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Peptide affinity chromatography media that bind N^{pro} fusion proteins under chaotropic conditions

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ABSTRACT

To design a generic purification platform and to combine the advantages of fusion protein technology and matrix-assisted refolding, a peptide affinity medium was developed that binds inclusion body-derived N^{pro} fusion proteins under chaotropic conditions. Proteins were expressed in *Escherichia coli* using an expression system comprising the autoprotease N^{pro} from Pestivirus, or its engineered mutant called EDDIE, with C-terminally linked target proteins. Upon refolding, the autoprotease became active and cleaved off its fusion partner, forming an authentic N-terminus. Peptide ligands binding to the autoprotease at 4 M urea were screened from a combinatorial peptide library. A group of positive peptides were identified and further refined by mutational analysis. The best binders represent a common motif comprising positively charged and aromatic amino acids, which can be distributed in a random disposition. Mutational analysis showed that exchange of a single amino acid within the peptide ligand caused a total loss of binding activity. Functional affinity media comprising hexa- or octapeptides were synthesized using a 15-atom spacer with terminal sulfhydryl function and site-directed immobilization of peptides derivatized with iodoacetic anhydride. The peptide size was further reduced to dipeptides comprising only one positively charged and one aromatic amino acid. Based on this, affinity media were prepared by immobilization of a poly amino acid comprising lysine or arginine, and tryptophan, phenylalanine, or tyrosine, respectively, in certain ratios. Binding capacities were in the range of 7–15 mg protein mL^{-1} of medium, as could be shown for several EDDIE fusion proteins. An efficient protocol for autoproteolytic cleavage using an on-column refolding method was implemented.

1. Introduction

Affinity chromatography has been employed with great success for analytical and preparative applications whenever complex mixtures of biological samples had to be separated [1–3]. An elegant method for extending the application range of affinity chromatography has been the introduction of fusion tags by means of recombinant protein expression technology [4,5]. With this approach, the target protein is fused to a protein or peptide where a specific and functional affinity pair exists. A specific tag often facilitates easy identification or even quantification; in addition, it provides the use of single-step purification with the effect of high purity [6–8]. Often, fusion protein technologies are used to increase expression levels for recombinant protein production. In particular, for peptides and small proteins, low expression levels are a common limitation of conventional expression systems. Along with increased expression titers, there is often improved solubility of the fusion proteins [4,9,10].

Previously, we reported the development of a novel prokaryotic expression system called N^{pro} fusion technology, which makes use of the autoproteolytic function of N^{pro} from classical swine fever virus [11]. Proteins or peptides expressed as N^{pro} fusion proteins are deposited as inclusion bodies (IBs) yielding very high titers. Upon in vitro refolding by switching from chaotropic to kosmotropic conditions, the fusion partner is released from the C-terminal end of the autoprotease by self-cleavage, leaving the target protein with an authentic N-terminus. The kinetics and refolding properties dependent on the fusion partner have also been described recently [12,13]. In contrast to conventional refolding kinetics, the N^{pro} mutant EDDIE follows a kinetics in which yield and rate are

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independent of the initial protein concentration. Throughout this paper, refolding and cleavage are considered as concurrent processes designated as autoproteolytic cleavage or autoproteolysis.

It is obvious that such a self-cleaving fusion construct is ideally suited for the application of a tag. For instance, if a his-tag is fused to the N-terminus of the autoprotease, it can be used for detection and purification in the denatured state as long as the fusion protein is intact. Upon refolding, the autoprotease cleaves at its C-terminus, and a chemical or enzymatic removal of the tag from the target protein is not required. The tag can simply be removed concurrently during the depletion of the autoprotease, for instance by precipitation or chromatographic methods. Moreover, his-tagged proteins expressed as inclusion bodies have been successfully applied for on-column refolding on immobilized metal ion affinity chromatography (IMAC) media [14]. However, two considerations have led us to search for a different approach. First, his-tag applications are protected by patents, and second, the binding capacity of IMAC media is significantly reduced in the presence of reducing agents because of a strong affinity with the immobilized metal ions. Conversely, the use of high concentrations of reducing agents is often required for solubilization and complete denaturation of inclusion body-derived proteins. Our efforts therefore targeted development of the combined use of N^{pro} as an autoprotease system and, concurrently, as a tag itself. The intention was to identify peptides, screened with a combinatorial library that can bind N^{pro} fusion proteins under chaotropic conditions. We also addressed the general question of whether or not it is possible to identify an affinity pair that functions under chaotropic conditions. In this work, we describe the screening of peptide ligands and immobilization strategies for the development of peptide affinity media binding N^{pro} autoprotease fusion proteins in 4 M urea under denaturing conditions. The paper first describes screening for peptides against the N^{pro} wild-type, with the same approach subsequently being applied using the tailor-made mutant EDDIE, which has better solubility and improved autoproteolytic cleavage properties. Capture of an EDDIE fusion protein comprising a 2-kDa polypeptide as the fusion partner is also described along with on-column refolding procedures as a potential process application.

2. Materials and methods

2.1. N^{pro} fusion protein production

Recombinant protein expression, fermentation, isolation of IBs, and chromatographic purification of N^{pro} fusion proteins are described elsewhere in detail [11,12]. Proteins were extracted from IBs by suspension 1:5 in dissolution buffer containing 10 M urea and 50 mM Tris at pH 8.0, supplemented with 5 mM monothioglycerol (MTG) as reducing agent. IBs were allowed to dissolve for at least 30 min. The protein solution was then centrifuged (Centrifuge 5415R, Eppendorf AG, Hamburg, Germany) for 30 min at 13,200 rpm and 4 °C. Afterwards, the supernatant was removed using a 10-mL syringe (Omnifix®, B. Braun Melsungen AG, Germany) and filtered using 0.80-μm and 0.22-μm pore diameter filter units (Millipore, Billerica, USA). Protein concentration was measured using UV/vis spectrophotometry (Cary 50 UV-Vis spectrophotometer, Varian, Victoria, Australia) at 280 nm. For binding assays or chromatographic experiments, the solutions were buffer exchanged or diluted with equilibrium buffer to obtain the specified protein concentration.

2.2. Spot synthesis

Cellulose-bound peptide libraries were semi-automatically prepared according to the method first published by Frank [15], slightly modified as described by Pflegerl et al. [16] and Duerauer et al. [17]. Briefly, peptides were immobilized C-terminally on cellulose membranes via double β-alanine anchors, and a conventional 9-fluorenylmethoxycarbonyl (Fmoc) technique was used for further peptide assembly. All amino acids as well as PyBOP® were purchased from Novabiochem (Läuflingen, Switzerland). After N-terminal acetylation of the last amino acid and cleavage of side-chain protection groups by treatment with trifluoroacetic acid (TFA), membranes were used immediately or dried and stored at −20 °C. Prior to usage, membranes were conditioned with 20% (v/v) methanol for at least 2 h.

2.3. Biotinylation of samples

Protein samples were provided in phosphate buffered saline (PBS), pH 7.3, at a concentration of around 0.5–3 mg mL^{-1}. In case of N^{pro} fusion proteins, the biotinylation reaction was carried out in the presence of 4 M urea to retain samples in their denatured state. Succinimidyl 6-(biotinamido)-hexanoate (Sigma, Vienna, Austria) was dissolved in dry dimethylformamide (DMF) at a concentration of 20 mmol. This stock solution was added to the sample solution to obtain a 5-fold molar excess. The reaction mixture was incubated for 2 h at room temperature or alternatively overnight at 4 °C. Reaction byproducts were removed by gel filtration using PD10 columns (GE Healthcare, Uppsala, Sweden).

2.4. Binding assays

2.4.1. General procedure

Spot membranes were washed three times with incubation buffer and blocked with a 3% bovine serum albumine (BSA) solution overnight. In the initial project phase, a solution of 20 mM sodium acetate, 0.5 M urea, 150 mM NaCl, and 0.1% Tween 20, pH 5.5, was used as the incubation buffer. Later, the standard incubation buffer contained 100 mM phosphate, 4.0 M urea, 150 mM NaCl, and 0.1% Tween 20, pH 7.3. Changes to this standard buffer concerned different concentrations of urea and detergents, e.g., sarcosin instead of Tween as the detergent, guanidinium hydrochloride (GuHCl) instead of urea as the chaotrop, and the addition of MTG. Biotin-labeled samples were diluted to a concentration in the range of 0.1–5 μg mL^{-1} in incubation buffer containing 1% BSA. After a 1-h incubation with the sample, the membranes were washed three times with incubation buffer and once more with incubation buffer without urea. After incubation of N^{pro}-pep6His wildtype samples, a horseradish peroxidase (HRP)-conjugated Penta His antibody (Qiagen) was diluted 1:2000 in the provided blocking reagent and incubated for 1 h, followed by 3–4 washes with blocking reagent. Biotin-labeled samples were reacted with a streptavidin-HRP conjugate (Sigma, Vienna, Austria), diluted 1:2000 into phosphate buffer containing 0.8 M NaCl, for 15 min. Subsequent detection was carried out using the Super Signal™ West Pico chemiluminescence detection system (Pierce, Rockford, IL, USA) and Lumi-Imager™ (Boehringer, Mannheim, Germany). All buffer components were purchased from Merck (Vienna, Austria).

2.4.2. Competition assay

Competition assays were carried out according to the standard procedure with a pre-formulated solution containing 1.0 μg mL^{-1} of biotin-labeled EDDIE–pep6His and a 10- or 100-fold excess of unlabeled protein.

2.5. Solid phase peptide synthesis

Solid phase peptide synthesis was performed on a 433A peptide synthesizer (Applied Biosystems, Vienna, Austria) with 1-hydroxy-1H-benzotriazol/N,N′-dicyclohexylcarbodiimide

(HOBt/DCC)-activation of Fmoc-protected amino acids (Bachem, Bubendorf, Switzerland). Peptides were synthesized on a 4-hydroxymethyl-phenoxymethyl-copolystyrene-1% divinylbenzene resin (HMP resin, Wang resin). Protecting groups for side chains were tert-butyl for tyrosine, serine, and threonine; O-tert-butyl for glutamic acid and aspartic acid; tert-butoxycarbonyl for lysine; and tryptophan and trityl for cysteine, histidine, asparagine, and glutamine. For the coupling of the first amino acid, 4-dimethylaminopyridine was used as a catalyst. After coupling of the first amino acid, a capping step was accomplished using benzoic anhydride. Deprotection of the Fmoc group was performed with 20% piperidin. Side-chain deprotection and cleavage from the resin were carried out by reaction with a cleavage mixture containing 95% TFA, 2.5% water, and 2.5% triisopropylsilane. After being washed with dichloromethane, the crude peptide was purified by repeated ether precipitation followed by lyophilization. The peptides were further purified by RP-HPLC on a Luna 15μ C18(2) 250 mm × 21.2 mm column (Phenomenex, Torrance, CA, USA) with P 3500 pumps (GE Healthcare, Uppsala, Sweden), using a linear gradient of 5–50% acetonitrile vs. water (0.1% TFA) at 30 mL min^{-1}. Purity was confirmed by analytical RP-HPLC with an HP 1090 liquid chromatograph (Hewlett Packard, USA) using a Luna 3μ C18(2) 100 mm × 4.6 mm column (Phenomenex, Torrance, CA, USA) with a linear gradient of 1% acetonitrile per minute. Homogeneity and identity were verified by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (ThermoBioanalysis, Hempstead, UK).

2.6. Preparation of peptide affinity sorbents

2.6.1. Preparation of spacer arms on affinity sorbents

A total of 10 g of Fractogel® epoxy (M) (Merck, Darmstadt, Germany) was reacted with 50 mL 1 M diaminodipropylamine (DADPA) for 48 h at room temperature. After the reaction, the gel was washed with 50 mL of 10 mM HCl and three times with 50 mL of water. The gel was resuspended in water, pH was adjusted to 7.0 by addition of 0.1 M NaOH, and 2 g of succinic anhydride were added. After 30 min of gentle stirring, the pH was adjusted to 7.0 by addition of 10 M NaOH, and another 2 g succinic anhydride were added. After another 30 min of stirring, the gel was washed with 50 mL of 0.1 M NaOH, 50 mL PBS, and three times with 50 mL water and 20% ethanol. The spacer synthesis on sepharose-based media was performed in the same manner. Reactions were carried out at a 5-mL scale. HiTrap N-hydroxysuccinimide (NHS) activated Sepharose HP and Epoxy Sepharose 6B were purchased from GE Healthcare (Uppsala, Sweden). Actigel B Ultraflow 6 was obtained from Sterogen (Arcadia, CA, USA). After suction drying, gels were stored at 4 °C until further use. Gels prepared in this manner are referred to as DADPA-SA media.

2.6.2. Activation of the carboxy groups and immobilization of peptides

A total of 3 g of wet sorbent, corresponding to approximately 3 mL of swollen gel, was washed twice with 6 mL of acetonitrile. Activation was performed with 6 mL of 0.1 M succinimidyl-trichloroethylcarbonate and 0.1 M triethylamine dissolved in acetonitrile for 3 h. The gel was subsequently washed with acetonitrile and 1 mM HCl. Peptides were dissolved in coupling buffer at a concentration of 3 mg mL^{-1}. Then, 6 mL of the peptide solution was rapidly added to the gel and reacted for 24 h. Alternatively, in case of poor solubility, the peptides were dissolved in DMF containing 0.1 M triethylamine. Coupling yield was determined by RP-HPLC of samples before and after coupling. All reagents for activation were purchased from Sigma.

2.6.3. Site-directed immobilization of peptides

For site-directed immobilization through the C-terminus, peptides were synthesized with an additional C-terminal lysine residue. The ε-amino group of this lysine was reacted with iodoacetic anhydride and coupling of this reactant onto media with terminal thiol groups (Fractogel-DADPA-IT). Briefly, 300 mg of N-acetylated peptide with C-terminal lysine (for instance, Ac-AFYRWYAK) were dissolved in 3 mL DMF containing 65 μL diisopropylethylamine. The solution was then cooled on ice. A total of 130 mg of iodoacetic anhydride was dissolved in 1.5 mL DMF and added to the peptide solution. The reaction was stopped after 1 min by addition of 1 mL of formic acid. The solution was then diluted with water to a final DMF concentration of 30% and purified by preparative RP-HPLC as described before. Fractions were freeze-dried and analyzed by mass spectrometry to identify mono-, di-, and poly-substituted reaction products.

Fractogel-DADPA was prepared as described above, and the 10-g wet gel was subsequently washed three times with PBS buffer. The gel was then reacted with 10 mg mL^{-1} iminothiolane dissolved in PBS buffer for 2 h. A total of 250 mg of the peptide–iodoacetic acid derivative was dissolved in 15 mL of 20 mM MES buffer, pH 6.0, containing 30% DMF. This solution was then reacted with the gel for 3 h. Coupling yield was determined by analyzing samples before and after coupling with RP-HPLC. Remaining thiol groups on the gel were blocked with 1 mg mL^{-1} iodoacetamide solution for 2 h. The matrix prepared in this manner is referred to as Fractogel-DADPA-IT-AFYRWYAK. All reagents for activation and derivatization were purchased from Sigma.

2.6.4. Preparation of an affinity matrix with poly amino acid ligands

A total of 10 g Fractogel epoxy was washed three times with coupling buffer (20 mM sodium carbonate buffer containing 150 mM sodium chloride and 10 mM triethylamine, pH 11.0). Then, 100 mg of poly(lysine, tryptophan), 4:1 (PolyKW, Sigma) were dissolved in 10 mL coupling buffer and reacted with the gel for 24 h at 40 °C. Coupling efficiency was determined by measuring the absorbance at 280 nm of the poly(lysine, tryptophan) solution before and after coupling. The gel was then reacted with 0.5 M ethanolamine to block any remaining epoxy groups. The matrix that resulted from this preparation is referred to as Fractogel-polyKW.

Alternatively, the described procedure was carried out with Actigel B Ultraflow 4 epoxy, and this matrix is referred to as Actigel-polyKW. In addition, the described procedure was carried out with Epoxy Sepharose 6B, yielding a matrix referred to as Sepharose-polyKW.

Activation of polyKW gels was performed by incubation of the gels with 25% glutaraldehyde in phosphate buffer, pH 7.0, for 12 h at room temperature.

2.6.5. Preparation of peptide affinity media by direct on-resin peptide synthesis

Instead of an HMP-resin, Fractogel-DADPA or Toyopearl-EDA was used and peptide synthesis was performed as described above.

2.6.6. Immobilization of peptides on CIM epoxy

Peptides were dissolved in 100 mM Na_2CO_3 buffer, pH 10.0, containing 0.15 M NaCl. CIM-epoxy monolithic disks were obtained from BIA Separations (Ljubljana, Slovenia). A disk (volume 0.34 mL) was mounted in a cartridge supplied by the manufacturer, and the peptide solution was slowly pumped through the disk using a P1 pump (GE Healthcare) in circulation mode for 48 h at room temperature. Coupling yield was determined by RP-HPLC of samples before and after coupling. After coupling, any remaining epoxy groups were blocked with 0.5 M ethanolamine, pH 10.0, for 48 h.

2.6.7. Amino acid analysis

Peptide supports were dried and subjected to amino acid hydrolysis with 6 M hydrochloric acid at 115 °C for 20 h. The resulting amino acids were analyzed on a 5-μm Hypersil column (Forschungszentrum Seibersdorf, Vienna, Austria) 4 mm in diameter and 250 mm in length, using a Hewlett-Packard HP 1050 Series system with a Shimadzu RF 535 variable wavelength fluorescence detector. Pre-column derivatization was performed with o-phthaldehyde. Cys and Trp cannot be detected by this method because these amino acids are destroyed during hydrolysis.

2.7. Chromatographic procedures

2.7.1. Batch adsorption and adsorption isotherms

Batch adsorption experiments were performed to determine the equilibrium binding capacities and adsorption isotherms, respectively. The peptide affinity media were divided into 50-μL portions using Media Scout® ResiQuot (Atoll, Weingarten, Germany), packed into a Microlute™ plate (Porvair Sciences, Leatherhead, UK), which consists of 96 chambers with a 2-mL reaction volume and ceramic frits at the bottom. Matrices were equilibrated with 4 M urea, 50 mM Tris, and 100 mM NaCl, pH 7.3, containing either 0 or 5 mM MTG. Buffer was exhausted using a vacuum pump (Diaphragm Vacuum Pump, Vacuubrand, Wertheim, Germany). IBs were dissolved as described before. Buffer exchange of the sample solution into equilibrium buffer was achieved using PD10 columns (GE Healthcare). Protein solutions at different concentrations and volumes were applied to the media and incubated for 12 h. Sample solution was extracted by suction and transferred into microtiter plates, and the protein concentration was determined using a GENios Pro™ UV reader (Tecan, Crailsheim, Germany) at 280 nm. The binding capacity was calculated by a mass balance considering the applied amount of protein, the medium volume, and amount of protein determined in the supernatant. Maximum binding capacity and affinity constants were determined by fitting the data points with the Langmuir isotherm using TableCurve 2D v5.0 (SPSS, Erkrath, Germany).

2.7.2. Column experiments

Chromatographic experiments were performed using the ÄKTA explorer (GE Healthcare, Uppsala, Sweden) controlled by UNICORN software version 5.10. Chromatography media were packed into Tricorn columns (GE Healthcare) with inner diameters of 5 or 10 mm at a column height of 5 cm. Matrices were equilibrated with equilibrium buffer containing 4 M urea, 50 mM Tris, 100 mM NaCl, and 5 mM α-MTG at pH 7.3. Protein samples from IBs were diluted with equilibration buffer, or IB solutions were directly loaded onto the column allowing 10 min residence time. Unbound material was washed out with equilibrium buffer. Conditioning was performed using three column volumes (CVs) of conditioning buffer (0.8 M urea, 50 mM NaAc, 0.25 M sucrose, 2 mM EDTA, and 20 mM MTG at pH 8.5). Refolding was then performed using three CVs of refolding buffer (0.8 M urea, 1.5 M Tris, 0.25 M sucrose, 2 mM EDTA, 20 mM MTG, and 0.1% sarcosine, pH 8.5) over 3–6 h. The column was regenerated using 0.5 M NaOH. Fractions of the flow-through, conditioning, refolding, and regeneration steps were collected and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and RP-HPLC.

2.7.3. Analytical methods

SDS-PAGE was performed on NuPage®-Bis-Tris 4–12% gradient gels (Invitrogen, Carlsbad, CA, USA) in the Xcell II™ Mini-Cell (Invitrogen, Carlsbad, CA, USA). SDS-PAGE was performed with MES SDS running buffer, prepared as described by the supplier, at a constant 200 V and 400 mA for 50 min. Samples were prepared with NuPage®-LDS sample buffer supplemented with 0.1 M dithiothreitol. SeeBlue® Plus2 pre-stained standard markers were purchased from Invitrogen. Proteins were detected using the Colloidal Blue stain kit (Invitrogen). Intensities of the EDDIE–pep6His and EDDIE bands were determined using Image Analysis Software of the Lumi-Imager (Boehringer Mannheim, Mannheim, Germany).

RP-HPLC analysis of pep6His was performed using a Jupiter™ C-4 column (2 mm × 150 mm, 5 μm, 300 Å; Phenomenex, Torrance, CA, USA) on an Agilent 1100 series system (Waldbronn, Germany) using an additional SecurityGuard™-cartridge.

3. Results and discussion

A main target of the N^{pro} fusion system is the expression of peptides and small- to medium-size proteins fused to the C-terminus [11]. Subsequently, these targets are released by exact auto-cleavage after the C-terminal cysteine residue, which occurs upon refolding of the denatured N^{pro} fusion protein. Our goal was to screen for peptide affinity ligands to develop an affinity chromatography system that can bind N^{pro} fusion proteins independently of the fusion partner. Furthermore, this chromatography system should also serve as a platform for on-column refolding and cleavage. As the prime model partner for screening, we selected a small peptide with the sequence H_2N-SVDKLAAALEHHHHHH-COOH (pep6His). It has been shown that cleavage efficiency depends to a certain degree on the nature of the first amino acid of the target protein. Small, polar amino acids achieve higher cleavage rates and yields in contrast to large, hydrophobic amino acids present at the first position of the fusion partner [11,13]. This peptide enables a good cleavage rate because of the serine at the first position and facilitates easy detection and purification through the his-tag. Screening was performed by combinatorial peptide libraries using the spot synthesis technique, a solid phase peptide synthesis-based method performed on cellulose or synthetic

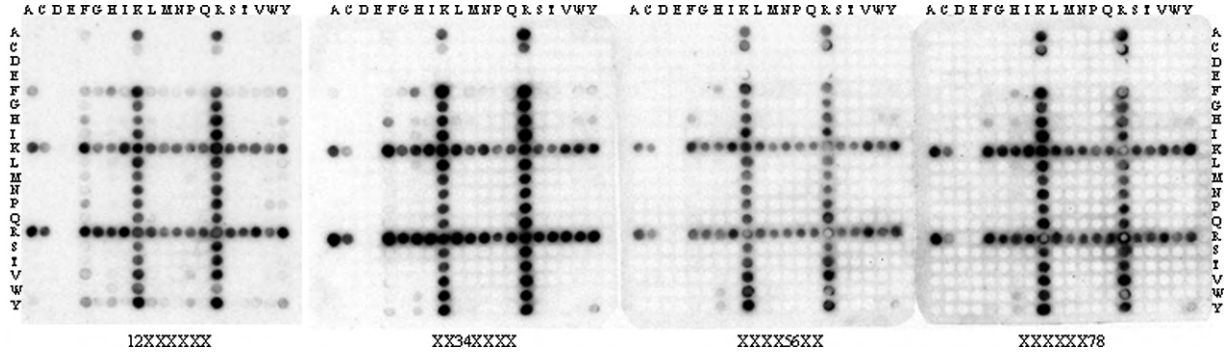

Fig. 1. Dual-positional scan of EDDIE–pep6His on a combinatorial octapeptide library. Biotinylated EDDIE–pep6His from IBs was applied at a concentration of 5 μg mL^{-1} in PBS and 4 M urea, 0.1% Tween 20. Detection was performed with streptavidin-HRP conjugate.

membranes [15,18,19]. A group of peptides, which shared negatively charged and hydrophobic amino acids as a common motif, could be identified to bind N^{pro}-6his under denaturing conditions of 4 M urea. Affinity media with immobilized peptides were able to bind N^{pro} fusion proteins. (A detailed description of the screening procedure and affinity media preparation is provided in the supplemental data.) However, when refolding was initiated by switching from chaotropic to kosmotropic conditions, precipitation of wildtype N^{pro} occurred and columns were irreversibly blocked. These results were in line with those of the batch refolding experiments using different N^{pro} fusion proteins. In the course of the project, the N^{pro} autoprotease wildtype was engineered by site-directed mutagenesis, and a tailor-made mutant, called EDDIE, was developed. EDDIE features improved autoproteolytic and solubility properties, is less hydrophobic, and has a lower isoelectric point compared to the wildtype. This improvement was achieved by exchange of basic and hydrophobic amino acids (arginine, valine, leucine, isoleucine, phenylalanine) with acidic or hydrophilic residues (glutamic acid and threonine). Also, the number of cysteines was reduced.

As a consequence of this substantial mutation, screening for peptide ligands had to be restarted. Fig. 1 shows the screening results with EDDIE–pep6His using a dual positional scan strategy with an octapeptide library.

The spot pattern was similar to that observed with N^{pro} wildtype, except that instead of the acidic amino acids, basic residues (lysine and arginine) were found to be essential for binding. This result was not too surprising because it reflected only the exchange of the respective amino acids on the N^{pro} and EDDIE mutant, respectively. Based on the screening results, a peptide with the sequence AFYRWYA was arbitrarily defined as a starting point or designated standard for a further sequence refinement. The following questions were addressed in this next screening round: (1) How far can sequence and size of the peptide, respectively, be reduced while still retaining the ability to bind EDDIE? (2) How sensitive is the reaction to negatively charged amino acids? (3) What are the influences of detergents, reducing agents, and type and concentration of chaotrope on the binding reactions? (4) How specific are the binding reactions?

To investigate these questions, peptides with specific features were synthesized and screened under various binding conditions (Fig. 2). The selected peptides represented dipeptides comprising one positively charged and one aromatic acid (KW, KY, KF, RW, peptides 1, 3, 5, and 7) as well as tripeptides into which a negative charge was introduced by addition of aspartic or glutamic acid (KWD, KYE, KFD, RWD, peptides 2, 4, 6, and 8). Peptides 9 and 10 were tetrapeptides comprising two aromatic amino acids, alanine and either lysine or arginine. Peptide 11 contained an additional aspartic acid. Peptide 12 represented the standard peptide AFYRWYA and peptide 13 the control with an additional aspartic acid. Peptides 14–20 were modifications of the standard peptide with exchange of one or two amino acids. Screening was performed with biotinylated EDDIE–pep6His at a concentration of 0.1 $\mu g\,mL^{-1}$. The light exposure time was always kept at a minimum to discriminate between different peptides. At this point, we again note that the obtained signals are not quantitative and represent relative binding strengths. To identify a non-binder, higher protein concentrations and prolonged light exposure were applied. If under these conditions binding was still not observed, respective spots were classified as non-binding peptides (data not shown). Fig. 2A–C shows binding assays at different detergent, reducing agent, and urea concentrations.

Amazingly, peptides could be reduced to the size of a dipeptide, and binding still occurred with such peptides containing a positively charged and one aromatic amino acid (peptides 1, 3, 5, and 7). A combination of, for instance, KK or FF resulted only in very weak binding (data not shown). The results show that the peptide–EDDIE

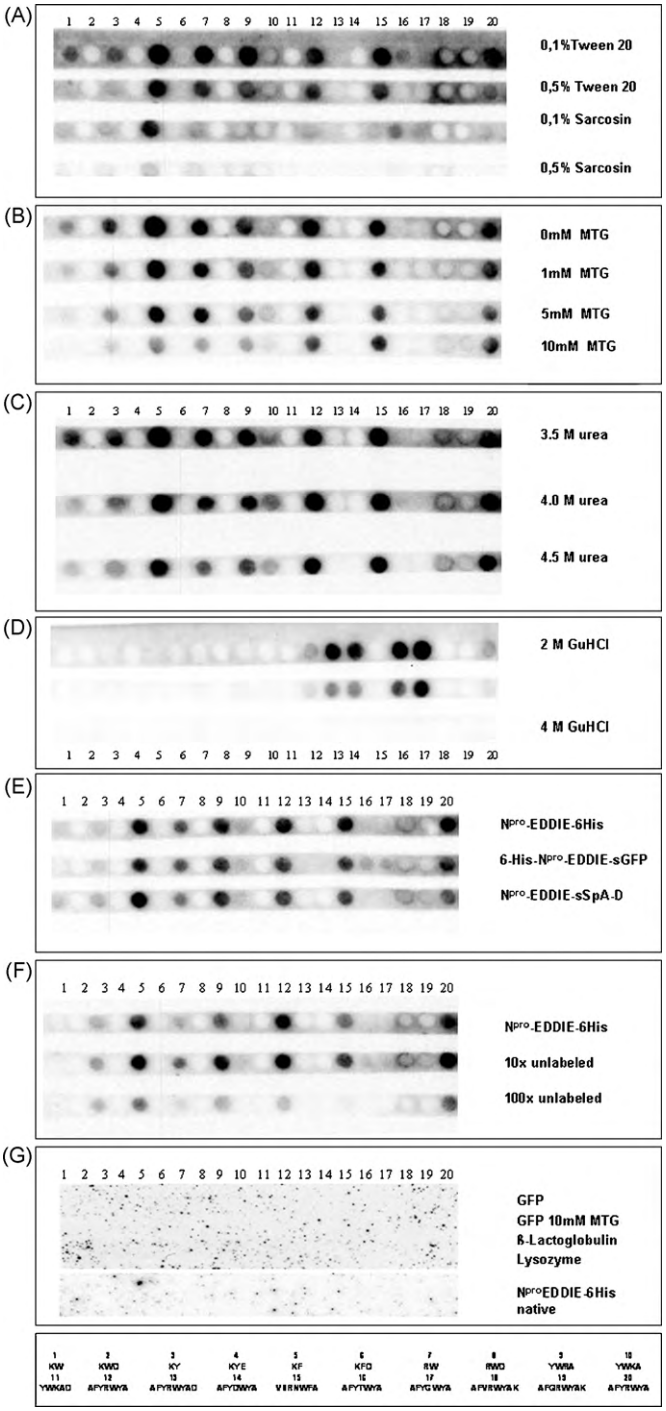

Fig. 2. Screening of selected peptides with biotinylated samples under various buffer conditions. Standard buffer conditions were PBS, 4 M urea, and 0.1% Tween 20; additives and alternative buffers are given in the respective figure. If not indicated elsewhere, the sample was EDDIE–pep6His at a concentration of 0.5 $\mu g\,mL^{-1}$. (A) Influence of detergents at different concentrations. (B) Influence of reducing agent MTG. (C) Influence of urea concentration. (D) Screening at GuHCl instead of urea as chaotropic agent. (E) Influence of fusion partner; peptide pep6His, green fluorescence protein (GFP), and *Staphylococcus aureus* protein A domain D (sSpA-D). (F) Competition assay of biotinylated EDDIE–pep6His with excess of unlabeled protein. (G) Binding assay with denatured model proteins GFP, β-lactoglobulin, and lysozyme. Assay with native biotinylated EDDIE–pep6His. Peptide sequences: (1) KW, (2) KWD, (3) KY, (4) KWD, (5) KF, (6) KFD, (7) RW, (8) RWD, (9) YWRA, (10) YWKA, (11) WWKAD, (12) AFYRWYA, (13) AFYRWYAD, (14) AFYDWYA, (15) VSRNWFA, (16) AFYTWYA, (17) AFYGWYA, (18) AFVRWYAK, (19) AFQRWYAK, and (20) AFYRWYA.

Table 1
Peptide affinity media against EDDIE fusion proteins based on hexa- or octapeptides.[a]

Nr.	Base matrix and activation	Ligand	Coupling buffer	Peptide applied (mg mL^{-1} gel)	Coupling yield (%)	Ligand density (mg mL^{-1})	Binding EDDIE–pep6His
C25	Fractogel-DADPA-SA	AFYRWY	PBS	11	91	10	–
C28	Fractogel-DADPA-SA	Ac-AFYRWYAAKKK	Direct synthesis			16–22[*]	Unspecific
C31	Fractogel-DADPA-SA	Ac-AFYRWYAA	Direct synthesis			34–45[*]	Unspecific
C37	NHS-Sepharose HP	AFYRWYA	PBS/DMF	15	54	8.1	–
C38	NHS-Sepharose HP	Ac-AFYRWYAK	PBS/DMF	9.4	50	4.7	–
C39	Epoxy Sepharose	AFYRWYA	0.1 M Na_2CO_3, 0.5 M NaCl pH 11	11.5	63	7.3	–
C40	Epoxy Sepharose	Ac-AFYRWYAK	0.1 M Na_2CO_3, 0.5 M NaCl pH 11	11.5	78	9.0	–
C45	Fractogel-DADPA-SA	AFYRWYAK	PBS/DMF	15.4	63	9.7	–
C47	Fractogel-DADPA-SA	AFYRWYA	PBS	20.6	68	15.8	–
C53	Actigel 6 DADPA-SA	AFYRWYA	PBS	15.1	99	15	–
C68	Actigel B Ultraflow 4	Ac-AFYRWYAK	0.1 M Na_2CO_3, 0.5 M NaCl pH 11	40	46	18.5	–
C69	Fractogel-epoxy (M)	Ac-AFYRWYAK	0.1 M Na_2CO_3, 0.5 M NaCl pH 11	40	88	35.3	–
C70	Fractogel-polyK (C67)-ALD	Ac-AFYRWYAK	PBS, 10% DMF	40	100	40	+
C71	Actigel B Ultraflow 4-polyK (C66)-ALD	Ac-AFYRWYAK	PBS, 10% DMF	40	63	25.3	–
C74	Superdex 200 BDGE-DADPA-SA	Ac-AFYRWYAK	PBS, 10% DMF	30	42	12.7	–
C75	Superdex 75 BDGE-polyK-ALD	Ac-AFYRWYAK	PBS, 10% DMF	25	100	25	–
C77	Fractogel-polyK-BDGE	Ac-AFYRWYAK	0.1 M Na_2CO_3, 0.5 M NaCl pH 10	20	45	9	–
C100	Fractogel –DADPA-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	10	77	7.7	+
C102	Fractogel –DADPA-IT	Ac-YWRAK-IAc	MES CB pH 6.0	20	100	20	12.5 (7.6)
C103	Fractogel –DADPA-IT	Ac-AFSTWYAK-IAc	MES CB pH 6.0	20	100	20	–
C104	Fractogel –DADPA-IT	Ac-AFQRWYAK-IAc	MES CB pH 6.0	20	100	20	9.4 (6.7)
C105	Fractogel –DADPA-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	20	100	20	9.6 (6.7)
C106	Fractogel-KKK-IT	Ac-AFQRWYAK-IAc	MES CB pH 6.0	20	100	20	9.0 (6.2)
C107	Fractogel-KKK-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	20	100	20	9.7 (5.8)
C108	Fractogel-polyK-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	20	100	20	4.2 (2.6)
C111	Sepharose 6B DADPA-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	22	100	22	2.8 (2.1)
C112	Profinity-DADPA-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	25	100	25	5.5 (5.4)
C113	Fractogel-DADPA-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	29.1	84	24.3	10.8 (6.7)
C115	Sepharose-polyK-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	20	100	20	6.3 (4.3)
C119	Superdex 30 DADPA-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	11.6	100	11.6	4.2 (3.1)
C120	Superdex 75- DADPA-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	11.6	100	11.6	5.5 (3.6)
C121	Superdex 200- DADPA-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	11.6	100	11.6	7.4 (5.9)
C123	Fractogel-DADPA-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	24.8	100	24.8	13.3 (6.4)
C124	Superdex 30 DADPA-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	25	100	25	5.2 (4.3)
C125	Superdex 75 DADPA-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	25	100	25	7.0 (4.0)
C126	Superdex 200 DADPA-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	25	100	25	n.d.
C137	Fractogel-DADPA-IT	Ac-AFYRWYAK-IAc	MES CB pH 6.0	42	80	33.5	14.2 (8.1)
C139	Fractogel-DADPA-IAcAH	Ac-AFYRWYAC	MES CB pH 6.0	22	84	18.6	6.8 (4.5)
C140	Sepharose-DADPA-IAcAH	Ac-AFYRWYAC	MES CB pH 6.0	22	43	9.5	n.d.

[a] Binding capacities were determined by column experiments (–, no binding; +, weak binding of a 300 μg sample pulse) or batch adsorption. Binding capacities were determined at initial protein concentrations of 5 mg mL^{-1} EDDIE–pep6His and 3 mg mL^{-1} (values given in parenthesis). *Abbreviations*: Ac, acetylated; ALD, aldehyde; BDGE, butandioldiglycidylether; DADPA, diaminodipropylamine; DMF, dimethylformamide; EDA, ethylenediamin; IT, imminothiolane; IAc, iodoacetylated; MES CB, MES coupling buffer: 20 mM MES 150 mM NaCl, 20% DMF, pH 6.0; NHS, N-hydroxysuccinimide; PBS, phosphate buffered saline; SA, succinic anhydride; TEA, triethylamine.

[*] Determined by amino acid analysis.

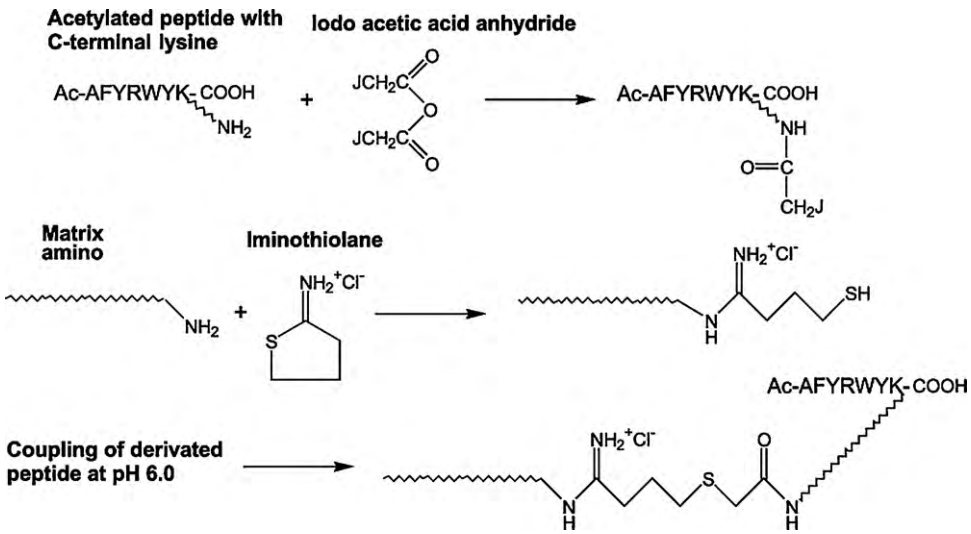

Fig. 3. Preparation of peptide affinity media by site-directed immobilization. Acetylated peptides with C-terminal lysine were reacted with iodo acetic acid anhydride. Base matrices with amino function were reacted with imminothiolane resulting in free sulfhydryl groups which reacted rapidly with the iodine group of the derivatized peptide.

interaction is very sensitive to the negatively charged amino acids glutamic acid and aspartic acid, which can completely suppress binding (peptides 2, 4, 6, and 8), even when a positively charged amino acid is present (peptides 13 and 14). In addition, binding under reducing conditions was less strong (compare lanes 1 and 2). For peptides without any charge but with tryptophan and tyrosine, the binding strength was significantly reduced (peptides 16 and 17). The strongest binding among all combinations was obtained for peptide 5 (KF) and peptide 12 (AFYRWYA). In general, binding strength decreased with increasing concentrations of detergents, reducing agents, and urea.

Screening was also performed in the presence of GuHCl instead of urea as the chaotrope (Fig. 2D). GuHCl is a stronger denaturing compound than urea, and the interaction was much weaker at the same concentration range. A striking observation was the fact that only octapeptides comprising negatively charged amino acids (peptides 13 and 14) or peptides without positive charges (peptides 16 and 17) could be identified as binders. Di- and tetrapeptides did not bind EDDIE–pep6His at all. Presumably, the positive charge of GuHCl shielded the carboxy groups of glutamic and aspartic acid. Overall, GuHCl does not seem to be a useful alternative to urea, particularly because the dipeptides did not show binding and these peptides represent a preferred ligand type for affinity chromatography, as will be shown below.

Fig. 2E–G shows the results of the probe for specificity of the interaction. Fig. 5E confirms the specificity of the peptides against the EDDIE moiety. The spot pattern was essentially the same regardless of the fusion partner (peptide pep6His, GFP, or sSpA-D). A competition assay, shown in Fig. 5F, was performed to test for non-specific binding of peptides to the biotin residue. At a 100-fold excess of unlabeled EDDIE–6His, the signal intensity was significantly reduced, indicating that the peptides had bound to the protein and no non-specific adsorption phenomenon was taking place. At a concentration of 1 $\mu g\,mL^{-1}$ and a 10-fold excess of unlabeled protein, there was obviously still enough biotinylated EDDIE–6His present to produce the same signal intensity that was achieved without addition of competitor. This result was very likely because the binding capacity of a spot was unknown and usually the working range for incubation was chosen to be in excess of sample to produce strong signals.

Fig. 2G shows negative controls with other proteins (GFP, β-lactoglobulin, and lysozyme) performed under denaturing conditions of 4 M urea. As can be seen, no (non-specific) interaction took place. The last row in Fig. 2G shows the interaction with native EDDIE in pure PBS buffer. Surprisingly, there was no binding observable. A possible explanation would be that the interaction site(s) is/are located in the interior of the EDDIE molecule and thus is/are only accessible when the molecule is denatured.

The next step in the design of a functional affinity system was the development of an appropriate immobilization strategy. Previous research had identified ligand presentation and accessibility as the most crucial criteria for generating a functional affinity medium with a short peptide as an affinity ligand [20–24]. At an early development stage, binding efficiency was measured by injecting a 100-μL sample pulse containing 3 $mg\,mL^{-1}$ EDDIE–pep6His onto a 1-mL column, and binding was classified as complete (+++), weak (++, +), or none (−). Later, binding capacities were determined precisely by batch adsorption experiments.

Based on the screening results of the spot membranes, the peptide AFYRWYA was selected as a target. Table 1 gives a comprehensive summary of the most relevant matrices prepared by different immobilization strategies using this peptide.

At first, the successful strategy using Fractogel with the extended spacer arm and performing immobilization through the N-terminus was retried (C25), but binding of EDDIE could not be obtained. Direct synthesis of the peptide on the chromatography medium with (C28) and without a lysine-tree (C29) resulted in rather high ligand densities, as determined by amino acid analysis. Both media strongly bound EDDIE, but the binding seemed to be a rather non-specific adsorption because other proteins were bound in considerable amounts, as well. Next, immobilizations were carried out on commercial NHS Sepharose HP and Epoxy Sepharose (C37–C40, C45), as well as on custom-made gels with an extended spacer arm (C43–45, C47), applying the introduction of organic solvent during the immobilization procedure as well as changing the direction of the peptide through a C-terminal lysine immobilization. None of these attempts, however, resulted in a substantially increased ligand density or binding of EDDIE. Immobilization was then tried at extremely high starting concentrations of peptide (40 $mg\,mL^{-1}$ of medium; C68, C69); with this approach, a ligand density of 35 $mg\,mL^{-1}$ could be achieved on Fractogel. Regardless of this excellent coupling yield, EDDIE did not bind. To achieve a better steric presentation of the ligand, a novel spacer type was introduced by immobilization of a 30-kDa poly amino acid (poly lysine). The residual ε-amino groups were reacted with glutaraldehyde, and subsequently the peptide could be immobilized at a high ligand

Table 2
Peptide affinity media against EDDIE fusion proteins based on polyamino acids.[a]

Nr.	Base matrix and activation	Ligand	Coupling buffer	Peptide applied (mg mL^{-1} gel)	Coupling yield (%)	Ligand density (mg mL^{-1})	Binding EDDIE–pep6His
C59	Fractogel-epoxy (M)	polyKW, polyKY	0.1 M Na_2CO_3, 0.5 M NaCl pH 10	15	100	15	n.d.
C60	Actigel B Ultraflow 4	polyKW, polyKY	0.1 M Na_2CO_3, 0.5 M NaCl pH 10	15	100	15	n.d.
C61	Fractogel-polyKW-KY-(C59)ALD	AFYRWYA	PBS	20	100	20	+
C62	Actigel B Ultraflow 4-polyKW-KY-(C60)ALD	AFYRWYA	PBS	20	100	20	–
C63	Actigel B Ultraflow 4	polyKY	0.1 M Na_2CO_3, 0.5 M NaCl pH 10	15	85	12.8	+
C64	Actigel B Ultraflow 4	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 10	10	100	10	++
C65	Fractogel-epoxy (M)	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 10	10	100	10	+++
C66	Actigel B Ultraflow 4	polyK	0.1 M Na_2CO_3, 0.5 M NaCl pH 10	10	100	10	n.d.
C67	Fractogel-epoxy (M)	polyK	0.1 M Na_2CO_3, 0.5 M NaCl pH 10	10	100	10	n.d.
C72	Fractogel-epoxy (M)	polyKW, polyKY	0.1 M Na_2CO_3, 0.5 M NaCl pH 10	10	100	10	++
C73	Actigel B Ultraflow 4	polyKW, polyKY	0.1 M Na_2CO_3, 0.5 M NaCl pH 10	10	100	10	++
C76	Fractogel	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 10	40	56	22.5	–
C92	Actigel B Ultraflow 4	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 11	5	100	5	+
C93	Actigel B Ultraflow 4	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 11	15	100	15	++
C94	Actigel B Ultraflow 4	polyKY	0.1 M Na_2CO_3, 0.5 M NaCl pH 11	5	100	5	+++
C95	Actigel B Ultraflow 4	polyKY	0.1 M Na_2CO_3, 0.5 M NaCl pH 11	10	100	10	+++
C116	Superdex 30-BDGE	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 11	10	56	5.6	12.0 (8.9)
C117	Superdex 75-BDGE	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 11	10	59	5.9	10.9 (7.1)
C118	Superdex 200-BDGE	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 11	10	62	6.23	8.6 (5.4)
C127	Epoxy High Flow	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 8.0; 40 °C	15	89	13.3	n.d.
C128	Epoxy High Flow	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 8.0; 40 °C	5	75	3.7	n.d.
C129	Epoxy Sepharose 6B	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 8.0; 40 °C	15	79	11.9	8.0 (4.9)
C130	Epoxy Sepharose 6B	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 8.0; 40 °C	5	85	4.2	4.7 (3.7)
C131	Fractogel epoxy	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 8.0; 40 °C	15	98	14.6	13.3 (8.8)
C132	Fractogel epoxy	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 8.0; 40 °C	5	98	4.9	9.8 (5.7)
C133	Profinity epoxy	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 8.0; 40 °C	15	97	14.6.	11.0 (7.7)
C134	Profinity epoxy	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 8.0; 40 °C	5	98	4.9	12.8 (7.6)
C135	Actigel B Ultraflow 4	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 8.0; 40 °C	15	98	14.7	13.5 (7.6)
C136	Actigel B Ultraflow 4	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 8.0; 40 °C	5	94	4.7	11.4 (4.7)
C138	Superdex 200-BDGE	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 8.0; 40 °C	10	100	10	11.6 (7.2)
C141	Fractogel epoxy	polyKW	0.15 M Na-Ac, pH 3.0; 40 °C	15	44	6.6	n.d.
C150	Toyopearl AF-Epoxy	polyKW	0.1 M Na_2CO_3, 0.5 M NaCl pH 9	15	89	13.4	10.9 (7.1)
C152	Toyopearl AF-Epoxy	polyKY	0.1 M NaOH, 0.5 M NaCl, pH 12	15	n.d.	n.d.	n.d.
C153	Fractogel Epoxy (M)	polyKY	0.1 M NaOH, 0.5 M NaCl, pH 12	15	n.d.	n.d.	n.d.
C160	Fractogel Epoxy (M)	polyKF	DMF, 50 mM TEA	5	n.d.	n.d.	11.5 (9.9)
C161	Fractogel Epoxy (M)	polyKF	DMF, 50 mM TEA	15	n.d.	n.d.	13.0 (11.9)
C162	Fractogel Epoxy (M)	polyRW	DMF, 50 mM TEA	15	72	10.8	9.3 (7.4)

[a] Binding capacities were determined by column experiments (–, no binding; +, weak binding, ++, moderate binding; +++, strong binding of a 300 μg sample pulse) or batch adsorption. Binding capacities were determined at initial protein concentrations of 5 mg mL^{-1} EDDIE–pep6His and 3 mg mL^{-1} (values given in parenthesis). *Abbreviations*: Ac, acetylated; ALD, aldehyde; BDGE, butandioldiglycidylether; DMF, dimethylformamide; PBS, phosphate buffered saline; TEA, triethylamine.

density of 40 mg mL^{-1} gel (C70). Despite this excellent immobilization yield, still only a very weak binding of EDDIE could be achieved.

Taking together all the results so far and considering all the explored possibilities, it could be assumed that an unknown factor was somehow responsible for suppressing the interaction of the immobilized peptide with EDDIE. The screening studies on the membranes have shown that the introduction of a negatively charged group, despite 150 mM NaCl in the buffer, could completely suppress binding. Taking a closer look at the immobilization chemistry yielded the possibility that residual carboxy groups might be present. These groups stem from hydrolysis of the active esters (NHS), which is always a competing side reaction to the actual immobilization reaction and which is practically unavoidable.

This hypothesis was tested by the development of a new, site-directed immobilization chemistry. The complete reaction scheme is shown in detail in Fig. 3. The peptide was N-terminally acetylated and synthesized with a C-terminal lysine. The ε-amino group of this lysine was specifically reacted with iodine acetic anhydride, resulting in a terminal iodine function. Fractogel was reacted with DADPA and subsequently modified with iminothiolane, resulting in a terminal sulfhydryl function. Coupling of the purified, derivatized peptide was performed at pH 6.0. The technique was applied for several peptides and different types of matrices (C100–C140), and all of them, depending on ligand density and peptide, showed a reasonably good binding of EDDIE–pep6His. Binding capacities were in the range of 4–14 mg mL^{-1} gel under the experimental conditions applied. It was also possible to reverse the reaction scheme by synthesizing the peptide with a terminal cysteine and functionalizing the spacer with an iodine (C139, 140). However, ligand densities and binding capacities could not be further improved.

A second realization of functional affinity media could be achieved by using poly amino acids as ligands. The rationale behind the introduction of a poly amino acid spacer initially was to improve the steric presentation of the peptide ligand. Coincidentally, several types of compounds are commercially available as polymers comprising one, two, or three different amino acids, with varying ratios of respective amino acids and also differing in molecular weight, ranging from about 10 to 150 kDa. Obviously, poly amino acids comprising the identified key amino acids (lysine, arginine and tryptophan, phenylalanine) were selected for investigations. At first, these types of compounds were intended for use only to "promote" the binding reaction and further immobilize peptides (C61). However, it turned out that these compounds could be used as ligands per se. A variety of media were prepared applying different base matrices, ligand types, and ligand densities (Table 2). Binding capacities were in the same range as for the peptide media. PolyKW was generally preferred because it has a much better solubility than, for instance, polyKF, which is only soluble in organic solvents.

A set of media was further investigated, and adsorption isotherms with EDDIE–pep6His were determined. Data points determined by batch adsorption experiments were fitted by the Langmuir isotherm:

$$q = \frac{q_m K_a C}{1 + K_a C} \quad (1)$$

As can be seen in Fig. 4A and B, all isotherms are more or less shallow and have a maximal binding capacity q_m between 10 and 15 mg mL^{-1}. The binding strength, expressed by the equilibrium association constant K_a, is influenced by the ligand density and basically reflects the results from the screening experiments where KF was found to be a stronger binder than KW (Table 3). In general, gels with such shallow isotherms are not well suited for capturing proteins from dilute solutions. However, in the present case, the feed stock represents dissolved inclusion bodies resulting in a high protein concentration in the load and thus the maximal capacity of the medium can be exploited. The achievable binding capacities are comparatively high for affinity systems, with the exception of protein A affinity media, which have binding capacities up to almost 70 mg IgG mL^{-1} gel [25–27].

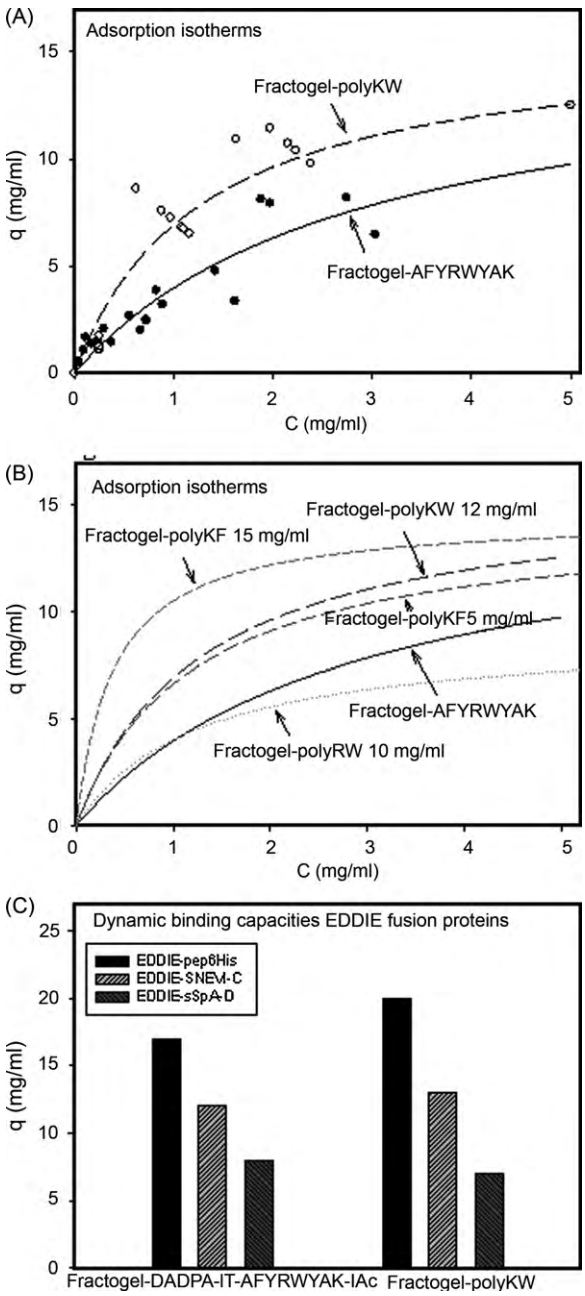

Fig. 4. Capacities of peptide affinity media with EDDIE–pep6His. (A) Data points and Langmuir fit for Fractogel-polyKW and Fractogel with the peptide ligand AFYRWYAK prepared by site-directed immobilization. (B) Adsorption isotherms of different peptide affinity media. (C) Dynamic binding capacities of Fractogel-polyKW and Fractogel-DADPA-AFYRWYAK-IAc determined with proteins EDDIE–pep6His, EDDIE-SNEVi-C, and EDDIE–sSpA-D.

Dynamic binding capacities for other fusion proteins were determined in column experiments (Fig. 4C). Accordingly, two additional fusion proteins were investigated, namely EDDIE–SNEVi-C and EDDIE–sSpA-D. SNEVi-C is another short peptide and sSpA-D is the D-domain of *Staphylococcus aureus* protein A. The peptide fusion showed almost the same capacity as EDDIE–pep6His while that of the protein A domain was somewhat lower.

Table 3
Adsorption isotherm parameters of selected peptide affinity media determined with EDDIE–pep6His.

Matrix	Ligand density (mg mL^{-1})	Equilibrium binding capacity, q_m (mg mL^{-1})	Equilibrium association constant, K_a (mL mg^{-1})
Fractogel-DADPA-IT-AFYRWYAK-IAc	25	15.3	0.35
Fractogel-polyKW	12	17.2	0.71
Fractogel-polyKF	5[a]	14.5	0.85
Fractogel-polyKF	15[a]	14.5	2.6
Fractogel-polyRW	12	9.0	0.8

[a] Nominal ligand density; actual ligand desnity could not be determined.

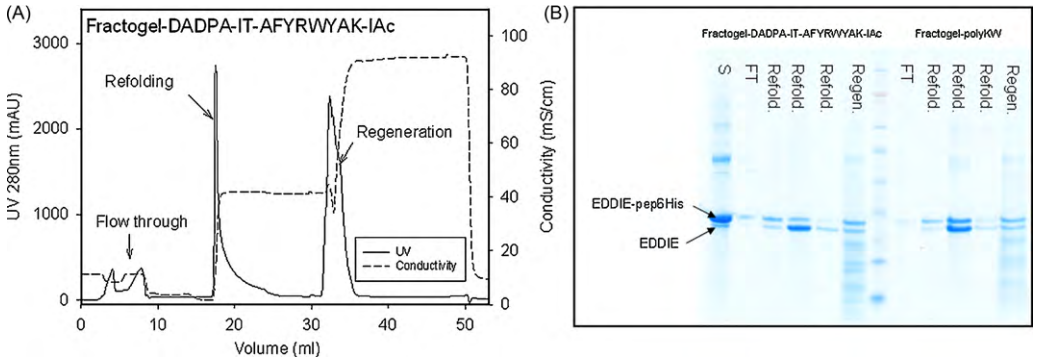

Fig. 5. On-column refolding of EDDIE–pep6His. (A) Representative chromatogram of Fractogel-DADPA-IT-AFYRWYAK-IAc at a saturation of 15 mg of EDDIE–pep6His mL^{-1} of gel. (B) SDS-PAGE of fractions processed on Fractogel-DADPA-IT-AFYRWYAK-IAc and Fractogel-polyKW.

As mentioned earlier, the ultimate goal of this study was to develop an on-column refolding process applying the N^{pro} fusion concept of autoproteolytic cleavage upon refolding. IBs comprising EDDIE–pep6His were dissolved at 10 M urea and loaded at 4 M urea. After a washing step, refolding buffer was applied to initiate the autoproteolysis. As described previously, N^{pro} and EDDIE require high concentrations of a positively charged buffer component for refolding and cleavage. The addition of such a component (Tris or GuHCl) affected immediate desorption; however, effective autoproteolysis occurred concurrently with the elution step and proceeded in the collected fraction.

Fig. 5A shows a representative chromatogram of Fractogel-DADPA-IT-AFYRWYAK-IAc at a column load of 15 mg EDDIE–pep6His mL^{-1} gel. Regeneration was performed with 0.5 M NaOH, which removed residual protein and mainly DNA from the column. Fig. 5B shows SDS-PAGE of fractions from on-column refolding (OCR) performed on DADPA-IT-AFYRWYAK-IAc and Fractogel-polyKW, respectively. The product, the 16 amino acid peptide pep6His, cannot be detected by this method, so quantification was performed based on the intensities of the EDDIE–pep6His and EDDIE bands. RP-analysis of refolding and regenerate fractions showed that the peptide was only found in the refolding fraction, and it can be assumed that the peptide that corresponds to the cleaved EDDIE in the regenerate fraction could also be recovered. The identity of pep6His was confirmed by RP-analysis of the refolding fraction after acidic precipitation of EDDIE in comparison with a synthetic peptide (supplemental Fig. 5) and by mass spectrometry analysis. Table 4 gives a summary of OCR experiments on different matrices at different column loadings.

In general, the matrices did not differ significantly in performance. The gel with the octapeptide showed slightly high step yields but also lower recovery in the refolding fraction. However, this was not really relevant because the peptide was recovered in the refolding fraction and the ratio of fusion protein and EDDIE in the regenerate fraction was about 40–60% for all matrices. The step yields for autoproteolysis were remarkably high, reaching 90% at lower loading. Such a high value suggests that the matrix contact acts as a folding helper, despite the fact that refolding and cleavage occurred during and after elution. In comparison with batch refolding of the same fusion protein, similar yields of 40–60% could be obtained [12,13], but the concentrations at which auto-

Table 4
On-column refolding of EDDIE–pep6His on peptide affinity media. Fractions were analyzed by SDS-PAGE and cleavage efficiency was determined.[a]

Matrix	Load 5 mg mL^{-1}				Load 10 mg mL^{-1}			
	Elu	Rec	Reg	Y	Elu	Rec	Reg	Y
Fractogel-AFYRWYAK	91.6	58	38.0	48	84.4	67	38.4	48
Fractogel-polyKW	83.2	68	38.1	48	70.4	67	38.4	39
Fractogel-polyKF 5 mg mL^{-1}	81.6	65	32.0	43	60.1	66	30.0	29
Fractogel-polyKF 15 mg mL^{-1}	92.2	75	31.5	56	–	–	–	–
	Load 15 mg mL^{-1}				Load 20 mg mL^{-1}			
	Elu	Rec	Reg	Y	Elu	Rec	Reg	Y
Fractogel-AFYRWYAK	80.5	68	39.8	46	81.0	59	40.5	43
Fractogel-polyKW	63.8	77	40.9	37	60.0	74	40.7	34
Load (IBs dissolved)	17.5 ± 4							

[a] Elu, eluate; Rec, recovery; Reg, regenerate; Y, yield; all values given in %.

proteolysis could be performed were much higher for the OCR process. A column loading of 15 mg mL^{-1} and an elution fraction volume of one column volume resulted in a protein concentration of 10.5 mg mL^{-1} at a protein recovery of 70%. Furthermore, this fraction contained peptide stemming from the EDDIE fraction that could only be desorbed by NaOH. OCR experiments with EDDIE–SNEVi-C and EDDIE–sSpA-D gave similar results (data not shown).

The question arises of how peptides can bind N^{pro} or EDDIE fusion proteins under denaturing conditions. We assume that a limited amount of secondary structure is preserved even in the presence of 4 M urea. Hiller et al. have shown that in the presence of urea, hydrophobic clusters are still present [28]. Another contribution could be the formation of hydrogen bonding, although urea is generally considered a hydrogen bond breaker.

4. Conclusions

The present work covered the screening of peptide ligands and further successful transfer to a functional affinity system for the autoprotease N^{pro} and its mutant EDDIE. First of all, it is notable that peptides could be designed that can bind N^{pro} and EDDIE at 4 M urea. Such interactions have been explained as being based on hydrophobic clusters. However, in the present case, charged amino acids also have been found to play an essential role. It was even more striking that the peptide size could be drastically reduced to dipeptides without losing binding efficiency. Unfortunately, the three-dimensional structure of both N^{pro} and EDDIE are unknown; thus, a mechanism and location of the binding site are not accessible. The rather flexible behavior has so far prevented NMR- or crystal-based structure determination. On the other hand, this behavior might be the key to the fact that peptide ligands with high affinity and specificity could be found at all.

The development of an affinity system for EDDIE fusion proteins, which have potential preparative and industrial applications, turned out to be quite tricky because of the sensitivity for negative charges. The developed site-directed immobilization strategy could solve this problem, but this technique is certainly not attractive for a large scale because of its complexity and the high amounts of peptide required. With respect to an industrial application, affinity gels using poly amino acid ligands seem much more attractive. OCR for a fusion protein comprising a small target peptide could be developed, and the protocol seems straightforward for other types of small polypeptide fusion partners. Application of OCR using larger proteins with more complex refolding properties as fusion partners of EDDIE has yet to be studied.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.07.074.

References

[1] I.M. Chaiken, M. Wilchek, I. Parik, Affinity Chromatography and Biological Recognition, Academic Press, New York, 1983.
[2] P. Cuatrecasas, M. Wilchek, C.B. Anfinsen, Proc. Natl. Acad. Sci. U.S.A. 61 (1968) 636.
[3] J. Turkova, Affinity Chromatography, Elsevier, Amsterdam, 1978.
[4] J. Arnau, C. Lauritzen, G.E. Petersen, J. Pedersen, Protein Expr. Purif. 48 (2006) 1.
[5] A. Einhauer, A. Jungbauer, J. Biochem. Biophys. Methods 49 (2001) 455.
[6] E. Hochuli, W. Bannwarth, H. Dobeli, R. Gentz, D. Stuber, Biotechnology 6 (1988) 1321.
[7] J.-C. Janson, L. Ryden, Protein Purification: Principles, High Resolution Methods, and Applications, Wiley-VCH, New York, 1998.
[8] D.B. Smith, K.S. Johnson, Gene 67 (1988) 31.
[9] G. Hannig, S.C. Makrides, Trends Biotechnol. 16 (1998) 54.
[10] D.S. Waugh, Trends Biotechnol. 23 (2005) 316.
[11] C. Achmüller, W. Kaar, K. Ahrer, P. Wechner, R. Hahn, F. Werther, H. Schmidinger, M. Cserjan-Puschmann, F. Clementschitsch, G. Striedner, K. Bayer, A. Jungbauer, B. Auer, Nat. Methods 4 (2007) 1037.
[12] W. Kaar, K. Ahrer, A. Dürauer, S. Greinstetter, W. Sprinzl, P. Wechner, F. Clementschitsch, K. Bayer, C. Achmüller, B. Auer, R. Hahn, A. Jungbauer, Biotechnol. Bioeng. 104 (2009) 774.
[13] R. Ueberbacher, A. Dürauer, K. Ahrer, S. Mayer, W. Sprinzl, A. Jungbauer, R. Hahn, Process Biochem. 44 (2009) 1217.
[14] X. Geng, C. Wang, J. Chromatogr. B 849 (2007) 69.
[15] R. Frank, Tetrahedron 48 (1992) 9217.
[16] K. Pflegerl, R. Hahn, E. Berger, A. Jungbauer, J. Peptide Res. 59 (2002) 174.
[17] A. Duerauer, E. Kopecky, E. Berger, M. Seifert, R. Hahn, A. Jungbauer, J. Biochem. Biophys. Methods 66 (2006) 45.
[18] A. Kramer, U. Reineke, L. Dong, B. Hoffmann, U. Hoffmueller, D. Winkler, R. Volkmer-Engert, J. Schneider-Mergener, J. Peptide Res. 54 (1999) 319.
[19] U. Reineke, R. Volkmer-Engert, J. Schneider-Mergener, Curr. Opin. Biotechnol. 12 (2001) 59.
[20] R. Hahn, K. Amatschek, E. Schallaun, R. Necina, D. Josic, A. Jungbauer, IJBC 5 (2000) 175.
[21] R. Hahn, E. Berger, K. Pflegerl, A. Jungbauer, Anal. Chem. 75 (2003) 543.
[22] R. Hahn, A. Podgornik, M. Merhar, E. Schallaun, A. Jungbauer, Anal. Chem. 73 (2001) 5126.
[23] P.Y. Huang, R.G. Carbonell, Biotechnol. Bioeng. 47 (1995) 288.
[24] H. Yang, P.V. Gurgel, R.G. Carbonell, J. Peptide Res. 66 (2005) 120.
[25] R. Hahn, P. Bauerhansl, K. Shimahara, C. Wizniewski, A. Tscheliessnig, A. Jungbauer, J. Chromatogr. A 1093 (2005) 98.
[26] R. Hahn, R. Schlegel, A. Jungbauer, J. Chromatogr. B 790 (2003) 35.
[27] R. Hahn, K. Shimahara, F. Steindl, A. Jungbauer, J. Chromatogr. A 1102 (2006) 224.
[28] S. Hiller, G. Wider, L.L. Imbach, K. Wüthrich, Angew. Chem. Int. Ed. 47 (2008) 977.