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High Affinity Synthetic Molecular Binders for Proteins

Design, Synthesis and Evaluation

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Abstract
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This thesis describes the design and synthesis of small molecule derivatives and their polypeptide conjugates as high affinity binders for proteins: the D-dimer protein (D-dimer), a biomarker for diagnosis of thromboembolic diseases; human myeloperoxidase (MPO), a biomarker for cardiovascular diseases; and chitinases, potential targets for asthma therapy. The interactions between the synthetic binder molecules and those proteins were evaluated by surface plasmon resonance (SPR) biosensor analysis and fluorescence spectroscopy. Competition SPR experiments or other methods proved that the small molecule components of the binder molecules were critical for binding and specifically bound to the original binding site of small molecules. The binder molecules consisted of a 42-residue helix-loop-helix polypeptide conjugated to a small molecule via aliphatic spacers of suitable length. The small molecules could be any type of moderately binding structure. In the binder development for the D-dimer, the tetrapeptide GPRP with a dissociation constant K_d of 25 μM was used and the affinity of 4C15L8GPRP obtained was estimated to be approximately 3 nM. In the binder development for MPO, salicylhydroxamic acid (SHA) with K_d of 2 μM was used and the affinity of 4C37L34C11SHA obtained was estimated to be approximately 0.4 nM. In the binder development for chitinases, a theobromine derivative (pentoxifylline) with a K_d of 43±10 μM was used and the affinity of 4C37L34-P obtained was estimated to be considerably higher than that of pentoxifylline. The binder molecules were identified from a 16-membered pool of candidates obtained by conjugating the small molecules to each member of a set of 16 designed polypeptides. The affinities were greatly enhanced by 2-3 orders of magnitude, compared to the small molecule. The polypeptides did not bind to the proteins with measurable affinities. The discovery of these new synthetic binders for protein targets can pave the way to diagnostic tests *in vivo* or *in vitro*, independent of antibodies.

Keywords: polypeptide, conjugates, D-dimer, myeloperoxidase, chitinase

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To my family

Do it now!
– Laboratory inspirer

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Ramapanicker, R.[+], Sun, X. J.[+], Viljanen, J., Baltzer, L. Powerful binders for the D-dimer by conjugation of the GPRP peptide to polypeptides from a designed set-illustrating a general route to new binders for proteins. (Under review *Bioconjugate. Chem.*,+ equally contributed)

II Sun, X. J., Yang, J., Norberg, T., Baltzer, L. A synthetic polypeptide conjugate from a 42-residue polypeptide and salicylhydroxamic acid binds human myeloperoxidase with high affinity. *J. Pep. Sci.*, 2012, DOI: 10.1002/psc.2459.

III Sun, X. J., Winander, C., Karlsson, M., Fromell, K., Johansson, G., Stenlid, J., Baltzer, L. Polypeptide conjugates that bind chitinases. (manuscript)

Contribution claim

Paper I: Designed and performed all the bioanalytical work except fluorescence titration and contributed in part to the manuscript writing.

Paper II: Participated in the project development. Designed and performed all the synthetic and bioanalytical work. Wrote the draft of the manuscript.

Paper III: Participated in the project development. Designed and performed most of the synthetic and bioanalytical work. Wrote the draft of the manuscript.

Contents

Abbreviations

Acm	acetamidomethyl
AgOTf	silver trifluoromethanesulfonate
Alloc	allyloxycarbonyl
An+Nc	*Aspergillus nidulans* and *Neurospora crassa*
AR	ankyrin repeat
BA	benzenesulfonamide
$BM(PEG)_2$	1,8-bismaleimidodiethyleneglycol
CA	carbonic anhydrase
CD	circular dichroism
CN-PAGE	clear-native polyacrylamide gel electrophoresis
Cr+Nc	*Clonostachys rosea* and *Neurospora crassa*
CRP	C-reactive protein
D	Dalton
DCC	*N,N*'-dicyclohexylcarbodiimide
DCM	dichloromethane
DIC	*N,N*′-diisopropylcarbodiimide
DIPEA	*N,N*′-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
eq.	equivalent
F108-PDS	2-pyridyl disulfide-modified pluronic F108
Fmoc	fluorenylmethyloxycarbonyl
Fs+Nc	*Fusarium sporotrichoides* and *Neurospora crassa*
HCAII	human carbonic anhydrase isozyme II
HOBt	1-hydroxybenzotriazole
HOCl	hypochlorous acid
HPLC	high-performance liquid chromatography
ITC	isothermal titration calorimetry
K_d	dissociation constant
MALDI-TOF	matrix assisted laser desorption ionization time-of-flight
MBP	maltose binding protein
MEM	2-methoxyethoxymethyl
MEMCl	2-methoxyethoxymethyl chloride

MPO	myeloperoxidase
MS	mass spectrometry
Mtt	4-methytriphenylmethyl
NMP	*N*-methyl-2-pyrrolidone
NMR	nuclear magnetic resonance
P_{20}	polyoxyethylene (20) sorbitan
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
$Pd(PPh_3)_4$	tetrakis(triphenylphosphine)palladium(0)
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RU	resonance units
S. griseus	*Streptomyces griseus*
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHA	salicylhydroxamic acid
SPPS	solid-phase peptide synthesis
SPR	surface plasmon resonance
T. viride	*Trichoderma viride*
t-Boc	*tert*-butyloxycarbonyl
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
UV	ultraviolet
4MU	4-methylumbelliferone
4MU-1	4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide
4MU-3	4-methylumbelliferyl β-D-*N*,*N*′,*N*″-triacetylchitotriose
Å	angstrom

Amino acid	Three letter code	One letter code	Amino acid	Three letter code	One letter code
Alanine	Ala	A	Isoleucine	Ile	I
Arginine	Arg	R	Leucine	Leu	L
Asparagine	Asn	N	Lysine	Lys	K
Aspartic acid	Asp	D	Norleucine	Nle	J
Cysteine	Cys	C	Phenylalanine	Phe	F
Glutamine	Gln	Q	Proline	Pro	P
Glutamic acid	Glu	E	Trptophan	Trp	W
Glycine	Gly	G	Tyrosine	Tyr	Y
Histidine	His	H	Valine	Val	V

1. Introduction

Similar to other macromolecules, *e.g.* nucleic acids and polysaccharides, proteins are essential parts of organisms, and they participate in almost every activity inside or outside cells. All functions of proteins are performed by interactions with other molecules, from small sized ions and small molecules such as cofactors, to macromolecules (*e.g.* antibodies, membrane proteins). Understanding how proteins work inside or outside cells in their individual functions via various interactions between molecules, and finding solutions for various protein malfunction-induced diseases (*e.g.* cardiovascular diseases) is one of the most important aims for the researchers in academia and industry.

Protein-molecule interactions are commonly observed in Nature, for example, antibody-antigen interactions and hormone-receptor interactions. One can also synthesize molecules that mimic proteins in their specific interactions and this is a challenging field. Decades ago, chemical synthesis was applied as a tool in molecular biology, *e.g.* oligonucleotides, peptides, carbohydrates and designed proteins were synthesized by different methods on solid phase or in solution. Chemical synthesis has been proved to be an efficient approach to further diversify structure and function of biopolymers. The quest for knowledge drives chemists to synthesize and utilize new molecules to unravel Nature's principles in molecular biology and biochemistry, pharmacology or bio-analytical chemistry [1-5].

The aim of this thesis work was to develop high affinity synthetic binder molecules for protein targets as a proof-of-principle demonstration of a novel protein binder concept by using small molecules and a set of polypeptides. The focus was on synthetic protein binder development via side chain modification of a defined set of polypeptides with different types of small molecules. Technically, the work mainly included organic synthesis of small molecules, their polypeptide conjugation, and evaluation of polypeptide conjugate binding to different proteins or macromolecules. Paper I focused on the tetrapeptide GPRP; Paper II focused on the substrate SHA; paper III focused on the inhibitor pentoxifylline, the β-blockers C4B3 and C5B1, and technique development relevant for complex biological media.

1.1 Protein binders

The term “protein binder” here refers to the molecules that bind to proteins and includes antibodies and other proteins, nucleic acids, peptides, polysaccharides and small organic molecules. The interest in protein binders is growing rapidly. They can be any kind of molecules from natural products to synthetic molecules and range in size from large proteins to small molecules. They can have important applications in clinical diagnostic and drug development as well as in basic research [6-15]. Most protein binders are produced by *in vivo* techniques, and antibodies are prepared by *in vivo* immunization or from large phage display libraries based on such antibodies. As an interesting example of a pharmaceutically important antibody, OKT3 became the first monoclonal antibody to be approved for *in vivo* therapy in 1986. Over 25 antibodies have now been approved for human therapy and more than 200 antibodies are in development worldwide for a wide range of diseases [5, 16, 17]. However, antibodies still have some disadvantages such as expensive manufacturing, batch to batch variation, and cross-reactivity with unrelated antigens as well as comparably large sizes, compared to many other alternatives. Therefore, development of new binder molecules with small sizes and high affinity and selectivity has been becoming important antibody alternatives. Some examples of protein-protein interactions and alternative binder molecules will be discussed below.

1.1.1 Protein-protein interactions

Well-studied examples of protein-protein interactions, such as those between antibody and antigen or hormone and receptor, has uncovered some implications for rational design of synthetic molecules [18-21]. Due to the many conformations of polypeptides, proteins fold in their native conformation, paying an entropy penalty. Structural studies of antibody-antigen complexes showed that the binding interfaces were large but that often only a small number of side chains were important for the binding. Aromatic residues, particularly Tyr and Trp were more abundant here than on the average protein surface, and Arg residues were also overrepresented [19]. Probably, Tyr residues provided a large hydrophobic surface, π-π interactions and hydrogen bonding through the hydroxyl groups, while Arg residues had three hydrophobic methylene carbon atoms and a guanidinium moiety that can contribute to binding by hydrogen bonding and salt bridge formation. Various conformational changes were also observed that probably serve to optimize the fit, for example, side chain movements, segment movement, and domain movements.

In hormone-receptor complexes, the interfaces were also large, but with only a few amino acid residues critical for the binding (the so called ‘hot spot’ concept). An alanine scan revealed that 8 of 31 residues (K, L, P, R, K,

T, F, R) accounted for ~85% of the binding affinity on the hormone side while 9 of 33 residues (R, E, I, W, I, P, I, D and W) accounted for virtually all of the binding energy on the receptor side of the contact interface between human growth hormone (hGH) and its receptor (hGHbp) [22]. The hydrophobic contacts were crucial at the interface, while hydrophilic interactions were less important. Binding affinity was maintained by a small cluster of contact residues. Polar residues appeared less important for affinity; on the other hand, they were probably important for solubility and specificity.

1.1.2 Alternative binder molecules

Synthetic molecules and biomolecules offer high affinity and good selectivity and can therefore replace the antibodies. Examples include affibodies and nanobodies [4], recombinant proteins and synthetic peptides [9, 12], ankyrin repeats proteins (ARP) [13] as well as aptamers [14, 15]. In the binding interface between ARP and maltose binding protein (MBP), a high content of aromatic residues (*e.g.*Tyr, 28%) were found in the X-ray structure. Many small molecule modulators (inhibitors, stabilizer or inducers) of protein-protein interactions have also been developed, for example, the interfacial inhibitors raltegravir targeting HIV-1 protease, peptides and peptidomimetics [6-8]. Those small molecules were identified out of large chemical libraries by screening, computational approaches and/or phage display techniques [43].

1.2 Binder design

A few decades ago, King *et al* proposed that different lengths of aliphatic chains connected to benzenesulfonamide would increase the affinity for human carbonic anhydrase II (HCAII), in comparison to that of benzenesulphonamide alone [23]. In 1994, Whitesides *et al* proposed an inhibitor design that spacer-linked molecules carrying primary (inhibitor) and secondary ligands would bind stronger than the inhibitor alone, as long as the spacer did not interact strongly in an unfavorable manner [24, 25]. In recent years, Baltzer *et al* developed a concept for protein recognition by conjugating 42-residue helix-loop-helix polypeptides to small molecules. The concept was illustrated in the case of HCAII where a binder molecule was formed that bound the enzyme with high affinity [26-30].

The polypeptide conjugates were formed from polypeptides from a 16-membered set of 42-residue sequences that were linked to small organic molecules via an aliphatic spacer or directly to a lysine side chain. The conjugation was shown to enhance the affinity by 3-4 orders of magnitude compared to that of the small molecule whereas the polypeptide did not bind in a detectable way. It was based on the idea that 1) the small molecule could

bind to the binding site, contributing significantly to the affinity and selectivity; 2) the aliphatic chain might also contribute to the affinity; and 3) that the 42-residue polypeptide scaffold would contribute significantly to affinity and selectivity. How do they bind together? First the small molecule binds to its binding site, and then the polypeptide interacts. The polypeptide is assumed to adapt to the surface of the protein. When interacting, the unordered polypeptide will be reorganized by excluding the hydrophilic groups from the binding interface and being more complementary to the surface of protein, Fig. 1.1, right. Although no high resolution X-ray crystal structure of such a polypeptide-protein complex is available yet, an NMR study of a polypeptide conjugate-protein complex indicated that the residues affected included hydrophobic, polar and charged ones and were located in the central hydrophobic cluster and on the protein surface close to the active site and polypeptide bound in a helix-loop-helix motif and benzenesulphonamide bound to active site when polypeptide-benzenesulphonamide conjugates bound to HCAII with high affinity [28]. Once a small molecule binder with moderate affinity for a protein is identified, it can be conjugated to a set of sixteen 42-residue polypeptides. Both the conjugation site and the polypeptide charge are varied in a systematic way, Fig. 1.2 [31-33].

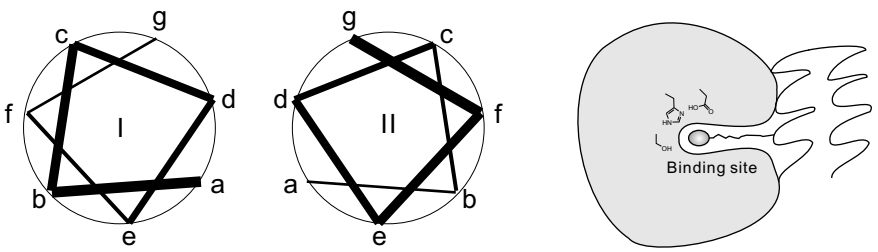

Figure 1.1 Schematic picture of the heptad repeat pattern $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$ of the 42-residue helix-loop-helix polypeptides (left two figures) and the protein binder concept (right): conjugating small molecules with polypeptide scaffolds to afford protein binders that recognize the protein with high affinity and good selectivity.

The 16 membered set of 42-residue polypeptides were designed *de novo* to form helix-loop-helix motifs having some amphiphilic propensity. The helix-loop-helix motif can be conveniently described in terms of the heptad repeat pattern $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$, Fig. 1.1, left. Hydrophobic residues such as residues Leu and Phe were introduced in a and d positions and kept in all sequences to possibly provide the binding energy by hydrophobic interactions with the protein. Charged residues such as residues Arg, Asp and Glu were introduced at the dimer interface positions b and e and at the solvent exposed positions c, f and g for salt-bridge formation in the folded state, in a different pattern in each sequence to give different total charges. Charged residues could possibly increase the selectivity during protein recognition or binding and increase the solubility of the polypeptide. The residue used for function-

alization such as Lys was introduced in different positions for connecting the small molecules. The helix-loop-helix character and dimerization of similar polypeptides were previously characterized by CD, NMR spectroscopy and ultracentrifugation [34, 88]. All N-terminal amino groups were acetylated and only one free lysine residue was available for linking the small molecules. In the present work, expanding the protein binder concept, different types of small molecules, depending on the chosen protein target, were conjugated to the side chain of a lysine residue on the 42-residue polypeptides.

Seq. name	N-Terminus	C-terminus	Charge
1C15L8	AcNEADLEA**K**IRHLAE**K**LEARGPEDAEQLAEQLARAFEAFARAG	-OH	-7
1C10L17	AcNAADLEAAI**K**HLAEAL**K**ERGPEDCEQLAEQLARAFEAFARAG	-OH	-7
1C25L22	AcNEADLEAAIRHLAEALEARGP**K**DA**K**QLAEQLARAFEAFERAG	-OH	-6
1C37L34	AcNEADLEAAIRHLAERLEARGPADAAQLAEQLAA**K**FE**K**FARAG	-OH	-5
2C15L8	AcNEADLEA**K**IRHLAE**K**LAARGPVDCAQLAEQLARAFEAFARAG	-OH	-4
2C10L17	AcNAADLEAAI**K**HLAEAL**K**ARGPVDCAQLAEQLARAFEAFARAG	-OH	-4
2C25L22	AcNEADLEAAIRHLAEALAARGP**K**DCKQLAEQLARAFEAFARAG	-OH	-4
2C37L34	AcNAADLEAAIRHLAERLAARGPVDCAQLAEQLAA**K**FE**K**FARAG	-OH	-3
3C15L8	AcNAADJEA**K**IRHLAE**K**JAARGPVDCAQJAEQLARRFEAFARAG	-NH2	-1
3C10L17	AcNAADJEARI**K**HLAERJ**K**ARGPVDCAQJAEQLARAFEAFARAG	-NH2	-1
3C25L22	AcNAADJEAAIRHLAERJAARGP**K**DC**K**QJAEQLARAFEAFARAG	-NH2	-1
3C37L34	AcNAADJEAAIRHLAERJAARGPVDCAQJAEQLAR**K**FE**K**FARAG	-NH2	-1
4C15L8	AcNAADJEA**K**IRHLRE**K**JAARGPRDCAQJAEQLARRFERFARAG	-NH2	+2
4C10L17	AcNAADJEARI**K**HLRERJ**K**ARGPRDCAQJAEQLARAFERFARAG	-NH2	+2
4C25L22	AcNAADJEARIRHLRERJAARGP**K**DC**K**QJAEQLARAFERFARAG	-NH2	+2
4C37L34	AcNAADJEARIRHLRERJAARGPRDCAQJAEQLAR**K**FE**K**FARAG	-NH2	+2

Figure 1.2 The sixteen 42-residue polypeptide sequences used for binder development shown with total charges and sites of functionalizations. K in bold and underlined style was functionalized with ligands, K in underlined style was functionalized with a chromophore. The names 4C37L34 or 4D37L34 implies that chromophores 7-methoxycoumarinyl (C) or dansyl (D) were linked to the side chain of lysine at the 37^{th} position and ligands were linked to the side chain of lysine at the 34^{th} position. The "4" is to indicate the total charge of the peptide (here +2 after conjugations).

1.3 Evaluation methods

Understanding molecular interactions are important for the understanding of the biological mechanisms of life. Various methods have emerged to characterize biomolecular interactions, such as X-ray crystallography, isothermal titration calorimetry (ITC), nuclear magnetic resonance (NMR), enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR) and fluorescence spectroscopy. In this work, SPR biosensor technology, fluorescence spectroscopy and bead-based pull down experiments were used to study interactions between proteins and polypeptide conjugates. In the following sections, SPR biosensor technology, fluorescence spectroscopy, and bead-based pull down experiments will be discussed briefly.

1.3.1 SPR biosensors

Surface plasmon resonance (SPR) biosensor technology offers highly sensitive real-time monitoring and label-free quantitative analysis of affinity [35, 36, 86].

The principle is shown in Fig. 1.3. The SPR phenomenon arises under the condition of total internal reflection when monochromatic and polarized light strikes a conductive metal layer such as gold layer at the interface between a solid support phase and ambient medium (with different refractive indexes) at a certain incident angle [37, 38]. The intensity of reflected light is attenuated to produce a sharp shadow (called SPR). The incident light angle observed in the shadow is called the SPR angle. When an analyte flows over the ligand-immobilized surface and binds to the ligand, an angle shift ($\Delta\Theta_{sp}$) is observed, due to changes of the refractive index close to the gold surface. This angle shift ($\Delta\Theta_{sp}$) will be detected as SPR signals expressed in resonance unit (RU). A sensorgram is obtained by monitoring the changes of RU as a function of time.

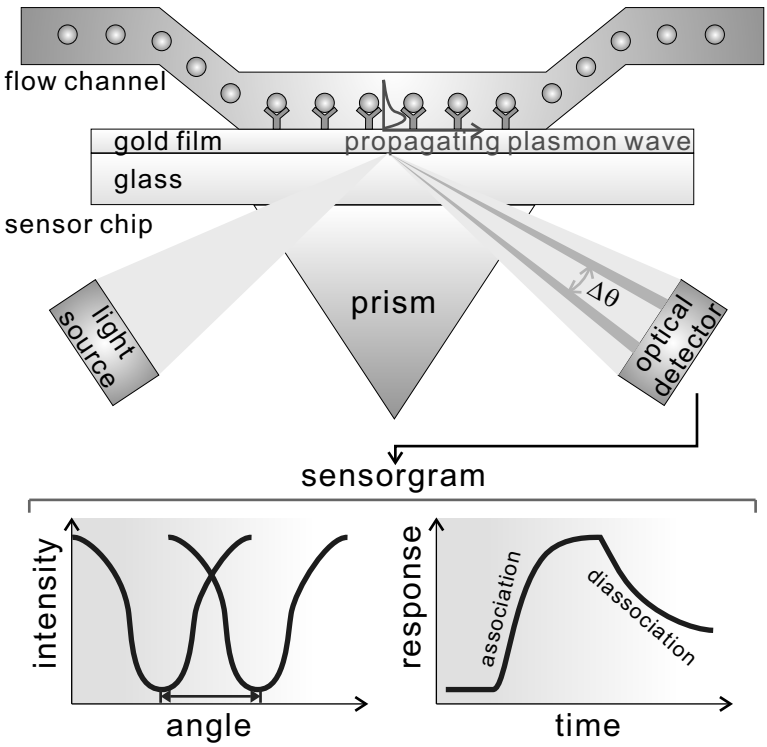

Figure 1.3 Principle of a SPR biosensor. When the analyte flows over the ligand-immobilized surface and binds to the surface, the refractive index close to the surface is changed. An angle change ($\Delta\Theta$) is observed (bottom row, left) which corresponds to a signal response and is plotted against time (bottom row, right) [37, 38].

For the interactions between protein P and binder B,

$$P + B \underset{}{\overset{K_d}{\rightleftharpoons}} PB$$

and

$$K_d = \frac{[P][B]}{[PB]} = \frac{([P]_{total} - [PB])[B]}{[PB]} \quad (1.1)$$

where $[P]_{total}$ is the total concentration of protein, [PB] is the concentration of the protein-binder complex and [B] is the concentration of free binder. When determining steady-state affinities with the Biacore instrument, Eq. 1.1 can be held as:

$$K_d = [B]\frac{(R_{\max} - R_{eq})}{R_{eq}} \quad (1.2)$$

where [PB] can be measured directly as the steady state response R_{eq}, proportional to the surface concentration of the binder bound by proteins. Concentration of the free protein ($[P]_{total}$-[PB]) can be measured as (R_{max}-R_{eq}), where R_{max} is the response when the binder binds to all proteins.

SPR biosensor technology has the practical advantages of less sample amount, high sensitivity (nM), real-time detection and label-free quantitative analysis. In this work, the SPR biosensor technique was used to estimate the affinity of binder molecules and to determine whether the small molecules on binders were bound to the binding sites of proteins (paper I and II).

1.3.2 Fluorescence spectroscopy

Being of relatively high sensitivity, fluorescence spectroscopy is also important for the study of molecular interactions.

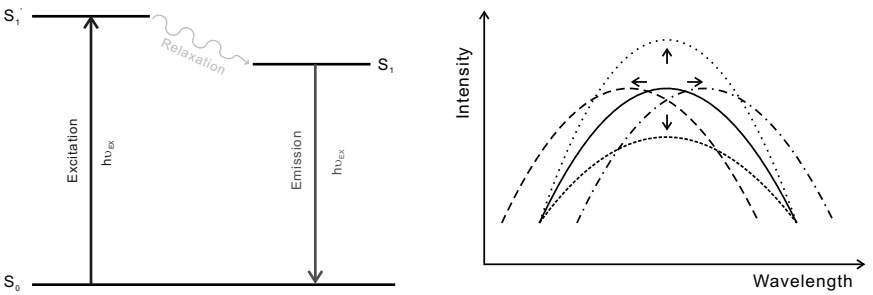

Figure 1.4 Illustration of the fluorescence process (excitation, relaxation, emission) (left) and sensing application on molecular interactions by using intensity change or wavelength shift between free and bound form of analyte (right).

Fluorescent dyes absorb energy from excitation light and emit light with a lower energy (longer wavelength). This process is shown in Fig. 1.4, left

[39]. The difference in wavelength is called the Stokes shift. The emitted light is detected as fluorescence emission. The intensity of the emission depends on the extinction coefficient of absorption and the quantum yield, and it also depends on the stability of the excited state of the molecules. Photobleaching, collisional quenching and self-quenching may be observed. The high sensitivity of fluorophores to the surrounding environment, such as solvent polarity, pH change, conformational changes or interactions with adjacent fluorophores or molecules can be used for various applications, such as monitoring the binding between molecules.

When determining the binding affinity in a 1:1 binding mode with fluorescence spectroscopy, the observed fluorescence intensity F_{obs} can be expressed as:

$$F_{obs} = \frac{F_{bound}[PB] + F_{free}[B]}{[B]_{Total}} = \frac{F_{bound}[P] + F_{free} \bullet K_d}{[P] + K_d} \quad (1.3)$$

where F_{bound} is the fluorescence intensity of binder bound to protein and F_{free} is the fluorescence intensity of the free peptide. $[B]_{Total}$ is the total concentration of binder. [P] is the concentration of free protein. [P] can be further expressed for fitting as:

$$[P] = -\frac{[B]_{Total} + K_d - [P]_{Total}}{2} + \sqrt{\left(\frac{[B]_{Total} + K_d - [P]_{Total}}{2}\right)^2 + K_d \times [P]_{Total}} \quad (1.4)$$

where $[P]_{Total}$ is the total concentration of protein.

In this work, the fluorescence intensity changes due to binding of fluorophore-labeled polypeptides to proteins were used, Fig. 1.4, right (paper I). Conjugated fluorophores, including 7-methoxycoumarinyl, dansyl and fluoresceinyl, were used to monitor whether polypeptide binders could bind to proteins and further to determine affinities (paper I). The properties of the fluorophores are listed in Table 1.1 [40, 89].

Recently, many fluorometric assays of enzymes have emerged. Some non-fluorescent enzymatic substrates can be converted to fluorescent products, which allow for sensitive detection of enzymatic activity. For example, 4MU-3 is a non-fluorescent substrate. After enzymatic cleavage, the product 4MU is strongly fluorescent at basic pH, Fig. 1.5. The fluorogenic substrate 4MU-3 was used in paper III.

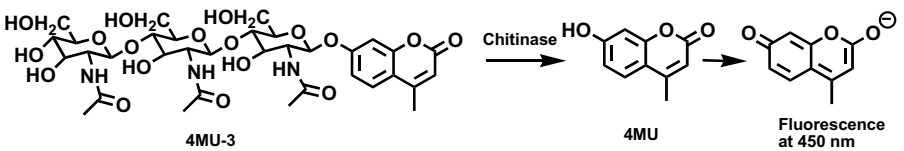

Figure 1.5 Conversion of **4MU-3** to **4MU** by enzymatic cleavage.

Table 1.1 *Properties of fluorophores*

Fluorophore	**Excitation** max (nm)	**Emission** max (nm)	**Extinction coefficient** ($M^{-1}cm^{-1}$)	**Solvent**
Fluoresceinyl	494	518	>80,000	-
Dansyl	335	526	4,600	Methanol
7-methoxycoumarinyl	358	410	26,000	Methanol
4MU	360	450	12,200	-

1.3.3 Bead-based pull down experiments

ELISA is one of the most popular enzyme immunoassay (EIA) systems currently in use, although many variations in the basic EIA concept have been designed [41, 42, 44]. The ELISA has been widely used as a bioanalytical tool. The basic principle of a sandwich ELISA is illustrated in Fig. 1.6. Antibodies against the analyte of interest are adsorbed onto a solid support, usually the walls of the wells of a microtiter plate and the sample solution is introduced to allow the analyte to be captured by the antibody. After washing, an enzyme-conjugated antibody specific for the analyte is introduced. The enzyme produces a signal indicating the presence of the analyte on the wall.

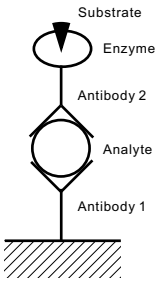

Figure 1.6 Illustration of the principle of a sandwich ELISA.

Pluronic derivative F108-PDS is coated onto polystyrene beads to reduce nonspecific binding and also to functionalize the surface with reactive groups *e.g.* disulfides, and then binder molecules with free thiols are conjugated by disulfide-sulfide exchange. To measure, the sample solution is introduced and the analyte is adsorbed ("pull down") from solution if it binds to the molecules conjugated to the beads. After washing, the beads are treated with reducing agents to release the binder molecules and their captured analytes. The captured analytes are analyzed by fluorimetry or CN/SDS-PAGE, depending on the nature of the analyte.

In this work, this method was used to discover binder molecules for chitinases (paper III).

2. Polypeptide-GPRP conjugates [paper I]

2.1 D-Dimer Protein

The D-dimer protein is a fibrin degradation product and is found in the blood after a blood clot is degraded by fibrinolysis. It consists of two crosslinked fragments of the fibrinogen protein (MW 200 kD). The D-dimer protein is used for diagnosis of thromboembolic diseases. In addition, it can be used in the diagnosis of the blood disorder disseminated intravascular coagulation [45-51]. The peptide derivative GPRP-amide prevents the polymerization of fibrin monomers and binds to fibrinogen and its D-dimer fragment with a K_d of approximately 25 μM. The binding occurs on the surface and is due to electrostatic interactions and hydrogen bonding, Fig. 2.1 [50, 52-55].

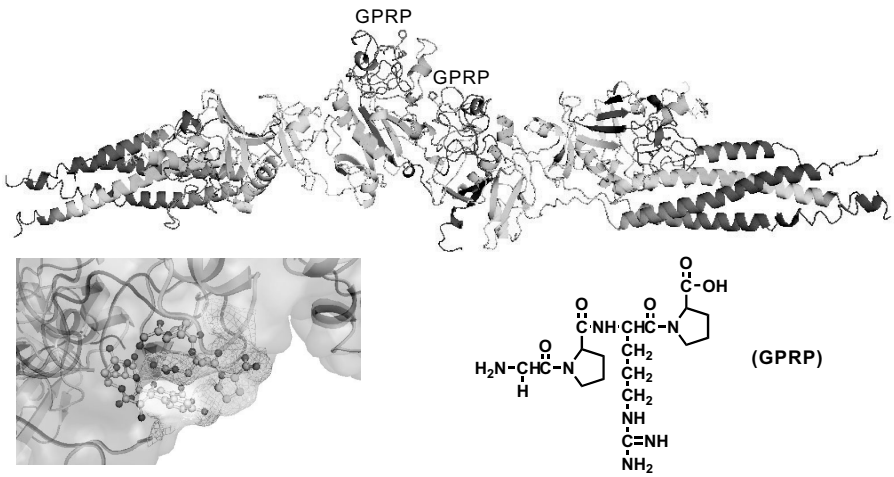

Figure 2.1 Cartoon structure of D-dimer protein with two GPRP ligands (top) and expansion of the GPRP binding sites (bottom, left) [50]. The structures were visualized by PyMOL 1.10 using X-ray structure of D-dimer protein (PDB entry: 1FZB).

2.2 Design and synthesis of GPRP ligands and conjugates

The X-ray crystal structure of the GPRP-D-dimer complex showed that the GPRP bound to the 'knot' close to the surface of the protein, Fig. 2.1, bot-

tom, left. To increase the affinity, it was decided to conjugate GPRP derivatives to polypeptides as was previously done successfully with other ligands [31]. The N-terminal residue Gly significantly contributed to binding affinity by hydrogen bonding and charge-charge interactions of the α-amino group. Therefore, the 6-carbon aliphatic conjugation spacer was connected to the C-terminal residue Pro. The spacer-linked GPRP 4-nitrophenyl ester **2.2** was synthesized by reacting 4-nitrophenol with compound **2.1**, which was synthesized by manual SPPS using Fmoc chemistry. Gly-preloaded 2-chlorotrityl resin was used to make the synthesis simple. The side chains of residues Arg and Gly were protected by Pbf and Boc groups. The 42-residue polypeptides were synthesized on a peptide synthesizer using Fmoc

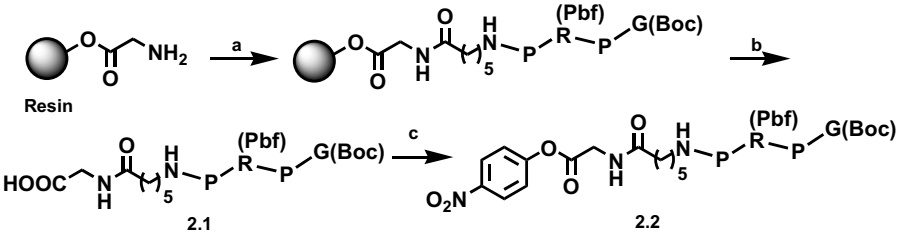

Figure 2.2 Synthesis of **2.2**. Conditions: a. Coupling: 2 eq. PyBOP, 2 eq. amino acids, 5 eq. DIPEA; Fmoc-deprotection: 20% piperidine in DMF; b. 1.5% TFA in DCM (v/v), 5 min; c. dry acetonitrile, 4-nitrophenol, 20% pyridine, DIC.

chemistry. One of the lysine side chains was Alloc-protected and was selectively deprotected by $Pd(PPh_3)_4$ on the solid phase and then modified by dansyl chloride or activated 7-methoxycoumarin-3-carboxylic acid. The polypeptides were cleaved from the resin, purified by HPLC and identified by MALDI-TOF-MS. The 4-nitrophenyl ester of GPRP (**2.2**) was conjugated to each polypeptide of a 15 membered set of 42-residue polypeptides in the presence of 10% pyridine and 0.5% DIPEA in DMSO solution and the acid-labile protection groups were finally cleaved by TFA to afford conjugates for D-dimer protein recognition, Fig. 2.3, left. The Acm group on residue Cys was deprotected by silver trifluoromethanesulfonate (AgOTf) in acidic aqueous solution. Due to the two binding sites on each half of the D-dimer protein, crosslinked polypeptide conjugates were also designed and synthesized by the reaction of the free thiols of two polypeptides with the bifunctional crosslinking agent $BM(PEG)_2$ (spacer arm 14.7 Å), Fig. 2.3, right.

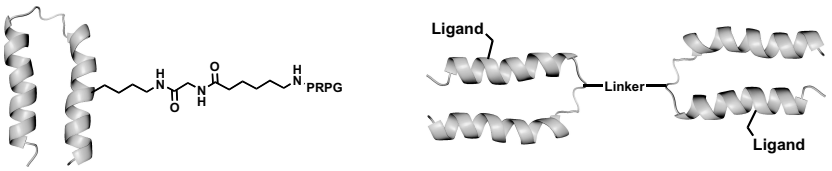

Figure 2.3 Cartoons of polypeptide conjugates 4C15L8GPRP (left) and $BM(PEG)_2$ crosslinked polypeptide conjugates 4C15L8GPRP-X-4C15L8GPRP (right).

2.3 Binding study

The D-dimer protein was covalently immobilized on a sensor chip by a standard procedure. A quick screening of 15 binder molecules using 0, 1, 10, 100 nM concentrations was performed using a Biacore 2000 instrument, Fig. 2.4. The concentration of binder molecules was estimated from weighing (20-30% error). As shown in Fig. 2.4, at 100 nM concentrations, the uptakes were large for some conjugates and at 10 nM concentration, uptake was still observed. The 3- and 4-series binder molecules were bound by the D-dimer

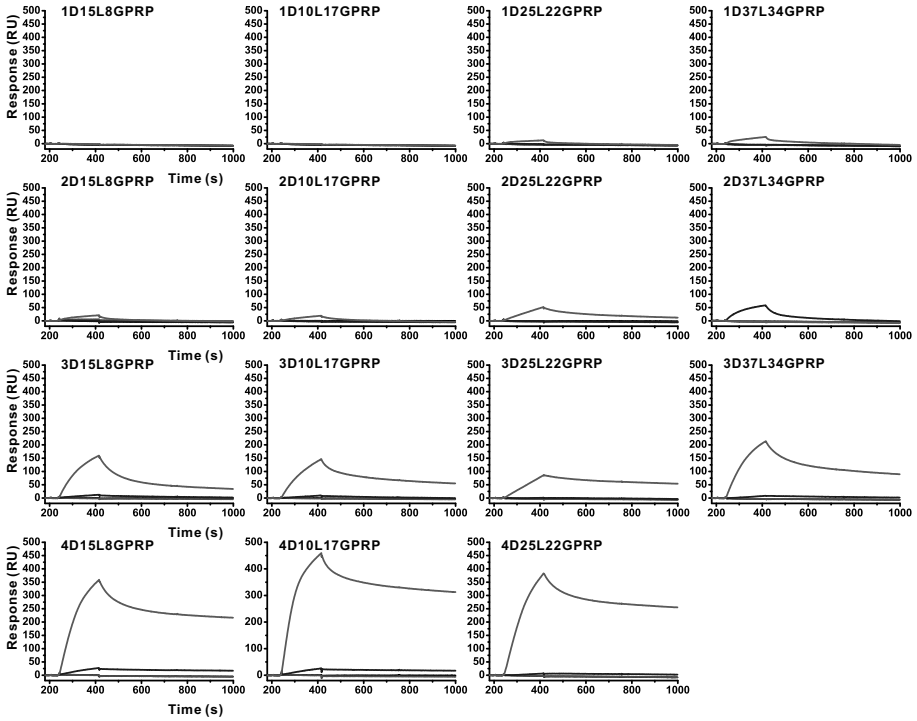

Figure 2.4 Sensorgrams from preliminary screening of 15 polypeptide conjugates (0, 1, 10, 100 nM concentrations) with immobilized D-dimer protein.

protein. The 3- and 4-series binder molecules were selected for further analysis. Fig. 2.5 shows the sensorgram panels for the interaction between 7 binder molecules and the D-dimer protein at seven concentrations (0, 5, 10, 20, 40, 80, 160 nM, concentrations determined by amino acid analysis). Assuming a simple 1:1 binding mode, K_d values were obtained by processing of binding curves, Table 2.1. In spite of the fact that the binding did not reach saturation and that the kinetic analysis was complex, the data showed that the polypeptide conjugates enhanced the affinity by about 4 orders of magnitude, compared to GPRP alone. The 4-series of binders had 6-9 nM affinity and the 3-series of binders had 15-42 nM affinity. To further improve the affinity by dimerisation, $(PEG)_2$ crosslinked binder molecules were

prepared and analyzed, Fig. 2.6, Table 2.1. The binding affinities of the crosslinked binders were further enhanced compared to the non-crosslinked binders. Disulfide-crosslinked binders without the $(PEG)_2$ chain were also tested. The binder 4C25L24GPRP gave better performance (2 fold higher).

Table 2.1 Approximate K_d *for conjugates binding to D-dimer protein from SPR interaction analysis.*

Binder	Kd (nM)	Binder	Kd (nM)
3D15L8GPRP	42	3D15L8GPRP-X-3D15L8GPRP	11
3D10L17GPRP	95	3D10L17GPRP-X-3D10L17GPRP	24
3D25L22GPRP	37	3D25L22GPRP-X-3D25L22GPRP	4290
3D37L34GPRP	15	3D37L34GPRP-X-3D37L34GPRP	32
4D15L8GPRP	6	4D15L8GPRP-X-4D15L8GPRP	13
4D10L17GPRP	7	4D10L17GPRP-X-4D10L17GPRP	2
4D25L22GPRP	9	4D25L22GPRP-X-4D25L22GPRP	3

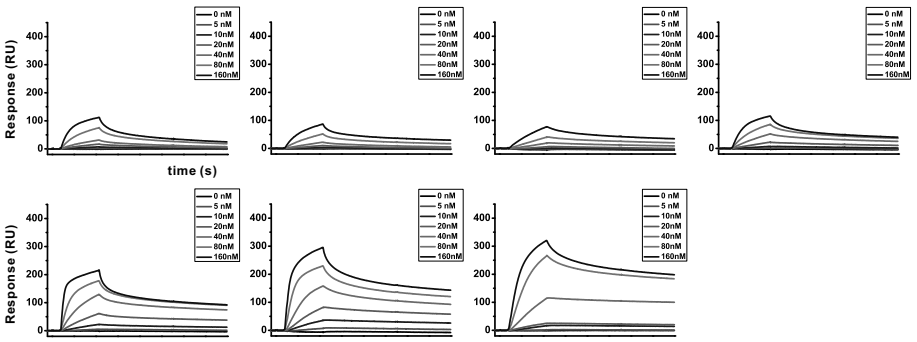

Figure 2.5 Sensorgrams for the best 3- and 4- series conjugates (0, 5, 10, 20, 40, 80, 160 nM concentrations) with immobilized D-dimer protein.

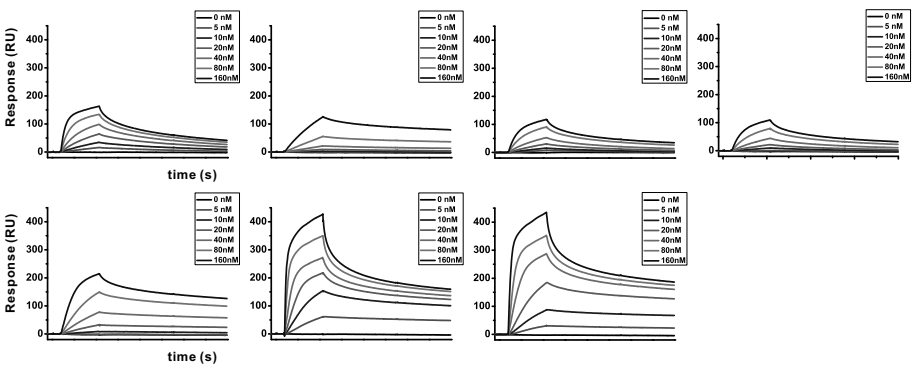

Figure 2.6 Sensorgrams for $BM(PEG)_2$ cross-linked 3- and 4-series conjugates (0, 5, 10, 20, 40, 80, 160 nM concentrations) with immobilized D-dimer protein.

Affinities of polypeptides without GPRP ligands were evaluated with the Biacore instrument for comparison to polypeptide-GPRP conjugates. For the

polypeptides without ligands, insignificant responses (0-5 RU) were observed, Fig. 2.7.

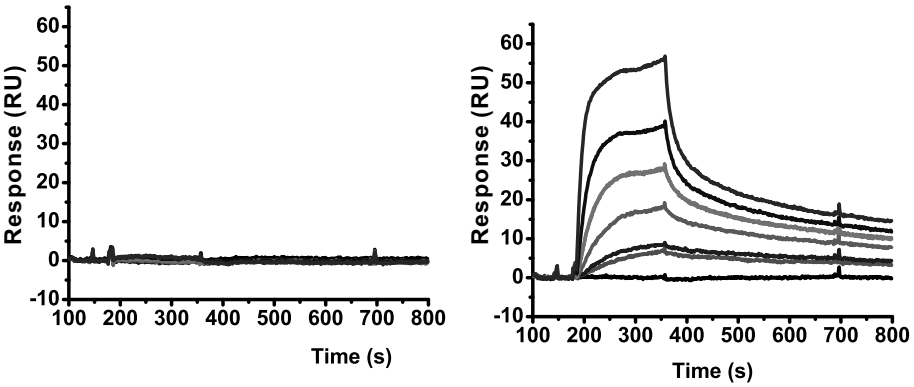

Figure 2.7 Sensorgrams for the polypeptide 4D15L8NH_2 (left) and the polypeptide-GPRP conjugate 4D15L8GPRP (right).

2.4 Competition study

To investigate whether the GPRP ligand of the polypeptide conjugates bound to the GPRP binding site, competition experiments were designed. A series of concentrations of free GPRP were mixed with a fixed concentration of a polypeptide conjugate (100 nM) and the solutions were flushed over freshly immobilized D-dimer protein. A series of solutions (0, 1, 10, 100, 1000 μM) of free GPRP and binder molecules, respectively, were used as controls, Fig. 2.8. As seen, mM concentrations of GPRP could completely suppress the uptake of the binder molecule 4D10L17GPRP (100 nM). With increasing GPRP concentrations, the uptake of polypeptide conjugate was decreased as a function of GPRP concentration, with 50% suppression at approximately 50 μM. The 500-fold concentration excess of GPRP over binder that was needed for 50% inhibition indicated that the affinity of the binder was about 3 orders of magnitude higher than that of GPRP. This study also showed that the GPRP on the binder was important for binding and was specifically bound to the GPRP binding site.

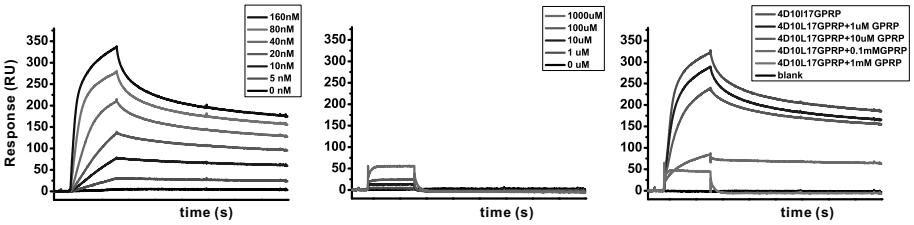

Figure 2.8 Sensorgrams for competition experiments. Left sensorgrams: 4D10L17GPRP (0, 5, 10, 20, 40, 80, 160 nM); middle sensorgrams: GPRP (0, 1, 10, 100, 1000 μM); right sensorgrams:100 nM 4D10L17GPRP with different concentrations of GPRP (0, 1, 10, 100, 1000 μM)

2.5 The affinity of 4C15L8GPRP

The SPR interaction analysis was based on covalently immobilized D-dimer protein. To exclude the effect of linking the protein to a surface, another method, fluorescence titration in solution, was used. Due to the low sensitivity of the dansyl fluorophore already present on the polypeptide conjugates, fluorescein-5-maleimide (FM) was conjugated to the thiol group on 4D15L8GPRP. Different concentrations from 1.0 nM to 1.5 μM of D-dimer protein were mixed with 100 nM FM-conjugated 4D15L8GPRP. Fluorescence spectra were recorded at an emission wavelength of 510-600 nm and an excitation wavelength of 495 nm. The intensity at 525 nm was measured as a function of D-dimer protein concentration and K_d was estimated to 3 nM by fitting Eq. 1.4 (chapter 1) to the experimental results under the assumption of a 1:1 binding model, Fig. 2.9. This showed that the binder had 4 orders of magnitudes higher affinity to the D-dimer protein than GPRP alone. The results agreed reasonably well with those from the SPR biosensor analysis, where an immobilized protein was used.

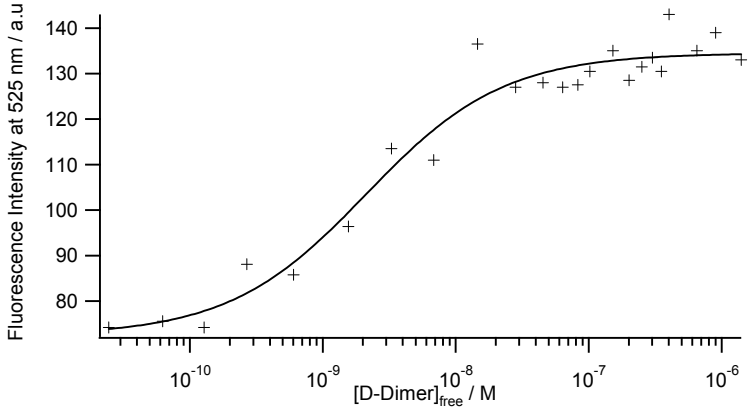

Figure 2.9 Titration of FM-conjugated 4D15L8GPRP with the D-dimer protein in phosphate buffered saline at pH 7.5.

2.6 Summary

Polypeptide binders for D-dimer protein, a marker for thrombosis diseases, were designed and synthesized by conjugation of a GPRP derivative to a set of 42-residue polypeptides. The binding properties of the conjugates were investigated by fluorescence titration and SPR biosensor analysis. The K_d of the conjugate 4D15L8GPRP was found to be 3 nM by fluorescence titration. The affinities of the 4-series binders were 3-4 orders of magnitude higher than that of GPRP alone. The binding was completely inhibited by free GPRP at mM concentrations, indicating that GPRP significantly contributed to the binding. The affinity of a $BM(PEG)_2$-crosslinked polypeptide conjugate was enhanced 2-4 fold, compared to its non-crosslinked counterpart.

3. Polypeptide-SHA conjugates [paper II]

3.1 Myeloperoxidase

Myeloperoxidase plays an important role in the innate immune defense system. It is found in the azurophilic granules of leukocytes, in monocytes and in a certain type of macrophages and it can constitute up to 5% of the neutrophile protein by weight [56]. In the presence of hydrogen peroxide, MPO catalyzes the formation of reactive intermediates or radical species, *e.g.* HOCl in the presence of chloride ion, which can kill the invading microorganisms [56-58]. However, an excess of the reactive intermediates can react with macromolecules, *e.g.* unsaturated lipid and DNA and lead to tissue damage or initiation and propagation of acute or chronic vascular inflammatory diseases. In recent years, evidence has been found for a link between MPO and cardiovascular diseases (CVD), and MPO has been suggested as a potential diagnostic marker, together with CRP [59-63].

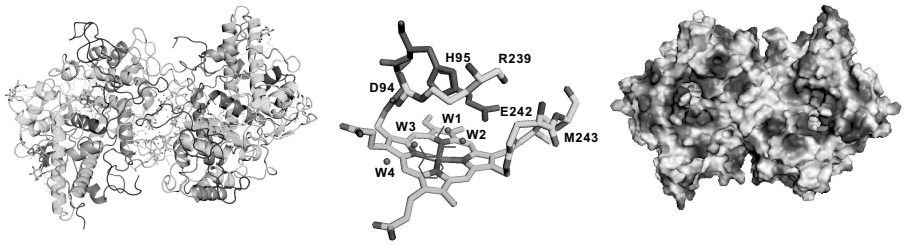

Figure 3.1 Structures of human dimeric MPO (left), SHA binding cavity (middle) and electrostatic potential map of MPO (right) [65]. The structures were visualized by PyMOL 1.1. from the X-ray structure PDB ID: 1MHL.

MPO is a dimeric, heme-containing enzyme (pI > 9.2, MW 146 kD). Each half contains two polypeptides (14.5 and 58.5 kDa) connected by intra-chain disulfides, Fig. 3.1, left [56]. The heme group is covalently linked to the polypeptide chain and located in a cavity, about 15 Å in depth and approximately 10 Å in diameter for solvent access via open channel [58]. Salicylhydroxamic acid (SHA), a substrate analogue inhibitor with a K_d of 2 μM, binds to the heme cavity, Fig. 3.1, middle [64, 65].

3.2 Design and synthesis of SHA derivatives and conjugates

According to the protein binder concept and considering the structure of the MPO-SHA complex, polypeptide-SHA conjugates were designed, Fig. 3.2. The structure of the MPO-SHA complex showed that the SHA aromatic ring could be substituted without affecting the binding significantly, therefore an aliphatic spacer could be linked to the *meta* position of SHA, Fig. 3.2.

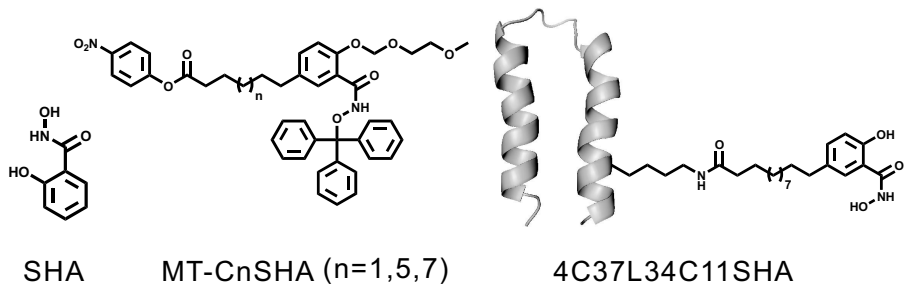

Figure 3.2 Structure of salicylhydroxamic acid SHA (left), the SHA derivatives MT-CnSHA (middle) and a cartoon of the polypeptide binder (4C37L34C11SHA) obtained by conjugating SHA with a 42-residue polypeptide via an aliphatic spacer (right).

Due to the hydrogen donors ($pK_a(NH_2OH)$ =7.43 and $pK_a(OH)$ > 9.0) and the difficulty of using an unprotected SHA active ester, an initial strategy involving nucleophilic reaction of a COOEt/Me ester on the polypeptide with NH_2OH and a conjugation reaction in aqueous solution was used. However, the conjugation in aqueous solution failed, probably due to the poor solubility of the active ester ethyl 2-hydroxy-5-[10'-(4''-nitrophenoxy)-10-oxodecanyl] benzoate in water. Conjugation in DMSO solution together with a weak base afforded polypeptide conjugates with the ligand ethyl 2-hydroxy benzoate. However, nucleophilic reaction on the polypeptide with aqueous NH_2OH (pH>10) failed, probably due to the instability of the peptide or the fluorophore under strongly basic conditions. Therefore, another synthetic strategy was developed involving conjugation of a fully protected SHA derivative MT-CnSHA to the polypeptide, followed by a final acidic deprotection, Fig. 3.2 and 3.3.

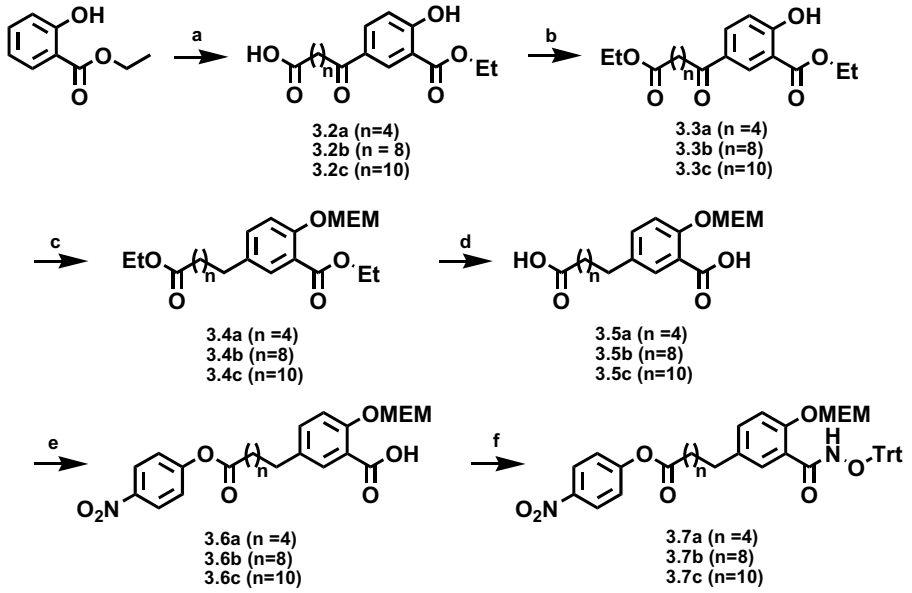

Figure 3.3 Synthesis of compound **3.7a**, **3.7b** and **3.7c**. Conditions: a) $AlCl_3$, $ClCO(CH_2)_nCOCl$, dichloroethane, -20 to 15 °C to rt, 30-50%; b) Zn/Hg, 6 M HCl, ethanol, reflux, 70-85%; c) NaH, THF, MEMCl, 0°C, 70-99%; d) 1M NaOH, H_2O:MeOH (1:1), 60°C, 89-99%; e) 4-nitrophenol, DCC, DMAP, dry DCM, rt, o.v.; f) HOBt, DIC, NH_2OTrt, NEt_3, dry DCM, 0°C 15-30% in the total e and f steps.

3.2.1 Synthesis of SHA derivatives

Synthesis of the SHA derivatives **3.7a**, **3.7b** and **3.7c** is shown in Fig. 3.3. The acid-labile groups MEM and Trt were employed for OH and NHOH protection, respectively. The synthesis started with ethyl salicylate, which was subjected to Friedel-Crafts acylation together with Fries rearrangement with an excess of $ClCO(CH_2)_nCOCl$ (n = 4, 8, 10) [67-69]. The yield of the first step was significantly improved (up to 50%), compared to Friedel-Crafts reaction of other aromatic ethers. The carbonyl group close to the aromatic ring was then reduced by a Clemmensen reduction, followed by an esterification in a one-pot reaction (Zn/Hg amalgam, HCl, water and alcohol) in a yield of up to 80%. The hydroxyl group on the aromatic ring was protected by a MEM group, followed by saponification of both carboxylic groups. Because of the difference in acidicity of the two carboxylic acids (pK_a of PhCOOH ~ 3.0, pKa of CH_3COOH ~ 5.0), the esterification with 4-nitrophenol mainly afforded the monosubstituted aliphatic ester product, together with a trace of the diester. The ^1H NMR shift differences before and after esterification clearly verified the identity of the monoester product, Fig. 3.4. Because of difficulties in purification of the monoesters **3.6a**, **3.6b** and **3.6c**, the crude compounds were directly subjected to the next step, reaction with O-tritylhydroxylamine. The purified major products **3.7a**, **3.7b** and **3.7c**

were obtained in up to 30% yield. The Trt group significantly affected the chemical shifts for the MEM protons of **3.7a**, **3.7b** and **3.7c**, confirming the aromatic hydroxamic acid structure, Fig. 3.4.

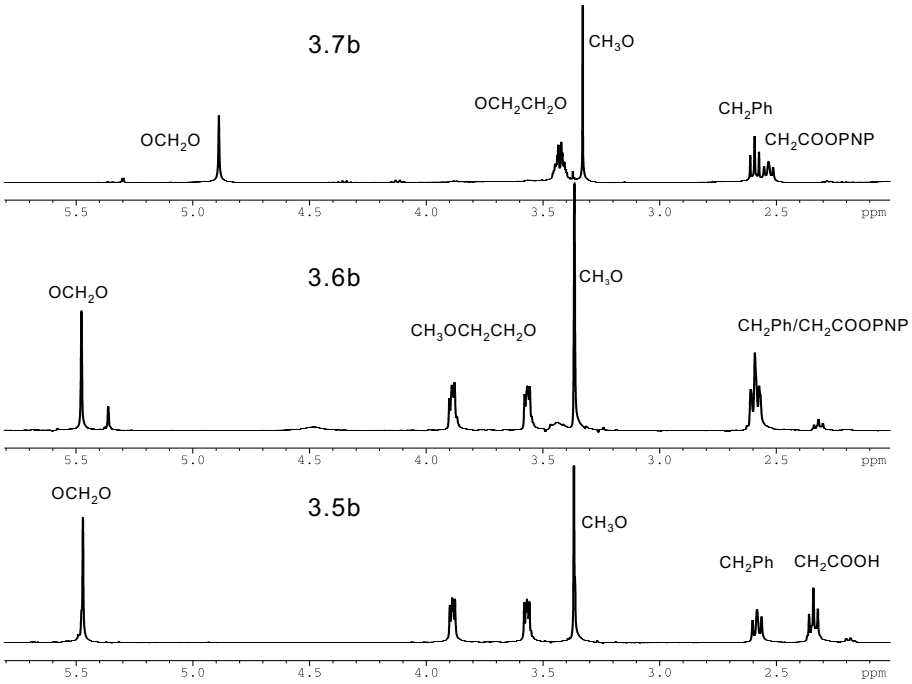

Figure 3.4 Regional ^{1}H NMR spectra of compounds **3.5b**, **3.6b** and **3.7b** at 2.0-6.0 ppm. Similar chemical shift changes were also observed for **a** and **c** analogs.

3.2.2 Synthesis of polypeptide conjugates

Due to the low solubility of compounds **3.7 a**, **b** and **c** in water, the conjugation reaction was not successful in aqueous solution. 4-nitrophenyl esters **3.7a**, **3.7b**, and **3.7c** were conjugated to the side chain of lysine on the polypeptides in DMSO solution to afford MEM and Trt-protected polypeptides in the presence of the weak base triethylamine or DIPEA, Fig. 3.5. The reactions were monitored by analytical HPLC. Some deprotection of the Trt group in the HPLC mobile phase containing 0.1% TFA was observed. Finally, the MEM and Trt groups were deprotected by a mixture of TFA: TIS: H_2O (95:2.5:2.5) to afford the final polypeptide conjugates, which were purified by semi-preparative HPLC and identified by MALDI-TOF-MS. A total of 40 conjugates were prepared from **3.7a**, **3.7b** and **3.7c** and 16 polypeptides, Fig. 1.2. Free Cys thiol groups on the polypeptide conjugates 4C37L34C11SHA and 4C15L8C9SHA were obtained by deprotection of Acm in the presence of AgOTf, TFA and anisole, followed by DTT treatment in acetic acid-water.

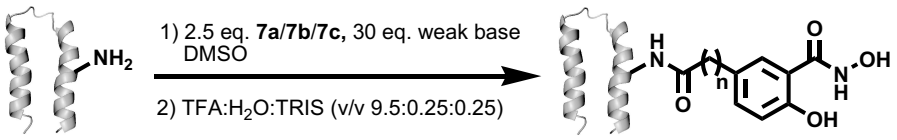

Figure 3.5 Conjugation reactions with active esters **3.7a**, **3.7b** and **3.7c**.

3.3 Binding study

Some of the polypeptide conjugates had poor solubility in aqueous solution at pH 7.4. Therefore, buffers containing 0-3% DMSO was used. A low intensity of the 7-methoxycoumarin fluorescence at 410 nm in 0.5 μM concentration was observed. It was probably due to the effect of Ph-OH, NHOH and heme groups on the stability of the excited fluorophore. Therefore, SPR biosensor technology was employed for binding evaluation. Sixteen polypeptide conjugates with 9-carbon aliphatic spacers were preliminarily screened, Fig. 3.6. Fig. 3.6 shows that 4-series conjugates with a 9-carbon spacer bound to MPO-immobilized chip at nM range concentrations, especially 4D37L34C9SHA. However, the 1-, 2- and 3-series conjugates showed little or no binding at nM concentrations on the same chip, μM concentrations of 1-, 2-, 3-series binders were therefore used. The response did not reach steady state and the curve fitting was poor in a 1:1 binding mode. Based on these data, the relative affinities were estimated. The positively charged polypeptides had much higher affinity than the negatively charged ones.

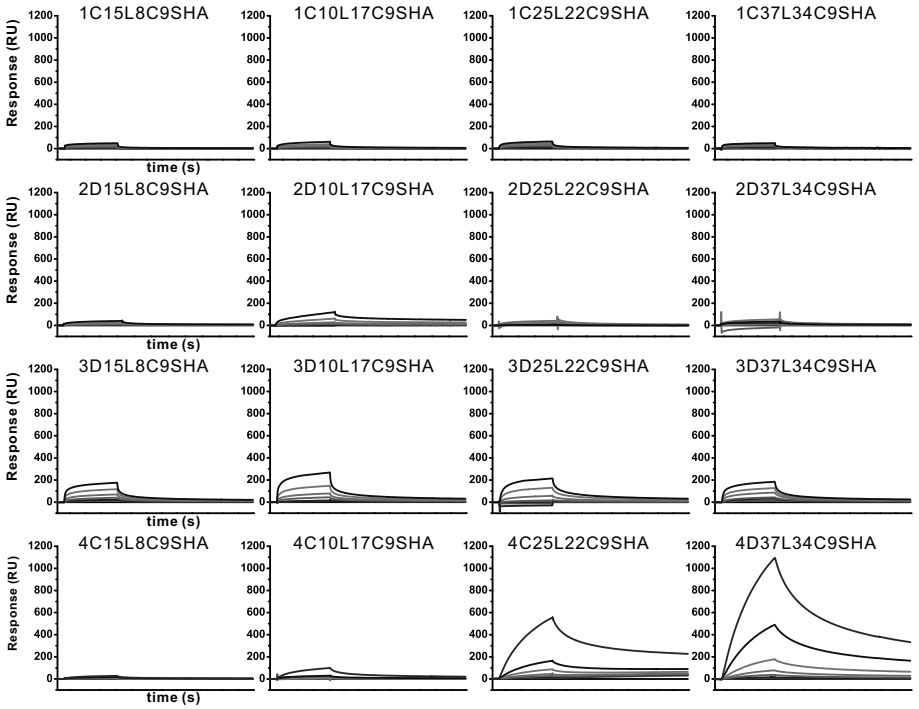

Figure 3.6 Sensorgrams for polypeptide conjugates with 9-carbon spacers and immobilized MPO. 1-, 2-, 3-series of binder molecules were at μM concentrations (0.5-32 uM), while 4-series of binder molecules were at nM concentrations (5-640 nM).

To further enhance the affinity for MPO, 3- and 4-series binder molecules with 11-carbon atom spacers were prepared and used for binding analysis with immobilized MPO using the same chip as before, Fig. 3.7. This showed that 4-series polypeptides with 11-carbon spacers were absorbed more strongly than the corresponding ones with 9-carbon spacers in nM range concentrations. Little or no absorption for 3-series polypeptides was observed. These data again indicate that the more positively charged polypeptide conjugates bound to MPO more strongly, compared to the negatively charged polypeptide conjugates. Conjugates 4C37L34C11SHA performed better than its shorter chain analog 4C37L34C9SHA (2-fold higher), while 4C25L22C11SHA was enhanced 6-fold, compared to 4C25L34C9SHA. MPO isoform C was also used for binding analysis, Fig. 3.8. As before, the 4-series polypeptide conjugates had better affinity as the 3-series polypeptide conjugates. The MPO is a highly basic protein. The binding can be partly explained by the electrostatic potential picture of MPO, Fig. 3.1. Part of the binding probably resulted from charge-charge interactions with the negatively charged surface on the cavity entrance.

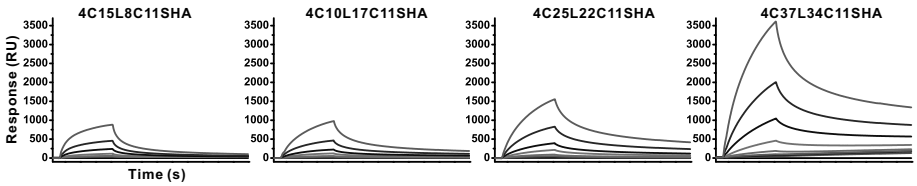

Figure 3.7 Sensorgrams for 4-series polypeptide conjugates with 11 carbon aliphatic spacer and immobilized MPO at nM range concentrations (5-320 nM).

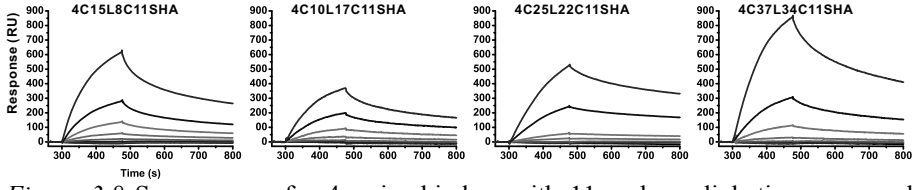

Figure 3.8 Sensorgrams for 4-series binders with 11 carbon aliphatic spacer and immobilized MPO isoform C. Little or no absorption of polypeptide conjugates was observed with 3-series binders at nM concentrations (5-320 nM).

3.4 Competition study

To investigate whether the small molecule SHA bound to the active site of the MPO heme cavity, competition experiments were carried out by flushing a mixture of binder molecules and a series of concentrations of free SHA over MPO immobilized on the surface. In this experiment, the regeneration was performed with buffer for 20 min, to avoid possible formation of "acidic MPO". For 100 nM binder molecule solutions containing 0.0625 mM-4 mM SHA, the binding of 4C37L34C11SHA was suppressed to 50% between 0.125 mM and 0.25 mM, Fig. 3.9. Presumably, at 50% inhibition, the concentration of the SHA-MPO complex was equal to the concentration of the binder-MPO complex. The K_d can be estimated to be 0.8-0.4 nM from these competition experiments. This is about four orders of magnitude higher than for SHA alone. This indicates that SHA is critical for binding and that is bound to the active site of MPO.

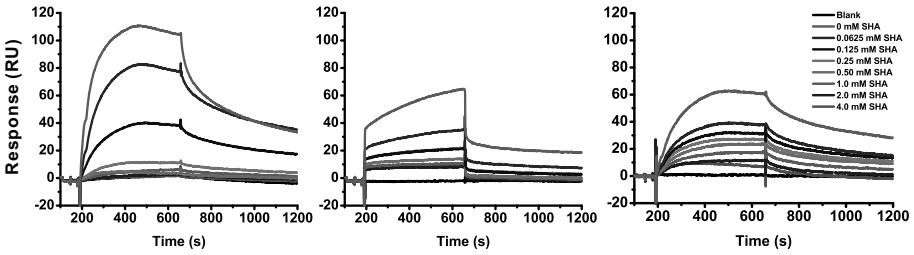

Figure 3.9 Competition experiments with different concentrations of SHA and immobilized MPO. Left sensorgrams: 0, 5, 10, 20, 40, 80, 160, 320 nM of 4C37L34C11SHA. Middle sensorgrams: 0, 62.5, 125, 250, 500, 1000, 2000, 4000 μM of SHA . Right sensorgrams: 100 nM 4C37L34C11SHA with a series of concentrations (0, 62.5, 125, 250, 500, 1000, 2000, 4000 μM) of SHA. The sensorgrams were obtained by subtraction of individual SHA responses.

3.5 Summary

Active ester derivatives of salicylhydroxamic acid containing MEM and Trt groups were designed and synthesized. These were conjugated to a set of polypeptides in organic solvent. The interactions between the polypeptide conjugates and immobilized MPO were evaluated by SPR. Positively charged polypeptide conjugates showed higher affinity than the negatively charged or neutral ones. Competition experiments verified that the conjugated SHA bound to the MPO binding pockets and was critical for binding. The affinity of the best conjugates was estimated to be about four order magnitudes higher than that of SHA alone. The binders in this work could be a starting point for the future development of diagnostics, drugs and medical imaging agents.

4. Polypeptide conjugates recognizing **chitinase** (paper III)

4.1 Chitinases

The enzyme chitinase hydrolyzes the glycosidic bonds of chitin, a β-(1,4)-linked homopolymer of *N*-acetylglucosamine, the second most abundant polysaccharide in nature after cellulose. Chitinases belong to the glycosyl hydrolase family 18 and 19 of enzymes that are related but differ slightly in amino acid sequence, structure and mechanism [70-71]. Chitinases can be found in a variety of organisms from prokaryotes to human and they play an important role in the defense against fungal pathogens, in catabolism and morphogenesis. Recently, an acidic mammalian chitinase has been suggested as a potential target for asthma therapy [72-74].

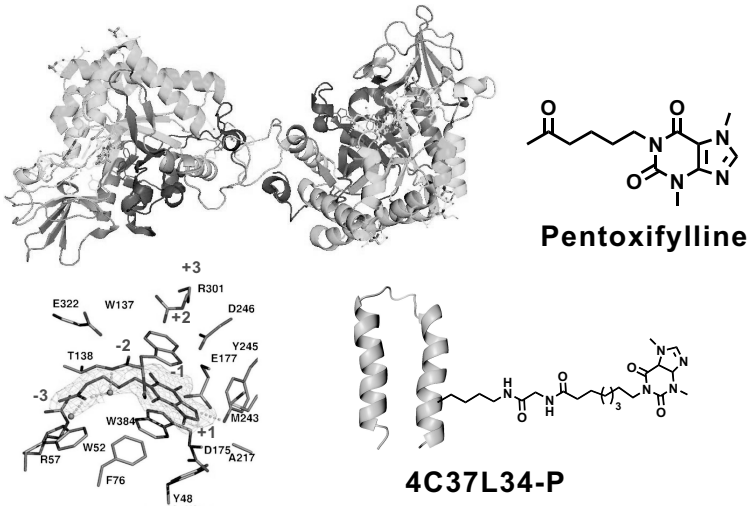

Figure 4.1 Illustration of binder development for chitinases. The binding mode of pentoxifylline in the chitinase from *A. fumigatus* shows that the xanthine group contributes significantly to the affinity via hydrophobic interactions with Trp residues [83]. The polypeptide conjugate 4C37L34-P was synthesized by the reaction of the only free lysine residue of the polypeptide with **4.2**. The structures were visualized by Pymol 1.1 by X-ray structures (PDB ID: 2A3C).

Many forms of chitinases *e.g.* ChiA, B and C are found in the fungus *A. fumigatus*, at least four chitinases are produced by the bacterium *Serratia marcescens*, and there are at least three chitinases found in *Trichoderma harzianum* [75-80,87]. It is difficult to identify the precise number of chitinases based only on biochemical methods. For example, some of the chitinases occur in multiple forms on an SDS-PAGE gel. Some chitinase structures are not well characterized because of the inaccessibility of purified forms. *Endo*chitinases are defined as enzymes that cleave the β-(1,4)-glycosidic bonds randomly at internal bonds in the chitin and *exo*chitinases are enzymes that split the β-(1,4)-glycosidic bonds progressively from the non-reducing end of chitin. Fig. 4.2 shows SDS-PAGE and CN-PAGE gels of cell lysates, the latter after in-gel enzymatic analysis with the substrate 4-methylumbelliferyl β-D-*N*,*N*′,*N*″-triacetylchitotriose (4MU-3), which is a substrate used for detection of *endo*-chitinase activity. The CN-PAGE gel shows one strong fluorescent band and some weak fluorescent bands from the commercially available chitinase from *T. viride*, indicating at least two chitinolytic enzymes and some weak bands from *S. griseus*. In addition, the chitinase substrate 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (4MU-1), which was one of the substrates for *exo*-chitinase activity, was used for enzymatic activity analysis. Both substrates were hydrolyzed, indicating the presence of both *endo*-chitinases and *exo*-chitinases. In this work, polypeptide conjugates were synthesized, with the aim to find specific binders for chitinases.

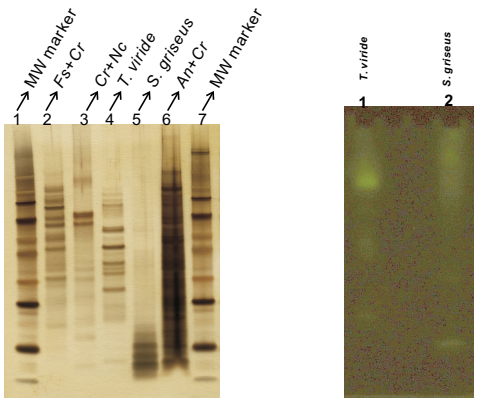

Figure 4.2 SDS-PAGE of cell lysates from *Fs+Cr*, *Cr+Nc*, *An+Cr* and chitinases from *T.viride* and *S. griseus* (left) and CN-PAGE after in-gel enzymatic activity analysis with the substrate 4MU-3 (right).

Chitinases have many different structures. However, they have a common catalytic domain with a tunnel-like active site groove and a substrate-binding domain. In family 18 chitinases, the catalytic domain usually contains a conserved DXDXE structural motif for catalysis and the substrate-binding domain for chitin usually contains exposed aromatic residues that form a long

groove extending to the active site [76, 81]. For example, chitinase B from *S. marcescens* had an about 40 Å long, 15 Å wide and 20 Å deep catalytic domain from the -3 to the +2 subsites and a 55 Å long continuous aromatic residues chitin binding domain, connecting to the catalytic domain [76, 81, 82]. Pentoxifylline, Fig. 4.1, a known pharmaceutical, binds to the -1 subsite of *A. fumigatus* ChiB, with a dissociation constant K_d of 43±10 μM, mimicking the binding mode of the natural product allosamidin [83, 84].

4.2 Design and synthesis of ligands and conjugates

In order to develop a polypeptide conjugate for the recognition of chitinases, pentoxifylline, was conjugated to polypeptides from a designed set. In addition, C4B3, an inhibitor of the glycosidase family 7 cellobiohydrolase [85], and C5B1, an analogue of C4B3, were tested. Peptides conjugated to acetyl groups were used as negative controls, Fig. 4.3. The small molecule ligands were linked to the 3- and 4-series of polypeptides presented in Fig.1.2 via site selective lysine acylation using the 4-nitrophenyl esters **4.2**, **4.8** and **4.13,** Fig. 4.4, 4.5 and 4.6. Attempts to further enhance selectivity and affinity were made by conjugating the small molecules pentoxifylline and C4B5 or C5B1 to two free lysine side chains on the same polypeptide. The underlying reason was that the two molecules are expected to bind to the protein in different positions and thus it might be possible to obtain cooperative effects.

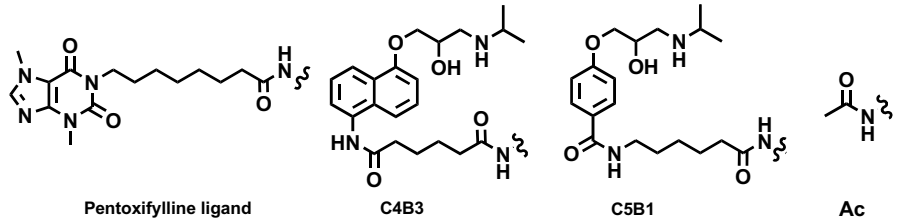

Figure 4.3 Structures of the small molecule ligands pentoxifylline ligand, **C4B3**, **C5B1** and **Ac**. The former three are shown linked to aliphatic spacers.

4.2.1 Synthesis of a pentoxifylline derivative

The 4-nitrophenyl ester **4.2** was synthesized by alkylation of theobromine with 8-bromooctanoate [90, 91], saponification and re-esterification with 4-nitrophenol in a total yield of 43%, Fig. 4.4. Compared to the analogous reaction with 8-bromooctanic acid, the methyl ester gave less purification problems and higher yields.

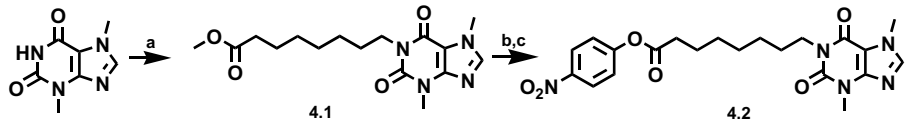

Figure 4.4 Synthesis of **4.2**.Conditions: a)1.1 eq. NaH, DMSO, 30 min, then methyl 8-bromooctanoate, 70°C, 85%; b) 2 eq. NaOH, MeOH, 50°C, 76%; c) DCC, 4-nitrophenol, DMAP, DCM, rt, overnight, 66%.

4.2.2 Synthesis of a C4B3 derivative

The 4-nitrophenyl ester **4.8** was synthesized in sex steps. The total yield was 6%, Fig. 4.5. The six-carbon aliphatic spacer was selectively linked to the amino group of 5-amino-1-naphtol in a fair yield, followed by reaction with epichlorohydrin to give the epoxide **4.4** in a 49% yield [66, 92]. Nucleophilic opening with isopropylamine then gave **4.5** in a 88% yield. To avoid reaction with the 4-nitrophenyl ester in the final step, the NH group in **4.5** was protected by a Boc group to give **4.6** in a 78% yield. Saponification and re-esterification with 4-nitrophenol gave the target compound **4.8** in a 91% yield.

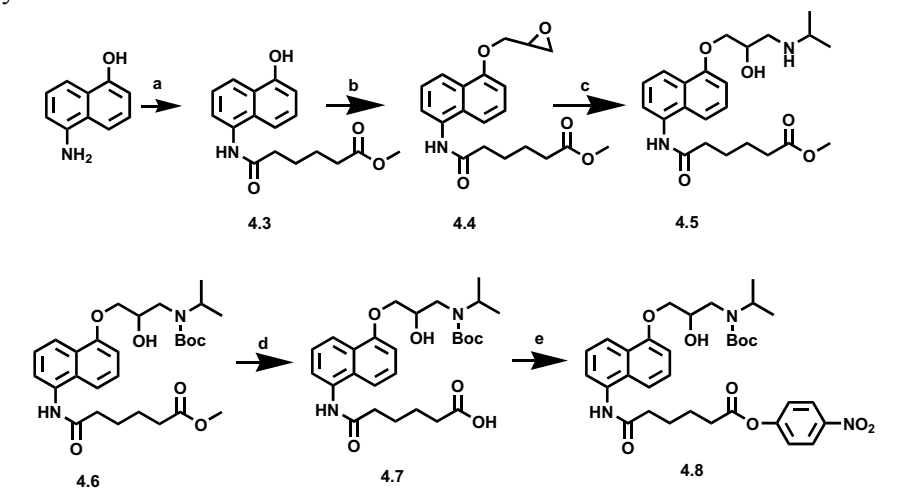

Figure 4.5 Synthesis of **4.8**. Conditions: a) 1 eq. 5-(methyloxycarbonyl)-pentanoic acid chloride, CH_2Cl_2, $NaHCO_3$, 0°C, overnight, 18%; b) 3 eq. epichlorohydrin, 3 eq. K_2CO_3, acetonitrile, reflux, overnight, 49%; c) 10 eq. NH_2iPr, MeOH, rt, overnight, 88%; c) Boc_2O, CH_2Cl_2, NEt_3, rt, overnight, 78%; d) LiOH in MeOH/THF /H_2O (2:1:2), rt, overnight, 98%; e) 1.1 eq. DCC, 1.1 eq. 4-nitrophenol, dry CH_2Cl_2, 0.1 eq. DMAP, 91%.

4.2.3 Synthesis of a C5B1 derivative

The 4-nitrophenyl ester **4.13** was synthesized in seven steps in a total 14% yield, Fig. 4.6, by a reversed synthetic route, compared to that of compound **4.8**. First, methyl 4-hydroxybenzoate was transformed to **4.11** via alkylation

with epichlorohydrin, nucleophilic epoxide opening with isopropylamine [92] and protection of the amino group with a Boc group. The obtained methyl ester **4.11** was saponified and the acid was coupled with methyl 6-aminohexanoate to give **4.12** in a 52% yield. Finally, saponification and re-esterification with 4-nitrophenol gave the target compound **4.13** in a 91% yield.

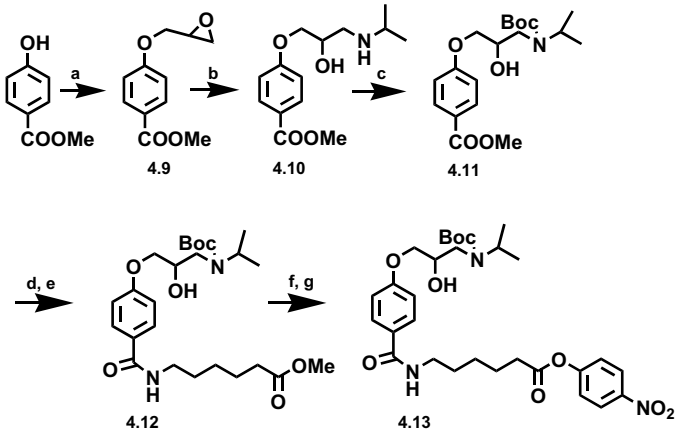

Figure 4.6 Synthesis of **4.13**. Conditions: a) 3 eq. epichlorohydrin, 3 eq. K_2CO_3, acetonitrile, reflux, overnight, 71%; b) 10 eq. NH_2iPr, MeOH, rt, o.v., 82%; c) Boc_2O, CH_2Cl_2, NEt_3, rt, *o.v.*, 81%; d) LiOH in MeOH/THF/H_2O, rt, o.v.,67%; e) methyl 5-aminohexanoate hydrochloride, *N*,*N*'-diisopropylcarbodiimide, DCM, 52%; f) 1M NaOH/MeOH, 50°C, 95%; g) 1.2 eq. DIC, 1.2 eq. 4-nitrophenol, dry DCM, 0.2 eq. DMAP, 91%.

4.2.4 Synthesis of polypeptide conjugates

The twenty-four 3-series and 4-series of polypeptide conjugates and their corresponding acetylated polypeptides used as controls were prepared via acylation of the side chain of lysine with the 4-nitrophenyl esters **4.2**, **4.8**, **4.13,** or 4-nitrophenyl acetate. Polypeptide conjugates with two ligands pentoxyfylline and C4B3 or C5B1 on the same polypeptide were also prepared by employing three orthogonally protected lysine side-chains on the polypeptide using the protections groups Mtt, Boc and Alloc.

4.3 Identification of binder molecules for chitinases

Assays for chitinases are of considerable interest but purified chitinases are difficult to access. The identification of good binder molecules for chitinase assays therefore had to be developed in a somewhat different way compared to that various chitinases had been readily available. The strategy was to use

bioconjugated particles for pull-down experiments equipped with a range of binder molecules and then to identify the extracted enzymes with SDS-PAGE or CN-PAGE combined with enzymatic assays, Fig. 4.7. Synthetic binder molecule candidates prepared as described above were conjugated to F108-PDS-coated polystyrene beads by disulfide bond exchange, cell lysates were incubated with the conjugated particles in so-called pull-down experiments and enzymatic assays for chitinolytic enzymes were employed to identify enzymatic activity by sensitive fluorimetric methods. The substrate 4MU-3, Fig. 1.5, can be used to assay chitinase activity by measuring the fluorescence of the reaction product 4MU. The aglycone 4MU is released by enzymatic hydrolysis and ionizes under basic conditions, *e.g.* at pH > 10 to give a highly fluorescent anion. The fluorescence of 4MU can be detected at the emission wavelength of 450 nm, Fig. 1.5. The extracted enzyme-binder complex can be released from the beads by chemical methods and analyzed by SDS-PAGE or CN-PAGE in combination with substrates that provide an optical signal after enzymatic hydrolysis.

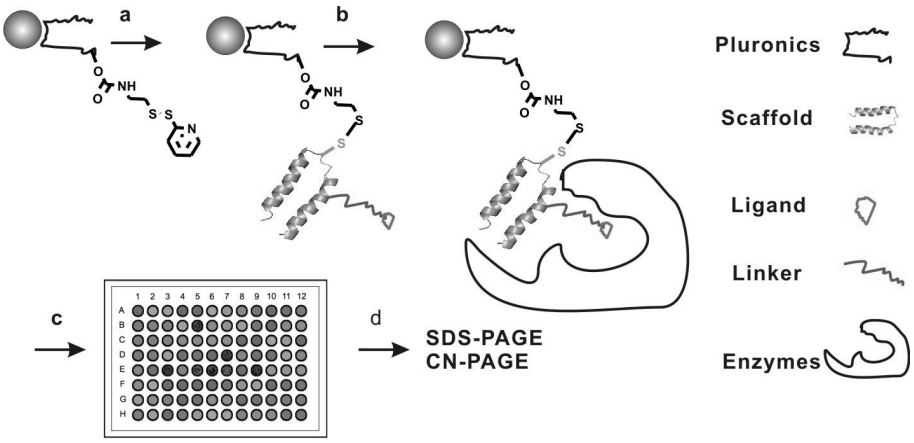

Figure 4.7 Illustration of the pull down experiment. a. a binder molecule candidate carrying a free thiol group is conjugated to beads by disulfide-sulfide bond exchange; b. cell lysates are added to the conjugated beads and incubated; c. after washing and removal of the lysates, the intact beads or released extracted enzymes are analyzed by a fluorimetric enzymatic assay; d. the extracted enzymes are analyzed by SDS-PAGE or CN-PAGE.

4.4 Screening

In order to identify the best binder molecules, 32 polypeptide conjugates were prepared from the eight 3- and 4-series of polypeptides, the three small molecule active esters **4.2**, **4.8**, **4.13** and 4-nitrophenyl acetate. An Acm protection group at the side chain of a Cys residue was selectively removed to

allow immobilization via the reaction of the free thiol group with the activated disulfide bond on the beads. Cell lysates from *Aspergillus*

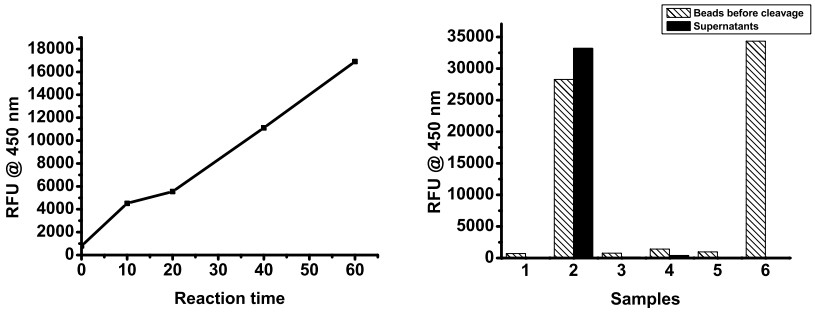

Figure 4.8 The enzymatic degradation of 4MU-3 as a function of time catalyzed by chitinase from *T. viride* (left) and evaluation of binder molecule candidates by enzymatic assay (right). 1 blank; 2 4C37L34-P; 3 4C37L34-C4B3; 4 4C37L34-C5B1; 5 4C37L34-Ac; 6 positive control chitinase from *T. viride*.

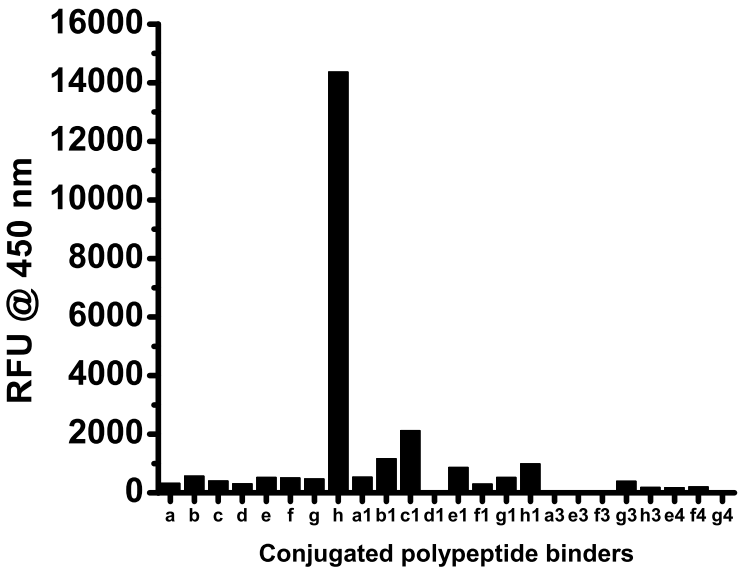

Figure 4.9 Screening of cell lysates for chitinase binders from *An+Nc* with polypeptides conjugated to the small molecules pentoxyfylline (P), C4B3 and C5B1. Conjugated polypeptides: a. 3C15L8-P; b. 3C10L17-P; c. 3C25L22-P; d. 3C37l34-P; e. 4C15L8-P; f. 4C10L17-P; g. 4C25L22-P; h. 4C37L34-P; a1. 3C15L8-C4B3; b1. 3C10L17-C4B3; c1. 3C25L22-C4B3; d1. 3C37l34-C4B3; e1. 4C15L8-C4B3; f1. 4C10L17-C4B3; g1. 4C25L22-C4B3; h1. 4C37L34-C5B1; a3. 3C15L8-C5B1; e3. 4C15L8-C5B1; f3. 4C10L17-C5B1; g3. 4C25L22-C5B1; h3. 4C37L34-C5B1; e4. 4C15L8-PL34-C4B3; f4. 4C10L17-PL34-C4B3; g4. 4C25L22-PL34-C4B3.

nidulans and *Neurospora crassa* (*An+Nc*) containing several chitinases were exposed to pull-down experiments. 4MU-3 was used as a substrate for the enzymatic assays, and a commercially available chitinase mixture from *T. viride* was used as positive control. To determine the conditions required for the analysis, an enzymatic reaction was carried out using chitinase mixture from *T. viride* and 4MU-3 where the increase in fluorescence was monitored over time. The reaction was allowed to proceed for one hour to generate sufficient fluorescence for detection with a good signal to noise ratio. In order to establish that the fluorescence produced did not emanate from unspecific binding of enzymes to the beads, the extracted enzyme-binder complex was cleaved from the beads by reduction of the disulfide bond by TCEP. The suspension was centrifuged and the supernatant was analyzed by the enzymatic assay and compared to the crude particle suspension with regards to enzymatic activity by fluorescence. The results show that the fluorescence at the wavelength of 450 nm was not only detected in supernatant but also in the particle suspension in equal proportions, Fig 4.8, right. It indicated that the active chitinases were extracted by the binder molecules not by nonspecific binding. Only one binder molecule, 4C37L34-P, gave rise to strong fluorescence intensity at the wavelength of 450 nm after pull-down experiment and enzymatic hydrolysis of 4MU-3, Fig. 4.9. The reasons can only be the subject of speculation; maybe the best binders inhibit the enzymes and maybe there is only one good binder molecule in the set of candidates.

4.4.1 Cleavage study

The cleavage of the disulfide bond connecting the polypeptide conjugates to the beads was optimized by employing two different reducing agents. When DTT was used in phosphate buffer at pH 6.0, no enzymatic activity was observed in the supernatant obtained after centrifugation and removal of the beads while it was detected in the bead suspension. When using TCEP, strong enzymatic activity was observed in the supernatant as well as in the bead suspension, Fig. 4.10. The supernatant was analyzed by CN-PAGE followed by in-gel activity measurement. One strong fluorescent band was observed, which indicated that at least one enzyme was extracted. SDS-PAGE showed multiple bands and either pentoxifylline is not specific for chitinases or there is nonspecific binding to other proteins by the beads. The multiple extracted proteins in combination with the small amounts of enzyme extracted made further analysis with MALDI-TOF-MS difficult. The extracted chitinase has therefore not yet been identified. However, the results clearly show that the binder 4C37L34-P recognizes chitinolytic enzymes and can extract them from a complex mixture.

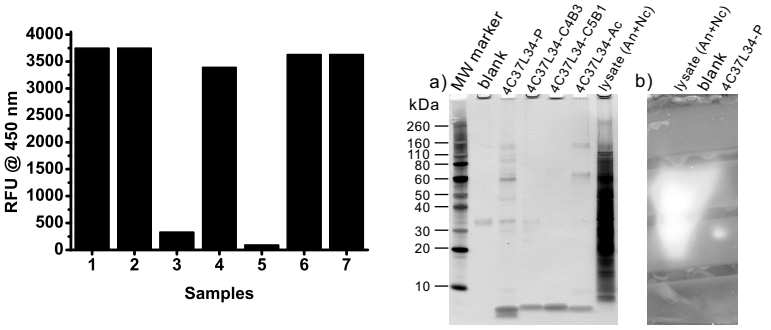

Figure 4.10 Enzymatic activities of bead suspensions and supernatants after cleavage by DTT and TCEP (left) and SDS-PAGE and CN-PAGE analysis of extracts (right). Left panel 1. particle suspension after treatment with DTT; 2. particle suspension after treatment with TCEP; 3. supernatant after DTT cleavage; 4. supernatant after TCEP cleavage; 5. control buffer; 6. positive control: chitinase from *T. viride*; 7. positive control: lysate from *An+Nc*. Right panel a) SDS-PAGE of extracts from *An+Nc* lysate, b) CN-PAGE of extract from *An+Nc*.

4.5 Competition study

To further investigate how the polypeptide conjugates bind to the enzyme, competition experiments with pentoxifylline equipped with a spacer 1-(7'-carboxyheptyl)-3,7-dimethylxanthine (P-7) were carried out employing supernatants from lysate obtained from *An+Nc* after cleavage from the beads. Three concentrations, 17.7 mM, 1.77 mM and 0.177 mM of the pentoxifylline derivative with DMSO as a positive control were used to compete with the binding of 4C37L34-P to chitinases in the extracts. The mixture was incubated for 1.5 hours and then subjected to enzymatic analysis. High mM concentration of P-7 inhibited enzymatic activity by 59%, Fig. 4.11. This showed that the binder molecule 4C37L34-P binds to the binding pocket of pentoxifylline as designed. Since it is very unlikely that chitinases are present in mM concentrations we also conclude that the affinity of 4C37L34-P is very much higher than that of pentoxifylline although an exact comparison has to await further analysis. Presumably the affinity enhancement is on the order of 2-3 orders of magnitude although it could be higher.

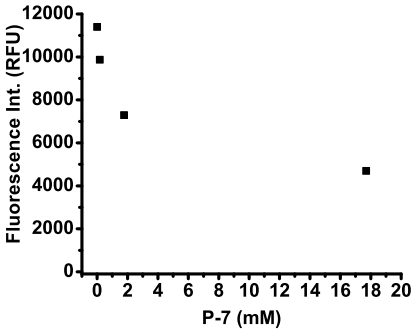

Figure 4.11 Competition between P-7 and 4C37L34-P for chitinases in lysates from *An+Nc*.

4.7 Specificity

To further investigate the specificity and sensitivity of the binder 4C37L34-P, lysates and mixtures of chitinases from *Fusarium sporotrichoides* and *Neurospora crassa* (*Fs+Nc*), *Clonostachys rosea* and *Neurospora crassa* (*Cr+Nc)*, *T. viride* and *S. griseus* were used for pull-down experiments. The results indicated that the binder can extract chitinolytic enzymes from *Fs+Cr* and *Cr+Nc* and family 19 chitinolytic enzymes from *S. griseus*, Fig. 4.12, left. The obtained extracts were subjected to SDS-PAGE, Fig. 4.12, right. Compared to the blank control samples, three sharp bands were observed in lane 4, 5 and 6. The reason is not clear. It was probably due to the fact that the small molecule pentoxifylline did not bind specifically to a certain chitinase but to several chitinases. This may be an advantage if combined with mass spectrometric analysis of chitinases from biological samples.

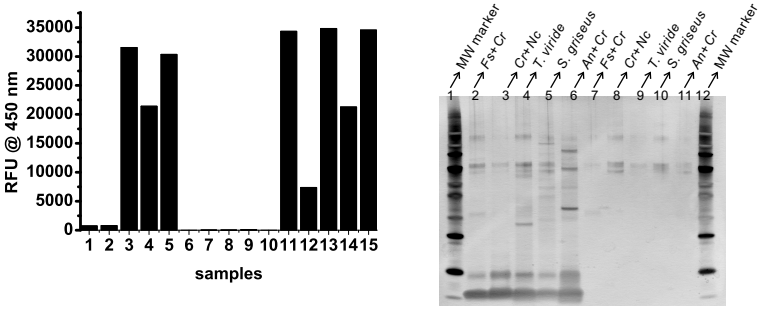

Figure 4.12 Enzymatic activity after extraction by 4C37L34-P (left) and SDS-PAGE of extracts (right). Left panel, 1. *Fs+Cr*; 2. *Cr+Nc*; 3. *T. viride*; 4. *S. griseus*; 5. *An+Nc*; 6-10. Blanks; 11. control (*Fs+Cr*); 12. control (*Cr+Nc*); 13. control (*T. viride*); 14. control (*S. griseus*); 15. control (*An+Nc*). Right panel, SDS-PAGE of extracts.

4.8 Applications

The developed methodology was used in a systematic search for binders for a chitinase mixture from *T. viride*. Eight polypeptide-pentoxifylline conjugates were attached to particles via disulfide bonds and screened for chitinase affinity, Fig. 4.13. As before, the conjugate 4C37L34-P was found to be an efficient binder for chitinases. However, the conjugate 4C10L17-P also gave rise to high enzymatic activity in the extract. The extracts were further analyzed by SDS-PAGE and CN-PAGE followed by in-gel enzymatic analysis, showing that at least one active chitinase was extracted.

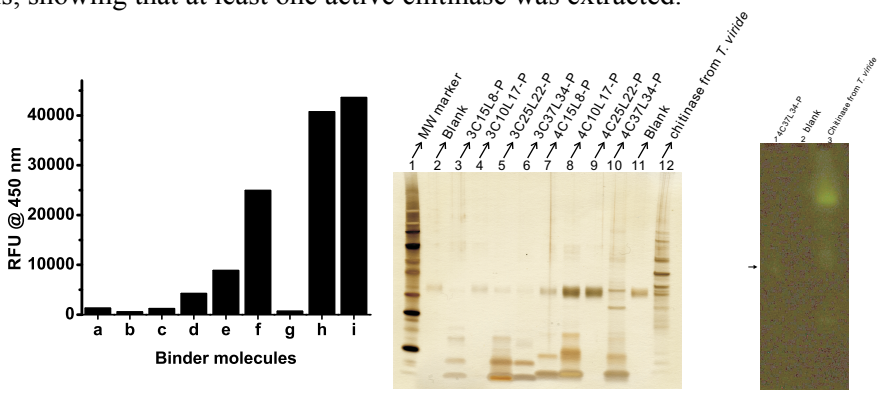

Figure 4.13 Screening of eight 3-series and 4-series pentoxifylline conjugates with chitinase from *T. viride* (left) and analysis by SDS-PAGE and CN-PAGE (right). Left panel shows enzymatic activity in extracts by the following polypeptide conjugates. a. 3C15L8-P; b. 3C10L17-P; c. 3C25L22-P; d. 3C37L34-P; e. 4C15L8-P; f. 4C10L17-P; g. 4C25L22-P; h. 4C37L34-P; i. positive control chitinase from *T. viride*; The intensities shown are those after subtraction of intensities from spontaneous hydrolysis of substrates. Right panel, SDS-PAGE of extracts (middle) and CN-PAGE followed by in-gel activity assay of extracts (right).

4.9 Summary

Polypeptide conjugates were synthesized, attached to particles and used in extraction of chitinases from cell lysates. The synthetic polypeptide conjugate 4C37L34-P was found to be a binder of some family 18 and 19 chitinases. Recognition of extracted active enzymes by 4C37L34-P was investigated. Competition experiment with pentoxifylline equipped with a spacer showed that 4C37L34-P was bound to the binding pocket of pentoxifylline and the affinity was estimated to be 2-3 orders of magnitude higher than that of pentoxifylline.

5. Conclusion and Outlook

In paper I, GPRP-linked polypeptides were investigated by SPR and it was found that they tightly bound to D-dimer protein with low nM affinity, 3-4 orders of magnitude higher than GPRP. Dimeric polypeptide conjugates were found to bind with even higher (2-5 fold) affinity. Competition experiments showed that the GPRP part of the conjugates bound to the binding cleft of the D-dimer protein and was critical for binding. The affinity of the selected binder 4C15L8GPRP was further confirmed by fluorescence titration in solution, and the data obtained were in agreement with the data from the SPR analysis.

In paper II, active ester derivatives of SHA with 9 and 11 carbon aliphatic spacers were designed, synthesized and used to prepare more than 20 polypeptide conjugates. The interactions between immobilized MPO and the polypeptide conjugates were investigated by SPR. Competition experiment indicated that the SHA bound to the heme distal cavity of MPO and was important for binding. The affinity of the polypeptide 4C37L34C11SHA was estimated to be 0.4 nM, about four orders of magnitude higher than that of SHA alone.

In paper III, active ester derivatives of pentoxifylline, C4B3 and C5B1 with aliphatic spacers were designed, synthesized and used for preparing polypeptide conjugates. These were used in pull-down experiments for the screening of chitinases in a complex biological media and in the study of specificity and sensitivity of the binders. The polypeptide conjugate 4C37L34-P was found to be a tight binder of chitinases, with 2-3 orders of magnitude higher affinity than that of the constituents alone. In competition assays, the small molecule P-7 was found to be an inhibitor of binding between the polypeptide conjugate and chitinases. This work paves the way for another generation of protein binders, perhaps useful in drug discovery and other applications.

In the future, high affinity synthetic binders could be used in *in vivo* or *in vitro* clinical diagnostics as well as in therapy. Easily available large peptide libraries, small peptide derivatives or sugar derivatives could be tried as polypeptide conjugation molecules in the future search for new specific binders of proteins or other biomolecules.

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7. Summary in Swedish

Molekylär igenkänning (bindning) spelar en viktig roll i de olika processer som pågår innanför och utanför levande celler. Utveckling av syntetiska bindarmolekyler som alternativ till naturens bindarmolekyler (t.ex. antikroppar) är ett hett forskningsområde inom kemisk biologi. Syntetiska molekyler är relativt enkla och billiga att producera jämfört med antikroppar, och kan trots en lägre molekylvikt uppvisa en hög bindningstyrka och selektivitet. De kan användas som bioanalytiska verktyg inom klinisk diagnostik *in vivo* eller *in vitro*, och kanske också i framtiden som läkemedel.

I detta arbete presenteras en ny typ av syntetiska molekyler (konjugat) som kan binda till proteiner. Dessa har framställts genom att sammanbinda (konjugera) en polypeptid med en liten molekyl som har svag eller måttlig förmåga att binda till ett målprotein. Sammanbindningen åstadkoms genom att utnyttja en reaktion mellan en lysin-sidokedja i polypeptiden och ett reaktiv esterderivat av den lilla molekylen. Genom denna sammanbindning kan man, med rätt vald polypeptid, mycket dramatiskt öka förmågan att binda till målproteinet.

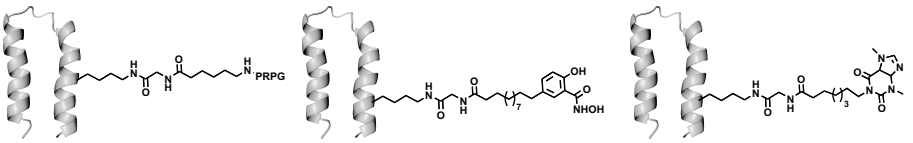
Valet av den lilla molekylen är också en viktig del i processen. Det kan röra sig om en peptid, ett kolhydrat, en nukleotid, eller en annan typ av organisk molekyl som man vet fungerar som enzyminhibitor, enzymsubstrat eller -substratanalog, men det kan också vara en molekyl som av helt andra orsaker är känd för att binda med svag eller måttlig styrka till målproteinet (K_d = milli- till mikromolar). Man kan också försöka selektera en förut okänd målprotein-bindande molekyl ur kommersiella kemiska bibliotek eller med hjälp av tekniker som fag-display.

I detta arbete beskrivs utvecklingen av effektiva bindarmolekyler till tre olika målproteiner (se även figuren nedan). Bindarmolekylerna syntetiserades och deras bindningsförmåga utvärderades med hjälp av biosensorer baserade på ytplasmonresonans eller latexkulor, eller med fluoresensspektroskopi. De tre målproteinerna var:

1. D-dimer protein, en biomarkör för blodpropp (tromboemboli), som man vet binder till den lilla peptiden Gly-Pro-Arg-Pro med måttlig styrka. Flera konjugat mellan denna tetrapeptid och olika polypeptider syntetiserades, och ett av dem (4C15L8GPRP) uppvisade en 3-4 tiopotenser högre bindningstyrka till D-dimerproteinet än enbart tetrapeptiden. Bindningstyrkan uppskattades till 3 nmolar med hjälp av ytplasmonresonans och fluoresensspektroskopi.

2. Myeloperoxidase (MPO), en biomarkör för hjärt-kärlsjukdom, som man vet binder till salicylhydroxamsyra (SHA) med måttlig styrka. Reaktiva esterderivat av SHA syntetiserades, och konjugerades till flera olika polypeptider. Ytplasmonresonans-mätningar visade, att konjugat med nettopositiv laddning band starkare till MPO än konjugat med nettonegativ eller ingen laddning. För ett av konjugaten (4C37L34C11SHA) uppskattades bindningstyrkan till 400 pmolar, vilket är 4 tiopotenser bättre än för enbart SHA. Denna typ av bindare till MPO borde i framtiden kunna användas i tidig diagnostik av hjärtsjukdomar, och då med fördel i kombination med bindare som vi tidigare utvecklat till CRP, en annan biomarkör för sjukdomen.

3. Chitinaser, en familj av likartade enzymer som spjälkar polysackariden kitin men som också binder svagt till xantinderivat, t.ex. koffein och teobromin. Konjugat mellan polypeptider och ett xantinderivat undersöktes, och ett av konjugaten, 4C37L34-P, befanns binda till olika typer av kintinaser med två-tre tiopotenser högre styrka än enbart xantin. Dessa typer av konjugat kan finna användning för att påvisa närvaro av kitinasenzym i biologiska media eller mikroorganismer.



4C15L8GPRP 4C37L34C11SHA 4C37L34CT1-7

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