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Beneficial properties of single-domain antibody fragments for application in immunoaffinity purification and immuno-perfusion chromatography

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Abstract

We explored the possibility to apply single-domain antibodies from *Camelidae* for immunoaffinity purification of the ice structuring protein (ISP) from *Lolium perenne*, which modifies ice crystal growth and therefore has potential application in medicine, biotechnology, agriculture and (frozen) foods. Using phage display together with an appropriate selection method, a group of candidate fragments was isolated from a llama-derived immune library. Affinity chromatography using a purposely selected antibody coupled to a matrix yielded a completely pure and functional ISP. Due to the extreme refolding capabilities and physical stability of single-domain antibodies, the affinity matrix could be regenerated more than 2000 times without loss of capacity, while the fragment's monomeric nature permitted an efficient elution of antigen. The results of this study show that highly pure proteins can be recovered from biological material in a single-step process.

Keywords: Single-domain antibody fragment; Phage display; Immunoaffinity chromatography; Ice structuring protein; Protein purification

1. Introduction

Affinity chromatography is a powerful technique for the analysis and purification of (bio)molecules. The method relies on the specific high-affinity interaction between a ligand, i.e. the molecule immobilized on a solid support, and a ligate, i.e. the molecule present in a mobile phase. The ligate is captured by the ligand from the solution containing impurities and subsequently the molecule of interest is released from the complex under conditions affecting the interaction. This single-step procedure enables a rapid and efficient purification, whereas traditional methods utilize a series of successive chromatographic separations to achieve the same degree of purity.

This study describes the use of immunoaffinity chromatography for the purification and quantification of an ice structuring protein (ISP). ISPs, also known as antifreeze proteins [1], exhibit a diversity of functions including depressing freezing temperatures (thermal hysteresis), suppressing or modifying ice crystal growth and inhibition of ice recrystallization. This class of proteins naturally occurs in a variety of species including fish, insects and plants [2,3]. The target molecule of this study has been discovered in the *over-wintering* perennial ryegrass *Lolium perenne* [4]. Grass ISP has less effect on depressing freezing temperatures than other known ISPs, but it is a better inhibitor for ice recrystallization [5]. The opportunities for applications of ISPs based on their ability to inhibit ice recrystallization include improvement of storage of medical materials such as blood and organs [6], but many applications could be thought of in the field of the frozen food industry [2,3].

We examined the use of single-domain antibody fragments for affinity purification. Animals belonging to the species of *Camelidae* contain a high fraction of heavy-chain

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antibodies in their blood, which are not associated with light chains [7]. The variable heavy-chain regions of the heavy-chain antibodies (indicated by VHH), the so-called single-domain antibody fragments, represent the smallest antigen binding domains derived from antibodies [8], with good binding characteristics and unusual high physical and thermal stability due to their single-domain nature [9]. The fragments are expressed efficiently in microorganisms such as the yeast *Saccharomyces cerevisiae* [10], thus offering the potential for cost-effective application of single-domain antibody fragments in industrial purification processes.

2. Material and methods

2.1. Library construction and selection of ISP-specific VHH

A llama was immunized [9] at days 0, 28 and 76 with 1 mg of ISP of *L. perenne*-produced in the yeast *Pichia pastoris* using the secretion vector pPIC9. With RNA extracted from peripheral blood lymphocytes, a phage display library was generated as was described before [10].

The phage library was selected via the biopanning method [11] by coating of *P. pastoris* produced ISP (10 μg/ml at round one and 5 and 1 μg/ml for the second round) or via the "in solution" selection method [12] with biotinylated ISP derived from *S. cerevisiae* (marker-free cloned by integration in the ribosomal locus of the genome of strain VWk 18 gal1 [13]). For this, the protein extract from 1 ml of fermentation broth of the ISP secreting clone was prepared by spin dialysis with a Microcon YM3 filter (Millipore, Billerica, US) and biotinylated with NHS-LC-biotin (Pierce Biotechnology, Inc., Rockford, US).

Individual clones were screened by testing culture supernatants in ELISA [11] on streptavidin-captured biotinylated ISP using the anti-myc monoclonal antibody 9E10 for detection of bound VHH [10]. After fingerprint analysis with *Hin*fI (New England Biolabs) a number of different ISP positive clones were identified for sequence analysis (Baseclear BV, Leiden, The Netherlands) and further characterizations with purified VHH, such as Western blot using *E. coli*-produced ISP (cloned in pET32).

2.2. Affinity purification with VHH coupled matrices

The VHH encoding gene was recloned in the *E. coli* production vector pUR5850. This vector is derived from pHEN1 [14], but lacks the gene 3 and contains a hexahistidine tail for Immobilised Metal Ion Affinity Chromatography [15] and an additional carboxy terminal tag sequence of 15 amino acids, which encodes a C-terminal tag leading to in vivo biotinylation [16]. The antibody fragment was isolated from inclusion bodies and after refolding [17,18] purified via its C-terminal hexahistidine tag on TALON column material according to the supplier's instructions (Clontech, PT1320-1 (PRO3469)).

For a pilot purification experiment, 1.6 mg VHH was immobilized on 1.0 ml TALON. The column material was incubated with 2 ml fermentation broth containing ISP produced with mating factor α signal sequence by *S. cerevisiae*. Following washing with sonication buffer (50 mM Na_2HPO_4, 10 mM Tris, 100 mM NaCl, pH 8.0), the bound antigen was eluted with 6 M guanidinium HCl (Pierce Biotechnology) in sonication buffer. The eluted fraction was dialyzed against water and analyzed on a Western blot using 200-fold diluted llama (pre- and) post-serum; bound Ig was detected with 2000-fold diluted rabbit anti-llama Ig and 4000-fold diluted anti-rabbit Ig alkaline phosphatase conjugate (Promega Corporation). The dialyzed material was used also for amino-terminal sequencing on a Porton LF3000 sequencer combined with a Beckman HPLC 125S and detector type 168 (Beckman Coulter, Fullerton, US).

To prepare a chromatography support with covalently coupled antibody, 12 mg of VHH in 21 ml buffer (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3) was immobilized to 2 g of CNBr-activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's protocol. The column material (final volume 7 ml) was packed in a XK 16/20 column (Pharmacia). After treatment with blocking buffer (0.1 M Tris, 0.5 M NaCl, pH 8.0) the affinity support was washed in an alternating fashion with phosphate buffered saline (PBS: 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4) as equilibration buffer and pH-adjusted PBS (pH 2.1) as elution buffer.

Grass ISP was purified from culture supernatant obtained from a 10-l fermentation of the *S. cerevisiae* clone (BAC BV, Naarden, The Netherlands). A sample of 4 ml was loaded in 8 min with a flow rate of 0.5 ml/min by introduction of equilibration buffer (PBS) in the sample loop. Following washing with equilibration buffer (PBS) at an increased flow rate (1 ml/min) during 60 min, the bound ISP was eluted with a pulse of elution buffer (PBS pH 2.1; 30 min at 1 ml/min) and immediately neutralized by the addition of 1 M $NaHCO_3$, pH 8.5.

The purification of ISP produced by *S. cerevisiae* was analyzed with SDS-PAGE followed by silver staining of the SDS-gel and eluted fractions were analyzed on a Western blot using 50,000-fold diluted llama (pre- and) post-serum; bound IgG was detected with 5000-fold diluted rabbit anti-llama Ig and 5000-fold diluted anti-rabbit horseradish peroxidase conjugate (Jackson ImmunoResearch, West Grove, USA). The ISP was also produced as a Trx-fusion protein in *E. coli* (cloned in pET32) and purified using the same column.

In order to find out which of the fractions contain most of the functional ISP, a modified recrystallization inhibition assay (SPLAT analysis) [5,19] was performed. Samples on cover slips were cooled rapidly to −40 °C and subsequently the temperature was raised to, and kept at, −6 °C. At −6 °C the ice crystal size was microscopically followed for 5 min.

The eluted material was dialyzed (Spectra/Por-3; cut-off 3.5 kDa) against water, lyophilized and used for amino-terminal sequencing and sequence analysis using the Alpha plus series 2 analyzer (Amersham Pharmacia Biotech).

2.3. Analysis with immuno-perfusion chromatography

The immunoaffinity chromatography matrix was prepared by coupling of 4.3 mg of VHH to 110 mg of POROS 20 AL according to the instructions of the supplier (Perseptive Biosystems). Matrix (100 μl) was packed in a column (diameter 2.1 mm; length 30 mm; Perseptive Biosysytems, PEEK) using an FPLC system (Pharmacia). Before analytical use, the column was washed several times with equilibration and elution buffer (see above).

The standards and samples were loaded during a 2-min period at a flow rate of 1 ml/min. The flow rate was increased to 2 ml/min for washing (2.5 min) and elution (1.5 min). After 2 min equilibration, the column was used for the injection of a new sample.

2.4. Biacore analysis

For analysis of the antigen–antibody interaction, a Biacore 3000 (Biacore AB, Uppsala, Sweden) was used in combination with a streptavidin-coated sensor chip (SA chip, Biacore). Approximately 1000 RU's of in vivo biotinylated VHH were immobilized on different flow cells and grass ISP was injected at a flow rate of 5 μl/min. For affinity measurements, approximately 100 RU's of VHH 9C and a control VHH were immobilized on different flow cells. ISP was injected in different concentrations; k_a and k_d values were calculated using BIAevaluation software.

3. Results

3.1. Selection of single-domain antibody fragments for affinity purification

For the isolation of antigen-specific antibody fragments via phage display a llama was immunized with *L. perenne* ISP produced in *P. pastoris*. *P. pastoris* produces ISP in different *N*-glycosylated forms. The obtained phage library with 2.8×10^6 clones encoding short hinge and 6.5×10^6 clones with long hinge-derived VHH was selected via the biopanning procedure using antigen-coated immunotubes [11]. After two rounds of selection, different ISP specific fragments were identified, which recognized the *E. coli*- and *P. pastoris*-produced antigen on Western blot (data not shown). Unfortunately, the soluble form of ISP was not recognized, and therefore these antibodies were not suitable for affinity purification.

Therefore, the strategy for selection of antibodies was changed: the antigen was biotinylated and allowed to interact with the phage-bound VHH in solution [12]. A high proportion (>95%) of ELISA-positive clones was found after the second round of selection (data not shown). Six clones with different *Hin*fI fingerprint patterns were sequenced (Table 1).

The most important requirements for affinity purification are first of all the efficient binding of the antigen, and secondly the effective elution of the antigen from the antibody–antigen complex. To analyze these features with surface plasmon resonance, four of the VHH were expressed with a carboxy terminal peptide extension, which leads to in vivo biotinylation by *E. coli* [16,20], and thereby permits the immobilization on a streptavidin-coated chip. Upon

Table 1
Amino acid sequences of the anti-ISP VHH

	FR1	CDR1	FR2	CDR2
	10 20 30	40	50 2A 60	
VHH 9C	QVQLQESGGGLVETGGSLRLSCAASGRTIS	SYTIG	WFRQAPGKEREFVS	HHFASGGVTDYADSVKG
VHH 5C	---------E--QA--------------L-	-----	-------NV----A	-------------F---
VHH 3D	------------QA--------------F-	-L-M-	--------V----A	-----------------
VHH 5D	------------QA--------V-----L-	---M-	--------V----A	-----------------
VHH 8D	------------QA--------------L-	---M-	----T---V----A	--------I--------
VHH 9F	------------QA--------------L-	-----	--------V-D-IA	-----------------

	FR3	CDR3	FR4	HINGE
	70 80 2ABC 90	100ABCDEFGHIJK	110	
VHH 9C	RFTISRDNAKNTVYLEMNSLKPEDTAVYYCAA	STFTISGYRALKAAYEYDY	WGQGTQVTVSS	AHHSEDPSS
VHH 5C	--------------------------------	-----P---N---------	-----------	---------
VHH 3D	--------------------------------	---SSP---N------F--	-----------	EPKTPK-QP
VHH 5D	--------------------------------	-------F-N------F--	-----------	---------
VHH 8D	--------------------------------	----H----E---------	-----------	EPKTPK-QP
VHH 9F	------------------V---------F---	----LP---Q---------	-----------	EPKTPK-QP

Included is the encoded hinge region; VHH 5C, 5D and 9C contain nine primer-encoded amino acids of the short hinge, the remaining fragments contain nine primer-encoded amino acids of the long hinge. Frameworks and complementarity determining regions are numbered and indicated with FR and CDR, respectively. Numbering follows Kabat et al. [34].

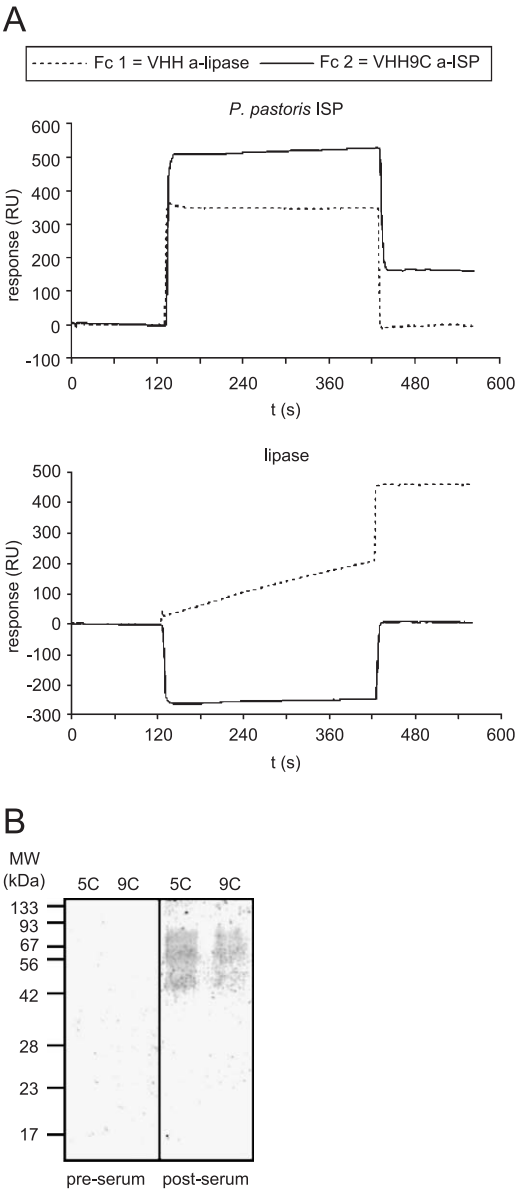

Fig. 1. (A) Specific binding of VHH 9C with *P. pastoris*-produced ISP as determined with surface plasmon resonance using the anti-ISP VHH immobilized on flow cell 2 and an anti-lipase VHH on flow cell 1. At $t = 130$ s *P. pastoris*-produced ISP (top panel) or lipase (bottom panel) was injected and injection was terminated at $t = 430$ s. (B) Western blot analysis of eluted fractions of a purification experiment using *S. cerevisiae*-produced ISP an VHH 5C and 9C coupled to a IMAC resin by their hexahistidine-tag. Blots with eluted fractions from ISP purifications with VHH 5C and VHH 9C were incubated with pre-immune (left) and post-immune (right) llama serum.

injection of ISP produced by *P. pastoris*, a good response was obtained, which turned out to be specific, since a biotinylated anti-lipase VHH did not show binding (Fig. 1A). When the culture supernatant of the relevant production strain was injected, again a specific response was gained and, even more important, the antigen could be eluted with a non-denaturing reagent (10 mM HCl). This analysis encouraged us to evaluate the antibody fragments for affinity purification.

3.2. Affinity purification of ISP with single-domain antibody fragments

In a pilot experiment, the performance of the fragments 5C and 9C for purification of ISP was tested. The VHH were immobilized on an IMAC matrix via their hexahistidine tail and incubated with culture broth containing ISP. After washing, the antigen was eluted with 6 M guanidinium-chloride, which denatures antigens and antibodies and thereby disrupts the binding between both molecules, but does not affect the interaction between the hexahistidine tag of the VHH and the metal-ion attached to the column matrix. The purification procedure was evaluated by Western blot analysis using pre- and post-immune serum for detection (Fig. 1B). In the eluted fractions, a smear of protein with a molecular weight between 40 and 160 kDa

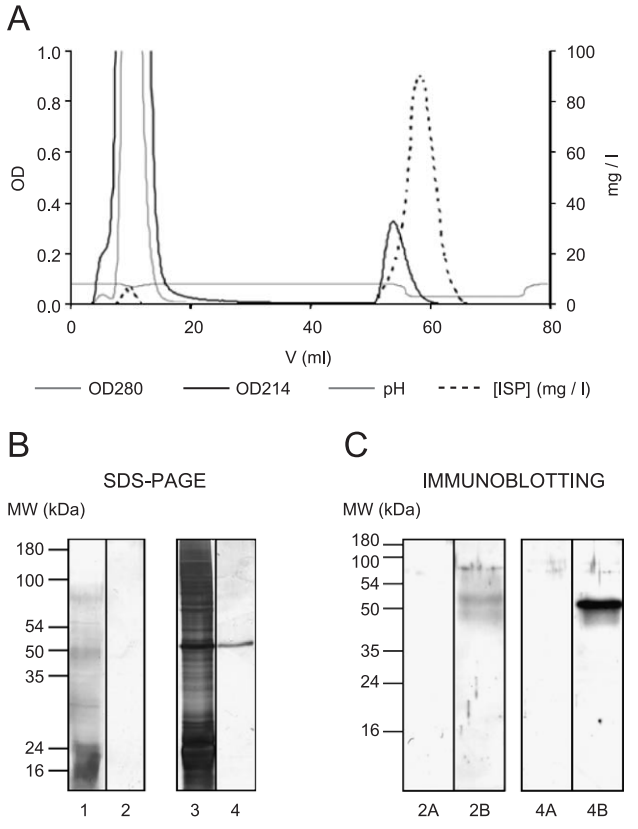

Fig. 2. Immunoaffinity purification of ISP using covalently coupled VHH 9C and *S. cerevisiae*-produced ISP. (A) Chromatogram; 280 and 214 nm detector signals, the pH profile and the quantity of ISP as measured with Immuno-Perfusion Chromatography are shown. (B) Electrophoretic analysis of the fractions from the immunoaffinity purification of ISP expressed by *S. cerevisiae* and *E. coli*. Silver-stained SDS-gel: (1) *S. cerevisiae* culture supernatant; (2) eluted protein; (3) *E. coli* lysate; (4) eluted protein. (C) Western blot analysis of the eluted protein fractions. Detection of eluted proteins with llama sera: (2A) *S. cerevisiae*-produced ISP-eluted protein fraction, incubated with pre-immune serum; (2B) *S. cerevisiae*-produced ISP-eluted protein fraction, incubated with post-immune serum; (4A) *E. coli*-produced ISP-eluted protein fraction, incubated with pre-immune serum; (4B) *E. coli*-produced ISP-eluted protein fraction, incubated with post-immune serum.

was detected by post-immune serum only, revealing the successful purification of ISP in its glycosylated forms produced by *S. cerevisiae*. The most convincing proof was obtained by amino-terminal sequencing of the eluted protein fraction: the first seven residues of ISP from *L. perenne* were identified, although traces of co-eluted antibody could be detected (data not shown).

The possibility of coupling VHH covalently via its primary amino groups to CNBr-activated Sepharose was examined with VHH 9C. To obtain sufficient amounts of pure ISP, a 7 ml column was prepared (coupling efficiency >95%), that was used more than 60 times until now. During purification, the antigen was bound at neutral pH and eluted with acid. The fractions of a representative purification run with *S. cerevisiae*-produced material (chromatogram is shown in Fig. 2A) were analyzed with a quantitative HPLC assay for ISP (see next paragraph), revealing a recovery of at least 85%. Ice recrystallization inhibition assays, in which the capacity of maintaining small ice crystals at sub-zero temperatures is determined, proved that the majority of the functional ISP was recovered after elution (Fig. 3). Due to it's amino acid composition, ISP cannot be stained with Coomassie Brilliant Blue or with silver staining. In order to evaluate the purification it was decided to produce the ISP as a Trx-fusion protein in *E. coli*. SDS-PAGE followed by silver staining and Western blot analysis showed that the protein was successfully purified from both *S. cerevisiae* culture supernatant and *E. coli* cell lysate (Fig. 2B,C). Amino-terminal sequencing of the eluted fractions confirmed the presence of the ISP from *L. perenne* without traces of leaking VHH or other contaminating proteins. The absence of contaminating proteins was confirmed by amino acid analysis after acidic hydrolysis of the eluted protein: the experimentally determined content of amino acids closely matched the expected composition of the protein, which is an indication of the purity of the protein.

To get an idea of the parameters determining the advantageous performance during immunoaffinity chromatography, it was decided to measure the on- and off-rates of the antigen binding by VHH 9C. The found k_a and k_d values were $(2.4 \pm 0.4) \times 10^5$ mol^{-1} s^{-1} and $(9.8 \pm 3.1) \times 10^{-4}$ s^{-1}, respectively, as determined with BIAcore analysis, resulting in a k_d of $(4.2 \pm 1.9) \times 10^{-9}$ M.

3.3. Development of a quantitative ISP assay with Immuno-Perfusion Chromatography

Immuno-Perfusion Chromatography is an HPLC-based method using styrene–divinylbenzene co-polymers coated with a cross-linked polyhydroxylated polymer (POROS) as matrix for covalent coupling of proteins [21,22]. The size of the pores in beads made from the polymers combines a high capacity of immobilized ligand with a high flow rate, resulting in a very rapid analysis with a large dynamic range.

VHH 9C was coupled with an efficiency of approximately 85% to POROS AL 20 Perfusion medium via primary amino groups. The injection of a sample, washing and subsequent elution including regeneration was performed in less than 8 min. A calibration curve was obtained by injecting standards in different dilutions. Since ISP does not contain aromatic residues, ISP was detected at the wave-

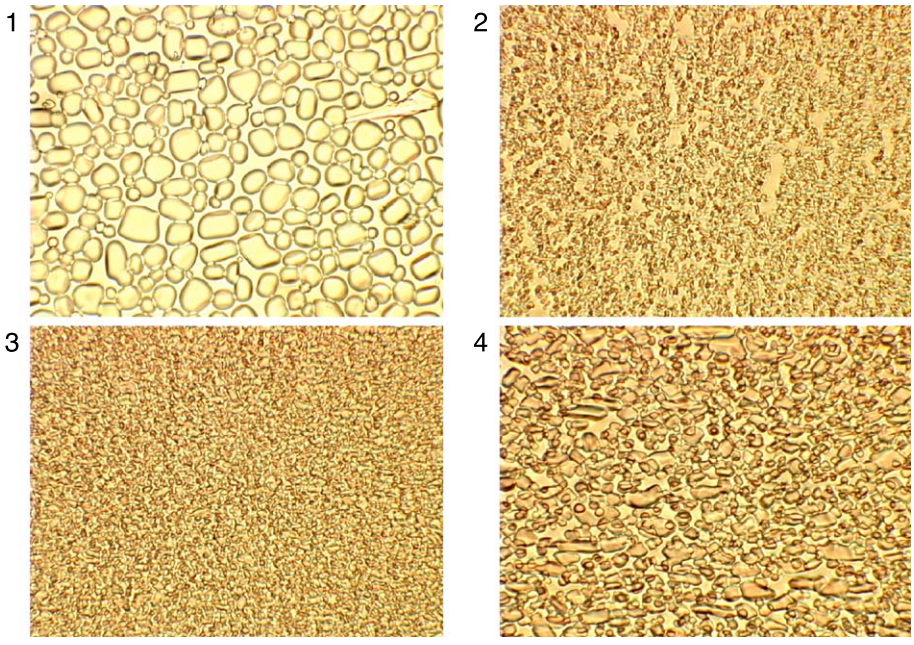

Fig. 3. Recrystallization inhibition assay with the fractions from the immunoaffinity purification of *L. perenne* ISP produced by *S. cerevisiae*. Samples were cooled to −40 °C and subsequently the temperature was raised to −6 °C. The recrystallization at −6 °C was followed microscopically and images were taken after 5 min: (1) blank; (2) end-of-fermentation sample; (3) eluted protein fraction; (4) non-binding protein sample.

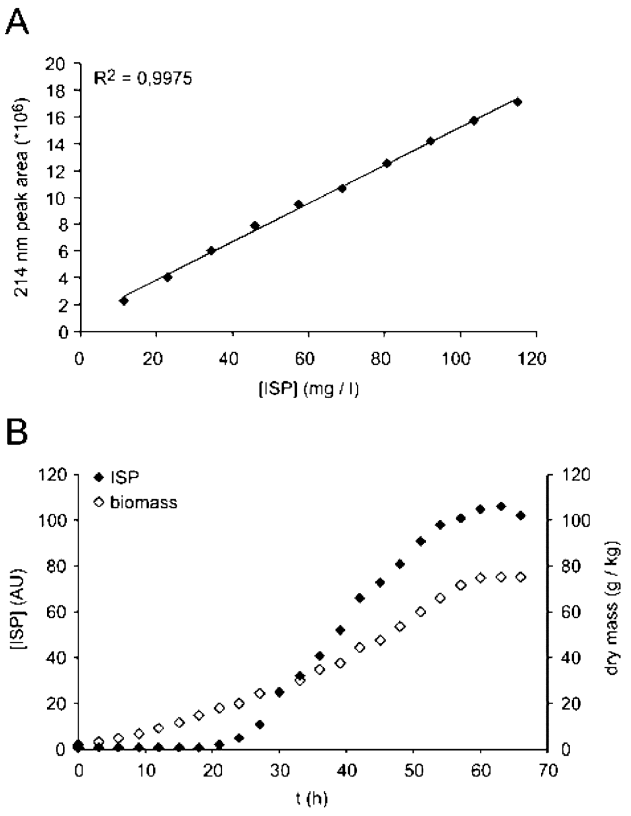

Fig. 4. Quantitative analysis with Immuno-Perfusion Chromatography of ISP produced by *S. cerevisiae*. (A) Calibration curve, injection of different dilutions of ISP; (B) biomass accumulation and levels of ISP in arbitrary units (AU) during fermentation.

length (214 nm) used for measurement of peptide bonds. A calibration plot showing the linear correlation between ISP concentration and the 214 nm peak area is shown (Fig. 4A). This same column has now been used for more than 2000 repeat analyses without capacity loss and is still performing well. The immuno-perfusion column was successfully used to measure production levels during fermentation of an ISP producing *S. cerevisiae* strain (Fig. 4B).

4. Discussion

The recently identified ISP from *L. perenne* [4] might be of interest for application in products and processes, but in order to evaluate its performance, the protein should be obtained in a pure form. The application of ISP in products or processes is economically only feasible when the ISP can be recovered efficiently and on a large scale, from natural sources or fermentation processes.

In this paper, we have explored the utility of single-domain antibody fragments for application in immunoaffinity purification and immuno-perfusion chromatography. When applying phage display technology for the isolation of single-domain antibodies suitable for immunoaffinity chromatography the method of selection influenced the outcome considerably. Coating of ISP to an immunotube, as is done in the biopanning procedure, delivered only VHH recognizing the coated antigen, but no VHH suitable for our purposes. Changing the selection method by using soluble (biotinylated) antigen solved this problem and only a group of clonally related antibody fragments (VHH 9C and its derivatives) was retrieved from the same immune library, which were not isolated via biopanning. With an alternative strategy resembling the epitope-shielding method [23], in which the library was selected against ISP captured by coated VHH 9C (data not shown), the antibody fragments previously found with biopanning were obtained again, suggesting that the side of the antigen containing the epitope of VHH 9C is not accessible when coated, probably due to the interaction with the solid surface.

The followed selection approach with soluble antigen mimics the intended application of affinity purification, where the immobilized antibody fragment should capture antigen from solution. Phage display technology enables the isolation of antibodies with specific characteristics by adapting the circumstances of selection accordingly.

The selected antibodies have the sequence characteristics of single-domain fragments derived from heavy-chain antibodies (Table 1). The typical residues at positions 37 (Phe in VHH instead of Val in VH), 44 (Glu or Val instead of Gly), 45 (Arg instead of Leu) and 47 (Phe instead of Trp) [24,25] are located in the area, that in classical antibodies forms the interface with the VL domain, thereby making this side of the variable region of heavy-chain antibodies more hydrophilic. In addition, the anti-ISP VHH have the typical long CDR3 (19 amino acid residues) [24]. All selected fragments belong to family 1 according to the classification of Harmsen et al. [26]. All selected VHH are probably clonally related [26], meaning that these are derived from a common ancestor. The differences are caused by somatic mutations, which are introduced during the in vivo maturation process. It is rather surprising to see that the same type of VHH has been found with both the long and short hinge isotype [7]. This might suggest that this type of isotype switching takes place during affinity maturation, although a PCR artifact occurring during the amplification of the immune repertoire cannot be excluded.

The reproducibility of the purification of ISP produced by *S. cerevisiae*, using the immunoaffinity matrix with VHH 9C coupled to CNBr-activated Sepharose, was investigated by performing 60 consecutive purification runs with a single batch of fermentation broth. Identical chromatograms and reproducible yields of functional ISP were obtained after a non-denaturing acid elution (pH 2.0), thereby illustrating the robustness of the antibody fragments. This was surprising, since the chance of success for finding traditional monoclonal antibodies suitable for affinity purification is rather low [27]. Most of the time, harsh elution conditions have to be applied to completely remove the antigen from the column, what

might damage the biological activity of the antibody and the eluted protein. In similar ways, as described in this paper, we have developed one-step affinity purifications for other proteins (glutathione S-transferase, mouse and human antibodies). As with ISP, there was no extensive screening procedure needed to find suitable candidate fragments, suggesting that the nature of the single-domain antibody determines the superior behaviour for affinity chromatography.

First of all, we think that the true monomeric nature is important for efficient elution of the antigen. This is based on the finding that the affinity of the antibody fragment is in the nanomolar range, similar to what has been found for monoclonal antibodies. In addition, the off-rate is relatively low, in the order 10^{-3} s^{-1}. This is comparable to what has been published for (monovalent Fab's derived from) monoclonal antibodies but the main difference is the avidity. Monoclonal antibodies of the IgG class with two binding sites, bind antigen with increased avidity leading to very low off-rates. The poor dissociation is the result of rebinding, in other words, when an antigen dissociates from the complex it is immediately captured by the neighbouring binding site. The avidity effect might also be a potential problem for single-chain Fv's, because these molecules tend to form multimers [28,29]. Others [30,31] as well as we [9] showed by gel filtration and mass spectrometry that VHH do not aggregate and are expressed as monomers. Recently it was reported that a VH derived from a monoclonal antibody recognizes human midkine effectively even without its natural light chain partner [32]. When used for affinity purification, the antigen could be recovered via a mild elution method (0.5 M NaCl), thereby indicating that the dissociation kinetics of a monomeric fragment are more favourable for an efficient elution.

Secondly, the earlier reported physical and thermal stability [9], or better, the capability of VHH to refold completely after denaturation [33], enables the apparent infinite reusability of the column. With a single perfusion column, improved production mutants of *S. cerevisiae* could be identified as well as numerous end-of-fermentation samples quantified, resulting in an impressive number of over 2000 runs until now without loss of capacity.

As expected [9], the example with ISP showed that VHH have superior characteristics for affinity purification and diagnostic applications. The results of this study show that highly pure proteins and glycoproteins can be recovered from biological material in a relatively facile one-step process.

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