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Kinetic studies on aminopeptidase M-mediated degradation of human hemorphin LVV-H7 and its *N*-terminally truncated products

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Abstract: The human hemorphin LVV-H7 belongs to the class of μ-opiod receptor-binding peptides, which also exhibits significant affinity to insulin-regulated aminopeptidase (IRAP) thereby affecting IRAP inhibition. The inhibitory potency towards IRAP is of pharmaceutical interest for the treatment of Alzheimer's disease. Consecutive *N*-terminal cleavage of the first two amino acid residues of LVV-H7 affects a drastic increase of the binding affinity (V-H7) but ultimately leads to its complete abolition after cleavage of the next amino acid residue (H7). Therefore, we investigated LVV-H7 truncation by aminopeptidase M (AP-M) identified as a LVV-H7 degrading enzyme potentially regulating hemorphin activity towards IRAP *in vivo*. Using a selective quantitative multi-component capillary zone electrophoretic method (CZE-UV), we analyzed the AP-M-mediated subsequent proteolysis of the hemorphins LVV-H7 (L^{32}-F^{41}), VV-H7 (V^{33}-F^{41}), and V-H7 (V^{34}-F^{41}) *in vitro*. Incubations were carried out with synthetic hemorphins applied as single substrates or in combination. Maximum velocities (V_{max}), catalytic constants (turnover numbers, k_{cat}), and specific enzyme activities (EA) were calculated. L^{32} cleavage from LVV-H7 happens more than two-times faster (k_{cat}: 140 $min^{-1} \pm 9\%$, EA: 1.0 U/mg $\pm$ 9%) than V^{33} cleavage from VV-H7 (k_{cat}: 61 $min^{-1} \pm 10\%$, EA: 0.43 U/mg $\pm$ 10%) or V^{32} deletion from V-H7 (k_{cat}: 62 $min^{-1} \pm 8\%$, EA: 0.46 U/mg $\pm$ 8%). In contrast, we showed that H7 (Y^{35}-F^{41}) was neither degraded by porcine AP-M nor did it act as an inhibitor for this enzyme. Determined turnover numbers were in the same dimension as those reported for dynorphin degradation. This is the first time that AP-M-mediated truncation of natural underivatized LVV-H7 and its physiological metabolites was analyzed to determine kinetic parameters useful for understanding hemorphin processing and designing hemorphin-derived drug candidates. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: aminopeptidase M; CZE; hemorphin; IRAP; LVV-H7; enzyme kinetics

INTRODUCTION

The endogenous decapeptide hemorphin LVV-H7 (L^{32}-F^{41}, MW_{av} 1308.5 Da) is intra- and extracellularily produced from the human hemoglobin-β chain (primary Swiss Prot accession No. P68771). It is cleaved from its precursor by, e.g. cathepsin D [1], chymotrypsin-like enzyme and pepsin [2], pancreatic elastase [3], and rat liver lysosomal proteases [4]. LVV-H7 and its *N*- and *C*-terminally truncated products belong to the class of nonclassical Tyr-Pro μ-opioid receptor-binding peptides exhibiting a number of physiological activities relevant for, e.g. blood pressure regulation [5,6], analgesia, inflammatory response [7], and performance of learning and memory [5,8,9]. Therefore, hemorphin LVV-H7 has become an interesting lead for pharmaceutical purposes.

Structure-activity studies have shown that the *N*-terminus of hemorphin peptides (see Table 1) is essential for the binding properties of receptors and enzymes thus affecting their regulatory potency [8,10]. Lammerich *et al.* reported on the affinity of natural hemorphins to the G-protein coupled receptor bombesin subtype 3 (hBRS-3) which is potentially involved in lung cancer growth [10]. It was shown that VV-H7 (V^{33}-F^{41}) has a 4-fold higher affinity than LVV-H7, whereas V-H7 (V^{34}-F^{41}) had no detectable agonistic activity when measuring the Ca^{2+} – release in hBRS-3 overexpressing chinese hamster ovary and NCI-N417 lung cancer cells. Furthermore, the binding of hemorphins to the insulin-regulated aminopeptidase (IRAP) is dependent on their *N*-terminal peptide length which is found to play a role in Alzheimer's disease and in memory deficits [5,8,9,11]. Hemorphins mimic angiotensin IV (AT4) with respect to the binding of IRAP (also known as AT4 receptor), thus inhibiting its catalytic activity towards the cleavage of peptide hormone substrates such as oxytocin and Met- or Leu-enkephalin [8,11,12]. Whereas LVV-H7 and its truncated form VV-H7 (V^{33}-F^{41}) exhibit similar affinities to IRAP, the metabolite V-H7 (V^{34}-F^{41}) possesses a 10-fold increased affinity. In contrast additional loss of V^{34} (H7, V^{35}-F^{41}) results in the eradication of binding. Nevertheless, hemorphins themselves do not serve as substrates for IRAP [8,12]. In contrast to *N*-terminal

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deletion, truncation of the *C*-terminus does not seem to play an important role in the affinity to IRAP.

These two examples demonstrate essential differences in physiological effects of LVV-H7 and its *N*-terminally truncated metabolites. Therefore, discussion of the regulatory role of LVV-H7 has to take into consideration the hemorphin-degrading enzymes that modify the biological activity. Detailed knowledge on LVV-H7 catabolism and the corresponding enzyme kinetics is essential for understanding related physiological processes and pharmacological efficacy. So far, only a few studies have focused on hemorphin catabolism in, e.g. pure ACE solution [13], in rat brain and blood *in vivo* after microdialysis [2], or *in vitro* in subcellular fractions from rat brain [14], and different mammalian plasma [15,16]. In comprehensive plasma stability studies, we have recently shown that LVV-H7 is *C*-terminally truncated by ACE and additionally it is *N*-terminally hydrolyzed by the aminopeptidase M (AP-M, EC 3.4.11.2). This enzyme causes subsequent cleavage of at least three *N*-terminal amino acids producing the hemorphins VV-H7, V-H7, and H7, each of them exhibiting individual regulatory potency as discussed above [13,16] (Figure 1). AP-M, which is also named alanyl-AP [17], CD13 [18], membrane alanyl-AP or AP-N [19], is an ubiquitous *O*- and *N*-glycosylated [20] Zn^{2+} – dependent metallo-exopeptidase with broad specificity. It is anchored in plasma membranes and also found as a soluble homodimer in human plasma [21–24]. This enzyme potentially participates in precursor maturation in the secretory pathway or at the extracellular level of specialized cells [25]. Furthermore, it is used as a marker for acute myeloid leukemia and plays a role in tumor invasion [18,26]. Only very little is known about hemorphin truncation by AP-M so far [16]. Therefore, we applied a novel validated CZE method [27] to analyze AP-M-mediated proteolysis of both the original underivatized LVV-H7 and its metabolites VV-H7, V-H7, and H7 to compare reaction velocities and substrate stabilities.

MATERIALS AND METHODS

Chemicals

Hemorphin peptide substrates LVV-H7 (L^{32}-F^{41}) and its *N*-terminal degradation products VV-H7 (V^{33}-F^{41}), V-H7 (V^{34}-F^{41}), and H7 (Y^{35}-F^{41}) were synthesized by Fmoc-chemistry and purified by HPLC. The peptide content of the products was 70–72% (w/w) as determined by amino acid analysis after acidic hydrolysis (Table 1). AP-M from porcine kidney (EC 3.4.11.2) was from Calbiochem (Bad Soden, Germany). Hydroxypropyl-methylcellulose (HPMC) for CZE analysis and α-cyano-4-hydroxycinnamic acid for MALDI-TOF MS analysis were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (ACN, gradient grade), phosphoric acid (H_3PO_4, guaranteed reagent), and trifluoroacetic acid (TFA, Uvasol) were from Merck (Darmstadt, Germany).

Incubation of LVV-H7 and its Metabolites VV-H7, V-H7, and H7 with Aminopeptidase M

To analyze aminopeptidase M-mediated degradation of LVV-H7 and its metabolites VV-H7, V-H7, and H7, peptide substrates (110 µM) were incubated at 37 °C in 1 mM NaH_2PO_4, pH 8.0 buffered enzyme solution (42.6 nM) as single substrates. Aliquots (20 µl) of these mixtures were analyzed by CZE-UV and MALDI-TOF MS after 0, 1, 2, 3, 4, 5, 8, 10, 15, and 20 min of incubation following the sample preparation procedure described below. In addition, a peptide mixture containing V-H7 and H7 in a molar ratio of 2 : 1 was incubated using the same buffer and enzyme concentration. Samples were taken at distinct time points up to 20 min. To determine the concentration-dependent period of half-change of LVV-H7, the peptide was incubated under the conditions described above starting with initial concentrations of 110, 55, 27.5, and 13.8 µM. Samples (20 µl) for quantitative CZE-UV analysis were taken after distinct periods of incubation. All incubations described above were carried out in triplicate.

Sample Preparation

For sample preparation, 20 µl-aliquots were mixed with 5 µl 0.1% (v/v) TFA and precipitated subsequently by adding 50 µl

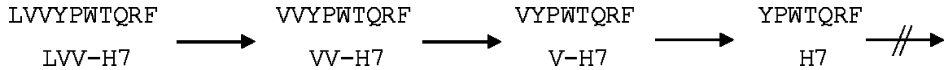

Figure 1 Subsequent truncation of human hemorphin LVV-H7 by aminopeptidase M. H7 is not further degraded by the used porcine aminopeptidase M.

Table 1 Analytical data for synthesized hemorphin peptides

Name	Sequence	$MW_{av\ (theor)}$ [Da]	$MW_{av\ (measur)}$ [Da]	Purity [%]	Peptide content [%]
LVV-H7	L^{32}VVYPWTQRF^{41}	1308.5	1308.7	> 99	72
VV-H7	V^{33}VYPWTQRF^{41}	1195.4	1195.5	> 98	70
V-H7	V^{34}YPWTQRF^{41}	1096.3	1096.2	> 98	70
H7	Y^{35}PWTQRF^{41}	997.2	997.2	> 99	72

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of cooled acetonitrile (4 °C) to terminate the reaction. After vigorous shaking and centrifugation (15 min, 12 000 × g) two aliquots of 30 µl each were either stored at −20 °C or diluted immediately with 0.1% TFA (v/v) (60 µl) to reduce the relative amount of acetonitrile and salts. The diluted samples were analyzed by CZE and MALDI-TOF mass spectrometry.

CZE-UV

Measurements by CZE for the simultaneous quantification of LVV-H7 and its *N*-terminal metabolites VV-H7, V-H7, and H7 were carried out using the validated method described in detail earlier [27]. In brief, separations were carried out at constant voltage (18 kV) after hydrodynamic anodic injection of the sample stored in a cooled autosampler (10 °C). Separations were performed at 25 °C in an uncoated fused silica capillary (57 cm × 75 µm) using 0.1 M H_3PO_4, 0.02% (w/v) HPMC (pH 2.5) as buffer. All CZE-UV analyses were performed on a P/ACE MDQ system (Beckman Coulter, Fullerton, CA, USA) controlled by the accompanying software version 2.2. Peptides were UV-detected at 214 nm by an integrated diode array detector. Quantification of analytes was achieved by external calibration curves using the peak area method. The method was validated in terms of recovery, precision, accuracy, ruggedness, linear range, and lower limits of detection and quantification [27].

MALDI-TOF MS

Qualitative mass spectrometric detection of peptides from the incubation mixtures was carried out in the linear positive ion mode on a Voyager DE-Pro instrument (1.2 m flight tube, 337 nm laser, Applied Biosystems, Darmstadt, Germany) supported by the Biospectrometry Workstation 5.1 software for controlling and the Data Explorer 4.0 program for data analysis. Analyses were performed using the dried droplet technique [16].

RESULTS AND DISCUSSION

N-terminally truncated forms of LVV-H7 exhibit individual physiological properties with regard to receptor or enzyme affinities [8,10]. As recently shown in *in vitro* plasma stability studies, subsequent *N*-terminal truncation of LVV-H7 is catalyzed by AP-M [16] producing at least three proteolytic products as depicted in Figure 1. Therefore, we decided to investigate the enzymatic conversion by AP-M of both natural underivatized LVV-H7 and its metabolites acting as substrates for that exopeptidase, which potentially regulates *in vivo* activity of hemorphins as known for the opiate receptor-binding dynorphins [28]. To the best of our knowledge, no kinetic data for AP-M-mediated degradation of that hemorphins are available so far, although information on enzyme kinetics are of importance for unraveling physiological processes and designing potential hemorphin-derived drug candidates.

Commercially available AP-M from porcine kidney was used as a model enzyme for the human analogue because the amino acid sequences of both species (pig: primary Swiss Prot accession No. P15145; human: primary Swiss Prot accession No. P15144) exhibit a sequence identity of 78.8% as determined by the LALIGN software and the BLOSUM62 matrix including conserved regions of metal binding and active sites [29].

LVV-H7, VV-H7, V-H7, and H7 were simultaneously quantified as underivatized peptides by a selective multi-component CZE-UV method described recently [27]. Within the upper micromolar range (6–150 µM), this procedure was robust, precise ($CV_{repeatability} \leq 8\%$, $CV_{reproducibility} \leq 11\%$), and accurate (accuracy: −8–4%) with 7 µM as the lower limit of quantification (LLOQ). Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) was used for substrate and product identification (data not shown) [16]. Many other techniques for investigating the activity of several aminopeptidases make use of unnatural amino acid substrates either labeled with fluorescent groups [30,31] or radioactive markers [32]. In contrast, our method allows the detection of structurally unchanged hemorphins. Owing to the relatively high LLOQ (7 µM) of the used CZE-UV method, elevated substrate concentrations in the upper micromolar range were required for reliable measurement [27]. All incubations were carried out with hemorphin concentrations (110 µM) exceeding a 2500-fold excess when compared to AP-M molarity causing enzyme saturation and thus zero-order kinetics. Therefore, measurement in the steady-state phase of unsaturated enzyme conditions as usually chosen for the determination of kinetic parameters was not feasible. As a result of these conditions, we assumed that V_{ini} was very close or identical to V_{max} of enzymatic catalysis (Table 2). This assumption was supported by the fact that (i) the concentrations of VV-H7 (Figure 2(b)) and V-H7 (Figures 2(c) and (e)) decreased linearly within the entire period of incubation, and that (ii) initial degradation velocities of LVV-H7 applied in four different concentrations (14–110 µM) were identical within the given precision (Figure 2(f)), and that (iii) the corresponding apparent periods of half-change of LVV-H7 truncation rose linearly with the applied concentration (Figure 2(f)). Furthermore, Michaelis–Menten constants (K_M) for different dynorphin peptides truncated by rat AP-M are between 0.02 and 8 µM [28] leading to the assumption that K_M-values for the tested hemorphins might be in the same range. With this estimate, hemorphin concentrations applied corresponded to approximately 10–4000 K_M affecting enzyme saturation and zero-order kinetics. The magnitude of V_{ini} was determined by the slope of linear regression of the substrate concentration-time curve within the first 5 min of incubation characterized by smallest relative product concentrations as well as paramount substrate excess (80–100% of total hemorphins). Corresponding specific enzyme activities were assessed considering a molecular weight of 140 kDa for

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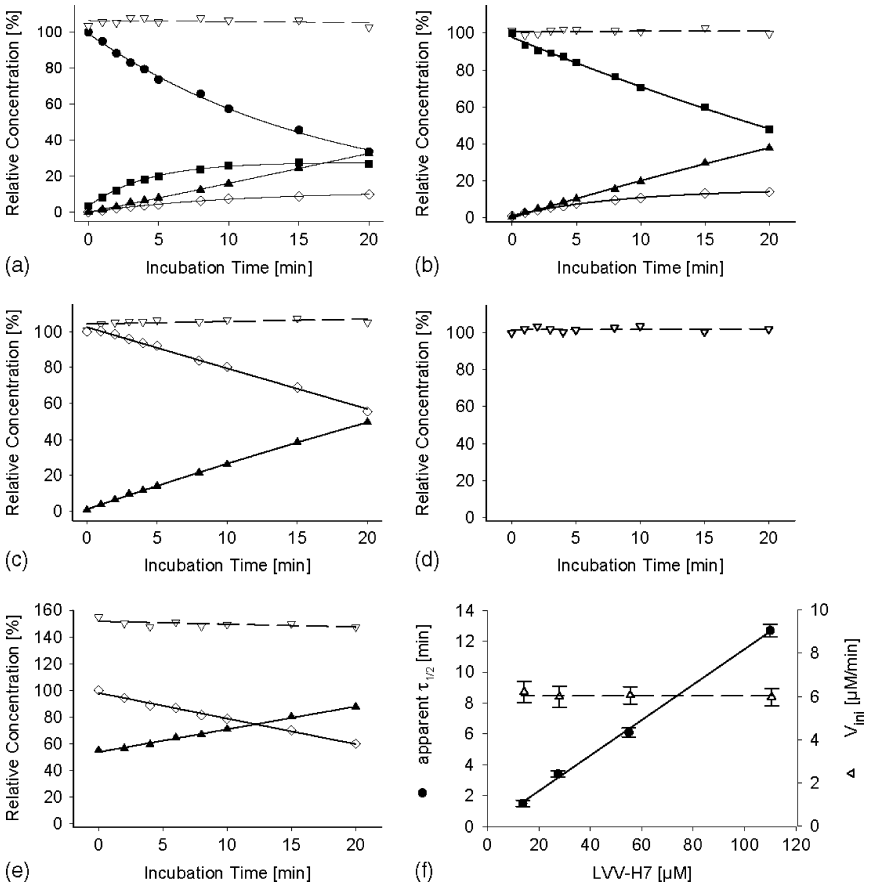

Figure 2 Representative concentration-time profiles and kinetics of aminopeptidase M-mediated degradation of human LVV-H7 and its *N*-terminally truncated products VV-H7, V-H7 and H7. Aminopeptidase M (0.04256 μM) mediated degradation of (a) LVV-H7 (L^{32}-F^{41}); (b) VV-H7 (V^{33}-F^{41}); (c) V-H7 (V^{34}-F^{41}); (d) H7 (Y^{35}-F^{41}); and (e) V-H7 incubated in the presence of H7 (initial molar ratio of 2 : 1). Open triangles: total concentration of all detected hemorphins; black circles: LVV-H7; black squares: VV-H7; open diamonds: V-H7; black triangles: H7 and (f) apparent periods of half-change (black circles) and corresponding initial velocities (open triangles) of LVV-H7 applied in different concentrations. Incubations were carried out in 1 mM NaH_2PO_4, pH 8.0 at 37 °C.

Table 2 Characteristics of hemorphin substrates and enzyme kinetics for the degradation by aminopeptidase M

Substrate	Name	App. $\tau_{1/2}$ (min)	$V_{ini} \approx V_{max}$ (μM/min)	k_{cat} (min^{-1})	Specific AP-M activity (U/mg)
L^{32}VVYPWTQRF^{41}	LVV-H7	12.7 ± 1.3	5.97 ± 9%	140 ± 9%	1.00 ± 9%
V^{33}VYPWTQRF^{41}	VV-H7	19.5 ± 1.1	2.59 ± 10%	61 ± 10%	0.43 ± 10%
V^{34}YPWTQRF^{41}	V-H7	22.5 ± 1.2	2.73 ± 8%	62 ± 8%	0.46 ± 8%
Y^{35}PWTQRF^{41}	H7	no degrad.	no degrad.	no degrad.	no degrad.
[a] V^{34}YPWTQRF^{41}	V-H7	23.8 ± 0.9	2.48 ± 9%	58 ± 9%	0.42 ± 9%

[a] in the presence of H7.
App. $\tau_{1/2}$, apparent (measured) period of half-change; $[E]_T$, total enzyme concentration (0.04256 μM); no degrad., no degradation detectable; k_{cat}, catalytic constant (turnover number) $k_{cat} = V_{max}/[E]_T$; MW_{av}, averaged molecular weight; specific enzyme activity is given in units/mg enzyme, U, unit, one unit is defined as the amount of AP-M that causes the degradation of 1 μmol hemorphin per min; V_{ini}, initial velocity determined between 1 and 4 min of incubation assuming zero-order kinetic of degradation; V_{max} maximum velocity of substrate degradation. Numbering of hemorphin residues is according to the precursor protein human hemoglobin-β (primary Swiss Prot accession No. P68771). All data were obtained from triplicate measurements.

AP-M [33] thereby assuming that both catalytic sites of the homodimer (MW 280 kDa) act independently and with identical specificity and velocity [28].

As demonstrated in Figure 2(a), LVV-H7 was degraded by AP-M producing *N*-terminally truncated products as schematically shown in Figure 1. LVV-H7

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was cleaved releasing VV-H7 as its primary major product followed by the generation of V-H7 and of H7 in the end (Figure 2(a)). Additional peptide metabolites were not produced as obvious from the constant sum of all detected hemorphin concentrations during each incubation (Figure 2(a)–(e)). Accordingly, each product, except H7 (Figure 2(d)), acted as a substrate for AP-M.

The described proteolytic cascade (Figure 1) reflects the situation of competitive enzyme inhibition by at least two products, which hampers the determination of kinetic constants. To reduce the complexity of the incubation mixture and to make kinetic investigations more reliable, we incubated the relevant *N*-terminal metabolites of LVV-H7 as single substrates in individual experiments (Figure 2(b)–(d)). Similar to LVV-H7, VV-H7 was also truncated generating V-H7 and H7 successively (Figure 2(b)). Incubation of V-H7 revealed that this substrate was truncated yielding exclusively H7 (Figure 2(c)), which itself was resistant against proteolytic cleavage as indicated by the constant concentration within the monitored incubation period (Figure 2(d)). However, to test the potential inhibitory activity of H7 we coincubated V-H7 and H7 in an initial molar ratio of 2 : 1 (Figure 2(e)) in order to compare the resulting velocity of substrate decrease with that obtained from V-H7 alone. As illustrated in Figure 2(c) and (e) no significant differences in the degradation rate were observed demonstrating the lack of fundamental inhibitory potential which is in conformity with previous results [34]. In consequence, quantitative interpretation of both concentration-time profiles resulted in nearly identical values for the initial velocity (V_{ini}), catalytic constant (k_{cat}, turnover numbers), and specific AP-M activity for V-H7 truncation (Table 2).

As listed in Table 2 the initial cleavage of L^{32} from LVV-H7 ($k_{cat} = 140\ min^{-1} \pm 9\%$) occurred 2.3-fold faster than the truncation of V^{33} from VV-H7 ($61\ min^{-1} \pm 10\%$) indicating a significant lower stability of LVV-H7 against proteolysis. Interestingly, Garreau *et al.* reported that LVV-H7 and VV-H7 act as competitive porcine AP-M inhibitors with identical potencies slowing the hydrolysis of the synthetic substrate alanine-*p*-nitroanilide (Ala-*p*Na) [34]. These findings indicate that both hemorphins exhibit similar binding affinities to AP-M when accumulating the Michaelis–Menten complex but differ in their kinetic constants for product generation. Furthermore, our results showed similar catalytic constants for the deletion of V^{33} from VV-H7 ($61\ min^{-1} \pm 10\%$) and of V^{34} from V-H7 ($62\ min^{-1} \pm 8\%$) revealing similar proteolytic stability against AP-M activity. Therefore, the rate of production and degradation of V-H7 were very similar resulting in a nearly constant V-H7 concentration course after the initial phase (from 5 to 20 min) as illustrated in Figure 2(a) and (b). These degradation kinetics underline that AP-M is an enzyme with broad specificity which is known to cleave preferentially at the *C*-terminus of alanine residues but also hydrolyzes amide bonds to other amino acids [30]. Consecutive hemorphin substrate degradation by truncating Y^{35} from H7 did not occur because of the adjacent proline residue [34,35]. In contrast, incubations in mammalian plasma *in vitro* showed the additional appearance of the metabolite W^{37}-F^{41} [15,16] possibly caused by the cleavage of the intact dipeptide $Y^{35}P^{36}$ by AP-M. Whether this discrepancy is due to the specific properties of porcine AP-M or to the different incubation matrices or to the activity of another plasma enzyme (dipeptidyl peptidase IV, DPP IV) remains to be elucidated.

The specific AP-M activity (Table 2) for cleaving the initial *N*-terminal amino acid residue from LVV-H7, VV-H7, and V-H7 (1 , 0.43 and 0.46 U/mg) appeared to be quite low when compared to synthetic labeled amino acid substrates but are relatively high when compared to the truncation of other physiological peptides. The manufacturer characterized the applied AP-M enzyme charge with a specific activity of 17.3 U/mg for the hydrolysis of Leu-*p*Na thus considerably deviating from our hemorphin-derived values. However, such differences may also occur to a much higher degree as reported by Bawab *et al.* [36]. They showed that AP-M from hog kidney degraded Leu-*p*Na with a 2-fold higher activity than for Ala-*p*Na and with a 5700-fold higher activity than determined for Leu-enkephalin, which is a well-studied peptide substrate for AP-M [36]. Specific activities similar to those in our study were obtained for AP-Ms purified from porcine muscle (3.9 U/mg), mucosa (3 U/mg), and kidney (3.8 U/mg) when hydrolyzing Leu-enkephalin [37]. In contrast, Bausback *et al.* determined much lower activities for the truncation of Leu-enkephalin (0.0156 U/mg) and Met-enkephalin (0.0183 U/mg) by AP-M from vascular plasma membrane [38] which might be due to an insufficiently purified enzyme. The dimensions of the catalytic constants of our study (Table 2) are in good accordance to those published by Savafi *et al.* for the truncation of dynorphin peptides by rat AP-M being in the range from 4 to 240 min^{-1}. At least our data support earlier findings [16] that LVV-H7 in plasma is degraded more rapidly by ACE (k_{cat}: 3000–13 000 min^{-1}) [13] than by AP-M (k_{cat}: 140 min^{-1}).

Further kinetic studies should focus on hemorphin degradation *in vitro* in, e.g. body fluids and tissues representing suitable biological systems for the analysis of LVV-H7 degradation under more complex physiological conditions. As obvious from the data discussed above kinetic investigations using the natural hemorphin substrates are crucial in characterizing the formation of LVV-H7 from hemoglobin precursors and the subsequent enzymatic degradation.

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CONCLUSIONS

The study described herein was performed to analyze *N*-terminal proteolytic cleavage of the natural underivatized hemorphin substrates LVV-H7, VV-H7, V-H7, and H7 by AP-M. Obtained kinetic data for the calculation of maximum velocity of hemorphin degradation, catalytic constants of AP-M (turnover numbers), and related specific activities will improve the understanding of the regulatory role of hemorphins and may be considered in future pharmaceutical development aiming to introduce LVV-H7-based drug candidates. From the more technical side of view, the present study demonstrates the highly reliable suitability of CZE-UV procedures applied for selective and simultaneous quantification of structurally unchanged endogenous peptides thus avoiding the use of unnatural fluorescent or radioactive labels.

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