



## Full Length Research Paper

# Fibrinolysis with a new microplasmin purified of human plasma

Agustín N. Joison<sup>1</sup>, Gustavo Baiardi<sup>2</sup>, Rocío Donalizio<sup>2</sup> and Federico Gallo<sup>3</sup>

<sup>1</sup>Faculty of Chemical Sciences, Catholic University of Córdoba.

<sup>2</sup>Institute of Biological and Technological Research (IIBYT-CONICET), National University of Córdoba, Faculty of Chemical Sciences, Catholic University of Córdoba,

<sup>3</sup>Institute of Vascular Research

Corresponding Author E-mail: [ajoison2001@yahoo.com.ar](mailto:ajoison2001@yahoo.com.ar) TE: +54-351-4807421

## ABSTRACT

Thrombosis, disease characterized to formation of blood clots, is a cause of death frequently associated with myocardial infarction (IAM), stroke, deep-vein thrombosis, and pulmonary embolism. Microplasmin is a low-molecular-weight protein termed with a molecular weight (Mr) of 28 KDa obtained by Plasmin autolysis process in an alkaline solution. Microplasmin was prepared from pooled human plasma by acetic acid precipitation (pH 6.1) in buffer sodium borate (pH 8.0). Microplasmin was purified with affinity and anionic interchange chromatography. A protein peak with most of the fibrinolytic activity of microplasmin adsorbed in the lysine-Sepharose column and eluted after washing the column with 200 mM of  $\epsilon$ -aminocaproic acid. The second peak eluted with 150 mM with fibrinolytic activity was pooled to gel electrophoresis analysis. Protein named microplasmin with two chains was insolated of human plasma showed fibrinolysis in vitro clot respect control.

**Keywords:** Plasminogen- microplasmin- chromatography- electrophoresis- fibrinolysis.

## INTRODUCTION

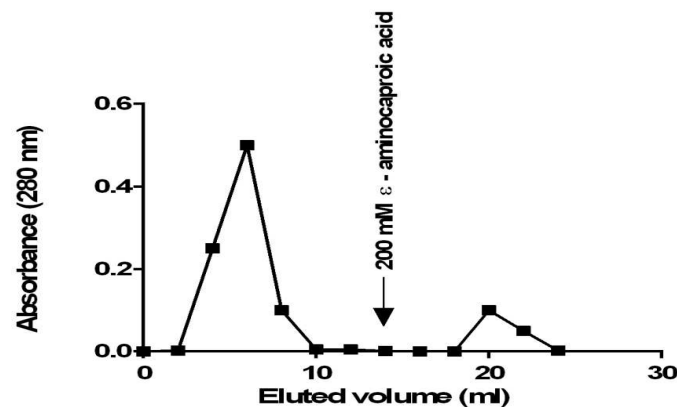
Thrombosis, disease characterized to formation of blood clots, is a cause of death frequently associated with myocardial infarction (MI), stroke, deep-vein thrombosis, and pulmonary embolism. Many fibrinolytic agents available in the clinic today were purified using the different plasminogen activators. However, these agents commonly have undesired side effects, including gastrointestinal bleeding. At respect, the search for safer fibrinolytic enzymes from a variety of potential sources is ongoing from as human, bovine tissues or bacterium, plants and fungus (Kim et al., 2015).

Microplasmin is a low-molecular-weight protein termed with a molecular weight (Mr) of 28 KDa obtained by Plasmin autolysis process in an alkaline solution and it is slightly more positively charged and hydrophobic molecule than plasminogen (Liu et al., 2015). Microplasmin has a B chain of plasmin consisting of 230 amino acids connected by disulfide bonds with other

peptide COOH-terminal portion of the A chain of plasmin consisting of 31 amino acid residues (Wu et al., 1987).

Microplasmin is a novel thrombolytic that may possess an improved benefit/risk ratio compared to tissue-plasminogen activator, due to potential improved efficacy (based on direct-acting mechanism) and improved safety (based on rapid alpha 2-antiplasmin inactivation, decreased bleeding risk, and neurovascular protective effect). Potential advantages microplasmin is a direct-acting thrombolytic that dissolves blood clots without the need for free plasminogen in the circulation Human microplasminogen, which lacks the five 'kringle' domains of plasminogen was expressed with high yield in *Pichia pastoris*. It was purified, converted to microplasmin and equilibrated with 5 mmol/ L citrate, pH 3.1, yielding a stable preparation (Nagai et al., 2003).

A novels fibrinolytic enzymes has been isolated from some earthworm species such as *Escherichia Coli*,



**Figure 1.** Elution of the alkali treated human microplasmin. A second peak with fibrinolytic activity was eluted with 25 mM  $\epsilon$ -aminocaproic acid. The solid line is absorbance at 280 nm.

*Lumbricus* or *Eisenia fetida* and were thoroughly characterized because the genes encoding been identified and the recombinant enzymes expressed strong fibrinolytic activity both in vitro and in vivo studies (Phan et al., 2011). The purpose of this study was to develop new methods for the preparation of pure microplasmin with fibrinolytic activity.

## MATERIAL AND METHODS

Microplasmin was prepared from pooled human plasma by acetic acid precipitation (pH 6.1) in buffer sodium borate (pH 8.0). The sample was applied to a L-lysine Sepharose (Sigma, USA) column (1.0 X 30 cm). Microplasmin was eluted with 6-Aminocaproic acid (Sigma, USA) 200 mM. To eliminate the 6-Aminocaproic acid the microplasmin sample was dialyzed against buffer sodium borate (pH 8.0). After dialysis the eluted was chromatographed by anionic interchange with mono Q HR 16/10 (GE Healthcare, Germany) column (0.5 X 20 cm) and eluted utilizing increase gradient of ionic strength 100, 200 and 1000 mM buffer sodium borate (pH 8.0). Native PAGE was carried out on microplasmin diluted in sample buffer (375 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 0.0025% (w/v) bromophenol blue, pH 8.6). Staining gel (2%) in buffer Tris-HCl 375 mM, separating gel (10%) in buffer Tris-HCl 375 mM pH 8.8 and Tris-glycine running buffer (25 mM Tris, 192 mM glycine, pH 8.3). SDS-PAGE was carried out on microplasmin previous heat 70 °C with 10% SDS and 1% DTT 10 minutes and then diluted in sample buffer (SDS 10%, 375 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 0.0025% (w/v) bromophenol blue, pH 8.6). Staining gel (2%) in buffer Tris-HCl 375 mM, separating gel (10%) in buffer Tris-HCl 375 mM pH 8.8 and Tris-glycine running buffer (10% SDS, 25 mM Tris, 192 mM glycine, pH 8.3). After

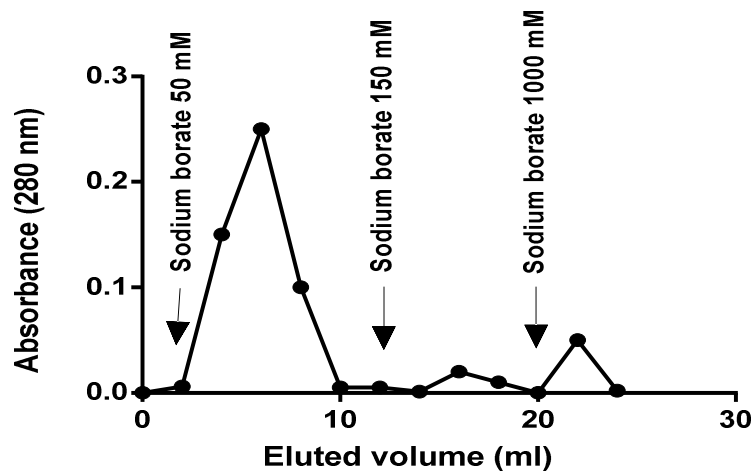
electrophoresis the gels were fixed in 30% Methanol, 10% acetic acid for at least 24 hours. After this the gels were rinsed twice in 20% ethanol, for 10 minutes for each wash, and then twice in water, for 10 minutes for each wash and sensitized by soaking (one at a time only) for one minute in 0.8 mM sodium thiosulfate. Gels were rinsed twice for 1 minute for each wash in water. Finally gels were impregnated with 12mM silver nitrate during 20 minutes and revealed with 0.27% of sodium carbonate. Fibrin clot was prepared with fibrinogen (sigma, USA), thrombin (Wiener lab, Argentina) 100 NIH UI/ml, for use this stock solution (100 NIH units/ml) was diluted in 150 mM NaCl to 20 NIH units/ml.  $\text{Ca}_2\text{Cl}$  (Biopack, Argentina) 250 mM. Microplasmin and sodium borate 150 mM used as control were applied in fibrin clot as drops of 30  $\mu\text{l}$  in 5 minute intervals; The weights were recorded in an analytical balance (OHAUS, USA) in each of one of intervals.

## Statistic analysis

The t unpaired test and lineal correlation analysis were used to compare the weight of fibrin clot between microplasmin and control. The values with  $P < 0.05$  were considered to be statistically significant.

## RESULTS

After purification in alkaline solution, the protein was purified by affinity chromatography and a protein peak with most of the fibrinolytic activity of microplasmin in adsorbed in the lysine-Sepharose column and eluted after washing the column with 200 mM of  $\epsilon$ -aminocaproic acid (Figure 1). The protein was dialyzed from the inhibitor with 200 mM sodium borate 50 mM pH 8.0, and a fibrinolytic activity protein was recovered.



**Figure 2.** Elution of the alkali treated human microplasmin. A second peak with fibrinolytic activity was eluted with 150 mM sodium borate pH 8.0. The solid line is absorbance at 280 nm.



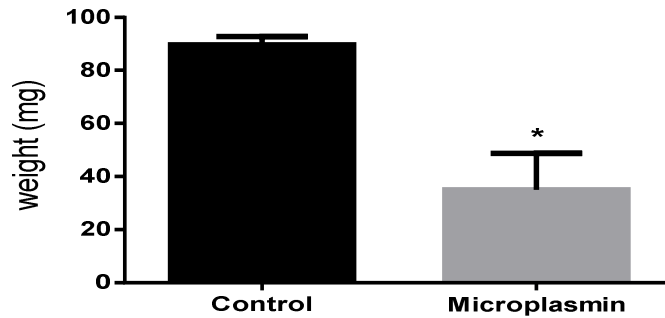
**Figure 3.** Native 10 % polyacrylamide gels of microplasmin. Lanes: 1, 3 and 4 native microplasmin after ionic interchange; Lane 2 native microplasmin before purification.

The protein fraction of the second peak of affinity chromatography after dialyzed was pooled and immediately applied to a mono Q Sepharose column. Three peaks were obtained with 50, 150 and 1000 mM buffer sodium borate pH 8.0 respectively. The second peak eluted with 150 mM with fibrinolytic activity was pooled to gel electrophoresis analysis (Figure 2). Microplasm in before purification and Protein fraction of 150 mM sodium borate peak was analyzed with native PAGE. A single band with apparent Mr of 67 Kdal was

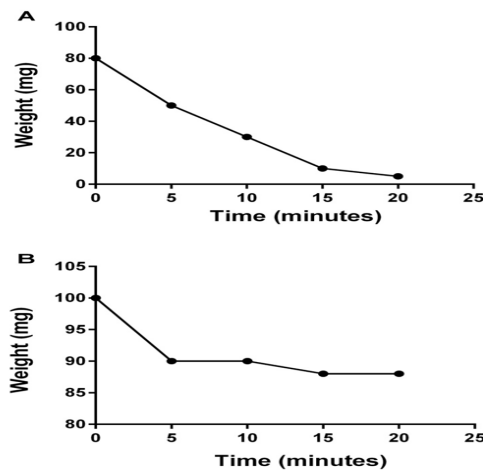
revealed with silver stain (Figure 3). When protein fraction of 150 mM sodium borate was analyzed with SDS PAGE in reduction conditions with DDT and after anionic chromatography, two bands with apparent Mr of 35 and 30 Kdal were revealed with silver stain (Figure 4). After purified microplasm in added fibrin clot showed a decrease in the weight respect control  $35.00 \pm 13.78$  vs  $89.60 \pm 3.19$ ; ( $F = 18.70$ ),  $t = 3.86$ ,  $P < 0.05$  (Figure 5). When determine the effect of microplasm in, the size of the fibrin clot treated was smaller respect to sodium



**Figure 4.** SDS with DTT 10 % polyacrylamide gels of microplasmin. Lanes: 1band of 35 Kdal; 2 and 3band of 30 Kdal (200 µg); 4 sodium borate buffer; 5 band of 30 Kdal (100 µg); 6 molecular weight standard (Ovalbumin: 50 Kdal, Carbonic Anhydrase: 34.3 Kdal, Soybean Trypsin Inhibitor: 28.8 Kdal, Myoglobin: 22 Kdal, Lysozyme: 20.7 Kdal, Aprotinin: 6 Kdal).



**Figure 5.** Weight values of fibrin clots (mg) respect to microplasmin and sodium borate treatment. The values correspond to the mean ± SEM. \* P < 0.05.



**Figure 6.** Lineal correlation between weights of clots (mg) and time (minutes) with microplasmin (Panel A) and control (Panel B).

borate buffer in 25 minutes (Figure 7 a, b, c, d, e).Microplasmin analysis showed a negative lineal correlation significant between the weight of clots and

time,  $r = - 0.97$ ,  $P < 0.05$  (Figure 6 A); Control not showed lineal correlation (Figure 6 B).



**Figure 7.**Changes of size of fibrin clots produced by microplasmin activity (left) and sodium borate buffer as control (right).

## DISCUSSION

Methods have been developing to improving fractionation strategies with human plasma samples, and many different techniques are used and applied until today. A current popular approach is IAD, in which antibodies are used to capture the most abundant proteins. Immunodepletion can facilitate the analysis of the next tier of proteins by eliminating some of the most abundant proteins. Actually, protein precipitation strategies, discriminated by size or size/pI, respectively, have also been widely applied prior to study of protein characterization by electrophoresis(Liu et al., 2016). This

protein fraction has low concentration in human plasma with high stability in pH above 8.0, so the isoelectric precipitation using acetic acid in alkaline buffer is a good strategy to isolate it.

Effective chromatography requires proteins to be in their native state, it is critical to select non-denaturing precipitating agents (e.g., ammonium sulfate, ethanol, and PEG) for compatibility with the downstream depletion step. Ammonium sulfate precipitation has been explored as a method for depleting some highly abundant proteins from blood plasma(Mahn and Ismail, 2011). In our study acetic acid is a weak acid and we used it because does not produce denaturing during the precipitation process.

In this way other advantages is that the method is quickly and safe to obtain the intact structure of protein.

Studies on fibrinolytic proteins showed that the amount of plasminogen retained by lysine-Sepharose decreased and at the same time, the amount of plasminogen that passed through the lysine-Sepharose column unabsorbed gradually increased. Others as active plasmin increased with time of reaction in the unabsorbed fraction from lysine-Sepharose and decreased in the retained fraction and microplasmin was obtained from microplasminogen with a catalytic amount of Urokinase. The microplasmin sample thus obtained consisted of two polypeptide chains as analyzed by HPLC after reduction and carboxymethylation (Shi and Wu, 1988). Scientist obtained microplasmin when incubate recombinant human  $\mu$ PIg with recombinant human rt-PA to used in vitreolysis (Chen et al., 2008). Studies on Over-Expression and Purification of active serine proteases and their variants from *Escherichia coli* Inclusion Bodies refolded microplasminogen protein must be activated to purify it by affinity chromatography. uPA, also a serine protease, cleaves microplasminogen specifically at the Arg15-Val16 bond, leaving a conformational change takes place, leading to an active-site exposure in microplasmin (Parry, 2002). Otherwise with previous authors that suggest that the microplasmin is not bounded to lysine sites on sepharose and therefore is not adsorbed to the column, our funding about of this method using plasma where introduces a cleavage by plasminogen digestion under alkali buffered conditions to produce a protein fraction different to plasmin, and this microplasmin bind to lysine-Sepharose so the lysine-binding site is clearly present in the molecule.

A peptide of Mr 26 Kdal was detected by SDS gelelectrophoresis of the reduced microplasmin. Results of gel filtration and SDS of non-reduced microplasmin showed that the Mr of microplasmin is 29 Kdal. In this way these results are consistent with the conclusion that microplasmin have an intact B chain and a small peptide chain of 31 amino acids from the A chain (Wu et al., 1987). The specific fragmentation of our protein fraction molecule similar to plasmin after precipitation in alkaline pH produced two chains, one of them have fibrinolytic activity and the other we suspected probably have regulation of this activity or the binding site.

A fibrin-specific thrombolytic with Streptokinase and Urokinase showed that tubular plasma clot in vitro decreased its volume of 192 mm<sup>3</sup> to 18 mm<sup>3</sup> after 90 minutes of activity lytic (Viennet et al., 2014). Others studies showed that there was also a significant difference in median clot weight between clot without fibrinolytic agent and with Streptokinase but the median clot weight showed little or no difference when increase the doses post Streptokinase incubation (Doig and Sternberg, 1995). Wu Chen study the fibrinolysis in vitro of novel recombinant  $\mu$ PIg and showed a significant

linear correlation between the diameter squares of lysis zone and fibrinolytic activity (Chen et al., 2016). Clot lysis initiated with microplasmin showed that first period of 5 minutes the 20% lysis of clot was slightly less, but after this time was faster than control. Normally the mechanism of fibrinolysis within the clot happen in a first stage when of plasminogen bind to fibrin lysine sites and in a second stage conversion of plasminogen into plasmin with subsequent lysis of the clot, contrary the microplasmin does not need to activate or bind to specific sites, therefore the times for lysis of clots decrease.

## ACKNOWLEDGEMENTS

To all staff of chemistry faculty, Córdoba Catholic University we really appreciate all your efforts.

## Conflict of interest

The authors have not conflict of interest.

## REFERENCES

- Chen W, Huang X, Ma XW, Mo W, Wang WJ, Song HY (2008). Enzymatic vitreolysis with recombinant microplasminogen and tissue plasminogen activator. *Eye (Lond)*.22 (2): 300-307
- Chen W, Li Y, Chen P, Wu M, Wang L, Zhang H, Wang L (2016). In vitro fibrinolysis and antithrombosis characterizations of novel recombinant microplasminogen with RGD and GPRP peptides. *J Thromb Thrombolysis*.42 (1): 118-126
- Doig A J, Sternberg MJ (1995). Side-chain conformational entropy in protein folding. *Protein Sci*.4 (11): 2247-2251
- Kim D W, Choi JH, Park S E, Kim S, Sapkota K, Kim S J (2015). Purification and characterization of a fibrinolytic enzyme from *Petasites japonicus*. *Int J Biol Macromol*.72 1159-1167
- Liu R, Zhao B, Zhang Y, Gu J, Yu M, Song H, Yu M, Mo W (2015). High-level expression, purification, and enzymatic characterization of truncated human plasminogen (Lys531-Asn791) in the methylotrophic yeast *Pichia pastoris*. *BMC Biotechnol*.15 (50): 50
- Liu Z, Fan S, Liu H, Yu J, Qiao R, Zhou M, Yang Y, Zhou J, Xie P (2016). Enhanced Detection of Low-Abundance Human Plasma Proteins by Integrating Polyethylene Glycol Fractionation and Immunoaffinity Depletion. *PLoS One*.11 (11): e0166306
- Mahn A, Ismail M (2011). Depletion of highly abundant proteins in blood plasma by ammonium sulfate precipitation for 2D-PAGE analysis. *J Chromatogr B Analyt Technol Biomed Life Sci*.879 (30): 3645-3648
- Nagai N, Demarsin E, Van Hoef B, Wouters S, Cingolani D, Laroche Y, Collen D (2003). Recombinant human microplasmin: production and potential therapeutic properties. *J Thromb Haemost*.1 (2): 307-313
- Parry MA (2002). Over-expression and purification of active serine proteases and their variants from *Escherichia coli* inclusion bodies. *Curr Protoc Protein Sci*.Chapter 21 (21): Unit 21 11
- Phan TT, Ta TD, Nguyen DT, Van Den Broek LA, Duong G T (2011). Purification and characterization of novel fibrinolytic proteases as potential antithrombotic agents from earthworm *Perionyx excavatus*. *AMB Express*.1 (1): 26
- Shi GY, Wu H L (1988). Isolation and characterization of microplasminogen. A low molecular weight form of plasminogen. *J Biol Chem*.263 (32): 17071-17075
- Viennet C, Laurensou C, Goydadin AC, Favier B, Muret P, Humbert P (2014). Development of an in vitro fibrin clot model to evaluate

fibrinolytic agents for wound care application. J Wound Care.23 (2): 66-67, 70, 72

Wu H L, Shi G Y,Wohl R C, Bender M L(1987). Structure and formation of microplasmin. Proc Natl Acad Sci U S A.84 (24): 8793-8795

## **ABBREVIATIONS**

**IAD:** immunoaffinity depletion.

**MI:** myocardium infarction.

**Mr:** molecular weight.

**PAGE:** polyacrylamide gel electrophoresis.

**PEG:** polyethylene glycol.

**PIg:** plasminogen.

**rt-PA:** recombinant tissue plasminogen activator.

**SDS:** sodium dodecyl sulfate.

**uPA:** Urokinase plasminogenactivator.

**μPIg:** microplasminogen.

**DTT:** Dithiothreitol