Preparation of active kallikrein7 for structural studies on inhibitor binding

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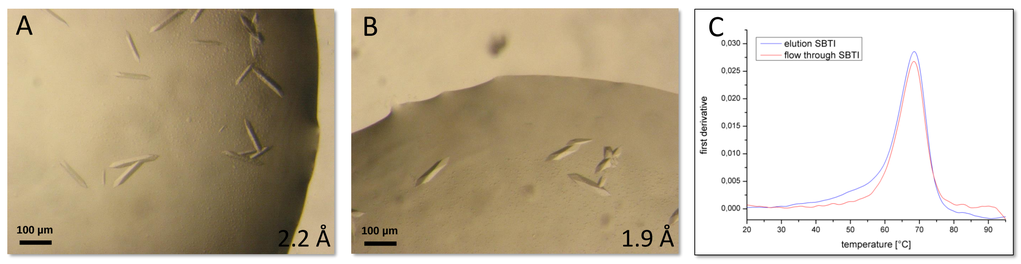

Fig.1: (A) Crystals of hK7 from the elution of the SBTI column, (B) Crystals of hK7 from the flow through of the SBTI column, (C) melting point determination by nano DSF (differential scanning fluorimetry) of hK7 from the elution of the SBTI column and from the flow through.

Physiological background

Human Kallikrein 7 (hK7) is a member of the human tissue kallikreins, a protein family consisting of 15 chymotrypsin- or trypsin- like serine proteases. Its dysregulation leads to pathophysiological inflammation processes in the skin, which causes diseases such as psoriasis or Netherton syndrome. It is also overexpressed in ovarian, breast and melanoma cancers. Furthermore, hK7 can cleave insulin chain A and B. Therefore, it has been proposed that hK7 plays a role in adipose-induced insulin resistance.

Small molecule inhibitors for hK7

Currently, small molecule inhibitors for hK7 are developed for the treatment of kallikrein7 associated diseases. Coumaric acid derivatives inhibit hK7 to a nM range by blocking the active site of the protease[1]. We aim to understand the mode of inhibition of these compounds by X-ray crystallography. So far, we could obtain crystals by co-crystallization that diffract to 1.9 Å but structure analysis showed only very weak and uninterpretable electron density in the active center of hK7.

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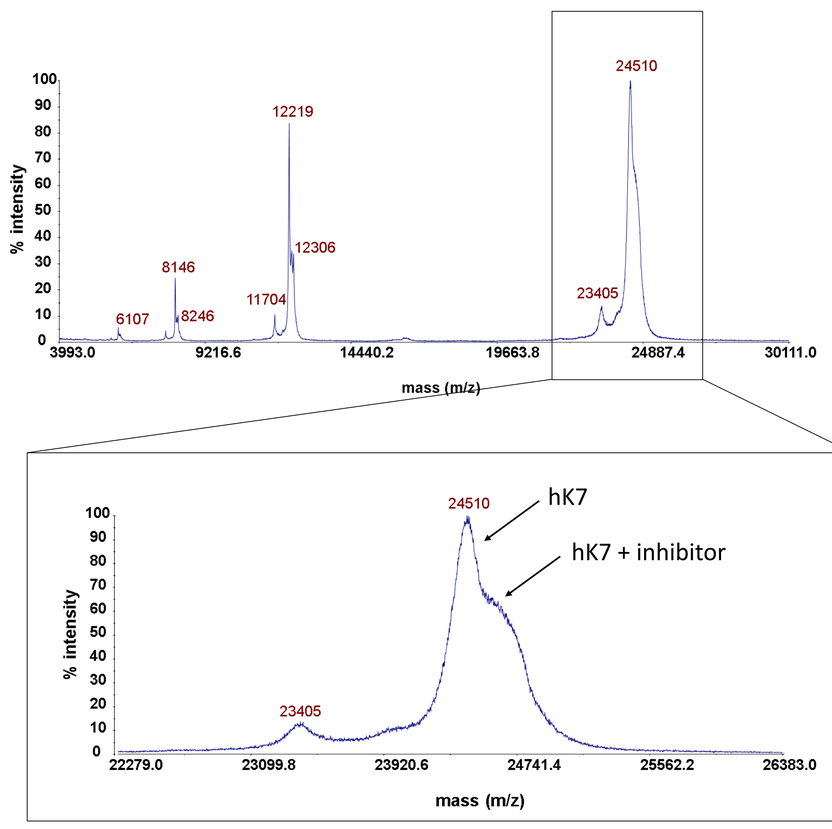

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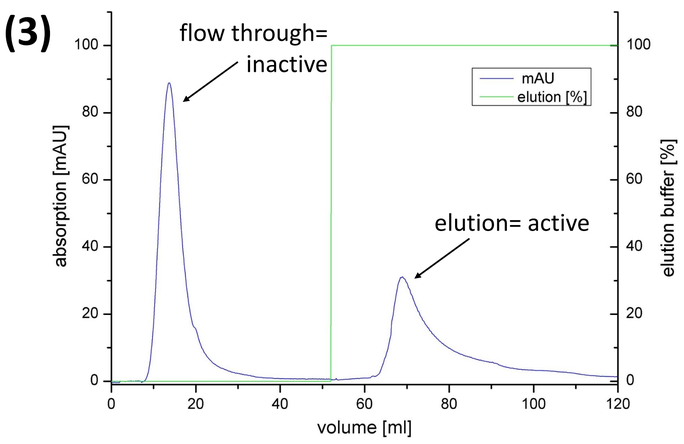

Mass spectrometry analysis

Mass spectrometry analysis of dissolved cocrystals showed a mixture of molecules which probably bound the inhibitor and unbound hK7 molecules (Fig. 2). Since the inhibitor was kept in excess over the protease during incubation, we had to assume that a part of our protein preparation is inactive.

preparation of active hK7

Recently we optimized the purification of the recombinant hK7 to separate active from inactive kallikrein after refolding the protease from inclusion bodies. By performing an affinity chromatography with immobilized soybean trypsin inhibitor (SBTI), which binds specifically to the active site of hK7, we could purify the active hK7 molecules (Fig.3). The activity of the protease was validated by incubation with the fluorogenic peptide substrate NFF3. This showed a highly active protease in the elution of the SBTI column and inactive protein in the flow through. The elution is even more active than commercially available hK7 (Table 1).

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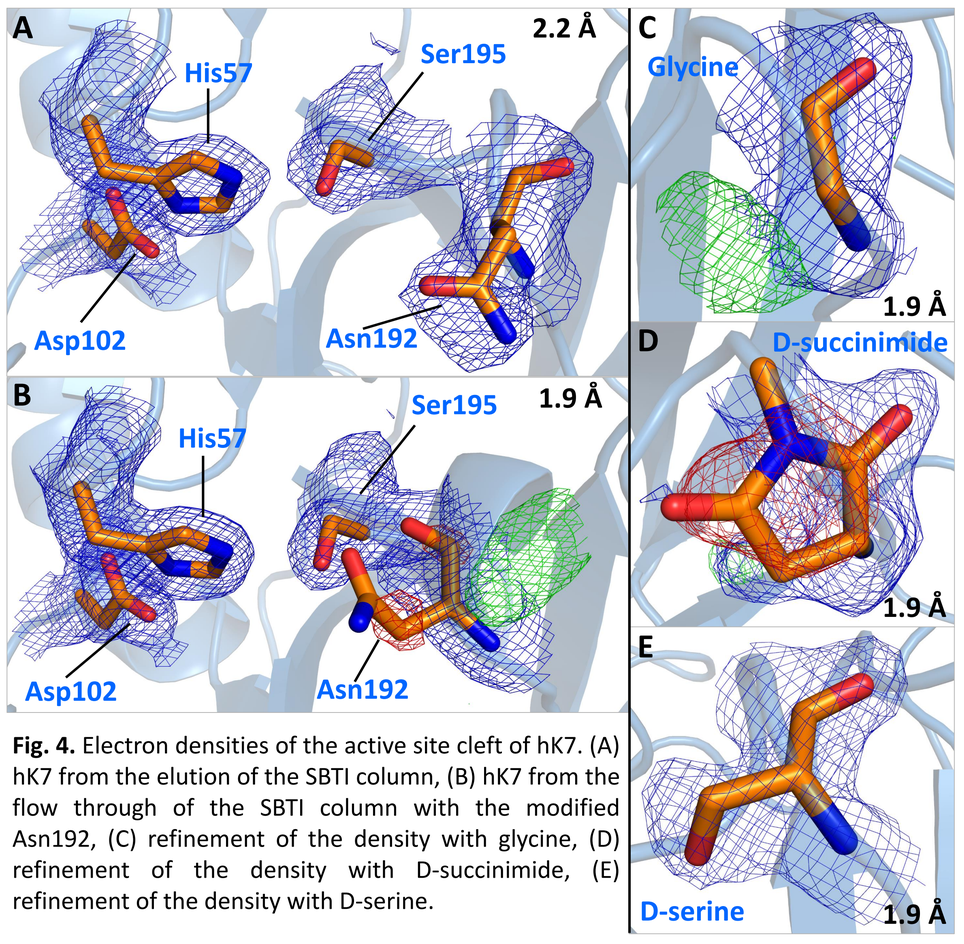

Fig. 4. Electron densities of the active site cleft of hK7. (A) hK7 from the elution of the SBTI column, (B) hK7 from the flow through of the SBTI column with the modified Asn192, (C) refinement of the density with glycine, (D) refinement of the density with D-succinimide, (E) refinement of the density with D-serine.

active site analysis

To figure out why the protein preparation is partly inactive, both species were crystallized and the electron densities compared. The only difference we observed was a modification at the Asn192 (Fig. 4.A and B), which is an amino acids framing the active site cleft of hK7. The electron density suggests the presence of a D- amino acid (Fig.4.C). The most likely modification would be the deamidation of the Asn forming a D-succinimide and indicating a slowly degradation of the protease[2]. Unfortunately D-succinimide does not fit the electron density (Fig.4.D). Best fits a D-serine (Fig. 4.E) but still it could not be explained what modification happened to Asn192 and why this results in an inactive protease.

Protein	Specific activity [(pmol/min)/µg]
hK7 before SBTI	140.15
hK7 elution SBTI	369.86
hK7 flow through SBTI	2.87
hK7 (commercial)	150

Table 1: Activity measurements of different hK7 preparations. The protease was incubated with the fluorogenic peptide substrate NFF3 (Mca-RPKPVE-Nva-WR-K(Dnp)-NH2) for 5 min. The fluorescence was detected by a FlexStation microplate reader.

conclusion and outlook

These experiments indicated a partly inactive protein preparation, although crystallization and melting point determination indicated correctly folded protein (Fig.1). With this new purification strategy we could establish a recombinant production of hK7 without the activation of a propeptide what is usually necessary for commercially purchased hK7. Our current efforts focus on the characterization of inhibitor binding to the fully active hK7 preparation and the characterization of the modification causing inactivity of the protease.

References

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[2] Yang, H. *et al.* (2010). Electrophoresis 2010, 31, 1764–1772.

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