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# Secretion Of A Neuropeptide-Metabolizing Enzyme Similar To Endopeptidase 22.19 By Glioma C6 Cells

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**Summary:** An endopeptidase capable of metabolizing a number of neuropeptides and generating [Met$^5$] and [Leu$^5$] enkephalin from enkephalin-containing peptides is secreted by glioma C6 cells. This neutral endopeptidase that is likely to be a thiol protease, has a Mr of 71KDa and is effective only towards oligopeptides. Its specificity towards neuropeptides is identical to that of soluble endopeptidase 22.19. Moreover, when a partially purified preparation of enkephalin-generating enzyme secreted by glioma C6 cells was submitted to immunoblotting, an antiserum against purified brain endopeptidase 22.19 recognized a single band at Mr of 71 KDa. These data suggest that the soluble endopeptidase 22.19 may be secreted by glioma C6 cells thus allowing its participation in the biotransformation of opioid peptides in the CNS. 

Enkephalin can be generated either by processing of its precursors inside secretory granules or by biotransformation of one bioactive enkephalin-containing peptide into another, a process that most likely occurs outside the cell (1). An interesting example of opioid biotransformation is the conversion of dynorphin $B_{1\text{-}29}$ into dynorphin B by secreted dynorphin-converting endopeptidase of a rat anterior pituitary lactotrophic cell line (2). A subsequent step

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Abbreviations: NT= neurotensin; Dyn= Dynorphin; BK= bradykinin; ME= [Met$^5$] enkephalin; LE= [Leu$^5$] enkephalin; BAM 18P= bovine adrenal medula 18 amino acid residues peptide; LPH= lipotropin; QF-ERP$_7$= quenched fluorescent enkephalin-related peptide [ortoaminobenzoil-GGFLRRV-N-(2,4,dinitrophenyl) ethylenediamine]; DFP= diisopropyl fluoro phosphate; DTNB= 5,5-dithio*bis* (2-nitrobenzoic acid); cFP-AAF-pAB= N-[1-R,S-carboxy-2-phenylethyl]-Ala-Ala-Phe-4-aminobenzoate; D-MEM/F$_{12}$= Dulbecco's modified Eagle's medium/F12; ECPs= enkephalin-containing peptides; G6PD= Glucose-6-Phosphate-Dehydrogenase.

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of dynorphin biotransformation could involve the conversion of dynorphin-related peptides into [Leu$^5$] enkephalin by the enkephalin-converting activity of endopeptidase 22.19 (3).

Endopeptidase 22.19 or endo-oligopeptidase A is a soluble thiol activated endopeptidase (4) that is able to convert small enkephalin-containing peptides (ECPs = 8 to 13 amino acid residues) derived from pro-enkephalin, prodynorphin and pro-opiomelanocortin into [Met$^5$] or [Leu$^5$] enkephalin (3,5,6,7). The presence of this enzyme in enkephalin-rich areas of the CNS of rat brain (8) and in retinal cells containing [Leu$^5$] enkephalin (7) prompted us to hypothesize that endopeptidase 22.19 is involved in the biotransformation of opioid peptides. On the other hand, the association of a predominantly intracellular endopeptidase with biotransformation events most likely occurring outside the cell does not favour this hypothesis. Recent findings, however, suggesting that glial cells may play a role in the secretion of processing enzymes (9) lend support to the conception that soluble enzymes participate in biotransformation.

In the present report, we describe the generation of [Leu$^5$] and [Met$^5$]enkephalin from a number of ECPs by a secreted endopeptidase obtained from a glioma C6 cell line. Our results indicate that the enkephalin-generating enzyme secreted by this cell line has similar Mr and specificity as endopeptidase 22.19. Moreover, a monospecific antibody against this thiol endopeptidase recognized a single band on an immunoblot of glioma C6 cell secreted proteins.

## Material and Methods

Synthetic peptides metorphinamide, neurotensin, luliberin, and substance P were purchased from Cambridge Research Biochemicals; dynorphin $A_{1-13}$, dynorphin $A_{1-17}$, BAM 18P, ß-neoendorphin and α-endorphin were from Peninsula Laboratories Ltd; bradykinin, bradykinin$_{1-5}$, bradykinin$_{6-9}$, neurotensin$_{1-8}$, neurotensin$_{9-13}$, and quenched fluorogenic substrate QF-ERP$_7$ were synthesized by solid phase method by Prof. L. Juliano of the Escola Paulista de Medicina, Sao Paulo, Brazil. Cultured medium (mixture D-MEM/$F_{12}$) was from Gibco BRL (USA). Fetal calf serum was from Cutilab, Campinas, Brazil. Soluble metalloendopeptidase (EC.3.4.24.15) specific inhibitor (cFP-AAF-pAB), was a generous gift of Dr. Marian Orlowski to Dr. Charles Lindsley (Escola Paulista de Medicina São Paulo, Brazil). Antibody raised against rabbit brain endopeptidase 22.19 was obtained as previously described (10).

Glioma C6 cells cultures were maintained in D-MEM/$F_{12}$ medium containing 10% fetal calf serum. Cells were grown in plastic flasks of 80 cm$^2$ in a humidified atmosphere consisting of 5% $CO_2$ and 95% air. The culture medium of subconfluent cells (approximately 1.5x10$^7$ cells /flask) was removed and cells washed five times with PBS buffer (1.27mM NaCl; 3.8 mM KCl; 1.1 mM $Na_2HPO_4$; 0.48 mM $KH_2PO_4$) without detaching them from the dishes. The cells were incubated at 37°C (see legend to Figure 1) and at appropriate time intervals the medium was collected by centrifugation (2,000 g/ 3min) and supernatants filtered through a 0.45 µm membrane (Millex-HV; Millipore, Bedford, MA. USA). The endopeptidase 22.19-like activity found in the incubation medium will be referred to as secreted endopeptidase or simply **endo-s**. After the last sample was withdrawn, cellular integrity was evaluated by trypan blue dye (0.1%) exclusion using a hemocytometer. In parallel experiments, after washing the cultures five times with PBS, cells were scraped from surface with a rubber policeman and homogeneized in a Potter-Elvehjem homogeneizer system and the

homogenate was centrifuged at 25,000 g x30 min. The supernatant fraction was used as the cytosolic fraction.

Endopeptidase 22.19 activity was fluorimetrically measured by incubating the enzyme preparations with a quenched fluorescent dynorphin-derived peptide(QF-ERP$_7$) as substrate(11). One mU of endopeptidase 22.19 activity is defined as the amount of enzyme that is able to cleave 1 nmol of QF-ERP$_7$ per min at 37°C and pH 7.5.

G6PD was determined by the method of Lee et al., (12).

The peptide cleavage site in enzymatic hydrolysis was determined by reverse phase chromatography of the hydrolyzate. Peptides (30 nmol) were incubated with 0.04 mU of Endopeptidase 22.19 activity in 200 µl of 20mM Tris-HCl buffer, pH7.5, and the reaction was allowed to proceed at 37°C for 10-30 min. Aliquots (50µl) of the reaction mixture were periodically withdrawn and subjected to high performance liquid chromatography (HPLC) as previously described (3). The products of peptide hydrolysis were determined by comparison with the elution profiles of synthetic neuropeptide fragments of known concentrations.

The Mr of endo-s was determined by gel filtration chromatography on a 1.6 X 97 cm Sephacryl S-200 column as described (13). The sample containing 2.46 mg of protein (4.5 mU of endopeptidase 22.19 activity) was applied to the column and eluted at a constant flow rate of 30 ml/h in 20 mM Tris-HCl buffer, pH 7.4 containing 0.125 M NaCl. The column was calibrated with Mr standards (Pharmacia, Uppsala, Sweden).

Immunoblotting was carried out essentially as described by Paik et al.,(14).

Protein determination was according to Lowry et al., (15) using BSA as standard.

## Results and Discussion

The endopeptidase 22.19-like activity in the PBS medium of the glioma C6 cells (endo-s) progressively increases between 30 to 90 min of incubation, declining thereafter (Figure 1). The peak of endo-s activity (0.06 ± 0.02 mU/10$^6$ cells) occuring at 90 min of incubation is about 20% of the initial value of the intra-cellular endopeptidase 22.19 activity (0.29 ± 0.03 mU/10$^6$ cells). This latter activity progressively decreases to 55% of its initial value after 90

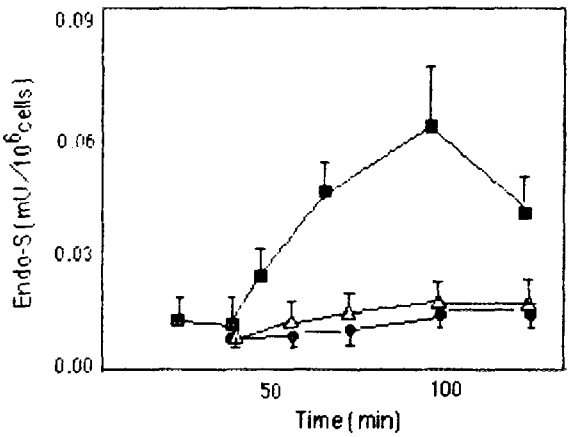


Figure 1. Time course for secretion of endopeptidase 22.19 -like enzyme from cultured glioma C6 cells. Each point represents the mean and the SEM of 4-5 separate determinations. The incubation of cultured cells, the enzyme preparation and assay for endopeptidase 22.19 activity are described in methods. Incubation media: ■ =PBS; Δ=PBS containing 1.2 mM $Ca^{++}$; ●= D-MEM/F12.

min incubation (data not shown). Parallel measurements of G6PD activity, a reference for cytoplasmic enzymes, indicates that 8% of this enzyme was present in the incubation medium upon 90 min incubation (data not shown). This fact indicates that most endopeptidase 22.19 found in the incubation medium may be secreted by the glioma C6 cells and it is not simply derived from cell lysis occuring during the incubation period. This interpretation is also supported by the Trypan blue dye exclusion which shows less than 6% of cell death upon 120 min incubation.

The rate of endo-s secretion by glioma C6 cells was significantly reduced when D-MEM/$F_{12}$ instead of PBS was used as incubation medium (Figure 1). This reduction of endo-s secretion may be explained by the presence of $Ca^{++}$ in the D-MEM/$F_{12}$ medium since the addition of 1.2 mM $Ca^{++}$ to PBS reduced the release of endo-s by 10 fold (Figure 1). This is apparently paradoxal to many other secretory systems in which extracellular $Ca^{++}$ is required for secretion (16). However, it is known that there are secretory systems in which extracellular $Ca^{++}$ either is not necessary [constitutive secretory processes (17)] or even reduces the rate of secretion [in the rat parathyroid cell line (18)]. Further studies with the system described here could lead to new insights into the role of calcium in protein secretion.

Similarly to endopeptidase 22.19 (3,6,7), the secreted enzyme is able to convert 8-13 amino acid opioid peptides derived from pro-enkephalin (metorphinamide), pro-dynorphin (β-neoendorphin) and pro-opiomelanocortin (β-lipotropin 61-69) into [$Met^5$] or [$Leu^5$] enkephalin (Table1). An exception to this rule is dynorphin $A_{1-13}$ which otherwise is a good competitive inhibitor for both intracellular endopeptidase 22.19 (19,11) and endo-s (Table 2).

For most opioid peptides used, crude endo-s is able to hydrolyze other peptide bonds in addition to the M-R, M-T and L-R,

**Table 1 - Relative Rates of Hydrolysis of Neuropeptides by Endo-S**

| Peptides | Structures | Rates of Hydrolysis (%) | Products* | | | |
|---|---|---|---|---|---|---|
| | | | ME | LE | $BK_{1-5}$ | $NT_{1-8}$ |
| Metorphinamide | $YGGFMRRVNH_2$ | 100 | 0.44 | - | - | - |
| β-Neoendophin | YGGFLRKP | 51 | - | 0.58 | - | - |
| β-$LPH_{61-69}$ | YGGFMTSEK | 31 | 0.90 | - | - | - |
| BAM 18P | YGGFMRRVGRPEWWMDYQ | 9 | 0.00 | - | - | - |
| Dynorphin $A_{1-17}$ | YGGFLRRIRPKLKWDNQ | 6 | - | 0.00 | - | - |
| α-Endorphin | TGGFMTSEKSQTPLVT | 4 | 0.00 | - | - | - |
| Dynorphin $A_{1-13}$ | YGGFLRRIRPKLK | 8 | - | 0.00 | - | - |
| Bradykinin | RPPGFSPFR | 28 | - | - | 0.75 | - |
| Neurotensin | <ELYENKPRRPYIL | 10 | - | - | - | 0.38 |

* mol/mol of peptide hydrolyzed.

**Table 2-Inhibition of Endo-S by Peptides and Other Compounds**

| Peptides | Concentration (μM) | Activity* (%) |
|---|---|---|
| None | - | 100 |
| Bradykinin | 30 | 25 |
| Neurotensin | 30 | 32 |
| Dynorphin $A_{1-13}$ | 5 | 8 |
| Substance P | 30 | 95 |
| Luliberin | 30 | 100 |
| α-Endorphin | 30 | 100 |
| **Other Compounds** | **(mM)** | **(%)** |
| EDTA | 5.0 | 120 |
| DFP | 5.0 | 60 |
| DTNB | 1.0 | 0 |
| cFP-AAF-pAB | 1.0 | 100 |

*The effect of peptides or other compounds on endopeptidase 22.19 activity was measured by the inhibition of hydrolysis of QF-ERP7 caused by the addition of peptide (without pre-incubation) or compound (15 min pre-incubation at 22°C) to the reaction mixture.

generating non-identified products. These cleavages suggest that crude endo-s encompasses other peptidase(s) in addition to endopeptidase 22.19 since the latter enzyme only produces a single cleavage on ECPs (3). This contaminating peptidase(s) may also explain why large peptides such as dynorphin $A_{1-17}$, BAM-18P, α-endorphin, which are not substrates for endopeptidase 22.19 (3,19), are degraded to a lesser extent not forming [Leu$^5$] or [Met$^5$] enkephalin.

Table 1 also shows that similarly to endopeptidase 22.19 activity (10,20), endo-s hydrolized bradykinin and neurotensin at F-S (75%) and R-R bonds (38%), respectively. Moreover, the presence of luliberin, substance P, or α-endorphin in the reaction medium does not affect endopeptidase 22.19 activity whereas, the presence of bradykinin, dynorphin $A_{1-13}$ or neurotensin significantly reduces endo-s activity (Table 2). These results indicate that only the alternative substrates of endopeptidase 22.19 (19) are able to affect endo-s activity.

Additional support for the identity of endo-s and endopeptidase 22.19 is the immunoblot presented in Figure 2, showing that in glioma C6 culture medium the antiserum against rabbit brain endopeptidase 22.19 and not the pre-immune serum recognizes a single band corresponding to the Mr of endopeptidase 22.19 (10).

Another enzyme which displays similar specificity towards neuropeptides is the soluble metallo-endopeptidase or endopeptidase 24.15 (20). However, the results presented in Table 2 indicate that endo-s is probably a cysteine-endopeptidase and not a metallo- or

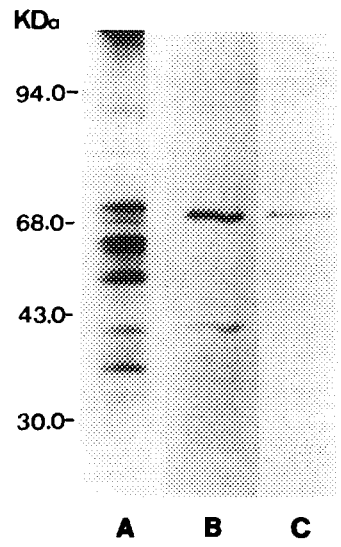


Figure 2. SDS-PAGE of 0.22 mg of crude endo-s (A) and 0.1mg of endo-s partially purified by gel filtration on Sephacryl S-200 (B). The gel was stained with Coomassie Brilliant Blue.( C): Western blot of crude endo-s (0.22mg of protein) which was transferred from a SDS-PAGE to nitrocellulose membrane. The membrane was subsequently incubated with polyclonal anti-endopeptidase 22.19 diluted 1:250 and then with 1:3.000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG for 12 h at room temperature.

serine-endopeptidase since it is not inhibited by the chelating agent EDTA nor by the serine specific inhibitor DFP but it is completely inhibited by DTNB, a thiol reacting compound (21). Moreover, endo-s can be distinguished from endopeptidase 24.15 because it is not inhibited by cFP-AAF-pAB, a specific inhibitor of endopeptidase 24.15 (22). On the other hand, the Endo-S activity is not affected by luliberin and substance P (Table 2) which are known to be substrates for endopeptidase 24.15 (22, 23).

The findings described in this report led us to conclude that endopeptidase 22.19 may be secreted by glial cells thus allowing its participation in the biotransformation of opioid peptides.

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