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Matrix-assisted refolding of autoprotease fusion proteins on an ion exchange column: A kinetic investigation

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ABSTRACT

Matrix-assisted refolding is an excellent technique for performing refolding of recombinant proteins at high concentration because aggregation during refolding is partially suppressed. The autoprotease N^{pro} and its engineered mutant EDDIE can be efficiently refolded on cation-exchangers. In the current work, denatured fusion proteins were loaded at different column saturations (5 and 50 mg mL^{-1} gel), and refolding and self-cleavage were initiated during elution. The contact time of the protein with the matrix significantly influenced the refolding rate and yield. On POROS 50 HS, the refolding rate was comparable to a batch refolding process, but yield was substantially higher; at a protein concentration of 1.55 mg mL^{-1}, an almost complete conversion was observed. With Capto S, the rate of self-cleavage increased by a factor of 20 while yield was slightly reduced. Processing the autoprotease fusion protein on Capto S at a high protein loading of 50 mg mL^{-1} gel and short contact time (0.5 h) yielded the highest productivity.

1. Introduction

Refolding of denatured proteins performed on chromatographic supports is a potential process alternative to the common technique of renaturation based on dilution of inclusion body (IB) suspensions. This type of process is usually performed in stirred tank reactors at low to moderate protein concentrations [1–5]. Dilution into refolding buffer initiates refolding by reducing the chaotrope concentration, which stems from the solubilization of the IBs. In addition, it provides a spatial statistical separation of folding protein molecules in solution and thus keeps the competing side reaction of aggregate formation at low levels. Alternatively, methods have been developed to increase refolding yield at higher protein concentration and minimize aggregation. Besides diafiltration [6,7], dialysis [8], micelles [9], and two-phase systems [10], the most prominent method has been matrix-assisted refolding (MAR) on stationary phases. This technique is frequently also referred to as on-column refolding. Basically all modes of chromatography have been successfully applied for MAR [11–14]. The underlying mechanisms are insignificantly different for each mode, but generally the concept of MAR has been explained as (i) shifting thermodynamic equilibria towards the refolded product, (ii) providing a spatial separation of the protein intermediate states, (iii) enabling a convenient buffer exchange into refolding buffer while removing chaotropes, and (iv) exerting a matrix effect that acts as a sort of folding helper similar to a chaperone.

The concept of expressing proteins or peptides of interest in the form of fusion proteins has frequently been applied previously [15–18]. A further improvement was the development of N^{pro} fusion technology [19], a system that uses the autoproteolytic activity of the autoprotease N^{pro} from classical swine fever virus [20]. Proteins and peptides expressed as N^{pro} fusion proteins are first deposited as IBs. Upon in vitro refolding by switching from chaotropic to kosmotropic conditions, the fusion is released from the C-terminal end of the autoprotease by self-cleavage, leaving the target protein with an authentic N-terminus. The wildtype N^{pro} was further genetically engineered by exchange of 11 amino acids, resulting in the so-called mutant EDDIE with improved solubility and refolding and cleavage yield. Various proteins and peptides have been produced with this expression system at very high expression levels. For all of these proteins, an authentic N-terminus has been confirmed. Dilution refolding of EDDIE fusion proteins in stirred tanks has been performed at protein concentrations of up to 3.9 mg mL^{-1} with a cleavage yield of approximately 60% [21]. With fusion to small target peptides lacking a distinct three-dimensional

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(3D) structure, refolding and cleavage yield were independent of initial protein concentration. This behavior differed from conventional first-order refolding kinetics, in which yield strongly depends on initial protein concentration because of an aggregation reaction of higher order. In the case of a large protein as the fusion partner, the reaction was dominated by the kinetics of the fusion partner and thus resulted in the typical decrease of refolding yield at higher initial concentrations.

MAR of a fusion protein comprising EDDIE and fused polypeptides of about 2 kDa in size on ion exchange resins has previously proved to be effective and powerful [22]. EDDIE requires a high concentration of positively charged ions to exert its autoproteolytic activity, which is characterized by a renaturation and subsequent self-cleavage reaction. Short polypeptides, the fusion partners in that previous investigation, commonly refold within a very short period of time, if they refold at all. Thus, the buffer composition must be tailored only with respect to EDDIE. Because of the high salt concentrations required by EDDIE, the refolding reaction occurred in the mobile phase during the elution rather than being adsorbed in the stationary phase. In addition, the main part of the refolding and cleavage reaction was not completed within the elution step, and refolding continued in the collected samples. Distinct matrix effects on protein recovery and refolding efficiency depending on the nature of the stationary phase have been found, but a clear correlation with matrix properties could not be established. Consistent with findings of batch refolding in a stirred tank, the overall yield of cleaved and recovered peptide did not drop with increasing protein concentrations, not even at a saturation capacity of approximately 50 mg fusion proteins mL^{-1} of resin. In such cases, the eluate concentration was around 15 mg mL^{-1}.

Machold et al. [23] previously demonstrated that the choice of stationary phase can significantly influence refolding properties. In recent work, Chen and Leong studied MAR of α-fetoprotein on ion exchangers and obtained a similar result [24]. The refolding yield of the off-column refolding, as they designated it, was substantially higher than refolding in the adsorptive state. However, thorough comparison on the basis of refolding kinetics has not been performed. Especially, the kinetics of the refolding reaction, which occurs simultaneously with the autoproteolytic cleavage reaction in the case of N^{pro} fusion proteins, has not been determined and compared to conventional batch refolding reactions. This latter comparison is the focus of the current work.

This investigation involved use of a model peptide (2 kDa) called sSNEVi-C, representing the inhibitory peptide of senescence-evasion-factor with a cysteine residue at the C-terminus, fused to the autoprotease EDDIE [25]. We have investigated the kinetics of refolding and cleavage in the eluate fractions in terms of yield and quantitative rate constants, applying a model developed by Kaar et al. [21]. The influence of column saturation, type of stationary phase, and contact time with the matrix were the main parameters to be studied. In addition, productivities were calculated for these different process conditions and were evaluated with respect to a preparative process application. For the stationary phases, two resins with different matrix backbone properties were chosen: Capto S, based on a hydrophilic matrix, and POROS HS, consisting of a hydrophobic backbone.

2. Materials and methods

2.1. Equipment and chemicals

Chromatographic experiments were performed with ÄKTAexplorer (GE Healthcare, Uppsala, Sweden) controlled by UNICORN software version 5.10. The ion exchange media, Capto S, was purchased from GE Healthcare (Uppsala, Sweden), and POROS® HS 50 micron was purchased from Applied Biosystems (Foster City, CA, USA). All chemicals were purchased from Merck and Sigma (Steinheim, Germany), respectively, if not otherwise indicated.

2.2. Recombinant protein expression and IB isolation

The recombinant protein EDDIE-sSNEVi-C was overexpressed in *Escherichia coli* BL 21 with a pET30a plasmid (Novagen, Madison, WI, USA) containing the corresponding coding gene [19]. *E. coli* fed-batch cultivation was performed with a semi-synthetic medium on a 5-L scale according to Clementschitsch et al. [26]. sSNEVi-C is a 20 amino acid peptide with the sequence KVAHPIRPKPPSATSIPAIC. Isolation of IBs was performed with an APV 2000 lab homogenizer (Invensys, Albertslund, Denmark) as described previously [21]. Protein concentrations were determined by reading UV at 280 nm on a Cary 50 UV–VIS Spectrophotometer (Varian, Palo Alto, CA, USA) using the theoretical extinction coefficient of EDDIE-sSNEVi-C ($\varepsilon = 1.162$ at 1.0 mg mL^{-1}). A correction factor of 0.9, determined by reading 280/260 absorption, was introduced to account for DNA impurities.

2.3. Refolding of basic fusion proteins using cation-exchange supports

The chromatographic refolding process of EDDIE-sSNEVi-C was performed using two different cation-exchange matrices: Capto S and POROS HS. These resins (Capto S: 4.32 mL, POROS HS: 4.16 mL) were packed into Tricorn columns (GE Healthcare) with inner diameters of 10 mm. Matrices were equilibrated with equilibrium buffer containing 4 M urea, 50 mM sodium acetate (NaAc), and 5 mM α-monothioglycerol (MTG) at pH 5.0. Proteins were extracted from IBs by suspending them at a 1:5 ratio in dissolution buffer containing 10 M urea, 50 mM NaAc, and 50 mM Tris at pH 5.0, supplemented with 100 mM MTG. IBs were allowed to dissolve for at least 1.5 h. 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 filter units with pore diameters of 0.80 μm and 0.22 μm, respectively (Millipore, Billerica, MA, USA). Protein concentration was measured on a Cary 50 UV–VIS Spectrophotometer at 280 nm. The protein solution was then diluted in dissolution buffer to a final concentration of 5 mg mL^{-1}. Aliquots of the protein (5 and 50 mg protein mL^{-1} gel) were applied to the columns, allowing 10 min of 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 6). 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 7.5) over 0.5, 1, and 7 h, respectively. 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).

2.4. Batch refolding

IBs were extracted and prepared as described for autoprotease fusion proteins using cation-exchange supports but using dissolution buffer containing 5 M urea instead of 10 M urea. An aliquot of the protein solution was then diluted in dissolution buffer to a final concentration of 7.7 mg mL^{-1}. The remaining protein solution was concentrated to final concentrations of 24.1 and 47.3 mg mL^{-1}, respectively using an Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA, USA). The filter device was centrifuged

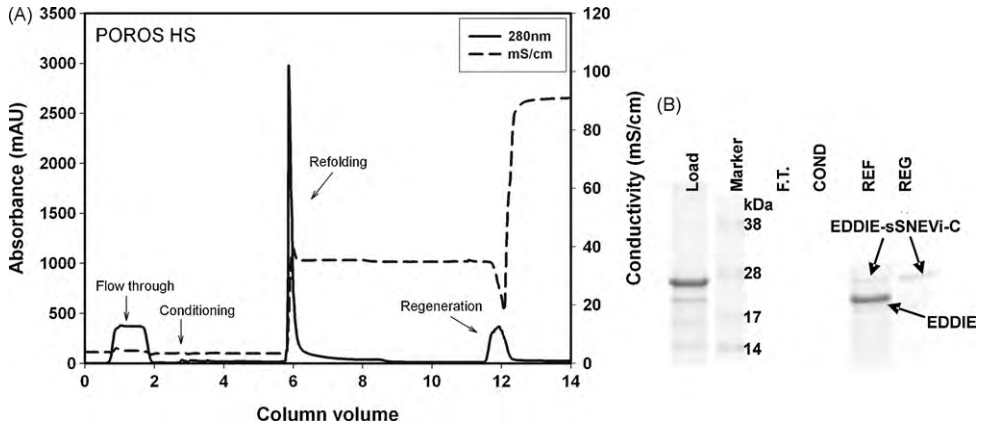

Fig. 1. (A) Representative chromatogram of MAR process using POROS 50 HS at a column loading of 5 mg EDDIE-sSNEVi-C per mL resin. Equilibration was performed using acidic buffer (pH 5) containing 4 M urea. A conditioning step was performed by applying buffers containing 0.8 M urea at pH 6. Refolding and elution buffer contained 0.8 M urea and 1.5 M Tris (pH 7.3). Refolding was performed with 3 CVs over 0.5 h. The column was regenerated with 0.5 M NaOH. (B) SDS-PAGE of chromatography fractions. Lane 1, load; lane 2, marker; lane 3, flow-through fraction (FT); lane 4, conditioning fraction; lane 5, refolding fraction; lane 6, regeneration fraction.

(Centrifuge 5810R, Eppendorf AG, Hamburg, Germany) for 3.5 h at 4000 rpm and 4 °C. The protein solutions with different concentrations were diluted with a refolding buffer to final protein concentrations of 1.35, 4.30, and 8.45 mg mL^{-1}. The final refolding buffer contained 0.8 M urea, 1.5 M Tris, 0.25 M sucrose, 2 mM EDTA, and 20 mM MTG at pH 7.5.

2.5. 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-sSNEVi-C and EDDIE bands were determined using Image Analysis Software of the Lumi-Imager (Boehringer Mannheim, Mannheim, Germany).

3. Results and discussion

Refolding and cleavage of EDDIE-sSNEVi-C was studied on the two selected cation-exchange columns, POROS 50 HS and Capto S. These particular two resins were chosen to represent different base matrix properties, a matrix with a hydrophobic polystyrene backbone in case of POROS, and a hydrophilic agarose backbone with a grafted tentacle-like dextran layer in the case of Capto. In this study, the influence of the stationary phase was investigated with respect to matrix type and protein loading, with the main focus on the contact time of the protein with matrix and its impact on refolding and cleavage properties. The kinetics of the cleavage reaction was then monitored in the collected eluate fractions. These results were compared to autoproteolytic processing by MAR with the conventional stirred tank process.

Panels A and B in Fig. 1 show a typical MAR run and analysis of fractions by SDS-PAGE. The column, POROS 50 HS, was equilibrated with acidic buffer containing 4 M urea. After loading of the sample, a wash with a conditioning buffer was applied to reduce the urea concentration and increase pH. Refolding and autoproteolytic cleavage were initiated during the desorption step with the refolding buffer and completed after elution. Finally, the remaining protein was desorbed with 0.5 M NaOH. Fractions were analyzed by SDS-PAGE to monitor the refolding reaction (Fig. 2A). The target peptide cannot be detected by this technique, but a mass balance could be established by quantifying the mass fraction of fusion protein and cleaved EDDIE in terms of the band intensities determined by image analysis. As shown previously by reversed-phase HPLC, the cleaved peptide is fully recovered in the eluate fraction, even

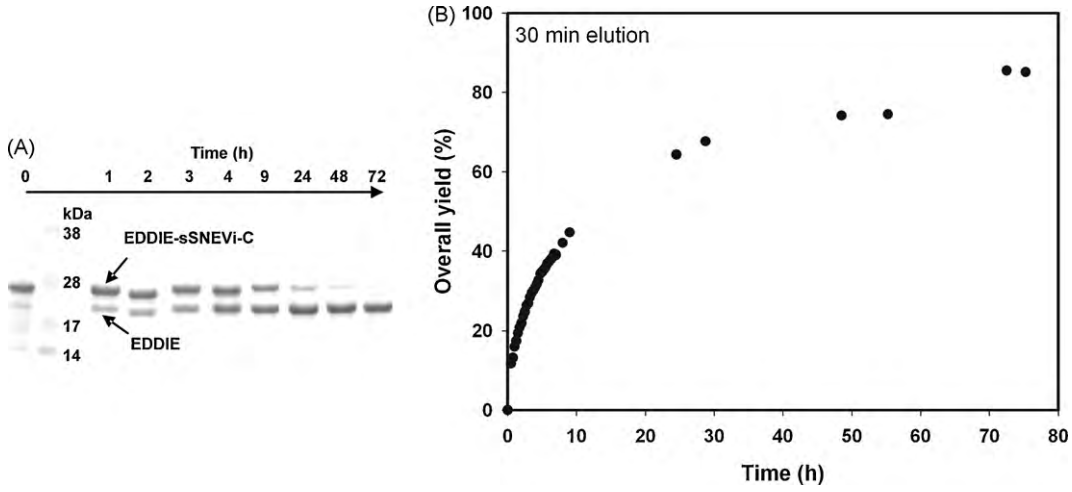

Fig. 2. (A) SDS-PAGE of refolding and cleavage of EDDIE-sSNEVi-C in refolding fraction over a time period of 72 h. (B) Overall yield Y_{REF} (%) calculated according to Eq. (1) starting at the beginning of elution over a time period of about 72 h. Data points of overall yields were fitted by Eq. (2).

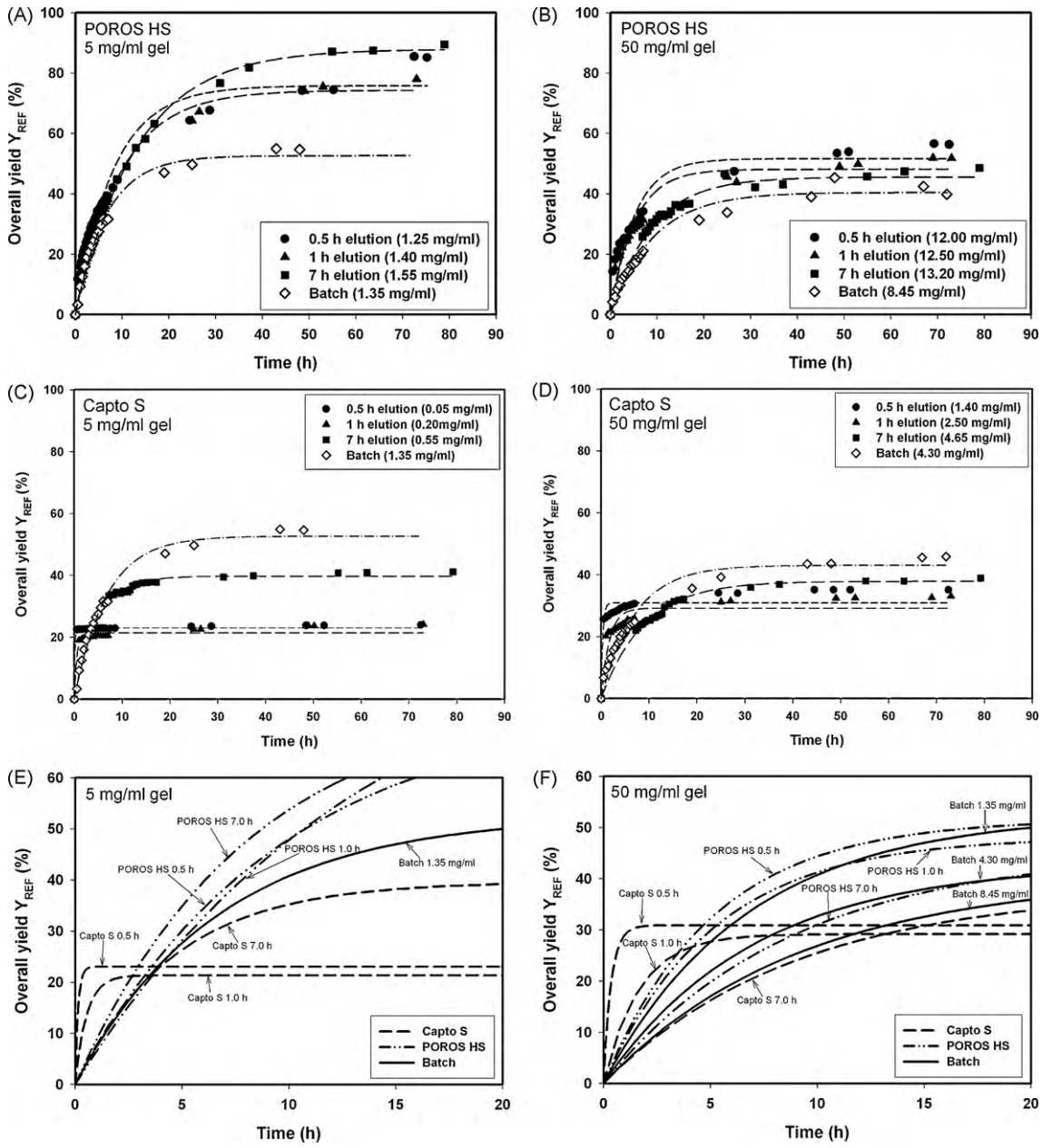

Fig. 3. Refolding and cleavage kinetics of EDDIE-sSNEVi-C achieved with MAR applying different elution times (0.5, 1.0, and 7.0 h) compared to batch refolding. MAR performed on both POROS 50 HS at a column loading of (A) 5 mg fusion protein mL^{-1} gel and (B) 50 mg fusion protein mL^{-1} gel, and Capto S at column loadings of (C) 5 mg fusion protein mL^{-1} gel and (D) 50 mg fusion protein mL^{-1} gel. Comparison of the early time course of refolding and cleavage kinetics of both resins at (E) low and (F) high column saturation. Data points of overall yields Y_{REF} (%) were calculated according to Eq. (1) and fitted using Eq. (2).

when the corresponding EDDIE moiety was found in the regenerate [22]. Taking this into account, the overall yield Y_{REF} can be calculated as follows:

$$Y_{REF} = \frac{(E_{REF} \cdot R_{REF}/100) + (E_{REG} \cdot R_{REG}/100) - E_{LOAD}}{FP_{LOAD}/(FP_{LOAD} + E_{LOAD})} \quad (1)$$

where E is the mass fraction of EDDIE, R the recovery, and FP the mass fraction of the fusion protein. The subscripts LOAD, REF, and REG indicate the fraction loading sample, refolding fraction, and regenerate fraction, respectively. To ensure correct mass balance, the amount of prematurely cleaved EDDIE (E_{LOAD}) has to be taken into account and subtracted accordingly.

The corresponding kinetics of the autoproteolytic cleavage in the eluate fraction based on Eq. (1) is shown in Fig. 2B. The refolding and cleavage time course are described by a model previously developed by Kaar et al. [21]:

$$Y(t) = \frac{k_1}{k_1 + k_2} - \frac{k_1}{k_1 + k_2} \cdot \exp(-(k_1 + k_2) \cdot t) \quad (2)$$

In this model, refolding (and cleavage) is independent of protein concentration and can be modeled by two first-order rate constants, k_1 (the refolding and cleavage constant) and k_2 (a constant accounting for a misfolding reaction that does not lead to aggregation other than with typical protein refolding reactions). The validity of this model has been demonstrated for several EDDIE fusion proteins with oligopeptides exhibiting a fast refolding kinetics [27]. In case of larger proteins as fusion partners, the refolding kinetics are dominated by the fusion partner and other models, considering an aggregation reaction of 2nd or higher order, have to be applied to describe the refolding process [28].

MAR experiments as described above were performed on POROS 50 HS and Capto S at column saturations of 5.0 and 50 mg fusion protein mL^{-1} of resin, respectively. The contact time of the protein with the matrix was adjusted by varying the elution flow-rate. Times tested were 0.5, 1.0, and 7.0 h whereas the elution volume was fixed at 3.0 CV. Kinetics of both MAR and batch refolding are shown in Fig. 3. Fig. 3A–D contains the experimental data points of overall yield calculated according to Eq. (1) and fits according to Eq. (2). Fig. 3E and F depicts the early time course of the autoproteolytic reaction. In the latter case, for better illustration only the fits are shown. Depending on the recovery in the eluate fraction, the protein concentration ranged between 0.05 and 13.2 mg mL^{-1}, respectively. For comparison, batch processing was performed at the same concentration range. In the case of the higher column saturation, the comparable batch process based on dilution refolding was performed at 8.5 mg mL^{-1}. For this purpose, the dissolved IBs had to be concentrated to a protein concentration of up to 50 mg mL^{-1}. An even higher concentration was possible but resulted in a very viscous protein suspension and thus was not regarded as of technical relevance.

Kinetic constants of the autoproteolytic reaction were derived from Eq. (2). A full list of all calculated parameters is given in Table 1. The most relevant parameters cleavage yield, overall yield and refolding and cleavage constants k_1 depending on the protein concentration in the eluate fraction are shown in Fig. 4. Fig. 4A demonstrates the effect of the matrix contact on the autoproteolytic reaction. The cleavage yield of fusion proteins in the eluate of MAR samples is significantly enhanced reaching almost complete conversion in a concentration range of ~1.5–4 mg mL^{-1}. However, for POROS this was the case at the lower column saturation of 5 mg mL^{-1}, whereas for Capto the column saturation was 50 mg mL^{-1}. In case of Capto S the recovery in the eluate was low with a trend of better recovery at longer contact time. The cleavage yield was around 70%. Processing on POROS at 50 mg mL^{-1} resulted in protein eluate concentrations of approximately 13 mg mL^{-1} with cleavage yield of 60–70%. In all cases, the cleavage yield was higher than in batch processes, regardless the concentration.

With regard to a preparative process, the overall yield (including the recovery) and the reaction time represented by the kinetic constant k_1 are of higher importance. These parameters represented as a function of protein concentration are shown in Fig. 4B and C. Evidently there are big differences between the two gels. The autoproteolytic process on POROS is distinguished by a very high overall yield even at eluate protein concentrations of close to 14 mg mL^{-1}. The kinetics of the autoproteolysis was in a relatively narrow range around $k_1 = 2 \times 10^{-5}\ s^{-1}$ which was equal or slightly higher than rate constants determined for the batch processing.

Compared to the results obtained on POROS 50 HS, significant differences could be observed for Capto. The overall yield was lower in all cases while the kinetics of the reaction was very fast for the shorter contact times with rate constants in the range of $10^{-4}\ s^{-1}$. Interestingly, the recovery was quite low at short contact times and increased gradually with longer contact, which occurred at both low and high protein saturation. The constants obtained for the longer elution times of 7.0 h were around $10^{-5}\ s^{-1}$, which was in the same range as the POROS eluates.

Table 1
Results of MAR on POROS 50 HS and Capto S at low and high column saturation and batch refolding at different protein concentrations.

	mg protein mL^{-1} gel load					
	5 mg mL^{-1} gel			50 mg mL^{-1} gel		
	0.5	1.0	7.0	0.5	1.0	7.0
POROS HS						
In vivo cleavage (%)	19	19	19	17	19	19
Recovery in eluate (%)	83	94	99	81	85	89
Cleavage yield in eluate at 0 h (%)	26	29	50	30	32	41
Cleavage yield in eluate at 24 h (%)	78	76	81	63	60	56
Cleavage yield in eluate at 48 h (%)	87	84	90	70	64	60
Cleavage yield in eluate at 72 h (%)	98	88	91	73	66	62
Cleavage yield in regenerate (%)	40	0	0	23	31	28
Protein concentration (mg mL^{-1})	1.20	1.40	1.55	12.00	12.55	13.20
Overall yield (%)	85	78	91	56	52	49
k_1 (s^{-1})	2.6×10^{-5}	2.1×10^{-5}	1.9×10^{-5}	2.8×10^{-5}	2.6×10^{-5}	1.4×10^{-5}
k_2 (s^{-1})	8.4×10^{-6}	7.4×10^{-6}	1.5×10^{-6}	2.6×10^{-5}	2.8×10^{-5}	1.7×10^{-5}
Capto S						
In vivo cleavage (%)	20	20	20	19	19	19
Recovery in eluate (%)	3	11	37	10	17	31
Cleavage yield in eluate at 0 h (%)	30	30	55	20	28	47
Cleavage yield in eluate at 24 h (%)	57	54	68	91	81	83
Cleavage yield in eluate at 48 h (%)	67	63	71	100	87	88
Cleavage yield in eluate at 72 h (%)	70	66	71	100	90	91
Cleavage yield in regenerate (%)	38	36	42	42	37	32
Protein concentration (mg mL^{-1})	0.05	0.20	0.55	1.40	2.50	4.65
Overall yield (%)	24	24	41	35	33	39
k_1 (s^{-1})	4.6×10^{-4}	1.1×10^{-4}	2.4×10^{-5}	2.4×10^{-4}	4.8×10^{-5}	1.2×10^{-5}
k_2 (s^{-1})	1.6×10^{-3}	4.0×10^{-4}	3.7×10^{-5}	5.4×10^{-4}	1.2×10^{-4}	1.9×10^{-5}

Batch	Protein conc. (mg mL^{-1})		
	1.35	4.30	8.45
In vivo cleavage (%)	20	23	26
Cleavage yield in eluate at 24 h (%)	60	53	51
Cleavage yield in eluate at 48 h (%)	64	57	60
Cleavage yield in eluate at 72 h (%)	–	58	55
Overall yield (%)	55	46	40
k_1 (s^{-1})	2.2×10^{-5}	1.7×10^{-5}	1.2×10^{-5}
k_2 (s^{-1})	2.0×10^{-5}	2.3×10^{-5}	1.8×10^{-5}

0.5, 1.0 and 7.0 indicate elution time (h).

Table 2
Productivity of MAR experiments on Capto S and POROS HS compared to batch refolding. Productivity was calculated according to Eq. (3) considering different reactor volumes (V_r): 1 CV ($P_{1\,CV}$); 2.75 CV eluate volume ($P_{2.75\,CV}$); 3.75 CV sum of both ($P_{3.75\,CV}$). $M_0 = C_0 \cdot V_0$ represents the initial mass of peptide (mg) and M the mass of produced peptide (mg).

	Elution time (h)	C_0 (mg mL^{-1})	Yield, $t_{s<1\%}$ (%)	M_0 (mg)	M (mg)	Time (h)	Peptide			Protein		
							$P_{1\,CV}$ (mg mL^{-1} h^{-1})	$P_{2.75\,CV}$ (mg mL^{-1} h^{-1})	$P_{3.75\,CV}$ (mg mL^{-1} h^{-1})	$P_{1\,CV}$ (mg mL^{-1} h^{-1})	$P_{2.75\,CV}$ (mg mL^{-1} h^{-1})	$P_{3.75\,CV}$ (mg mL^{-1} h^{-1})
CaptoS 5 mg mL^{-1} gel	0.5	0.05	23	1.50	0.34	2.20	0.036	0.013	0.010	0.372	0.135	0.099
	1.0	0.20	21	1.50	0.31	4.00	0.018	0.007	0.005	0.187	0.068	0.050
	7.0	0.55	35	1.50	0.52	17.90	0.007	0.002	0.002	0.070	0.025	0.019
CaptoS 50 mg mL^{-1} gel	0.5	1.40	31	15.16	4.70	4.90	0.222	0.081	0.059	2.280	0.829	0.608
	1.0	2.50	27	15.16	4.09	8.60	0.110	0.040	0.029	1.132	0.412	0.302
	7.0	4.65	29	15.16	4.40	22.70	0.045	0.016	0.012	0.460	0.167	0.123
POROS HS 5 mg mL^{-1} gel	0.5	1.20	68	1.46	0.99	19.60	0.012	0.004	0.003	0.125	0.045	0.033
	1.0	1.40	65	1.46	0.95	21.65	0.011	0.004	0.003	0.108	0.039	0.029
	7.0	1.45	79	1.46	1.15	34.50	0.008	0.003	0.002	0.083	0.030	0.022
POROS HS 50 mg mL^{-1} gel	0.5	12.00	47	14.96	7.03	15.05	0.112	0.041	0.030	1.153	0.419	0.308
	1.0	12.55	43	14.60	6.28	15.24	0.099	0.036	0.026	1.017	0.370	0.271
	7.0	13.20	37	14.60	5.40	24.10	0.054	0.020	0.014	0.553	0.201	0.148
		C_0 (mg mL^{-1})	Yield, $t_{s<1\%}$ (%)	M_0 (mg)	M (mg)	Time (h)		P (mg mL^{-1} h^{-1})[a]			P (mg mL^{-1} h^{-1})[a]	
Batch		1.34	46	1.74	0.80	13.85		0.004			0.046	
		4.27	36	5.53	1.99	12.70		0.012			0.124	
		8.46	31	10.96	3.40	13.60		0.019			0.198	

[a] Batch processing was performed in ~12 mL corresponding to the volume of the elution fraction (2.75 CV).

Fig. 4. Plot of the most relevant parameters of MAR on POROS HS and Capto S, respectively applying different contact times (0.5, 1, 7 h): (A) cleavage yield (%) in eluate, (B) overall yield (%) and (C) refolding and cleavage rate k_1 (s^{-1}) versus protein concentration (mg mL^{-1}) in the eluate.

In an industrial environment, productivity is of major importance, so we have analyzed our data in this context. For evaluation, the point at which the change of overall yield was less then 1% was defined as the cut-off, and the reaction was regarded as completed. Productivity was calculated according to:

$$P = \frac{YC_0V_0}{V_r t} \quad (3)$$

where P is the productivity (mg mL^{-1} h^{-1}); C_0 (mg mL^{-1}) and V_0 (mL) the initial protein concentration and sample volume, respectively; Y the overall yield of the autoproteolytic process defined

by Eq. (1); V_r the reaction volume (mL); and t the process time (h). V_r can be regarded in three ways: first, considering only CV to characterize the chromatographic column as a reactor; second, considering only the elution volume, which represents 2.75 CV, comparing it to a batch refolding process; and third, considering both CV and elution volume (3.75 CV) to provide a basis for calculations assuming a large industrial requirement where the total space yield is important. Another variation for calculations includes productivity of produced peptides, which is the actual product but represents only approximately 10% of the fusion protein. Productivity of protein refolding considering the entire fusion protein is particularly interesting for comparison with other protein refolding processes. Table 2 provides a summary of all productivities calculated. Processing on Capto S at 50 mg mL^{-1} protein loading and a short contact time was superior in terms of productivity to all other conditions, mainly because of the matrix effect, which resulted in a very high rate constant at an acceptable yield of 31%. The same conditions on POROS gave a productivity that was roughly half, but the yield was higher at 47%. In a production scenario, the decision of whether to use POROS or Capto will depend on fermentation capacities and waste costs. Data in the literature concerning the productivity of refolding are rare. Compared to data on MAR of α-fetoprotein published by Chen and Leong [24], productivities for EDDIE-sSNEVi-C were about 6-fold higher. Obviously, the complexity of the protein plays an important role. MAR of α-lactalbumin was performed at 3-fold higher productivity compared to the process presented here, but in that case, a pure protein was used instead of IBs [23].

4. Concluding remarks

The work presented here demonstrated that MAR conditions can significantly influence the refolding reaction of a protein. The refolding and cleavage kinetics of the autoprotease fusion protein EDDIE-sSNEVi-C could be increased by a factor of 20 under specific process conditions. In another case, cleavage yield could be enhanced up to almost 100%. Both refolding and cleavage kinetics and overall yield depended on matrix type and process conditions. A comparison of the MAR process with conventional dilution refolding revealed the superior performance of this chromatographic method. The process investigated here may represent a case study for industrial production of polypeptides in a size range of 20–50 amino acids. In this range, recombinant production becomes more and more competitive compared to conventional solid-phase peptide synthesis.

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