Available online at www.sciencedirect.com
ScienceDirect
Protein Expression and Purification 53 (2007) 112–118
Protein Expression & Purification
www.elsevier.com/locate/yprep

Proteolytic hydrolysis and purification of the LRP/alfa-2-macroglobulin receptor domain from α-macroglobulins

Daniel Iván Barrera [a], Luisa Marina Matheus [b], Torgny Stigbrand [c], Luis Fernando Arbeláez [a,*]

[a] Grupo de Investigación en Biomoléculas, Universidad de Pamplona, Pamplona, Colombia
[b] Unidad de Bioquímica, Facultad de Medicina, Universidad del Rosario, Bogotá, Colombia
[c] Department of Immunology, Umeå University, Umeå, Sweden

Received 19 October 2006, and in revised form 6 December 2006
Available online 16 December 2006

Abstract

A new, easier and efficient purification method, using Sephacryl and DEAE-Sephacel, of the C-terminal fragment of two α-macroglobulins, α_2-M and PZP, is presented. Two larger peptides were identified for each protein as the C-terminal fragment, with molecular weights of ~30 kDa and the N-terminal sequences were determined to be SSTQDTV for α_2-M and VALHLS for PZP. The smaller peptides with molecular weights of 18 kDa correspond to a shorter C-terminal sequence of these proteins, and they were determined to be EEFPFA for α_2-M and ALKVQTV for PZP, with no interfering sequences detected. The results confirmed the discriminatory capacity of the purification procedure and the purity of the fragments. This new methodology facilitates biological studies of α-macroglobulins, and will enable elucidation of the role the C-terminal region may exert to eliminate α-macroglobulin-proteinases complexes from the circulation by the LRP/receptor.

Keywords: PZP; α-Macroglobulins; C-terminal region; Chymotrypsin

Both pregnancy zone protein (PZP)[1] and α_2-macroglobulin (α_2-M) belong to the denominated α-macroglobulin (α-Ms) subgroup family, which also contains the complement subgroup comprising the complement factors C3, C4 and C5 [1]. PZP shares 71% aminoacid homology with α_2-M and 24% with C3. The aminoacid sequence identity between complement factors C3 and C4 is 29% [2]. PZP, α_2-M, C3 and C4, but not C5, contain an internal thiolester [3]. α-Ms are synthesized as 180–200 kDa monomers, and are held together by disulphide bonds [4]. PZP was initially detected by starch gel electrophoresis, when sera from pregnant women were compared with sera from their newborn babies and non-pregnant women [5]. Expression of PZP is hormonally induced during pregnancy, and the levels reach as high as 1 mg/ml [6]. The increment occurs at the end of the first trimester after which the serum levels remain rather constant at a plateau level. The protein is synthesized exclusively by maternal tissues including the liver and connective tissue cells. All humans have high concentrations of α_2-M, and hitherto there is no reported case of its absence. During pregnancy the levels of α_2-M increase approximately 20%, but α_2-M is not induced by estrogens [7,8]. The putative biological role of α_2-M as a proteinase inhibitor is based on the fact that this protein can be cleaved to generate a "bait region" [9] by proteinases from all four classes of proteinases [10–14].

The interaction of plasmin and trypsin with α_2-M has been studied both *in vivo* and *in vitro,* and these proteases

* Corresponding author. Fax: +577 5685304x156.
E-mail address: luifer@unipamplona.edu.co (L.F. Arbeláez).

[1] *Abbreviations used:* PZP, pregnancy zone protein; α_2-M, α_2-macroglobulin; DTBN, 5,5′-dithiobis-(2 nitrobenzoic acid); PTI, pancreatic trypsin inhibitor; PMSF, phenylmethylsulfonyl fluoride; MA, methylamine; DMSO, dimethylsulfoxide; HRP, horseradish peroxidase; EDTA, ethylenediaminetetraacetic acid; CT, α-chymotrypsin; PEG, polyethylene glycol.

1046-5928/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2006.12.008

were found to bind differently to α_2-M depending on the concentration of the proteases. Both of these α_2-M-complexes were found to be cleared from the circulation by a receptor on the cell surface, and in this process the C-terminal domain of the α-Ms plays a pivotal role [15]. For this reason, the C-terminal domains of PZP and α_2-M were isolated and sequenced, and they share 85% homology. Even though the differences between these domains are small, immunological assays with monoclonal antibodies are available [16–18]. There are also major differences in other common domains, especially in the dimer contact surfaces [19]. PZP and α_2-M also seem to have different functions, since PZP only inhibits kallikreins, while α_2-M inhibits the major part of the enzymes involved in the coagulation and fibrinolytic pathways [20–22].

The importance of the C-terminal domain relies on the fact that this region makes direct contact with the LRP/α_2-M receptor, which eliminates the α-Ms-proteinase complexes from the circulation [15]. The receptor binding domain of the α-Ms has been demonstrated to be involved in the interaction with the LRP-α_2-M receptor, which internalizes multiple ligands such as α_2-M- and PZP-proteinase complexes [23,24] as well as complexes of the tissue plasminogen activator and urinary-type plasminogen activator with plasminogen activator inhibitor type-1 [25,26]. Several syndromes and pathological states are related to these proteins, i.e., multiple sclerosis, and conformational aberrations in α_2-M limit the capacity of this protein to eliminate systemic proteases [27]. In the case of rheumatoid arthritis, α_2-M affects the cytokine level involved in the inflammatory process [28,29].

In this report a new significantly improved method for isolation of the C-terminal fragment of α_2-M and PZP, modified from the methods of Arbeláez et al. [16] and Jensen et al. [19] is presented. The method can be used for the purification of the receptor binding domain.

Materials and methods

Chemicals and enzymes

All buffer substances, salts and other chemicals employed were of the highest available purity.

5,5′-Dithiobis-(2 nitrobenzoic acid) (DTBN), bovine pancreatic trypsin inhibitor (PTI), ε-amino caproic acid, α-chymotrypsin (EC 3.4.21.1) (CT), phenylmethylsulfonyl fluoride (PMSF), methylamine (MA), dimethylsulfoxide (DMSO), aprotinin and *p*-nitrophenyl-*p*′-guanidinobenzoate hydrochloride, horseradish peroxidase (HRP) were from Sigma Co. Ethylenediaminetetraacetic acid (EDTA), polyethylene glycol 6000 (PEG) and zinc chloride were purchased from Merck. DEAE-Sephacel and Sephacryl S-200 HR were supplied by Amersham Biosciences. Amido Black stain and Opti 4CN kit were purchased from Bio-Rad. The monoclonal antibodies KF-2 and KG-3 were kindly provided by Dr. Torgny Stigbrand from the Umeå University in Sweden.

Active site titration

Following the methodology proposed by Chase and Shaw [30], CT yields 0.93 mol active site/mol enzyme when titrated with *p*-nitrophenyl-*p*′-guanidinobenzoate hydrochloride. The concentration was determined by absorption at 280 nm employing $(\varepsilon^{1\%})_{1cm} = 20$ and a molecular mass of 25 kDa [30].

Protein purification procedure

PZP and α_2-M were purified according to the method described by Arbeláez and Stigbrand [16], from fresh pregnancy plasma obtained at the Erasmo Meoz Hospital (Cúcuta, Colombia). The purified proteins were concentrated to 3 mg/mL using an Amicon device and pelleted (dropping protein solution into liquid nitrogen). The pellets were stored at −81 °C until use. The thiolester activity of the proteins was 98% as determined by titration with DTNB following incubation with MA [31]. The native PZP was 98% dimeric and 2% tetrameric. The concentration of PZP and α_2-M were determined by absorption at 280 nm employing $(\varepsilon^{1\%})_{1cm} = 8.2$ and a molecular mass of 360 kDa [32] for PZP; and $(\varepsilon^{1\%})_{1cm} = 8.9$ [33] and a molecular mass of 720 kDa for α_2-M [34].

Enzyme linked immunosorbent assay of α-Ms

Five milligrams of the monoclonal anti PZP and anti α_2-M antibodies RK35 and B6, respectively, were conjugated with 10 mg of HRP using the method described by Engvall [35]. Microtiters plates (96 well, Nunc, Denmark) were coated with antibodies H3 and B7, respectively, in 0.1 M $NaHCO_3$ overnight at 5 °C. The plates were washed with 1% NaCl and 0.05% Tween 20. The human α-Ms antigens were diluted to levels between 0 and 100 ng/mL of human α-Ms. The plasma samples and purification steps were added to the plates coated with antibodies and were incubated for 2 h at room temperature. The plates were washed as above and the HRP conjugated antibodies RK35-HRP and B6-HRP, diluted 1:2000 were added and the plates were incubated for 2 h at room temperature, the plates were washed again and developed by addition of 200 μL of *o*-phenylendiamine containing H_2O_2 in citrate-phosphate buffer, pH 5.0, for 15 min. The reaction was stopped by addition of 50 μL of 3 M sulphuric acid and the Abs at 420 nm was determined by a Thermomax microplate reader from GTF, Gothenburg, Sweden. The amount of α_2-M and PZP found in the starting batch of plasma by ELISA, were used as the 100% protein in plasma, the amount founded in each purification step was calculated in % from the amount found in the starting material. The purifications procedures are summarized in Tables 1 and 2.

Purification of the C-terminal fragments of PZP and α_2-M

PZP (3.3 mL (10 mg)) or α_2-M were dialyzed overnight against PBS (20 mM sodium phosphate, 0.15 M NaCl, pH