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Crystallization and preliminary crystallographic studies of human kallikrein 7, a serine protease of the multigene kallikrein family

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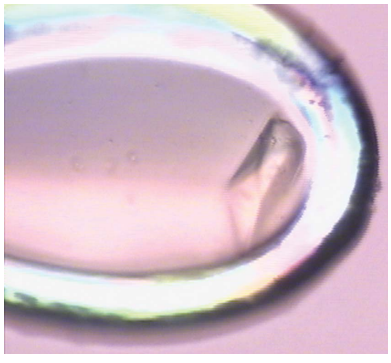

Human kallikreins are a group of serine proteases of high sequence homology whose genes are grouped as a single cluster at chromosome 19. Although the physiological roles of kallikreins are generally still unknown, members of the kallikrein family have been clearly implicated in pathological situations such as cancer and psoriasis. Human kallikrein 7 (hK7) has been shown to be involved in pathological keratinization, psoriasis and ovarian cancer. In order to gain insight into the molecular structure of this protein, hK7 was crystallized after recombinant production in its folded and active form using a periplasmic secretion vector in *Escherichia coli*. The crystals belonged to the rhombohedral space group *H*32 and diffracted to 2.8 Å. The phase problem was solved by molecular replacement using the mouse kallikrein-related protein neuropsin. Completion of the model and structure refinement are under way.

1. Introduction

Proteases have been shown to play many essential roles in an extremely wide variety of physiological functions such as degradation of the extracellular matrix (Werb, 1997), neural degeneration (Tsirka *et al.*, 1995) and zymogen activation (Stennicke & Salvesen, 2000). Among more than 500 proteases contained into the human degradome (Puente *et al.*, 2003), the kallikrein (KLK) gene family displays the largest contiguous cluster of enzyme-coding genes within the human genome. This cluster, localized on chromosome 19q13.4 (Yousef, Chang *et al.*, 2000), comprises 15 genes (KLK1–KLK15). Although KLKs generally share 30–50% sequence similarity at the nucleotide and amino-acid levels (Diamandis *et al.*, 2000), the so-called 'classical kallikreins' (KLK1, KLK2 and KLK3) are by far the most homologous, sharing 73–84% nucleotide and 61–77% amino-acid sequence similarity (Henttu & Vihko, 1989).

The proteins encoded by the KLK genes (hKs) are expressed in the glandular epithelium of many organs. Kallikreins are synthesized as zymogens which can be converted to the active protein by protease digestion (Magklara *et al.*, 2003). Other proteolytic enzymes, either metalloproteinases or serine proteases, can also activate the hKs (Takada *et al.*, 1985). Hydrolysis of the propeptide induces conformational changes in both the active site and the substrate-binding specific pocket of the hKs. The physiological roles of kallikrein are generally still unknown, except for the cases of the tissue kallikreins hK1 and hK3 (Bhoola *et al.*, 1992; Lilja, 1985). However, dysregulation of KLK synthesis have been described for both steroid-dependent (ovarian, breast and testicular carcinomas; Magklara *et al.*, 2001; Yousef *et al.*, 2002) and nonsteroid-dependent (lung adenocarcinomas, pancreatic cancer or lymphoblastic leukaemia; Bhattacharjee *et al.*, 2001; Roman-Gomez *et al.*, 2004) tumours. In cancer pathophysiology, KLKs seem to at least be involved in extracellular matrix degradation, angiogenesis, invasion and metastasis (Tschesche *et al.*, 1989; Plendl *et al.*, 2000). Surprisingly, high levels of kallikrein expression seem to be a positive tumour prognosis factor in some cases (Borgono & Diamandis, 2004).

Human kallikrein 7 (hK7) was initially cloned from the stratum corneum of human skin (Hansson *et al.*, 1994). It was classified as a serine protease (Yousef, Scorilas *et al.*, 2000). Subsequently, hK7 (previously named stratum corneum chymotryptic enzyme; SCCE) was shown to be involved in pathological keratinization, psoriasis and

ovarian cancer (Tanimoto *et al.*, 1999; Ekholm & Egelrud, 1999). Zymogen pro-hK7 (27 525 Da, 253 amino-acid residues; Gene ID 5650) is converted by another kallikrein, hK5 (previously termed SCTE). This mechanism constitutes the first described kallikrein activation cascade (Caubet *et al.*, 2004). Recent discoveries clearly show that domains of the LEKTI protein inhibit the activity of both hK5 and hK7 in the nanomolar range (Egelrud *et al.*, 2005), which points towards the existence of a complex physiological regulation loop in the case of the hK5/hK7 couple.

The crystal structures of several human kallikreins, hK1 (Katz *et al.*, 1998), hK4 (Debela *et al.*, 2006), hK6 (Bernett *et al.*, 2002) and pro-hK6 (Gomis-Ruth *et al.*, 2002), have been determined. The overall fold of the hKs resembles that of the chymotrypsin-like serine proteases. It consists of two juxtaposed β-barrels and two α-helices, with the catalytic active site located between the two β-barrels (Katz *et al.*, 1998; Gomis-Ruth *et al.*, 2002). Three conserved residues, termed the 'catalytic triad' (a histidine, an aspartic acid and a serine), are the essential components of the catalytic site. A group of 10–12 cysteine residues that form either five (hK1, hK2, hK3 and hK13) or six (hK4, hK12 and hK15) disulfide bonds are also highly conserved. Despite these similarities, remarkable sequence differences run parallel to the respective substrate specificities of the various hKs (Debela *et al.*, 2006). The crystallization and preliminary X-ray analysis of recombinant hK7 reported here represents the first step towards the determination of this enzyme structure. The results should provide an important insight into the structural basis of the enzymatic properties of this protein.

2. Materials and methods

2.1. Cloning, expression and purification of hK7

A cDNA coding for the full hK7 (Gene ID 5650) was used as a template for creating by PCR-amplification the mature hK7 (without the signal peptide and the propeptide; 24 849 Da, 226 amino-acid residues) fused to a C-terminal His tag (NDTMKHHHHHH) flanked by *Eco*RI and *Hin*dIII restriction sites. The amplified PCR product was inserted into the multicloning site of the secretion expression vector pRHO (Fernandez-Tornero *et al.*, 2002). All enzymes used for recombinant DNA techniques were from New England Biolabs and plasmid DNA-isolation kits were from Roche. At all necessary stages, DNA sequencing was employed to ascertain the accuracy of the construction. *Escherichia coli* strain BL21(DE3)SS (Fernandez-Tornero *et al.*, 2002) was transformed using the pRHO-hK7-HisTag vector. Cell cultures for protein expression were carried out at 310 K in LB medium supplemented with ampicillin. Once the culture reached an OD_{600} of 0.7, protein expression was induced by adding isopropyl β-D-thiogalactopyranoside to the culture (to a final concentration of 1 m*M*). At this point, the temperature was lowered to 288 K and the cells were grown for a further 48 h. The supernatant was collected by centrifugation (5260*g*, 20 min) and the presence of hK7 was ascertained by Western blot analysis using an anti-His_6 peroxidase-coupled mouse antibody (Roche). N-terminal sequencing was carried out on a microsequencer (Applied Biosystems 494) to confirm the identity of the protein detected by Western blot analysis. For purification, the supernatant was directly batch-incubated overnight at 277 K with SP Sepharose Fast Flow (General Electric) at pH 8.5 and hK7 was eluted from the slurry using a linear 0–1 *M* NaCl gradient in 50 m*M* sodium phosphate buffer pH 8.0 after packing the slurry into a chromatography column. hK7-containing fractions identified by Western blotting were pooled and applied onto a column containing 15 ml nickel-chelating Sepharose equilibrated in the same buffer containing 40 m*M* imidazole to avoid nonspecific protein binding. The protein was eluted with a linear 40–500 m*M* imidazole gradient in the same buffer. The final purification step was carried out using a reversed-phase column (Source 15RPC; Pharmacia) using an acetonitrile gradient in aqueous 0.1% trifluoroacetic acid. hK7 eluted from this last column in an almost single peak and was extensively dialyzed against 50 m*M* sodium phosphate pH 7.5 and lyophilized until future use. Protein purity was assessed at this stage by Coomassie blue-stained SDS–PAGE (Fig. 1*a*). The activity of the recombinant enzyme was assayed using the fluorogenic peptide Mca-R-P-K-P-V-E-Nval-W-R-K(Dnp)-NH_2 (R&D Systems). The homogeneity of hK7 in solution was determined by dynamic light-scattering (DLS) measurements in a DynaPro instrument (Protein Solutions Ltd) with protein concentrations in the range 0.5–1 mg ml^{-1}.

2.2. Crystallization, data collection and processing

Crystallization experiments were carried out at 295 K using the sitting-drop vapour-diffusion method in 96-well Greiner plates with a

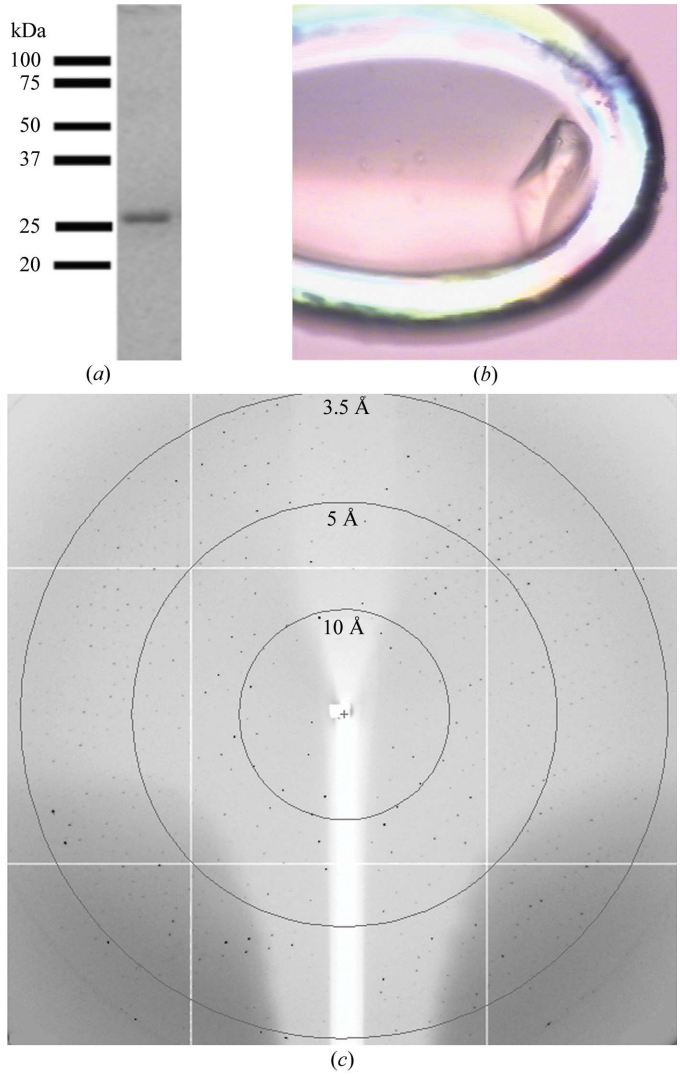

Figure 1
(*a*) SDS–PAGE of the final hK7 preparation before setting up crystallization trials. (*b*) Optimized rhombohedral crystal of recombinant hK7 obtained using the sitting-drop vapour-diffusion method. (*c*) Representative diffraction pattern of hK7 obtained using synchrotron radiation, showing a maximum resolution of 2.8 Å.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	*H*32
Unit-cell parameters (Å)	$a = b = 113.6$, $c = 326.4$
Resolution (Å)	50.0–2.8 (2.95–2.80)
V_M (3 molecules per ASU; Å^3 Da^{-1})	2.60
R_{merge}† (%)	16.2 (41.5)
Measured reflections	81666 (12115)
Unique reflections	19424 (2816)
Multiplicity	4.2 (4.3)
Completeness (%)	99.0 (99.5)
$\langle I/\sigma(I)\rangle$	9.4 (2.1)

† $R_{merge} = \sum_{\mathbf{h}}\sum_{l}|I_{\mathbf{h}l} - \langle I_{\mathbf{h}}\rangle|/\sum_{\mathbf{h}}\sum_{l}\langle I_{\mathbf{h}}\rangle$, where $I_{\mathbf{h}l}$ is the lth observation of reflection **h** and $\langle I_{\mathbf{h}}\rangle$ is the weighted average intensity for all observations l of reflection **h**.

variety of commercially available screens (Hampton Research, Jena Bioscience and Nextal). The initial hK7 protein concentration was 8 mg ml^{-1} in 50 m*M* sodium phosphate pH 7.5. Nanodrops were prepared using a Honeybee X8 Cartesian crystallization robot by mixing 0.1 µl protein solution with 0.1 µl crystallization buffer. Several conditions gave small crystals in the initial screens that were subsequently improved by refining these conditions and adapting them to standard drops (1 µl). Crystals suitable for diffraction experiments were transferred from the crystallization drop into a cryoprotectant solution using nylon loops and were rapidly flash-cooled to 100 K in liquid nitrogen. The cryoprotectant solution consisted of the crystallization solution supplemented with 25%(*v*/*v*) glycerol in place of an equivalent volume of buffer. For data collection, a single crystal was analysed using the synchrotron-radiation source at the ESRF (Grenoble, France). A complete data set was collected using a Quantum ADSC detector in 100 frames with an oscillation range of 1° to a maximum resolution of 2.8 Å. The diffraction data were integrated using *MOSFLM* (Leslie, 2006) and scaled using *SCALA* (Evans, 2006). Further analyses were performed using programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

We have expressed and purified mature human kallikrein 7 (hK7) in *E. coli* cultures using the pRHO T7-secretion vector (Fernandez-Tornero *et al.*, 2002). The protein-purification protocol from the supernatant rendered an active hK7 preparation that showed up a single band on SDS–PAGE (Fig. 1*a*), corresponding to an unglycosylated polypeptide of ∼27 kDa (the exact molecular weight of hK7 calculated from the gene sequence is 25 596 Da). The final yield was 0.2 mg purified protein per litre of culture. This is the first time that a human kallikrein, either as zymogen or mature protein, has been expressed properly folded in its active form in *E. coli*.

Crystals of recombinant hK7 were grown at pH 7.5 by the sitting-drop vapour-diffusion method from several different crystallization conditions that contained ammonium sulfate as the main precipitant. Improved crystals were subsequently obtained with a precipitant solution consisting of 3.2 *M* ammonium sulfate in 0.1 *M* HEPES pH 7.0. Crystallization was reproducible under these conditions, with crystals of maximum dimensions 50 × 50 × 100 µm appearing after one month (Fig. 1*b*). X-ray data were collected from a single crystal at 100 K at the European Synchrotron Radiation Facility to a maximum resolution of 2.8 Å (Fig. 1*c*). Crystals belong to the rhombohedral space group *H*32, with unit-cell parameters $a = b = 113.6$, $c = 326.4$ Å (hexagonal setting). Data-collection statistics are summarized in Table 1. Estimation of the protein content using the Matthews coefficient calculation (Matthews, 1968) suggested that two or three hK7 subunits were most likely to be present, giving solvent contents of 68.8% or 53.2% and V_M values of 4.0 or 2.6 Å^3 Da^{-1}, respectively. A self-rotation function using *MOLREP* (Vagin & Teplyakov, 1997) did not show any significant noncrystallographic axes (twofold or threefold axes), perhaps owing to their relative parallel (or close to parellel) orientation to the crystallographic axes. Structure elucidation of hK7 will provide important clues towards understanding the enzymatic properties of this protein.

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