4 Heterologous expression und purification of human ET-1 in *E. coli*

4. 1 Introduction

4.1.1 Strategies for heterologous protein expression in *E. coli*

When setting up a process for production of a recombinant protein, the normal approach is to first try to express the protein of interest in *E. coli*. Alternative expression systems are used only if the product is biologically inactive after production due to lack of essential post-translational modifications, incorrect folding or when the recovery of the native protein is too low.

E. coli is a popular and well understood system for heterologous protein expression. The major advantages of this expression system are low costs, homogeneity of the recombinant proteins, short generation time (20 min) and short delay of expression. This system offers the possibility to rapidly test a number of different constructs and to optimize expression levels before undertaking the purification. There are at least four different expression options: One option is the direct expression, but the problem may be that the *E. coli* cytoplasm is a reducing environment, and proper disulfide bonds formation is difficult to ensure, particularly when the correct folding of the protein requires formation of disulfide bridges. The second option, the expression of the protein of interest as fusion protein, can overcome insolubility and/or instability problems with small peptides. Moreover, fusion expression has purification advantages based on affinity chromatography. Proteins used as solubilizing fusion partners include Thioredoxin (Trx), N-utilizing substance A (NusA), the maltose-binding protein (MBP), DsbA and GST (Sorensen & Mortensen, 2004). The third option is targeting of peptides or proteins for secretion to the periplasmatic space. The periplasm offers a more oxidizing environment, where proteins tend to fold better. Moreover, the periplasmic space harbours foldases involved in the formation of disulfide bonds and isomerization of the proline imide bonds (Missiakas *et al.,* 1995). Frequently used signal sequences include those derived from the *E. coli* periplasmic proteins PhoA and MalE, the outer membrane proteins OmpA and LamB (Blight *et al.,* 1994), β-lactamase (Kadonaga *et al.,* 1984) and DsbA (Collins-Racie *et al.*, 1995). The major drawbacks of the secretion strategy are the limited expression quantity, since only 0.1-0.2% of total cell protein are secreted and inability for posttranslational modifications of proteins. As a fourth option, *E. coli* can also be used for production of the heterologous protein in inclusion bodies. These dense particles, containing

precipitated protein of interest are very easy to purify. Their formation depends on protein synthesis rate and growth conditions. Moreover, the accumulation in these inclusion bodies protects the recombinant protein from proteolytic degradation, the yield is high, the recombinant protein is relatively pure and easy to separate. One disadvantage is that their formation is hard to control. Another disadvantage is that accumulated protein is inactive and functional *in vitro* refolding and renaturation after purification is a required step.

4.1.2 Previous production strategies of ET-1 in the literature

In *E. coli* expression of peptides or proteins below 7 kDa is known to be hampered by protein instability and degradation attributed to an unstructured state in solution that is susceptible to cellular proteases (Marston, 1986). Accordingly, direct expression of the 21 amino acid ET-1 has not been successful (Watanabe *et al.,* 1989). To circumvent this limitation, the peptide can be expressed as minor part of a fusion protein which aids in overall stability and also solubility.

For the recombinant expression and purification of BigET-1, several systems have been developed (Fig. 4.1), which employed fusions of bigET-1 to alkaline phosphatase (Becker *et al*., 1994), maltose binding protein (Fassina *et al*., 1994), or the N-terminal domain of beta-galactosidase (Yasufuku *et al*., 1992; Ohashi *et al.*, 1991; Ohashi *et al*., 1994), respectively. Following expression of the fusion protein, bigET-1 was released from the fusion partner by cleavage using either trypsin (cleavage after Lys or Arg residues; Yasufuku *et al*., 1992; Ohashi *et al*., 1994; Fassina *et al*., 1994), or Factor Xa (cleavage site after Ile-Glu-Gly-Arg; Ohashi *et al*., 1991) or a combination of collagenase (cleavage site after Gly-Pro in (Gly-Pro-Ala)$_4$) and dipeptidylpeptidase IV (removal of Gly-Pro; Becker *et al*., 1994)). Finally, from the intermediate bigET-1, recombinant ET-1 was described to be obtained by proteolysis with pepsin (Yasufuku *et al*., 1992) or with α-chymotrypsin (Fassina *et al*., 1994). As apparent from the multitude of reports, no consensus has been reached as to the optimal expression, purification and proteolysis strategy. In addition, a direct expression strategy of mature ET-1 - i.e. omitting the bigET intermediate - has been missing so far.

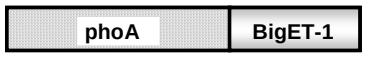
Expression construct	Molecular weight	Structure	Yield	Reference
ETB-42P	8,65 kDa		0,4 mg ET-1/l 0,6 mg ET-1/l	(Okashi et al., 1991) (Yasufuku et al., 1992)
ETB-50P	7,8 kDa		0,9 mg BigET-1 (1-38)/l	(Okashi et al., 1994)
AP-BigET-1	52 kDa		1,1 mg BigET-1 (1-38)/l	(Becker et al., 1994)
MBP-BigET-1	51 kDa		3,1 mg ET-1/l	(Fassina et al., 1994)

Fig. 4.1. Schematic representation of the expression constructs used for production of bigET-1 in *E. coli*. β-Gal, N-terminus of β-galactosidase; phoA, alkaline phosphatase; MBP, maltose binding protein.

4.1.3 Previous production strategies for recombinant ET-1 in our group

In previous attempts the following expression vectors were examined to achieve expression of ET-1 in *E. coli* (Fig 4.2). The expression construct Trx-BigET-1 (Fig 4.2A) comprising a fusion of Thioredoxin to BigET-1 yielded about 45 mg/l of fusion protein. Thioredoxin was chosen as the fusion partner because it is small in size (12 kDa) and because of its complete solubility in *E. coli*. However, the fusion protein formed inclusion bodies and enterokinase treatment did not lead to the formation of an appreciable amount of bigET-1 (M. Hiller, diploma thesis 2001). The expression construct GST-BigET-1 (Fig. 4.2B) has been constructed to direct the expression of BigET-1 as a GST fusion protein. Although the fusion protein was synthesized in large amounts, it accumulated in the form of inclusion bodies and it proved to be difficult to release ET-1 by two successive cleavage steps (J. Wolkenhauer, pers. communication). To reduce the formation of inclusion bodies, bigET-1 was directed to the periplasm (construct DsbC-ET-1; Fig. 4.2C). However, the yield of fusion protein was very low, possibly due to limited capacity for secretion of proteins into the periplasm (G. Lipok, pers. communication).

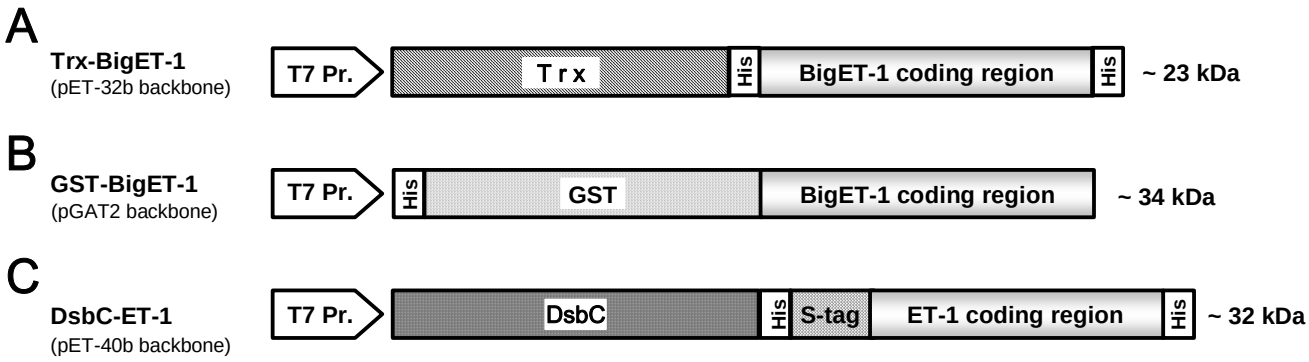


Fig. 4.2. Schematic representation of previous expression constructs used for production of human ET-1 in *E. coli*. The boxes shown are not drawn to scale and construct designations used in the text are given on the left.

In conclusion, the expression systems used thus far are not suitable to yield milligram amounts of ET-1 fused to a protein partner. In this work, an expression system was used to directly express the ET-1 (as opposed to BigET-1) as a Trx-ET-1 fusion protein in *E. coli*. Moreover, protease cleavage sites that do not leave extra amino acid residues on the peptide of interest were employed to release ET-1 from the fusion protein.

4.1.4 Aim of study

The focus of the work was to establish an efficient and cost effective system for expression in *E. coli* to produce ET-1, purified in an active form and in sufficient quantities. In order to investigate structural or dynamical properties of the ET_BR/ET-1 complex by solid-state NMR, isotopic labelling has been indispensable in aiding assignments of resonances, and in obtaining the structural information that forms the basis for NMR structure and determination both in solution and in solid state. A major prerequisite for NMR application is the need to isotopically label ET-1 to achieve spectral dispersion of chemical shifts in multiple dimensions and to obtain a higher intrinsic sensitivity for detection. The solid-state NMR approach chosen in this work requires the synthesis of several isotope-labelled ET-1 variants (see Chapter 5). To obtain isotopically enriched proteins, we opted for growth of bacterial expression strains on isotope-enriched media with the goal to yield active ET-1 in sufficient quantities (ca. 1 mg ET-1/l culture) for purification of the ET_BR produced in *P. pastoris* (see Chapter 3).

4.2 Materials and experiments

4.2.1 Cloning of expression vectors

The vector modification and cloning of fragments were done according to the methods of Sambrook *et al*. (1989). The expression plasmid pET32b-TrxXaET-1 was constructed by replacing the Enterokinase recognition site by a Factor Xa site in the expression vector pET32b-TrxEKET-1: In a first cloning step a 93 bp PCR amplification product using the primers 5' *Nsp*V Xa ET-1 (5'-TTCGAAATCGAGGGAAGGTGCTCCTGCTCGTCCCTGATG-3') and 3'-*Hin*dIII(Stop)ET-1-overhang (5'-CCCAAGCTTTCACCAAATGATGTCCAGGTGGCAG-3') and pET32b-TrxEKET-1 as template was cloned via T/A overhangs into the vector pGEMTeasy (Promega) to produce the intermediate pGEMTeasy-Xa-ET-1. In a second cloning step, this intermediate was cut with *Bst*BI and *Hind*III and the insert was cloned into the plasmid pET32b-Trx-EKET-1 previously opened with *Bst*BI and *Hin*dIII. The 12 bp Factor Xa site was introduced by the 5' primer. The product pET32b-TrxXaET-1 was sequence-verified in both directions using the 5' and 3' primers as sequencing primers.
To construct the expression plasmid pQE30XaET-1 the ET-1 sequence was amplified from pET32b-TrxET-1 by PCR using the primers 5' ET-1 (5'-TGCTCCTGCTCGTCCCTGATGGATAAAGAG-3') and 3' HindIII(Stop)ET-1 (5'-AAGCTTTCACCAAATGATGTCCAGGTGGCAGAAGTAGACACA-3'). The PCR product (72 bp) was cut with *Hin*dIII and subcloned into the expression vector pQE30Xa (Qiagen) previously opened with *Stu*I and *Hin*dIII. In frame ligation at the fusion junction was verified by sequence analysis in both directions using the forward sequencing primer pQE30RBS (5'-CGGATAACAATTTCACACAG-3') and the reverse primer pQErev (5'-GTTCTGAGGTCATTACTGG-3'). Clonings were carried out in either the *E. coli* strains XL1Blue or DH5α.

4.2.2 Expression of Trx-EK-ET-1 and Trx-Xa-ET-1 fusion proteins

Proteins were expressed as Trx-EK-ET-1 or as Trx-Xa-ET-1 fusion proteins using an *E. coli* BL21 (DE3) host (Novagen) and M9 minimal medium (see Table A1, Appendix). Prior to growth on this medium starter cultures were grown overnight in 5 ml LB medium containing 100 μg/ml ampicillin at 37°C. The overnight bacterial cultures were adapted for 24h in 50 ml M9 minimal medium containing 0.5 g/l NH_4Cl and 3g/l glucose at 37°C using 1% inoculum.

On the following day, adapted cultures were subcultured using 2x 500 ml M9 minimal medium and 1% inoculum. For ^{15}N enrichment, M9 was supplemented in the final culture with 0.5 g/l ^{15}N-NH_4Cl as the sole source of nitrogen. For uniform ^{13}C enrichment, additionally 3g/l $^{13}C_6$-glucose were provided as the sole source of carbon and for partial ^{13}C enrichment 3 g/l 1,3-^{13}C-glycerol and 2 g/l $NaHCO_3$ or 3 g/l 2-^{13}C-glycerol and 2 g/l $NaH^{13}CO_3$ were supplemented. After growing the cells to an OD_{600} of 1.0-1.1, the cultures were induced for protein overexpression with isopropylthiogalactoside (IPTG; 1 mM) and the temperature was reduced to 25°C. Cells were harvested 4 hours later, washed once with 0.9% NaCl, pelleted and stored at -70°C.

4.2.3 Fermentation protocol

The production of large amounts of TrxXaET-1 by fermentation was carried out in a BioFlow 3000 fermentor (New Brunswick Scientific) equipped with a 10 l bioreactor. The batch medium had the same composition as the medium used for shake cultures. An overnight 500 ml *E. coli* M9 culture was used to inoculate the 10 L fermenter vessel containing 9.5 l M9 minimal medium with 0.4% glucose or 0.3% glycerol and grown under constant agitation (500 rpm), aeration (2.5 litres/h) was checked by on oxygen electrode and held at 30%. The temperature was controlled at 37°C. The pH was at 7.0. Ammonium hydroxide solution was used as the base solution to adjust the pH. After 5 hours of batch culture, the optical density reached 1. Protein expression was induced by addition of 1 mM IPTG at an OD_{600} of 1.1. After 4 h induction at 25 °C, cells were harvested.

4.2.4 Purification of Trx-EK-ET-1 and Trx-Xa-ET-1 fusion proteins

All work was carried out at 4°C. *E. coli* cells were thawed and resuspended in lysis buffer (20 mM Tris/HCl pH 8.0, 300 mM NaCl, 10 mM imidazole, 2 mM β-mercaptoethanol) and a protease inhibitor cocktail (“complete”; Roche Diagnostics), broken in a French pressure cell (SLM Amingo) and centrifuged two times at 22000 rpm for 20 min to remove all insoluble cellular debris. Supernatant was loaded onto a 50 ml column of Ni-NTA superflow resin (Qiagen). Following pre-equilibration with lysis buffer and loading of the supernatant, the column was washed with 3 column volumes of the lysis buffer and then with 2 column volumes of lysis buffer containing 50 mM imidazole. Elution of the fusion protein was carried out by lysis buffer containing 100 mM imidazole. Following to concentration of the fusion protein (Vivaspin concentrator, 5 kDa molecular mass cutoff, Vivascience), a second purification step using 6 ml anion exchanger ResourceQ (Amersham Pharmacia) was

performed. The ResourceQ column was loaded, washed with buffer containing 20 mM Tris/HCl pH 7.0, 1 mM EDTA and 2 mM β-mercaptoethanol. Fractions containing purified fusion protein were eluted with buffer containing additionally 450 mM NaCl and concentrated to a volume of 30 ml as described before. After confirming homogeneity of the purified protein by SDS-PAGE, the concentration of Trx-Xa-ET-1 was determined by UV spectroscopy (280 nm; $\varepsilon = 21273\ M^{-1}cm^{-1}$).

4.2.5 Proteolytic cleavage and purification of ET-1 from the fusion protein

For cleavage with enterokinase (Novagen) the purified Trx-EK-ET-1 protein (1-2 mg/ml) was digested using 1.6 U of enterokinase for 0.1 mg fusion protein at 37°C in buffer containing 20 mM Tris/HCl (pH 7.4), 50 mM NaCl, 2 mM $CaCl_2$, 0.5 M urea and 2 mM β-mercaptoethanol for 16-24 h. For cleavage with Factor Xa the purified Trx-Xa-ET-1 protein (1-2 mg/ml) was digested using 1 U of Factor Xa (Qiagen) for 0.1 mg fusion protein at 23°C for 16-24 h in buffer containing 20 mM Tris/HCl (pH 7.2), 50 mM NaCl, 1 mM $CaCl_2$ and 2 mM β-mercaptoethanol. Following proteolytic cleavage, fragments were separated by RP-HPLC (LDC-Analytical, Pickering Laboratories), using a phenomenex Jupiter C18 preparative column (250 mm x 21.2 mm, 300Å, 5 μm spherical particle size) with a flow rate of 5 ml/min and UV-detection at 220 nm. The column was equilibrated with 10% acetonitrile in ddH_20/0.1% TFA until a stable baseline was attained. 5 ml of 1-2 mg/ml protein was subsequently loaded onto the column. The products were eluted with a gradient of acetonitrile from 10% to 95% over 70 min. 6-8 runs were performed, and a total of up to 5-6 mg of purified ET-1 was recovered. Fractions collected from the preparative run were analytically assayed using the same column and synthetic ET-1 as external standard. Pure fractions were pooled, lyophilized to a white powder and stored at -20°C. The concentration of purified ET-1 was determined by UV spectroscopy (280 nm; $\varepsilon = 2793\ M^{-1}\ cm^{-1}$). Synthetic ET-1 was prepared by Fmoc solid phase synthesis in the group of Dr. M. Beyermann (FMP, Berlin).

4.2.6 Preparation and solubilization of inclusion bodies of GST-bigET-1

Each gram of pelleted cells was resuspended in 8 ml lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, protease inhibitor cocktail) and lysed by French press. Crude inclusion bodies were recovered by centrifugation at 20000 g for 20 min, and washed twice with washing solution (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% (v/v) Triton X-100). Each 0.1 g of washed inclusion bodies was solubilized in 1 ml of 8 M urea containing 20 mM Tris-HCl, pH

8.0, and 150 mM NaCl . The resulting solution was incubated with agitation for 2 h at 8°C, and the insoluble component was removed by centrifuging at 20 000 g for 10 min. The preparation contained more than 90% GST-bigET-1, as judged by SDS-PAGE.

4.2.7 MALDI-TOF MS

Molecular weights of compounds were determined by a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis using a Voyager-DE STR Biospectrometry workstation with delayed extraction and reflectron capability (PE Biosystems) and α-cyano-4-hydroxycinnamic acid as the matrix. Mass spectra were obtained in the positive ion mode. 20 kV accelerating voltage was used. To calculate the efficiency of isotope incorporation, the ratios of the signal intensities of the observed monoisotopic mass and the theorectical monoisotopic mass of complete uniformly labelled ET-1 were calculated. Values are expressed as m/z. In addition the obtained isotope distribution of the uniformly labelled ET-1 was compared with a predicted distribution using the isotope simulator IsoPro 3.0 (http://members.aol.com/msmssoft/). For reduction of cysteine residues, the ET-1 sample was incubated with a 30-fold molar excess of Tris(2-Carboxyethyl)-Phosphine Hydrochloride (TCEP; Pierce) in acetic acid/H_2O (40:60 v/v), pH 2.5 at room temperature for 30 min. TCEP can reduce disulfide protein samples under acidic conditions, substantially more effective than DTT, and cannot form covalent interactions with cysteines. The samples were subjected immediately to MALDI-TOF measurements.

4.2.8 NMR spectroscopy

For all NMR experiments, measurements were performed using a Bruker DRX600 spectrometer at 298 K. Samples (unlabelled, uniform [^{15}N and ^{13}C] or selective ^{13}C labelled ET-1) were dissolved in 0.5 ml d_3-acetic acid/H_2O (40:60 v/v) at pH 2.5 to give a final peptide concentration of 0.7-1.7 mM. A total correlation spectroscopy (2D-TOCSY) experiment was performed, collecting 1024-2048 points in f2 and 400-600 points in f1. A ^{15}N-HSQC spectrum was recorded with 1024 (t1) x 512 (t2) complex points in each dimension and spectral width of 3012 Hz (^{15}N) x 10000 Hz (^{1}H). Backbone Cα, HN, N and side chain Cβ resonances were assigned, using triple resonance experiments (HNCACB and HN(CO)CACB pair). For all experiments performed, quadrature detection in the indirect dimensions was achieved using time-proportional phase incrementation (TPPI) and solvent suppression was carried out by using the presaturation method or using the Watergate pulse sequence. The spectra were processed with the software package XWINNMR version 2.6 (Bruker), whereas

the assignment of the resonances was carried out using Sparky version 3.100 (T.D. Goddard & D.G. Kneller, University of California).

4.2.9 Activity assay

Competition radioligand binding assays were performed to determine the affinity of ET-1 for ET_BR. Membrane suspensions were incubated for 2 h at 25°C in 100 µl of Tris-BAME buffer containing 100 pM ^{125}I-ET-1 and progressively higher concentrations of unlabelled ET-1. After incubation, free ^{125}I-ET-1 was separated from the bound ^{125}I-ET-1 using vacuum filtration.

4.3 Results

4.3.1 Novel expression vectors for ET-1 production in *E. coli* – an overview

Expression of ET-1 in *E. coli* by four bacterial expression vectors listed below was examined (Fig 4.3). In constructs 4.3 A, the ET-1 genes were only fused to the His-tag and a protease recognition sequence, which should facilitate purification and give the constructs a reasonable minimal size to be expressed. They mainly differ in the promoter, i. e. different strength of transcription regulation, vector backbone and the protease recognition site. For the case that these short constructs are not expressed, the ET-1 genes were alternatively fused to the well-expressed *E. coli* thioredoxin domain (constructs 4.3 B & C). The recognition sites of enterokinase and Factor Xa were chosen because Enterokinase (EK) and Factor Xa are, according to their specificity (EK, DDDDK↓; Factor Xa, IEGR↓), the only commercially available proteinases that could cleave the fusion protein to ET-1 without additional amino acids at its NH_2-terminus.

The main focus of this work was on the constructs TrxEKET-1 and TrxXaET-1.

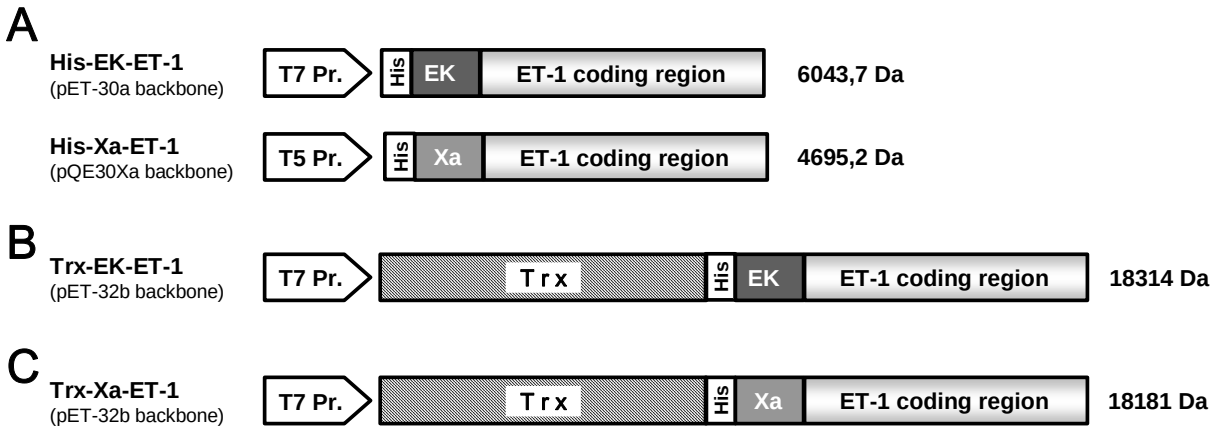


Fig. 4.3. Expression vectors for ET-1 used in this study. His, His6-tag; EK, enterokinase; Xa, factor Xa; Trx, thioredoxin; Pr., promoter.

4.3.2 Evaluation of direct ET-1 expression constructs

Since the most direct way of production of ET-1 would be the direct overexpression of the ET-1 cDNA, fused to a protease-cleavable purification tag, this strategy was envisaged at first. The ET-1 gene was introduced as N-terminal His-tagged fusion proteins, either under control of the T5 or T7 promoters (see constructs shown in Fig. 4.3A). Analysis of expression of ET-1 by Western Blot showed that specific ET-1 product could not be detected (data not shown), possibly due to proteolytic degradation in the host. These results showed that straightforward systems were not suitable for direct ET-1 overexpression.

4.3.3 Evaluation of a previous GST-BigET-1 construct

To evaluate the strategy of expressing BigET-1 as partner of a fusion protein, the construct GST-BigET-1 (Fig. 4.2B) was chosen. Previous studies had already shown that expression of GST-BigET-1 results in inclusion bodies (J. Wolkenhauer, pers. communication). As a variant strategy to reduce the formation of inclusion bodies, the temperature for induction with IPTG was reduced from 37°C to 25°C. However, inclusion bodies were still recovered and solubilized in 8 M urea. The fusion protein was recovered and purified to >95% homogeneity in a single-step affinity chromatography on Ni(2+)-nitrilotriacetic acid (NTA) agarose. Purified GST-ET-1 was obtained at a yield of 225 mg/l of culture. However, processing of the fusion to release ET-1 using limited trypsin digestion (1:100 (w/w) ratio of protease to target) proved to be too inefficient, considering also the low fraction of BigET-1 (4 kDa) in the GST-

BigET-1 fusion protein (32 kDa), to obtain appreciable amounts of ET-1. Therefore, approaches using a large size fusion partner were discontinued.

4.3.4 Results with the expression construct Trx-EK-ET-1

Since previous attempts to express intact human ET-1 in bacteria were unsuccessful, the ET-1 peptide was expressed as a thioredoxin fusion. Thioredoxin was selected as fusion partner because of its small size (109 amino acids), and its property to act as a chaperone to protect undesirable aggregation during expression (LaVallie *et al*., 1993; Kern *et al*., 2003).

4.3.4.1 Expression using Trx-EK-ET-1 results in soluble protein

For expression of ET-1 fused to the C-terminus of thioredoxin, ET-1 was cloned into pET32b and introduced into *E. coli* BL21(DE3) (Fig. 4.3B). Upon induction with IPTG at OD_{600}=0.8 in LB medium, the Trx-EK-ET-1 accumulated to high levels within the cells and upon separation of the soluble and insoluble cell extracts, the fusion protein was found primarily in the soluble fraction. In preliminary experiments, the cytoplasmic production of Trx-EK-ET-1 was compared in *E. coli* strain BL21(DE3) and its isogenic derivative OrigamiB(DE3), which displays a more oxidizing cytoplasm. Production level of Trx-EK-ET-1 was similar when using OrigamiB(DE3), suggesting that disulfide formation was not more efficient in this strain and that disulfide formation was not the limiting step for producing high levels of Trx-EK-ET-1.

4.3.4.2 Purification of Trx-EK-ET-1

The fusion of ET-1 peptide with His6 allows rapid purification using Ni-NTA affinity. Protein purification was done under native conditions and the use of 2 mM β-mercaptoethanol in all buffers should prevent oxidation and possible inactivation of the fusion protein following to bacterial cell disruption. The purification steps (see Fig. 4.4 A) show that one major protein species with an apparent molecular mass of 19 kDa was obtained. Further analysis by MALDI-TOF confirmed a single molecular species of 18300 ± 300 Da, which is consistent with the predicted molecular mass for Trx-EK-ET-1. In addition, the fusion protein was detected mainly as monomer, a small part was detected as dimer. Although the resulting protein was pure, it proved to be inherently unstable and could not be stably stored undergoing degradation at 4°C within 24-48 h (detected as two additional peaks in MS spectra of around 12 kDa). Because of degradation, anion exchange chromatography as a second purification step was applied. Degraded fragments could be removed in the flow through of

the chromatographic fragtions. Finally, from 1 liter of culture, 60-80 mg of TrxET-1 was typically purified.

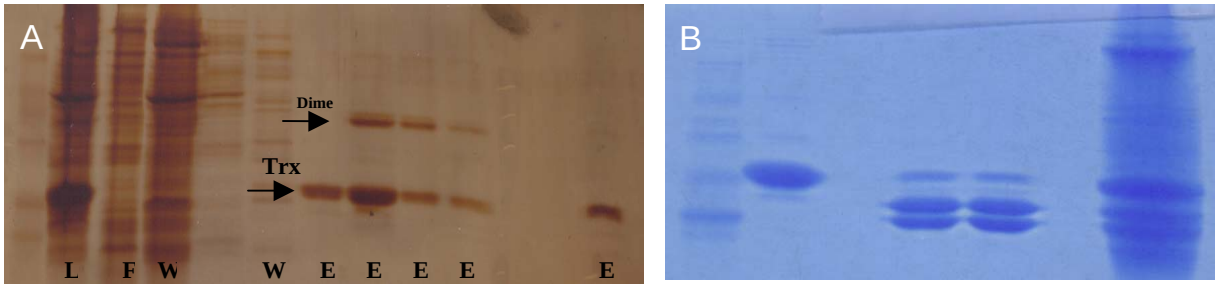


Fig. 4.4. (A) FPLC purification on Ni-NTA Superflow. TrxEKET-1 (~18 kDa) was purified from cleared lysate (0.8 liters M9). Left: silver-stained SDS-Gel. L: lysate, F: flow-through, W: wash, W1: 50-mM wash, E1-E4: eluate. Total yield was about 40 mg. (B) Cleavage of TrxEKET-1 with enterokinase. Left lane: before cleavage; middle lanes: during cleavage; right lane: aggregation after cleavage.

4.3.4.3 Cleavage of Trx-EK-ET1 fusion protein by enterokinase

Downstream processing of the fusion protein to obtain native ET-1 requires enzymatic removal of the fusion partner. In Trx-EK-ET1, the fusion partner was designed to be removed from the ET-1 moiety using enterokinase digestion. The cleavage reaction was followed by reducing SDS-PAGE (Fig. 4.4 B). At 37°C Trx-EK-ET-1 was nearly completely digested, yielding two products, one of 12 kDa and the other of 16 kDa. The 12 kDa species resembled that already present in the uncleaved sample and is most likely a degraded variant of the fusion protein, proteolysed by *E. coli* enzymes. The intensity of this band increased in the course of the cleavage reaction, suggesting that a recognition motif in the spacer competed effectively with the enterokinase recognition site. To enhance cleavage, the protein solution was slowly dialyzed against cleavage buffer containing 0.5 M urea at 4°C and subsequently the dialyzate was subjected to cleavage. A problem encountered under these conditions was the occurrence of aggregation, most probably because under these partially denaturing conditions unfolding intermediates are formed, which tend to self-associate or aggregate (Fig. 4.4 B, right lane).

4.3.4.4 Purification of ET-1

Once the purified fusion protein has been cleaved, the resulting fragments were purified by RP-HPLC. Separation of the cleavage by reverse-phase HPLC gave rise to the chromatogram shown in Fig.4.5A. Previously, synthetic ET-1 was used as a standard and eluted with an

acetonitrile concentration of 24% (R_f= 50). The Trx fusion partner was well resolved from ET-1. The peak corresponding to ET-1 after enterokinase cleavage was not as sharp as that of the native ET-1 but corresponded to that of native ET-1 in terms of retention time on HPLC, suggesting that it is a mixture of conformers with slightly different retention times. The final yield of ET-1 peptide from 60 mg of TrxET-1 was 0.5 mg as determined by UV spectroscopy corresponding to 6% recovery with respect to the theoretical yield.

In conclusion, despite successful efforts to obtain the fusion protein expressed as soluble fraction (and possibly folded into a native conformation), the percentage recovery of the ET-1 peptide was relatively low, which appears to be due to loss in the enzyme digestion step.

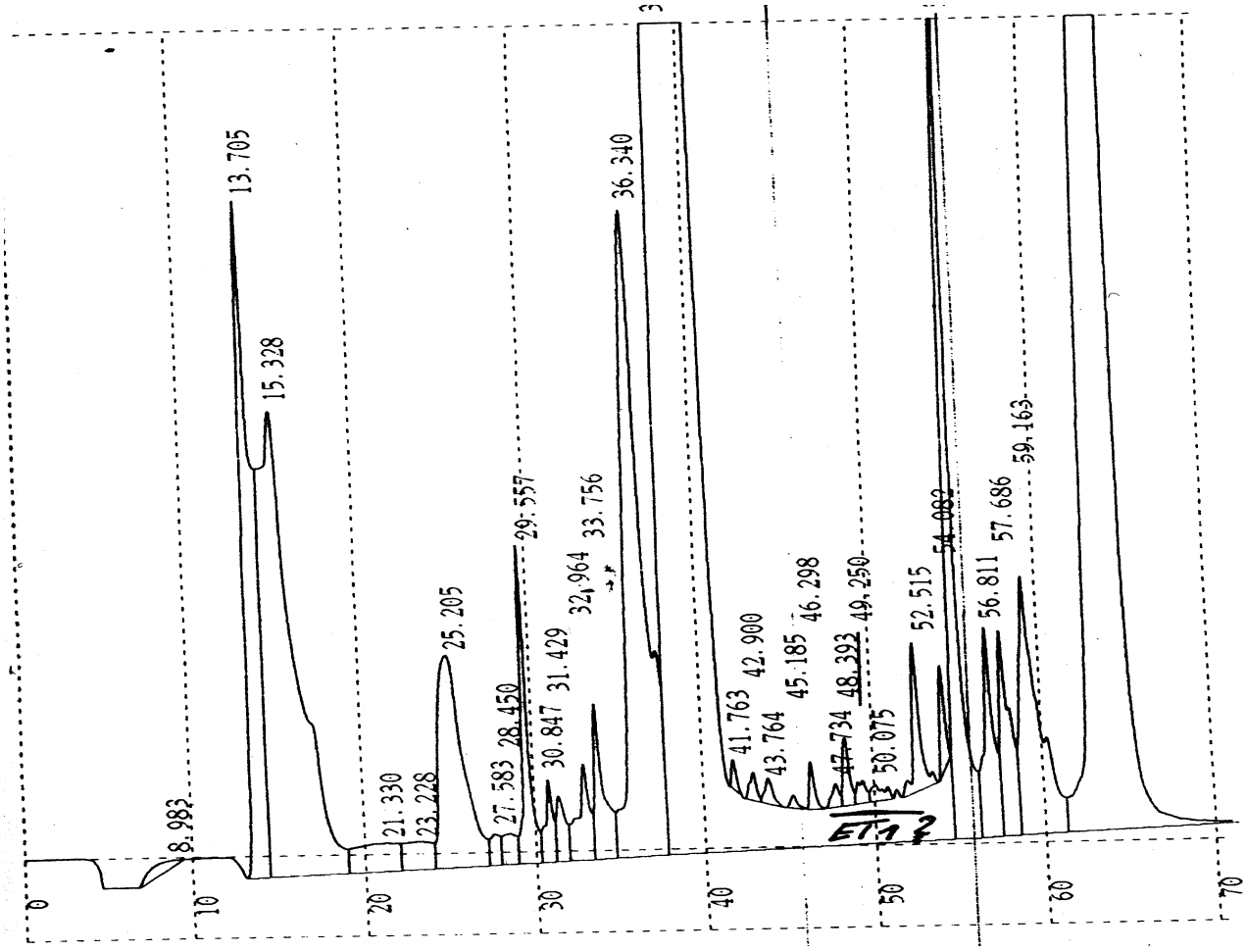


Fig. 4.5. RP-HPLC chromatogram of Trx-EK-ET-1 after cleavage with enterokinase. The sample was loaded onto a C18 column equilibrated with 10% solvent B (80% acetonitrile, 0.1% TFA) in solvent A (0.1% TFA) and separated using a 70-min linear gradient of 10-95% solvent B in solvent A, at a flow rate of 5 ml/min. The area corresponding to purified ET-1 is labelled with a line.

4.3.8 Characterization of recombinant ET-1 by MALDI-TOF MS and analytical RP-HPLC

To identify ET-1 in the collected elution fractions, these fractions were subjected to MALDI-TOF. The identity of the correctly folded ET-1 was determined by comparing HPLC retention time of the folding products with the reference synthetic ET-1. The recombinant ET-1 peptide's molecular mass was confirmed to within 0.1% of the theoretical value (2490.9 Da). With lower intensities, adducts of ET-1 with sodium ions were detected at m/z 2512.8. MALDI-TOF analysis showed that the recombinant ET-1 eluted as a broad peak and separation leaded to peak tailing. This problem may have its origin in the ET-1 molecule's secondary and/or tertiary structure.

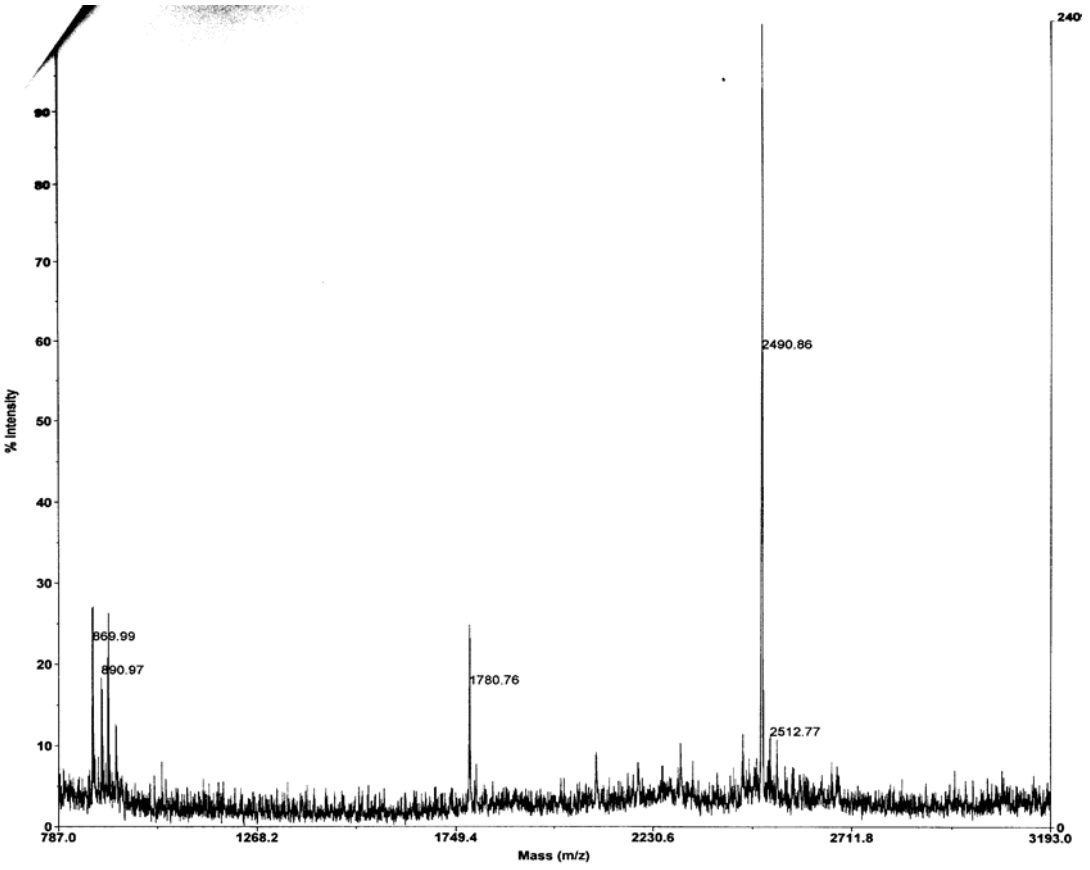


Fig. 4.6. MALDI-TOF analysis of purified ET-1 after enterokinase cleavage.

To investigate the disulfide pattern obtained in the purified ET-1, the product fraction, previously determined by MALDI-TOF MS to be relatively pure, was compared with a reference sample by means of analytical RP-HPLC. The ET-1 loaded was recovered as one major peak with trace impurities. The retention times on RP-18 HPLC were identical with synthetic ET-1 under the same conditions (Fig. 4.7). Therefore, it was concluded that both the

recombinant and synthetic peptides contain 4 intramolecular disulfide bonds with the same pattern of connectivity.

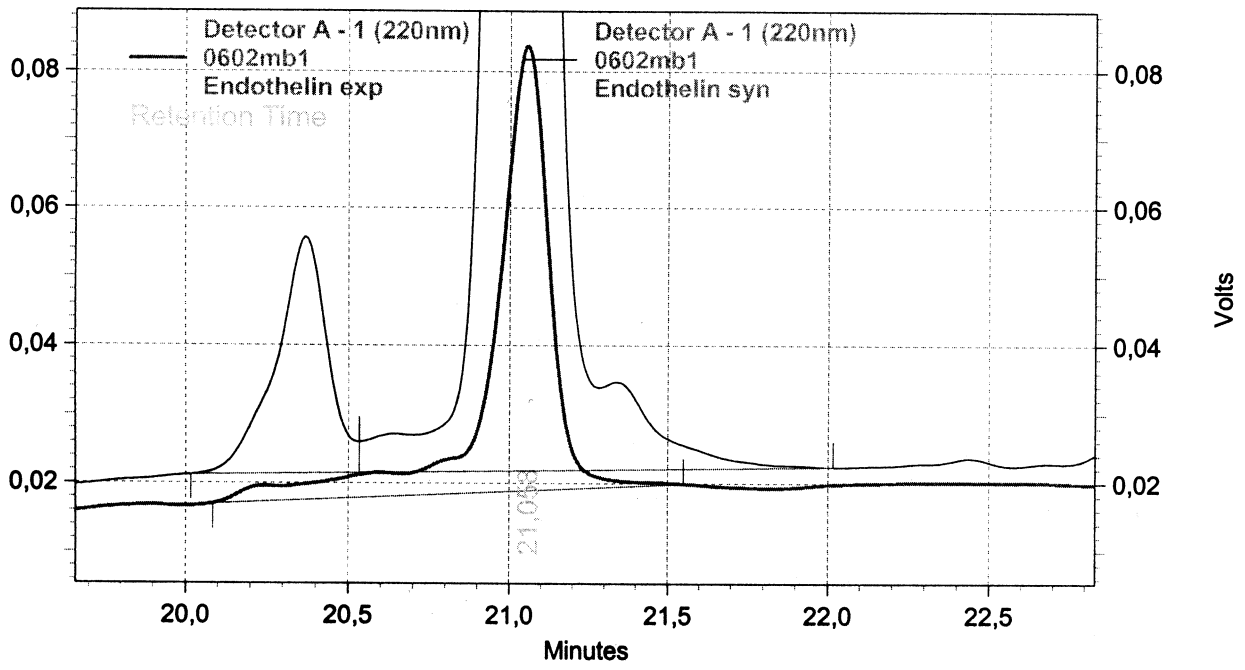


Fig. 4.7. Comparison of synthetic and recombinant ET-1 by RP-HPLC. Overlay of chromatograms obtained following RP-HPLC of recombinant ET-1 (dark line) and synthetic ET-1 (pale line).

4.3.9 Activity assay

In order to be able to directly compare recombinant *versus* synthetic affinities of ET-1, radioligand competition assays between recombinant and synthetic ET-1 to the ET_BR, prepared from Sf9 cells that express ET_BR-GFP, were conducted. This assay is based on the competitive inhibition of ^{125}I-ET-1 binding by the recombinant or synthetic homologue to be tested. Figure 4.8 shows the concentration-binding curves of the synthetic active ET-1 and the recombinant ET-1. Receptor binding affinities were calculated as IC_{50} values (the IC_{50} value represents the ET-1 concentration required for 50% inhibition *in vitro*). Synthetic ET-1 was employed at concentrations varying from 10^{-11} to 10^{-6} M and exhibited an IC_{50} of 0.3 nM. Recombinant ET-1 also produced concentration-dependent [^{125}I]ET-1 binding, but was 5-fold less potent than synthetic ET-1 (IC_{50}= 6.6 nM).

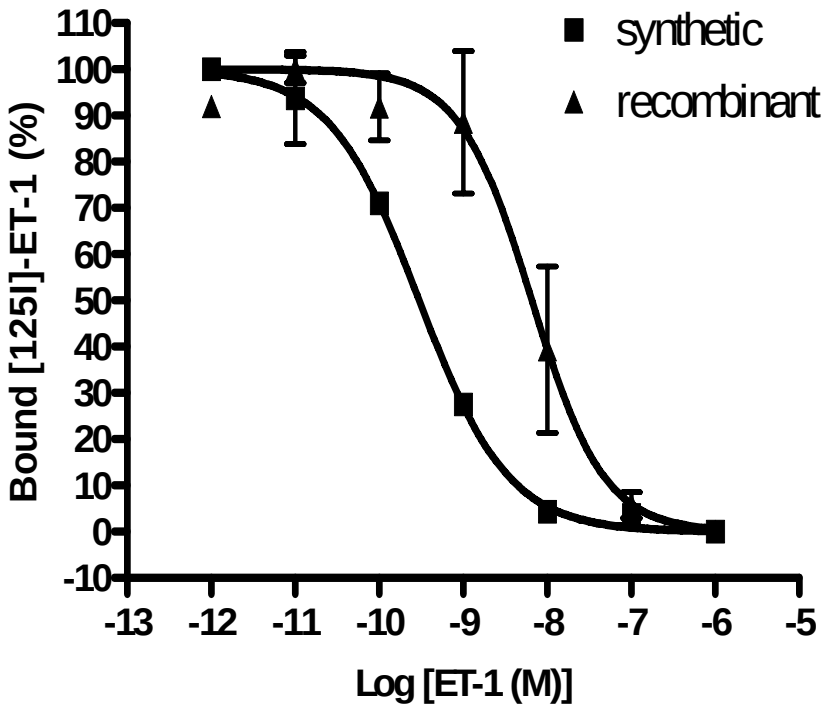


Fig. 4.8. Inhibition by ET-1 of the specific [^{125}I]ET-1 binding to ET_BR-GFP. Ligand binding activities were performed at 5 µg of membrane protein/ml. Increasing concentrations of ET-1 were added to receptor expressed in Sf9 cell membranes to compete with 100 pM [^{125}I]ET-1. Competing peptides were synthetic or recombinant ET-1.

The thioredoxin fusion system was successful in expressing Trx-EK-ET-1, and the expression was high-level. However, removal of the fusion partner to produce ET-1 was technically difficult, most likely due to the dependence on neighbouring residues of the enterokinase recognition site. Moreover, the activity of the purified recombinant ET-1 was determined to be not identical to that of the chemically synthesized peptide. Therefore, further optimization of the Trx-EK-ET-1 approach was discontinued and, to evaluate a different proteolysis strategy, the enterokinase site was exchanged for that of Factor Xa (see construct Fig. 4.3C).

4.3.5 Results with the expression construct Trx-Xa-ET-1

4.3.5.1 Expression and isotopic labelling using the construct Trx-Xa-ET-1

To devise a alternative cleavage strategy using the previously shown approach to fuse ET-1 to Thioredoxin, the construct pET32bTrxXaET-1 was generated. As shown in Fig. 4.3C, this expression vector encodes a fusion of ET-1 to the C-terminus of thioredoxin, a hexa-Histidine tag for affinity purification and a Factor Xa cleavage site between the fusion partners.

Upon expression of the construct in the strain *E. coli* BL21(DE3), the soluble and insoluble cell extracts were separately analyzed by SDS-PAGE. A protein species exhibiting approximately the molecular mass calculated for the His6-TrxXaET-1 (18.1 kDa) was found to be accumulated to a large extent in the soluble fraction (Fig. 4.9A). The soluble fraction was purified in two successive steps first by Ni-NTA affinity chromatography and subsequently by anion exchange chromatography. In the majority of eluate fractions analyzed from the final purification step by SDS-PAGE (Fig. 4.9B), a single protein species with an apparent molecular mass of about 18 kDa was detected. A higher resolution of the molecular mass of this protein species was additionally determined by MALDI-TOF. Within the molecular range of 2 kDa to 20 kDa, a predominant molecular species of 18,100 ± 100 Da was observed, consistent with the molecular mass calculated for TrxXaET-1. An additionally observed protein species of apparently the double size (lane 8 in Fig. 4.9B) resulted possibly from the formation of dimers covalently linked by cysteine disulfide bonds, since both protein species were recognized by an antibody directed against the His6 tag (data from Western Blot analysis not shown).

Conditions for expression and purification were initially optimized in M9 minimal medium. Finally, the optimized parameters (as described in Materials and Methods) were applied for selective isotope labelling supplementing M9 medium with ^{15}N-NH_4Cl as sole source of nitrogen and ^{13}C-glucose, 1,3 ^{13}C-glycerol and 2 ^{13}C-glycerol, respectively, as sole source of carbon. The efficiency of the labelling procedure relies on a very low basal expression of the cloned target gene during the growing phase. The T7 polymerase-based pET system provides such an efficient and stringently controlled expression of the target peptide.

The total concentration of expressed and purified fusion protein was determined by UV spectroscopy. The yield varied between 80 mg/l (^{13}C-glucose), 61 mg/l (1,3 ^{13}C-glycerol) and 65 mg/l (2 ^{13}C-glycerol), respectively, (Table 4.1) pointing towards a higher metabolic efficiency of glucose compared to glycerol as sole carbon source.

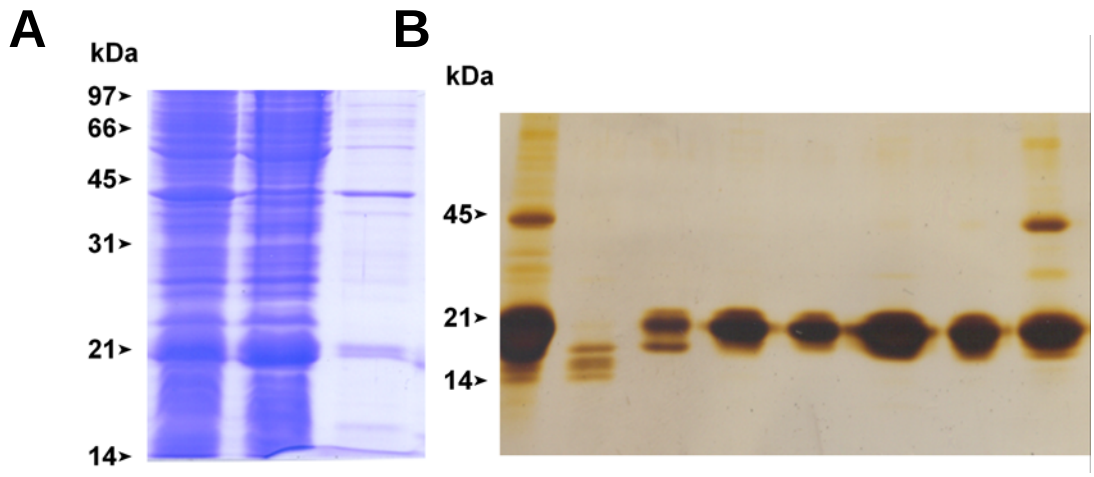


Fig. 4.9. (A) Expression of soluble Trx-Xa-ET-1 in BL21(DE3). Lane1: total cell lysate, lane 2: soluble cell lysate, lane 3: inclusion bodies fraction. (B) FPLC purification on ResourcQ. The pooled Ni-NTA fractions containing Trx ET-1 was purified. Silver-stained SDS-Gel: pooled Ni-NTA fractions (lane 1, left), flow-through (lane 2), wash fraction (lane 3), eluate fractions (remaining lanes).

4.3.5.2 Cleavage of the Trx-Xa-ET-1 fusion protein by Factor Xa and RP-HPLC analysis

Our goal was to isolate from the fusion protein a cleavage product with a primary sequence identical to authentic ET-1. Therefore we decided to select a proteolysis strategy using Factor Xa that is known to cleave with high specificity N-terminally to the tetrapeptide Ile-Glu-Gly-Arg thereby converting the purified precursor to the 21 amino acid ET-1. The conversion efficiency of the fusion protein to the cleavage product was approximately 90% as estimated from SDS-PAGE (data not shown). To separate the cleavage products, we employed preparative RP-HPLC and to identify the correct 21 amino acid ET-1 species, we made use of synthetically available ET-1 peptide as an external reference. Separation chromatograms of the isotopically labeled Trx-Xa-ET-1 cleavage products in comparison to synthetic ET-1 show that both samples gave rise to a single and sharp peak elution at an acetonitrile concentration of 24% corresponding to a retention time of 44.5 min. The identity in terms of retention time confirmed the recovery of a fragment with biophysical properties (hydrophobicity) identical to synthetic endothelin. Furthermore, this result strongly suggests conformational identity of both species with respect to their three-dimensional structures.

The yield of purified ET-1 was determined by UV spectroscopy (Table 4.1). Taking into account the molar weight ratios of fusion protein and released peptide, the total yield of purified ET-1 with respect to purified Trx-Xa-ET-1 was as high as 57%. This result

demonstrates for the first time that isotopically labelled ET-1 can be efficiently produced employing a single cleavage step in mg quantities.

Table 4.1 Comparison of recombinant ET-1 protein production yield.

	$^{13}C_6$ Glucose		**1,3-^{13}C-Glycerol**		**2-^{13}C-Glycerol**	
	Total protein	Yield	Total protein	Yield	Total protein	Yield
Fusion protein (Trx-Xa-ET-1)	64 mg (3.5 µmol)		61 mg (3.3 µmol)		65 mg (3.5 µmol)	
Isolated ET-1	5 mg (2µMol)	57%	3,6 mg (1.4 µMol)	42%	2,0 mg (0.8 µMol)	23%

Fusion proteins were isolated from 0.8 l (^{13}C-glucose) and 1 l (^{13}C-glycerol) cultures. Total protein [mg], molarity [µmol] and yield (%) are given.

4.3.5.3 MALDI-TOF analysis of labelled ET-1

An important question for applications using isotopically labelled proteins is to what extent the isotopes were incorporated. To analyze the extent of ^{15}N and ^{13}C incorporation, we subjected the purified ET-1 samples to a MALDI-TOF mass spectrometry. Whereas ^{15}N-NH_4Cl and ^{13}C-glucose substrates are expected to result in uniform labelling of all N and C atoms, respectively, the ^{13}C from 1,3-^{13}C-glycerol and 2-^{13}C-glycerol, when these compounds are used as carbon sources, is expected to be partially incorporated into different amino acids (McDermott *et al*., 2000).

We calculated the mass of ET-1 containing all four cysteine residues in the oxidized state to be at 2491.03 Da. This value is in good accordance to the major peak for recombinant non-labelled ET-1 (m/z = 2490.67; Fig. 4.10A) considering an error range of 0.5 Da generally assumed for peptide MALDI-TOF measurements in reflector mode. This results confirms the identity to authentic ET-1 of the RP-HPLC-purified cleavage product.

An analysis of isotopic incorporation is based on the shift in m/z for the isotopically labeled ET-1. However, also the reduction of the four cysteines would lead to an incremental increase in peptide mass and therefore compromise calculation of incorporation grade. To determine the extent of disulfide bond formation in recombinant ET-1 from the change in the m/z ratio upon disulfide reduction, we compared the mass spectra before and after reduction for samples of both the recombinant unlabelled and labelled ET-1. A comparison of the mass spectra obtained for purified unlabelled ET-1 and its reduced derivative (enlarged monoisotopic mass profiles shown in Fig. 4.10B and 4.10D) confirms a difference in m/z of 4 a.m.u. whereas the overall distribution of the multiplet seemed to be unaffected. This result

indicates that the cysteine residues in recombinant ET-1 are homogenously oxidized (two intrachain disulfide bonds) whereas the cysteine residues in the TCEP-treated sample are homogenously reduced.

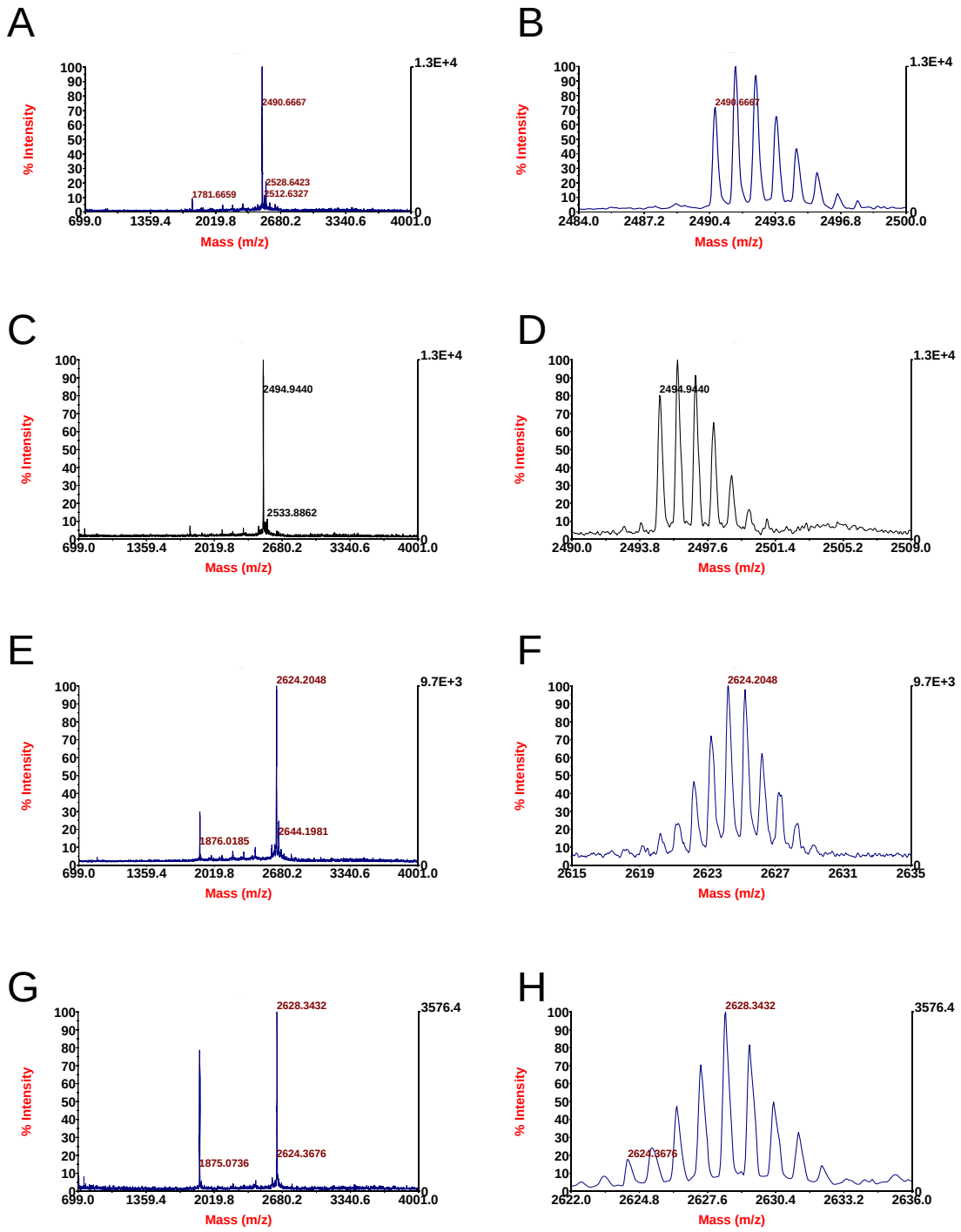


Fig. 4.10. MALDI-TOF mass spectrometry analysis of purified ET-1.
Mass spectrum of recombinant unlabelled ET-1 before (A) and after reduction (C). Associated sodium and potassium adducts may be responsible for side peaks at *m/z* 2512.63 and 2528.64, respectively. Enlarged view shows the monoisotopic mass profile of oxidized (B) and reduced (D) unlabelled ET-1. An isotopic multiplet results from unique abundances of naturally occuring C, H, N, O and S isotopes. This shows the mass shift of 4 Da. Mass spectrum of uniformely $^{13}C/^{15}N$ labelled recombinant ET-1 before (E) and after reduction (G) with enlargements showing the same species (F) or its reduced derivative (H).

The MALDI-TOF spectrum for ET-1 that has been uniformely labelled using ^{15}N-NH_4Cl and ^{13}C-glucose as substrates shows a predominant peak with a mass (m/z) of 2624.09 Da (Fig. 4.10E). Assuming complete isotope incorporation, we calculated the monoisotopic mass for this species to be at 2624.31 Da which is in good accordance to the experimentally obtained m/z value. Upon reduction, an increase in 4 a.m.u. was also observed for ^{13}C and ^{15}N-labelled ET-1 (compare muliplet profiles in Fig. 4.10F (oxidized) and Fig. 4.10H (reduced)) confirming that the recombinant species contains homogenously oxidized cystein residues. To determine the percent incorporation of ^{13}C and ^{15}N isotopes, we applied two different methods: First, we compared the intensity ratios of peaks at incrementally (-1 a.m.u.) lower masses to the peak at 2624 Da (complete labelling). Secondly, we compared our experimental monoisotopic mass distribution profile to a predicted profile using the isotope simulator IsoPro 3.0. Both methods indicated an average ^{13}C and ^{15}N labelling of $\geq$ 98%.

In an analogous way, we subjected ET-1 samples labelled with 1,3-^{13}C-glycerol and 2-^{13}C-glycerol to MALDI-TOF spectrometry and observed predominant species at 2583.23 Da and 2558.91 Da, respectively (mass spectra are listed in Appendix 5). With respect to ^{15}N incorporation, conditions of bacterial expression were as described before and thus a similar degree of ^{15}N incorporation may be assumed. However, the pattern of ^{13}C-labelling in these ET-1 species is complex and dependent on the anabolism of individual amino acids (McDermott *et al.*, 2000) preventing accurate determination of the degree of ^{13}C incorporation.

4.3.5.4 NMR analysis of labelled ET-1

NMR spectroscopy can provide insight on purity, conformation and folding states of the purified peptide, as additional information over the mass spectrometric results. For synthetic ET-1, the sequence-specific ^{1}H-NMR assignments as determined by DQF-COSY have already been described (Dalgarno *et al.*, 1992), and provided a reference to NMR spectra of our samples. Chapter 5 gives an introduction to NMR.

From ^{1}H-NMR TOCSY experiments we determined the chemical shifts for synthetic and recombinant ET-1 (Fig. 4.11A). First, we compared the ^{1}H-NMR chemical shifts for synthetic ET-1 to published ^{1}H-NMR assignments (Dalgarno *et al.*, 1992) and could confirm their identities. Second, the spectra of both the synthetic and recombinant ET-1 were overlayed. Since overlapping ^{1}H resonances were observed, we concluded that the conformational state of recombinant ET-1 is identical to synthetic ET-1. The proportion of the overlapping conformation as evaluated from the ratio of main to side resonances was at least 80% for both

synthetic and recombinant ET-1. Since synthetic ET-1 previously showed high affinity to the ET_BR in biochemical displacement assays (IC_{50} = 1.7 *10^{-10} M, Doi *et al.*, 1999; IC_{50} = 3*10^{-10} M; data of this work, see Fig. 4.8), we conclude from the identity of ^{1}H-NMR spectra that biochemically active ET-1 was obtained in our protocol.

To carry out assignment of resonances to the primary sequence, we conducted heteronuclear NMR experiments: The assignment of backbone (Cα, HN, N) and side chain Cβ resonances (Table 4.2) was inferred from ^{1}H-^{15}N HSQC (Fig. 4.11B) and additional HNCACB, HN(CO)CACB triple resonance experiments. The fingerprint region of ^{1}H-^{15}N HSQC spectrum showed a good dispersion of the amide-proton resonances in the region from 7.0 to 10 ppm. Therefore, we concluded that also the uniformely ^{13}C/^{15}N labelled ET-1 was present in the folded conformation.

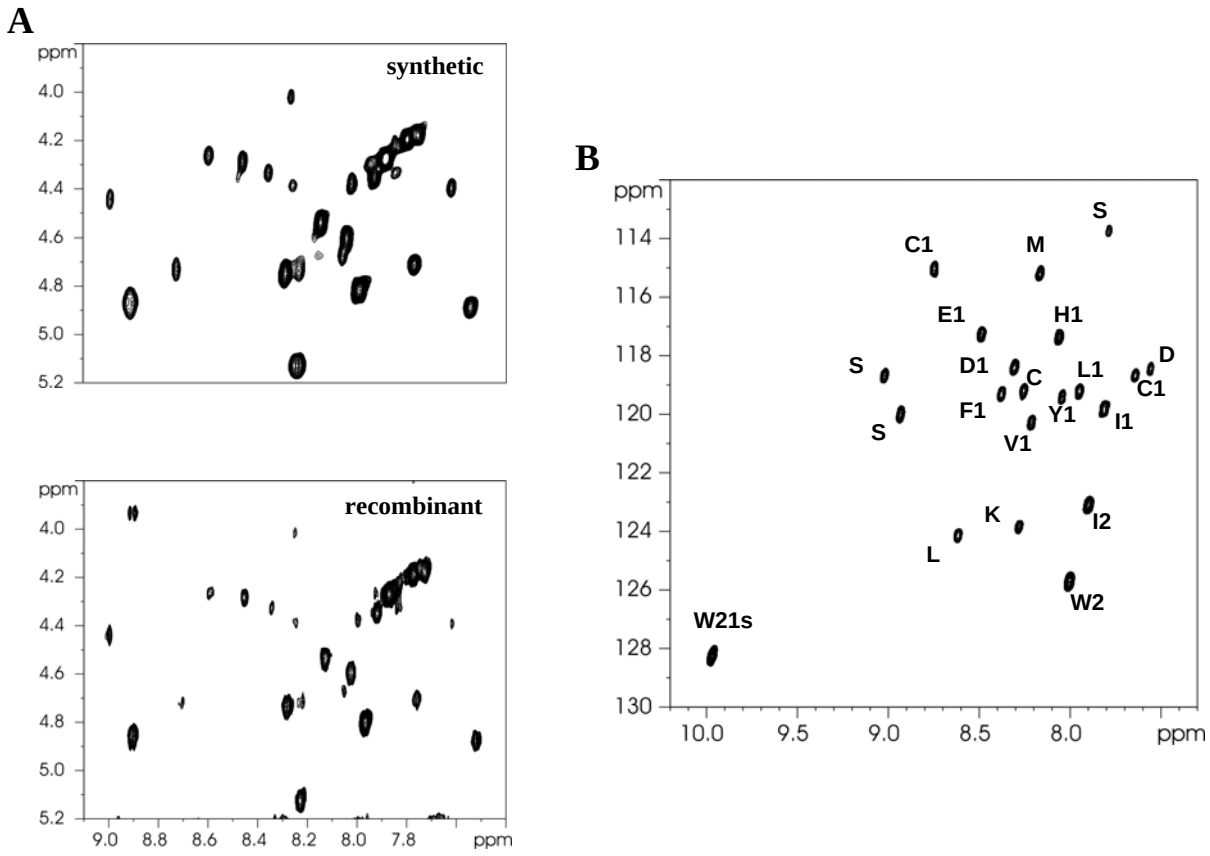


Fig. 4.11 NMR analysis of recombinant ET-1. (A) Overlay of NH fingerprint region of the 2D-TOCSY of recombinant (red) and synthetic (green) ET-1. (B) ^{1}H-^{15}N-HSQC of uniformly labelled ET-1. Backbone assignments are as indicated

Table 4.2 Resonance assignments for uniformely $^{13}C/^{15}N$ labeled ET-1.

Residue	Chemical shift (ppm)			
	Cα	Cβ	NH	N
Cys[1]	52.7	36.4		
Ser[2]	54.9	62.3	8.9	120.0
Cys[3]	50.9	39.7	8.3	119.2
Ser[4]	57.2	60.6	9.0	118.7
Ser[5]	54.7	61.8	7.8	113.7
Leu[6]	53.5	38.7	8.6	124.1
Met[7]	52.8	29.6	8.2	115.2
Asp[8]	49.6	35.3	7.6	118.5
Lys[9]	57	29.7	8.3	123.9
Glu[10]	56.1	25.9	8.5	117.3
Cys[11]	57.1	38.0	7.6	118.6
Val[12]	63.8	29.1	8.2	120.3
Tyr[13]	58.1	35.4	8.0	119.5
Phe[14]	58.4	36.6	8.4	119.3
Cys[15]	52.5	36.5	8.7	115.1
His[16]	53.9	24.9	8.1	117.4
Leu[17]	52.9	39.7	7.9	119.2
Asp[18]	50.4	35.3	8.3	118.4
Ile[19]	58.2	36.2	7.8	119.8
Ile[20]	58.1	36	7.9	123.1
Trp[21]	53.6	27	8.0	126.3

4.3.5.5 Scaling up production of ET-1 by fermentation

To obtain larger quantities of fusion proteins, switching from shake-flask expression to fermentation was undertaken, which in some cases, can even give increases in yield. Scale-up studies were conducted in BioFlo® 3000 benchtop fermenters (New Brunswick Scientific) with 10 L working volume. The optimal conditions previously established for shaking cultures were applied to the fermenter cultures.

Scale-up studies were conducted in BioFlo® 3000 benchtop fermenters (New Brunswick Scientific) with 10 L working volume to evaluate how expression would be affected by the transition from shake-flask culture to a bioreactor. The maximal biomass obtained were 40 g *E. coli* cells wet weight/ litre of M9 medium containing glucose and 29 g *E. coli* cells wet weight/l of M9 medium containing glycerol, respectively, and the yields were comparable to that in 1 l shake culture. However, protein production was lower and carbon-source independent, with an average yield of 40 mg of purified Trx-Xa-ET-1 fusion protein/litre of culture in both M9 media, compared to 60-80 mg previously obtained with shake-flask culture (Table 4.1). One reason for reduced yield may be that for the present study, fermentation could only run in a batch mode, meaning that the growth of the cells is limited by the nutrients

present in the medium at the time of inoculation. Most of the carbon source ends up in cell mass in such a way that substantially less desired fusion protein is obtained.

4.4 Discussion

To produce sufficient quantities of ET-1 labelled with NMR sensitive isotopes, several criteria must be met, including high yield, low costs, scalability of host and keeping number of steps as low as possible. Substantial experimental effort has been expended to establish a recombinant expression system for ET-1. Previous studies employed the following strategies:

(1) The production method described by Yasufuku *et al.* consisted of expression of a β-galactosidase-bigET-1 fusion, solubilization of inclusion bodies, single-step purification and sequential trypsin and pepsin digestions to release at first bigET-1 and then ET-1. Finally, ET-1 was purified from the enzymatic digestions by RP-HPLC. Although this method is cost-efficient and straightforward, only 0.6 mg/l ET-1 were obtained, mainly due to low expression level of the fusion protein.

(2) The method described by Fassina *et al*., consisted of expression of a MBP-bigET-1 fusion, trypsin treatment to release bigET-1, a single-step affinity chromatography, cleavage of bigET-1 with α-chymotrypsin to release ET-1 and purification of ET-1 by RP-HPLC. Although this method is relative efficient (3.1 mg/l ET-1), the overall process yield is low due to the large size of the fusion protein (51 kDa). In addition, the chromatography step requires preparation of a hydropathic peptide complementary to bigET-1 and immobilization of this peptide to the affinity column.

(3) The method described by Becker *et al*., consisted of expression of an AP-bigET-1 fusion, solubilization of inclusion bodies, two-steps of enzyme digestions with collagenase and dipeptidylpeptidase IV each followed by RP-FPLC purification to release bigET-1. Similar to Fassina *et al.*, the size of the AP-bigET-1 fusion is large (52 kDa), and the overall process costs are substantial (two step enzyme digestion, fermentation). This protocol yielded 1.1 mg/l bigET-1, which may be further processed to ET-1. Interestingly, with this method two disulfide conformers of bigET-1 in a molar ratio of 3:1 could be separated using RP-FPLC. For chemically synthesized ET-1, also two conformers were separated with HPLC in the same ratio (Kumagaye *et al*., 1988).

(4) Previous fusion constructs used in our group (Fig. 4.2; Trx-BigET-1, GST-BigET-1, DsbC-ET-1) commonly led to a low yield or produced proteins as inclusion bodies. Inclusion

bodies necessitate complex treatments including denaturation, refolding, and several purification steps which further decrease the quantity of the final product. By these approaches labelling of ET-1 with stable isotopes in quantities needed for receptor purification would have been prohibitively expensive. Therefore, these approaches were not further pursued.

In comparison to these protocols, the method described in the present work shows the following advantages: ET-1 was produced as minor partner of a rather small (18.1 kDa) Trx fusion protein. The purification and proteolysis strategies are straightforward and cost-effective. From 1 L of bacterial culture, we purified up to 5 mg ET-1 to homogeneity with a high overall process yield (up to 57%).

With respect to proteolysis strategies, cleavage sites for Enterokinase and Faktor Xa were evaluated. The cleavage efficiency of ET-1 was to a large extend affected by the nature of the protease recognition sequence. The presence of the enterokinase recognition sequence rendered the protein labile leading thus to enhanced susceptibility towards proteolysis. However, using Factor Xa proteolysis, difficulties with instability were not encountered. Finally, the TrxXaET-1 fusion protein approach showed applicable for use in recombinant ET-1 labelling and expression, with high purity of product and high product yield. An expression system that permits the production of sufficient quantities of ET-1 (mg scale) from 1 liter of culture was developed. The protocol is simple, achieves a high level of biosynthetic incorporation.

In conclusion, this work describes the application of a thioredoxin fusion strategy for production, affinity purification and isotope labelling of ET-1. Isotopically labelled peptides aid in resolving peptide resonances in NMR spectra, and are required to analyze the interaction of small peptides with proteins (e.g. to resolve the structure of a receptor-bound ligand; Luca *et al.,* 2003) using NMR. The expression strategy reported here should prove to be valuable for the recombinant production of isotopically labelled short peptides in general.