Optimized Protocol for Amyloid-β Extraction from the Brain

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Abstract. Brain levels of amyloid-β (Aβ) peptides are frequently assessed in transgenic mouse models of Alzheimer’s disease (AD). Currently, a considerable number of sequential Aβ extraction protocols exist [1–5], which differ mainly in the buffers used for homogenization and the number of extraction steps. In general, the procedure involves, at each step, homogenization of the tissue sample in an appropriate buffer followed by centrifugation, removal of supernatant, and re-homogenization of the pellet in other buffer used in the next step of the sequential process. However, only one previous attempt has been made to ascertain if these procedures are able to extract the whole amount of Aβ peptides present in these different fractions obtained from a brain tissue sample [6]. Indeed, it has been shown that the relative amounts of Aβ extracted from transgenic mouse and human brains are influenced by the extraction protocol [2].

Brain levels of amyloid-β (Aβ) peptides are frequently assessed in transgenic mouse models of Alzheimer’s disease (AD). Currently, a considerable number of sequential Aβ extraction protocols exist [1–5], which differ mainly in the buffers used for homogenization and the number of extraction steps. In general, the procedure involves, at each step, homogenization of the tissue sample in an appropriate buffer followed by centrifugation, removal of supernatant, and re-homogenization of the pellet in other buffer used in the next step of the sequential process. However, only one previous attempt has been made to ascertain if these procedures are able to extract the whole amount of Aβ peptides present in these different fractions obtained from a brain tissue sample [6]. Indeed, it has been shown that the relative amounts of Aβ extracted from transgenic mouse and human brains are influenced by the extraction protocol [2].

The present project was prompted by the finding that increasing the volume of homogenization buffer in our former extraction protocol produced substantial increments in the amount of peptide extracted from a given sample. This finding suggested that the extraction capacity of the buffers, at the usual 10% weight/volume proportion, could become saturated, leading to a dramatic underestimation of the real amount of Aβ peptide present in the sample. It could imply large differences in the estimations of total Aβ levels determined on identical samples depending on the protocol used and cause an apparent lack of effects for potential therapies aimed to reduce brain amyloid burden. In this context, several studies have reported reductions in the immunohistochemical amyloid loads without simultaneous changes in the biochemical loads measured by ELISA [7–11]. For these reasons, additional work on this issue was necessary.

Thus, we hypothesized that greater volumes of buffer for homogenization and eventual repetition of each extraction step could result in a more complete and accurate extraction of Aβ from brain tissue. Therefore, we carried out two series of experiments to

Keywords: Alzheimer’s disease, amyloid-β peptides, animal models, ELISA, tissue extraction, tissue homogenization

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Biotech, Zaragoza, Spain), were used as detection anti-
N-terminal monoclonal antibody was used as cap-
tion) were collected. Concentrations of brain Aβ in
formic acid; the resultant supernatants (insoluble frac-
tion) were re-suspending the remaining pellets in 70%
extracted re-suspending the remaining pellets in 70%
size” (ES) was calculated, as the mean difference in

Due to the low n, no attempt was made to calcu-
late the p value for the comparisons between different
extraction volumes. Only the statistical variable “effect
size” (ES) was calculated, as the mean difference in

Data are expressed as mean ± SEM. n = 3 mice per volume group.

Table 1

<table>
<thead>
<tr>
<th>Volumes (mL)</th>
<th>Aβ40</th>
<th>Aβ42</th>
<th>TBS (pg)</th>
<th>TX (pg)</th>
<th>AF (pg)</th>
<th>Total brain (pg/mL) TBS (pg/mL)</th>
<th>TX (pg/mL)</th>
<th>AF (pg/mL)</th>
<th>Total concentration (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>10.3 ± 1.8</td>
<td>10.2 ± 3.5</td>
<td>192.4 ± 18.05</td>
<td>212.9</td>
<td>17.1 ± 2.2</td>
<td>17.0 ± 6.1</td>
<td>321.0 ± 30.1</td>
<td>354.8</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>28.0 ± 9.50</td>
<td>9.7 ± 1.50</td>
<td>160.0 ± 14.33</td>
<td>197.7</td>
<td>46.7 ± 6.3</td>
<td>56.1 ± 2.5</td>
<td>240.0 ± 23.9</td>
<td>320.0</td>
<td></td>
</tr>
<tr>
<td>1.8 ± 1.47</td>
<td>33.6 ± 2.17</td>
<td>299.8 ± 4.48</td>
<td>352.2</td>
<td>21.8 ± 1.5</td>
<td>33.6 ± 2.2</td>
<td>300.0 ± 4.9</td>
<td>355.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>41.2 ± 5.00</td>
<td>17.2 ± 2.63</td>
<td>289.9 ± 32.30</td>
<td>349.1</td>
<td>42.5 ± 1.5</td>
<td>17.2 ± 2.6</td>
<td>250.0 ± 32.3</td>
<td>349.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>72.6 ± 1.09</td>
<td>115.1 ± 19.06</td>
<td>392.2 ± 22.20</td>
<td>357.9</td>
<td>36.3 ± 0.5</td>
<td>57.6 ± 5.5</td>
<td>196.0 ± 11.1</td>
<td>290.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>88.7 ± 5.5</td>
<td>38.7 ± 3.58</td>
<td>377.2 ± 58.9</td>
<td>504.6</td>
<td>44.4 ± 2.8</td>
<td>19.4 ± 1.8</td>
<td>188.0 ± 29.1</td>
<td>252.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>117.9 ± 6.25</td>
<td>203.6 ± 31.5</td>
<td>364.1 ± 1.85</td>
<td>617.4</td>
<td>17.4 ± 0.2</td>
<td>50.0 ± 0.9</td>
<td>159.0 ± 3.8</td>
<td>230.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>206.9 ± 18.4</td>
<td>99.7 ± 9.00</td>
<td>614.4 ± 15.0</td>
<td>921.0</td>
<td>51.7 ± 4.6</td>
<td>24.9 ± 2.3</td>
<td>154.0 ± 3.9</td>
<td>230.3</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>96.8 ± 11.95</td>
<td>260.7 ± 29.34</td>
<td>340.6 ± 41.65</td>
<td>698.1</td>
<td>16.1 ± 2.0</td>
<td>43.4 ± 4.9</td>
<td>56.8 ± 7.0</td>
<td>116.4</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>328 ± 12.8</td>
<td>158.4 ± 33.55</td>
<td>1285.7 ± 248.05</td>
<td>1772.9</td>
<td>54.8 ± 2.1</td>
<td>28.4 ± 6.0</td>
<td>214.0 ± 4.1</td>
<td>295.5</td>
<td></td>
</tr>
</tbody>
</table>

The amount of Aβ peptides in the supernatants pro-
duced by the three-step (TBS, TX, and FA fractions) 
extraction procedure using different buffer volumes 
are summarized in the Table 1. The total ELISA measur-
able brain Aβ levels were higher as brain tissues were 
and 6 mL. Aβ peptides were sequentially extracted from 
frozen hemi-brains (159.97 ± 10.03 mg) in a three-step protocol. Brains were 
were homogenized in TBS buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing a cocktail 
of protease inhibitors (Complete, Roche Diagnostics). Homogenates were centrifuged (175,000 × g, 30 min, 
and supernatants (soluble fraction) were col-
lected. The pellets were then re-homogenized in 
TBS plus 1% Triton X-100, centrifuged as above, 
and the resultant supernatants (membrane-bound frac-
tion) were collected. Insoluble, plaque-bound Aβ was extracted re-suspending the remaining pellets in 70%
formic acid; the resultant supernatants (insoluble frac-
tion) were collected. Concentrations of brain Aβ peptides were quantified using a β-amyloid ELISA kit (ABtest Kit, Aracon Biotech, Zaragoza, Spain) as described elsewhere [12]. In brief, an anti-Aβ N-terminal monoclonal antibody was used as cap-
ture antibody and two highly specific anti C-terminal polyclonal antibody, pAB002 and pAB031 (Aroncon Biotech, Zaragoza, Spain), were used as detection anti-
body for Aβ40 and Aβ42. The concentrations of Aβ40 and Aβ42 were calculated using standard curves for 
both peptides by comparing the sample’s absorbance with the absorbance of known standard concentrations. 

In the first experiment, we used three 8-month-
old Tg2576 transgenic mice per assayed extraction volume of 0.6, 1, 2, 4, and 6 mL. Aβ peptides 
were sequentially extracted from frozen hemi-brains 
were differences between the two Aβ peptides. Thus, 
while Aβ42 levels (in pg) were higher as brain tissues were 
homogenized in higher buffer volumes. In particular, 
about a 6-fold higher level (in pg) in total Aβ40 + Aβ42 
ocurred in the higher extraction volume (6 mL) when 
compared with the lower one (0.6 mL). In statistical 
results, the ES of the homogenization volume on brain 
Aβ levels between these two extraction volume groups 
was 9.9. However, it is worth mentioning that there 
were differences between the two Aβ peptides. Thus, 
while Aβ42 levels (in pg) were higher as brain tissues were 
homogenized in higher volumes (ES: 0.6 versus 1 mL, 5.42; 1 versus 2 mL, 3.37; 2 versus 4 mL, 9.59; 
and 4 versus 6 mL, 5.3), Aβ40 levels were only higher in 
the homogenization volume of 2 mL, from which 
peptide levels were much smaller (ES: 0.6 versus 1 mL, 
6.21; 1 versus 2 mL, 4.13; 2 versus 4 mL, 0.9; and 
4 versus 6 mL, 1.5).

We must emphasize that the concentration of Aβ42 (in pg/mL) remained approximately the same for all 
extraction volumes tested, which strongly suggests 
a saturation of the buffer solution. The concentra-
tion of Aβ40 in TBS and TX fractions were also 
approximately the same for all the extraction volumes. However, the concentration of Aβ40 in the FA fraction 
was substantially lower in the 4 mL volume, suggesting 
that a near complete extraction of the insoluble Aβ40 was 
achieved with 2 mL (Table 1). 

These results suggested that homogenization of 
brains in a low volume of buffer was inadequate and 
led to dramatic underestimation of brain Aβ levels. It
was apparent from our results (Table 1) that the use of higher volumes of homogenization buffer resulted in the measurement of higher amounts of brain Aβ. However, as a plateau was not reached for the extracted peptide with any volumes used in this experiment, it could not be assured that a complete extraction was achieved by homogenizing the brain tissue in 6 mL of homogenization buffer, particularly for Aβ42. Then, a complementary approach was necessary to achieve a more complete peptide extraction since the use of extraction buffer volumes over 6 mL is unadvised for technical reasons. Thus, in our second series of experiments we repeated each extraction step five times with the same buffer before going onto the next step in the extraction protocol.

For this experiment, we used another three 8-month-old Tg2576 mice. Brain tissues were homogenized in 6 mL of TBS and centrifuged as mentioned before. The supernatants were removed (fraction TBS1) and the pellets re-homogenized again in 6 mL of the same TBS...
to obtain the supernatant (fraction TBS2) and so on up to obtain five TBS fractions from each brain. The same procedure was followed with TX and FA buffers to obtain five TX fractions and five FA fractions from each brain. All supernatants were assayed for Aβ.

Results are shown in Fig. 1. They indicate that substantial additional soluble and membrane-bound Aβ was extracted from the first three fractions obtained by re-homogenizing the remaining pellets in the same buffer. The levels of soluble and membrane-bound Aβ in the fourth and fifth fractions were below the limit of quantification by the ELISA method and were considered negligible. Insoluble Aβ required only one homogenization step with FA to be practically entirely extracted.

This “extra” soluble Aβ extracted in the successive repetitions with the same buffer is unlikely to come from the membrane-bound Aβ since quantification of the TX1 fraction resulted in Aβ levels similar to those obtained in the control protocol, in which the five previous re-homogenizations in TBS were not performed (TX-control, Fig. 1). The same held for the “extra” membrane-bound Aβ, which is unlikely to come from insoluble Aβ (FA1 fraction) as levels obtained in the first repetition were also very similar to those obtained with the control protocol (FA1 versus FA control, Fig. 1). Moreover, these results support the idea that the extraction capacity of the TBS and TX buffers becomes saturated in the former protocol, leading to a dramatic underestimation of brain peptide content. In addition, it seemed reasonable to assume that with the control protocol, the TX and FA fractions could contain considerable soluble and membrane-bound Aβ, respectively, which had not been extracted in the previous step. This could additionally lead to a misinterpretation of the relative distribution of peptide amounts among the different fractions.

In conclusion, the total Aβ extraction from brain tissue requires: 1) a high volume of homogenization buffer and 2) repeated re-homogenization with the same buffer before continuing to the next step in the extraction protocol. Our results suggested that a three extraction protocol with three repetitions of re-homogenization with TBS and TX and one of FA using a volume of homogenization buffer of 6 mL should be adequate for the near complete ELISA measurable Aβ extraction from the brain. Nevertheless, because of the possible complex peptide-matrix interactions, the ideal homogenization volume and number of repetitions should be empirically adjusted for every set of buffers and characteristics of the tissue (species, age, stage of disease).

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REFERENCES


