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Selection and optimization of proteolytically stable llama single-domain antibody fragments for oral immunotherapy

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Abstract We previously demonstrated that oral application of the recombinant single-domain antibody fragment (VHH) clone K609, directed against *Escherichia coli* F4 fimbriae, reduced *E. coli*-induced diarrhoea in piglets, but only at high VHH doses. We have now shown that a large portion of the orally applied K609 VHH is proteolytically degraded in the stomach. Stringent selection for proteolytic stability identified seven VHHs with 7- to 138-fold increased stability after in vitro incubation in gastric fluid. By DNA shuffling we obtained four clones with a further 1.5- to 3-fold increased in vitro stability. These VHHs differed by at most ten amino acid residues from each other and K609 that were scattered over the VHH sequence and did not overlap with predicted protease cleavage sites. The most stable clone, K922, retained 41% activity after incubation in gastric fluid and 90% in jejunal fluid. Oral application of K922 to piglets confirmed its improved proteolytic stability. In addition, K922 bound to F4 fimbriae with higher affinity and inhibited fimbrial adhesion at lower VHH concentrations. K922 is thus a promising candidate for prevention of piglet diarrhoea. Furthermore, our findings could guide selection and improvement by genetic engineering of other recombinant antibody fragments for oral use.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhoea and mortality in recently weaned piglets. ETEC infections can be prevented by passive administration of antibodies binding to ETEC fimbriae of the F4 type, which results in prevention of bacterial adherence to the intestinal mucosa (Nagy and Fekete 1999). The aim of our study is to use single-domain antibody fragments (VHHs) for such immunotherapy (Harmsen et al. 2005). VHHs are derived from camelid heavy chain antibodies and are naturally devoid of light chains (Hamers-Casterman et al. 1993). They have a high physicochemical stability (Van der Linden et al. 1999) and are well expressed in microorganisms (Frenken et al. 2000). We previously isolated six F4 fimbriae binding VHHs from yeast VHH expression libraries. One of these VHHs, K609, effectively inhibited in vitro ETEC adhesion to the intestinal mucosa but poorly protected piglets against ETEC infection after oral administration (Harmsen et al. 2005).

The effectiveness of oral immunotherapy is often complicated by partial proteolytic degradation of antibodies within the gastro-intestinal (GI) tract (Schmidt et al. 1989; Wiedemann et al. 1990; Yokoyama et al. 1993; Reilly et al. 1997). Here, we have shown that this was also the case for K609. Proteolysis of ingested proteins is initiated by the action of pepsin in the stomach and continued in the duodenum by the action of trypsin and chymotrypsin. These proteases have different primary cleavage site specificities that are dependent on particular amino acid sequence patterns in their substrates. Specificity is furthermore determined by the accessibility of such putative protease cleavage sites within the folded protein (Keil 1992). Thus, proteolysis is most efficient on flexible, surface-exposed regions of proteins such as the antibody hinge region (North 1989). Degradation of antibodies therefore initially results in $F(ab')_2$, Fab and Fc fragments. The $F(ab')_2$ and Fab fragments can retain some of their neutralizing activity in the GI tract, which is, however, lost upon further proteolysis of these fragments. Antibody fragments with different variable domains differ in their sensitivity to proteolysis (Reilly et al. 1997).

The use of recombinant antibody fragments for oral immunotherapy opens the possibility of selection and engineering of proteolytically stable forms. Among the

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many methods to improve the physicochemical stability of enzymes and recombinant antibody fragments, DNA shuffling is often used (Kurtzman et al. 2001). This method relies on the random recombination of different gene segments by DNA fragmentation and polymerase chain reaction (PCR) reassembly. However, this method has never been used for increasing proteolytic stability. Here, we describe (1) the phage display selection of anti-F4 VHHs with significantly increased resistance to in vitro proteolysis, (2) the further improvement in proteolytic stability by DNA shuffling and (3) the in vivo stability of the optimized VHH after oral application to piglets.

Materials and methods

Strains and genetic constructs

E. coli XL1-Blue (Stratagene, La Jolla, CA) was used for routine recombinant DNA manipulation. *E. coli* TG1 (Stratagene) was used for phage display purposes. *Saccharomyces cerevisiae* SU51 (*can1*; *his4–519*; *leu2–3*, *−112*; *cir*$^{+}$) was used for VHH production as described previously (Van der Vaart 2002).

Phage display selection of F4 fimbriae binding VHHs

The VHH repertoire of a llama that was previously immunized with F4 fimbriae (Harmsen et al. 2005) was recovered from peripheral blood lymphocytes as recently described (Frenken et al. 2000) and separately inserted into the *Pst*I–*Hin*dIII cut phage display vector pUR4676 and the *Pst*I–*Bst*EII cut yeast expression vector pUR4584 as described before (Harmsen et al. 2002). pUR4584 is a 2-μm-based *LEU2*-marked plasmid suitable for *GAL7* promoter-controlled expression of VHHs fused to the yeast invertase signal peptide that does not encode a C-terminal c-myc tag but is further identical to pUR4585 (Harmsen et al. 2000). VHH clones were transferred between pUR4676 and pUR4584 using the *Pst*I and *Bst*EII restriction sites. Phage display selections of clones producing VHHs binding to F4 fimbriae were performed using a single round of biopanning (McCafferty and Johnson 1996) using Maxisorb immunotubes (Nunc, Roskilde, Denmark) coated with 1, 0.1 or 0.01 μg/ml F4 fimbriae.

Sequencing and sequence analysis

Single-domain antibody fragment encoding regions of pUR4676-derived plasmids were subjected to automated DNA sequencing as described previously (Harmsen et al. 2000). The deduced VHH amino acid sequences were aligned according to the International ImMunoGeneTics Information System (IMGT) (Lefranc 2004) for alignment, numbering and complementarity-determining region (CDR) definition of immunoglobulins. Putative protease cleavage sites were predicted based on the VHH amino acid sequence using a Web-based tool (http://www.expasy.org/tools/peptidecutter) and the following cleavage site definitions (Keil 1992). Chymotrypsin cleaves C-terminal to Phe, Tyr and Trp but not N-terminal to Pro residues. Trypsin cleaves C-terminal to Arg and Lys but not N-terminal to Pro residues. The pepsin cleavage site was defined as it occurs above pH 2.

In vitro F4 fimbriae villous epithelium adhesion inhibition assay

The ability of anti-F4 VHHs to inhibit binding of F4 fimbriae to isolated brush borders (BBs) was determined using an inhibition enzyme-linked immunosorbent assay (ELISA). BBs were isolated from the small intestine of an F4ac-receptor-positive piglet as described previously (Sellwood et al. 1975). The protein content of the isolated BB preparation was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Polystyrene Polysorb 96-well plates (Nunc) were coated with 6 mg/l BBs in 0.05 M carbonate/bicarbonate buffer, pH 9.6. Subsequently, 100 μl 0.1 mg/l biotinylated F4-fimbriae in phosphate-buffered saline (PBS) containing 1% ovalbumin (Sigma, Saint Louis, MO; grade V) and 0.05% Tween 20 was pre-incubated with a twofold dilution series of purified VHH for 1 h at room temperature, transferred to the BB-coated plate and incubated for another hour. Bound biotinylated F4 fimbriae were then detected using peroxidase-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA) and the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB). A control incubation without VHH resulted in an extinction at 450 nm of 1.2. The 50% inhibitory concentration (IC_{50}) was calculated by interpolating the VHH concentration, resulting in 50% of this extinction value.

DNA shuffling for improving proteolytic stability

A secondary phage display library of anti-F4 VHHs was generated by DNA shuffling according to Adey et al. (1996) using equal amounts of the K609, K712 and K719 VHH encoding inserts of pUR4584-derived plasmids that were obtained by PCR amplification using primers BOLI5 (5′-ATTATTTTAGCGTAAAGGATGGGG-3′) and BOLI6 (5′-CCTTTTCCTTTTGGCTGGTTTTGC-3′). These shuffled fragments were then inserted into phage display vector pUR4676 using the *Pst*I and *Bst*EII sites. A single round of phage display selection on immunotubes coated with 0.01 μg/ml F4 fimbriae was performed as described above but using phage incubated in jejunal or gastric fluid prior to selection. Based on preliminary experiments we chose a GI fluid concentration that resulted in a decrease in antigen binding capacity in phage ELISA to 10% of an untreated control.

Affinity measurements

Surface plasmon resonance study of VHH interactions with F4 fimbriae was performed on a BIAcore 3000 instrument (BIAcore, Uppsala, Sweden). F4 fimbriae were immobilized on a BIAcore CM5 sensor chip using an amine coupling kit (BIAcore). Then, concentration series of VHHs were applied to the chip in 10 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES)-buffered saline, pH 7.4, [containing 3.4 mM Titriplex III, 0.15 M NaCl and 0.05% surfactant P20 (BIAcore)] at 25°C and a flow rate of 50 µl/min. After each measurement, residual VHH was removed using 100 mM HCl. Association and dissociation rate constants (K_a and K_d) were calculated using a 1:1 Langmuir binding model and BIAevaluation 3.0 software (BIAcore). K_D was calculated by dividing K_d by K_a.

In vitro proteolytic stability of VHHs

Gastric or jejunal fluid was derived from 4-week-old specific pathogen-free piglets. In initial experiments (Table 1) we used freshly prepared GI fluids from a single piglet. Later, we pooled the jejunum content of 5 piglets and the stomach content of 15 piglets. The pooled stomach content had a pH of 2.8. All samples were clarified by centrifugation and stored as aliquots at −20°C. For assays with purified proteases we used porcine pepsin (Sigma), bovine trypsin (Roche Applied Science, Mannheim, Germany) and bovine chymotrypsin (Merck). Incubations with pepsin were performed in 10 mM HCl (pH 2) and with the other two proteases in 1 mM Tris–Cl (pH 8.0), 20 mM $CaCl_2$.

For quantitative measurements a 2-µg amount of purified yeast-produced VHH was incubated in 20 µl of gastric or jejunal fluid or protease solution and in PBS (control) for 1 h at 37°C. The reaction was stopped by the addition of 200 µl PBS containing 1% skimmed milk, 1 g/l Pefabloc SC (Roche Applied Science), 1 µM pepstatin A and 100 mM Tris–Cl (pH 8.5). The amount of functional VHH was then determined by antigen-specific ELISA as follows: Polystyrene 96-well plates (Greiner, Solingen, Germany) were coated with 5 µg/ml F4 fimbriae in 0.05 M carbonate/bicarbonate buffer, pH 9.6. Residual sites were blocked for 2 h with 1% skimmed milk in PBS. After incubation with serial twofold dilutions of VHHs, bound VHHs were detected with peroxidase-conjugated rabbit anti-llama immunoglobulins and staining with 3,3′,5,5′ tetramethylbenzidine. Antibody titres were calculated by interpolating the VHH concentration, resulting in an extinction at 450 nm of 0.3. The percentage functional VHH remaining after proteolysis relative to incubation in PBS was then calculated.

For semi-quantitative measurements 1 µl of *E. coli* culture supernatant containing soluble VHH was incubated in 20 µl GI fluid and in PBS and analysed by ELISA at a single tenfold dilution as described above. The ratio of the extinction at 450 nm of the GI fluid sample and the PBS control was taken as a measure for proteolytic stability.

Table 1 Proteolytic stability of VHHs in gastrointestinal fluids

Clone	Stability in stomach (%)	Stability in jejunum (%)
K3	<1	<1
K27	<0.5	<0.5
K609	<0.1	0.3
K719	<0.1	1.3
K807	0.13	<0.1
K812	0.18	1.2

In vivo proteolytic stability of VHHs

Ten piglets were weaned at 3 weeks of age and fed a standard piglet feed ad libitum. Two groups of five piglets received either yeast-produced K609 or K922 that was purified by affinity chromatography using protein A or Ni-NTA (Van der Vaart 2002), respectively, 1 week later. Each piglet received 5 ml PBS containing 0.1 g CoEDTA and 0.5 mg VHH by injection into the throat. CoEDTA was included as a non-degradable and non-absorbed tracer. The piglets were killed 3 h later, and the stomach and duodenum content were collected entirely, whereas the jejunum content was collected in six to eight segments of about 1 m. The posterior small intestinal segment was defined as the ileum. The pH of the collected stomach content was determined. Then, proteolytic activity was stopped by adding 0.1 volume 1 M Tris–Cl (pH 8.5) and 0.01 vol 1 mM pepstatin A to gastric samples and 0.01 vol 10 g/l Pefabloc SC to other GI samples. The samples were then clarified by centrifugation. The amount of functional VHH in these samples was determined by antigen-specific ELISA using purified VHHs as a standard. The cobalt concentration was determined by inductively coupled plasma atomic emission spectroscopy (ISO 11885; International Organization for Standardization; http://www.iso.ch/iso/en/). The proteolytic stability of VHHs in each segment was then calculated by the formula $\left[\frac{V_{out}C_{in}}{C_{out}V_{in}}\right]$ ×100 (%), where V and C represent VHH, and cobalt concentrations in the mixture given to the piglets (in) and in the GI contents (out). Statistical analysis was performed using an unpaired one-sided Student's t test.

Nucleotide sequence accession numbers

Nucleotide sequences encoding unique VHHs were submitted to the European Molecular Biology Laboratory (EMBL) database (Fig. 1). Note that clones K609 and K719 were isolated previously (Harmsen et al. 2005).

Results

Proteolytic susceptibility of F4 fimbriae binding VHHs

A large fraction of six previously isolated F4 fimbriae binding VHHs (Harmsen et al. 2005) is degraded after in

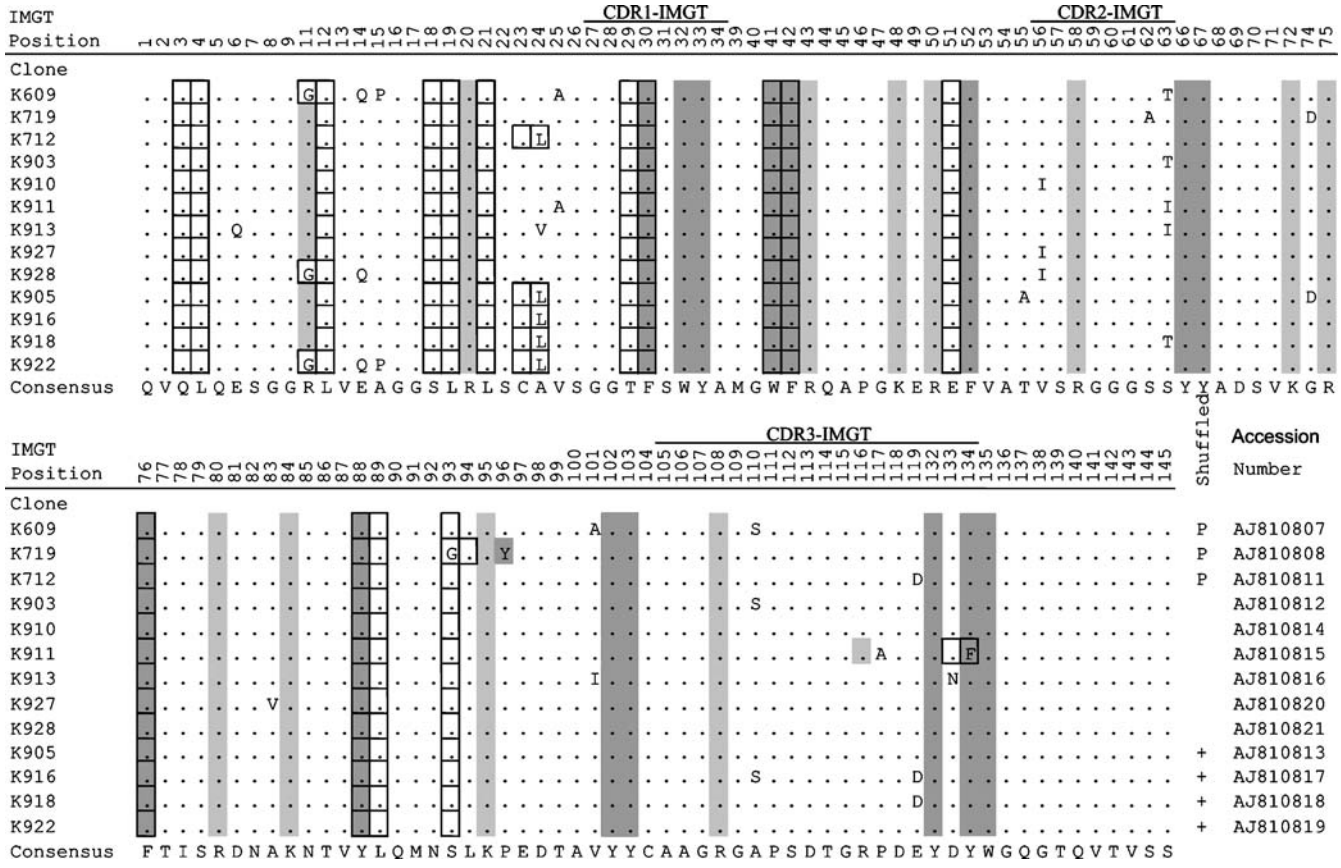

Fig. 1 Multiple amino acid sequence alignment of F4 fimbriae binding VHHs. Residue numbering and CDR definitions are according to the IMGT numbering system (Lefranc 2004). *Dots* indicate amino acid residues that are identical to the consensus sequence. Four clones were obtained by DNA shuffling (indicated by a "+" sign) of three parental clones (indicated by *P*). Predicted protease cleavage sites are indicated in *grey* (trypsin), *dark grey* (chymotrypsin) or by *boxing* (pepsin) of the residue forming the C terminus after cleavage

vitro incubation in either gastric or jejunal fluid from newly weaned piglets (Table 1). The K609 VHH was not inactivated by incubation in 0.1 M HCl (pH 1) or in gastric fluid that was not pH-neutralized, but which contained pepstatin A to inhibit pepsin activity (results not shown). This indicates that VHH inactivation in gastric fluid was not caused by the low pH of this sample but due to proteolysis.

Isolation of F4 binding VHHs with increased proteolytic stability by phage display

After a single round of phage display selection from a previously immunized llama, about 50% of the individual clones bound F4 fimbriae. After semi-quantitative measurements of proteolytic stability of 721 F4-binding clones, we selected the 12 apparently most stable clones which could also inhibit fimbrial adhesion to the intestinal mucosa.

Sequencing of their VHH encoding region revealed that clone K712 was isolated three times, and clone K910 was isolated four times, resulting in seven unique clones. The CDR3 of VHHs generally is most variable (Harmsen et al. 2000) and is directly involved in antigen binding. The seven clones and the previously isolated clones K609 and K719 all had similar CDR3 sequences, and their overall sequence differed by only one to ten amino acid residues (Fig. 1). This suggests that they have the same epitope specificity. The seven unique VHHs were produced in yeast and purified for further quantitative characterization. In comparison to K609, they had a 7- to 138-fold increased stability in gastric fluid (Fig. 2a). Some clones also showed a small, at most twofold, increased stability in jejunal fluid (Fig. 2b). It should be noted that we now, and in subsequent experiments, used GI fluids pooled from several piglets that resulted in less potent VHH inactivation than the samples from a single piglet used previously (cf. K609 in Table 1 and Fig. 2). Furthermore, all newly isolated clones inhibited fimbrial adhesion to the intestinal mucosa at lower VHH concentrations (Fig. 3c).

Improvement of proteolytic stability by DNA shuffling

A second-generation phage display library was generated by DNA shuffling of three VHHs: K609, K712 and K719. For stringent selection of proteolytically stable VHHs this library was treated with gastric or jejunal fluid prior to

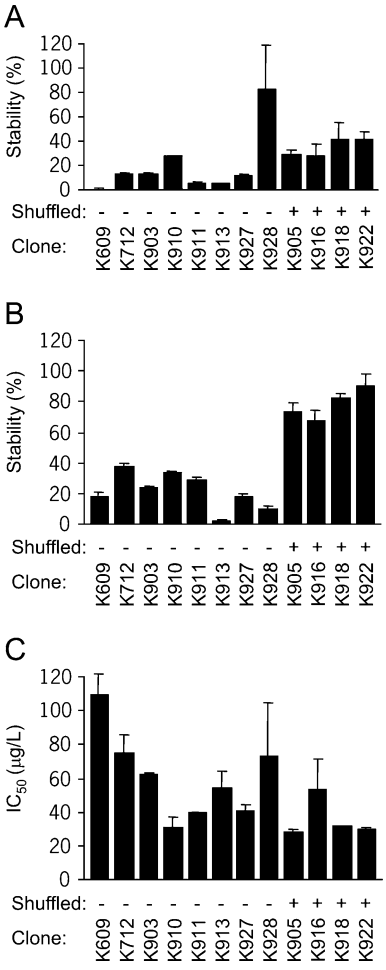

Fig. 2 In vitro proteolytic stability and adhesion inhibiting activity of VHH domains. VHHs were incubated in gastric (**a**) or jejunal (**b**) fluid for 1 h at 37°C, after which the percentage residual VHH as compared to a mock-treated control was determined by ELISA. **c** The VHH concentration resulting in 50% inhibition of adhesion of F4 fimbriae to brush-border membranes was determined by ELISA. *Bars* are means±standard deviation of three replicate samples. The four clones obtained by DNA shuffling are indicated by a "+" sign

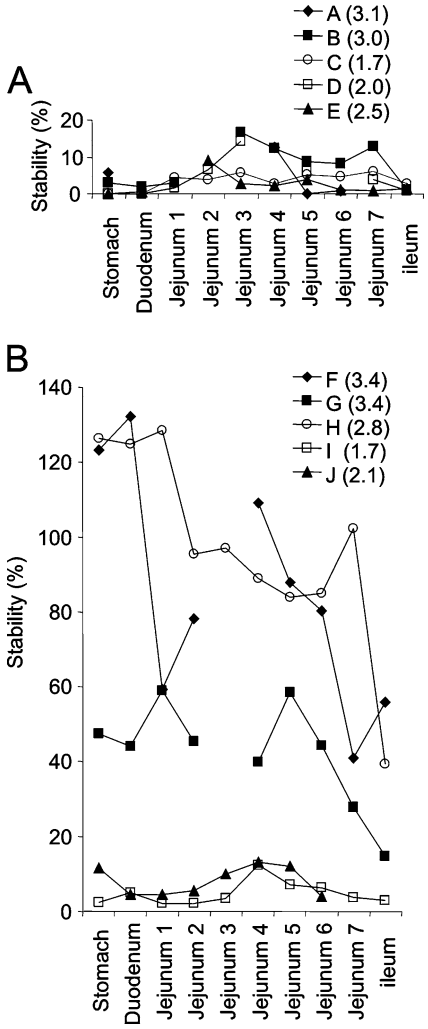

Fig. 3 In vivo proteolytic stability of VHH fragments K609 (**a**) and K922 (**b**) 3 h after oral application to groups of five piglets. Individual piglets are indicated by *letters* with gastric pH between parentheses. The proteolytic stability of VHHs was determined in different GI segments and corrected for dilution effects. For some segments stability could not be determined because a sufficient amount of GI content could not be collected

biopanning, which resulted in 161 F4-binding clones. The four clones with the highest proteolytic stability (K905, K916, K918 and K922) were expressed in yeast and characterized as described in the previous section. They had a 1.5- to 3-fold increased stability in both gastric and jejunal fluid and inhibited fimbrial adhesion to the intestinal mucosa more effectively than the most stable parental clone, K712 (Fig. 2).

Clone K905 contains a non-parental alanine residue at position 55 (Fig. 1) that presumably was introduced during the PCR amplification step of the shuffling process. All shuffled clones contain variable parts of the most stable parental clone K712. Only the leucine at position 24 is consistently present. However, this residue is lacking in highly stable clones that were not shuffled such as K910 and K928. In a further attempt to specify residues that determine proteolytic susceptibility, we predicted pepsin, trypsin and chymotrypsin cleavage sites based on the amino acid sequence of all 13 VHHs (Fig. 1). However, we did not find a strong correlation between proteolytic stability and absence of particular putative cleavage sites. For example the glycine at position 11, which is predicted to be a pepsin cleavage site, occurs in both clones that are sensitive (K609) and relatively resistant (K928 and K922) to incubation in gastric fluid (cf. Figs. 1 and 2). Furthermore, the leucine at position 24 that was retained in all shuffled clones, which are relatively stable, was predicted to be a pepsin cleavage site. Only the glycine at position 93 of clone K719 could be associated with its high pepsin sensitivity (Table 1). However, for most clones, we were unable to identify the stabilizing mutations.

Clone K922 was selected for further analysis because of its high overall proteolytic stability. Furthermore, K922 bound F4 fimbriae with increased affinity as compared to its three parental clones, predominantly due to a decreased dissociation rate constant (Table 2).

Table 2 Kinetics of VHH interaction with F4 fimbriae

Clone	$K_a \times 10^5$ (M^{-1} s^{-1})	$K_d \times 10^{-4}$ (s^{-1})	K_D(nM)
K609	11	6.6	0.61
K712	18	1.9	0.11
K719	6.3	1.0	0.16
K922	18	0.8	0.04

We compared the proteolytic stability of K609 and K922 after incubation with different concentrations of purified pepsin, trypsin or chymotrypsin (Table 3). K609 was substantially degraded already at the lowest pepsin concentration, whereas about 80% remained intact at 100-fold higher concentrations of trypsin or chymotrypsin. K922 degradation required a 100-fold higher pepsin concentration to reach the same level as observed with K609. K922 was not degraded at the highest trypsin or chymotrypsin concentration tested. Thus, as compared to K609, K922 has a substantially increased resistance against degradation by pepsin and a moderately increased resistance against degradation by trypsin or chymotrypsin.

Proteolytic stability after oral application to piglets

The proteolytic stability of K609 and K922 was analysed in vivo in different segments of the piglet GI tract 3 h after oral application. For both VHHs, degradation is already high in the stomach (Fig. 3). VHH that has moved further along the GI tract is not substantially further degraded or is even degraded to a lesser extent in some piglets such as piglets B and D (Fig. 3a). Assuming that VHH degradation occurs predominantly in the stomach, the decreased VHH degradation further along the GI tract could be due to a faster stomach transit. Different piglets show considerable variation in both VHH proteolytic stability and gastric pH, which appears to be lower in piglets with higher VHH degradation. However, despite this high variation in stability, K609 is clearly more rapidly degraded in vivo as compared to K922 (cf. Fig. 3a,b). In support of this conclusion, the amounts of K609 and K922 within the stomach differed significantly (P=0.043).

Table 3 Proteolytic stability of K609 and K922 after incubation with specific proteases

Protease		Percentage residual VHH (mean±standard deviation)			
Type	Concentration (mg/ml)	K609		K922	
Pepsin	0.1	0.08	±0.007	21	±1
Pepsin	0.01	0.7	±0.1	39	±3
Pepsin	0.001	17	±4	94	±7
Trypsin	0.1	76	±5	102	±8
Trypsin	0.01	102	±8	98	±11
Trypsin	0.001	104	±19	102	±7
Chymotrypsin	0.1	80	±13	105	±4
Chymotrypsin	0.01	94	±11	101	±12
Chymotrypsin	0.001	99	±7	104	±4

Discussion

We have shown that the K609 VHH is rapidly degraded within the GI tract of piglets. The primary site of degradation appears to be the stomach, since less functional VHH is found after in vitro incubation in gastric fluid (0.6%) as compared to jejunal fluid (18%). Furthermore, K609 was much more susceptible to degradation by pepsin than trypsin or chymotrypsin. Furthermore, 3 h after feeding K609 to piglets, functional VHH levels were decreased to below 6% already within the stomach and did not significantly decrease further along the GI tract. This difference in the extent of in vivo and in vitro proteolysis may be caused by the presence of other proteins in feed in vivo, which compete with VHHs for proteolysis. It is well known that digestion of specific proteins can be reduced by the addition of a high amount of other proteins (Wiedemann et al. 1990; Morgavi et al. 2000). The in vivo K609 stability varied considerably between individual piglets and appeared to be dependent on the gastric pH. This correlation is probably due to the pH dependence of pepsin activity, which is optimal at pH 2 (Becker and Rapp 1979), and further suggests that cleavage by pepsin is the primary cause of K609 degradation. A large variation in gastric pH and proteolytic activity between different piglets is commonly observed (Low 1982; Schmidt et al. 1989) and can also explain the difference in in vitro K609 stability using GI fluids derived from different piglets (Table 1 and Fig. 2).

K609 and five other VHHs were previously isolated from a small library of yeast-produced VHHs without selection for stability (Harmsen et al. 2005). Here, we isolated seven VHHs with a 7- to 138-fold increased in vitro stability in gastric fluid by isolating a 100-fold higher number of antigen specific clones from larger VHH libraries using phage display that were subsequently screened for proteolytic stability. Furthermore, we obtained an additional, at most threefold, increased stability by DNA shuffling. Thus, we have shown that relatively proteolytically stable VHHs can be isolated from larger libraries by careful selections and further optimized by DNA shuffling.

These clones differed by only a few amino acids from K609 and had highly identical CDR sequences. This contrasts with the diversity of the six previously isolated VHHs, of which only two VHHs belonged to the K609 CDR group (Harmsen et al. 2005). That more stringent selection for proteolytic stability results in the selection of clones from a single CDR group suggests that CDRs are the primary site of proteolysis. This is in accordance with the general view that CDRs form flexible surface-exposed loops (Padlan 1994; Harmsen et al. 2000), which is a structure that is more susceptible to proteolysis (North 1989).

However, the newly selected clones still varied considerably in proteolytic stability. These clones had different amino acid substitutions that were scattered around the VHH sequence and not located at predicted protease cleavage sites, indicating that increased resistance to proteolysis was not caused by elimination of sites determining primary protease specificity. A scattered pattern of amino acid substitutions is commonly found in recombinant antibodies selected for improved affinity or physicochemical stability (Daugherty et al. 2000; Jermutus et al. 2001; Zahnd et al. 2004). It has been reported that amino acid substitutions outside the CDRs that result in increased affinity can stabilize CDR loops in a conformation that is more suitable for antigen binding (Foote and Winter 1992; Padlan 1994; Wedemayer et al. 1997). Possibly, the higher proteolytic stability of the newly selected VHHs is similarly caused by reduced flexibility of CDR loops. Such a suggestion is in accordance with earlier observations that proteolytic and thermal stability of proteins generally are correlated because unfolded proteins expose flexible regions that are more sensitive to proteolysis (Arnold and Ulbrich-Hofmann 1997; Amin et al. 2004). Furthermore, the proteolytic stabilization of certain enzymes by protein engineering was due to reduced flexibility of the protein region that was prone to proteolysis (Frenken et al. 1993; Markert et al. 2001).

The high in vitro proteolytic stability of a clone obtained by DNA shuffling, K922, was confirmed in vivo. As was found for K609, most residual K922 degradation occurred in the stomach of piglets with low gastric pH values, again suggesting that proteolysis is due to pepsin activity. However, K922 degradation is far lower than the 90% degradation of egg antibodies that are successfully used for treatment of piglet diarrhoea (Wiedemann et al. 1990). Thus, the limited K922 degradation may not preclude effective oral immunotherapy. Furthermore, K922 had a tenfold higher affinity and inhibited fimbrial adhesion at threefold lower VHH concentrations than K609, which already gave limited protection (Harmsen et al. 2005). Therefore, K922 is a promising candidate for oral immunotherapy of piglet diarrhoea.

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