In vitro degradation of angiotensin II (A-II) by human placental subcellular fractions, pregnancy sera and purified placental aminopeptidases

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Abstract. The degradation of angiotensin II (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8: A-II) by human placental particulate and soluble fractions, pregnant and non-pregnant sera, and highly purified placental enzymes such as placental leucine aminopeptidase P-LAP (microsomal), retroplacental serum P-LAP (soluble), aminopeptidase A and post-proline endopeptidase, was studied by measuring liberated amino acids by high performance liquid chromatography. Placental particulate and soluble fractions degraded A-II almost completely into single amino acids. The purified P-LAP (microsomal) actively liberated five amino acids from the N-terminal. The placental particulate fraction containing P-LAP (microsomal) also actively liberated these amino acids. The purified aminopeptidase A liberated Asp^1 very actively as expected. When the ratio of the velocity of liberation of each amino acid to P-LAP activity measured with leu-p-nitroanilide as a substrate was calculated, placental soluble fraction liberated Asp^1 very actively, but the liberation rate of Asp^1 with the purified P-LAP (soluble) was very low. Therefore it seems that the enzyme in the placental soluble fraction and pregnancy serum responsible for the Asp^1 liberation is not P-LAP (soluble), but aminopeptidase A. The mixture of purified P-LAP (soluble) and aminopeptidase A showed higher liberation rate of Arg^2 and Val^3 than that with purified aminopeptidase A alone, demonstrating that once the N-terminal Asp^1 was liberated, the P-LAP (soluble) attacks the shorter peptide (angiotension III) very actively. It was concluded that P-LAP (microsomal) together with aminopeptidase A seem to contribute greatly to the degradation of A-II in pregnant women.

The concentration of angiotensin II (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8: A-II) in the serum of pregnant women is higher than that in non-pregnant women (Weir et al. 1973). The highest concentration of the hormone can be found in arterial cord blood (Vallotton et al. 1976). The higher concentration of A-II in arterial cord blood than in venous cord blood suggests that A-II is generated mainly in the foetus itself and partly destroyed within the placenta. It is easy to infer that the inactivation of A-II is also enhanced during pregnancy under the conditions of increased A-II production.

Despite studies on the metabolism of A-II in rat tissues (Regoli et al. 1968; Tonnaer et al. 1983), little is known about its metabolism in human placenta and pregnancy serum. In addition the enzyme responsible for the degradation of A-II during pregnancy has not been exactly identified. The present paper deals with the proteolytic degradation of A-II into amino acids by subcellular fractions prepared from human placenta, pregnancy serum, and aminopeptidases highly purified...
from human placenta and retroplacental serum in an attempt to identify enzymes responsible for A-II degradation in pregnant women.

Materials and Methods

Normal non-pregnant and pregnant sera were obtained at the Nagoya university Hospital. Synthetic A-II was obtained from the Protein Research Foundation, Osaka, Japan. All other chemicals for high performance liquid chromatography (HPLC) were of analytical grade commercially available.

**Tissue preparation**

Human placenta (25 g) obtained after normal delivery was immediately rinsed gently with chilled saline. The placental tissue was rapidly dissected and homogenized in 10 vol of ice-cold 0.05 M sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl and 0.25 M sucrose, using a Polytron homogenizer at setting 5. After centrifugation at 4°C (600 × g for 10 min) the pellet obtained was discarded and the supernatant (190 ml) was centrifuged (10 000 × g for 30 min). The supernatant obtained was centrifuged again at 105 000 × g for 60 min. The resulting supernatant and pellet were used as the soluble fraction and the particulate fraction, respectively.

**Purification of enzymes**

P-LAP (soluble), P-LAP (microsomal), aminopeptidase A and post-proline endopeptidase were purified according to our methods described elsewhere (Sakura et al. 1981; Mizutani et al. 1981, 1982, 1984).

**Assay**

Enzyme activities of P-LAP, aminopeptidase A and post-proline endopeptidase were measured according to our methods (Mizutani et al. 1976, 1981, 1984). Protein was determined according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

**Incubations**

Each crude and purified enzyme was incubated with 130.8 nmol of A-II at 37°C in 300 μl of 50 mM sodium-phosphate buffer (pH 7.5) containing 150 mM NaCl. At various time intervals following the start of incubation, 30 μl sample of the incubation medium was taken out and the reaction was stopped by adding 30 μl of ethanol. Protein concentrations used in experiments are shown in Table 1.

**Determination of amino acids by HPLC**

A LC-4A pump, a fluorometric detector type RF-530, and a Chromatopac C-R 2A recorder (Shimazu Co. Ltd., Kyoto, Japan) were used. A packed column with 7.5 μm strongly acidic cation-exchange resin of styrene-divinyl

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Protein concentration in incubation mixture (µg/300 µl)</th>
<th>P-LAP¹ (nmol/min/mg protein)</th>
<th>Aminopeptidase A² (nmol/min/mg protein)</th>
<th>Post-proline³ endopeptidase (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzymes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placental particulate fraction</td>
<td>680</td>
<td>10.0 (680)*</td>
<td>5.5 (3740)*</td>
<td></td>
</tr>
<tr>
<td>Placental soluble fraction</td>
<td>268</td>
<td>5.48 (1469)</td>
<td>0.88 (236)*</td>
<td></td>
</tr>
<tr>
<td>Non-pregnant serum</td>
<td>2720</td>
<td>0.262 (713)</td>
<td>0.15 (408)*</td>
<td></td>
</tr>
<tr>
<td>Pregnant serum at term</td>
<td>2700</td>
<td>3.274 (8840)</td>
<td>0.73 (1971)</td>
<td></td>
</tr>
<tr>
<td>Purified enzymes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-LAP (microsomal)</td>
<td>0.8</td>
<td>11 150 (8920)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Placental aminopeptidase A</td>
<td>0.6</td>
<td>–</td>
<td>12 600 (7560)</td>
<td>–</td>
</tr>
<tr>
<td>P-LAP (soluble)</td>
<td>20</td>
<td>7000 (140 000)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Placental post-proline endopeptidase</td>
<td>8</td>
<td>–</td>
<td>1760 (14 080)*</td>
<td></td>
</tr>
</tbody>
</table>

¹ P-LAP activity was measured with 0.8 mM of leu-p-nitroanilide in the presence of 0.02 M l-methionine.
² Aminopeptidase A activity was measured with 5.5 mM of Asp-β-naphthylamide in the presence of 10 mM CaCl.
³ Post-proline endopeptidase activity was measured with 0.1 mM of succinyl-gly-pro-methylcoumarineamide.
* Total activity present in incubation mixture (pmol/min) in parenthesis.
benzen copolymer (4 × 15 cm I.D., Shimazu Co. Ltd., Kyoto, Japan) was used. Chromatography was performed essentially according to the method of Ishida et al. (1981). Since each crude enzyme liberated some constituent amino acids of A-II, amounts of these amino acids were subtracted from the quantities of identified amino acids in each experiment.

Results

Table 2 shows the velocity of amino acid liberation from A-II by the crude preparations and highly purified enzymes. Placental particulate and soluble fraction almost completely degraded A-II into single amino acids. The placental soluble fraction liberated Asp\(^1\) more actively than other amino acids. A-II degrading activity in pregnancy serum was higher than that in non-pregnancy serum.

Purified P-LAP (microsomal) liberated six amino acids actively except His\(^6\) and Pro\(^7\). Purified aminopeptidase A liberated Asp\(^1\) most actively, followed by Arg\(^2\) and Val\(^3\). Purified P-LAP (soluble) liberated five amino acids from the N-terminal, but their liberation velocities were very low. Post-proline endopeptidase cleaved the C-terminal Phe\(^8\) specifically.

Since it is likely that after N-terminal Asp\(^1\) is eliminated, the shorter peptide chain may be attacked by P-LAP (soluble), the mixture was incubated with aminopeptidase A alone, with P-LAP (soluble) alone and with the combination of the two enzymes as shown in Fig. 1. The mixture of purified aminopeptidase A and P-LAP (soluble) showed higher liberation rates of Arg\(^1\) and Val\(^3\) than that with the purified aminopeptidase A alone.

Since the velocity of amino acid liberation by enzymes depends upon the activity of enzyme preparation, we have tried to calculate the velocity of liberation of each amino acid to P-LAP activity measured with leu-p-nitroanilide as a substrate (Table 3). The ratio for Asp\(^1\) was high with the placental particulate fraction, placental soluble fraction and purified P-LAP (microsomal), whereas it was low with the purified P-LAP (soluble).

Discussion

Placental particulate and soluble fractions almost completely degraded A-II into single amino acids, although the velocity of each amino acid liberation was different (Table 2). Tonnaer et al. (1983) showed that A-II was rapidly metabolized by rat brain synaptosomal soluble fraction, but not by membrane fraction.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Velocity of amino acid liberation (pmol/min/mg protein)</th>
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<tbody>
<tr>
<td></td>
<td>Asp(^1)</td>
</tr>
<tr>
<td>Crude enzymes</td>
<td></td>
</tr>
<tr>
<td>Placental particulate fraction</td>
<td>4720</td>
</tr>
<tr>
<td>Placental soluble fraction</td>
<td>4110</td>
</tr>
<tr>
<td>Non-pregnant serum</td>
<td>16</td>
</tr>
<tr>
<td>Pregnant serum at term</td>
<td>50</td>
</tr>
<tr>
<td>Purified enzymes</td>
<td></td>
</tr>
<tr>
<td>P-LAP (microsomal)</td>
<td>2.10 × 10⁶</td>
</tr>
<tr>
<td>Placental aminopeptidase A</td>
<td>2.78 × 10⁵</td>
</tr>
<tr>
<td>P-LAP (soluble)</td>
<td>1081</td>
</tr>
<tr>
<td>Placental post-proline endopeptidase</td>
<td>0</td>
</tr>
</tbody>
</table>

* The velocity was calculated from 4–5 plots checking the linear increase in each amino acid.
We previously showed that aminopeptidase A is present in human placenta and increased in pregnancy sera (Mizutani et al. 1981; Sakura et al. 1983). It is not surprising that aminopeptidase A cleaves the N-terminal Asp\textsuperscript{1}, but unexpectedly it also cleaves Arg\textsuperscript{2} and Val\textsuperscript{3} (Table 2).

Purified P-LAP (microsomal) competes with purified aminopeptidase A for Asp\textsuperscript{1} liberation velocity (Table 2). Purified P-LAP (microsomal) had the highest activity in degrading A-II among the purified enzymes tested (Table 2). It liberated six amino acids except His\textsuperscript{6} and Pro\textsuperscript{7} at almost the same rate. This pattern of amino acid liberation was very similar to that of the placental particulate fraction. The similar ratio patterns for the particulate fraction and P-LAP (microsomal) to P-LAP activities measured with leu-p-nitroanilide as a substrate suggest that the particulate fraction contains a large amount of this enzyme (Table 3).

A-II degrading activity with P-LAP (soluble) purified from retroplacental serum was low (Table 2). Purified P-LAP (soluble) gave a lower value than placental soluble fraction and pregnancy serum (Table 3). This result suggests that the high amino acid liberating activity found in the soluble fraction is not due to this enzyme, but probably due to aminopeptidase A (Table 2).

Our study showed that the purified post-proline endopeptidase specifically hydrolyzes the C-terminal Phe\textsuperscript{8} (Table 2). Recently, we showed that human placental post-proline endopeptidase is localized in the cytosol fraction (Mizutani et al.)

\[ \begin{align*}
\text{Table 3.} \\
\text{Ratio of amino acid liberation velocity of P-LAP activity measured with leu-p-nitroanilide.} \\
\end{align*} \]
1984). Therefore, the Phe\textsuperscript{8} liberation observed with the placental soluble fraction may be at least partly due to this enzyme (Table 2).

Taking into consideration the human placental weight (about 500 g) and total serum volume of full term pregnancy women (about 3 litre), we can compare the total A-II degrading activity in serum with that in the placenta. A-II degrading activity in a whole placenta was calculated from the data with the crude preparations (Table 2) to be more than 10 times as high as that in whole pregnant serum (42 times calculated for Asp\textsuperscript{1} liberation; 14 times for Arg\textsuperscript{2}; 15 times for Val\textsuperscript{3}; 79 times for Tyr\textsuperscript{4}; 73 times for Ile\textsuperscript{5} and 74 times for Phe\textsuperscript{8}). Therefore A-II inactivation seems to be achieved mainly in the placenta in pregnant women.

Our present study suggests that the A-II degrading activity found in the placental particulate fraction is mainly due to P-LAP (microsomal), and the activities in the placental soluble fraction and pregnant serum are mainly due to aminopeptidase A. Once the N-terminal Asp\textsuperscript{1} is liberated, it appears that P-LAP (soluble) attacks the shorter peptide (angiotensin III) actively. These placental enzymes may regulate the A-II concentration in both maternal and foetal sera.

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References


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