Prolyl carboxypeptidase purified from human placenta : its characterization and identification as an apelin-cleaving enzyme

Prolyl carboxypeptidase purified from human placenta: its characterization and identification as an apelin-cleaving enzyme.

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Running title: PRCP identified as an apelin-cleaving enzyme

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List of abbreviations: α-MSH, α-melanocyte-stimulating hormone; ACE2, angiotensin-converting enzyme 2; Ala, alanine; Phe, phenylalanine; hPRCP, purified human prolyl carboxypeptidase; HUVEC, human umbilical vein endothelial cells; ITC, isothermal titration calorimetry; Pro, proline; PRCP, prolyl carboxypeptidase; (pyr)-apelin-13, pyroglutamated apelin-13; rhPRCP, recombinant human prolyl carboxypeptidase; Val, valine; Z, N-benzyloxycarbonyl

Abstract

Background: The proteolytic regulation of peptides involved in feeding behavior is poorly understood. Prolyl carboxypeptidase (PRCP) is particularly known for its role in body weight control by converting the anorexigenic peptide, α-melanocyte-stimulating hormone 1-13 into the inactive form 1-12. The purpose of this study was to characterize purified human PRCP, to investigate its substrate specificity and to discover novel substrates linked to obesity. Pyroglutamated apelin-13, ghrelin, enterostatin and obestatin were investigated since these are feeding-regulating peptides with potential cleaving sites for PRCP.

Methods: PRCP was purified from human placenta and identified using western blotting and mass spectrometry. The kinetic parameters of purified and commercially available PRCP for known and potential peptide substrates were determined and compared using a RP-HPLC activity assay, isothermal titration calorimetry and mass spectrometry.

Results: PRCP was purified 575-fold from human placenta and succesfully identified as human lysosomal Pro-X carboxypeptidase. Purified and recombinant PRCP had similar substrate specificity with angiotensin III as the substrate of preference. Pyroglutamated apelin-13 was observed to be a novel substrate for human PRCP *in vitro* and PRCP-dependent cleavage was shown in a human umbilical vein endothelial cell culture experiment. Other potential substrates e.g. obestatin, ghrelin and enterostatin were not hydrolyzed by PRCP.

Conclusion: These results show that placenta is a good source of human PRCP and that PRCP removes the C-terminal phenylalanine from

pyroglutamated apelin-13. For the first time, PRCP is identified as an apelin-cleaving enzyme. This finding adds evidence to the hypothesis that PRCP plays a role in energy homeostasis.

Keywords: Energy homeostasis, Prolyl carboxypeptidase, (Pyr)-apelin-13, Enzyme kinetics, Substrate specificity

1. Introduction

Worldwide, overweight and obesity are growing health problems with insufficient treatment options [1–3]. Although numerous environmental, metabolic and genetic factors leading to this disorder have already been described, many aspects of its pathophysiology remain to be explored [4]. Among these are the possible influence of fetal and neonatal glucose homeostasis on metabolism and feeding behavior later in life [5]. Several peptide neurotransmitters and hormones are involved in the regulation of food intake and energy balance [6]. While there is considerable knowledge about their synthesis and release, the enzymes that degrade these peptides are poorly understood. Recent findings indicate that the enzyme prolyl carboxypeptidase (PRCP, angiotensinase C, EC 3.4.16.2) plays a role in body weight control by metabolizing neuropeptides that cause a loss of appetite, i.e. 'anorexigenic' peptides [7]. PRCP hydrolyzes these peptides by cleaving off a single C-terminal amino acid when alanine (Ala) or proline (Pro) are in the penultimate position [8]. In addition, altered PRCP is likely to play a role in the pathophysiology of Type 2 Diabetes Mellitus [9].

In the past, the involvement of PRCP in the regulation of food intake was mainly attributed to the inactivation of a single anorexigenic peptide, α-melanocyte-stimulating hormone (α-MSH) 1-13. PRCP cleaves off the C-terminal valine (Val) of α-MSH 1-13 thereby generating an inactive form α-MSH 1-12 [10]. Thus, inhibition of PRCP might increase the bioavailability of central α-MSH 1-13, resulting in reduced food intake and eventually in decreased body weight. Therefore, targeting PRCP could be a new therapeutic approach to treat obese patients [11]. In 2011, Diano *et al.*

discovered that PRCP-null mice were leaner than their wild-type variant and that the administration of PRCP inhibitors in wild-type and obese mice decreased food intake, independent of peripheral or central administration [10]. This finding suggests that besides central α-MSH 1-13, PRCP could also regulate α-MSH 1-13 in the periphery or other feeding-regulating peptides. To further improve insight in the regulation of food intake by PRCP, we searched for additional putative substrates with Ala or Pro in the P1 position and found apelin, enterostatin, ghrelin and obestatin. These peptides have been related to body weight control and have sequences that theoretically are susceptible to cleavage by PRCP (Table 1) [12–16].

So far, human PRCP has only been purified from kidneys and neutrophils [17,18]. Searching the literature, a few reports on the purification of PRCP from other species were found (overview in the Supplementary Material) [19–22]. The enzyme is found both intracellularly in lysosomes and extracellularly as a cell membrane-bound (e.g. endothelial cells) and soluble protein [23]. PRCP is not only known for its high expression in kidney, liver and lung, it is also abundantly present in placenta [24]. For this reason, we decided to purify human PRCP from placental tissue. The aims of this study are to use both purified human natural PRCP (hPRCP) and recombinant human PRCP (rhPRCP) to investigate the truncation of potential novel PRCP substrates. Based on their amino acid sequence pyroglutamated apelin-13 ((pyr)-apelin-13), the predominant isoform of apelin in several peripheral tissues and human plasma [25,26], enterostatin, ghrelin and obestatin were investigated. This *in vitro* study determines the efficiency of cleavage of these peptides in comparison with previously identified substrates such as angiotensin II,

angiotensin III, des-Arg^9-bradykinin and N-benzyloxycarbonyl-L-proline-L-phenylalanine ((Z)-Pro-Phe) [10,27–29].

2. Results

2.1 Protein purification

PRCP was purified about 575-fold from human placental tissues using a heating step, Q sepharose big beads, CM-sepharose and Concanavalin A-Sepharose, followed by concentration on a Hitrap Q and Hitrap SP column. From this 6-step purification procedure 0.2 mg of hPRCP was obtained, indicating that human placenta is a good source for PRCP. The purification scheme of the first four steps is shown in Table 2. hPRCP cleaves Z-Pro-Phe at a rate of 330 µmol/min/g protein. As shown on SDS-PAGE (Figure 1A), the purified sample showed a protein band at a molecular weight of 58 kDa, corresponding with the previously reported data on human PRCP [18].

2.2 Identification of the purified protein

Two different approaches were used to identify the purified protein as PRCP. First, a western blot experiment was performed using polyclonal rabbit antibodies against human PRCP. These antibodies reacted with one band for hPRCP at a molecular weight of 58 kDa and with one band for rhPRCP at 52 kDa (Figure 1B). Second, the mass spectrum of the digested 58 kDa band was compared with theoretical spectra of peptides in the SwissProt database. Based on the similarity between these calculated masses and the observed masses, a protein score of 74 was found for human lysosomal Pro-X carboxypeptidase (protein scores greater than 67 are considered significant ($p < 0.05$)). The identified peptides and their masses are presented in Table 3. The co-purified bands at 30 and 80 kDa were identified as human carbonic

anhydrase 2 (protein score of 201) and human serotransferrin (protein score of 517). Mass spectrometry-based identification of hPRCP was probably hampered by post-translational modifications of the protein e.g. glycosylation and therefore difficult to obtain.

2.3 Truncation of candidate substrates by hPRCP and rhPRCP

Analysis was performed via a MALDI-TOF mass spectrometric assay where the mass spectra of peptides incubated with hPRCP and rhPRCP were compared with spectra of the substrate blanks (Supplementary Material). As a negative and positive control we first investigated the cleavage of the amide and free acid form of α-MSH 1-13. As expected, the amide form was not hydrolyzed by PRCP. In contrast, incubation of the free acid form of α-MSH 1-13 with hPRCP and rhPRCP resulted in the appearance of an additional mass peak for α-MSH 1-12 (m/z 1566.91). Cleavage of enterostatin (m/z 497.32) by PRCP would lead to an additional product peak with a m/z of 341.12. No such or other product peaks were seen, thereby illustrating that this peptide is not a PRCP substrate *in vitro* since the C-terminal arginine was not cleaved off. Similar results were found for ghrelin (m/z 3371.30) and obestatin (m/z 2546.15) whose mass spectra remained unaltered upon incubation with hPRCP and rhPRCP for 2 hat 37 °C.

2.4 Substrate specificity study

Quantification of Phe by RP-HPLC was used to determine the kinetic parameters (K_m, k_{cat}) for a number of known natural substrates of PRCP (angiotensin II, angiotensin III, des-Arg^9-bradykinin) and for the synthetic substrate, Z-Pro-Phe. Verification of the results was done by repeating the experiment for angiotensin II and angiotensin III via isothermal titration

calorimetry (ITC). The hydrolysis of (pyr)-apelin-13 was also investigated via ITC (Figure 2A and 2B). The data show for the first time that (pyr)-apelin-13 is a novel substrate for PRCP *in vitro* and that the cleavage rate is comparable to that of the known *in vivo* substrates of PRCP. In addition, hPRCP and rhPRCP have a similar substrate specificity profile and the kinetic parameters obtained via two different methods, HPLC and ITC, are very comparable. These results are summarized in Table 4 and the Michaelis plots are all shown in the Supplementary Material.

2.5 (Pyr)-apelin-13 truncation by endothelial cells

To investigate whether PRCP is capable of cleaving exogenously administered (pyr)-apelin-13 in a cellular context, human umbilical vein endothelial cells (HUVEC) in the presence and absence of a PRCP inhibitor were treated with 100 µM (pyr)-apelin-13 for 2 h, 4 h and 8 h at 37 °C. The release of the C-terminal Phe from (pyr)-apelin-13 showed a linear increase in function of the incubation time (Figure 3: insert). A decrease in Phe formation (on average 17%) for each time point was observed when HUVECs were treated with a serine protease inhibitor KYP-2047 that is known to inhibit PRCP with an IC50 of 100 µM (Figure 3) [30,31]. These results show that PRCP is partially responsible for (pyr)-apelin-13 cleavage in this HUVEC cell culture experiment.

3. Discussion

In 1993, Tan *et al.* published that the mRNA expression of PRCP is relatively high in human placenta compared to brain or heart tissue [24]. However, the purification of PRCP from this natural source has never been reported. In this study, PRCP was purified from human placenta, identified with two different

approaches, and used to characterize its efficiency to cleave the known natural substrates as well as potential novel candidates.

First, the homogenate was heated to 60 °C to inhibit the activity of another lysosomal carboxypeptidase, cathepsin A [32]. This enzyme is sensitive to high temperatures while PRCP is not [19]. PRCP protein purification was mainly based on ion exchange and lectin affinity chromatography [21]. As PRCP has six potential N-glycosylation sites [27], a Concanavalin A-sepharose column was chosen to bind the glycosylated enzyme. This strategy has never been explored before and turned out to be very efficient. The resulting enzyme preparation has a predominant band on SDS-PAGE at a molecular weight of approximately 58 kDa and a specific activity of 330 µmol/min/g protein, based on the cleavage of the substrate Z-Pro-Phe. On western blot hPRCP and rhPRCP appeared at a slightly different molecular weight. The rhPRCP was expressed in a mouse myeloma cell line and has, according to the product data sheet, a predicted molecular mass of 54 kDa. Since PRCP is subjected to post-translational modification, differences herein could explain the band of rhPRCP to be situated somewhat lower on the membrane. Besides western blotting, in gel digest of the purified protein followed by mass spectrometric analysis was used to identify the purified protein as PRCP. No reports were found in the literature for a specific functional interaction between PRCP and the co-purified proteins.

The purification and characterization of the enzyme along with our previously optimized activity assay allowed us to perform substrate specificity studies [33]. The comparison of the kinetic parameters of angiotensin II, angiotensin III and des-Arg^{9}-bradykinin with earlier publications supports the finding that

angiotensin III is cleaved at a faster rate than angiotensin II *in vitro* [17]. Des-Arg^9-bradykinin also had a lower K_m value than angiotensin II. The synthetic substrate Z-Pro-Phe had a higher K_m value for PRCP (± 5 mM) purified from placental tissue compared to natural kidney PRCP (± 1 mM) [28]. Overall, our results show that hPRCP has a similar substrate specificity profile as rhPRCP. In addition, the kinetic parameters for angiotensin II and angiotensin III determined via two different methods, namely HPLC and ITC, were very comparable. An interesting observation is that the K_m values for the natural substrates of PRCP are rather high. However, *in vivo* studies have already shown that deletion or inhibition of PRCP increases the levels of angiotensin II and α-MSH 1-13 which provides evidence that these peptides are natural substrates for PRCP [11,34]. Therefore, we hypothesize that unknown cofactors enhance the cleavage efficiency of PRCP *in vivo* or that PRCP acts in a multimolecular complex rather than as a solitary enzyme. This mechanism has been shown for lysosomal neuramidase whose biological activity is enhanced by its interaction with protective protein/cathepsin A and β-galactosidase [35]. In the future, it is worthwhile to investigate this phenomenon for PRCP as well.

The most important goal of this study was to identify new substrates for PRCP which are also linked to energy homeostasis and metabolic disorders such as obesity. Therefore, we investigated the cleavage of four potential substrates, (pyr)-apelin-13, enterostatin, ghrelin and obestatin by human PRCP. In 1998, apelin was discovered as the endogenous ligand for the G protein-coupled receptor APJ [36]. Both apelin and its APJ receptor are widely expressed in the central nervous system and in peripheral tissues [37]. Moreover, PRCP

and apelin are both expressed in placenta so a possible role for PRCP in apelin cleavage is conceivable. Apelin is mostly known as an important regulatory peptide of the cardiovascular system by exerting inotropic and hypotensive effects [25]. Besides its cardiovascular role, apelin is secreted by adipocytes and a link between apelin, obesity and diabetes has been made, suggesting it has a metabolic function as well [38,39]. There are several active apelin forms but in this study we have focused on the predominant, pyroglutamated form [26]. Many studies have already investigated the effects of the administration of peripheral apelin in wildtype and obese mice. In both types of mice a decrease in body weight, plasma triglycerides and blood glucose was observed, suggesting a protective role for apelin [12,40,41]. This study indicates that besides angiotensin-converting enzyme 2 (ACE2, EC 3.4.17.23), PRCP is also capable of hydrolyzing (pyr)-apelin-13, however at a slower rate than ACE2 [42]. The efficiency of cleavage of (pyr)-apelin-13 by PRCP is comparable to that of the angiotensins. We also investigated the cleavage of apelin-17 and apelin-36 by hPRCP. These peptides are also cleaved at the C-terminus by hPRCP and it seems that the efficiency of cleavage of apelin-17 is approximately the same as (pyr)-apelin-13. In contrast, apelin-36 was cleaved at a slower rate (60-70% compared to (pyr)-apelin-13), Supplementary Material). (Pyr)-apelin-13 treatment of HUVECs resulted in the release of the C-terminal Phe in the medium, a hydrolysis that was partially due to PRCP activity. HUVECs were chosen as a model since laser scanning confocal microscopy showed that part of PRCP is constitutively expressed on the cell membrane of these cultured cells [23]. Although it has been reported that modifications at the C-terminus of apelin-

13 greatly influence its affinity for the APJ receptor and hypotensive effects [43–46], the physiological relevance of its cleavage by PRCP in fetal as well as later in life still has to be revealed in follow up studies.

The second anorexigenic peptide that caught our attention was enterostatin, which inhibits fat intake in various species. Enterostatin is a pentapeptide derived from pancreatic procolipase in the gastrointestinal tract and it appears in the circulation and lymph node after the consumption of food [47]. Similar to apelin, the inhibition of food intake by enterostatin is observed after central and peripheral administration [13,48]. Its has been shown that enterostatin is efficiently cleaved and inactivated by dipeptidyl peptidase IV [49]. Although this peptide has a Pro on the penultimate position, mass spectrometric analysis showed no cleavage by PRCP. This observation is in accordance with Craik *et al.*, who reported earlier that PRCP is incapable of hydrolyzing substrates with arginine in the S1' pocket [8]. Finally, we investigated the cleavage of obestatin and ghrelin, two gut peptides derived from proghrelin. Ghrelin is known to induce appetite but the function of obestatin in the regulation of body weight is still uncertain [50]. In 2005, Zhang *et al.* discovered that obestatin decreased food intake and body weight gain in rats [14]. Although these results seemed promising, recent studies failed to reproduce the shown anorexigenic effects, thereby raising questions about the actual function of obestatin [16,50–52]. Our interest in obestatin as a potential PRCP substrate was based on a prior publication which indicated that PRCP can also cleave peptides with an Ala at the P1 position [8]. However, we revealed that neither obestatin nor ghrelin are hydrolyzed at the C-terminus. A possible explanation could be that the C-terminus of obestatin

and ghrelin is not free to interact with the active site of the enzyme or that leucine and arginine are not tolerated at the P1' position.

In conclusion, our results indicate that human placenta is a good source for the purification of natural PRCP. The search for potential PRCP substrates involved in the regulation of energy homeostasis led us to identify pyroglutamated apelin-13 as a novel PRCP substrate. Placental PRCP as an additional apelin-metabolizing enzyme during pregnancy thus deserves further investigation. Our finding indicates that the role of PRCP in body weight control may not only be attributed to the truncation of α-MSH 1-13, but also to the truncation of other metabolism-regulating peptides in the periphery.

4. Materials and methods

4.1 Materials

Angiotensin II, angiotensin III and enterostatin were obtained from Pepscan (Lelystad, The Netherlands), des-Arg^9-bradykinin, α-melanocyte-stimulating hormone 1-13 (amide and free acid) and Z-Pro-Phe from Bachem (Bubendorf, Switzerland), ghrelin from Abcam (Cambridge, United Kingdom), (pyr)-apelin-13 from Bio-Connect BV (Huissen, The Netherlands), obestatin from Enzo Life Sciences (Antwerp, Belgium) and recombinant human PRCP from R&D Systems (Abingdon, United Kingdom). KYP-2047 (4-phenylbutanoyl-l-prolyl-2(*S*)-cyanopyrrolidine) was synthesized as reported by [53,54]. HUVECs were purchased from Clonetics (San Diego, CA), cell culture flasks and well-plates from Greiner Bio-One (Wemmel, Belgium). Endothelial cell growth medium (EGM™-2 BulletKit™) was obtained from Lonza (Verviers, Belgium) and TrypLE™ Express and PrestoBlue® Cell Viability Reagent from Life Technologies (Ledeberg, Belgium). Chromatographic packing materials and

columns were all purchased from GE Healthcare Life Sciences (Diegem, Belgium). Oriole fluorescent gel stain solution was obtained from Sigma and Trypsin Sequencing Grade from Roche Applied Science (Penzberg, Germany). For western blotting the primary rabbit antibody against human PRCP was purchased from Sigma-Aldrich (HPA017065), secondary HRP conjugated goat anti-rabbit antibody from Invitrogen (656120, Ghent, Belgium) and SuperSignal West Femto substrate kit from Thermo Fisher Scientific (Erembodegem, Belgium). C_{18} Ziptips were purchased from Millipore (Overijse, Belgium) and α-cyano-4-hydroxycinnamic acid from Sigma-Aldrich.

4.2 Protein purification

Human placental tissues were collected for the purification of proteolytic enzymes such as PRCP. All mothers (GZA Hospital Antwerp, Belgium) gave oral informed consent for use of their anonymized placental tissue. The board of Gynecologists and the medical director of the hospital gave their written consent for use of this procedure. Placental tissue was washed with ice-cold PBS, cut and pooled before freezing at -80 °C.

Upon purification, human placental tissues were thawed and homogenized (Polytron 1200E) on ice in 2 L of 50 mM acetate buffer (pH 5), containing 10 mM EDTA and 1% octylglucoside to solubilize PRCP from all cellular compartments. Nuclei and cell debris were removed by centrifugation at 10000 *g* for 30 min at 4 °C in a Sorvall RC-5 superspeed centrifuge with the SS-35 rotor (Analis, Namur, Belgium). The supernatant was collected and the sediment was homogenized and centrifuged a second time under the same conditions. The supernatants were combined, fractionated and stored at -80 °C until further processing.

The homogenate supernatant (212 ml) was thawed and heated at 60 °C for 30 min to inactivate cathepsin A activity [28]. Precipitated proteins were removed by centrifugation as described above. The supernatant (210 ml) was applied onto a Q sepharose big beads column (2.6 x 28 cm). The bound proteins were eluted with 50 mM sodium acetate buffer (pH 5) containing 1 M NaCl at 3 mL/min. At pH 5 PRCP was not adsorbed onto the column and the PRCP containing fractions could be loaded directly onto a CM-sepharose column (2.6 x 23 cm) previously equilibrated with 50 mM sodium acetate buffer (pH 5). The bound proteins including PRCP were eluted with a linear gradient from 0 to 1 M NaCl in 50 mM sodium acetate buffer (pH 5) at 2 mL/min. The peak of PRCP activity appeared around 0.4 M NaCl. The PRCP containing fractions were combined (160 mL) and dialyzed at 4 °C against 2 L of a 20 mM sodium phosphate buffer (pH 6.6) containing 0.5 M NaCl to avoid non-specific ionic interactions. The dialysate (160 mL) was then applied on a Concanavalin A-sepharose column (2.6 x 5 cm), previously equilibrated with the same buffer. PRCP was eluted stepwise with increasing concentrations of methyl α-D-glucopyranoside (0 – 2 M in 20 mM sodium phosphate buffer, pH 6.6) at 2 mL/min. The column was heated to 45 °C to improve the elution. The PRCP containing fractions were dialyzed at 4 °C against 2 L of 50 mM sodium acetate buffer pH 5. To concentrate PRCP, the dialysate (35 mL) was applied to a Hitrap Q column (1 mL), immediately followed by a Hitrap SP column (1 mL) to which PRCP binds at pH 5. Elution took place by a linear increase in salt concentration (0 - 1 M NaCl) at 2 mL/min. The active fractions were pooled and dialyzed against 2 L of 50 mM sodium acetate buffer, pH 5.

4.3 PRCP activity measurement

PRCP activity was measured using a previously validated RP-HPLC assay [33,55]. The hydrolysis of the substrate Z-Pro-Phe by PRCP at 37 °C leads to the release of Z-Pro and Phe. The quantification of the peak height of either of these two products is proportional to the PRCP activity, which is expressed as units per gram (U/g) protein. One unit defines the amount of enzyme needed to hydrolyze 1 µmol of substrate per minute. Protein concentration was determined via the Bradford method with BSA as the standard protein [56]. The kinetic parameters ($K_{m,}$ k_{cat}) for angiotensin II, angiotensin III, des-Arg^9-bradykinin and Z-Pro-Phe were determined by incubating hPRCP and rhPRCP with different substrate concentrations and by quantifying the released Phe with RP-HPLC. The results were fitted to the Michaelis-Menten equation using GraphPad Prism 6 software (La Jolla, California, USA).

4.4 Gel electrophoresis

After each purification step, samples of the active fractions were collected, diluted in Laemmli sample buffer (4x) and boiled for 5 min before loading onto a 10% SDS-PAGE gel. In general, 0.5 - 15 µg of protein was loaded in each lane. Electrophoresis was performed at a constant voltage (200 V) for 45 min. Separated proteins were visualized by Oriole fluorescent gel stain solution (90 min staining in the dark) using the OptiGo (Isogen Life Sciences, De Meern, The Netherlands) and Proxima AQ-4 software (Isogen Life Sciences, The Meern, The Netherlands). Myosin (Mr 200 000), β-galactosidase (Mr 116 250), phosphorylase b (Mr 97 400), serum albumin (Mr 66 200), ovalbumin (Mr 45 000), carbonic anhydrase (Mr 31 000) and trypsin inhibitor (Mr 21 500) were used as the standard marker proteins.

4.5 Western blotting

After SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane (0.45 µm). Blocking of non-specific binding sites was achieved by placing the membrane in 5% skimmed milk in washing buffer (0.05 M Tris, 0.15 M NaCl, 0.01% Tween 20, pH 7.4) for 1 h at room temperature. The blot was then incubated overnight with rabbit anti-human PRCP antibodies (1:500 dilution), followed by a second incubation for 2 h with HRP conjugated goat anti-rabbit IgG's (1:5000 dilution). Chemiluminesecent detection was performed using the SuperSignal West Femto substrate kit. The protein bands were visualized via an OptiGo viewer and Proxima AQ-4 software.

4.6 In-gel digest

Proteins were separated by SDS-PAGE (10%) and stained with Coomassie Brilliant blue R. The band of interest (58 kDa) and two co-purified bands (30 and 80 kDa) were excised with a clean scalpel and cut into 1 mm pieces. The gel pieces were washed with Millipore water, 100 mM NH_4HCO_3 / acetonitrile (1:1, pH 8.0), and 100% acetonitrile to destain the gel pieces. The acetonitrile was aspirated and the pieces were dried at room temperature. Next, the disulfide bonds of the protein were reduced with 10 mM DTT for 45 min at 56 °C and thereafter alkylated with 55 mM iodoacetamide for 30 min at room temperature in the dark. Final washing (100% acetonitrile/ Millipore water, 1:1) and destaining steps (100% acetonitrile) were performed before the gel pieces were digested overnight with 12 ng/ µL Trypsin in digest buffer (25 mM NH_4HCO_3 pH 8, 2% acetonitrile) at 37 °C. The mixture of extracted peptides was acidified with 0.1% trifluoroacetic acid (pH<3) and stored at -20 °C until analysis.

4.7 Peptide mass fingerprint of the protein

The peptides were desalted and concentrated with C_{18} ZipTips and eluted directly on the MALDI-target using MALDI matrix solution 2 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid. Peptide identification was carried out using a 4800 Plus matrix assisted laser desorption ionisation Time-of-flight TOF/TOF^{TM} Analyzer (Applied Biosystems, Foster city, CA, USA). Peptide mass spectra were acquired in positive reflectron mode with 100 laser shots (intensity 4230) per MS-TOF spectrum and 1000 shots (intensity 4700) per MS/MS spectrum. For MS/MS up to 40 precursors with a mass range of 100-4000 Da and exceeding a S/N value of 30 were submitted. The search program GPS explorer was used to compare the generated MS and MS/MS peptide mass fringerprint data with theoretical peptide mass data of all human proteins stored in the SwissProt database. The search parameters were restriction of autolytic trypsin peptides with maximum one missed cleavage, peptide mass tolerance of ± 80 ppm, fragment ion mass tolerance to 0.3 Da, monoisotopic mass, cysteines modified by carbamidomethylation as a fixed modification and oxidation of methionine as a variable.

4.8 Truncation of candidate substrates by hPRCP and rhPRCP

The *in vitro* cleavage of enterostatin, ghrelin and obestatin could not be analyzed by the RP-HPLC activity assay due to the presence of the C-terminal arginine (174.2 g/mol) and leucine (131.2 g/mol). Instead, we used a mass spectrometric assay to investigate potential cleavage. hPRCP (4.2 nM) and rhPRCP (1 nM) were incubated with 75 µM (final concentration) of enterostatin, ghrelin and obestatin for 2 h at 37 °C, pH 5. The reaction was

stopped with 0.1% trifluroroacetic acid (pH<3), the peptides were desalted and concentrated with C_{18} ZipTips and eluted directly on the MALDI-target using MALDI matrix solution 2 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile. Substrate blanks (enterostatin: m/z 497.32, ghrelin: m/z 3371.30, obestatin: m/z 2546.15) were run to serve as a control. Hydrolysis of these potential substrates at the C-terminus would lead to additional product peaks in the mass spectrum (enterostatin: m/z 341.12, ghrelin: m/z 3215.1, obestatin: m/z 2432.95). Cleavage of angiotensin II (m/z 1046,63) and the free acid (m/z 1666.08) and amide form (m/z 1664.87) of α-MSH 1-13 (75 µM) by hPRCP (4.2 nM) and rhPRCP (1 nM) served as a positive and negative control.

4.9 Truncation of (pyr)-apelin-13 by hPRCP and rhPRCP

ITC was used to study the enzyme kinetics for the hydrolysis of (pyr)-apelin-13 by hPRCP and rhPRCP. ITC is based on the direct measurement of the heat generated or absorbed when a substrate is hydrolyzed [57]. (Pyr)-apelin-13 as well as rhPRCP and hPRCP were buffered in 100 mM sodium acetate containing 10 mM EDTA (pH 5) before start of the experiment. Analysis was performed on a MicroCal^{TM} PEAQ-ITC instrument (MicroCal, Malvern Instruments, Northamption, MA, USA) and the results were fitted with PEAQ-ITC analysis software (MicroCal, Malvern Instrumnets, Northampton, MA, USA) and the Origin7 software (OriginLab^{TM}, Northampton, MA, USA). The substrate is injected into the sample cell which contains the enzyme and the heat of the reaction is measured. The heat produced as a function of time is directly proportional to the conversion of the substrate into product and thus to the rate of the enzymatic reaction [58,59]. First, the total molar enthalpy

(ΔHapp) of the reaction was determined by injecting the substrate (pyr)-apelin-13 at a concentration of 1 mM to the sample cell that contained 54 nM rhPRCP. Sufficient time intervals between the injections were allowed to ensure that all of the substrate is converted to product. Complete conversion of a known amount of substrate to product gives ΔH_{app} (-216 cal/mol) of the reaction. In order to determine K_m and k_{cat} values for (pyr)-apelin-13, 10.9 mM of substrate was injected in multiple steps to the sample cell containing 50 nM rhPRCP or 301 nM hPRCP. Short time intervals between the injections were chosen so that steady-state conditions were preserved. The same strategy was used to determine the kinetic parameters (K_m, k_{cat}) for angiotensin II (ΔHapp = -367 cal/mol, 12.5 mM substrate, 50 nm rhPRCP,) and angiotensin III (ΔHapp = -1847 cal/mol 12 mM substrate, 50 nM rhPRCP) in order to verify the results generated with RP-HPLC.

4.10 (Pyr)-apelin-13 truncation by endothelial cells

HUVECs were grown in endothelial cell growth medium in T75 culture flasks at 37 °C in 5% CO_2. After reaching confluence, HUVECs were detached using TrypLE™ Express, counted and seeded at a density of 7000 cells/well in 96-well plates. Cells at passage numbers four to six were used. HUVECs were first treated with vehicle control or 400 μM KYP-2047 for 1 h at 37°C in 5% CO_2. Then, a vehicle control or 100 μM (pyr)-apelin-13 in the presence or absence of 400 μM KYP-2047 was added to the wells for 2 h, 4 h and 8 h at 37 °C in 5% CO_2. At the end of the treatment, the release of the C-terminal Phe of (pyr)-apelin-13 in the medium was quantified using RP-HPLC. The chosen concentrations of apelin and KYP-2047 were optimized based on prior publications [60], data on the IC50 value for PRCP [31] and the limit of the

detection of the RP-HPLC assay used to quantify the released Phe [33]. To exclude that any effects of apelin and KYP-2047 treatment were due to cell death, cell viability of HUVECs was determined using PrestoBlue® Cell Viability Reagent, according to manufacturer's instructions. In brief, PrestoBlue® reagent was added to the cells at a 1:10 ratio, followed by an incubation of 30 min at 37 °C. Hereafter, fluorescence at 570 nm was measured in an InfiniteTM 200 reader (Tecan Benelux, Mechelen, Belgium). More than 90% of HUVECs were still alive after apelin and KYP-2047 treatment for 8 h (data not shown).

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6. References

[1] R.S.M. Chan, J. Woo, Prevention of overweight and obesity: how effective is the current public health approach, Int. J. Environ. Res. Public Health. 7 (2010) 765–783.

[2] T.A. Nicklas, C.E.O. Neil, Prevalence of Obesity : A Public Health Problem Poorly Understood, AIMS Public Health. 1 (2014) 109-122.

[3] A.S. Kelly, S.E. Barlow, G. Rao, T.H. Inge, L.L. Hayman, J. Steinberger, et al, Severe obesity in children and adolescents: identification, associated shealth risks, and treatment approaches: a scientific statement from the American Heart Association, Circulation. 128 (2013) 1689–1712.

[4] N. Srivastava, R. Lakhan, B. Mittal, Pathophysiology and genetics of obesity, Indian J. Exp. Biol. 45 (2007) 929–936.

[5] T. Van Mieghem, R. van Bree, E. Van Herck, R. Pijnenborg, J. Deprest, J. Verhaeghe, Maternal apelin physiology during rat pregnancy: the role of the placenta, Placenta. 31 (2010) 725–730.

[6] J.D. Kim, S. Leyva, S. Diano, Hormonal regulation of the hypothalamic melanocortin system, Front. Physiol. 5 (2014) 1-7.

[7] S. Diano, New aspects of melanocortin signaling: a role for PRCP in α-MSH degradation, Front. Neuroendocrinol. 32 (2011) 70–83.

[8] A.J O'Donoghue, A.A. Eroy-reveles, G.M. Knudsen, J. Ingram, M. Zhou, J.B. Statnekov, et al, Global identification of Peptidase Specificity by Multiplex Substrate Profiling, Nat. Methods. 9 (2013) 1095–1100.

[9] T. Tabrizian, F. Hataway, D. Murray, Z. Shariat-madar, Prolylcarboxypeptidase Gene Expression in the Heart and Kidney : Effects of Obesity and Diabetes, Cardiovasc. Hematol. Agents Med. Chem. 13 (2015) 113–123.

[10] N. Wallingford, B. Perroud, G. Gao, A. Coppola, E. Gyengesi, Z. Liu, et al, Prolylcarboxypeptidase regulates food intake by inactivating α-MSH in rodents, J. Clin. Invest. 119 (2009) 2291-2303.

[11] J.K. Jeong, S. Diano, Prolyl carboxypeptidase and its inhibitors in metabolism, Trends Endocrinol. Metab. 24 (2013) 61–67.

[12] I. Castan-Laurell, C. Dray, C. Attané, T. Duparc, C. Knauf, P. Valet, Apelin, diabetes, and obesity, Endocrine. 40 (2011) 1–9.

[13] K. Berger, M.S. Winzell, J. Mei, C. Erlanson-Albertsson, Enterostatin and its target mechanisms during regulation of fat intake, Physiol. Behav. 83 (2004) 623–630.

[14] J.V. Zhang, P. Ren, O. Avsian-Kretchmer, C. Luo, R. Rauch, C. Klein, et al, Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake, Science. 310 (2005) 996–999.

[15] M. Kojima, K. Kangawa, Ghrelin : Structure and Function, Physiol. Rev. 85 (2005) 495–522.

[16] R. Nogueiras, P. Pfluger, S. Tovar, M. Arnold, S. Mitchell, A. Morris, et al, Effects of obestatin on energy balance and growth hormone secretion in rodents, Endocrinology. 148 (2007) 21–26.

[17] C.E. Odya, D.V. Marinkovic, K.J. Hammon, T.A. Stewart, E.G. Erdös, Purification and properties of prolylcarboxypeptidase (angiotensinase C) from human kidney, J. Biol. Chem. 253 (1978) 5927-5931.

[18] S. Xu, L. Lind, L. Zhao, B. Lindahl, P. Venge, Plasma prolylcarboxypeptidase (angiotensinase C) is increased in obesity and diabetes mellitus and related to cardiovascular dysfunction, Clin. Chem. 58 (2012) 1110–1115.

[19] T. Kakimoto, G. Oshima, H.S.J. Yeh, E.G. Erdös, Purification of lysosomal prolylcarboxypeptidase angiotensinase C, Biochim. Biophys. Acta. 302 (1973) 178–182.

[20] Y. Suzawa, B.Y. Hiraoka, M. Harada, T. Deguchi, (1995) High-performance liquid chromatographic determination of prolylcarboxypeptidase activity in monkey kidney, J. Chromatogr. B Biomed. Appl. 670 (1995) 152–156.

[21] K. Suga, K. Ito, D. Tsuru, T. Yoshimoto, Prolylcarboxypeptidase (Angiotensinase C): Purification and Characterization of the enzyme from Xanthomonas maltophilia, Biosco. Biotech. Biochem. 59 (1995) 298–301.

[22] I.A. Goptar, D.A. Shagin, I.A. Shagina, E.S. Mudrik, Y.A. Smirnova, D.P. Zhuzhikov, et al, A digestive prolyl carboxypeptidase in *Tenebrio molitor* larvae, Insect. Biochem. Mol. Biol. 43 (2013) 501-509.

[23] Z. Shariat-Madar, F. Mahdi, A.H. Schmaier. Identification and characterization of prolylcarboxypeptidase as an endothelial cell prekallikrein activator, J. Biol. Chem. 277 (2002) 17962–17969.

[24] F. Tan, P.W. Morris, R.A. Skidgel, E.G. Erdös, Sequencing and Cloning of Human Prolylcarboxypeptidase (Angiotensinase C), J. Biol. Chem. 268 (1993) 16631-16638.

[25] J.J. Maguire, M.J. Kleinz, S.L. Pitkin, A.P. Davenport, (Pyr1)apelin-13 identified as the predominant apelin isoform in the human heart: vasoactive mechanisms and inotropic action in disease, Hypertension. 54 (2009) 598–604.

[26] E.Y. Zhen, R.E. Higgs, J.A. Gutierrez, Pyroglutamyl apelin-13 identified as the major apelin isoform in human plasma, Anal. Biochem. 442 (2013) 1–9.

[27] J. Mallela, J. Yang, Z. Shariat-Madar, Prolylcarboxypeptidase: a cardioprotective enzyme, Int. J. Biochem. Cell Biol. 41 (2009) 477–481.

[28] C.E. Odya, D.V. Marinkovic, K.J. Hammon, T.A. Stewart, E.G. Erdös, Purification and properties of prolylcarboxypeptidase (angiotensinase C) from human kidney, J. Biol. Chem. 253 (1978) 5927–5931.

[29] S.M. Chajkowski, J. Mallela, D.E. Watson, J. Wang, C.R. McCurdy, J.M. Rimoldi, et al, Highly selective hydrolysis of kinins by recombinant prolylcarboxypeptidase, Biochem. Biophys. Res. Commun. 405 (2011) 338–343.

[30] A.J. Jalkanen, J.V. Leikas, M.M. Forsberg, KYP-2047 penetrates mouse brain and effectively inhibits mouse prolyl oligopeptidase, Basic Clin. Pharmacol. Toxicol. 114 (2014) 460–463.

[31] A.J. Jalkanen, T.P. Piepponen, J.J. Hakkarainen, I. De Meester, A.M. Lambeir, M.M. Forsber, The effect of prolyl oligopeptidase inhibition on extracellular acetylcholine and dopamine levels in the rat striatum, Neurochem. Int. 60 (2012) 301-309.

[32] J.K. Mcdonald, B.B. Zeitman, S. Ellis, Detection of a lysosomal carboxypeptidase and a lysosomal dipeptidase in highly-purified dipeptidyl aminopeptidase I (cathepsin C) and the elimination of their activities from preparations used to sequence peptides, Biochem. Biophys. Res. Comm. 46 (1972) 62–70.

[33] K. Kehoe, R. Verkerk, Y. Sim, Y. Waumans, P. Van der Veken, A.M. Lambeir, et al, Validation of a specific prolylcarboxypeptidase activity assay and its suitability for plasma and serum measurements, Anal. Biochem. 443 (2013) 232–239.

[34] J. Tamaoki, F. Sugimoto, E. Tagaya, K. Isono, A. Chiyotani, K. Konno, Angiotensin II 1 receptor-mediated contraction of pulmonary artery and its modulation by prolylcarboxypeptidase, J. Appl. Physiol. 76 (1994) 1439–1444.

[35] E.J. Bonten, A. d'Azzo, Lysosomal neuraminidase. Catalytic activation in insect cells is controlled by the protective protein/cathepsin A, J. Biol. Chem. 275 (2000) 37657–37663.

[36] K. Tatemoto, M. Hosoya, Y. Habata, R. Fujii, T. Kakegawa, M. Zou, et al, Isolation and Characterization of a Novel Endogenous Peptide Ligand for

the Human APJ Receptor, Biochem. Biophys. Res. Comm. 476 (1998) 471–476.

[37] Y. Kawamata, Y. Habata, S. Fukusumi, M. Hosoya, R. Fujii, S. Hinuma, Molecular properties of apelin : tissue distribution and receptor binding, Biochim. Biophys. Acta. 1538 (2001) 162–171.

[38] K. Higuchi, T. Masaki, K. Gotoh, S. Chiba, I. Katsuragi, K. Tanaka, Apelin, an APJ receptor ligand, regulates body adiposity and favors the messenger ribonucleic acid expression of uncoupling proteins in mice, Endocrinology. 148 (2007) 2690–2697.

[39] A. Than, Y. Cheng, L. Foh, M.K. Leow, S.C. Lim, Y.J. Chuah, et al, Apelin inhibits adipogenesis and lipolysis through distinct molecular pathways, Mol. Cell Endocrinol. 362 (2012) 227–241.

[40] C. Dray, C. Knauf, D. Daviaud, A. Waget, J. Boucher, M. Buléon, et al, Apelin stimulates glucose utilization in normal and obese insulin-resistant mice, Cell. Metab. 8 (2008) 437–445.

[41] P. Yue, H. Jin, M. Aillaud, A.C. Deng, J. Azuma, T. Asagami, et al, Apelin is necessary for the maintenance of insulin sensitivity, Am. J. Physiol. Endocrinol. Metab. 298 (2010) 59–67.

[42] C. Vickers, P. Hales, V. Kaushik, L. Dick, J. Gavin, J. Tang, et al, Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase, *J. Biol. Chem.* 277 (2002) 14838–14843.

[43] A. Murza, A. Parent, E. Besserer-Offroy, H. Tremblay, F. Karadereye, N. Beaudet, et al, Elucidation of the structure-activity relationships of apelin: influence of unnatural amino acids on binding, signaling, and plasma stability, ChemMedChem. 7 (2012) 318–325.

[44] D.K. Lee, V.R. Saldivia, T. Nguyen, R. Cheng, S.R. George, B.F. O'Dowd, (2005) Modification of the terminal residue of apelin-13 antagonizes its hypotensive action, Endocrinology. 146 (2005) 231–236.

[45] A. Murza, K. Belleville, J. Longpré, P. Sarret, E. Marsault, Stability and degradation patterns of chemically modified analogs of apelin-13 in plasma and cerebrospinal fluid, Biopolymers. 101 (2014) 297–303.

[46] S. El Messari, X. Iturrioz, C. Fassot, N. De Mota, D. Roesch, C. Llorens-Cortes, Functional dissociation of apelin receptor signaling and endocytosis: implications for the effects of apelin on arterial blood pressure, J. Neurochem. 90 (2004), 1290–1301.

[47] C. Erlanson-Albertsson, D. York, Enterostatin-A Peptide Regulating Fat Intake, Obesity Research. 5 (1997) 360-372.

[48] L. Lin, M. Park, D.A. York, Enterostatin inhibition of dietary fat intake is modulated through the melanocortin system, Peptides. 28 (2007) 643–649.

[49] T. Karl, T. Hoffmann, R. Pabst, S. von Hörsten, Extreme reduction of dipeptidyl peptidase IV activity in F344 rat substrains is associated with various behavioral differences, Physiol. Behav. 80 (2003), 123–134.

[50] V. Vicennati, S. Genghini, R. De Iasio, F. Pasqui, U. Pagotto, R. Pasquali, Circulating obestatin levels and the ghrelin/obestatin ratio in obese women, Eur. J. Endocrinol. 157 (2007) 295–301.

[51] G. Gourcerol, D.H. St-Pierre, Y. Taché, Lack of obestatin effects on food intake: should obestatin be renamed ghrelin-associated peptide (GAP)?, Regul. Pept. 141 (2007) 1–7.

[52] L.M. Seoane, O. Al-Masadi, Y. Pazos, U. Pagotto, F.F. Casanueva, Central obestatin administration does not modify either spontaneous or ghrelin-induced food intake in rats, *J. Endocrinol. Invest.* 29 (2006) 13–15.

[53] E.M. Jarho, J.L. Venäläinen, J. Huuskonen, J.A.M. Christiaans, J.A. Garcia-Horsman, M.M. Forsberg, et al, A Cyclopent-2-enecarbonyl Group Mimics Proline at the P2 Position of Prolyl Oligopeptidase inhibitors, J. Med. Chem. 47 (2004) 5605–5607.

[54] P. Van der Veken, V. Fülöp, D. Rea, M. Gerard, R. Van Elzen, J. Joossens, et al, P2-substituted N-acylprolylpyrrolidine inhibitors of prolyl oligopeptidease: biochemical evaluation, binding mode determination, and assessment in a cellular model of synucleinopathy, J. Med. Chem. 55 (2012) 9856-9867.

[55] K. Kehoe, R. Brouns, V. Verkerk, S. Engelborghs, P.P. De Deyn, D. Hendriks, et al, Prolyl Carboxypeptidase Activity Decline Correlates with Severity and Short-Term Outcome in Acute Ischemic Stroke, Neurochem. Res. 40 (2014) 81-88.

[56] M.M. Bradford, A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, Anal. Biochem. 72 (1976) 248–254.

[57] Whitepaper, The use of ITC for obtaining enzyme kinetic constants, (2014)

[58] M.L. Bianconi, Calorimetry of enzyme-catalyzed reactions, Biophys. Chem. 1267 (2007) 59–64.

[59] M.J. Todd, J. Gomez, Enzyme Kinetics Determined Using Calorimetry : A General Assay for Enzyme Activity?, Anal. Biochem. 296 (2001) 179–187.

[60] Y. Lu, X. Zhu, G.X. Liang, R.R. Cui, Y. Liu, S.S. Wu, et al, Apelin-APJ induces ICAM-1, VCAM-1 and MCP-1 expression via NF-κB/JNK signal pathway in human umbilical vein endothelial cells, Amino Acids. 43 (2012) 2125–36.