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

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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

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[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.

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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

Table 1
Yield of PZP/ml of plasma in the different purification steps, as determined by ELISA.

Procedure step	Amount of PZP (mg)	PZP recovery (%)	Fold purification
1. Starting material (200 mL)	220	100	1
2. Lysine–Sepharose chromatography	220	100	1
3. Precipitation with PEG (first)	202	92	ND
4. Precipitation with PEG (second)	170	77	ND
5. DEAE-Sephacel chromatography (pooled samples)	140	64	8
6. Zn–Sepharose chromatography (pooled samples)	120	55	145

ND, not determined.

Table 2
Yield of α_2-M/ml of plasma in the different purification steps, as determined by ELISA

Procedure step	Amount of α_2-M (mg)	α_2-M recovery (%)	Fold purification
1. Starting material (200 mL)	480	100	1
2. Lysine–Sepharose chromatography	480	100	1
3. Precipitation with PEG (first)	450	98	ND
4. Precipitation with PEG (second)	410	85	ND
5. DEAE-Sephacel chromatography (pooled samples)	375	78	7
6. Zn–Sepharose Chromatography (pooled samples)	250	52	27

ND, not determined.

7.4), and the volume of the dialyzate was measured and the solution was made 0.1 M with MA and incubated for 2 h at room temperature. Protein solutions were applied to a Sephacryl S-200 column (120 × 2.5 cm) equilibrated with 0.1 M sodium phosphate, pH 8.0. Following chromatography, the protein fractions were concentrated to 5 ml. The PZP-MA and α_2-M-MA solutions were incubated at 37 °C for 5 min with CT in a 1:4 mol/mol ratio. The reaction was stopped by the addition of PMSF to a final concentration of 0.5 mM (dissolved in DMSO). Another exclusion chromatography was performed employing a Sephacryl S-200 column (120 × 2.5 cm), in this case equilibrated with 1200 mL of 0.1 M NH_4CO_3, pH 8.2. The C-terminal fragments were concentrated to 3 mg/ml using $(\varepsilon^{1\%})_{1cm} = 5.5$ as factor [34] and dialyzed for 24 h with a 10 mM acetic acid solution. The purification procedures are summarized in Tables 3 and 4.

Electrophoretic analysis

Gel electrophoresis was performed in non-denaturing (5% PAGE) and denaturing (7.5% and 12.5% SDS–PAGE) conditions according to Laemmli [36]. Protein samples of 5 μg were mixed with the sample buffer (±SDS) [36] in a 1:1 (vol/vol) ratio. For sample buffer with SDS, the proteins were allowed to react, before electrophoresis, with SDS and β-mercaptoethanol (10%) for 60 min at room temperature. Boiling of samples was avoided because the thiolesters in PZP and α_2-M are sensitive to thermolysis [37]. Proteins were visualized by staining with Coomassie Brillant Blue R, and silver staining. Bio-Rad´s silver stain high range molecular weight marker was employed: 200 kDa (myosin), 116.25 kDa (β-galactosidase), 97.4 kDa (phosphorylase b), 66.2 kDa (bovine serum albumin), 45 kDa (ovalbumin), and Bio-Rad´s prestained low range molecular weight marker:

Table 3
Yield and purity of the C-terminal peptide (from 10 mg of PZP–MA) in different purification steps, monitored spectrophotometrically at 280 nm

Procedure step	Total protein (mg)	Amount of C-terminal (mg)	C-terminal recovery (%)	Fold purification
1.Starting material (5 mL)	10	1.550	100	1
2. Sephacryl S-200 chromatography	9.638	1.499	96	1.037
3. PZP-MA digestion (CT)	9.638	1.499	96	ND
4. Sephacryl S-200 chromatography	8.8	1.368	88	1.136

ND, not determined.

Table 4
Yield and purity of the C-terminal peptide from 10 mg of α_2-M–MA in different purification steps, monitored spectrophotometrically at 280 nm

Procedure step	Total protein (mg)	Amount of C-terminal (mg)	C-terminal recovery (%)	Fold purification
1. Starting material (5 mL)	10	1.550	100	1
2. Sephacryl S-200 chromatography	9.638	1.499	96	1.037
3. α_2-M-MA digestion (CT)	9.638	1.499	96	ND
4. Sephacryl S-200 chromatography	8.8	1.368	88	1.136

ND, not determined.

103 kDa (phosphorylase b), 77 kDa (bovine serum albumin), 50 kDa (ovalbumin), 34.3 kDa (carbonic anhydrase), 28.8 kDa (soybean trypsin inhibitor), 20.7 kDa (lysozyme) was employed with Coomassie staining.

Protein sequence analysis

Sequence analysis for intact PZP and α_2-M protein (Fig. 1, lanes 2 and 3) and for C-terminals (Fig. 2, lanes 2 and 4, fragments 5 and 6) were performed. Proteins in the 5% native gel, and 7.5% and 12.5% SDS gel were transferred by electroblotting to a PVDF membrane (Bio-Rad) in a solution of 25 mM Tris with 192 mM glycine and 20% methanol, pH 8.3. Samples were blotted for 3 h. Transferred bands were visualized by incubation for 2–3 min with a solution of 0.1% amido black dissolved in distilled water. The membranes were destained in a solution containing 55% water, 35% methanol and 10% acetic acid. The sequencing was performed at Umeå University (Umeå-Sweden) employing the Edman degradation methodology.

Western blot analyses

Western blot analyses of the C-terminals of human α-Ms were performed. The bands of these domains were transferred by electroblotting to an Imobilon-P transfer membrane (Millipore) from 12.5% SDS gel by tank transfer in a solution of 25 mM Tris with 192 mM glycine and 20% methanol, pH 8.3. Samples were blotted overnight. After transfer, the blots were allowed to dry completely. For immunodetection the membranes were incubated for 1 h with primary monoclonal antibodies reactive with α_2-M and PZP C-terminals (KG-3 and KF-2, respectively) diluted 1:1000 in blocking buffer consisting of 1% BSA in PBS–T (phosphate-buffered saline: 10 mM Na-phosphate, pH 7.2, 0.9% NaCl and 0.05% Tween 20). After washing the membrane three times with the same buffer, the blot was incubated with the secondary polyclonal antibody (horseradish peroxidase-conjugated anti-mouse rabbit immunoglobulin) diluted 1:2000 in blocking buffer. After three washes using PBS–T, the proteins were visualized with the Opti 4CN kit (Bio-Rad).

Results

In Fig. 1a, lane 1 the α_2-M and PZP fractions obtained from the elution of DEAE-Sephacel are shown as they appear in a native polyacrylamide gel. The position of α_2-M and PZP are indicated for both proteins which were completely separated without any cross contamination. A purity of approx. 98% was achieved in both cases after elution of α_2-M and PZP from the Zn–Sepharose column as shown in Fig. 1b for PZP as visualized by silver staining. Tables 1 and 2 summarize the purification methods and steps as well as the fold purification for both PZP and α_2-M. Furthermore, only one band of 180 kDa was visualized using Coomassie staining of the denatured α_2-M-MA and PZP-MA (Fig. 2, lanes 1 and 5), as a corroboration of the purity of these samples and the results of the sequence analyses confirmed only one sequence in each sample. The yield and purity of the peptides are presented in Tables 3 and 4. The sequence of the N-terminal was TEPQYMV for PZP and SVSGKPQ for α_2-M (Table 5). The fragment F0 (Fig. 2, upper band in lane 2) corresponds to the complex

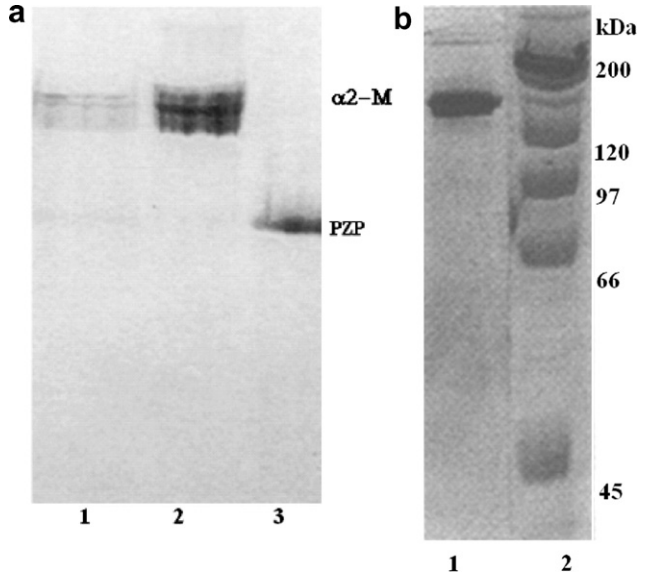

Fig. 1. (a) Electrophoresis of α-Ms in a 5% native polyacrylamide gel, lane 1, elution of both α_2-M and PZP from DEAE-Sephacel, lane 2 α_2-M and lane 3 PZP eluted by Zn–Sepharose chromatography and visualized by Coomassie staining and (b) SDS–PAGE (7.5%) of purified PZP. The samples were visualized by silver staining; lane 1 contains 3 μg of protein, lane 2 contains the molecular weight markers, as indicated in Materials and methods.

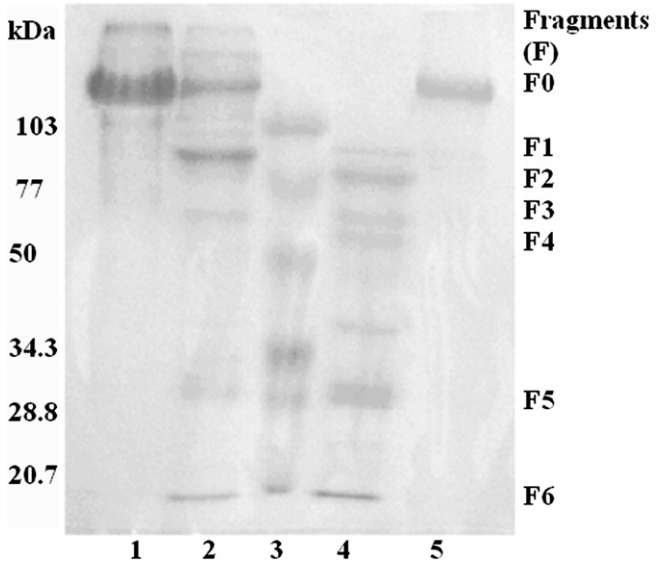

Fig. 2. SDS–PAGE (7.5%) of digestion products of α_2-M–MA and PZP–MA with chymotrypsin incubated for 5 min. at 37 °C. Lane 1, complex α_2-M–MA; lane 2, digested α_2-M–MA; lane 3, molecular weight standards as indicated in Materials and methods; lane 4, digested PZP–MA; lane 5, the complex PZP–MA.

α_2-M-MA (180 kDa) undegraded by the enzyme. No high molecular weight complexes were visualized because the β-cysteinyl-γ-glutamyl thiolesters were blocked by MA. CT cleavage of the α-Ms reveals the typical degradation fragments between 50 and 100 kDa corresponding to the "bait region" (Fig. 2, lanes 2 and 4, fragments 1–4), and the ~30 kDa fragment is obtained from the subsequent cleavage of this domain (Fig. 2, fragment 5 and peak 2 of the exclusion chromatography, Fig. 4) identified as the C-terminal fragment (~30 kDa). The N-terminals sequences were determined to be VALHALS for PZP and SSTQDTV for α_2-M, respectively. There results indicate the cleavage between the amino acids ^{1260}Thr-Val^{1261} in PZP (GenBank Accession No. NP_002855) and ^{1248}Phe-Ser^{1249} in α_2-M (GenBank Accession No. NP_000005). A peptide of 18 kDa (Fig. 2, lanes 2 and 4, fragment 6 and peak 3 in the exclusion chromatography, Fig. 3) in both proteins corresponds to a shorter N-terminal of these proteins, as demonstrated by sequence analysis. The N-terminal sequences were ALKVQTV and EEFPFA for PZP and for α_2-M, respectively (Table 5). This indicate a cleavage between the amino acids ^{1346}Phe-Ala^{1347} in PZP (GenBank Accession No. NP_002855) and ^{1336}Lys-Glu^{1337} in α_2-M (GenBank Accession No. NP_000005). The first peak in Fig. 3 corresponds to a mixture of components with molecular weights from 50 to 180 kDa. The total yield at the C-terminal purification procedure was approx 88%. The ~30 kDa C-Terminal fragments were transferred to a hydrophobic membrane and visualized by the antibodies KG-3 for the C-terminal fragment of α_2-M and KF-2 for the C-terminal fragment of PZP.

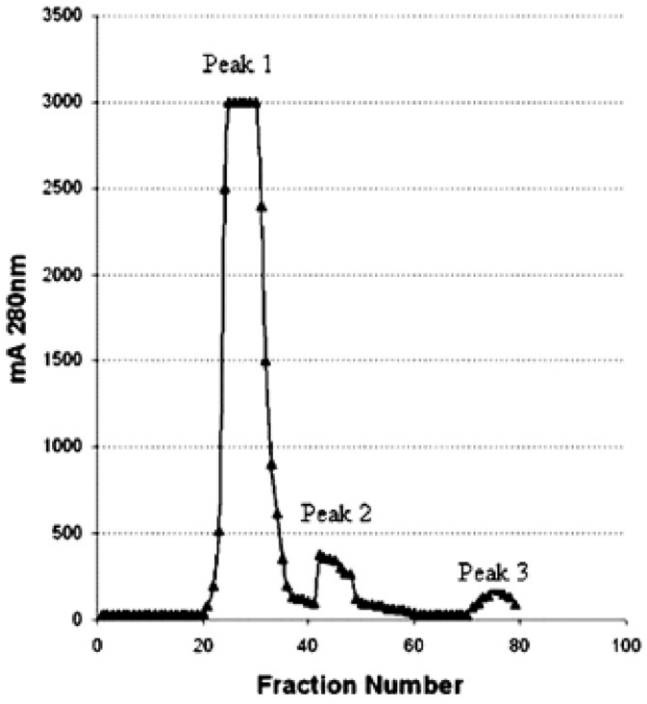

Fig. 3. Elution profile of PZP–MA digested with chymotrypsin from the Sephacryl S-200 column. Peak 1 consists of high molecular weight fragments, peak 2 is the C-terminal of PZP (30 kDa) and peak 3 is a subsequent degradation of this domain (18 kDa).

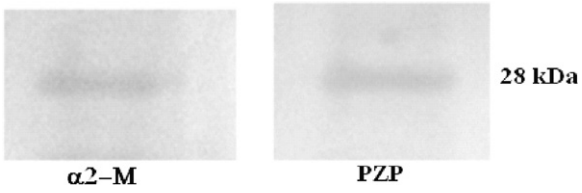

Fig. 4. The left band corresponds to the α_2-M C-terminal visualized by the KG-3 monoclonal antibody and the right band is the PZP C-terminal identified by the KF-2 monoclonal antibody.

Discussion

This paper presents a new, faster and efficient purification method of the C-terminal fragments of α_2-M and PZP, fragments with significance for the biological role of these proteins. Due to the homology between the C-terminal of α_2-M and PZP, it is of importance to determine the amount of cross contamination of the preparations. No PZP dimers were found in the α_2-M preparation; neither did α_2-M tetramers appear in the PZP sample which would indicate the presence of α_2-M or denaturation of PZP as judged by sequence analysis. The amount of α_2-M is less than 0.1% in the PZP preparation. Both proteins were purified to approx. 98% purity, and the determined sequences were identical to those previously reported [16].

The cleavage of the receptor binding domain of the α-M-MA by CT demonstrated more selective degradation than cleavages with other proteases, in which several different N-terminal sequences were found, indicating more than one cleavage site. Thomsen and Sottrup-Jensen identified different domains in α-Ms in which they found two fragments of the C-terminal domain when hydrolyzing the α-Ms with CT followed by PAGE–SDS electrophoresis [38]. The Phe^{1248}-Ser^{1249} cleavage of α_2-M by CT, is a typical cleavage site for this enzyme, found also in the autolysis and autolytic inactivation of CT [39]. Probably PZP is first cleaved at the same site as α_2-M, but is then further degraded by CT. PZP was demonstrated to be more sensitive to enzyme hydrolysis than α_2-M, as can be demonstrated in Fig. 2 lane 4, in which the 180 kDa band (F0) of PZP was totally degraded, while the one for α_2-M

Table 5
Aminoacidic sequences of the N-terminal region and C-terminal region of α-Ms (Fig. 3 peaks 2 and 3), determined using Edman degradation methodology

Purified protein	N-terminal sequence (180,000 Da)	Peak 2 N-terminal sequence (30,000 Da)	Peak 3 N-terminal sequence (18,000 Da)
PZP	TEPQYMV	VALHALS	ALKVQTV
α_2-M	SVSGKPQ	SSTQDTV	EEFPFA

was not (Fig. 2, lane 2 (F0)). The native and three-dimentional structure of these proteins protect them from degradation by enzymes. When α_2-M and PZP were treated with MA, and expose their C-terminals, this facilitates the hydrolyzation of the C-terminal and its further degradation. CT not only cleave aromatic sites, but this enzyme has been demonstrated to cleave other sites with different catalytic constants, and the cleavage of the fragments of ~30 and 18 kDa are in good agreement with earlier reported cleavages sites by CT [39–41]. These two C-terminal fragments present molecular weights in accordance with the sequences of the isolated fragments, which strongly suggests that these C-terminals, are similar or the same as these previously hydrolyzed with CT but not sequenced by Thomsen and Sottrup-Jensen [38]. This new method thus allows the purification of C-terminals of α-Ms in a selective and rapid way, avoiding the long 12 h incubation with papain which produces several of the low molecular weight fragments which decrease the final yield of the C-terminal fragment [16]. This new method produces only two fragments with high yield and it also requires lower amounts of α-Ms than any other known method. The procedure is simple and allows high recuperation of the C-terminal fragment (88%) which is superior to other methodologies [16,18,34]. The receptor binding domains of the α-Ms were also isolated with high purity and only two C-terminal sequences for each protein were identified, both, with different size belong to the C-terminal. This confirms the selective cleavage by CT, and explains the high recuperation of the total amount of C-terminals.

The interaction of the receptor binding domain of the α-Ms with the LRP/α_2-M receptor has been extensively studied, several theories on the mechanism of removal have been presented and all of them involve one, two or four of the α-Ms C-terminals participating in the internalization of the complexes. During the last years, the repetitive sequences of the cluster I and II of the LRP/ α_2-M receptor have been identified as the domain of interaction with the receptor binding domain [23] which is exposed when the α-Ms are complexed with proteinases. Furthermore, other ligands to the LRP/α_2-M receptor have been demonstrated not to compete with the binding of the α-M complexes with the receptor, indicating an unique interaction of the complexes of the α-Ms with the receptor [42–44].

This new purification method of the receptor binding domain will facilitate the final elucidation of the precise site of the interaction between the α-M complexes and the LRP/α_2-M receptor, because the purified C-terminals of the α-Ms have been hydrolyzed with only two cleavage sites. Both can be used in studies with the binding site of the receptor, especially by generation of a fully functional LRP deletion mutant (minireceptor) [23]. Furthermore the purified C-terminals can be used for purification of the minireceptor and the minireceptor can be used in the purification of *in vivo* complexes of α-Ms with proteinases. The ~30 kDa C-terminal as well as the 18 kDa will contributed to size-limit the C-terminal of the α-Ms and may facilitate the identification of those aminoacids in the C-terminal which play a pivotal role in the interaction with LRP/α_2-M receptor. This will contribute to a better understanding the biological role of these two proteins in man and animals.

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