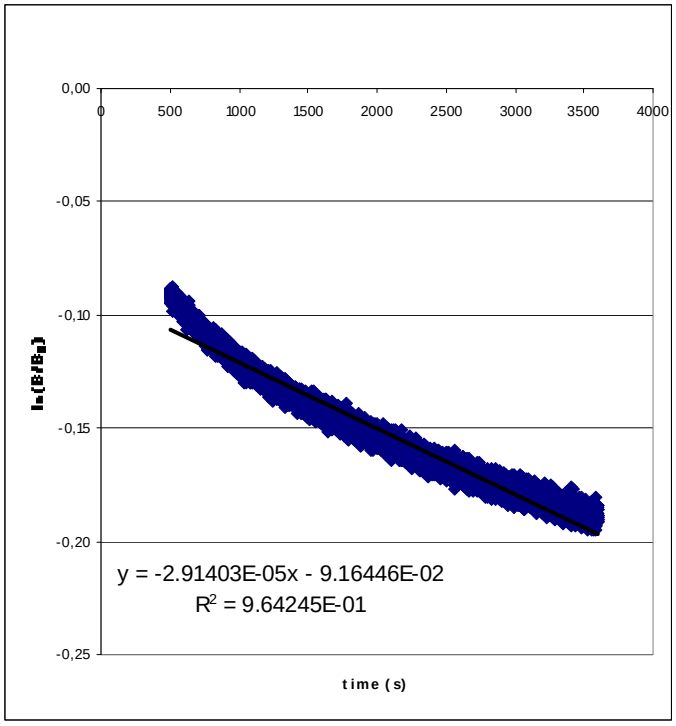
Appendix 4: *Protein Ladder, Fermantas*



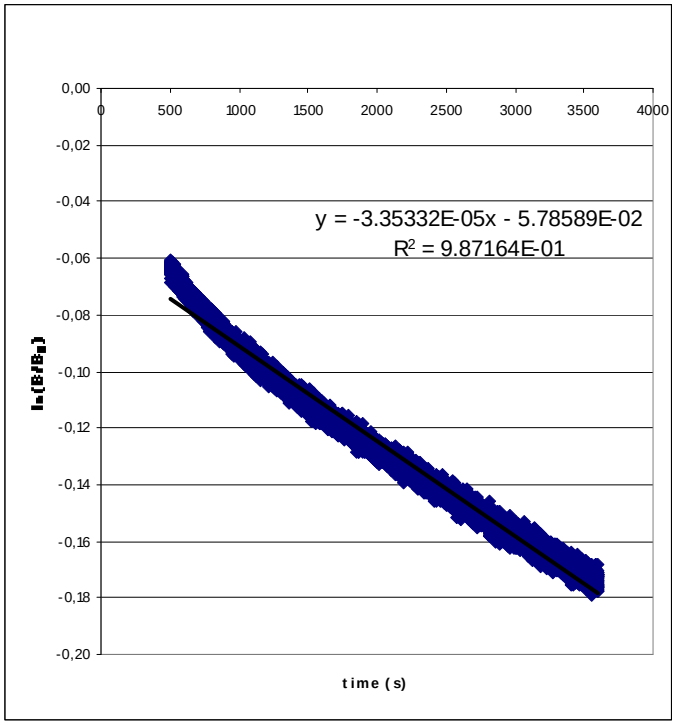
Appendix 5: *Protein Ladder –plus, Fermentas*



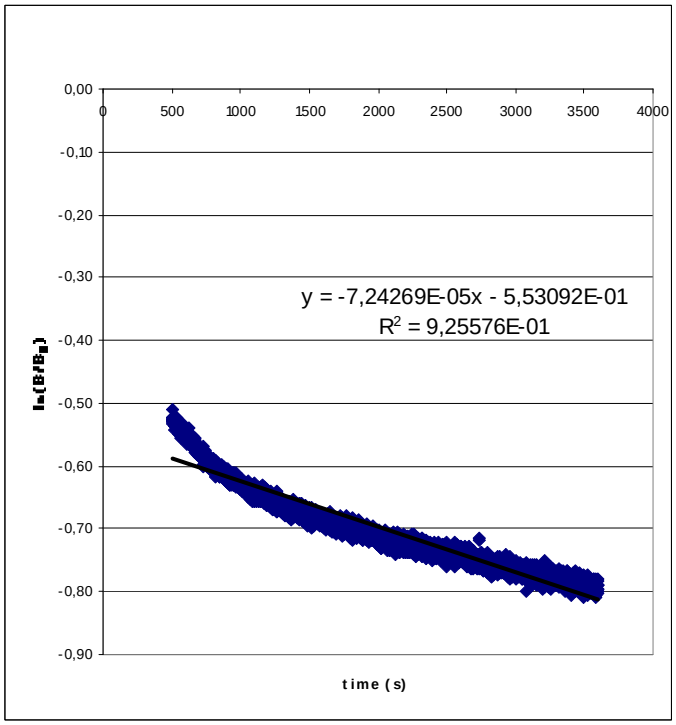
Appendix 6: *Determination of the dissociation rate constant of avidin by fluorescence spectrometry assay*



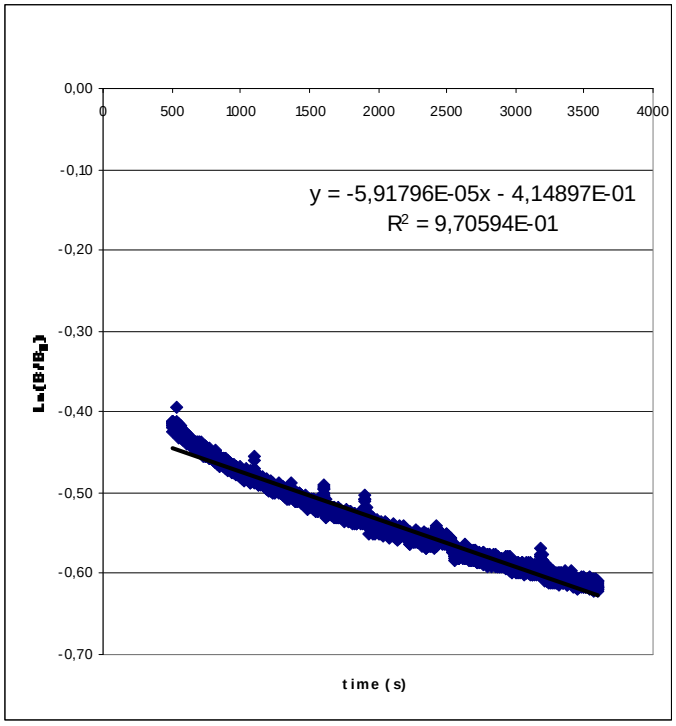
Appendix 7: *Determination of the dissociation rate constant of AVR4 by fluorescence spectrometry assay*



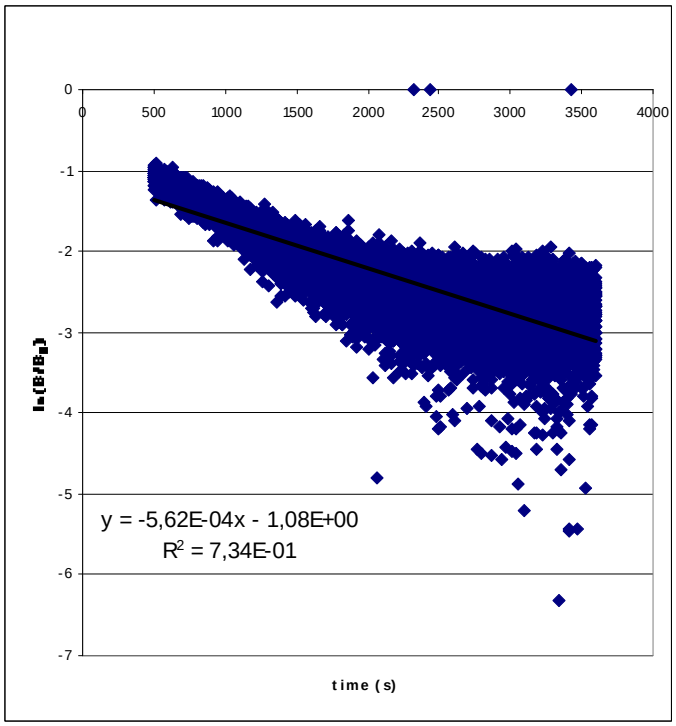
Appendix 8: *Determination of the dissociation rate constant of dcAA4 by fluorescence spectrometry assay; sample 4 (table 1): 25 nM protein with 50 nM Bf560–BTN*



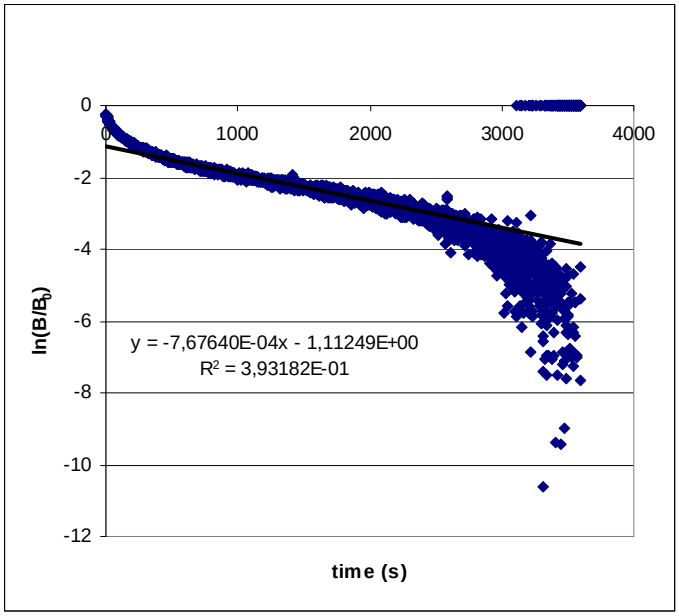
Appendix 9: *Determination of the dissociation rate constant of dcAA4 by fluorescence spectrometry assay; sample 5 (table 1): 200 nM protein with 50 nM Bf560–BTN*



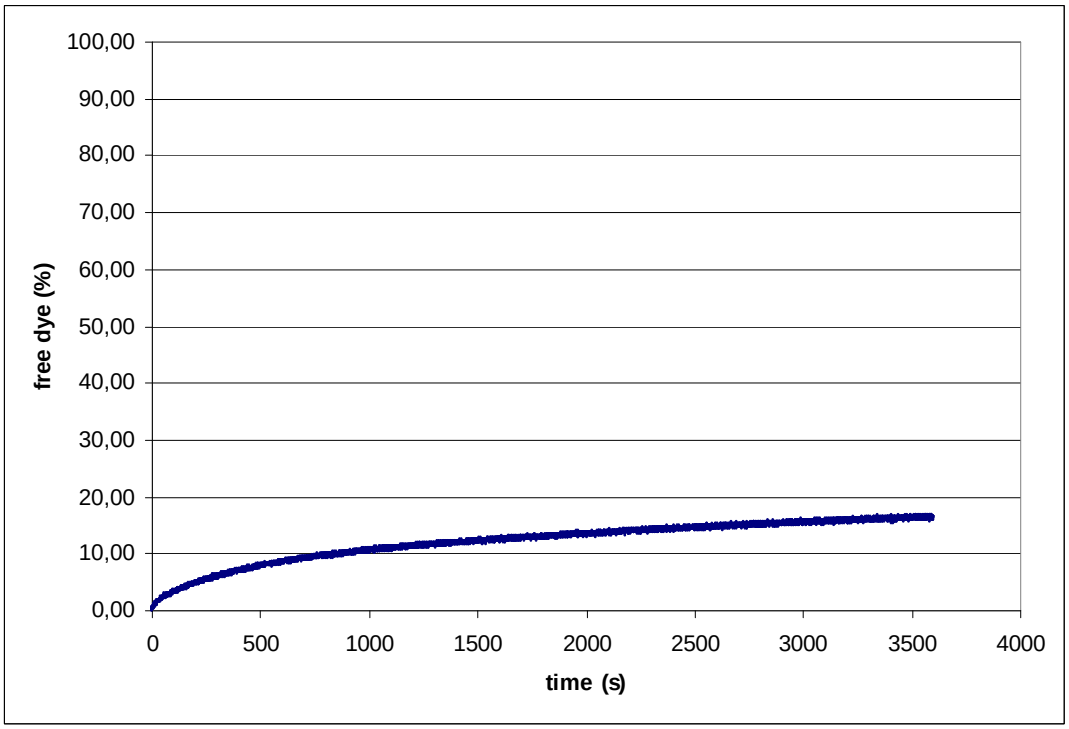
Appendix 10: *Determination of the dissociation rate constant of dcAA4 by fluorescence spectrometry assay; sample 6 (table 1): 50 nM protein presaturated with 25 nM d–biotin and released with 50 nM Bf560–BTN*



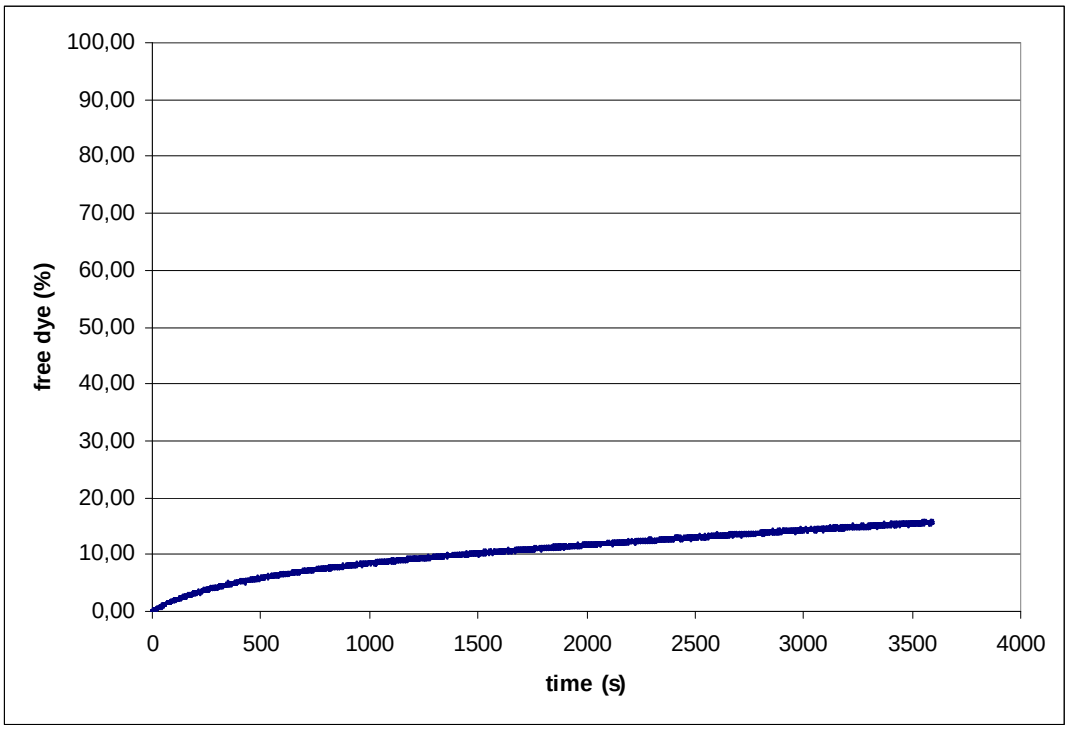
Appendix 11: *Determination of the dissociation rate constant of dcAA4 by fluorescence spectrometry assay, simulated data*



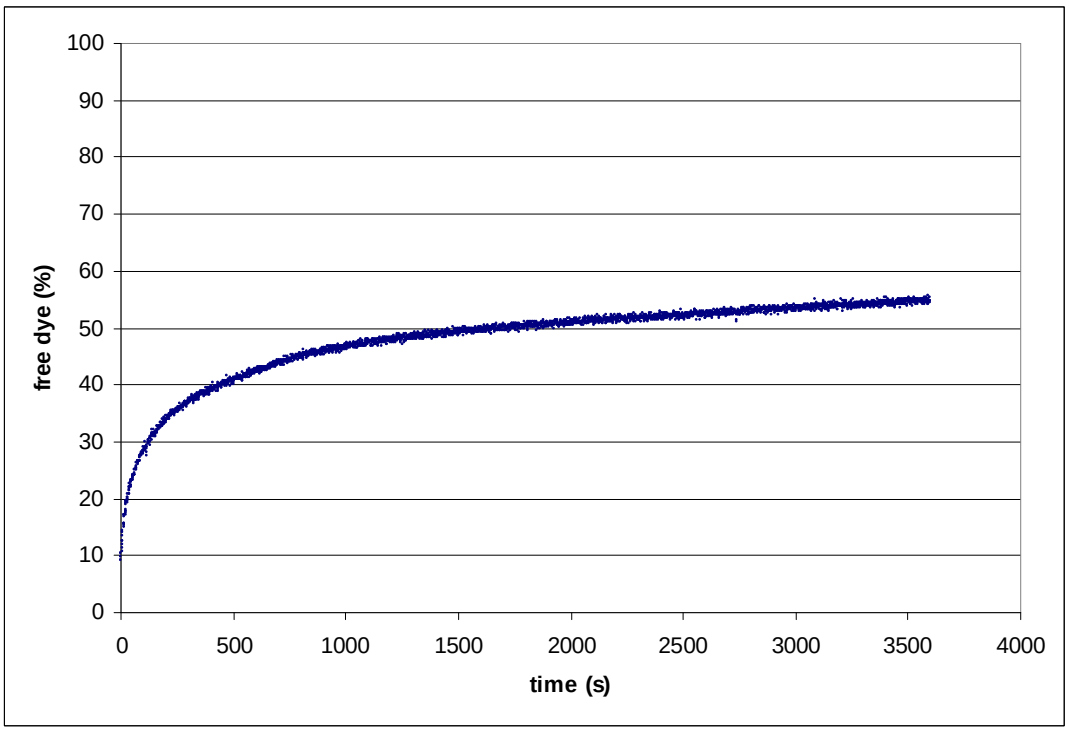
Appendix 12: *The relative amount of free Bf560–BTN released from the Avidin–Bf560–BTN complex after the addition of free biotin*



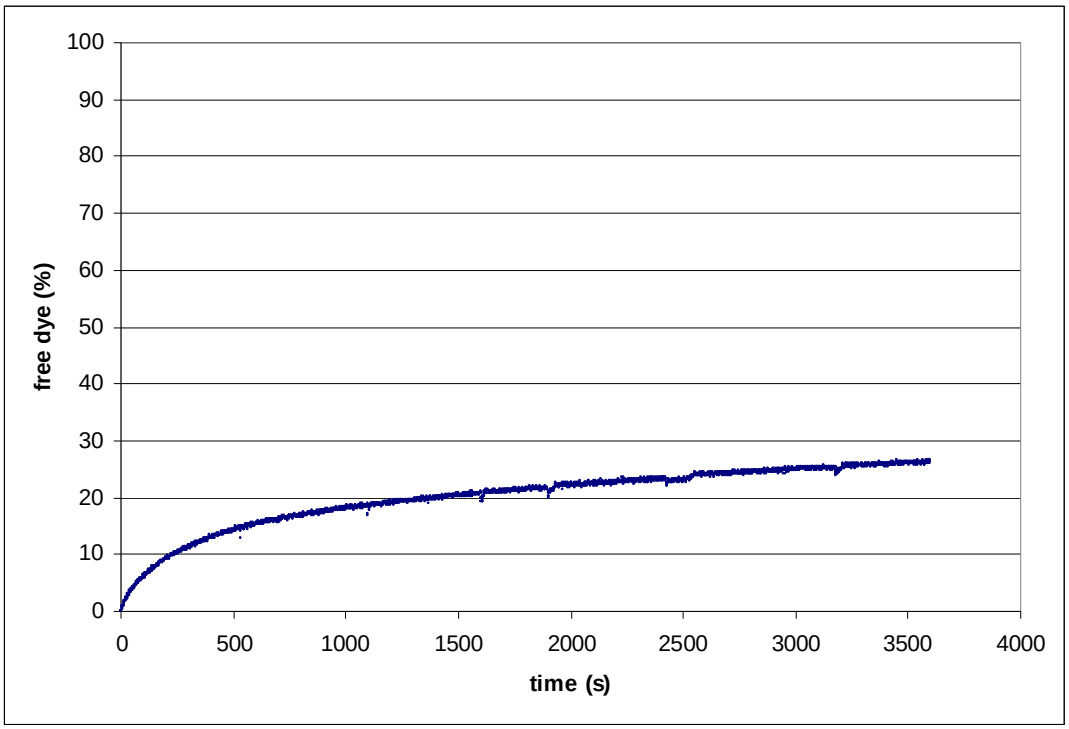
Appendix 13: *The relative amount of free Bf560–BTN released from the AVR4–Bf560–BTN complex after the addition of free biotin*



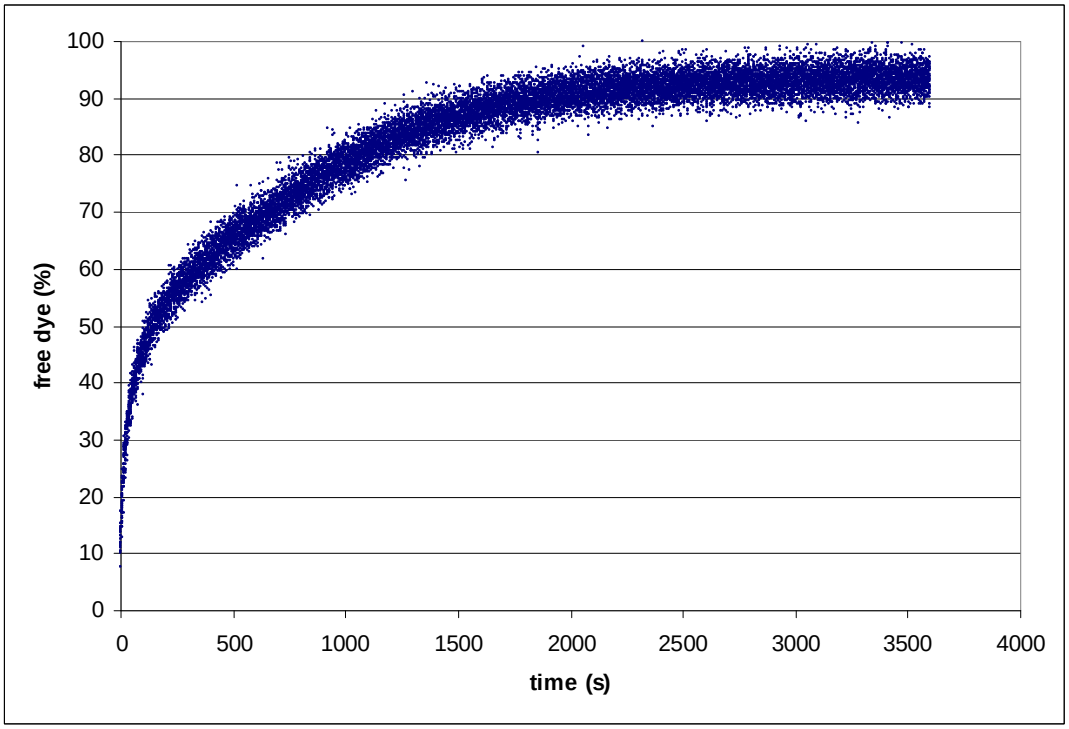
Appendix 14: *The relative amount of free Bf560–BTN released from the dcAA4–Bf560–BTN complex after the addition of free biotin (sample 4, table 1; 25 nM protein with 50 nM Bf560–BTN)*



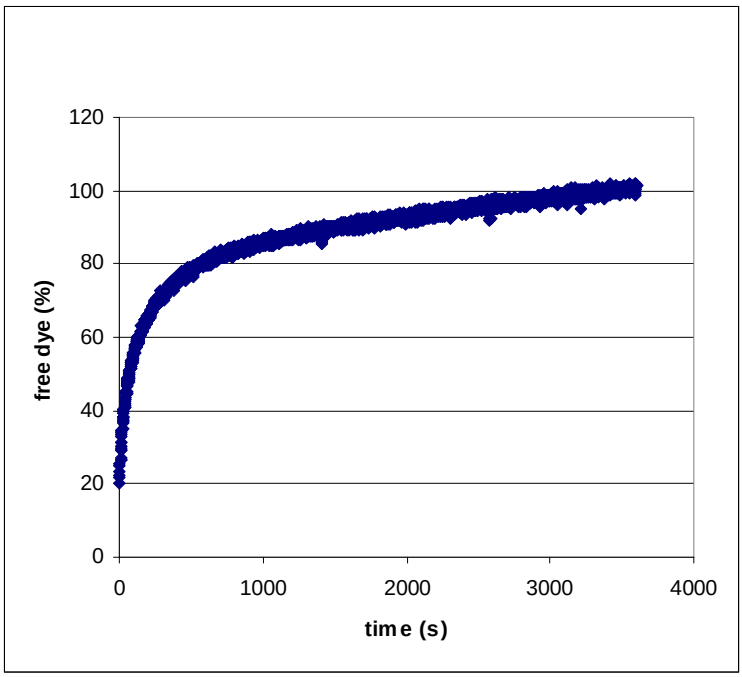
Appendix 15: *The relative amount of free Bf560–BTN released from the dcAA4–Bf560–BTN complex after the addition of free biotin (sample 5, table 1; 200 nM protein with 50 nM Bf560–BTN)*



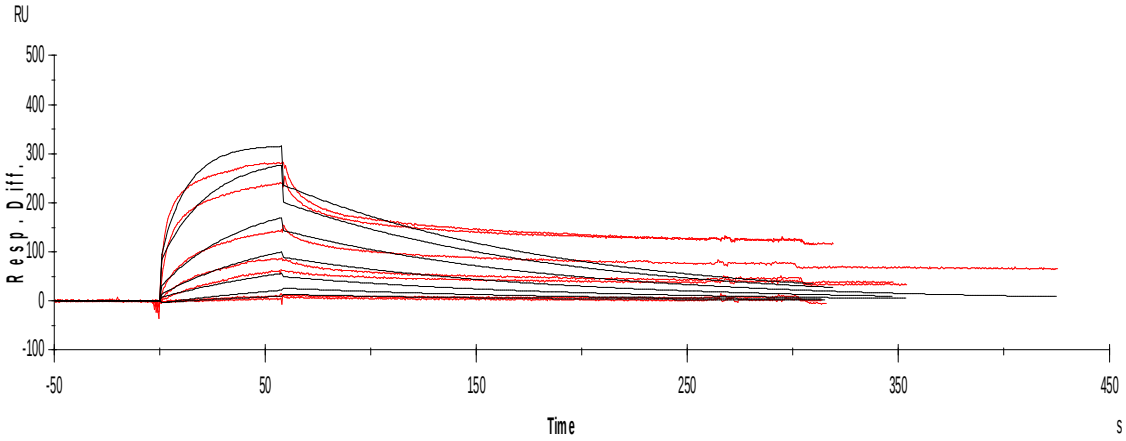
Appendix 16: *The relative amount of free Bf560–BTN released from the dcAA4–Bf560–BTN complex after the addition of free biotin (sample 6, table 1; 50 nM protein presaturated with 25 nM d-biotin and released with 50 nM Bf560–BTN)*



Appendix 17: *The relative amount of free Bf560–BTN released from the dcAA4–Bf560–BTN complex after the addition of free biotin, simulated data*



Appendix 18: *A BiaCore sensogram series obtained for AVR4 dilution series*



Appendix 19: *A BiaCore sensogram series obtained for dcAA4 dilution series*