PAPP-A from human atherosclerotic plaques is an active enzyme that cleaves IGFBP-4

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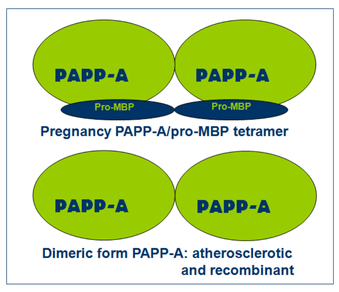
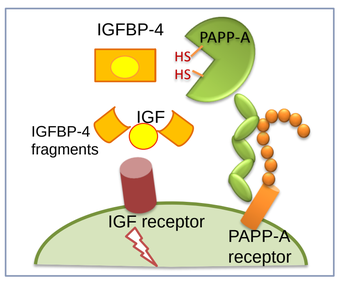
Introduction:

Pregnancy Associated Plasma Protein-A (PAPP-A) is a high-molecular-weight glycoprotein originally isolated from human pregnancy serum. PAPP-A is a member of metalloproteinase metzincin superfamily and exists in two isoforms: **heterotetrameric** 2:2 complex **PAPP-A/proMBP (htPAPP-A)** found in the blood of pregnant women and **homodimeric** form **(dPAPP-A)** that was recently found in unstable atherosclerotic plaques. It was shown that dPAPP-A is produced by activated cells of the immune system in unstable plaques and is released into the extracellular matrix and circulation.





Recent studies indicate that increased serum level of dPAPP-A is associated with development of acute coronary syndrome. Thus dPAPP-A was suggested as a marker of some cardiovascular diseases, such as unstable angina, myocardial infarction. PAPP-A is a metalloproteinase specific to IGF-binding protein-4. Cleavage of IGFBP-4 leads to release of free IGF and activation of IGF-dependent processes. It is known that proMBP is a unique and specific inhibitor of PAPP-A protease activity that acts through formation of disulfide bonds with PAPP-A in its active centre. Thus, htPAPP-A does not display protease activity.

It is still unknown whether atherosclerotic dPAPP-A is active protease, but it was shown that **recombinant dPAPP-A is an active enzyme** and cleaves IGFBP-4 in vitro. It was hypothesized that dPAPP-A in atherosclerotic plaques is an active enzyme cleaving IGFBPs complexed with IGF-1, thus releasing free IGF-1. Local growth of IGF-1 levels promotes weakening of the fibrous cap and plaque destabilization.

The aim of the current study was to investigate biochemical and enzymatic properties of dPAPP-A from human atherosclerotic plaques.

Materials and Methods:

Tissue dPAPP-A extraction: Samples of human atherosclerotic coronary vessels were stored at -70 C until used. Tissue form of dPAPP-A was extracted from atherosclerotic coronary arteries in 50 mM tris-HCl buffer, pH=7.5 in the presence of protease inhibitors and then it was purified by affinity chromatography using immobilized **MAb 4G11**. This matrix was used for purification of both tissue and recombinant dPAPP-A.

Recombinant dPAPP-A: Full-lenth PAPP-A expression plasmid was constructed by **Hytest LTD.** HEK 293 cells were transfected by the plasmid. The conditioned media from transfected cells were pooled and stored at -70 C.

Protein analysis: dPAPP-A from atherosclerotic coronary arteries (tissue dPAPP-A), recombinant dPAPP, and PAPP-A from human pregnancy serum were analyzed by **SDS-PAGE** in reducing conditions.

These proteins were also analyzed by **Western blotting** with several PAPP-A-specific mAbs: **18A10, 7A6 and 3C8**, and by liquid chromatography/tandem mass spectrometry analysis (**MS-MS**).

Sandwich immunofluorescent assay (IFA): For the pairs design we used MAbs obtained from **HyTest LTD** (Finland) with different specificity: MAbs ***10A5, 7A6*** specific to PAPP-A subunit; MAb ***5H9*** specific to proMBP, ***PAPP2*** specific to dPAPP-A.

Using this antibodies we have obtained 3 types of in-house assays:

1) specific to htPAPP-A (PAPP-A/proMBP): 5H9 – 7A6;
2) react with both htPAPP-A (PAPP-A/proMBP) and dPAPP-A proteins: 10A5 – 7A6;
3) Specific to dPAPP-A only: PAPP2 – 7A6.

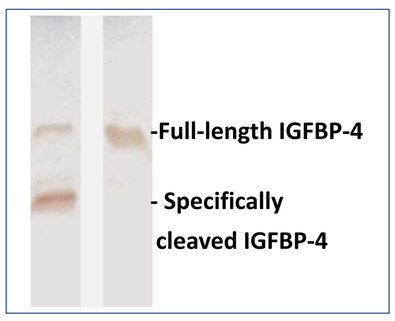


Figure 1 – Western blotting detection of proteolytic fragments of IGFBP-4 demonstrating recPAPP-A protease activity.
IGFBP-4 was treated with:
Lane 1, recombinant dPAPP-A;
Lane 2, without PAPP-A

We used detection antibodies labeled with Eu3+-chelate. The fluorescence was measured by Victor 1420 multilabel counter (Wallac-PerkinElmer, Finland).

Measurements of PAPP-A proteolytic activity:

Different types of PAPP-A was incubated at 37 C with recombinant IGFBP-4 contained in 50 mM Tris, 100 mM NaCl, 2 mM CaCl2, pH=7,5. Incubations were carried out in the presence of IGF-2. The samples after 13-15 hours of incubation were separated by reducing 15% SDS-PAGE. The degree of cleavage was detected by Western Blotting with anti-IGFBP-4 rabbit PAbs (Figure 1).

Results and Discussion:

We have elaborated a method of dPAPP-A isolation from human atherosclerotic tissue using affinity chromatography utilizing MAb 4G11 (HyTest LTD, Finland). Using SDS-PAGE analysis we have shown that the major band of protein with molecular mass about 200 kDa was PAPP-A subunit (Figure 2). We have suggested that purity of PAPP-A preparation was about 80%.

Comparing this endogenous protein with recombinant dPAPP-A and htPAPP-A, we have shown that in all cases PAPP-A subunit had equal apparent molecular masses about **200 kDa** being analyzed by SDS-PAGE. Identity of the proteins was confirmed by mass spectrometry analysis.

The analysis of proMBP content by Western blotting showed the **absence of proMBP** in tissue dPAPP-A (data not shown). In this experiment we used MAbs specific to PAPP-A subunit (18A10, 3C8, 7A6) or MAbs specific to proMBP (11E4, 5H9).

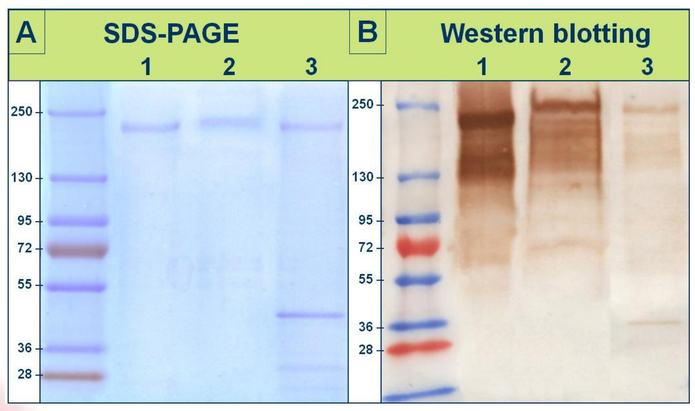


Figure 2 A – Reducing SDS-PAGE analysis tissue of PAPP-A purified from atherosclerotic arteries. Protein bands were visualized by Coomassie R250 staining.
Lane 1, heterotetrameric form of PAPP-A (PAPP-A/proMBP);
Lane 2, recombinant dPAPP-A;
Lane 3, tissue dPAPP-A.
ProMBP released from heterotetrameric PAPP-A upon reduction is not visible due to its high glycosylation.
B – Western blotting analysis of PAPP-A purified from atherosclerotic tissue. For immunostaining we used mixture of anti-PAPP-A MAbs (18A10, 7A6 and 3C8). Lines are noted as in Fig.2 A.

Sandwich type immunoassay ***5H9 - 7A6-Eu*** enabled us to demonstrate the absence of considerable amounts of heterotetrameric (PAPP-A/proMBP) complex in the PAPP-A preparation from atherosclerotic tissue as well as in the recombinant PAPP-A (Figure 3).

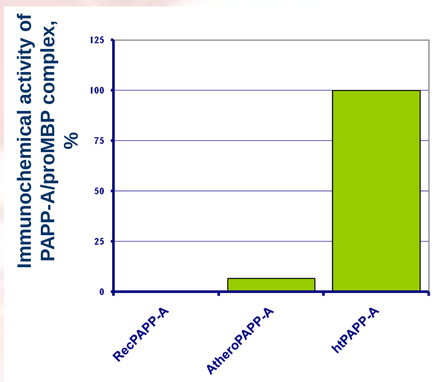


Figure 3 – Comparison of immunochemical activities of heterotetrameric PAPP-A (PAPP-A/proMBP) estimated by assay ***5H9 – 7A6*** in atherosclerotic tissue form of PAPP-A, recombinant PAPP-A and pregnancy form of PAPP-A (htPAPP-A)**.**

Concentration of total PAPP-A measured by reference assay ***10E1 – 7A6*** was accepted as 100%

It was hypothesized that if dPAPP-A from atherosclerotic tissue was uncomplexed with proMBP, dPAPP would be an active protease. For detection of proteolytic activity recombinant IGFBP-4 was treated by different types of PAPP-A and detected by Western blotting analysis.

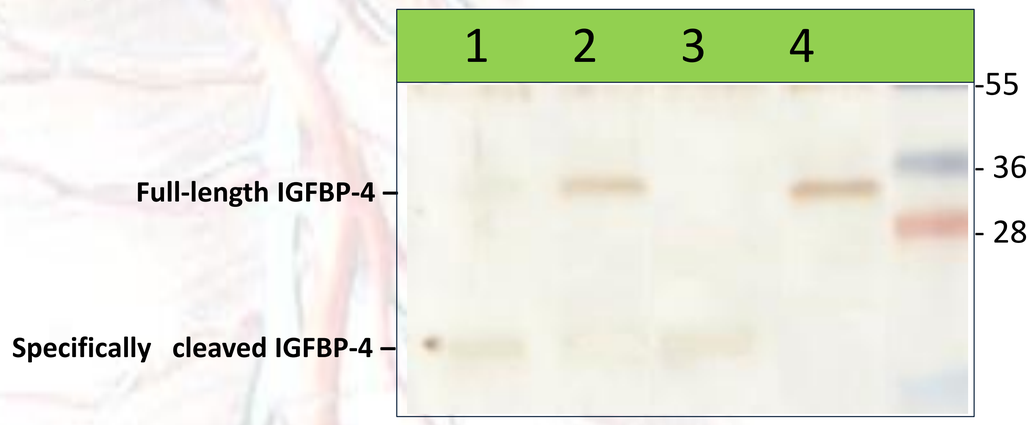


Figure 4 – Western blotting detection of proteolytic fragments of IGFBP-4 demonstrating PAPP-A protease activity. Rabbit anti-IGFBP-4 PAbs were used immunostaining.
IGFBP-4 was treated by:
Lane 1, recombinant dPAPP-A;
Lane 2, heterotetrameric form of PAPP-A (PAPP-A/proMBP);
Lane 3, atherosclerotic tissue dPAPP-A.
Lane 4, without PAPP-A

For the first time we have shown that endogenous dPAPP-A is an active protease that cleaves IGFBP-4 in the presence of IGF-1 in vitro (Figure 4) . Our findings support the hypothesis that enzymaticaly active dPAPP-A can participate in atherosclerotic plaque destabilization and rupture.

Conclusions:

1. **We have developed a method of dPAPP-A purification.**
2. **We have characterized dPAPP-A from human atherosclerotic tissue by SDS-PAGE.**
3. **We have shown the absence of proMBP in PAPP-A from human atherosclerotic tissue.**
4. **We have shown that dPAPP-A from human atherosclerotic tissue is an active enzyme that cleaves IGFBP-4.**

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