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Quantitation of active alpha-2-macroglobulin by trypsin protease zymography

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Abstract: A simple and reliable method for the determination of the concentration and function of alpha-2-macroglobulin (α_2M) by zymography was developed. The method is based on the covalent binding of α_2M and trypsin followed by non-reducing PAGE and zymography with gelatine incorporated in the electrophoretic gel. The results showed that α_2M binds trypsin in a concentration-dependent manner exhibiting a linear relation. The sensitivity of the method is 125 nM and the intra-assay coefficient of variation 4.2 %. Freezing of α_2M induces its partial denaturation, which could be seen as the reduction in the amount of functional molecule and its reactivity with trypsin. The reported method enables measurement of α_2M taking into consideration both its quantity and function, stressing the importance of the determination of the amount of physiologically active molecules and not just their presence in the sample. The method was further confirmed using α_2M from patients with end-stage renal disease who are known to be under increased oxidative stress and inflammation, which are expected to modify the structure of proteins.

Keywords: alpha-2-macroglobulin; functional assay; electrophoresis.

INTRODUCTION

Alpha-2-macroglobulin (α_2M) is a homotetrameric blood glycoprotein having molecular mass of 720 kDa.[1] Its concentration in adult humans is 4.64± ±1.42 μM (3.34±1.02 g L^{-1}) and decreases to 2.26±0.58 μM (1.63±0.42 g L^{-1}) in healthy elderly individuals.[2,3] The biological function of this protein is to act as a general protease inhibitor. Upon binding to protease, its structure changes, causing an entrapment of the protease. Steric hindrance of the protease and its catalytic site disables its interaction with large substrates, such as proteins. Proteolysis of small substrates, such as peptides, is, on the other hand, not affected. Upon

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complex formation, a covalent bond between α_2M and a protease is created. A molecule of native tetrameric α_2M can bind two small protease molecules (*e.g.*, trypsin) or one large one (*e.g.*, plasmin) at the same time.[4]

Hitherto, the methods to estimate the quantity of α_2M and its activity were separate procedures. The quantity is usually measured by immunochemical assays and the anti-protease activity of α_2M by measuring the activity of trypsin bound to α_2M using the chromogenic substrate BAPNA.[5] In brief, α_2M and trypsin are allowed to interact, then soy trypsin inhibitor is added to inhibit the activity of the remaining free enzyme, and the activity of α_2M-bound trypsin is further determined by **s**pectrophotometry**.**

A simple, reliable and cost-effective method for the determination of the concentration of active α_2M molecules is described in this paper. The measurement is based on the covalent binding of α_2M and trypsin followed by non-reducing PAGE and zymography with gelatine incorporated in the electrophoretic gel. Specific substances, such as BAPNA and soy trypsin inhibitor, are not necessary, contributing to the advantages of the method. It should be emphasized that monitoring of the relation quantity/activity becomes very important when the sample originates from an individual exposed to a stress or with a disease accompanied by post-translational modifications of proteins, such as diabetes, renal disease or cancer.[6]

EXPERIMENTAL

Materials

Alpha$_2$M was purchased from BioRad (Hercules, USA). A stock solution of trypsin was obtained from the Institute Torlak (Belgrade, Serbia) at the concentration of 107 μM. All other used chemicals were of analytical grade. Serum samples were collected from healthy adult individuals (n = 10) and from patients with end-stage renal disease (ESRD, n = 10) who underwent peritoneal dialysis in the Clinical Centre Serbia (CCS). The study was approved by the Ethical committee of CCS (Approval number 890/8).

Isolation and immunodetection of α_2M from human serum

Alpha$_2$M was isolated from each serum separately. Individual samples were used for the development of the assay, and to test the applicability of the assay on α_2M from patients, two pools were formed: one for healthy persons and one for patients with ESRD (containing equal quantities of α_2M from each sample in the group). The protein was isolated in two steps using a modified published procedure.[7] In the first step, serum (1.5 mL) was subjected to precipitation by 40 % ammonium sulphate (AS) solution. The supernatant was discarded, and the pellet dissolved in 50 mM PBS, pH 7.4. The remaining AS was removed by solvent exchange using 50 mM PBS, pH 7.4 and a centrifugal filter device (Millipore, Billerica, USA; 10 kDa cut-off) at 10000 g for 10 min. The protein solution was applied on a gel-filtration column filled with Sephadex G-200 (30×1.7 cm; Pharmacia, Uppsala, Sweden). The elution was performed with 50 mM PBS, pH 7.4, and the fraction at V_0 maximum was collected. The fraction contained α_2M and the concentration of the protein was determined using a BCA reagent kit (Pierce Biotechnology, Waltham, USA). The identity and the purity of the α_2M were confirmed by immunoblotting after SDS PAGE electrophoresis under reducing conditions (on 8

% gel). Proteins were transferred to a nitrocellulose membrane and incubated with primary goat anti-α_2M antibody (BioRad, Hercules, USA). For immunodetection, biotinylated secondary antibody (Vector, Burlingame, USA) and HRP-conjugated avidin (Vector, Burlingame, USA) were used. Proteins were visualised by autoradiography.

Determination of α_2M by zymography

Both commercial and isolated α_2M of human origin at different concentrations (from 125 to 1000 nM) were incubated with bovine trypsin at a concentration of 1000 nM (previously determined as the optimal concentration for zymography). The incubation lasted for 15 min at room temperature and after addition of a non-reducing sample buffer for another 30 min at 37 °C. Zymography was performed according to a published protocol, using a 10 % gel containing bovine 0.1 % gelatine.[8] After electrophoresis, the gel was washed in distilled water (2×1 min) and initially incubated in TNC buffer (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, pH 8.5) containing 2.5 % Triton X-100 (2×30 min, under constant shaking) and then in the same buffer without detergent (at 37 °C, overnight). The gel was stained for 20 to 40 min, depending on individual zymogram, in a fresh 0.2 % CBB solution and scanned. Densitometric analysis was performed using TotalLab software version 2.01 (Amersham BioSciences, Buckinghamshire, UK). In order to test the applicability of the method on partially denatured α_2M, two procedures for its denaturation were conducted prior to incubation with trypsin.[9] One was thermal (incubation at 100 °C for 5 min) and the other cryo (incubation at –20 °C overnight).

RESULTS AND DISSCUSION

The α_2M isolated from the serum samples contained the same molecular forms as the commercial α_2M, as could be seen from the protein stained by Ponceau S and the immunoblot shown in Fig. 1 (one representative example of the isolated α_2M is given). Under the electrophoretic condition, α_2M tends to degrade resulting in several fragments.

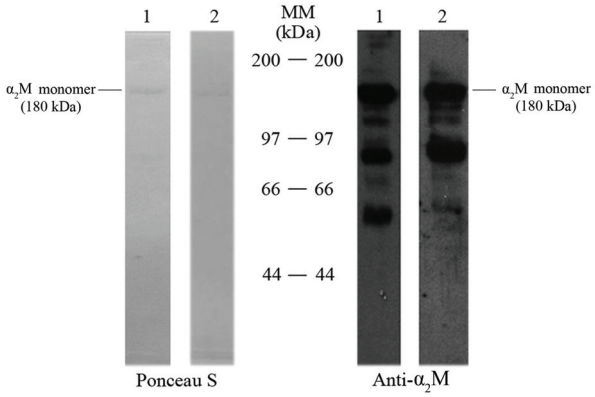

Fig. 1. A representative example of the isolated α_2M (1) and α_2M commercial standard (2) analysed by reducing SDS-PAGE (on 8 % gel) and immunoblotting. Samples stained with Ponceau S (left hand-side) and immunoblotted using anti-α_2M antibody (right hand-side).

A complex which forms between α_2M and trypsin includes covalent bonding,[10] and thus it is expected to remain intact under the non-reducing conditions

employed in SDS PAGE. The stability of the complex was confirmed and moreover, α_2M-bound trypsin retained its proteolytic activity towards co-polymerized gelatine, as could be seen in Fig. 2A (at three different concentrations of α_2M, 250, 500 and 1000 nM). In a control experiment, when α_2M was subjected to zymography alone, without pre-incubation with trypsin, no proteolytic signal was detected (Fig. S-1 of the Supplementary material to this paper), clearly indicating that all the activity in zymography originated from trypsin. Two specific signals related to the α_2M/trypsin complex were seen in the zymogram, the lower most probably originating from the α_2M-fragment/trypsin (the signal from trypsin alone can be seen at a much smaller mass, Fig. S-1). Based on the position of α_2M-fragment/trypsin complex, this α_2M-fragment most likely originates from a fragment of approximately 90 kDa (Fig. 1).

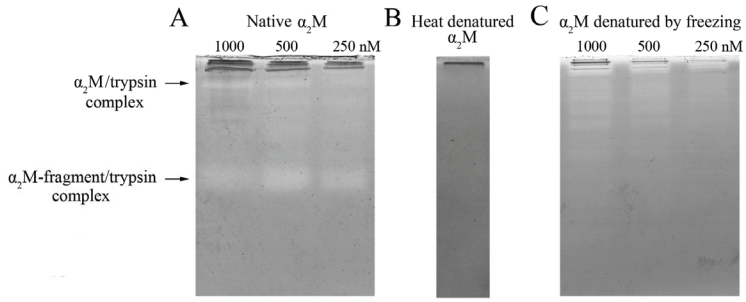

Fig. 2. Zymography of the native α_2M at three concentrations incubated with trypsin (A), heat denatured α_2M incubated with trypsin (B) and cryo-denatured α_2M at three concentrations incubated with trypsin (C).

Thermal denaturation of α_2M resulted in its inability to bind trypsin (Fig. 2B), while denaturation by freezing resulted in both aggregation of α_2M (precipitate formed upon thawing) and appearance of a number of proteolytic bands in the zymogram (Fig. 2C, at three different concentrations of α_2M, 250, 500 and 1000 nM). This last finding could be explained by structural alterations of α_2M, which were already recognised as a consequence of freezing.[9] The experiments with denatured α_2M confirmed the specificity of the method, as only functional α_2M molecules could be assayed. It is worth mentioning that this method could be employed to investigate the remaining activity of α_2M after its partial denaturation.

The intensity of the proteolytic bands seen with native α_2M (expressed in arbitrary densitometric units) was further analysed in relation to the concentration of α_2M and a linear dependence was seen undoubtedly only in the case of the upper band, originating from the intact α_2M/trypsin complex (Fig. 3A and 3B). The assay was optimised to satisfy the linear function in the range of α_2M con-

centrations from 125 to 1000 nM. A concentration of α_2M less than 125 nM could not be measured reliably.

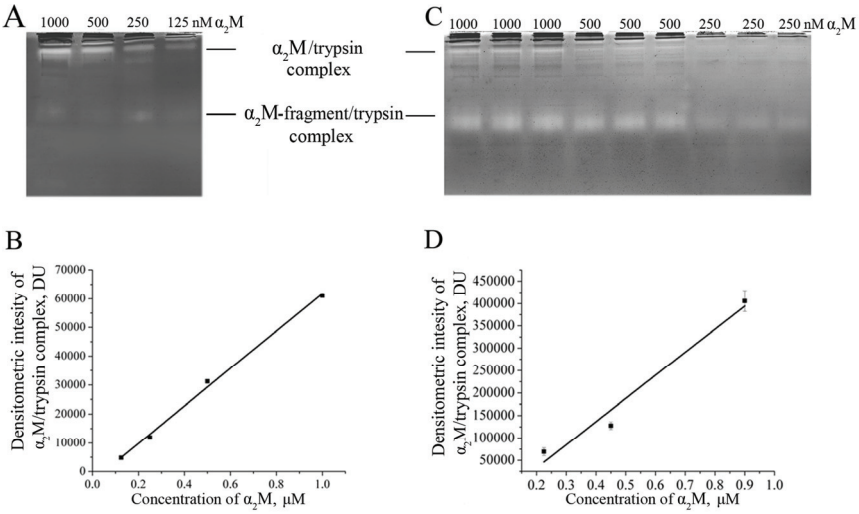

Fig. 3. Zymography of α_2M at four concentrations incubated with trypsin (A) and densitometric analysis of the signals originating from the α_2M/trypsin complex (B); zymographic assessment of the intra-assay coefficient of variation using three concentrations of α_2M in triplicate (C) and statistical analysis of the data (D).

Analytical validation of the method was further performed by assessing the intra-assay coefficient of variation (or repeatability) of the test. Samples (α_2M at three different concentrations) were subjected to zymography in triplicate and the results statistically analysed. The greatest coefficient of variation was calculated for the smallest concentration of α_2M and it was 4.2 %, which is within the accepted limit of the measurement uncertainty for this type of assay (Fig. 3C and 3D).

The same results and relations were obtained with the commercial α_2M and α_2M isolated from the serum from healthy persons. In order to test the behaviour of α_2M originating from other sources, the assay was performed with two pools of α_2M, one derived from healthy individuals and the other from patients with ESRD. When the same concentrations of α_2M were subjected to zymography, similar, yet somewhat different data were acquired (Fig. 4). The intensities of signals originating from α_2M from healthy persons were slightly higher than those from the patients, the difference being more pronounced as the concentration increased, resulting in a lower slope of the dependence (Fig. 4C). This difference is not unexpected since structural modifications of proteins in ESRD are well documented.[11] Therefore, the proposed method can reliably measure the amount of α_2M originating from healthy persons, whereas quantitation of α_2M from patients, which may contain structurally altered α_2M, could be seen more as

an estimation instead of a precise measurement, since a modified affinity may appear as a new variable.

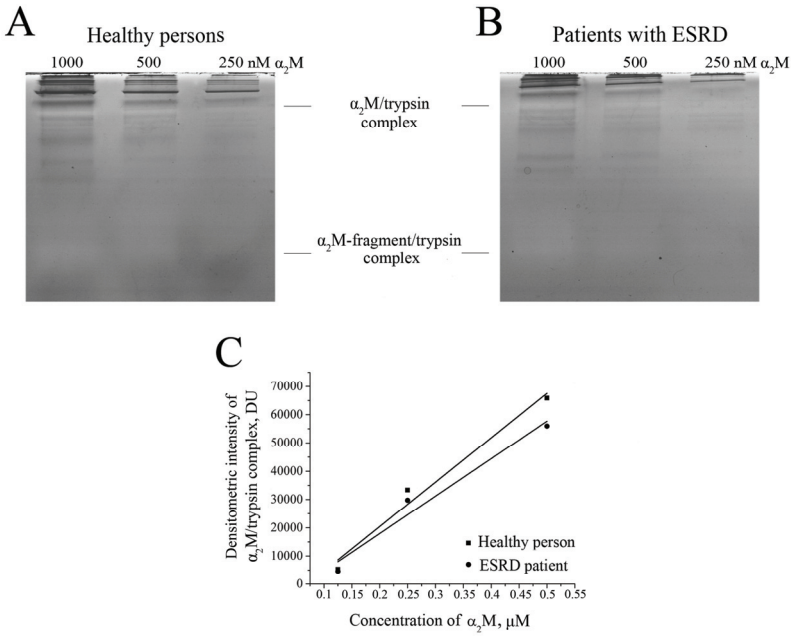

Fig. 4. Zymography of α_2M pools from healthy persons (A) and patients with ESRD (B) at three concentrations incubated with trypsin, and densitometric analysis of the signals originating from the α_2M/trypsin complex (C).

The zymographic assay described in this paper enables investigation of the concentration/structure/function relationship of α_2M in different pathologies as well as in the presence of modifying agents that may accompany them. This issue is important since studies on cancer, liver fibrosis, myocardial infarction and Alzheimer's disease have already recognised α_2M as a potential biomarker.[12–15]

CONCLUSIONS

A simple and reliable method for the determination of the concentration and function of alpha-2-macroglobulin (α_2M) by zymography was developed. The method is based on the covalent binding of α_2M and trypsin followed by non-reducing PAGE and zymography with gelatine incorporated in the electrophoretic gel. Analytical validation confirmed linear function, high sensitivity and low intra-assay coefficient of variation of the test. The method could also be employed to investigate the remaining activity of α_2M after its partial denaturation or structural modification due to disease.

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И З В О Д

ОДРЕЂИВАЊЕ АКТИВНЕ ФОРМЕ АЛФА-2-МАКРОГЛОБУЛИНА МЕТОДОМ ПРОТЕАЗНЕ ЗИМОГРАФИЈЕ ТРИПСИНОМ

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Развијена је једноставана и поуздана метода за одређивање концентрације и функције алфа-2-макроглобулина (α_2M) зимографијом. Метода се заснива на ковалентном везивању α_2M и трипсина, након чега следи нередукујућа полиакриламидна електрофореза и зимографија са желатином у електрофоретском гелу. Резултати су показали да постоји линерна зависност између концентрације α_2M и количине везаног трипсина. Осетљивост методе је 125 nM, док је коефецијент варијације унутар теста 4,2 %. Замрзавање α_2M изазива његову делимичну денатурацију, која се испољава кроз смањење удела функционалног молекула и његове реактивности према трипсину. Описана метода омогућава мерење α_2M узимајући у обзир његов квантитет и фукционалност, наглашавајући важност одређивања количине физиолошки активног молекула, а не само присутног у узорку. Валидност методе је потврђена са α_2M молекулима пацијената са крајњим стадијумом бубрежне болести, за које се зна да су под повећаним оксидативним стресом и инфламацијом, односно под процесима од којих се очекује да модификују структуру протеина.

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