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CHARACTERIZATION OF A SUBSTANCE P-GLY^{12} AMIDATING ENZYME IN HUMAN CEREBROSPINAL FLUID

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Enzyme activity capable of converting the glycine-extended substance P precursor, substance P-Gly^{12}, into substance P was purified from human cerebrospinal fluid. The conversion reaction was monitored by radioimmunoassay measurement of substance P formation. The chemical identity of the product was verified by reversed-phase HPLC. The enzyme reaction was stimulated by Cu(II) ion and ascorbic acid and inhibited by the presence of diethyldithiocarbamate. By HPLC molecular sieving, the major enzyme activity appeared as a protein of 26,000 molecular weight.

The undecapeptide substance P (SP; Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) is found in many areas of the central and peripheral nervous system (1). It has been given considerable attention because of its putative function in neurotransmission or neuromodulation. Substance P belongs to a family of structurally related peptides, the tachykinins, which exhibit similar biological activities and share a common C-terminal sequence. The C-terminal amino acid residue of the tachykinins is amidated. The primary structure of two substance P precursors, alpha- and beta-preprotachykinin, in bovine brain has been deduced by cDNA sequencing (2). Direct evidence for precursors of SP has been found in human and bovine brain (3). In the precursors, the SP sequence followed by a glycine residue in its C-terminal is flanked by pairs of basic amino acids, Arg-Arg and Lys-Arg, respectively. The release of SP from its precursor is likely to occur via several enzymatic steps, initiated by trypsin-like cleavages, liberating a dodecapeptide with a C-terminal glycine, SP-Gly^{12} (Fig. 1). The C-terminal Gly residue is enzymatically converted by an enzyme or enzymes capable of converting peptides terminating in -X-Gly into alpha-amidated products (-X-NH_2). Such enzymes have been purified from several sources (4-8) using various di- or tripeptidyl-glycine compounds as substrate. The reaction was stimulated in the presence of

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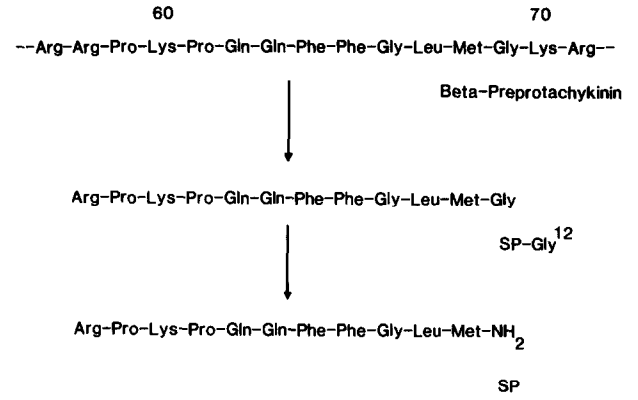

Fig. 1. Tentative processing of substance P precursor.

Cu(II) ion and ascorbic acid but inhibited by diethyldithiocarbamate. It also appeared from these studies that the alpha-amidating activity is heterogenous with regard to molecular size (6,8).

In the present study, we describe the purification and some characteristics of an enzyme present in human cerebrospinal fluid (CSF) and capable of producing SP from its glycine-extended precursor, SP-Gly^{12}. Large quantities of the fluid were processed by use of different chromatographic procedures and the product was characterized with regard to its biochemical and kinetic properties.

MATERIALS AND METHODS

Materials. The CSF material was collected from individual patients with suspected but not confirmed neurological deficits and was kept frozen at -70°C before being processed. Standard peptides used in this study were purchased from Bachem (Bubendorf, Switzerland) except for SP-Gly^{12} which was prepared by solid phase synthesis on a Boc-Gly-resin with the aid of Ppoc-amino acids, essentially as described previously (9). The crude peptide, obtained on HF-cleavage, was purified by preparative HPLC on a reversed-phase column to give a product of satisfactory purity. The chromatographic material, Sephadex G-25, Sephadex G-100 and DEAE Sepharose CL-6B were from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals and solvents were of analytical-reagent grade from commercial sources.

Enzyme purification. Prior to fractionation, CSF samples from 12 individual patients were thawed, collected in one pool (300 ml) and lyophilized. The freeze-dried material was reconstituted in 50 ml of 20 mM Tris-HCl, pH 7.8, and applied to a Sephadex G-100 column. The column (5 cm x 95 cm) was eluted with the same buffer, maintaining a flow rate of 80 ml/h. Fractions of 16 ml were collected and analyzed for UV-absorbance (280 nm) and enzyme activity. The active fractions were collected and further purified by ion exchange chromatography on a DEAE-Sepharose CL-6B column (5 cm x 20 cm). The enzyme-containing fraction was directly applied to the anion exchanger, which had been previously equilibrated with 20 mM Tris-HCl, pH 7.8. After sample application, the column was washed with one volume of the Tris-HCl buffer and subsequently eluted with a gradient of NaCl (0-0.5M) containing 20 mM Tris-HCl, pH 7.8. Fractions of 10 ml were collected at a flow rate of 120 ml/h and assayed for enzyme activity. The active material was desalted on Sephadex G-25 and lyophilized before further studies.

High performance liquid chromatography (HPLC). For molecular sieving, the HPLC-system (LKB, Bromma, Sweden) was equipped with an Ultropac TSK-G3000 column (7.5 mm x 600 mm). The column was eluted with 20 mM Tris-HCl buffer (pH 7.2), maintaining a flow rate of 0.5 ml/min, and 0.5 ml fractions were collected and analyzed for enzyme activity. For the reversed-phase separation, a Spherisorb ODS-2 C-18 column (4.6 mm x 250 mm, particle size 5 μm) was used. The elution was carried out with a linear gradient of acetonitrile (0 to 45%) containing 0.04% trifluoroacetic acid (TFA). The flow rate was 0.5 ml/min and fractions of 0.5 ml were collected and evaporated prior to radioimmunoassay (RIA).

Enzyme assay. The alpha-amidating activity was recorded by measuring the formation of SP from SP-Gly^{12}, using a specific RIA for the undecapeptide. All incubations were performed in Eppendorf tubes at 37°C. About 12 pmol of synthetic SP-Gly^{12} was incubated with the enzyme fraction together with $CuCl_2$ (10 μM) and ascorbic acid (80 μM) in a final volume of 100 μl buffered at pH 7.8 with 20 mM Tris-HCl. The reaction was terminated by the addition of 0.5 ml cold methanol, followed by centrifugation and the supernatant was evaporated before RIA-analysis.

Radioimmunoassay. The procedure has been described in detail in a preceding paper (10). The antibodies, principally recognizing the C-terminal part of SP, were raised in rabbits against the peptide-thyroglobulin conjugate, and (^{125}I-Tyr^8)-SP was used as tracer. The cross-reactivity with SP (3-11) and SP (5-11) was 100 and 60%, respectively. SP-Gly^{12}, SP free acid and the N-terminal SP fragments (1-7) and (1-8) cross-reacted in the RIA with less than 0.1%.

RESULTS

Molecular sieve chromatography on Sephadex G-100 yielded the SP-Gly^{12} amidating activity in a principal peak eluting at a calibrated molecular weight (for a protein) of around 25,000 dalton (Fig. 2). Further purification of the

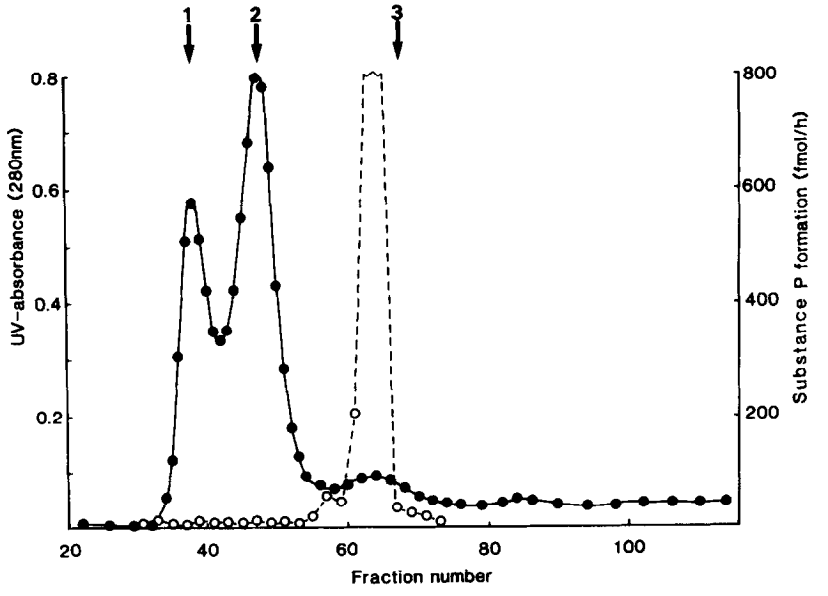

Fig. 2. Distribution of substance P-Gly^{12} amidating activity after molecular sieve chromatography (Sephadex G-100) of lyophilized human CSF (original volume, 300 ml). Standard proteins are indicated by arrows (1=human gamma-globulin, MW=160,000; 2=bovine serum albumin, MW=68,000; 3=prolactin, MW=22,000. For details, see text.

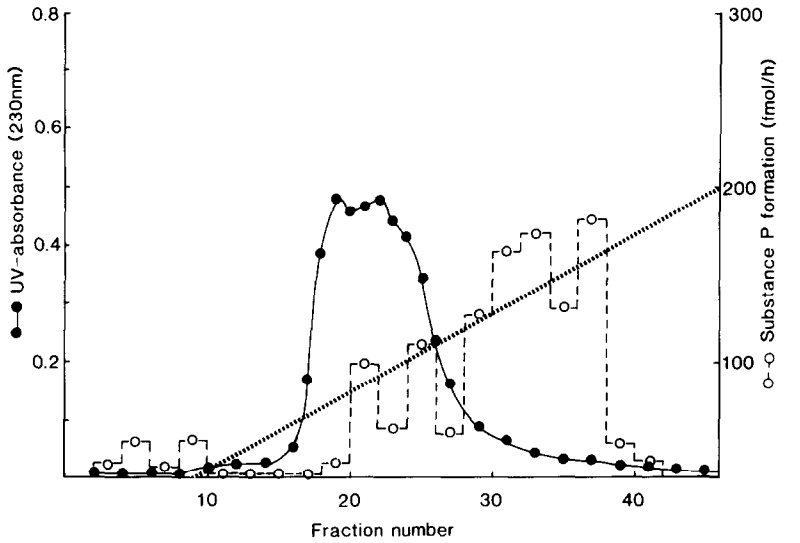

Fig. 3. Ion-exchange chromatography on DEAE-Sepharose CL-6B of enzyme activity from the Sephadex G-100 column (fractions 60-66). Elution was done with a linear gradient of NaCl (0-0.5M) as indicated. For details, see text.

amidating enzyme was achieved by ion-exchange chromatography on DEAE-Sepharose CL-6B. As shown in Fig. 3, the major activity was adsorbed to the ion exchanger and eluted at a comparatively high salt concentration. Fractions 29-38 were collected, desalted on Sephadex G-25 and lyophilized before further characterization studies. At this stage the enzyme was obtained in a yield of 3.7 µg per ml CSF. The specific activity was 1.4 pmol SP min^{-1} (mg protein)$^{-1}$.

In HPLC molecular sieving, the purified enzyme activity appeared as a homogenous peak with an apparent molecular weight of approximately 26,000 dalton (not shown). In order to confirm the structure of the product generated, SP, as well as an incubation mixture with the purified enzyme and SP-Gly^{12} were analyzed by reversed phase HPLC (Fig. 4). It can be seen in Fig. 4 that the major part of the immunoreactivity eluted from the column identically with the SP standard.

Table 1 summarizes the effects of certain agents on SP formation. The presence of diethyldithiocarbamate, which forms complexes with Cu(II) ion, was found to reduce product formation even at low concentrations. When added in excess, both the dipeptides Met-Gly and Tyr-Gly were found to inhibit the amidation of SP-Gly^{12}. The Met-Gly sequence showed higher potency.

DISCUSSION

A variety of neuropeptides have an alpha-amidated C-terminal amino acid. Amidation is usually essential for biologic activity (11). In fact, Mutt and co-workers took strategic advantage of this principle in the search of new biologically active peptides in tissue extracts (12).

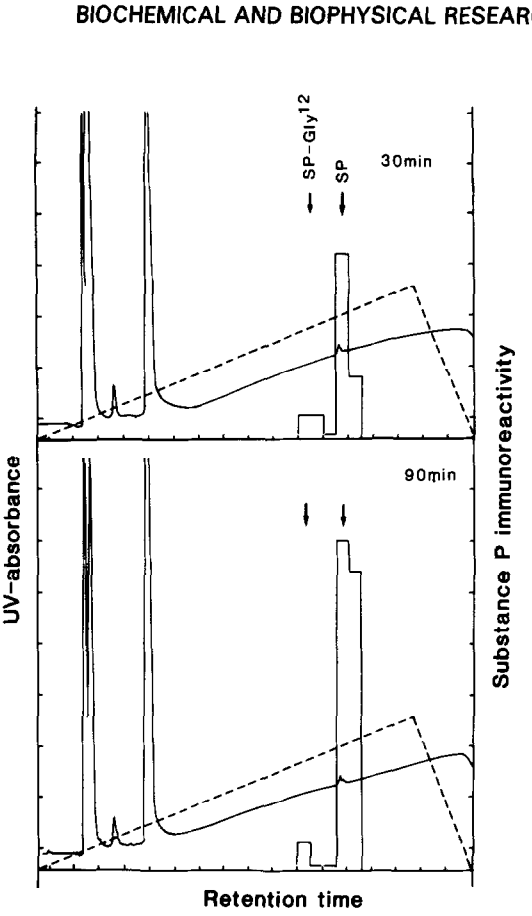

Fig. 4. Reversed phase HPLC of reaction mixtures of substance P-Gly^{12} (12 pmol) and the purified enzyme (2 ug). Incubation was performed at 37°C for 3 h. The elution positions of peptide standards are indicated (1=substance P-Gly^{12}; 2=substance P).

TABLE 1. Effects of diethyldithiocarbamate (DDC) and the dipeptides, Met-Gly and Tyr-Gly on substance P-Gly^{12} amidation

Inhibitor/dipeptide	Concentration (μM)	Inhibition of SP-Gly^{12} conversion (%)
DDC	1	30
	10	78
	100	97
Met-Gly	0.4	0
	4	13
	40	28
Tyr-Gly	0.4	0
	4	0
	40	11

Incubations were performed at 37°C for 90 min with 1 μg purified enzyme in a final volume of 50 μl. Substrate concentration, 4 μM. Values are given as means of three different determinations.

Previous studies have utilized simple model substrates such as D-Tyr-Val-Gly (4,7,8) or p-Glu-His-Pro-Gly (pro-TRH, 6). Peptidyl-Gly alpha-amidating activity was found in various tissues, including serum (7) and CSF (13). Here, the SP precursor, SP-Gly^{12}, was used since a major aim was to define those enzymes critically involved in the biosynthesis/degradation of SP, an approach which previously led to the discovery of an endopeptidase cleaving SP at the (7-8) and (8-9) bonds, in CSF (14) and in CNS tissue (unpublished).

The enzyme purified from CSF seems to belong to the same family as other alpha-amidating monooxygenases. For instance, it is stimulated by Cu(II) ion and ascorbic acid, confirming previous observations (6,7,8,13). However, previous studies have also indicated heterogenity. For instance, there may be two distinct forms of enzyme giving C-terminal amidation in rat brain and in bovine neurointermediate pituitary (8) and pituitary (6). The latter separated into two components of different molecular sizes (54,000 and 38,000 dalton). The activity found in human serum had an estimated molecular weight of approximately 60,000 dalton (7). The apparent molecular size of the SP-Gly^{12} amidating activity described in this study is only around 25,000 dalton. Whether the CSF enzyme represents an active fragment or subunit of a larger, perhaps native form of alpha-amidating activity, is not clear. Except for the serum variant (7), no other glycine-directed alpha-amidating enzymes in humans has yet been characterized with regard to molecular weight. A critical issue is the substrate specificity of the different isolated enzymes, which requires more testing.

The relevance of the presence of the present enzyme in the CSF compartment is not known. It could originate from passive leakage out of the neurons into the extracellular fluid and then diffuse into the CSF. It may also have been stored together with SP or other peptide precursors in synaptic vesicles and released with the peptide via exocytosis. Quantitative analysis of the enzyme in individual CSF samples in combination with RIA analysis of SP may give an indication whether or not the enzyme is co-secreted with this particular peptide and, if so, its quantitation in the fluid may give a measure of SP-ergic activity.

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