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Purification and characterization of leucine aminopeptidase from human cerebral cortex

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Aminopeptidases (EC 3.4.11) have been implicated both in the biotransformation of physiologically active peptides in the central nervous system, and in the degradation of peptides in the general cellular protein catabolic process. As part of a systematic characterization of aminopeptidases previously identified in human cerebral cortex (Lauffart & Mantle, 1986), we describe in this paper the purification to homogeneity (as judged by polyacrylamide-gel electrophoresis) and characteristics of leucine aminopeptidase; the specificity of this enzyme in degrading oligopeptides is also described.

Approximately 35 g of human cerebral cortex (mixed grey and white matter, obtained at autopsy within 15 h of death) was homogenized using an Ultra-Turrax homogenizer (2×30 s at 5000 rev./min). An homogenate was prepared in the following buffer (1:4, by vol.): 50 mm-glycine/NaOH buffer, pH 7.5, at 4°C. The homogenate was centrifuged at 20000 g for 30 min and the supernatant (120 ml; NaCl concentration adjusted to 0.1 M) applied to a column (50 cm×3 cm) of DEAE Sephadex A50, which was eluted with extraction buffer containing 0.1 M NaCl. Fractions were assayed in a medium (0.3 ml) containing 50 mm-glycine/NaOH buffer, pH 9.5, at 37°C, 5 mm-MgCl2 and 1.25 mm-leucyl-7-amido-4-methylcoumarin (Leu-AMC). After incubation at 37°C, ethanol (0.6 ml) was added and the fluorescence of the liberated AMC measured. Aminopeptidase activity eluting from the column under the above conditions was well separated from other aminopeptidases known to be present in cerebral cortex, which were retained on the column (Lauffart & Mantle, 1986). Fractions containing leucine aminopeptidase activity were pooled, concentrated by ultrafiltration (Amicon cell, 100000 Da molecular mass exclusion membrane) and applied to a gel filtration chromatography column (90 cm×1.5 cm) of Sephacryl S300, eluted with 50 mm-Tris/acetate buffer, pH 7.5, at 4°C/l mm-EDTA/1 mm-2-mercaptoethanol. Following assay as above, fractions containing leucine aminopeptidase activity were pooled, concentrated to a volume of approx. 1 ml (B15 miniconcentrator, Amicon) and further purified by preparative polyacrylamide-gel electrophoresis. Samples were run at 25 mA/gel slab for 24 h at 4°C, using 5% (w/v) slab gels (14 cm×10 cm) and a continuous Tris/acetate buffer system (0.1 M, pH 8, at 4°C). Protein staining was as described by Mantle et al. (1983). After gel sectioning, the enzyme was extracted by soaking the gel slices in assay buffer for 24 h at 4°C. A single protein staining band coinciding with enzyme activity was obtained.

Following purification as described above, leucine aminopeptidase had the following characteristics: optimum activity was at pH 9.5, in the presence of Mg2+ (5 mm) or Mn2+ (0.5 mm). Approximately 50% inhibition of activity was obtained with the following inhibitors: o-phenanthroline (5 mm), p-hydroxymercurationphensulfonate (5 μM), bestatin (0.05 μM), amastatin (0.5 μg/ml) and bacitracin (2 mm). Only the leucyl- and methionyl-36% relative activity) AMC derivatives were appreciably hydrolysed. The molecular mass of the enzyme was estimated as 720000 Da (gel filtration). A value for the Km for reaction of the enzyme with Leu-AMC was estimated as 1.25 mm for a Lineweaver–Burk plot of 1/V versus 1/[S] over the concentration range 0.2-4 mm. The specificity of leucine aminopeptidase on a series of oligopeptides was analysed by reverse-phase h.p.l.c. using a Waters automated system incorporating a Z-module radial compression unit and Nova-Pak C-18 cartridge (Smith & McDermott, 1984). Column elution was carried out with a linear gradient of acetonitrile (2-49% (v/v), 20 min at 1 ml/min) containing 11 mm-trifluoroacetic acid; product elution times were monitored by u.v. absorption at 241 nm. Peptide (10 μg) was incubated with enzyme (10 μl) in 50 mm-glycine/NaOH buffer, pH 9.5, at 37°C containing 5 mm-MgCl2 (total 50 μl) for 1 h at 37°C; the reaction was terminated by addition of 0.1% (v/v) trifluoroacetic acid (0.15 ml). The following peptides were hydrolysed: [N-terminal amino acids and hydrolysis rates (nmol/min per ml of enzyme) are shown in parentheses]: luteinizing hormone-releasing factor [Ser-Tyr-, 0.44]; [Leu]-enkephalin (Tyr-Gly-Leu-Arg-Phe-Met, 0.20); [Leu]-enkephalin (Tyr-Gly-Phe-Met-Leu-Arg-Phe-Met, 0.18). The specificity of the enzyme against N-terminal amino acids is thus broader for peptides than for the aminocyl-AMC derivatives. Peptides which were not appreciably hydrolysed by the enzyme included: neurotensin (pGlu-Leu-); somatostatin (Ala-Gly-Phe-Glu-Lys-Leu-Gly-Asp-Phe-Ala, 0.44); and cholecystokinin octapeptide (Asp-Tyr-Pro-Glu-Leu-Phe-His-Thr-Cys). In the hydrolysis of [Leu]-enkephalin by the enzyme, only the N-terminal Tyr residue was removed; the pH optimum for the latter was at 10.0 with a Km of 5.7 mm. The activity of the enzyme against [Leu]-enkephalin in the absence of Mg2+ (5 mm) or Mn2+ (0.5 mm) was less than 1% of maximum.

These results suggest that the characteristics of leucine aminopeptidase purified from human cerebral cortex correspond reasonably closely with those for similar enzymes previously characterized in other tissues (McDonald & Barrett, 1986). As far as we are aware, this is the first report describing the specificity of leucine aminopeptidase purified from human brain in degrading neuropeptides.

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Peptidases involved in the degradation of neurotensin by human brain synaptic membranes

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The functioning of neuropeptidases as neurotransmitters or neuromodulators requires a mechanism for their inactivation and this is thought to involve membrane-bound synaptic peptidases (Turner, 1986). Several rat synaptosomal peptidases have been shown to be involved in the degradation of neurotensin ([pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH] in vitro, the main points of hydrolysis being Arg<sup>-</sup>-Arg<sup>+</sup>, Pro<sup>16</sup>-Tyr<sup>14</sup> and Tyr<sup>11</sup>-Ile<sup>12</sup> (McDermott et al., 1983; Checler et al., 1983, 1986). This communication describes attempts to identify the peptidases involved in neurotensin inactivation in human brain.

Synaptosomes were prepared from human post-mortem brain (12 g of frontal cortex; post-mortem delay 15 h) by the method of Dodd et al. (1981). Crude synaptic membranes were prepared by lysis of the synaptosomes for 1 h at 4°C in 5 mM-Tris/HCl (pH 7.4) and pelleted by centrifugation (16 000 g, 15 min). The membranes were washed twice with 0.2 M-NaCl in 10 mM-Tris/HCl (pH 7.4) and twice with 10 mM-Tris/HCl (pH 7.4) at 4°C. Neurotensin (40 nM) was incubated with membranes (1.2 mg of protein/ml) in 10 mM-Tris/HCl (pH 7.4) at 37°C for 15-180 min. The reaction mixture was then acidified with an equal volume of 1% trifluoroacetic acid (TFA) and the degradation products were analysed by reversed-phase h.p.l.c. (Nova-Pak C<sub>18</sub> cartridge; 2.1-49% acetonitrile gradient over 20 min containing 1% trifluoroacetic acid (TFA) and 0.1% trifluoroacetic acid (TFA)).

Table 1. Effect of inhibitors on the degradation of neurotensin by human synaptic membranes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition (%)</th>
<th>Formation of neurotensin fragments (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>1-8</td>
</tr>
<tr>
<td>50 μM-CPE</td>
<td>55</td>
<td>100</td>
</tr>
<tr>
<td>10 μM-Thiorphan</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>10 μM-Captopril</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>6 μM-Dynorphin (1-13)</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>50 μM-CPE + 10 μM-thiorphan</td>
<td>78</td>
<td>100</td>
</tr>
</tbody>
</table>

Neurotensin was incubated with human synaptic membranes in the presence of the appropriate inhibitor. Extent of degradation and degradation products were analysed by reversed-phase h.p.l.c.

In summary, human synaptic membranes appear to have three main neurotensin-degrading enzymes, all metalloendopeptidases as reported for rat synaptic membranes (Checler et al., 1983). Captopril, an inhibitor of angiotensin-converting enzyme (Ondetti et al., 1977), had no overall effect on neurotensin degradation, but appeared to inhibit conversion of neurotensin (1-10) to neurotensin (1-8) (Checler et al., 1983). Dynorphin (1-13) was the most effective inhibitor; the pattern of product formation was consistent with inhibition of the endopeptidase 24.15-like enzyme (McDermott et al., 1986) and the Pro<sup>16</sup>-Tyr<sup>14</sup>-cleaving peptidase, while leaving endopeptidase 24.11 unaffected.

In order to further characterize these peptidases, human brain synaptic membranes were solubilized in 20 mM-Tris/HCl (pH 7.5) containing Triton X-100 (1%, v/v) at 4°C for 45 min and the extract was applied to an anion-exchange column (TSK-DEAE-5PW; 7.5 cm x 0.75 cm), eluting with a linear gradient of NaCl (0-0.5 M; 30 min) in 20 mM-Tris/HCl (pH 7.5) at 0.7 ml/min. Fractions (0.5 ml) were collected and assayed for neurotensin-degrading activity, the products being identified by reversed-phase h.p.l.c. Two peaks of activity were identified. The major peak (at 0.19 mM-NaCl) hydrolysed neurotensin to give the (1-10) and (11-13) fragments. A second peak of activity (at 0.23 mM-NaCl) hydrolysed neurotensin at Arg<sup>-</sup>-Arg<sup>+</sup> and corresponded to endopeptidase 24.15. The major peak of activity was subjected to size exclusion chromatography (TSK-G3000-SW; 60 cm x 0.75 cm) eluting with 0.1 M-Tris/acetic acid (pH 7.4). The neurotensin-degrading activity had an elution volume of 13.9 ml (K<sub>d</sub> 0.051) corresponding to a M<sub>d</sub> of 180 000, compared with 72 000 for the equivalent rat metalloendopeptidase (Checler et al., 1986).

In summary, human synaptic membranes appear to have three main neurotensin-degrading enzymes, all metalloendopeptidases as reported for rat synaptic membranes. It is not yet clear which is responsible for controlling neurotensin levels in vivo.

Abbreviations used: pGlu, pyroglutamic acid; TFA, trifluoroacetic acid; CPE-AAF-pAB, carboxyphenethyl-Ala-Ala-Phe-p-aminoanobenzole.