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Protein Expression and Purification 57 (2008) 290–302
Protein Expression & Purification
www.elsevier.com/locate/yprep

Purification and biochemical characterization of ovine α-1-proteinase inhibitor: Mechanistic adaptations and role of Phe^{350} and Met^{356}

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Received 5 June 2007, and in revised form 6 September 2007
Available online 1 October 2007

Abstract

The glycoprotein α-1-proteinase inhibitor (α-1-PI) is a member of the serpin super family that causes rapid and irreversible inhibition of redundant serine protease activity. A homogenous preparation of ovine α-1-PI, a 60 kDa protein was obtained by serially subjecting ovine serum to 40–70% $(NH_4)_2SO_4$ precipitation, Blue Sepharose, size-exclusion, and concanavalin-A chromatography. Extensive insights into the trypsin, chymotrypsin, and elastase interaction with ovine α-1-PI, point towards the involvement of Phe^{350} besides the largely conserved Met^{356} in serine protease recognition and consequent inhibition. The N-terminal of C-terminal peptides cleaved on interaction with elastase, trypsin, and chymotrypsin prove the presence of diffused sub-sites in the vicinity of Met^{356} and the strategically positioned Pro anchored peptide stretch. Further, human α-1-PI is more thermolabile compared to ovine α-1-PI, higher thermolability is mainly attributed to poorer glycosylation. The enzymatic deglycosylation of human and ovine α-1-PI results in diminished thermostability of the inhibitors, with sharp decrease in thermal transition temperatures but retaining their inhibitory potency. Homology modeling of the deduced amino acid sequence of ovine α-1-PI using the human α-1-PI template has been used to explain the observed inhibitor–protease interactions.

Keywords: Trypsin; Chymotrypsin; Elastase inhibition; Serpin; Reactive site analysis; Homology modeling; Deglycosylation; Thermal stability

The serpins comprise of a large family of proteins that are involved in the regulation of many fundamental physiological processes by maintaining the proteinase inhibitor–proteinase balance [1]. Mammalian serpins irrespective of their high sequence, structural, and mechanistic homology display noticeable differences in stability, specificity, and protease inhibitory activity. Serpins comprise 2% of total human plasma protein, 70% of which is the archetype of the family, α-1-PI [2]. α-1-PI placed on chromosome 14q32.1 along with α-1-antichymotrypsin, protein Z dependent proteinase-inhibitor and centerin, was initially observed in human plasma by Fermi and Pernossi [3]. Its major physiological role is to protect pulmonary elastin fibers from excessive degradation by neutrophil elastase. α-1-PI has been implicated in regulating vital fluid phase biological events viz. blood coagulation, fibrinolysis, complement activation, apoptosis, matrix remodeling, reproduction, tumor progression, and inflammatory response [4–6]. Genetically induced α-1-PI deficiency due to the z-mutation (Glu^{342}-Lys) and/or oxidative inactivation of the inhibitor active site leads to enzymatic degradation of lung connective tissue and ultimately pulmonary emphysema. The z-mutation results in delayed protein folding favoring the spontaneous formation of α-1-PI loop-sheet polymers and accumulation of the protein in the endoplasmic reticulum of the liver, leading to hepatic damage [7]. Low levels of circulating α-1-PI fail to protect the lungs against proteolytic attack and predispose the homozygote to early onset of pan-lobular emphysema, bronchiectasis, and asthma [8]. α-1-PI is the primary blood-borne serine protease inhibitor for a broad range of proteases: destructive neutrophil proteases including elastase, cathepsin G,

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1046-5928/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2007.09.013

proteinase-3, pancreatic elastase, trypsin, and chymotrypsin and skin and synovial collagenases [9–11]. α-1-PI is responsible for 80–90% of plasma leucocyte elastase inhibitory activity in man [12]. The intact inhibitor presents the reactive site, which mimics a good substrate, to the target protease as an exposed strained loop, much like the bait of a mousetrap [13]. Reaction with the cognate protease is influenced by approximately 20 amino acids flanking the semi-discreet scissile peptide stretch (–P3–P2–P1–P1′–P2′–P3′–) [14]. This proteolytic reaction generates a carboxyl-terminal fragment, which is reported to play a role in inflammatory processes [15,16].

Human α-1-PI, a glycoprotein contains 10% of its mass as carbohydrate [17]. Alteration in the proteinase inhibitor–proteinase balance in the lungs leads to emphysema. This can be alleviated by therapeutic use of synthetic inhibitors or by α-1-PI supplementation therapy, which has been licensed for treatment of α-1-PI deficient individuals with pulmonary emphysema. However, the lack of availability as well as stability, and susceptibility to oxidative impairment of human α-1-PI reduces the potential effectiveness. Human α-1-PI has been characterized extensively but not much is known about the comparative physical and biochemical properties of α-1-PI from related mammalian sources. Detailed investigations make it conceivable to obtain α-1-PI from unconventional sources such as ovine serum. The vast availability of ovine blood as well as the observed higher stability of ovine α-1-PI compels a thorough biochemical and structural characterization of the molecule, to understand the differences between human and related α-1-PIs. We present here the purification of ovine α-1-PI to homogeneity and its detailed biochemical characterization including the effect of deglycosylation on stability. The deglycosylation and activity–stability correlation is significant keeping in view the reduced antigenicity of the non-glycosylated proteins for therapeutic purposes. Met^{356}-Ser^{357} as in human α-1-PI is the key site for elastase and chymotrypsin inhibition but additionally Phe^{350}-Leu^{351} sub-site plays a pivotal role in the manifestation of trypsin specific inhibition. It is the synergistic understanding between these two subsites that allows α-1-PI to inhibit a vast array of serine proteases. The absence of a three-dimensional structure of ovine α-1-PI prompted us to build a homology model from the deduced amino acid sequence of ovine α-1-PI using the human α-1-PI as template to explain the interactions at the inhibitor–protease interface.

Materials and methods

Materials

Ovine blood was obtained from the local abattoir. Sephadex G-200 and concanavalin-A Sepharose were procured from Amersham Biosciences, Uppasala Sweden; Molecular weight markers for SDS–PAGE were from Bangalore Genei, Bangalore, India. α-*N*-benzoyl-DL-arginine-*p*-nitroanilide HCl (BAPNA)[1], *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTPNA), *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (NSAPNA), *N*-acetyl-DL-phenylalanine-β-naphthyl ester (APNE), TPCK-treated trypsin, TLCK treated bovine pancreatic α-chymotrypsin (3× crystallized, type II, EC 3.4.21.1), porcine pancreatic elastase (EC.3.4.21.36), 1,2-cyclohexanedione, chloramine-T, H_2O_2, 2,4,6-trinitrobenzenesulfonic acid (TNBS), human α-1-PI, Cibacron blue 3GA agarose, and analytical gel filtration markers were procured from Sigma–Aldrich, St. Louis, Missouri, USA. Citraconic anhydride was obtained from Pierce, Illinois. USA. Peptide-*N*-glucosidase-F (PNGase-F) was procured from New England Biolabs, USA. All the other chemicals used were of highest purity available commercially.

Purification of ovine α-1-PI

Ovine blood was allowed to clot at 25 ± 2 °C and serum collected by centrifugation at 2000*g*. All steps of purification were carried out at 4 °C. Ovine serum was fractionated by using $(NH_4)_2SO_4$ to a final concentration of 40% and centrifuged at 10,500*g* for 30 min. $(NH_4)_2SO_4$ was added to the supernatant to obtain 70% saturation. The precipitate obtained was resuspended in 0.05 M Tris–HCl buffer, pH 8.2 containing 0.05 M NaCl, and dialysed extensively ($2\ L \times 5$) against the same buffer. The retentate was applied to a Cibacron blue Sepharose 3GA column (1.5×30 cm) pre-equilibrated with 0.05 M Tris–HCl buffer, pH 8.2 containing 0.05 M NaCl. α-1-PI was obtained as the unbound fraction. The unbound fraction was loaded on to Sephadex G-200 column (1.5×90 cm) pre-equilibrated with 0.05 M Tris–HCl buffer, pH 7.4 containing 0.9% NaCl and 1 mM Cys. The active fractions were pooled, concentrated and loaded on to a concanavalin-A Sepharose column (1.5×10 cm) pre-equilibrated in 0.05 M sodium phosphate buffer, pH 6.0 containing 0.25 M NaCl and 1 mM Mg^{2+}, Ca^{2+}, Mn^{2+} ions. The elution of the bound α-1-PI was achieved using the same buffer containing 0.1 M mannose minus the metal ions. The fractions exhibiting α-1-PI activity were appropriately combined, dialysed against 0.05 M Tris–HCl buffer, pH 7.4, and concentrated.

Protease inhibitory assay

The amidase activity of trypsin and its inhibition was assayed using the chromogenic substrate BAPNA as described earlier [18]. The amidase activity of chymotrypsin and its inhibition was assayed using the chromogenic substrate BTPNA in 0.05 M Tris–HCl, pH 7.8 containing 5%

[1] *Abbreviations used:* TNBS, trinitrobenzenesulfonic acid; BAPNA, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide HCl; BTPNA, *N*-benzoyl-L-tyrosine-*p*-nitroanilide; NSAPNA, *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide; APNE, *N*-acetyl-DL-phenylalanine-β-naphthyl ester; RCL, reactive center loop.