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Chemical synthesis and evaluation of a backbone-cyclized minimized 2-helix Z-domain

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The Z-molecule is a small, engineered IgG-binding affinity protein derived from the immunoglobulin-binding domain B of *Staphylococcus aureus* protein A. The Z-domain consists of 58 amino acids forming a well-defined antiparallel three-helix structure. Two of the three helices are involved in ligand binding, whereas the third helix provides structural support to the three-helix bundle. The small size and the stable three-helix structure are two attractive properties comprised in the Z-domain, but a further reduction in size of the protein is valuable for several reasons. Reduction in size facilitates synthetic production of any protein-based molecule, which is beneficial from an economical viewpoint. In addition, a smaller protein is easier to manipulate through chemical modifications. By omitting the third stabilizing helix from the Z-domain and joining the *N*- and *C*-termini by a native peptide bond, the affinity protein obtains the advantageous properties of a smaller scaffold and in addition becomes resistant to exoproteases. We here demonstrate the synthesis and evaluation of a novel cyclic two-helix Z-domain. The molecule has retained affinity for its target protein, is resistant to heat treatment, and lacks both *N*- and *C*-termini.

Keywords: protein scaffold; Z-domain; two-helix protein; SPPS; IgG; affinity purification

Introduction

Owing to its high affinity toward immunoglobulins (Ig), staphylococcal protein A (SpA) is one of the most extensively studied proteins for chromatographic purification and detection of IgG. The *N*-terminal part of SpA contains five homologous domains (E, D, A, B, and C), which display affinity toward immunoglobulins [1]. The Z-domain is an engineered version of the B-domain [2], which is composed of 58 amino acids forming a three-helix bundle protein devoid of cysteine residues (Figure 1). The Z-domain has been shown to possess several advantageous properties such as high affinity binding to the Fc part of IgG and high stability toward pH extremes and denaturing agents [1]. The protein has also been subject to further protein engineering efforts, for example to make an alkali-stabilized version of the Z-domain suitable for affinity chromatography, where high resistance to repeated NaOH treatment for cleaning of the column is required [3]. In another study, a minimized version of the Z-domain composed of only two helices was generated, to reduce the size of the protein and identify the minimal requirements for immunoglobulin binding [4]. It can be seen from the crystal structure of the B-domain in complex with a human Fc fragment [5] that 11 residues on helices 1 and 2 are responsible for the interaction with Fc, whereas the third helix (helix 3) provides stability to the scaffold. In the minimization of the Z-domain, the third helix was omitted, creating an even smaller three-helix Z-domain structure with comparable affinity to human IgG [4]. The smaller size does not only simplify production through synthetic chemistry but it also facilitates additional chemical engineering of the affinity molecule.

In this study, a truncated Z-variant, denoted Z_{min}, has been synthesized and evaluated for affinity capture of human IgG (hIgG) on solid phase. The Z_{min} sequence is based on the previously reported minimized two-helix Z-domain, $Z^{34}C$ [6], with a few introduced modifications (Table 1). The aim was to produce an affinity molecule with decreased size, facilitated synthetic generation, and increased stability compared to previously reported Z-domains. After synthesis, the Z_{min} peptide was backbone-cyclized through native chemical ligation (NCL) [7]. The ligation reaction generates a native peptide bond between helices one and two, which stabilizes the two-helix conformation and eliminates possible degradation by exoproteases. A cysteine residue is introduced at the point of ligation, which can be employed as a chemoselective handle for protein labeling or immobilization. This native ligation technique differs from the strategy used to produce the previously reported two-helix Z-molecules, where a disulfide bridge links the first and second helix to compensate for the loss of the third stabilizing helix (Figure 1) [4,6,8]. The Z_{min} protein was synthesized by means of SPPS [9] and the affinity protein obtained was characterised by MS, CD, and surface plasmon resonance (SPR)-based biosensor studies. Further, the minimized binder was immobilized onto solid support for proof-of-principle affinity capture of human polyclonal IgG.

Materials and Methods

Materials

For SPPS of all peptides, the following Fmoc-protected amino acids with respective side-chain protecting groups were used: *tert*-

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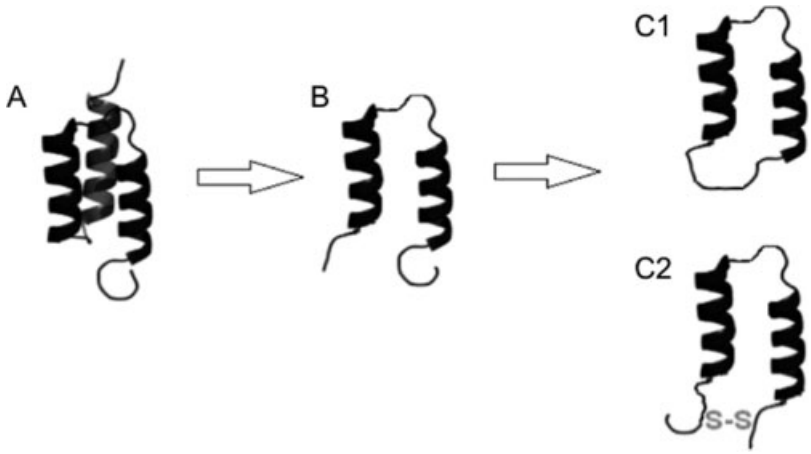

Figure 1. Strategy for the generation of two-helix Z-molecules. (A) The three-helix Z-domain with the helices numbered 1, 2, and 3. Removal of the third, stabilizing helix generates (B) a two-helix Z-domain (minimized Z-domain). The two-helix domain can be further stabilized through (C1) backbone ligation or (C2) the formation of a disulfide bridge.

butyloxycarbonyl (Boc) for Lys and Trp, *tert*-butyl (tBu) for Ser, Thr and Tyr, *tert*-butyl ester (OtBu) for Asp and Glu, trityl (Trt) for Gln, Cys, Asn and His and pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg. Other amino acids had unprotected side-chains. One Boc-protected amino acid was used, Cys with side-chain protecting group Trt.

Cys, Ala, Ser, Gly, Lys, Asn, Trp, His, Leu, Arg, and Thr together with Boc-protected Cys were from Novabiochem, Hohebrunn, Germany. Ile, Val, Pro, Phe, and Met were from Applied Biosystems, Warrington, UK. Asp, and Tyr were from Perseptive Biosystems, Warrington, UK and Glu and Gln were from Iris Biotech GmbH, Germany. Fmoc-amide resin (substitution: 0.66 mmol/g) was from Applied Biosystems. Activating reagents used: 1-hydroxybenzotriazole monohydrate (HOBt $\times$ H_2O) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) from Iris Biotech GmbH, Germany. Solvents used: *N*-methylpyrrolidone (NMP), DCM, *N*,*N*-DMF and DIEA from Applied Biosystems. Additional chemicals used: TFA from Apollo Scientific Ltd, Breadbury, UK, 4-nitrophenylchloroformate from Fluka, 1,2-ethanedithiol (EDT) from Aldrich Chemical Company, Milwaukee, USA, 4-mercaptophenylacetic acid (MPAA) and tris(2-carboxyethyl)phosphine hydrochloride solution (TCEP) from Sigma Aldrich. Triisopropylsilane (TIS), pyridine, guanidinium chloride, *tert*-butyl methyl ether, 2-propanol, and acetonitrile (AcN) were all from Merck Schuchardt OHG and acetic anhydride (Ac_2O) was from Alfa Aesar GmbH & Co, KG, Germany.

General Procedure for Peptide Synthesis

Peptides were assembled in a step-wise manner using a 433A Peptide Synthesizer (Applied Biosystems) with the SPPS Fmoc/tBu strategy on amide resin. The syntheses were carried out in 0.1 mmol scale and all the amino acids were coupled in tenfold excess. The protocol used was based on a standard protocol from the SynthAssist® 2.0 software (chemistry: FastMoc 0.1 Ω MonPrevPeak, Applied Biosystems). Real-time monitoring of the deprotection reaction was performed by measurement of conductivity. HOBt/HBTU (10 equiv.) in DMF was used to activate the amino acid α-carboxyl group before coupling, in the presence of DIEA (15 equiv.) in NMP. To avoid the formation of deletion peptides during synthesis, capping with Ac_2O of remaining free amines after the acylation step was performed.

Synthesis of Linear $Z^{34}C$ and Z_{min}

Assembly of $Z^{34}C$ was carried out on amide resin, according to the standard procedure described above. The synthesis of Z_{min} was carried out on amide resin functionalized with a diaminobenzoic acid (Dbz) linker. To attach the Dbz linker to the resin, the resin was swelled in DCM and the Fmoc group deprotected using 20% piperidine, followed by manual coupling of di-Fmoc-3,4-diaminobenzoic acid (5 equiv.) using HBTU/HOBt (5 equiv.) in DMF and DIEA (7.5 equiv.) in NMP. Coupling was performed in DCM/DMF (50/50). To achieve satisfactory coverage of Dbz on the resin, a second round of coupling was performed as described above. The *C*-terminal glycine residue of the peptide was coupled to the resin-bound Dbz manually in the same manner as coupling of Dbz. A ninhydrin test was performed to confirm the complete coupling of the amino acid. The remaining sequence, with Boc-protected cysteine as the *N*-terminal amino acid, was synthesized on an automated peptide synthesizer in accordance with the general synthesis procedure described above. However, Ac_2O capping of the uncoupled free amines was not performed, to avoid acetylation of the Dbz linker. After completed synthesis, Dbz was rearranged to *N*-acyl-benzimidazolinone (Nbz) in order to form a *C*-terminal ester. Briefly, 4-nitrophenylchloroformate (5 equiv.) in DCM was added (40 min) to the peptide-resin for specific acylation, followed by deprotonation and cyclization of the linker with 0.5 M DIEA in DCM [10].

Cleavage from the solid support was performed using the same protocol for both synthesized peptides. The peptide-resin was treated for 2 h with a cleavage solution containing TFA/EDT/H_2O/TIS (94 : 2.5 : 2.5 : 1) at room temperature. Three rounds of extraction of the peptides were carried out using *tert*-butyl methyl ether and H_2O (2 : 1) succeeded by filtration and lyophilization of the aqueous phase.

Purification and Analysis of Linear $Z^{34}C$ and Z_{min}

All linear peptides were purified and analyzed by RP-HPLC (Agilent Technologies). $Z^{34}C$ was purified using an elution gradient of 10–40% B (A: 0.1% TFA-H_2O, B: 0.1% TFA-AcN) for 20 min at a flow rate of 2.5 ml/min using a 10 mm $\times$ 250 mm C18 column with a particle size of 5 μm (semi-preparative, Reprosil Gold 300, Dalco Chromtech AB). Z_{min} was purified using an elution gradient

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of 20–55% B for 20 min using the same column as for $Z^{34}C$. The fractions collected were frozen and lyophilized. The identities of the peptides were confirmed by MALDI-TOF MS (biflex IV, Bruker Daltonics) or ESI-MS (6520 Accurate Mass Q-TOF LC/MS, Agilent Technologies), the latter coupled to RP-HPLC using a 300 µm × 5 mm C4 column, particle size 5 µm (Acclaim PepMap300, LC Packings – Genetech). The concentration of the peptides was determined by absorbance measurements using Eppendorf BioPhotometer (Eppendorf) and the concentration was calculated using Beer-Lambert's law (extinction coefficient = 1490 M^{-1} cm^{-1}). To confirm the concentration and assess the purity of the samples, SDS-PAGE analysis was performed (NuPAGE 4–12% Bis-Tris Gel, Invitrogen). Recombinantly produced Z_{wt} and Z_{IgA} (used as positive and negative control, respectively), both containing a *C*-terminal cysteine residue, were a kind gift from Affibody AB.

Cyclization and Purification of $Z^{34}C$ and Z_{min}

$Z^{34}C$ was cyclized by overnight incubation of the peptide in 5 mM NH_4Ac buffer, pH 9.2, to allow the two cysteine residues in the peptide sequence to form a disulfide bridge. To confirm the cyclization, the peptide was analyzed using ESI-Q-TOF-MS (data not shown) after which the sample was frozen and lyophilized. Cyclization of Z_{min} was carried out using a ligation buffer consisting of $Na_2H_4PO_4$/MPAA/TCEP (0.2 M : 0.2 M : 0.02 M) pH 7–7.5. The peptide was treated with ligation buffer for 2 h. Purification of the cyclized product was performed by two rounds of semi-preparative RP-HPLC using a gradient of 20–35% B for 2 min, followed by 35–55% B, at a flow rate of 2.5 ml/min with the same column and solvent system as described above for the linear peptides. After the first round of RP-HPLC, the peptide was dissolved in guanidinium chloride (6 M) and then purified by a second round of RP-HPLC. Alternatively, the peptide was purified by dialysis following cyclization. The dialysis was carried out using Slide-A-Lyzer® Dialysis Cassette (Fisher Scientific), 2.5 kDa, 2 × O.N. with 10 mM NaAc, pH 4.5, as dialysis buffer.

CD Analysis

CD spectra were recorded over a heat gradient (20–90 °C) on a Jasco J-810 spectropolarimeter (Jasco Scandinavia AB). The wavelength was measured in the range 190–250 nm in 0.1-nm intervals and the spectra were collected using a 0.1-cm quartz cell. The spectra were collected at 20 °C with a peptide concentration of 0.3 mg/ml. Each of the final CD spectra represents an average of three assembled scans.

Surface Plasmon Resonance Analysis of the Binding Kinetics

Surface plasmon resonance (SPR) analysis of the binding to human IgG was performed on a Biacore 2000 instrument (GE Healthcare). Dextran-coated CM5 sensor chips were activated by EDC/NHS, followed by modification of the succinimidyl esters with 2-(2-pyridinyldithio) (PDEA) ethaneamine Thiol Coupling Reagent (GE Healthcare). Z_{min} and the control proteins Z_{wt} and Z_{IgA} were diluted to a concentration of 10 µg/ml in 10 mM NaAc buffer, pH 4.5, and coupled to the sensor chip by thiol-disulfide exchange. The protein immobilization resulted in a response of approximately 50 response units (RU) for Z_{min}, 80 RU for Z_{wt}, and 70 RU for Z_{IgA}. The excess reactive groups were deactivated with cysteine/NaCl. Owing to the absence of a free thiol group in $Z^{34}C$, the domain could not be immobilized through thiol-disulfide exchange and

Table 1. The sequences of Z_{wt}, $Z^{34}C$, and Z_{min}

Domain	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
Z_{wt}	V	D	N	K	F	N	K	E	Q	Q	N	A	F	Y	E	I	L	H	L	P	N	L	N	E	E	Q	R	N	A	F	I	Q	S	L	K	D	P	S	Q	S	A	N	L	L	A	E	A	K	K	L	N	D	A	Q	A	P	K
$Z^{34}C$						-	-	M	Q	C	-	R	R	-	-	A	-	-	D	-	-	-	-	T	-	-	-	-	-	K	-	K	-	I	R	-	-	C																					
Z_{min}			C	G	-	S	-	-	-	-	R	R	-	-	-	A	-	-	D	-	-	-	-	T	-	-	-	-	-	K	-	K	-	I	R	-	E	G	te																				

Underlined amino acids represent helices 1, 2 and 3. In $Z^{34}C$, a disulfide bond can be created between C9 och C38. Z_{min} has a *C*-terminal thioester (te) for subsequent cyclization. The N6S and N23T substitutions in Z_{min} were introduced to facilitate chemical synthesis.

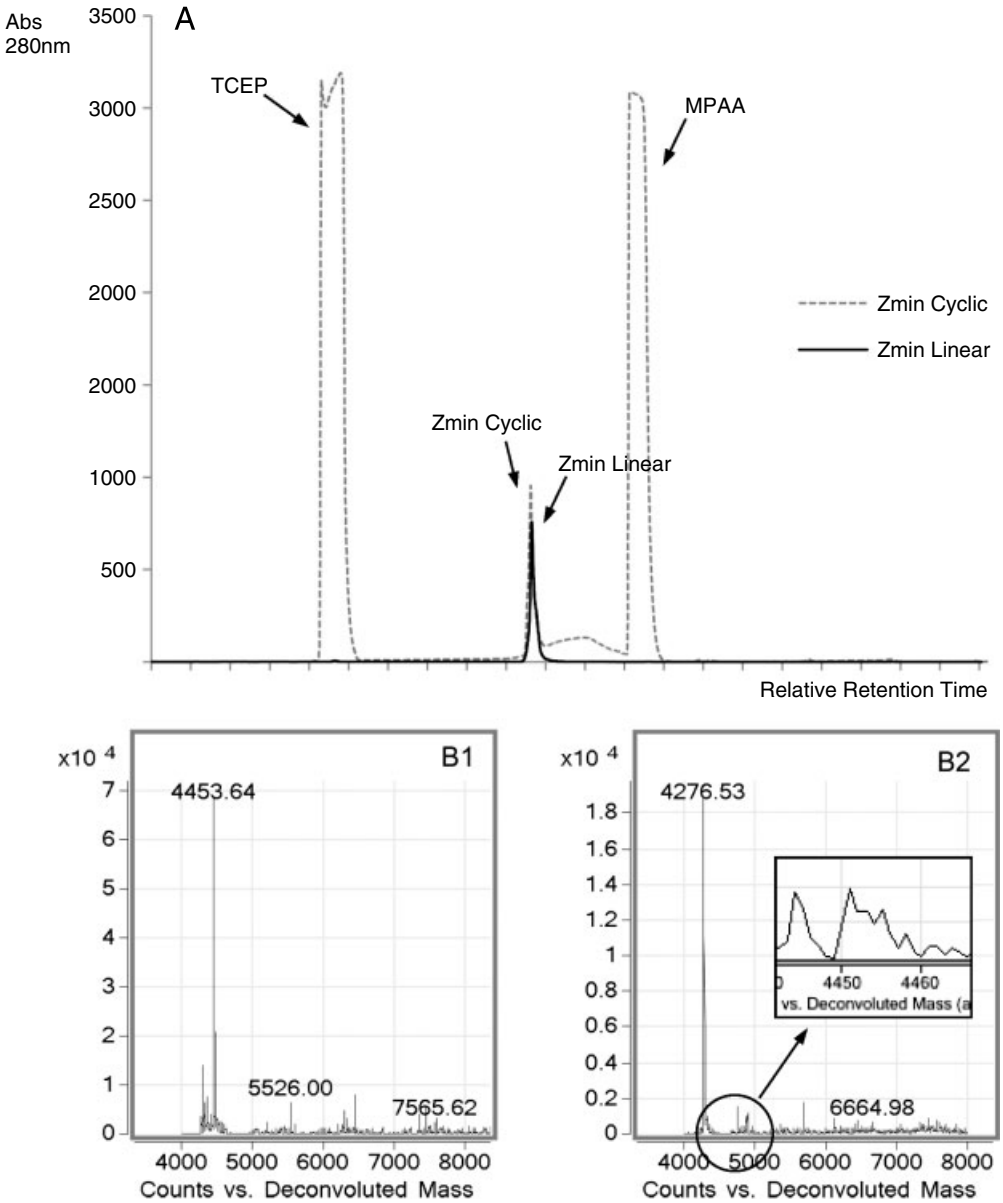

Figure 2. Cyclization, purification, and characterization of Z_{min}. After cyclization, Z_{min} elutes separately from the reducing agent TCEP and the catalyst MPAA. The cyclic peptide has approximately the same retention time as its linear precursor, when purified by RP-HPLC (A). No trace of the linear product (B1) can be detected after the cyclization step, hence all the linear peptide is assumed to convert to the cyclic Z_{min} (B2).

was therefore not included for comparison in the SPR studies. Analysis of the interactions was carried out at 25 °C with a flow rate of 25 µl/min in HEPES, EDTA, NaCl, Surfactant P20 buffer (HBS-EP) (100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1500 mM NaCl, 34 mM EDTA, 0.05% Tween 20, pH 7.4) and a 30 µl injection of sample at concentrations ranging from 2 to 15 nM. Regeneration of the surfaces between each sample injection was performed with a 20 µl injection of 15 mM HCl. All calculations were performed in the Biaeval software (GE Healthcare) and the K_D constant was determined with a χ^2 value below 1.

Affinity Capture of hIgG

To verify that the minimized Z-domain remains functional in an affinity application setup, Z_{min} was immobilized onto an iodoacetyl-activated agarose gel (Sulfo Link® Pierce) and used for affinity capture of hIgG. The gel bead slurry was equilibrated with Tris, NaCl, Tween-20 buffer (TST) (25 mM Tris, 1 mM EDTA, 200 mM NaCl 0.05% Tween 20, pH 8). Z_{min} was dissolved in TST buffer and gently mixed with the gel beads for 15 min. The gel bed was then allowed to settle for 30 min, followed by draining of the gel bed. The column was extensivley washed with TST buffer and the unreacted binding sites were blocked with 50 mM L-cysteine in TST buffer. The gel bed was finally washed with 5 mM NH_4Ac, pH 5.5 and stored in Phosphate buffered saline (PBS) with 0.05% NaN_3. To evaluate the affinity capture, hIgG was allowed to bind to gel medium for 1 h and the slurry was extensively washed with TST buffer and 5 mM NH_4Ac, pH 5.5. Elution was performed with 0.2 M HAc, pH 3.3, and fractions were collected, lyophilized, and analyzed by SDS-PAGE (Figure 4).

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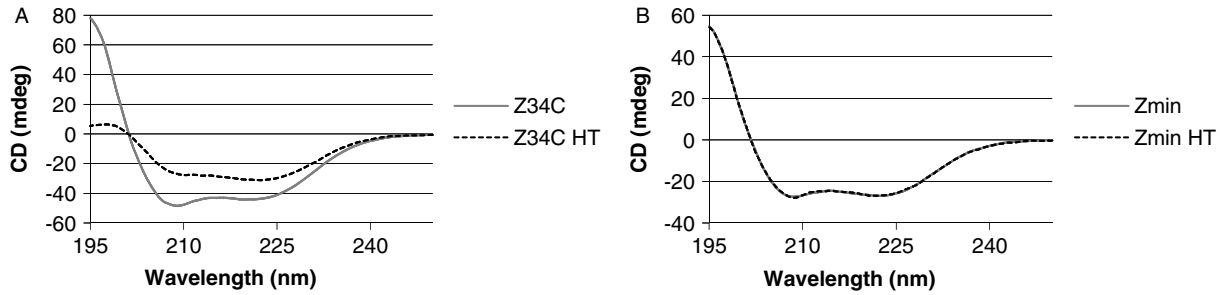

Figure 3. Circular dichroism spectra of $Z^{34}C$ and Z_{min} before and after 90 °C heat treatment (HT). A) $Z^{34}C$ before and after HT B) Z_{min} before and after HT. All spectra represent an average of three assembled scans.

Results and Discussion

Peptide Design and Synthesis

The presented two-helix Z_{min} domain is based on the previously reported sequence of $Z^{34}C$ [6]. $Z^{34}C$ was generated using structure-based design and phage display methods (Table 1). In $Z^{34}C$, two cysteine residues were incorporated at positions 9 and 38 of the sequence (all positions correspond to the original Z-domain, Table 1), resulting in a minimized two-helix structure stabilized by a disulfide bridge (Figure 1). In the Z_{min} sequence, a *C*-terminal thioester was instead introduced for backbone cyclization of the peptide. To facilitate ligation between the *N*- and *C*-terminal of the peptide, a glycine residue was introduced on each side of the conjunction site to increase flexibility at the linkage site. In an earlier synthesis of the Z-domain, bulky, trityl-protected asparagine residues were found difficult to couple [11] and to avoid the formation of deletion peptides in the synthesis, Asn6 and Asn23 were substituted with serine and threonine, respectively (Table 1). Substitution of these residues has earlier been shown tolerated by a Z-based scaffold [12]. These replacements also allow for the use of pseudoprolines as a way to further increase synthesis yield, although this strategy was not used in this study. As the linear Z_{min} precursor peptide is synthesized on a Dbz linker (which, to our experience, is not fully compatible with the use of Ac_2O capping as a way to reduce deletion peptide formation), it was important to consider strategies to improve the coupling yield.

After cyclization of the Z_{min} peptide by NCL, no linear precursor could be detected by ESI-MS analysis of the product and the reaction was thus considered quantitative (Figure 2). The experimentally determined molecular weights (MW) were 4276.5 Da for Z_{min} (theoretical MW: 4276.7 Da) and 4180.5 Da for $Z^{34}C$ (theoretical MW = 4180.7 Da).

CD Analysis

CD spectroscopy was used to analyze the helical content of Z_{min}, $Z^{34}C$, and Z_{wt}. All spectra showed the characteristics of α-helical structure with minima at 208 and 220 nm and a maximum between 194 and 196 nm. The molar ellipticity of Z_{min} was lower than for Z_{wt} [11] (data not shown), however this was expected since the minimized domain has only two helices as opposed to the original 3-helix Z-domain.

When exposed to heat treatment (HT) (20–90 °C gradient), both $Z^{34}C$ and Z_{min} gradually lost their secondary structure at approximately 55–65 °C with no sharp transition temperature (data not shown). Following heat exposure, the $Z^{34}C$ domain was unable to fully refold to its original helical structure, while Z_{min} rapidly adopted its native helical fold when cooled down to room temperature (Figure 3). These data suggest that the native peptide bond that links helices 1 and 2 in Z_{min} increases the ability of the protein to adopt a helical structure, compared to the disulfide bridge in $Z^{34}C$.

Surface Plasmon Resonance Analysis of the Binding Kinetics

Z_{min} was immobilized onto a sensor chip in order to study the interaction with hIgG in solution with hIgA used as a negative control. Z_{wt} and the engineered IgA-binding Z-variant Z_{IgA} [13], both with a *C*-terminal cysteine residue, were immobilized and utilized as positive and negative controls, respectively. Analysis of the binding kinetics shows that Z_{min} binds hIgG with comparable affinity to the positive control Z_{wt}, and has no measurable affinity for hIgA (Figure 4). The dissociation constant K_D of the interaction between Z_{min} and hIgG was determined to be 15 nM, compared to 3 nM for Z_{wt}. The determined dissociation constants are similar to those previously reported for the three-helix and two-helix Z-domains [6]. No interaction of hIgG with the negative control (Z_{IgA}) was observed.

Affinity Capture of hIgG

When covalently immobilized onto a commercially available solid phase coupling gel (Sulfo Link® Pierce), Z_{min} was able to efficiently capture hIgG (Figure 5). Covalent, site-specific immobilization was conveniently generated through the free thiol group on the cysteine residue (utilized for backbone cyclication through NCL, see Table 1). Elution of the bound protein was straightforward and a concentrated hIgG sample was easily obtained. These results show that the backbone-cyclized two-helix domain Z_{min} can be employed for affinity purification of immunoglobulins, demonstrating a potential application for the minimized protein.

Summary

This article presents a cyclic two-helix Z-molecule denoted Z_{min}. The two-helix structure is stabilized through a native peptide bond, linking the *N*- and *C*-termini of the protein. Z_{min} has an affinity toward its target IgG that is comparable to the original three-helix domain Z_{wt}. The backbone-cyclized Z_{min} protein readily refolds into its initial two-helix structure following HT and the free thiol group present at the ligation site (Table 1) can be utilized for directed immobilization onto a solid support.

The presented backbone-cyclized two-helix domain might be an alternative to the Z-domain and other SpA constructs for several applications, including affinity purification of immunoglobulins.

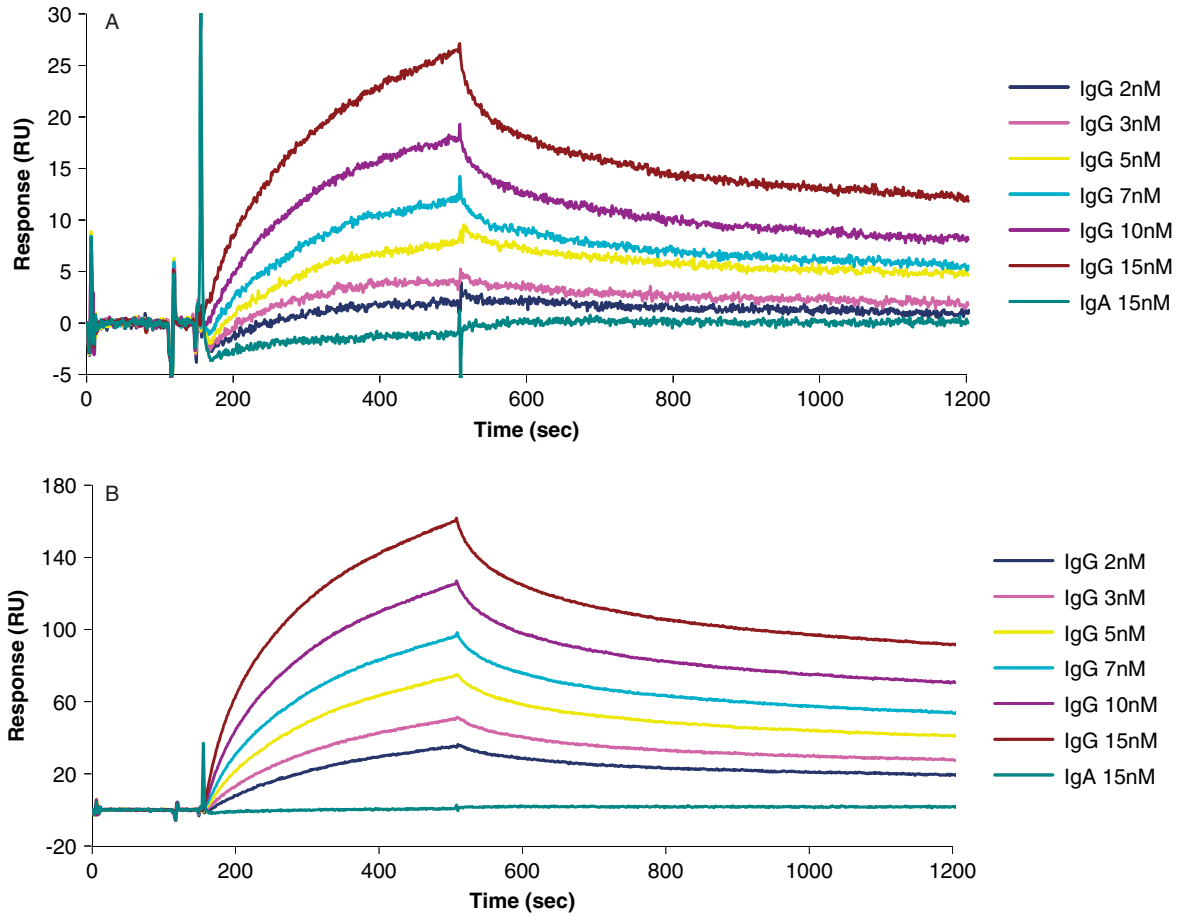

Figure 4. Surface plasmon resonance analysis of the interaction between hIgG and immobilized Z_{min} and Z_{wt}. Concentrations of hIgG: 2–15nM. Concentration of the negative control hIgA: 15nM. (A) Interaction between hIgG and immobilized Z_{min}, K_D = 15nM (B) Interaction between hIgG and immobilized Z_{wt}, K_D = 3nM.

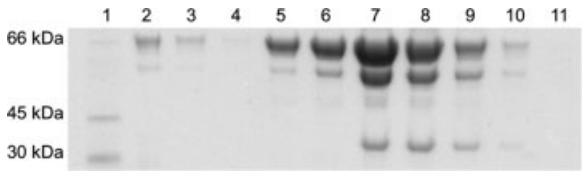

Figure 5. Purification of hIgG on solid phase by Z_{min} analyzed by SDS-PAGE. (1) Protein ladder, (2) hIgG reference, (3–4) wash steps, and (5–11) elution fraction.

Further, it has earlier been shown that 13 amino acids in the Z-domain can be randomized to produce large libraries, from which affinity molecules of different specificity can be selected [14]. These Z-based affinity proteins, denoted affibody molecules, have shown great promise for several affinity-based applications both *in vitro* and *in vivo* [15]. In line with the results here shown for Z_{min}, the third helix may be omitted from the affibody scaffold, creating a two-helix affibody molecule [16]. Such two-helix affibody scaffolds have been used as affinity reagents for *in vivo* imaging with promising results, and the authors speculate that the smaller two-helix (affibody) molecule has higher tissue penetration and a lower immunogenic potential than its three-helix counterpart [8]. By implementing the backbone cyclization strategy of the presented Z_{min} domain on the affibody scaffold, the increased stability and the lack of *N*- and *C*-termini, resulting in resistance to exoproteases, might provide the minimized molecule with certain advantages for possible future applications such as *in vivo* targeting and imaging.

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