crystallization papers

Purification and crystallization of the extracellular domain of human neutral endopeptidase (neprilysin) expressed in *Pichia pastoris*

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Neutral endopeptidase (NEP) is a mammalian zinc metalloprotease involved in the inactivation of a wide variety of regulatory peptides such as enkephalins and atrial natiuretic factor. The soluble extracellular domain of NEP (sNEP) was expressed in the methylotrophic yeast *Pichia pastoris*. The protein was purified to homogeneity and single crystals have been obtained. Enzymatic deglycosylation of the enzyme was essential for the production of crystals suitable for X-ray analysis for both the NEP–phosphoramidon binary complex and the apo enzyme.

1. Introduction

Neutral endopeptidase 24.11 (also known as neprilysin; NEP; CALLA, CD10) is a membrane-bound zinc metalloendopeptidase originally extracted from the brush border of rabbit kidney (Kerr & Kenny, 1974). NEP is involved in the inactivation of many biologically active peptides, including the enkephalins, tachykinins, endothelin and atrial natiuretic peptide (for a review, see Erdos & Skidgel, 1989; Roques *et al.*, 1993; Turner & Tanzawa, 1997). Owing to the physiological importance of NEP, a variety of inhibitors have been designed and some are currently in clinical development (Burnett, 1999). The enzyme is the prototype of a group of metallopeptidases, which includes the endothelin-converting enzymes (ECE-1 and ECE-2), the erythrocyte surface antigen KELL and the PEX gene product, comprising the M13 subfamily of mammalian neutral endopeptidases. Each of these proteins consists of a short N-terminal cytoplasmic domain followed by a single transmembrane helix and a large C-terminal extracellular domain that contains the active site (HYP Consortium, 1995; Emoto & Yanagisawa, 1995; Lee *et al.*, 1991; Shimada *et al.*, 1994).

Early structure–activity studies have shown that the bacterial zinc metalloprotease thermolysin (TLN) and NEP cleave the peptide bond at the N-terminus of a hydrophobic residue and are inhibited by the same type of molecules, such as phosphoramidon and thiorphan (Benchetrit *et al.*, 1987; Fulcher *et al.*, 1982; Hersh & Morihara, 1986). Although there is very limited overall homology between the primary sequences of NEP and TLN, statistically significant similarities are observed between TLN and the carboxy-terminal domain of NEP (Benchetrit

et al., 1988). It is here that the signatures of the TLN family of proteases, consensus sequences ^{583}HExxH^{587} and ^{646}ExxxD^{650}, have been identified (Matthews, 1988). Some of the most important residues present in the active site of TLN, identified crystallographically, can be aligned with equivalent residues in NEP (Tronrud *et al.*, 1986; Weaver *et al.*, 1977). Based on this alignment, site-directed mutagenesis experiments have confirmed several residues in the active site of NEP and a three-dimensional model of the active-site region of this enzyme has been constructed (Tiraboschi *et al.*, 1999 and references therein). However, in order to design new potent and selective inhibitors of NEP, it is important to have precise information about its active site. Although the enzyme has been expressed in large quantities, successful crystallization of NEP has remained elusive (Devault *et al.*, 1988; Fossiez *et al.*, 1992). In this manuscript, we report the expression, purification and crystallization of NEP produced in *P. pastoris*.

2. Materials and methods

2.1. Construction of *P. pastoris* secreting sNEP

The extracellular domain of human NEP (sNEP, residues 52–749) was amplified by PCR using vector pCISrENK (Gorman *et al.*, 1989) as template, cloned into the T/A cloning vector (Invitrogen) and sequenced. The primers used for the PCR were Fwd, 5′-GCATCGATG-*GATGATGGTATTTGCAAGTC*-3′; Rev, 5′-TTCTTTGGTACC*TCACCAAACCCGG-CACTTC*-3′ (italicized sequences denote nucleotides complementary to the NEP cDNA). The forward primer used for the PCR introduced a *Cla*I restriction site and consequently added the amino acids alanine and serine to the N-terminus of the protein. The

reverse primer used in the PCR introduced a *Kpn*I site following the stop codon. The insert was excized from the T/A vector by *Kpn*I and partial *Cla*I digestion and cloned into the *Cla*I/*Kpn*I site of pPICZαC (Invitrogen, San Diego, CA, USA). The vector pPICZα-sNEP contains a open reading frame coding for the *Saccharomyces cerevisiae* α-factor secretion signal and the entire sequence for the extracellular domain of NEP. Approximately 30 µg of purified pPICZα-sNEP was linearized with *Sac*I. All methods used standard techniques (Sambrook *et al.*, 1989). The digest was sequentially extracted with phenol and chloroform, precipitated with ethanol and resuspended in 10 µl H_2O. 7.5 µl of plasmid was mixed with 70 µl electrocompetent *P. pastoris* GS115 or KM71 cells prepared as described in the EasySelect Pichia Expression Kit, Version C (Invitrogen). Following electroporation (1 mm cuvettes, 750 V; Eppendorf electroporator) transformants were selected on YPDS plates containing zeocin (100 µg ml^{-1}). The plates were incubated for 4 d at 303 K. Ten zeocin-resistant colonies from each strain were screened for methanol utilization (Mut phenotype) as recommended by the manufacturer (Invitrogen). DNA from colonies was isolated and the presence of the NEP gene was determined by PCR.

2.2. Screening of transformants for secretion of sNEP

Ten KM71 and ten GS115 transformants were screened for the production of secreted sNEP. Colonies were grown for 24 h in 2 ml YPD medium plus zeocin (100 µg ml^{-1}) to an OD$_{600}$ of 8–10. Cells were pelleted by
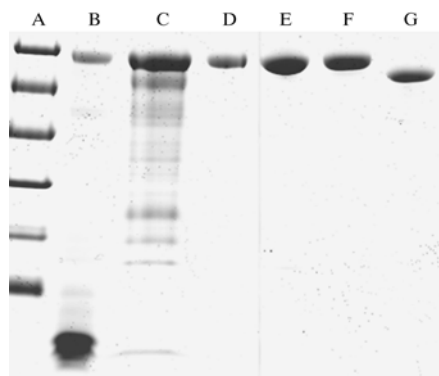

Figure 1
SDS–PAGE analysis of the NEP purification. The purity of the protein determined by HPLC is given in parentheses. Lane A, M_W standard; lane B, Sephacryl-200 (24.5%); lane C, phenyl Sepharose (46.3%); lane D, SourceQ (55.6%); lane E, MonoQ (92.6%); lane F, BioSec (96.3%); lane G, deglycosylated NEP.

centrifugation and resuspended in 20 ml YP medium (1% yeast peptone and 2% peptone) plus 3% methanol at a final OD$_{600}$ of 1. Cultures were grown at 303 K in 100 ml baffled flasks shaken at 250 rev min^{-1}. Aliquots were removed at various times and assayed for NEP activity and by SDS–PAGE. One colony (GS115; slow growth on MMH) was found with a particularly high level of activity and protein and was used for all further experiments.

2.3. Scaled-up expression

A single colony expressing sNEP was cultured in 100 ml YEPD plus zeocin (25 mg l^{-1}) for 96 h at 303 K on a rotatory shaker. The culture was mixed with sterile glycerol to 10% final concentration, placed in sterile 1 ml ampoules and frozen at 253 K before being transferred to a liquid-nitrogen storage tank. All subsequent fermentations were initiated using a new ampoule of cells. A 500 ml baffled shake flask containing 100 ml of YEPD medium plus zeocin (25 mg l^{-1}) was inoculated with 1 ml of cells and incubated for 24 h (303 K, 200 rev min^{-1}). Three shake flasks (2.5 l), each containing 1 l of the same medium, were then inoculated with 30 ml of the initial culture and subsequently incubated for 72 h (303 K, 200 rev min^{-1}). The cells were separated by centrifugation and resuspended in 2 l of BMMY (as described by Invitrogen, without methanol). A 10 l fermenter (Chemap FZ-2000) was sterilized with 8 l of BMMY (without methanol) and the cell suspension containing 50 ml of methanol and zeocin (25 mg l^{-1}) was added. Fermentation conditions were set at 303 K, 0.2 v.v.m. (volume air per volume fermentor per minute) aeration and 600 rev min^{-1} stirrer speed. The pH was regulated at 6.0 ± 0.1 by automatic addition of potassium hydroxide (4 M) or phosphoric acid [25%(*v*/*v*)] and the dissolved oxygen was maintained above 50% by automatic control of stirrer speed and aeration (*IRIS* fermenter control software, Infors AG). The actual concentration of methanol was measured twice daily by GLC and methanol was added in 50 ml aliquots when needed. After 90 h, the broth was cooled to 283–285 K.

2.4. Purification of sNEP

The supernatant from the 10 l fermenter of *P. pastoris* was passed through a 0.2 µm filter followed by a 500 kDa filter and finally concentrated to 300 ml by crossflow ultrafiltration (Skanette) using a 3 kDa microfiltration module (Skan AG). The concentrate was applied in 90 ml aliquots to

a Sephacryl 200 XK 50/100 column equilibrated with 25 m*M* Tris pH 8.0, 100 m*M* NaCl, 2 m*M* MgCl$_2$, 2 m*M* TCP, 5% glycerol, 0.02% NaN$_3$ (buffer *A*). Fractions containing sNEP were collected and brought to 1.5 *M* (NH$_4$)$_2$SO$_4$. Protein was applied to a phenyl Sepharose XK 26/30 column equilibrated with 25 m*M* Tris pH 8.0, 1.5 *M* (NH$_4$)$_2$SO$_4$, 1 m*M* MgSO$_4$, 0.02% NaN$_3$ (buffer *B*). Protein was eluted with 25 m*M* Tris pH 8.0, 1 m*M* MgSO$_4$, 5% glycerol, 0.02% NaN$_3$ (buffer *C*). The fractions were pooled, dialyzed against 25 m*M* Tris pH 8.5, 2 m*M* MgCl$_2$, 5% glycerol, 0.02% NaN$_3$ (buffer *D*) and loaded onto a SourceQ super performance 16/150 column. Active protein was found in the flow-through. The protein solution was dialyzed against 25 m*M* Tris pH 8.0, 2 m*M* MgCl$_2$, 5% glycerol, 0.02% NaN$_3$ (buffer *E*) then loaded onto a monoQ HR5/5 column equilibrated with buffer *E*. The elution profile showed a major peak at 20 m*M* and a minor peak at 60 m*M* NaCl which were indistinguishable both on HPLC and Superdex size-exclusion chromatography. Heterogeneity of the carbohydrate moieties may explain this elution profile; the major peak was used for all subsequent experiments.

For enzymatic deglycosylation, the protein was concentrated to 4–5 mg ml^{-1} and the buffer was changed to 25 m*M* sodium acetate pH 5.5, 100 m*M* NaCl, 5% glycerol, 0.02% NaN$_3$. Endo F1 was added to 0.05% of the NEP concentration and the mixture was incubated for 30 min at 293 K. After deglycosylation, the protein was concentrated and loaded onto a BioSec 16/600 column equilibrated with 25 m*M* Tris pH 8.0, 100 m*M* NaCl, 2 m*M* MgCl$_2$, 2 m*M* TCP, 5% glycerol, 0.02% NaN$_3$ (buffer *F*). The protein eluted as a single peak at a volume consistent with a monomeric protein. Analytical size-exclusion chromatography was performed on a Superdex-200 column (PC 3.2/30) using the SMART system (Pharmacia), with 50 µg of purified protein and buffer *F* as the mobile phase. HPLC analysis of the protein samples was performed on a Protein C4 150 mm column (Vydac) using a Hewlett Packard 1050 instrument. Purified proteins were completely desalted prior to mass-spectral analysis.

2.5. Crystallization

Prior to crystallization trials, the purified protein preparations were examined by dynamic light scattering at a concentration of 1 mg ml^{-1} using a DynaPro Molecular Sizing instrument (Protein Solutions). For crystallization trials, sNEP was concentrated

to approximately 20 mg ml^{-1} using Ultra-free Tangental membranes (Millipore) with a molecular-weight cutoff of 30 kDa. A sparse-matrix crystallization screen based on the original described by Jancarik & Kim (1991) was carried out using the vapour-diffusion technique on an automated crystallization workstation (Cyeberlab). In a typical experiment, 2 µl of protein was mixed with 2 µl of reservoir and stored at room temperature. For co-crystallization setups, the inhibitor phosphoramidon was added to the protein to give a final concentration of 1 mM. Crystal seeding was accomplished by either the streaking

method (Stura & Wilson, 1992) or by a seed dilution technique as described by Luft & DeTitta (1999).

2.6. Data collection

Crystals were placed in cryoprotectant solution corresponding to the reservoir condition containing either 40% PEG or 30% glycerol and were flash-frozen in liquid nitrogen. All crystals were measured at 120 K in a nitrogen stream (Oxford Cryosystems) using Cu $K\alpha$ radiation provided by a Nonius FR591 rotating-anode generator equipped with an Osmic mirror system. The

data were recorded on a MAR Research image-plate area detector and evaluated with the *XDS* software (Kabsch, 1988).

3. Results and discussion

The extracellular domain of human neutral endopeptidase (sNEP; residues 52–749) was expressed in *P. pastoris* and the protein was purified to homogeneity. The ectodomain of NEP was cloned into pPICZα-C to create a fusion with the α-mating factor signal sequence. To accomplish this, a *Cla*I restriction site was engineered, resulting in the addition of two additional amino acids (Ser, Met) at the N-terminus of sNEP. Both *P. pastoris* strains GS115 and KM71 were transformed with the linearized sNEP–pPICZα-C construct to create strains that expressed sNEP under the control of the AOX1 promoter and secreted the recombinant protein to the medium. Initial experiments to produce the protein in sufficient amounts in minimal media were unsuccessful. A modified protocol was employed whereby protein expression was accomplished using YP medium with 3% methanol (S. Masayuki, personal communication). Both SDS–PAGE and activity analysis demonstrated that one GS115 clone expressed approximately four times more sNEP into the medium (data not shown). The secretion level of sNEP was modest at 10 mg l^{-1}; however, it was adequate for crystallization studies and could be scaled up by fermentation.

The major difficulties in dealing with the modest level of expression in the yeast-peptone medium was that it limited the types of chromatography that could be used in the first step of the purification scheme. Following diafiltration, the protein solution was passed over a Sephacryl-200 size-exclusion column which removed the majority of contaminating peptides. Sequential chromatography on phenyl Sepharose, SourceQ, MonoQ and Biosec size-exclusion chromatography was used to purify the protein to 96.3% purity as judged by HPLC analysis (Fig. 1). The N-terminal sequence of purified sNEP indicated that the protein was efficiently processed by the STE13 signal peptidase. The kinetic properties of sNEP were similar to that of the native enzyme (data not shown).

The purified protein eluted from the analytical gel-filtration column with an elution volume consistent with a molecular mass of 95 kDa. The molecular-weight estimate obtained from light scattering was 100 kDa, which is in good agreement with gel-filtration experiments, indicating that
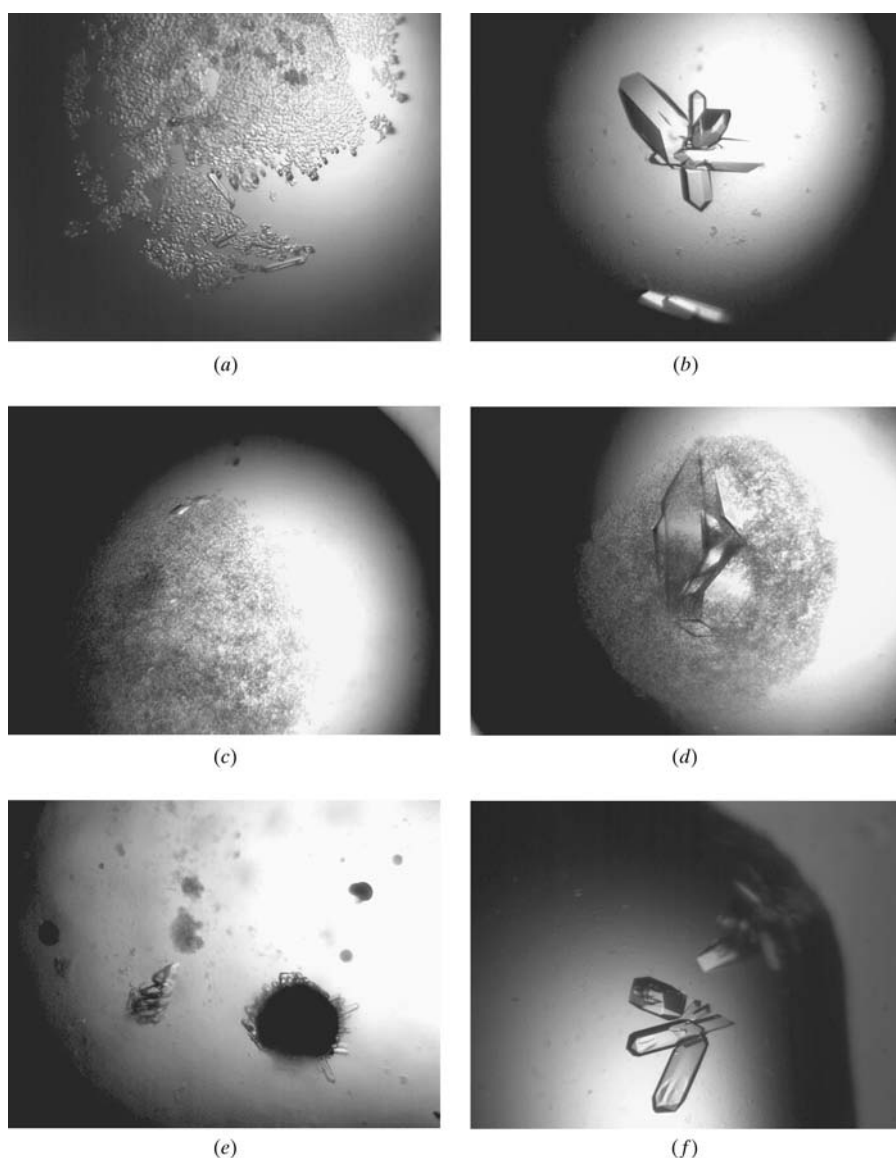
Figure 2
Photomicrographs of the NEP crystals. (*a*) Deglycosylated plus phosphoramidon grown in 25% PEG 3350, 100 mM bis-tris pH 6.5, 200 mM sodium chloride. (*b*) Identical conditions as (*a*) after seeding. (*c*) Glycosylated plus phosphoramidon grown in 25% PEG 3350, 100 mM bis-tris pH 5.5, 200 mM ammonium sulfate after one month. (*d*) Identical conditions as (*b*) after three months. (*e*) Deglycosylated native grown in 25% PEG 3350, 100 mM HEPES pH 7.5, 200 mM NaCl after one month. (*f*) Deglycosylated native grown in 25% PEG 3350, 200 mM ammonium acetate pH 7.5 after cross seeding.

Table 1
Data-collection statistics.

Values in parentheses refer to the outer resolution shell, 2.8–2.71 Å for apo Nep and 2.6–2.5 Å for the NEP–phosphoramidon binary complex.

	Apo NEP	NEP–phosphor-amidon binary complex
Space group	$P3_221$ (No. 154)	$P3_221$ (No. 154)
Data-collection temperature (K)	120	120
Unit-cell parameters a, c (Å)	107.2, 112.8	107.6, 112.8
Resolution range (Å)	20.0–2.71	20.0–2.5
No. of observed reflections	35740	91966
No. of unique reflections	19039	26702
R_{sym}† (%)	6.2 (23.9)	9.6 (37.4)
$I/\sigma(I)$	16.4 (3.9)	16.2 (3.1)
Completeness (%)	91.6 (82.4)	95.5 (79.9)

† $R_{sym} = \sum_h \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ and $\langle I(h) \rangle$ are the ith and mean measurements of the intensity of reflection h.

sNEP is a monomer in solution. Mass-spectral analysis of the purified protein resulted in a mass of 92 186 Da (predicted molecular mass is 80 086 Da), indicating that the protein is significantly glycosylated. Treatment of sNEP with Endo F1 glycosidase resulted in a decrease of approximately 10 kDa as estimated by SDS–PAGE (Fig. 1). The deglycosylated protein eluted from the analytical gel-filtration column at an elution volume corresponding to a molecular mass of approximately 80 kDa, which also correlates well with estimates from light-scattering experiments (76 kDa). Mass-spectral analysis of the Endo F1 treated protein gave a mass of 81 369 Da, indicating that a small degree of glycosylation remained.

Light-scattering analysis of both sNEP and the deglycosylated sNEP showed a narrow unimodal size distribution suggesting that they were suitable for crystallization trials. Three preparations of sNEP were used for crystallization: sNEP plus phosphoramidon, deglycosylated sNEP plus phosphoramidon and deglycosylated sNEP. The sNEP–phosphoramidon complex gave crystals in two conditions: thin needles or plates appeared after 1–2 weeks in 25% PEG 3350, 100 mM bis-tris pH 7.5, 200 mM ammonium sulfate and large bipyramids were subsequently observed after three months in almost identical conditions at pH 5.5 instead of 7.5. These crystals showed only weak diffraction to approximately 7.5 Å and were thus not considered suitable for further crystallographic analysis. Complexes of the deglycosylated sNEP–phophoramidon crystallized within 3 d in five conditions containing PEG and various salts over the pH range 5.5–7.5 and some crystals were already suitable for initial X-ray analysis. Attempts to reproduce these crystals using identical screen conditions were not immediately successful; some crystals appeared spontaneously at 277 K but were not suitable for further characterization. It appeared that nucleation was the critical factor; when drops containing 2 µl of protein and 2 µl of 25% PEG 3350, 100 mM bis-tris pH 6.5, 200 mM sodium chloride were seeded with crystals from the original screen, crystals were observed within 1 h. The seeding procedure was optimized to produce a supply of large single crystals and this form was used to initiate native and heavy-atom data collection. The deglycosylated NEP without inhibitor bound produced spherulites or microcrystals in only one condition: 25% PEG 3350, 100 mM HEPES pH 7.5, 200 mM NaCl after approximately 2–3 months at room temperature. This initial screen was subsequently cross-seeded with inhibited deglycosylated crystals and 17 out of the 24 conditions containing polyethylene glycol produced crystals. The best condition (25% PEG 3350, 200 mM ammonium acetate pH 7.5) after cross seeding was then optimized to produce crystals for X-ray analysis. Crystallization conditions and crystal morphology are shown in Fig. 2. Crystallographic data of deglycosylated sNEP and a sNEP–phosphoramidon binary complex are summarized in Table 1.

4. Conclusions

The extracellular domain of the human NEP was expressed in the methylotrophic yeast *P. pastoris* and the secreted protein was purified to homogeneity. Enzymatic deglycosylation and microseeding were essential for the production of crystals suitable for X-ray analysis. A structure determination is in progress and its completion should lead to valuable insights concerning the mode of action of this pharmacologically important enzyme.

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