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

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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.

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

Abbreviations: α-MSH, α-melanocyte-stimulating hormone; ACE2, angiotensin-converting enzyme 2; Ala, alanine; Phe, phenylalanine; hPRCP, purified human prolyl carboxypeptidase; HUVECs, 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.

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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 (Fig. 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].

Table 1
The amino acid sequence and length of the peptides as well as the PRCP cleavage place is depicted.

Peptides	Sequence	Length (AA)
(Pyr)-apelin-13	Glp-RPRLSHKGPMP↓F	13
Enterostatin	APGP↓R	5
Ghrelin	GSSFLSPEHQRVQQRKESKKPPAKLQP↓R	28
Obestatin	FNAPFDVGIKLSGVQYQQHSQA↓L	23
α-MSH 1-13	Ac-SYSMEHFRWGKP↓V	13
Angiotensin II	DRVYIHP↓F	8
Angiotensin III	RVYIHP↓F	7
Des-Arg^9-bradykinin	RPPGFSP↓F	8

Table 2
Scheme for purification of PRCP from human placenta.

Procedure	Volume (ml)	Total protein (g)	Total activity (U)	Specific PRCP activity (U/g)	Purification factor	Yield (%)
Homogenate	212	6.1	3.5	0.6		100
60 °C, 30 min	210	3.1	3.1	1.0	1.7	89
Q-sepharose	230	2.7	2.9	1.1	1.1	92
CM-sepharose	160	0.3	2.0	7.3	6.8	68
Concanavalin A	35	0.0004	0.1	330	45.4	6

One unit of enzyme activity represents the amount of enzyme needed to convert 1 μmol of substrate per minute under the assay conditions as described in [33].

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 (Fig. 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 > 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 (Fig. 2A and B). 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