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Purification of Substance P endopeptidase activity in the rat ventral tegemental area with the Äkta-Purifier chromatographic system ☆

Krister Karlsson*, Fred Nyberg

Division of Biological Research on Drug Dependence, Department of Pharmaceutical Biosciences, Uppsala University, P.O. Box 591, S-751 24, Uppsala, Sweden

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Abstract

The new chromatographic system Äkta-Purifier 10 (Amersham–Pharmacia Biotech), scaled for preparative HPLC, was used for the purification of Substance P (SP) endopeptidase activity in the ventral tegemental area (VTA) of the rat brain. SP endopeptidase previously identified and purified from human cerebrospinal fluid has been found to degrade the neuroactive peptide SP in a specific pattern. In this study we have recovered SP endopeptidase from the rat VTA following a purification scheme involving homogenization (ultrasonication) and extraction of the excised tissue, size-exclusion chromatography (Superdex 75 HR), and ion-exchange chromatography (Resource Q). In this way we were able to achieve a purification factor of almost 7500, based on specific activity. The obtained SP endopeptidase activity, was then subjected to characterization with regard to inhibition profile. The enzyme activity was monitored by following the conversion of SP to its N-terminal fragment SP(1–7) using a radioimmunoassay, specific for the heptapeptide product. On basis of inhibition profile it was possible to discern two different SP endopeptidase-like activities, one sensitive toward the protease inhibitor phosphoramidon (preparation A), and another non-sensitive to phosphoramidon or captopril (preparation B). The molecular masses of preparations A and B, as derived from sodium dodecyl sulfate–polyacrylamide gel electrophoresis, were found to be 90 000 and 76 000, respectively. Our data suggest that the purified phosphoramidon sensitive endopeptidase activity may be an enzyme that plays a major role in the conversion of SP to its bioactive fragment SP(1–7) in the rat VTA. This is likely to be identical to the previously known neutral endopeptidase (EC 3.4.24.11). However, this study also demonstrates the existence of a distinct endopeptidase activity with properties in agreement with rat spinal cord SP endopeptidase. In the context of previously shown altered levels of SP(1–7) in the VTA during morphine withdrawal both purified enzyme activities may turn out to be responsible.

Keywords: Sample preparation; Substance P endopeptidase; Enzymes

1. Introduction

Substance P (SP) is an 11-residue neuropeptide belonging to the tachykinin family. The peptide is involved in a large number of biological functions (for a review see Ref. [1]). These include transmission of pain signals [2], immunomodulation [3], and a variety of behaviors [1]. In the central nervous

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*Corresponding author. Tel.: +46-18-4714-167; fax: +46-18-501-920.

E-mail address: krister.karlsson@farmbio.uu.se (K. Karlsson).

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system (CNS) SP is synthesized as part of larger precursor-proteins known as preprotachykinins. The active peptide is released and subjected to maturation during the axonal transport under the influence of processing and amidating enzymes [4]. Following nerve-terminal release the peptide is probably inactivated or rather converted by a peptidase action. There are several peptidases that have been demonstrated to participate in hydrolyzing SP [5]. Angiotensin converting enzyme (ACE) [6] and neutral endopeptidase (NEP, EC 3.4.24.11) [7–9] are two proteases known to hydrolyze SP. These enzymes, however, appear rather unspecific regarding substrates and act on several other peptides as well. More specific enzyme activities capable of hydrolyzing SP are also described. A membrane bound enzyme was isolated and characterized from human brain [10]. This enzyme hydrolyzes SP between the Gln^6–Phe^7, Phe^7–Phe^8 and Phe^8–Gly^9 bonds. It was characterized as a neutral metallo–endopeptidase with a molecular mass of 40 000–50 000. Furthermore a similar enzyme denoted substance P degrading enzyme (SPDE) with a molecular mass of 70 000, was reported to exist in the rat spinal cord [11]. Another enzyme, substance P endopeptidase, with an apparent high specificity for SP was purified and characterized from human cerebrospinal fluid (CSF) [12]. An SP endopeptidase-like enzyme purified from rat spinal cord also supported the idea of a substrate specific enzymatic inactivation for the undecapeptide [13]. Interestingly the major fragments generated by SP endopeptidase and the SP endopeptidase-like enzymes are SP(1–7) and SP(1–8), which both have been demonstrated to exhibit biological effects [14–16], even reverse to the parent peptide [17]. Indeed, further characterization studies indicated SP endopeptidase to be different from other SP proteases [18].

The SP(1–7) fragment was recently shown to decrease the intensity of withdrawal reaction to opioids in mice [14]. In a recent study we found that the level of SP(1–7) was significantly increased in the ventral tegemental area (VTA) in the male rat during naloxone precipitated opioid withdrawal [19]. In a very recent study (unpublished) we observed increased activity in an SP endopeptidase-like activity in the VTA during morphine withdrawal in the male rat. This observation prompted us to attempt to purify and characterize this enzyme. However, due to limited amount of tissues (the VTA is a very small tissue in the rat brain) a requirement for this approach is a chromatographic system which allows micropurification of small quantities with high accuracy. For that purpose we applied the new chromatographic system Äkta-Purifier (version 10) from Amersham–Pharmacia Biotech, Sweden.

2. Experimental

2.1. Materials

Sprague–Dawley male rats (220–250 g) were used as tissue donors. The VTA was excised immediately after decapitation and kept frozen at −70°C until use. Substance P, phosphoramidon and captopril were purchased from Sigma (St. Louis, MO, USA). SP(1–7) and Tyr–SP(1–7) were prepared by Dr. G. Lindeberg, Department of Medical Chemistry, Uppsala University, Uppsala, Sweden. The electrophoresis markers, Kaleidoscope, catalogue No. 1610324 were obtained from Bio-Rad Labs. (Sundbyberg, Sweden). Protein amounts were assessed by Bio-Rad Protein Assay, catalogue No. 500-0006. All other chemicals and reagents were of analytical-reagent grade and purchased from various commercial sources.

2.2. Purification

All procedures were carried out at 5°C. Frozen rat ventral tegemental areas from 40 animals (410 mg) were thawed and homogenized in 20 m*M* sodium phosphate, pH 7.8 (1 ml/g) with an ultrasonic probe (Branson Sonifer cell disruptor B15) for 45 s. The homogenate was extracted in the same buffer for 5 min before centrifugation at 18 000 *g* for 10 min (Eppendorf Centrifuge 5417, rotor F45-24-11, Beckman). The supernatant was collected and kept frozen (−70°C) until use. At this stage the extract was aliquoted into three equal volumes. This means that we followed three identical preparation procedures from this point forward in the purification scheme, which allowed us to retrieve statistical reliability (Table 1). The extract was subsequently applied to

Table 1
Purification and recovery of substance P endopeptidase from rat VTA (values are given as means from three different preparations, see text for details)

Fraction	Total protein (mg)	Total activity (pmol/min)	Specific activity (pmol min^{-1} mg^{-1})	Purification factor
Extract	1.804	3.21	1.78	1
Size-exclusion chromatography				
I	0.134	9.66±3.05	72±23	40±12
II	0.00541	2.58±0.55	238±70	134±39
Anion-exchange chromatography				
A	0.00003	2.41±0.74	80 000±24 000	45 000±13 000
B	0.00025	3.33±0.32	13 000±1200	7500±700

size-exclusion chromatography (SEC) through a 30-μl sample loop, using the column Superdex 75 HR 10/30 (Amersham–Pharmacia Biotech) installed on the preparative high-performance liquid chromatography (HPLC) system Äkta-Purifier 10 (Amersham–Pharmacia Biotech). Elution with 0.04 *M* NH_4HCO_3 at a flow-rate of 0.5 ml/min was carried out and 0.5-ml fractions were collected. The fractions ware scanned for enzyme activity (see Fig. 1), and those indicated to contain SP endopeptidase activity were pooled for further separation on an anion-exchange chromatography system. Again the Äkta-Purifier 10 was used, now with a Resource Q, 1-ml column (Amersham–Pharmacia Biotech). In this chromatographic step the samples were eluted with 20 m*M* sodium phosphate, pH 7.4 using a linear gradient of 0–1 *M* KCl. The length of the gradient was set to 20 column volumes, and the flow was 4 ml/min. Fractions of 1 ml were collected and assayed for SP endopeptidase activity.

2.3. Enzyme assay

The enzyme activity was assessed by following the conversion of SP to its N-terminal fragment SP(1–7), using a specific radioimmunoassay (RIA) for this product. The substrate, SP (0.05 μg) was incubated with the enzyme-containing fraction together with a protease inhibitor mixture including phosphoramidon and captopril (15 μ*M*) at a final volume of 50 μl. Phosphoramidon is an inhibitor of NEP and captopril a typical ACE inhibitor. The reaction was held in a 37°C waterbath and was terminated after 45 min by adding 150 μl ice-cold MeOH–0.2 *M* HCl (2:1). The reaction vials were subsequently centrifuged for 10 min at 18 000 *g* and the supernatants were collected and tested for SP(1–7) content by RIA. The RIA was performed as described in Ref. [20]. Briefly, the antibodies were raised in rabbit against the peptide–thyroglobulin conjugate and the iodinated peptide was used as a tracer. The ^{125}I-labeled Tyr^0–SP(1–7) and the sample or unlabeled SP(1–7) (standard) were thus incubated with specific antibody against SP(1–7) in 1.5-ml Eppendorf tubes. The tubes were stored at 5°C until expected equilibrium had been established (>16 h). Separation of bound peptide from free peptide was carried out using a procedure based on the charcoal adsorption technique as described [20]. The quantity of bound tracer was determined by measuring the radioactivity in a gamma-counter. The detection limit of the RIA and the cross-reactivity of SP(1–7) related peptides in the RIA were as described [20].

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The purified enzyme preparations were analyzed by polyacrylamide gel electrophoresis (PAGE). In the presence of sodium dodecyl sulfate (SDS) according to a procedure described elsewhere [13]. Standard proteins were run in parallel to calibrate the gel for estimation of molecular size of the purified proteins. Prior to electrophoresis, the samples were desalted by reversed-phase HPLC.

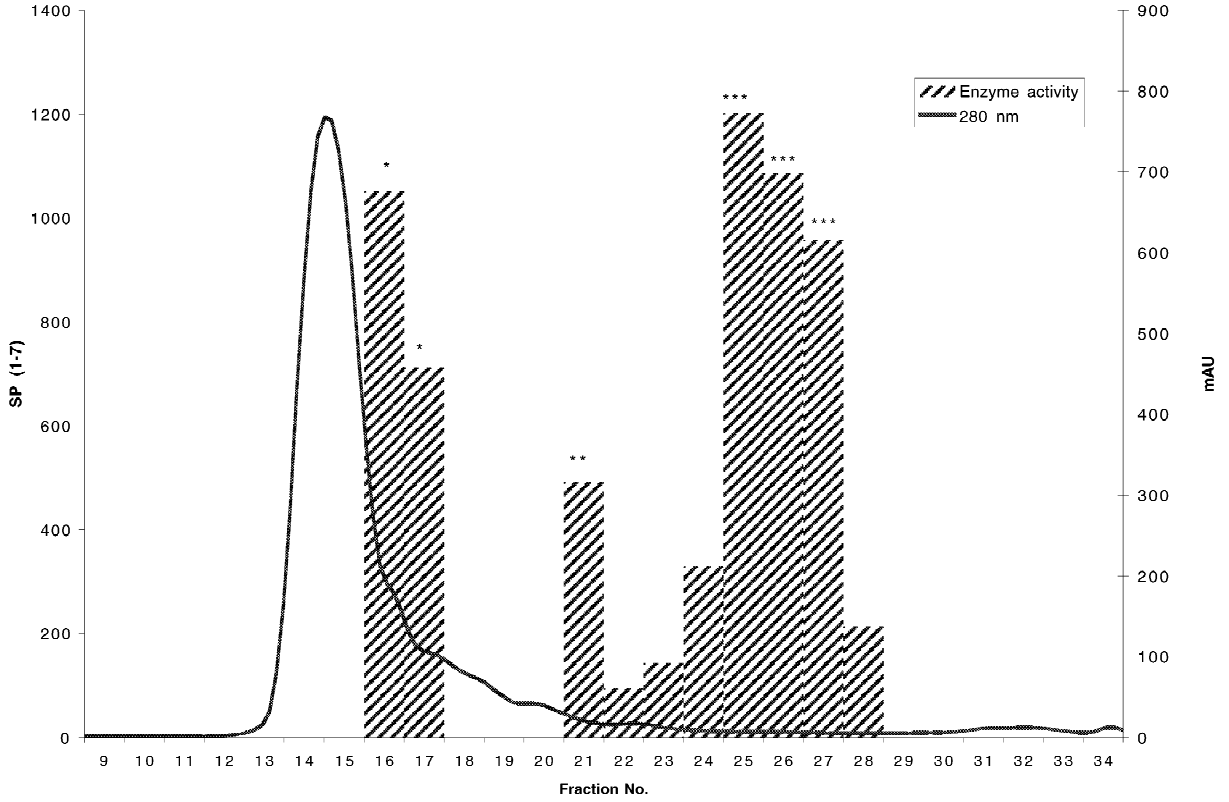

Fig. 1. Size-exclusion chromatography of the soluble fraction of rat ventral tegemental homogenate. The extract was applied to a Superdex 75 HR 30/10 column and eluted with 0.04 *M* NH_4HCO_3 at a flow-rate of 0.5 ml/min. Fractions of 0.5 ml were collected. *Peak I fractions (16–17), **peak II fractions (21), and ***peak III fractions (25–27) were pooled and each pool was further processed by anion-exchange chromatography as described in the text.

3. Results

Fig. 1 shows the distribution pattern of protein content and enzyme activity obtained when the soluble fraction from the extracted rat VTA was subjected to SEC. As shown, the SP endopeptidase activity was resolved in three major activity peaks; one appeared at fractions 16–17, another at fraction 21, and a third at fractions 25–27. All these three peaks were pooled into three fractions which were separately applied to high-performance anion-exchange chromatography. The result from this separation indicated that the major activity seen after ion-exchange chromatography originated from the early eluting material (fractions 16–17) in the gel filtration step. Only very little activity was recovered from ion-exchange chromatography of fractions 21 and 25–27 (not shown). In the case of the activity in fractions 25–27 it is likely that this is due to interference of low-molecular-mass compounds [e.g., salts, or even endogenous SP(1–7)] in the RIA procedure. The enzyme activity and protein profile recorded in this step are shown in Fig. 2. As can be seen in the figure, the major SP endopeptidase activity was found to elute ahead of gradient. At least two separate SP endopeptidase-like fractions were observed. Additional studies based on the inhibitor testing of activity found in the size-exclusion step suggested that a more phosphoramidon sensitive activity was present in fractions 16–17, whereas a captopril sensitive activity was recovered in fraction 21. Regarding the two activity peaks found in fractions 3 and 6 in the ion-exchange step (referred to as preparation A and preparation B,

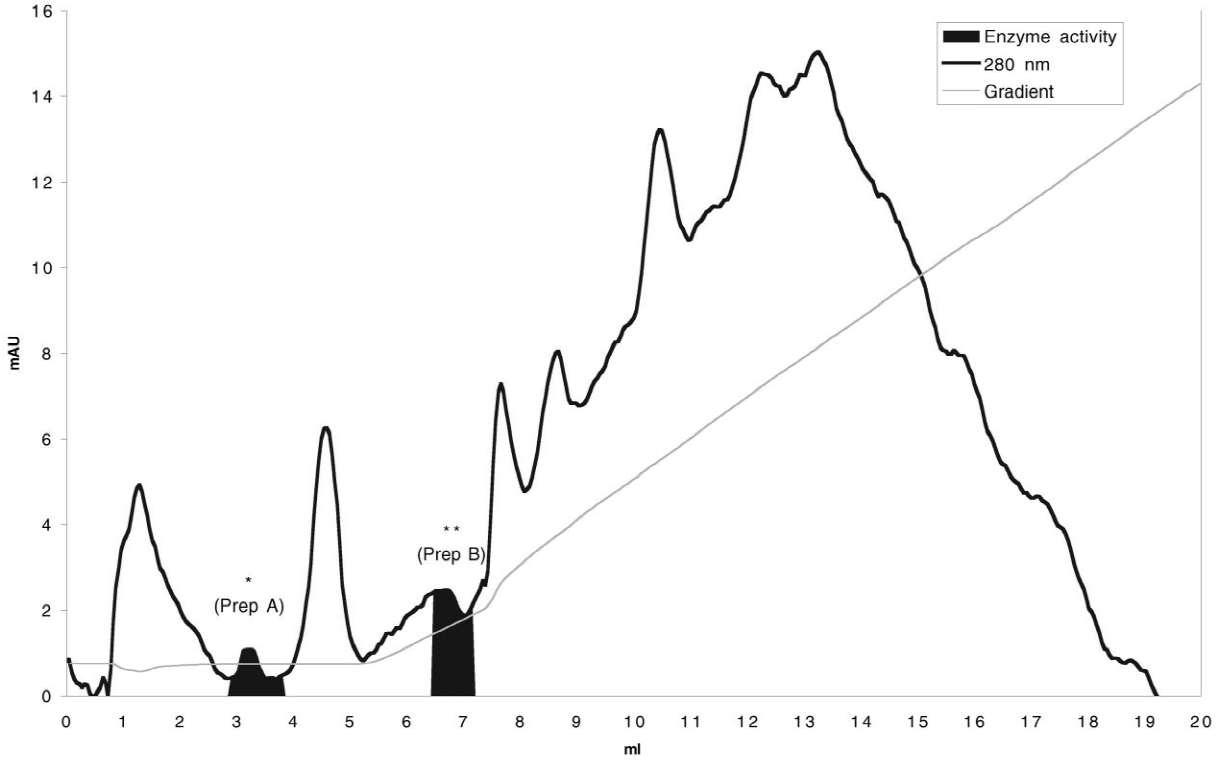

Fig. 2. High-performance anion-exchange chromatography on the material present in peak I from the size-exclusion step (Fig. 1). The pooled material was applied on a Resource Q (1-ml column) through a 1000-μl sample loop. The flow-rate was 4 ml/min and fractions of 1 ml were collected during the linear elution gradient of 0–1.0 *M* KCl, 20 m*M* sodium phosphate, pH 7.4. The enzyme activity was assayed as described in the text. *Preparation A (corresponding to fraction No. 3), and **preparation B (corresponding to fraction No. 6) were analyzed by SDS–PAGE (Fig. 3).

respectively) the sensitivity towards phosphoramidon remained only in preparation A. Preparation B was neither sensitive toward phosphoramidon nor captopril. By phosphoramidon (15 μ*M*) preparation A was inhibited 30% compared to control but was not affected by captopril. These findings indicate that it was possible to discern a NEP-like phosphoramidon activity (preparation A) from an SP endopeptidase-like activity (preparation B). By SDS–PAGE a major band was stainable for the SP endopeptidase-like preparation B (Fig. 3). Molecular masses (M_r) values of 90 000 and 76 000 were estimated for the enzyme preparations A and B, respectively. M_r for preparation A was determined from a Comassie stained gel of poor quality, not shown. Based on protein assay, the amount of protein in the crude extract originating from 410 mg tissue was estimated to be 1804 μg. In the purified preparations A and B the protein contents were assessed as 30 and 250 ng, respectively. The purification factor based on specific activity were almost 7500 for the SP endopeptidase-like activity (preparation B), see Table 1. The corresponding figure for preparation A was calculated to be 45 000.

4. Discussion

In this study we have purified two enzyme activities capable of degrading the undecapeptide Substance P. One of these activities was partly sensitive toward the NEP inhibitor phosphoramidon, while the other activity was not affected either by phosphoramidon or captopril. An ACE-like activity was detected after the gel filtration step but was only detected in negligible amounts after ion-exchange

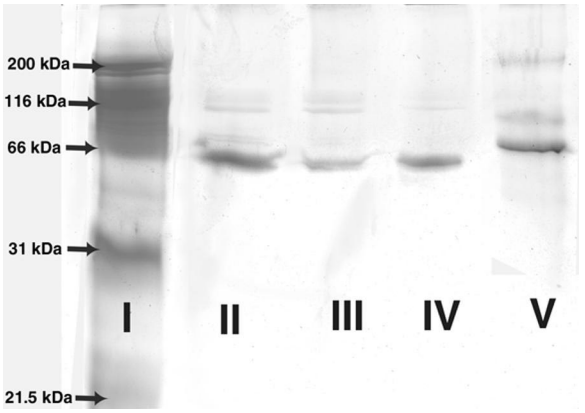

Fig. 3. SDS–PAGE was performed on preparations A and B from the anion-exchange step (Fig. 2). The preparations were lyophilized and resolved in 10 μl of sample buffer (62.5 m*M* Tris–HCl, pH 6.8; 10% (v/v) glycerol; 2% (w/v) SDS; 0.05% Bromphenol blue; water up to 8 ml) before run. Lanes: I=Bio-Rad Kaleidoscope, catalogue No. 1610324, II=0.5 μg bovine serum albumin (BSA), III=0.1 μg BSA, IV=0.25 μg BSA, V=preparation B. kDa=Kilodaltons.

chromatography. These enzymes seem to resemble the Substance P degrading activity previously found in the rat spinal cord [13], where two different SP endopeptidase-like activities were found. One of these was inhibited by phosphoramidon and captopril and the other was not affected by either phosphoramidon or captopril. As shown for the enzymes in the spinal cord, SP endopeptidase in the VTA is also present as a soluble protein. Thus, it is likely from this study that SP is to a large extent inactivated in the rat VTA by a NEP-like enzyme, but also that an SP endopeptidase-like activity significantly contributes to the hydrolysis of SP. Previous studies have shown SP endopeptidase to be more specific for SP and it could well be at least as significant for the SP regulation in that area, as suggested for the spinal cord.

This study also indicates that the Äkta-Purifier is a powerful system for small amounts of enzyme activity. An about 7000-fold (preparation A) or 40 000-fold (preparation B) purification factor in a two-step procedure demonstrates an impressing capacity of this system. Dealing with minute amounts of protein may complicate the handling of enzymes. Attachment of active material to the wall of the incubation vials and loss of enzyme material during the process may contribute to loss of activity and enzyme stability. Also during purification, the optimal conditions for enzyme activity may change.

The functional relevance of the purified enzymes preparations still remains to be clarified. However, as both of them have the ability to release SP(1–7) from SP it is obvious that both can modulate the signal of SP. In the case of morphine withdrawal, where the content of the fragment seems to increase it appears that the enzyme activity releases a compound counteracting the effect of the parent peptide. Thus, SP potentiates the intensity of the withdrawal reaction, while the fragment SP(1–7) has the opposite effect.

In conclusion, in the present work we have used the Äkta-Purifier to purify two enzyme activities from the rat VTA. Both enzymes hydrolyze SP to yield its bioactive SP(1–7) fragment. One of these peptidases resembles NEP whereas the other exhibits characteristics similar but non-identical to SP endopeptidase. The chromatographic system appears useful for this purpose but to fully elucidate the structure of the studied activities more tissue needs to be processed.

Acknowledgements

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