



HAL
open science

A small bispecific protein selected for orthogonal affinity purification

Tove Alm, Louise Yderland, Johan Nilvebrant, Anneli Halldin, Sophia Hober

► **To cite this version:**

Tove Alm, Louise Yderland, Johan Nilvebrant, Anneli Halldin, Sophia Hober. A small bispecific protein selected for orthogonal affinity purification. *Biotechnology Journal*, 2010, 5 (6), pp.605. 10.1002/biot.201000041 . hal-00552347

HAL Id: hal-00552347

<https://hal.science/hal-00552347>

Submitted on 6 Jan 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



A small bispecific protein selected for orthogonal affinity purification

Journal:	<i>Biotechnology Journal</i>
Manuscript ID:	biot.201000041.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	12-Apr-2010
Complete List of Authors:	Alm, Tove; Proteomics, Biotechnology Yderland, Louise; Proteomics, Biotechnology Nilvebrant, Johan; Proteomics, Biotechnology Halldin, Anneli; Proteomics, Biotechnology Hober, Sophia; Proteomics, Biotechnology
Primary Keywords:	Bispecific binder
Secondary Keywords:	Orthogonal purification
Keywords:	Phage display, ABD, Z domain



Research Article ((8636 words))

A small bispecific protein selected for orthogonal affinity purification

Tove Alm, Louise Yderland, Johan Nilvebrant, Anneli Halldin, and Sophia Hober*
School of Biotechnology, Department of Proteomics, Royal Institute of Technology,
AlbaNova University Center, Stockholm, Sweden

Key words: Bispecific binder, orthogonal purification, phage display, ABD, Z domain

*Corresponding Author

Sophia Hober, Professor
AlbaNova University Center
KTH, School of Biotechnology, Proteomics
106 91 Stockholm, Sweden
Fax: +46 5537 8481 Tele: +46 5537 8330

Email: sophia.hober@biotech.kth.se

ABD	Albumin binding domain
ABD*	Stabilized albumin binding domain
Amp	Ampicillin
BSA	Bovine serum albumin
CD	Circular dichroism
Cml	Chloramphenicol
CV	Column volume
HBS-EP	HEPES buffered saline
HSA	Human serum albumin
IMAC	Immobilized metal affinity chromatography
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline supplemented with tween
PBSTB 5%	PBST supplemented with 5% bovine serum albumin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPA	Staphylococcal protein A
TSB	Tryptic soy broth
TYE	Tryptone yeast extract
Z ₂ domain	Dimeric Z domain

Abstract

A novel protein domain with dual affinity has been created by randomization and selection. The small alkali stabilized albumin binding domain (ABD*) used as scaffold to construct the library, has affinity to human serum albumin (HSA) and is constituted of 46 amino acids of which 11 were randomized. To achieve a dual binder, the binding site of the inherent HSA-affinity was untouched and the randomization was made on the opposite side of the molecule. Despite its small size and randomization of almost 1/4th of its amino acids a bifunctional molecule, ABDz1, with ability to bind to both HSA and the Z₂ domain/protein A was successfully selected using phage display. Moreover the newly selected variant showed improved affinity for HSA compared to the parental molecule. This novel protein domain has been characterized regarding secondary structure and affinity to the two different ligands. The possibility for affinity purification on two different matrices has been investigated using the two ligands, the HSA-matrix and the protein A-based, MabSelect SuRe-matrix, and the new protein domain was purified to homogeneity. Furthermore, gene fusions between the new domain and three different target proteins with different characteristics were made. To take advantage of both affinities, a purification strategy referred to as orthogonal affinity purification using two different matrices was created. Successful purification of all three versions could efficiently be carried out by the orthogonal affinity purification strategy.

1 Introduction

Due to the high cost in producing and purifying proteins the production steps as well as the purification of the target protein need to be optimized. For high throughput production and purification of many proteins in parallel, general methods without the need for individual optimization are necessary. Recombinant techniques allow efficient production of target proteins and affinity chromatography offers a selective purification method. In order to achieve effective purification for a wide range of different proteins, a selective purification tag can be used. Different affinity interactions have been exploited for this purpose, e.g. enzyme-substrate, protein-metal, protein-carbohydrate, or protein-protein [1]. Depending on the choice of interaction partners the cost and complexity of the purification conditions will vary. The choice of affinity tag can also influence the amount of produced protein, its solubility, stability and function. A small tag is less likely to interfere with the target protein and its further applications. Another important aspect is that the tag should be robust and fold easily regardless of target protein. In order to achieve a target protein with high purity usually more than one purification step is necessary. To increase the selectivity of the purification method and thus enhance the purity of the sample, an orthogonal purification strategy would be beneficial. In classical orthogonal purification design, the succeeding separation characteristics are distinct from each

1
2
3
4
5
6 other, utilizing different characteristics of the proteins. Here we introduce the term
7
8 orthogonal affinity purification. Endowing one purification tag with more than one
9
10 useable affinity would increase the efficiency of the purification. The two binding
11
12 surfaces in the small bispecific tag could be used for subsequent purification without
13
14 any target specific optimization.
15
16

17
18 A small protein domain, historically used as affinity tag for protein purification, is the
19
20 albumin binding domain (ABD) [2]. ABD is a three helical bundle [3] derived from
21
22 the Streptococcal protein G [4]. To render this domain further usable in affinity
23
24 purification it has been engineered into a variant denoted ABD* with increased
25
26 resistance to alkaline conditions [5]. Hence, cleaning in place of the equipment
27
28 including the affinity matrix with ABD* coupled as ligand is possible. To gain more
29
30 information about the protein domain, directed mutagenesis was used to locate the
31
32 albumin-binding site. It was concluded to be positioned mainly in the second helix
33
34 [6]. This finding was further supported by structural investigations, using NMR and
35
36 X-ray crystallography [7, 8]. Also triple alanine mutants were made in helix one and
37
38 three at positions pointing away from the suggested binding site showing retained or
39
40 increased binding to human serum albumin (HSA) [6]. Thus indicating that positions
41
42 pointing away from the HSA binding surface would be possible to randomize to
43
44 create a library that renders selection of new binders with retained affinity to HSA
45
46 possible. Due to the improved characteristics of ABD* and its ability to bind HSA, it
47
48 was chosen as scaffold when creating the phage display library.
49
50
51
52
53

54
55 Another protein also frequently used for protein purification, both as affinity ligand
56
57
58
59
60

1
2
3
4
5
6 and as purification tag, is the Staphylococcal protein A (SPA) [9]. The high
7
8 selectivity has made protein A matrices commonly used for antibody purification [10]
9
10 and due to the availability of affinity matrices with protein A as ligand, one of the
11
12 domains responsible for IgG-binding was chosen as target in the phage display
13
14 selection.
15

16
17 Here we, for the first time, present a strategy that makes it possible to select small
18
19 proteinaceous binders capable to selectively bind two different proteins. Moreover
20
21 the possibility of purifying a selected and characterized variant from the ABD*-
22
23 library using two different affinity matrices was investigated. Finally, the selected
24
25 domain was introduced in the N-terminus of three different target proteins and
26
27 utilized as affinity tag in two succeeding purification steps.
28
29
30
31
32
33
34

35 **2 Materials and methods**

36 37 38 39 40 41 *2.1 Bacterial strains and oligonucleotides*

42
43 For recombinant- and phage work the *Escherichia coli* (*E. coli*) strain RRIΔM15 was
44
45 used [11]. The recombinant DNA techniques used were carried out essentially as
46
47 described by Sambrook et al. [12]. Oligonucleotides for the library construction were
48
49 synthesized at SGS (Scandinavian Gene Synthesis, Köping, Sweden). All other
50
51 primers were synthesized at MWG (MWG-BIOTECH AG, Ebersberg, Germany).
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 DNA restriction enzymes were bought from MBI Fermentas (MBI Fermentas,
7
8 Vilnius, Lithuania). Protein production was carried out in *E. coli* strain Rosetta(DE3)
9
10 (Novagen, Merck KGaA, Darmstadt, Germany) and all protein purifications were
11
12 carried out using an ÄKTAExplorer (GE Healthcare, Uppsala, Sweden).
13
14
15

16 17 18 *2.2 Library construction*

19
20 The phagemid vector pML was constructed from pKN [13] by introduction of an
21
22 *EcoRI*-site and a dummy fragment using restriction sites *MluI* and *XhoI*. pML was
23
24 restricted using *EcoRI* and *XhoI* and the restricted vector was separated on a 1%
25
26 agarose gel and extracted from the gel using QIAquick Gel Extraction Kit (QIAGEN,
27
28 Solna, Sweden). The library was assembled using oligonucleotides ABDlibCod3 and
29
30 ABDlibRev3 having complementary regions in the part encoding helix two. The
31
32 DNA-fragments were hybridized and extended using six temperature cycles with
33
34 AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), purified using
35
36 QIAquick PCR Purification Kit (QIAGEN) and restricted using *EcoRI* and *XhoI*.
37
38 After separation and extraction from a 3% low melt agarose gel the purified vector
39
40 and fragment were ligated using T4 DNA Ligase (New England BioLabs, Boston,
41
42 MA, USA). To remove contaminating proteins the ligation mixture was phenol-
43
44 chloroform extracted and thereafter ethanol precipitated. The plasmids were
45
46 transformed into electrocompetent RRIΔM15 using Bio-Rad Gene Pulser (Bio-Rad
47
48 Laboratories, Hercules, CA, USA) to yield a final library size of $\sim 10^7$ members.
49
50
51
52
53
54
55
56
57
58
59
60

2.3 Analysis of the ABD*-library

The library was analyzed by PCR amplification and DNA sequencing was performed on an ABI 3700 DNA Sequencer (Applied Biosystems).

To assess the retainment of HSA-binding in the library two rounds of phage selection against HSA were performed. HSA (Sigma-Aldrich, Steinheim, Germany) was biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, IL, USA) according to the manufacturer's recommendation. The selections were carried out as described in section 2.4 with a target concentration of 1 μ M with the exception that cycle 1 was performed with the target protein captured on paramagnetic beads during the binding step.

A western blot was done on phage stocks eluted from cycle 1 and 2 to analyze the enrichment of HSA-binders from the initial library. As a positive control cell lysate with the parental ABD* was included. The samples were reduced and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a 4-12% gradient NuPAGE Novex Bis-Tris gel (Invitrogen, Paisely, Scotland). After separation the proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) using an XCell II™ Blot Module (Invitrogen). The membrane was blocked with phosphate buffered saline supplemented with tween (50 mM phosphate, 100 mM NaCl, pH 7.2, 0.1% Tween 20) (PBST) also containing 1% gelatin. The membranes were incubated with biotinylated HSA (120 nM) for 1 h, followed by

1
2
3
4
5
6 washing (PBST), incubation with the secondary peroxidase-conjugated streptavidin
7
8 (DakoCytomation, Glostrup, Denmark) (0.6 g/l diluted 1:5000), and a final washing
9
10 step. Detection was carried out using a CCD-camera (Bio-Rad) with SuperSignal
11
12 West Dura Extended Duration Substrate (Pierce) according to the manufacturer's
13
14 protocol.

15
16
17 To investigate the degree of truncated pIII in relation to full length on the phage
18
19 surface, another western blot analysis was done as described above but detection was
20
21 carried out using a primary mouse antibody recognizing pIII (Nordic Biosite, Täby,
22
23 Sweden) (0.5 g/l diluted 1:1000) and a secondary peroxidase-conjugated rabbit
24
25 antibody (Sigma-Aldrich) (diluted 1:5000). The relative intensities of the bands were
26
27 compared using QuantityOne software (BioRad).
28
29
30
31
32
33

34 *2.4 Selection*

35
36
37 Four rounds of selection in solution against a dimeric Z domain (Z₂ domain) [14]
38
39 were performed. All tubes used in the selection were protein LoBind tubes
40
41 (Eppendorf, VWR International, Stockholm, Sweden) blocked with PBST
42
43 supplemented with 5% bovine serum albumin (BSA) (Sigma-Aldrich) (PBSTB 5%).
44
45 Streptavidin-coated or REGEN beads coated with neutravidin according to
46
47 manufacturer's recommendations (Dynal Biotech, Oslo, Norway) were used for
48
49 preselection and selection. Beads were washed in 2*500 µl PBST and blocked with
50
51 500 µl PBSTB 5%. Four series (A, B, C, and D) with different target concentration
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 were performed and to increase the stringency of each cycle the number of washes
7
8 and amount of Tween 20 in the washing buffer (PBST supplemented with 3% BSA)
9
10 was increased for each cycle, table 1. PBS was used in the last wash of each cycle.
11
12 For each round of selection a new phage stock was prepared by infection with helper
13
14 phage M13KO7 (New England BioLabs), followed by polyethylene glycol and
15
16 sodium chloride (PEG/NaCl) precipitation resulting in phage titers of 10^{11} - 10^{12}
17
18 cfu/ml. Each round of selection started with a negative selection using the
19
20 corresponding blocked paramagnetic beads. The supernatant from this step was
21
22 moved to a new tube and mixed with the biotinylated Z_2 domain at varying
23
24 concentrations, table 1, and incubated for two hours at room temperature (RT). The
25
26 binding phages were captured on 0.5 mg of paramagnetic beads and washed as
27
28 described above. For elution, incubation with 500 μ l of 0.05 M glycine-HCl pH 2.2 for
29
30 10 minutes at RT was performed. After elution the pH was adjusted with 450 μ l PBS
31
32 and 50 μ l 1 M Tris-HCl pH 8.0. The eluate was mixed with log phase RRI Δ M15 and
33
34 incubated at 37°C for 30 minutes. Thereafter the cells were spread on Tryptone Yeast
35
36 Extract (TYE) agar plates (15 g/l agar, 3 g/l NaCl, 10 g/l tryptone and 5 g/l yeast
37
38 extract), supplemented with 2% glucose and 100 mg/l ampicillin (Amp) (Sigma-
39
40 Aldrich) and incubated at 37°C over night. The cells were resuspended in tryptic soy
41
42 broth (TSB, 30 g/l) (Merck, Darmstadt, Germany). A fraction of the cell suspension
43
44 was used to create a new phage stock. Elution titers and number of phages introduced
45
46 in each cycle were determined by infection of log phase RRI Δ M15.
47
48
49
50
51
52
53
54
55
56
57
58
59
60

2.5 Cloning

Cloning of ABD-variants*

Colonies from cycle three and four were randomly picked from agar plates supplemented with 100 mg/l Amp for PCR screening and sequencing. Three constructs were chosen for further investigation; ABDz1, ABDz83, ABDz86 (ABDz_x, x=1, 83, 86). The ABD* library fragments were amplified by PCR, using Phusion High Fidelity DNA polymerase (New England BioLabs). The cystein in ABDz1 was replaced by a serine using primer ABDz1C25S and PCR-mutagenesis to create ABDz1C25S. The fragments were purified before restriction as described in section 2.2. The expression vector pHis (containing a T7-promoter, a His-tag, and a kanamycin resistance gene) and the fragments ABDz_x and ABDz1C25S were cleaved with the restriction enzymes *EcoRI* and *XhoI*. The purified fragments were ligated with pHis using T4 DNA Ligase (New England BioLabs) resulting in pHisABDz_x and pHisABDz1C25S. A dimeric version of ABDz1C25S was created using primers ABDdimerCod and ABDdimerRev with pHisABDz1C25S as template, resulting in a fragment with flanking *XhoI* restriction sites. The purified fragment and pHisABDz1C25S were restricted with *XhoI* and purified. The linear vector was dephosphorylated using Antarctic Phosphatase (New England BioLabs). Subsequent ligation resulted in pHisABDz1C25SSDim.

Cloning of target proteins in fusion with ABDz1

1
2
3
4
5
6 DNA fragments containing only ABDz1 were made by PCR, using primers
7
8 ABDz1Not1 and ABDz1Nco1, incorporating restriction sites Nco1 and Not1. The
9
10 restriction enzymes were used to cleave the purified PCR fragment and vectors
11
12 containing different target proteins; pZbABP141377, pZbABPHT875, and
13
14 pZbABPHT2375 [15]. Ligation of vector and ABDz1-fragment was performed at RT
15
16 using T4 DNA ligase (New England BioLabs) giving pABDz1-141377, pABDz1-
17
18 HT875 and pABDz1-HT2375. The plasmids were sequence verified.
19
20
21
22
23

24 *2.6 Protein production*

25
26
27 Protein production was essentially performed according to [15]. After protein
28
29 production for 18 hours, the cell suspensions were gently harvested (2400 g, 8 min,
30
31 4°C) and pellets were frozen for later use.
32
33
34
35
36

37 *2.7 Protein purification of His₆-tagged ABD*-variants*

38
39 The frozen pellets were resuspended in 25 ml running buffer (50 mM sodium
40
41 phosphate, 6 M Urea, 300 mM NaCl, pH 8.0) and cells were disrupted by sonication
42
43 (Vibra cell; Sonics and materials, Inc., Danbury, CT, USA) at 60% amplitude and
44
45 1.0/1.0 pulses for 3 minutes. Before loading on a 1 ml Talon resin column (Clontech
46
47 Laboratories, Inc., Mountain View, CA, USA) the samples were centrifuged (35000
48
49 g, 20 min, 4°C) and filtered (0.45 µm). The column was equilibrated with 5 column
50
51 volumes (CV) of running buffer and 20 ml of sample was loaded. After washing with
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 20 CV running buffer bound proteins were eluted with elution buffer (50 mM NaAc,
7
8 6 M Urea, 100 mM NaCl, 30 mM HAc, pH 4.5).
9

10 11 12 13 *2.8 Protein analysis*

14
15 The eluted proteins were analyzed by SDS-PAGE using a NuPAGE Novex Bis-Tris
16 4-12% gradient gel stained with GelCode Blue Stain Reagent (Pierce). NAP10
17 columns (GE Healthcare) were used for buffer exchange to HEPES buffered saline
18 (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA and 0.05% surfactant P20, pH 7.4)
19 (HBS-EP). Protein concentration was determined by absorbance measurements at 280
20 nm and amino acid analysis.
21
22
23
24
25
26
27
28
29
30
31

32 *2.9 Biosensor analysis*

33
34 Real-time biospecific interaction analysis between the selected variants and the target
35 protein, the Z₂ domain, as well as its original binding partner, HSA, was performed
36 on a Biacore 2000 instrument (GE Healthcare). The Z₂ domain was immobilized
37 (~130 RU) by thiol coupling through a C-terminal Cys and HSA was immobilized
38 (~2500 RU) by amine coupling onto the dextran layer of a CM5 chip according to the
39 manufacturer's instructions. All analytes were diluted in HBS-EP, and filtered
40 through a 0.45 µm filter. ABD* and IgG were included in all analyses as controls.
41
42 After every injection the surfaces were regenerated using 15 mM HCl. In the initial
43 study each variant (ABDzx, ABDz1C25S and ABDz1C25SDim) was diluted to ~500
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5 nM and injected over all surfaces at 50 $\mu\text{l}/\text{min}$. For kinetic studies a new chip was
6
7
8 made with an immobilization level of ~ 200 RU and ~ 2000 RU on the Z_2 -surface and
9
10 HSA-surface respectively. ABD* and ABDz1C25S were analyzed on the HSA-
11
12 surface and ABDz1 on the Z_2 -surface in concentrations ranging from 10 nM to 1000
13
14 nM at a flow rate of 50 $\mu\text{l}/\text{min}$. The association rate constant (k_a), the dissociation rate
15
16 constant (k_d), and the dissociation equilibrium constant (K_D) were estimated using
17
18 BIAevaluation 3.2 software (Biacore).
19
20
21

2.10 Circular dichroism

22
23
24
25
26
27 Analysis of secondary structure was performed using a J-810 spectropolarimeter
28
29 (JASCO, Tokyo, Japan). All samples were diluted to approximately 0.22 mg/ml
30
31 except ABD* that was diluted to 0.05 mg/ml in PBS. The samples were scanned in a
32
33 1 mm cell from 250 nm to 195 nm at a speed of 100 nm/min. An average from five
34
35 scans was calculated. Using the same sample the thermal stability was also
36
37 investigated by heating the sample from 20°C to 90°C using a temperature slope of
38
39 5°C/min. The circular dichroism (CD) signal at 221 nm was detected. Thereafter a
40
41 new CD spectrum was performed as previously described to investigate if the protein
42
43 regained its secondary structure.
44
45
46
47
48
49
50

2.11 Protein purification using HSA sepharose

51
52
53
54 The frozen pellets were resuspended in 25 ml running buffer (25 mM Tris-HCl, 200
55
56
57
58
59
60

1
2
3
4
5
6 mM NaCl, 1 mM EDTA, 0.05% Tween 20, pH 8.0) and supernatant containing
7
8 protein lysate was prepared as described in section 2.7. The protein lysates were
9
10 loaded on a column with 1 ml HSA Sepharose. The column was equilibrated with 10
11
12 CV of running buffer and 25 ml of the sample was loaded. After washing with 5 CV
13
14 running buffer and 5 CV washing buffer (5 mM NH₄Ac, pH 5.5) the bound proteins
15
16 were eluted with 0.5 M HAc, pH 2.8. The protein concentration in the eluted fractions
17
18 was determined by absorbance measurements at 280 nm. The eluted peaks were
19
20 analyzed using SDS-PAGE as described in section 2.8. To compare the binding
21
22 capacity of ABD* and ABDz1 to the HSA sepharose, equal amounts of previously
23
24 purified protein were loaded onto the column and purified as described above. In the
25
26 orthogonal purification setup, the two protein fractions with the highest absorbance
27
28 from the SuRe purification step were pooled and pH was adjusted to 7.5 before
29
30 loading on the HSA sepharose and purified as described above.
31
32
33
34
35
36
37
38

39 *2.12 Protein purification using MabSelect SuRe*

40
41 The frozen pellets were thawed and resuspended in 25 ml running buffer (20 mM
42
43 phosphate, 150 mM NaCl, pH 7.2) and the supernatants containing protein lysates
44
45 were prepared as described in section 2.7. The protein solution was loaded on a 1 ml
46
47 HiTrap MabSelect SuRe column (GE Healthcare). The column was equilibrated with
48
49 5 CV of running buffer and 20 ml of sample was loaded. After washing with 5 CV of
50
51 running buffer the proteins were eluted with 0.2 M HAc, pH 3.3. In the orthogonal
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 purification setup the two protein fractions with the highest absorbance from the HSA
7
8 purification step were pooled and pH was adjusted to 7.5 before loading on the SuRe
9
10 matrix and purified as described above. To investigate if it was possible to achieve
11
12 effective purification also under reducing conditions one sample was treated with 50
13
14 mM DL-Dithiothreitol (DTT) (Sigma-Aldrich) at 37°C for 30 minutes before
15
16 filtration and loading onto the column and thereafter the described purification
17
18 scheme was used. In an attempt to find milder elution the samples were eluted using
19
20 0.2 M HAc at pH 3.3, pH 4.0 and pH 4.5. The fractions of the eluted peaks were
21
22 pooled and analyzed as described in section 2.8. When purifying fusion proteins
23
24 using the SuRe matrix 0.2 M HAc at pH 3.3 was used as elution buffer.
25
26
27
28
29
30
31
32

3. Results

3.1 Rationale for the design of the library

33
34
35
36
37
38 Here we present a novel phage display library of $\sim 10^7$ variants constructed to create a
39
40 possibility to achieve small protein molecules that with high selectivity are able to
41
42 bind two different target molecules. For construction of the library, a small and stable
43
44 molecule with inherent and selective binding to a protein target, was desired. Due to
45
46 improved characteristics [5] and the inherent binding property, ABD* was chosen as
47
48 scaffold for the library. In an earlier mutational study [6] it was also shown that
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 despite mutations of nine amino acids in helix one and three, at positions distant from
7
8 the suggested binding site, the HSA-binding was retained or even improved.
9
10 Therefore these nine amino acids in helix one and three and two additional amino
11
12 acids, at the end of helix one and helix three respectively, were chosen for
13
14 randomization when building the library (Fig. 1a). The amino acids were randomized
15
16 using NNK-degeneracy coding for all 20 amino acids and the amber stop (TAG)
17
18 using 32 different codons. A visualization of the protein domain is shown in figure 1b
19
20 where the three helices are shown as ribbons and in figure 1c the molecular surface is
21
22 presented.
23
24
25
26
27
28

29 *3.2 Analyses of the unbiased ABD*-library*

30
31
32 To verify that the library was randomized in the correct positions and without bias for
33
34 certain amino acids, 150 clones were sequenced. Approximately 93% of the selected
35
36 clones were full-length library members with correct inserts. The remaining 7% were
37
38 plasmids without insert and these clones were omitted in the sequencing reaction. The
39
40 amino acid occurrence in the randomized positions of the full-length clones was
41
42 compared to the theoretically expected occurrence of each amino acid. The
43
44 distribution of each amino acid was found to be close to the theoretical value (data
45
46 not shown).
47
48
49

50
51 To investigate if the HSA binding was retained, a western blot was performed on
52
53 phage stocks from selections against HSA. ABD* was included in the western blot as
54
55
56
57
58
59
60

1
2
3
4
5
6 a positive control. A faint band can be seen in the lane with the naïve ABD*-library,
7
8 and the intensity of the band is increased after each selection cycle (Fig. 2a). This
9
10 confirms that the HSA-binding is retained in a large part of the represented protein
11
12 molecules despite randomization of almost 25% of the amino acids in the small
13
14 ABD* domain. The presence of truncated pIII-ABDlib in comparison with full-length
15
16 pIII encoded by the phagemide was also investigated by western blot. In the original
17
18 library 11% of the expressed pIII was the truncated (library) variant. After the first
19
20 and second selection cycle against HSA the subset of truncated pIII-ABDlib
21
22 increased to 17% and 37%, respectively, as determined by image analysis on western
23
24 blot data, indicating enrichment of phages expressing truncated pIII in fusion with
25
26 randomized protein domains (data not shown).
27
28
29
30
31
32
33

34 *3.3 Selection*

35
36 Isolation of Z₂-binders from the ABD*-library was achieved using phage display in-
37
38 vitro selection technology. After four rounds of biopanning against the Z₂ domain, the
39
40 same sequence was identified in all sequenced clones, ABDz1 (Fig. 2b). To
41
42 encounter additional variants, clones from the third round of biopanning were
43
44 sequenced. Of the 80 clones sequenced 84% were identical with the sequence found
45
46 after cycle four, ABDz1. Two additional sequences appeared once each, ABDz83 and
47
48 ABDz86. When sequencing clones from selection cycle two, ABDz1 was found only
49
50 once (corresponding to less than 1% of the sequenced clones). The three variants,
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 ABDz1, ABDz83, and ABDz86, have low sequence homology in the randomized
7
8 regions and all three were selected for further characterization.
9

10 11 12 13 *3.4 Purification and analyses of ABD*-variants* 14

15
16 The identified candidates were recloned into an expression vector containing the T7
17
18 promoter [16] and an N-terminal hexahistidyl purification tag [17]. After expression
19
20 of the proteins in Rosetta, the cells were lysed and the His-tagged ABD*-variants
21
22 were purified to homogeneity by immobilized metal affinity chromatography
23
24 (IMAC).
25

26
27 Biosensor analysis was used to verify the binding between the selected variants and
28
29 the target molecules, the Z₂ domain and HSA. The three variants, ABDz1, ABDz83,
30
31 and ABDz86, were diluted to ~500 nM and the capacity to bind both the Z₂ domain
32
33 and HSA was evaluated. The results showed that all three variants had retained HSA
34
35 binding and that one of the three variants, ABDz1, possessed Z₂-binding as well (Fig.
36
37 3a and 3b). Hence, ABDz1 was chosen for further studies as it had the desired dual
38
39 binding capacity and had the fastest on-rate when binding to HSA. Of the 11
40
41 positions randomized in ABDz1 two amino acids were conserved compared to the
42
43 parental sequence; arginine in position 29 and tyrosine in position 34. Interestingly, a
44
45 cysteine has been introduced in position 25 enabling disulfide formation between the
46
47 molecules. Both lysines were exchanged to histidines, also basic amino acids, but
48
49 with lower pKa. All acidic amino acids (positions 22, 30 and 58) were exchanged for
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 uncharged amino acids. The substitution in position 30 introduced a glycine in helix
7
8 one (Fig. 1b and 2b). In total, all mutations resulted in an increase in theoretical pI
9
10 from 5.8 to 6.4 and the net charge at neutral pH was decreased from -5 to -2.

11
12 By SDS-PAGE analysis it was concluded that ABDz1 appears as almost 100%
13
14 homodimer due to intermolecular disulfide bridge formation (data not shown). To
15
16 assess the importance of the dimerization for the binding to the Z₂ domain and HSA,
17
18 the ABDz1 was further engineered by replacing the cysteine in position 25 by a
19
20 serine creating a molecule denoted ABDz1C25S. To investigate the possible avidity
21
22 effects and as a comparison to the dimer formed through the disulfide bridge a
23
24 dimeric variant of ABDz1C25S was also created by head-to-tail dimerisation,
25
26 ABDz1C25SSDim. This construct excludes the N-terminal linker region of one
27
28 ABDz1C25S, and therefore the molecular weight will be 13.3 kDa whereas it is 14.4
29
30 kDa for the dimeric ABDz1.
31
32
33
34
35

36
37 Surface plasmon resonance was used to compare the binding of the four protein
38
39 molecules, ABDz1, ABDz1C25S, ABDz1C25SSDim, and ABD*, to HSA. The
40
41 concentrations were adjusted so that the number of available binding sites should be
42
43 equal in all samples. All three novel constructs showed faster on-rate than ABD*, and
44
45 ABDz1 had slower off-rate than all others (Fig. 3c). Avidity effects could be seen
46
47 both for the genetic dimer, ABDz1C25SSDim and the dimer formed by the cysteines
48
49 in ABDz1 since these dimers showed higher apparent affinity than the corresponding
50
51 monomer, ABDz1C25S. The dimers have similar affinity and the difference in
52
53 response can be explained by the difference in molecular weight between the two
54
55
56
57
58
59
60

1
2
3
4
5
6 protein constructs (dimer of ABDz1 14.4 kDa and ABDz1C25SDim 13.3 kDa). It
7
8 was also investigated if ABDz1 could retain the Z₂-binding after exchanging the
9
10 cysteine for a serine but only ABDz1 showed affinity to the Z₂ domain (Fig. 3d).

11
12 Kinetic studies were performed to investigate the binding of ABD* and ABDz1C25S
13
14 to HSA. To be able to use a one-to-one binding algorithm when calculating the
15
16 binding constants, ABDz1C25S was used instead of the dimer-forming ABDz1. Due
17
18 to the identical amino acids in the HSA-binding surface of the protein domains, the
19
20 HSA affinities of a monomeric ABDz1 and ABDz1C25S would be very similar. The
21
22 measured values for ABD* correspond well to earlier published data [5].
23
24 ABDz1C25S has approximately the same off-rate as ABD* but a faster on-rate and
25
26 therefore the calculated dissociation equilibrium constant is slightly stronger, low
27
28 nano molar, for ABDz1C25S (Table 2). A similar comparison for the binding to the
29
30 Z₂ domain is less appropriate since dimerization of ABDz1 is crucial for Z₂-binding.
31
32 However, kinetic analysis of the ABDz1 binding to the Z₂ domain using a one-to-one
33
34 model gave an apparent K_D of low micro molar (Table 2).

35
36 Using circular dichroism the structural contents of the ABD* variants were
37
38 determined. The structural characteristics of the selected variants were compared to
39
40 ABD* which is a three-helical bundle as determined by NMR [3], and the high alpha-
41
42 helicity is also confirmed by circular dichroism [5]. The curves from the CD analyses
43
44 showed that despite a retained α -helical secondary structure, the signals were much
45
46 lower than for the parental ABD*-molecule (data not shown). Hence, the alpha
47
48 helical content in the new molecule is much lower than that in ABD*. Also the
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 modified variant, ABDz1C25S has less α -helicity than the parental domain (data not
7 shown). Furthermore the thermal stability was investigated by comparing the curves
8 before and after heating the sample. No change could be detected indicating that the
9 molecules were able to refold and regain their structure.
10
11
12
13
14
15
16
17

18 *3.5 Protein purification using the inherent affinities*

19
20 An effective path to increase the purity of a proteinaceous target molecule is to purify
21 it in an orthogonal way and take advantage of different characteristics when planning
22 the purification strategy. Here we have investigated the possibility of purifying the
23 bispecific ABDz1 on two different affinity matrices. The first purification column
24 used contained HSA sepharose. The two proteins, ABD* and ABDz1, were
25 successfully purified from cell lysate using this matrix. To assess the efficiency in the
26 binding, ABDz1 was compared to ABD* by purifying equal amounts of protein in
27 subsequent purifications. The eluted peak for ABD* was smaller due to low
28 absorbance at 280 nm since the protein contains no tryptophan (Fig. 4a). SDS-PAGE
29 analysis of loaded and eluted sample from the purification showed that equal amounts
30 of protein were loaded on the column and that the matrices were able to capture all
31 loaded protein in both cases (Fig. 4b). Hence, the mutated variant, ABDz1, and
32 ABD* possess similar capacity to bind to the HSA sepharose.
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49

50 To investigate if ABDz1 could be purified on MabSelect SuRe, a protein A-derived
51 matrix, cell lysate was loaded on the column and captured protein was eluted using
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 pH 3.3 (Fig. 4c). This confirmed that the Z₂-binding capability introduced into
7
8 ABDz1 enabled purification on MabSelect SuRe. Different elution conditions were
9
10 used to investigate if a less harsh buffer than pH 3.3 was possible to use for elution of
11
12 ABDz1 from the SuRe matrix. The target protein was eluted in comparable amounts
13
14 when using pH 3.3 and pH 4.0, but at pH 4.5 a minor amount of the protein was
15
16 eluted (Fig. 4c). When cleaning the column with pH 3.3 the majority of the loaded
17
18 target protein was released (Fig. 4c). Analysis with SDS-PAGE confirmed amount
19
20 and purity of the eluted target protein. Thus the pH of the elution buffer can be raised
21
22 to 4.0 and still effective elution is accomplished.
23
24
25

26
27 To assess the importance of the disulfide bridge formed between the ABDz1
28
29 molecules for its binding to the Z₂-domain, DTT-treated sample was loaded onto the
30
31 MabSelect SuRe matrix. Successful purification could not be achieved, possibly due
32
33 to the reduced disulfide bridge (data not shown).
34
35
36
37
38

39 *3.6 Protein purification using an orthogonal purification strategy*

40
41 To challenge the usability of ABDz1 as purification tag, three different proteins with
42
43 dissimilar characteristics (Table 3) were genetically fused with the gene encoding
44
45 ABDz1 giving fusion proteins with the new binding domain in the N-terminus. The
46
47 three fusion proteins and the sole tag were produced in *E. coli* and thereafter purified
48
49 in two chromatographic steps. In the first purification step a column with HSA matrix
50
51 was used and the purification efficiency was therefore dependent on the capacity of
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 the tag to bind to HSA. The eluted peaks were collected and a sub fraction of each
7
8 peak was analyzed by SDS-PAGE. The rest of the eluted material was loaded on the
9
10 protein A derived matrix, MabSelect SuRe, and the eluted fractions were also
11
12 analyzed using SDS-PAGE. Moreover, it was investigated if the two matrices could
13
14 be used in reverse order with comparable result. The orthogonal affinity purification
15
16 principle was applied as described above on the three fusion proteins. This time the
17
18 first purification was carried out on the MabSelect SuRe matrix followed by HSA
19
20 sepharose chromatography. As can be seen in figure 5 a and b, a very pure product
21
22 can be obtained regardless of the characteristics of the target protein since the
23
24 interaction with the matrix only is dependent on the N-terminal purification domain.
25
26 Orthogonal affinity purification using the two affinities in ABDz1 can successfully be
27
28 applied regardless of the order in which the two affinities are used.
29
30
31
32
33
34
35
36
37

38 **4. Discussion**

39
40
41
42 A novel phage display library based on the improved ABD domain, ABD* (Fig. 1)
43
44 has been created. The library was used for selection of binders towards the Z₂
45
46 domain. The objective was to isolate a molecule with dual binding capacities to be
47
48 used as a highly specific tag in affinity chromatography. It was concluded that the
49
50 library size was approximately 10⁷ and the distribution of amino acids were found to
51
52 be close to the theoretical values. In spite of the extensive randomization, 11 of the 46
53
54
55
56
57
58
59
60

1
2
3
4
5 amino acids, enrichment of HSA-binders from the ABD* library was possible (Fig.
6
7
8 2a).

9
10 Selection of Z₂-binders from the library resulted in three different constructs with low
11
12 sequence homology. All three variants contained a cysteine in the middle or at the
13
14 end of helix one resulting in dimers formed by the disulfide bridge. However, only
15
16 one of them showed Z₂-binding, ABDz1 (Fig. 2b and 3b), and was therefore chosen
17
18 for further studies. Biosensor analysis of the affinity constants for ABDz1 binding to
19
20 the Z₂ domain immobilized on the chip surface using a monovalent model gave an
21
22 apparent K_D value of 0.4 μM (Table 2). The indication of moderate affinity was not
23
24 surprising due to the rather small size of the library (approximately 10⁷ variants) [18].
25
26 The Z₂-affinity dependence of the dimerization as well as the avidity effect gained by
27
28 the disulfide formation was investigated by replacing the cysteine with a serine,
29
30 creating ABDz1C25S. From this mutant a genetic dimer without cysteins was
31
32 created, ABDz1C25SDim. Unfortunately, neither ABDz1C25S nor ABDz1C25SDim
33
34 did show any Z₂-binding indicating that the conformation formed by ABDz1 through
35
36 the cystein-bridge is crucial for binding. The surface formed by the dimerisation is
37
38 not easily mimicked with a genetic head-to-tail dimerisation, which is shown by the
39
40 inability of ABDz1C25SDim to bind to the Z₂ domain. Sensorgrams acquired with
41
42 HSA on the surface show that the stronger response seen for ABDz1 compared to
43
44 ABDz1C25S (and ABD*) is mainly due to dimerisation since ABDz1C25SDim
45
46 shows almost equally strong response as ABDz1 to HSA (Fig. 3c). The somewhat
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 higher response seen for ABDz1 compared to ABDz1C25SDim is probably caused
7
8 by differences in molecular weight but also due to the slightly slower off-rate for
9
10 ABDz1 (Fig. 3c). Presumably, this effect is due to a more favorable conformation
11
12 formed by the dimer as well as avidity effects caused by the dual binding sites. A
13
14 closer examination of the kinetic constants for ABDz1C25S and ABD* showed that
15
16 there was almost no difference in the dissociation rate constant, k_d , whereas the
17
18 association rate constant, k_a , was higher for ABDz1C25S, and thus the calculated
19
20 dissociation equilibrium constant, K_D , was lower, than for ABD* (Table 2). The
21
22 faster on-rate can possibly be explained by the substitutions at the randomized
23
24 positions. At the randomized positions in ABDz1C25S all negative charges were
25
26 replaced by uncharged amino acids and an uncharged position was exchanged with a
27
28 positively charged amino acid resulting in a less negatively charged molecule than the
29
30 parental ABD*. Since HSA has negative net charge at the conditions used in the
31
32 analysis (-12) the faster on-rate determined could be due to a less negative net charge
33
34 of ABDz1C25S (-2) as compared to ABD* (-5). A similar charge affected behavior
35
36 has earlier been shown for other interaction pairs as well [19, 20]. When examining
37
38 the structural content of ABDz1 and ABDz1C25S using CD, a curve shape indicating
39
40 a-helicity was acquired (data not shown). However, the signal intensities were much
41
42 lower than for ABD* showing that the selected variants had a lower alpha helical
43
44 content. The substitution in position 30 introduced a glycine in helix one. Since
45
46 glycine is known to have low propensity for alpha helical structures this amino acid
47
48 could partly be responsible for the low helical content [21-23]. Also the histidines
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 acquired at the end of helix one and in the middle of helix three might destabilize the
7
8 structure as histidine has a low helix-stability rank [24]. In positions 57 and 58 in the
9
10 third helix two aromatic amino acids were found, which also could affect the α -
11
12 helicity. Even though the variants showed a lower structural content than ABD* they
13
14 demonstrated superior binding characteristics to HSA. Interestingly, some sequential
15
16 similarity can be seen in the binding surface of Z_{SpA-1} , a randomized variant of the Z-
17
18 domain with Z-binding ability [25, 26], and the randomized surface of ABDz1. Z_{SpA-1}
19
20 was selected from a library of Z variants (Z_{lib}) using the Z domain as target [25].
21
22 When comparing the interaction surface of Z_{SpA-1} with ABDz1 by placing two
23
24 ABDz1 molecules beside each other, helix one from the left molecule and helix three
25
26 from the right creates an interaction surface similar to Z_{SpA-1} . This, together with our
27
28 experimental data, indicates that dimerization of ABDz1 is not crucial to gain avidity
29
30 effects, but rather that two molecules are needed for the correct interaction surface to
31
32 be created. This also conveys that the apparent affinity should be evaluated by using
33
34 the concentration of the dimer instead of the monomer and thereby resulting in an
35
36 apparent affinity of 0.2 μ M. It has not been possible to detect binding between
37
38 ABDz1 and variants of the Z domain (selected from Z_{lib}) using surface plasmon
39
40 resonance (data not shown) indicating that the binding interphase of ABDz1 to the Z
41
42 domain is in the same area as for Z_{SpA-1} , but this remains to be further investigated.
43
44
45
46
47
48
49
50
51
52

53 The dual characteristics established in ABDz1 encouraged further experiments where
54
55
56
57
58
59
60

1
2
3
4
5
6 the two acquired affinities were used for protein purification (Fig. 4). Purifications of
7
8 ABDz1 using HSA as ligand on a sepharose matrix were shown to be as effective as
9
10 for ABD*. Since the affinity for the Z₂ domain was not as strong as for HSA,
11
12 purification on the protein A-derived matrix, MabSelect SuRe, would be more
13
14 challenging. However, the purification was successful and due to the moderate
15
16 affinity for the Z₂ domain, a strategy with milder elution conditions could be
17
18 established.
19
20
21
22
23

24 To challenge the new domain as an affinity tag in protein purification, three different
25
26 target proteins were genetically fused to the affinity domain. Target proteins with
27
28 different molecular weight and also different pI were chosen (Table 3) to evaluate if
29
30 the tag was affected by the characteristics of the attached protein. When utilizing the
31
32 bispecific affinity domain in a two-step chromatographic purification setup a very
33
34 high degree of purity was achieved for the target proteins (Fig. 5). Hence, the
35
36 constructed affinity domain showed usefulness in protein purification and the domain
37
38 was able to effectively interact with the two different matrices regardless of the
39
40 characteristics of the C-terminal target protein. Noteworthy is that the two affinity
41
42 chromatography steps can be used in any order.
43
44
45
46
47
48
49

50 In many applications very pure and active molecules are essential. Using an
51
52 orthogonal affinity purification strategy with two succeeding affinity purification
53
54 steps will greatly enhance the purity of the final product. A purification system built
55
56
57
58
59
60

1
2
3
4
5
6 on a genetic fusion to a purification tag is mainly suited for protein purification in
7
8 small to medium scale systems. The large benefit is the possibility for high
9
10 throughput purification of a wide variety of target proteins with different
11
12 characteristics without any target specific optimization. By knowing the
13
14 characteristics of the fused purification domain all details regarding the purification
15
16 setup can be planned in advance. The low pH necessary for the disruption of the
17
18 interaction between the affinity domain and the ligand on the matrix might, may for
19
20 some target proteins, be too harsh. This effect can in many cases be decreased
21
22 remarkably by immediately increasing the pH of the eluted sample. For some
23
24 downstream applications it is essential to remove the purification tag from the target
25
26 protein. This can be done by e.g. on-column digestion and has previously been shown
27
28 successful [27].
29
30
31
32
33
34
35

36 Here we, for the first time, have shown that a small molecule, consisting of only 46
37
38 amino acids, can be engineered to have dual affinities. Moreover, the domain could
39
40 be expressed in *E. coli* in fusion to three different target proteins. All these proteins,
41
42 as well as the domain itself, could efficiently be purified in a two-step affinity
43
44 chromatographic setup giving very pure protein. The achieved data show that the
45
46 novel purification tag is efficient and the orthogonal affinity purification strategy
47
48 gives highly pure protein. Also the similar behavior of the three different target
49
50 proteins purified here indicates that the tag is of general use regardless of the target
51
52 protein.
53
54
55
56
57
58
59
60

Acknowledgement

The authors thank Professor Per-Åke Nygren for scientific discussions and advice.

This project was funded by the Knut and Alice Wallenberg Foundation and the Swedish Research Council.

The authors have declared no conflict of interest.

For Peer Review

5. References

- [1] Hedhammar, M., Graslund, T., Hober, S. Protein engineering strategies for selective protein purification. *Chemical Engineering & Technology* 2005; 28: 1315-1325.
- [2] Nilsson, J., Stahl, S., Lundeberg, J., Uhlen, M. et al. Affinity fusion strategies for detection, purification, and immobilization of recombinant proteins. *Protein Expr Purif* 1997; 11: 1-16.
- [3] Kraulis, P. J., Jonasson, P., Nygren, P. A., Uhlen, M. et al. The serum albumin-binding domain of streptococcal protein G is a three-helical bundle: a heteronuclear NMR study. *FEBS Lett* 1996; 378: 190-194.
- [4] Olsson, A., Eliasson, M., Guss, B., Nilsson, B. et al. Structure and evolution of the repetitive gene encoding streptococcal protein G. *Eur J Biochem* 1987; 168: 319-324.
- [5] Gulich, S., Linhult, M., Nygren, P., Uhlen, M. et al. Stability towards alkaline conditions can be engineered into a protein ligand. *J Biotechnol* 2000; 80: 169-178.
- [6] Linhult, M., Binz, H. K., Uhlen, M., Hober, S. Mutational analysis of the interaction between albumin-binding domain from streptococcal protein G and human serum albumin. *Protein Sci* 2002; 11: 206-213.

- 1
2
3
4
5
6 [7] Johansson, M. U., Frick, I. M., Nilsson, H., Kraulis, P. J. et al. Structure,
7 specificity, and mode of interaction for bacterial albumin-binding modules.
8 *The Journal of biological chemistry* 2002; 277: 8114-8120.
9
10
11
12 [8] Lejon, S., Frick, I. M., Bjorck, L., Wikstrom, M. et al. Crystal structure and
13 biological implications of a bacterial albumin binding module in complex
14 with human serum albumin. *The Journal of biological chemistry* 2004; 279:
15 42924-42928.
16
17
18 [9] Uhlen, M., Guss, B., Nilsson, B., Gatenbeck, S. et al. Complete sequence of
19 the staphylococcal gene encoding protein A. A gene evolved through multiple
20 duplications. *The Journal of biological chemistry* 1984; 259: 1695-1702.
21
22
23 [10] Hober, S., Nord, K., Linhult, M. Protein A chromatography for antibody
24 purification. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007; 848: 40-
25 47.
26
27
28 [11] Ruther, U. pUR 250 allows rapid chemical sequencing of both DNA strands
29 of its inserts. *Nucleic Acids Res* 1982; 10: 5765-5772.
30
31
32 [12] Sambrook, J., Fritsch, E. F., Maniatis, T. *Molecular Cloning: A Laboratory*
33 *Manual*. Cold Spring Harbor Laboratory Press, New York 1989.
34
35
36 [13] Nord, K., Nilsson, J., Nilsson, B., Uhlen, M. et al. A combinatorial library of
37 an alpha-helical bacterial receptor domain. *Protein Eng* 1995; 8: 601-608.
38
39
40 [14] Nilsson, B., Moks, T., Jansson, B., Abrahmsen, L. et al. A synthetic IgG-
41 binding domain based on staphylococcal protein A. *Protein Eng* 1987; 1: 107-
42 113.
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3
4
5
6 [15] Alm, T., Steen, J., Ottosson, J., Hober, S. High-throughput protein
7 purification under denaturing conditions by the use of cation exchange
8 chromatography. *Biotechnol J* 2007; 2: 709-716.
9
10
11
12 [16] Studier, F. W., Moffatt, B. A. Use of bacteriophage T7 RNA polymerase to
13 direct selective high-level expression of cloned genes. *J Mol Biol* 1986; 189:
14 113-130.
15
16
17 [17] Hochuli, E., Bannwarth, W., Dobeli, H., Gentz, R. et al. Genetic Approach to
18 Facilitate Purification of Recombinant Proteins with a Novel Metal Chelate
19 Adsorbent. *Bio/Technology* 1988; 6: 1321-1325.
20
21
22 [18] Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M. et al. Isolation
23 of high affinity human antibodies directly from large synthetic repertoires.
24 *Embo J* 1994; 13: 3245-3260.
25
26
27 [19] Schreiber, G., Fersht, A. R. Rapid, electrostatically assisted association of
28 proteins. *Nat Struct Biol* 1996; 3: 427-431.
29
30
31 [20] Albeck, S., Schreiber, G. Biophysical characterization of the interaction of the
32 beta-lactamase TEM-1 with its protein inhibitor BLIP. *Biochemistry* 1999; 38:
33 11-21.
34
35
36 [21] Bishop, B., Koay, D. C., Sartorelli, A. C., Regan, L. Reengineering
37 granulocyte colony-stimulating factor for enhanced stability. *The Journal of*
38 *biological chemistry* 2001; 276: 33465-33470.
39
40
41 [22] Hecht, M. H., Hehir, K. M., Nelson, H. C., Sturtevant, J. M. et al. Increasing
42 and decreasing protein stability: effects of revertant substitutions on the
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3
4
5
6 thermal denaturation of phage lambda repressor. *Journal of cellular*
7
8 *biochemistry* 1985; 29: 217-224.
9
- [23] Pace, C. N., Scholtz, J. M. A helix propensity scale based on experimental
10
11 studies of peptides and proteins. *Biophysical journal* 1998; 75: 422-427.
12
13
- [24] O'Neil, K. T., DeGrado, W. F. A thermodynamic scale for the helix-forming
14
15 tendencies of the commonly occurring amino acids. *Science* 1990; 250: 646-
16
17 651.
18
19
- [25] Nord, K., Gunneriusson, E., Ringdahl, J., Stahl, S. et al. Binding proteins
20
21 selected from combinatorial libraries of an alpha-helical bacterial receptor
22
23 domain. *Nat Biotechnol* 1997; 15: 772-777.
24
25
26
- [26] Wahlberg, E., Lendel, C., Helgstrand, M., Allard, P. et al. An affibody in
27
28 complex with a target protein: structure and coupled folding. *Proc Natl Acad*
29
30 *Sci U S A* 2003; 100: 3185-3190.
31
32
33
- [27] Hedhammar, M., Jung, H. R., Hober, S. Enzymatic cleavage of fusion
34
35 proteins using immobilised protease 3C. *Protein Expr Purif* 2006; 47: 422-
36
37 426.
38
39
- [28] Hedhammar, M., Stenvall, M., Lonneborg, R., Nord, O. et al. A novel flow
40
41 cytometry-based method for analysis of expression levels in *Escherichia coli*,
42
43 giving information about precipitated and soluble protein. *J Biotechnol* 2005;
44
45 119: 133-146.
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Legends to figures

Figure 1

A) The original sequence of the three-helical ABD* [5] used as scaffold when building the library. The 11 randomized positions are indicated by increased font size. Numbering according to [3]. The shaded areas represent helix 1, 2, and 3 (H1, H2, and H3).

B) and C) The three-dimensional structure of ABD* as determined by NMR [3], protein data bank entry 1GJT, modulated by YASARA, shown as ribbon structure with the randomized positions numbered in circles (B) and molecular surface (C). Dark areas represent the randomized positions.

Figure 2

A) Western blot performed on phage stock from selections against HSA showing pIIIABDlib (27 kDa), lane 1, 2, and 3. ABD* (7 kDa) is included as a positive control, lane 4. A faint band can be seen in lane 1 which represents the HSA-binding phages in the original library and an increased intensity of the band can be seen after each round of selection, lane 2 and 3. Lane 2 shows the phage stock after the first selection cycle and lane 3 is after the second selection cycle. This confirms that the HSA-binding is retained in the scaffold despite randomization of almost 1/4th of the amino acids. Marker (M) in kDa.

1
2
3
4
5
6 B) The sequences of the selected variants ABDz1, ABDz83, and ABDz86. Only
7
8 ABDz1 shows affinity to the Z₂ domain. However, all three have retained HSA
9
10 binding. Numbering according to [3]. The 11 mutated positions are indicated by
11
12 increased font size. The shaded areas represent helix 1, 2, and 3 (H1, H2, and H3).
13
14
15
16
17

18 **Figure 3**

19
20 Sensorgrams from biospecific interaction analysis

21
22 A and B) Sensorgrams showing the binding of the selected ABD* variants to HSA
23
24 (A) or the Z₂ domain (B): ABDz1 (black line), ABDz83 (grey line), and ABDz86
25
26 (light grey line) (500 nM). In (A) HSA was immobilized on the surface. All three
27
28 selected variants, ABDz1, ABDz83, and ABDz86, show HSA-binding. In (B) the Z₂
29
30 domain was immobilized on the surface. One of the selected variants, ABDz1, shows
31
32 Z₂-binding.
33
34
35

36
37 C and D) Sensorgrams showing the binding of ABD* and variants thereof to HSA
38
39 (C) or the Z₂ domain (D): ABD* (black dashed line) (500 nM), ABDz1 (black line)
40
41 (500 nM), ABDz1C25S (grey dashed line) (500 nM), and ABDz1C25SDim (grey
42
43 line) (250 nM). The concentrations are adjusted so that an equal number of molecules
44
45 are available in each sample. In (C) HSA was immobilized on the surface.
46
47 ABDz1C25SDim shows almost equally strong response as ABDz1 to HSA indicating
48
49 that avidity effects are responsible for the stronger response for ABDz1 compared to
50
51 ABDz1C25S. In (D) the Z₂ domain was immobilized on the surface. The cysteine-
52
53 containing variant, ABDz1, shows Z₂-binding but neither the cysteine-free monomer
54
55
56
57
58
59
60

1
2
3
4
5
6 nor the cysteine-free dimer does. Thus the conformation created by the cysteine-
7
8 bridge seems to be essential for binding.
9

10 11 12 **Figure 4**

13 Chromatograms from ÄKTAEexplorer and SDS-PAGE gels

14
15 A and B) Elution profile of ABD* (dashed line) and ABDz1 (solid line) purified on
16
17 HSA sepharose using an ÄKTAEexplorer. To investigate the capacity of the selected
18
19 variant, ABDz1, to bind the HSA sepharose and compare the binding with the
20
21 parental ABD*, similar amounts of purified protein were loaded and eluted with pH
22
23 2.8 in subsequent purifications. The loaded sample and eluted peaks were analyzed
24
25 using SDS-PAGE. Approximately equal amounts of ABDz1 (7.4 kDa) and ABD*
26
27 (7.1 kDa) were loaded (lane 1 and 3 respectively) and eluted (lane 2 and 4
28
29 respectively). Marker (M) in kDa.
30
31
32
33
34
35

36 C and D) Elution profile of ABDz1 purified on MabSelect SuRe using an
37
38 ÄKTAEexplorer. Three protein lysates were pooled and divided into three equal
39
40 volumes and loaded on the column in three subsequent purifications. ABDz1 was
41
42 eluted using three different elution conditions, pH 3.3 (black line), 4.0 (dashed line),
43
44 and 4.5 (grey line). The target protein was eluted in comparable amounts for pH 3.3
45
46 and pH 4.0, but at pH 4.5 the elution of the target protein was less effective, peak 1
47
48 (P1). In the setup using elution buffer at pH 4.5 the remaining target protein was
49
50 released when washing with a buffer at pH 3.3, peak 2 (P2). Analysis of the loaded
51
52 sample and eluted peaks was done by SDS-PAGE. Identical samples of protein lysate
53
54
55
56
57
58
59
60

1
2
3
4
5 were loaded (lane 1) in all three setups and efficient elution of ABDz1 (7.4 kDa) was
6 achieved at pH 3.3 (P1) (lane 2) and pH 4.0 (P1) (lane 3). At pH 4.5 the elution was
7 less effective (P1) (lane 4) and the remaining protein was washed out with a buffer at
8 3.3 (P2) (lane 5). Marker (M) in kDa.
9
10
11
12
13
14
15
16
17

18 **Figure 5**

19 SDS-PAGE analysis of target proteins expressed in fusion with ABDz1

20 A) Lane 1-4 shows the protein lysates before purification. In lanes 5-8 results from
21 the first purification step using HSA matrix are shown. In lanes 9-12 results from the
22 second purification step by MabSelect SuRe is shown. Samples are loaded in the
23 following order: ABDz1-141377, ABDz1-HT875, ABDz1-HT2375 and ABDz1.
24
25
26
27
28
29
30
31

32 B) Lane 1-3 shows protein lysates before purification. Lanes 4-6 show the results
33 from the first purification step using MabSelect SuRe. Lanes 7-9 show the results
34 from the second purification step, HSA purification. Samples are loaded in the
35 following order: ABDz1-141377, ABDz1-HT875, and ABDz1-HT2375.
36
37
38
39
40
41

42 The molecular weight of respective protein is shown in table 3.

43 The orthogonal affinity purification setup using the bispecific ABDz1 gives very pure
44 protein regardless of target protein fused to the tag. The two chromatographic steps
45 can be used in any order.
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Tables

Table 1

Biopanning by phage display

	A	B	C	D	Number of washes	Tween %
Cycle 1	100 nM			100 nM (N)	3	0.1
Cycle 2	80 nM	20 nM	80 nM	80 nM	5	0.2
Cycle 3	80 nM	20 nM	50 nM	50 nM (N)	7	0.3
Cycle 4	80 nM	20 nM	20 nM	20 nM	10	0.4

Four series (A, B, C, and D) of biopanning with different target concentration were performed in four cycles (1, 2, 3 and 4). To increase the stringency in every selection round, the number of washes and the amount of tween was increased in each cycle. Streptavidin coated beads have been used if not stated (N) for neutravidin.

Table 2

Kinetic constants from surface plasmon resonance measurements

	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_D (M)
ABD*	3.5E+04	1.4E-03	4.0E-08
ABDz1C25S	2.7E+05	1.3E-03	4.8E-09

Ligand: HSA

	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_D (M)
ABDz1	1.1E+04	3.9E-03	4.0E-07

Ligand: Z₂ domain

Data have been evaluated using an one-to-one model. Due to the dimerization of ABDz1 the values describe the apparent affinity.

k_a =association rate constant

k_d =dissociation rate constant

K_D =dissociation equilibrium constant

Table 3

Characteristics of the target and fusion proteins used for evaluation of the bispecific affinity protein domain as a purification tag.

Name ^{a)}	Target protein			Fusion product	
	Uniprot ^{b)}	Mw ^{c)} [kDa]	Solubility Class ^{d)}	Mw ^{c)} [kDa]	pI ^{e)}
141377	B7Z3I5	17.4	4	23.7	8.5
HT875	P01040	10.8	3	17.1	4.9
HT2375	P00740	8.9	5	15.2	6.8

Affinity tag, ABDz1: 6.3 kDa, pI 6.7

ABDz1 (including an N-terminal His₆-tag): 7.4 kDa, pI 6.4

^{a)} The target protein represents a part of the Uniprot protein

^{b)} <http://www.uniprot.org>

^{c)} Mw = molecular weight

^{d)} Solubility class of protein fragment with N-terminal His₆ABP [28]

^{e)} pI = isoelectric point

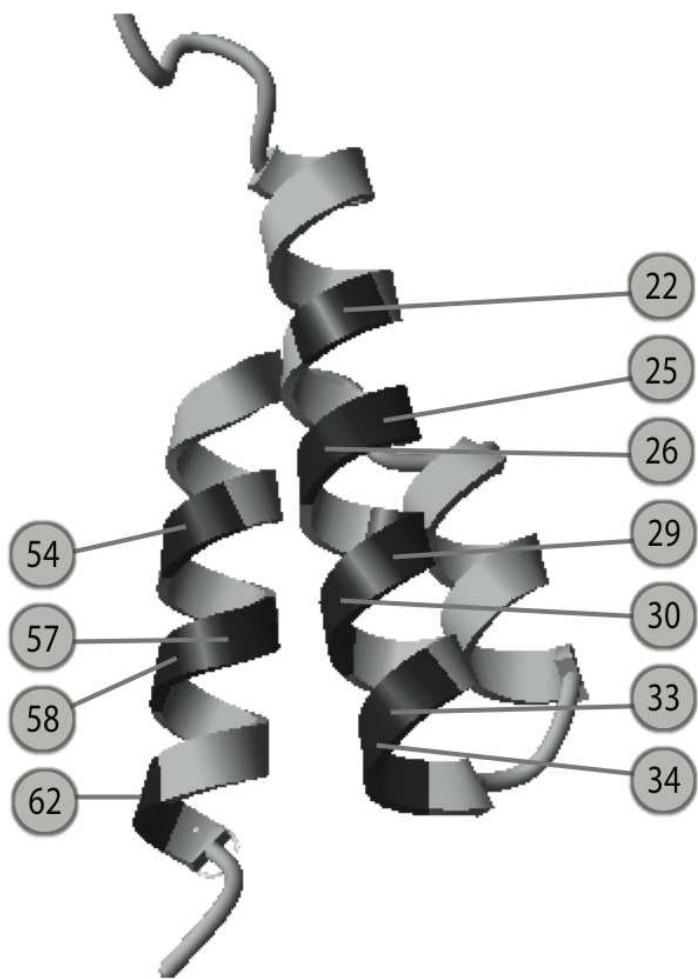


167x17mm (150 x 150 DPI)

For Peer Review

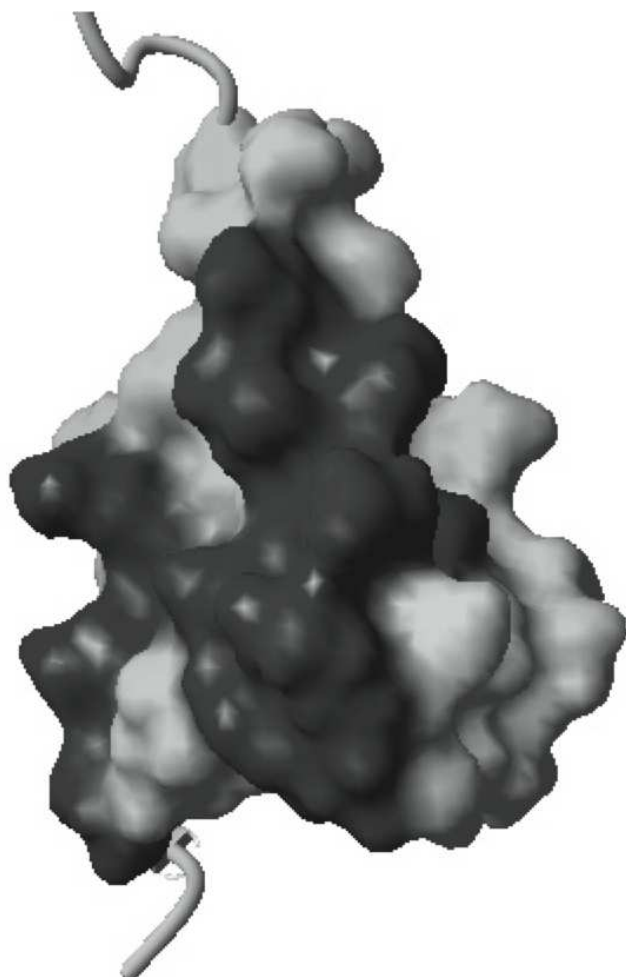
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



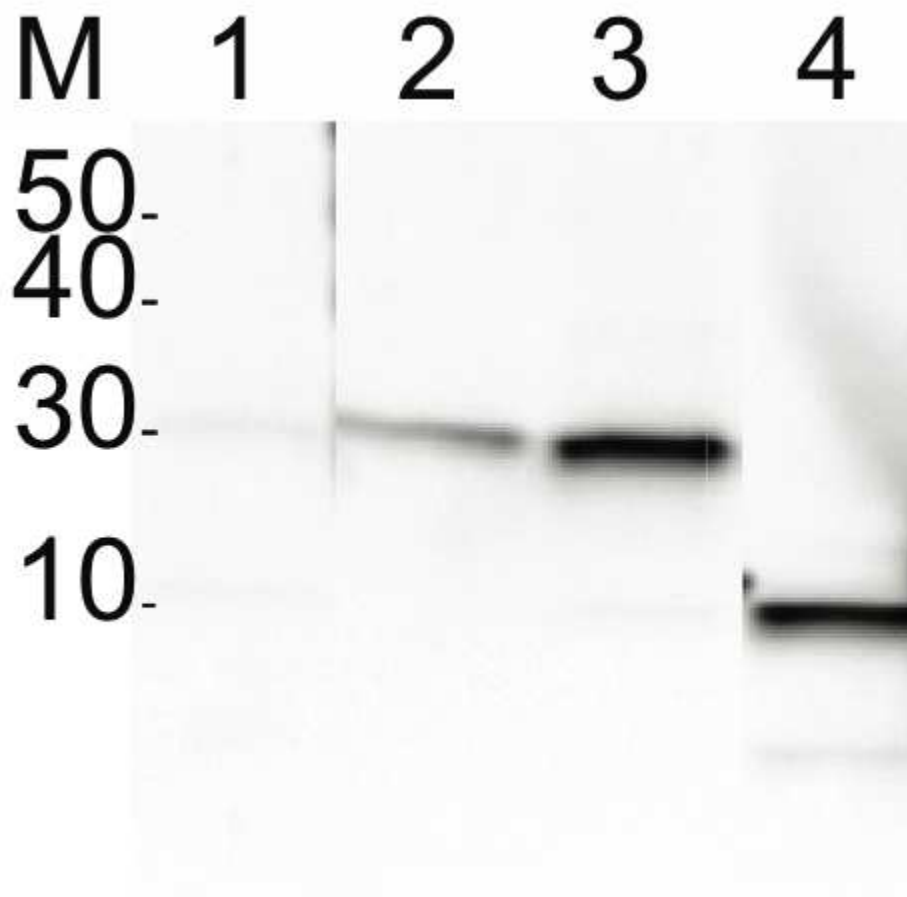
144x144mm (150 x 150 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



144x144mm (150 x 150 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



80x78mm (150 x 150 DPI)



ABDz1

H1													H2					H3																														
A	N	S	L	A	L	A	K	C	R	A	L	R	G	L	D	H	Y	G	V	S	D	Y	Y	K	D	L	I	D	K	A	K	T	V	E	G	V	H	A	L	W	F	E	I	L	Q	A	L	P
17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65

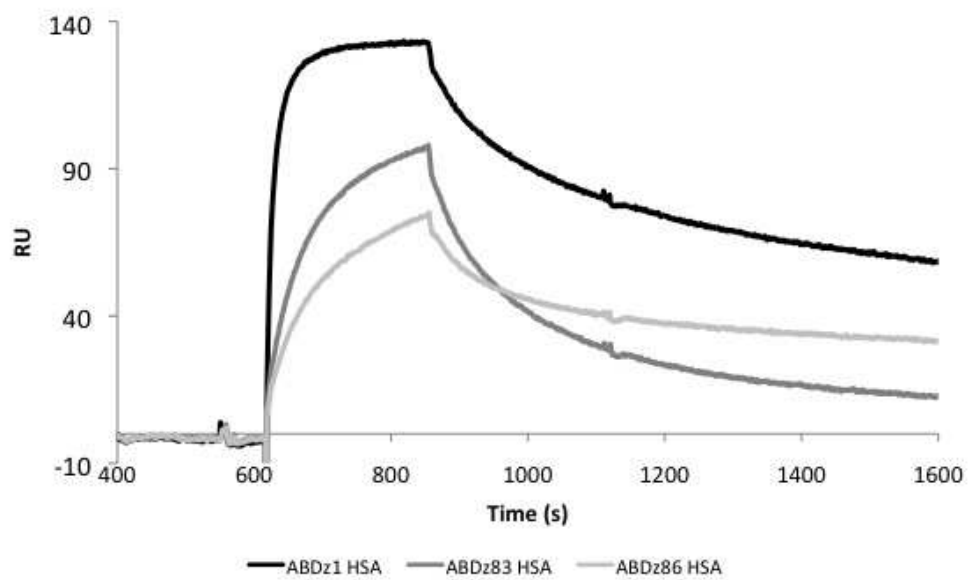
ABDz83

H1													H2					H3																														
A	N	S	L	A	F	A	K	D	W	A	L	W	R	L	D	S	C	G	V	S	D	Y	Y	K	D	L	I	D	K	A	K	T	V	E	G	V	S	A	L	L	E	I	L	M	A	L	P	
17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65

ABDz86

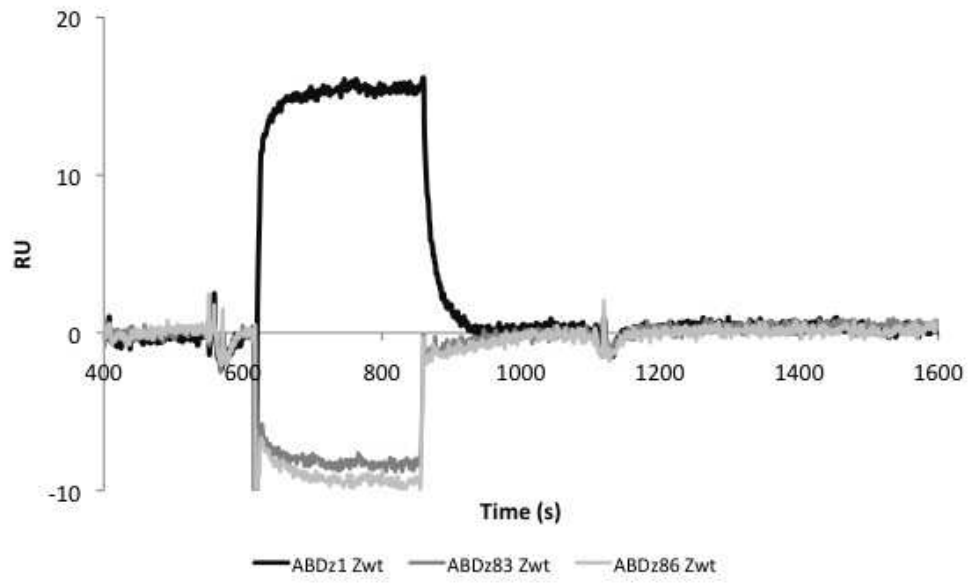
H1													H2					H3																														
A	N	S	L	A	R	A	K	C	L	A	L	H	A	L	D	L	D	G	V	S	D	Y	Y	K	D	L	I	D	K	A	K	T	V	E	G	V	S	A	L	T	L	E	I	L	H	A	L	P
17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65

166x91mm (150 x 150 DPI)

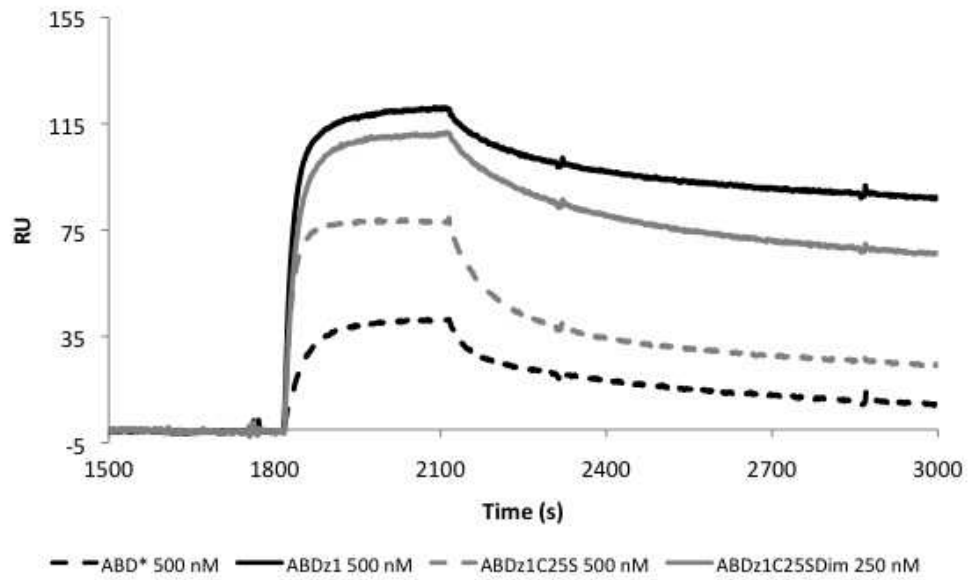


200x120mm (72 x 72 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

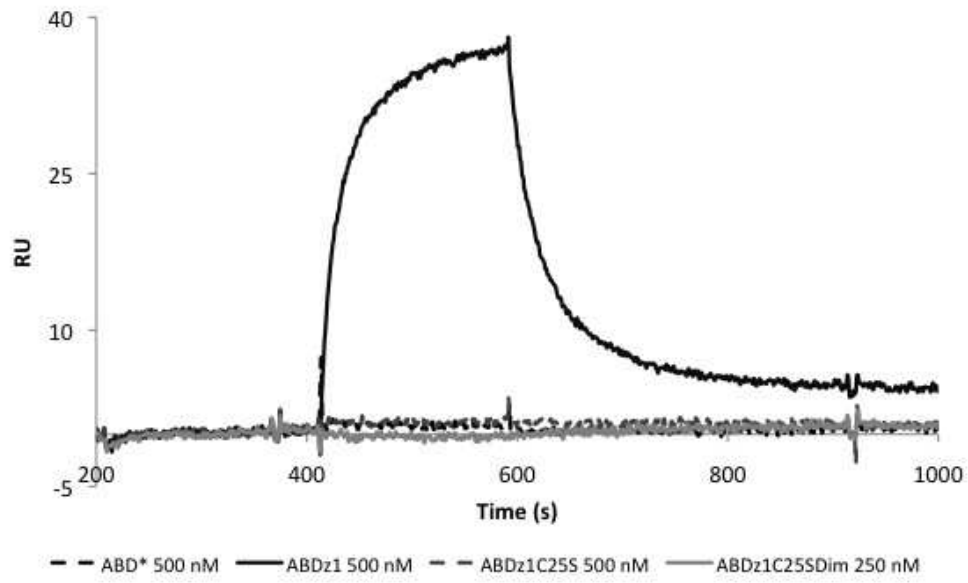


200x120mm (72 x 72 DPI)

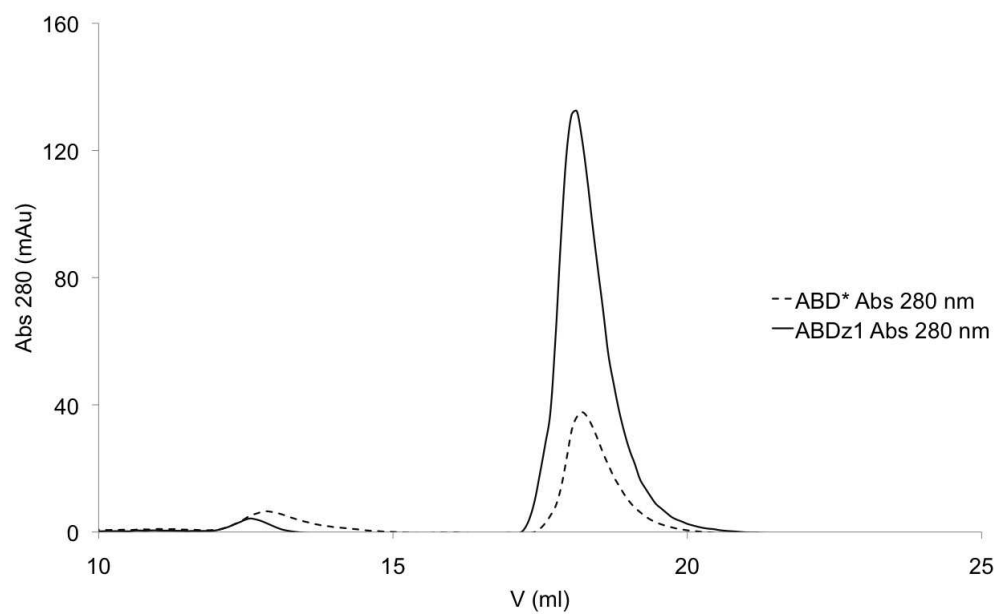


200x120mm (72 x 72 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

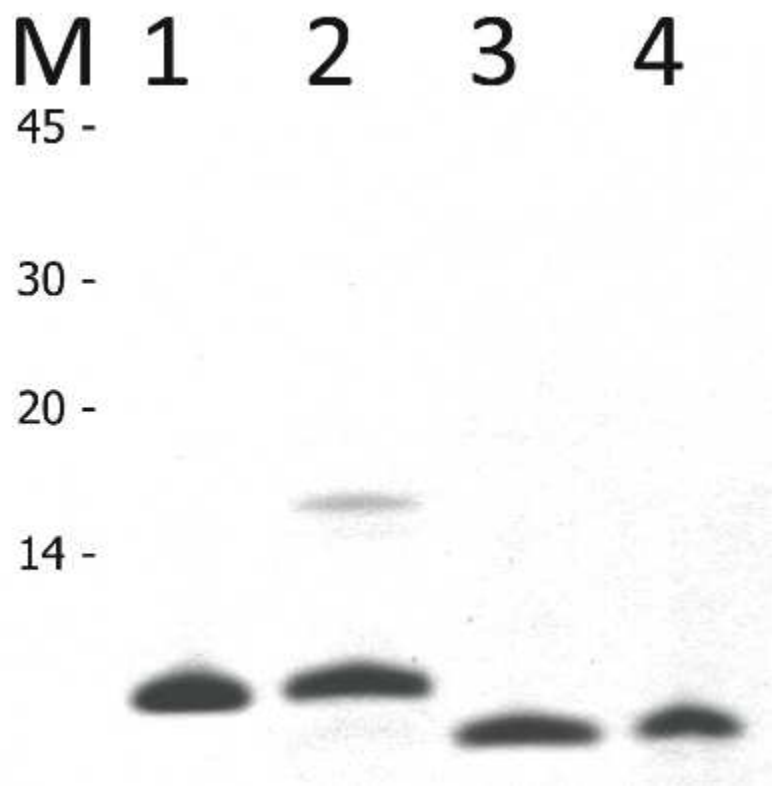


200x120mm (72 x 72 DPI)



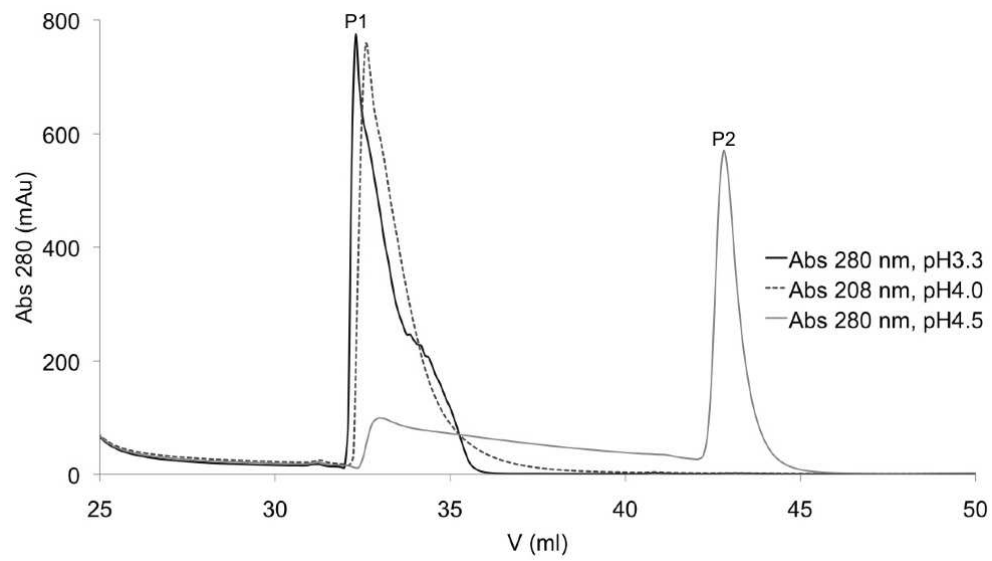
439x271mm (72 x 72 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



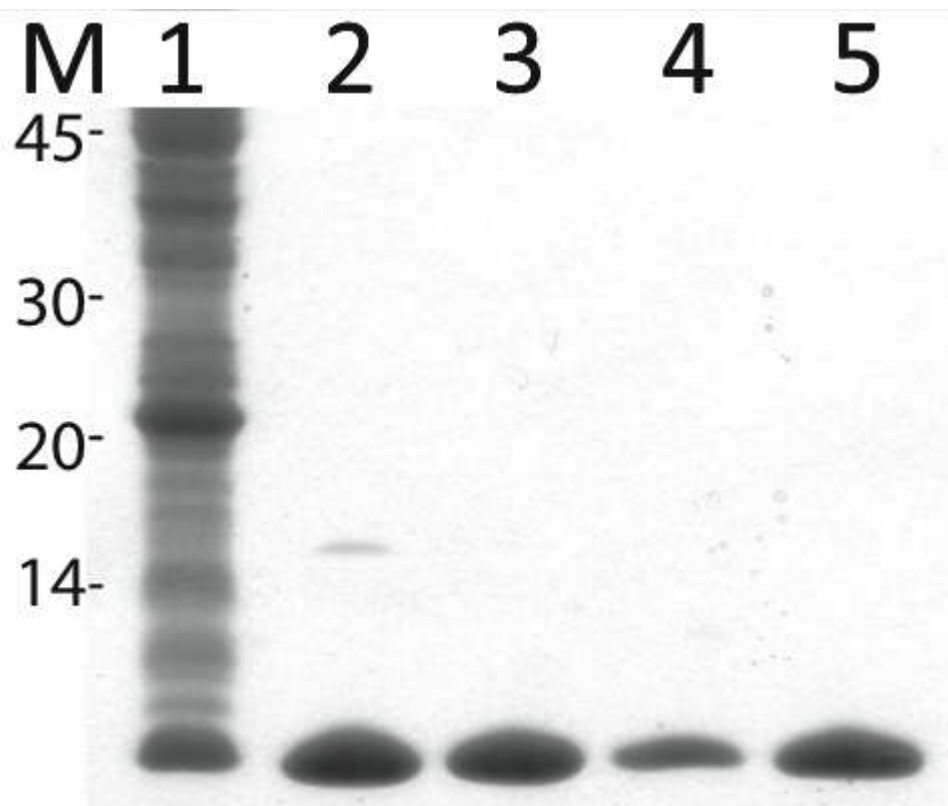
67x66mm (150 x 150 DPI)

review



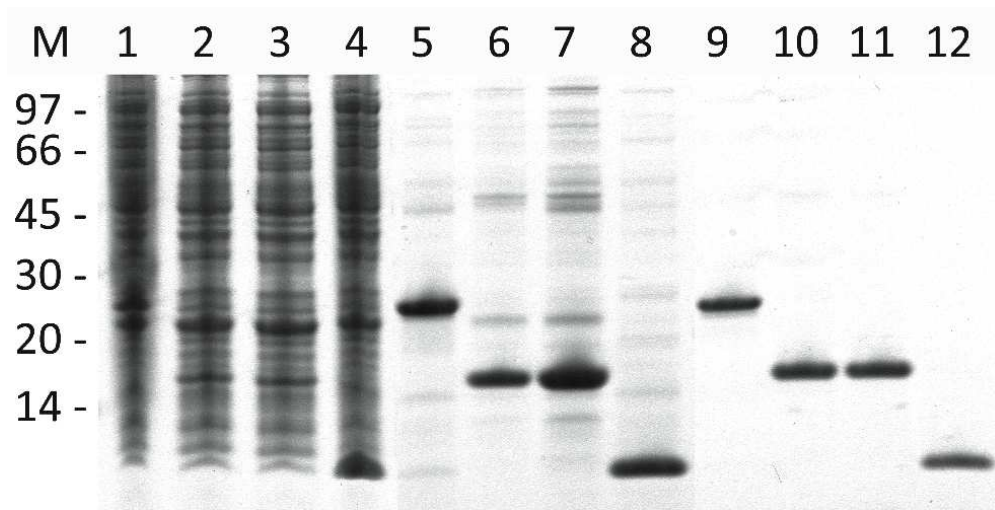
168x96mm (150 x 150 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



81x67mm (150 x 150 DPI)

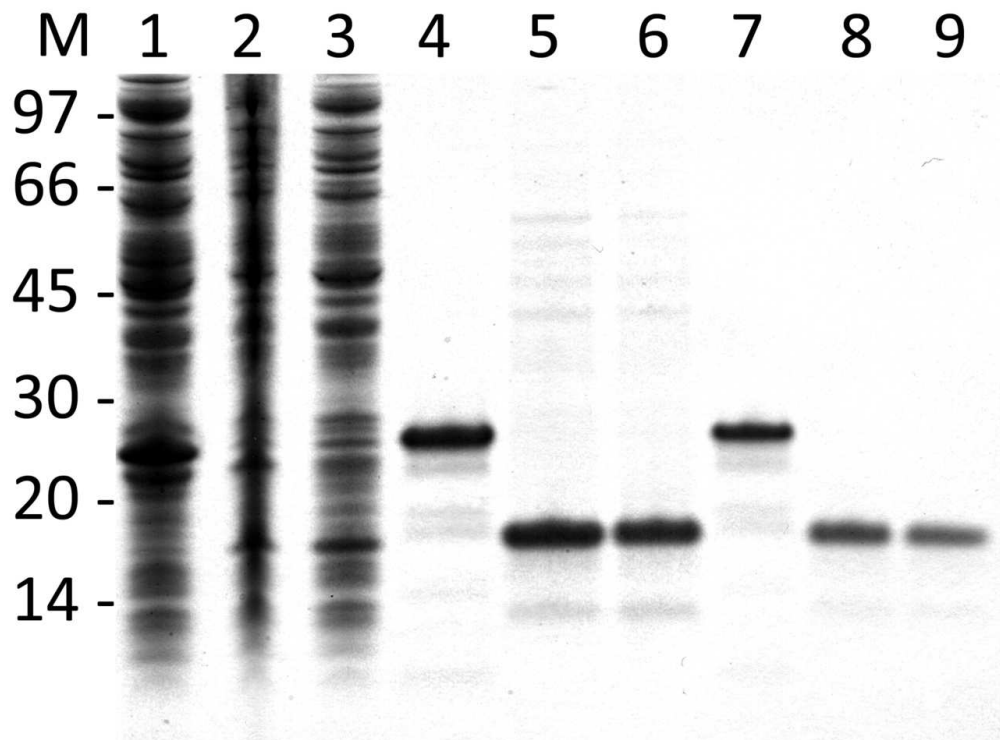
view



166x84mm (150 x 150 DPI)

Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



54x40mm (600 x 600 DPI)

Review