HHS Public Access

Author manuscript

*Biochem Biophys Res Commun*. Author manuscript; available in PMC 2019 October 22.

Published in final edited form as:

*Biochem Biophys Res Commun*. 2019 October 22; 518(4): 651–656. doi:10.1016/j.bbrc.2019.08.098.

# Mast cell peptidases (carboxypeptidase A and chymase)-mediated hydrolysis of human angiotensin-(1–12) substrate

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## Abstract

Angiotensin processing peptidases (carboxypeptidase A (CPA) and chymase) are stored in cardiac mast cell (MC) secretory granules in large quantity and are co-released into the extracellular environment after activation/degranulation. In the human heart, chymase is primarily responsible for angiotensin II (Ang II) generation from the alternate substrate angiotensin-(1–12) (Ang-(1–12)). We investigated the individual and combined hydrolytic specificity of CPA and chymase enzymes (1:1 and 1:⅓ ratio) in the processing of the human Ang-(1–12) (hAng-(1–12)) substrate. To determine the $K_m$ and $V_{max}$, the CPA and re-combinant human chymase (rhChymase) enzymes were incubated with increasing concentrations of hAng-(1–12) substrate (0–300 μM). We found that CPA alone sequentially metabolized hAng-(1–12) substrate into angiotensin-(1–9) (Ang-(1–9), 53%), Ang II (22%) and angiotensin-(1–7) (Ang-(1–7), 11%) during a 15min incubation. In the presence of rhChymase alone, $^{125}$I-hAng-(1–12) was directly metabolized into Ang II (89%) and no further hydrolysis of Ang II was detected. In the presence of both CPA + rhChymase enzymes (1:1 or 1:⅓ ratio), the amount of Ang II formation from $^{125}$I-hAng-(1–12) within a 5 min incubation period were 68% or 65%, respectively. In the presence of both (CPA + rhChymase), small amounts of Ang-(1–9) and Ang-(1–7) were generated from $^{125}$I-hAng-(1–12). The $K_m$ and $V_{max}$ values were 150 ± 5 μM and 384 ± 23 nM/min/mg of CPA and 40 ± 9 μM and 116 ± 20 nM/min/mg of rhChymase. The catalytic efficiency ($V_{max}/K_m$ ratio) was higher for rhChymase/hAng-(1–12) compared to CPA/hAng-(1–12). Compared to CPA, chymase has a much higher affinity to hydrolyze the hAng-(1–12) substrate directly into Ang II. In addition, Ang II and Ang-(1–7) are the end products of chymase and CPA, respectively. Overall, our findings suggest that the Ang II generation from hAng-(1–12) is primarily mediated by chymase rather than CPA.

### Keywords

Mast cell protease; Carboxypeptidase A; Chymase; Angiotensin-(1–12); Angiotensin I; Angiotensin II; Metabolism; Renin-angiotensin system; Angiotensinogen

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

## 1. Introduction

Mast cells (MCs) are the immune cells having abundance of electron-dense secretory granules filled with large amounts of preformed powerful chemical compounds called MC mediators. In addition to these mediators, MC secretory granules also store a number of MC-specific proteases including tryptase, chymase and carboxypeptidase A (CPA). Chymase and CPA proteases are stored in remarkably high amounts in human MC secretory granules (4.5 and 16 pg per MC in adult foreskin, respectively) [1,2]. MCs are present throughout the connective and mucosal tissues of the body. MCs involvement in the pathophysiology of cardiovascular disorders continues to receive attention among the scientific community [3–5]. Increased numbers of cardiac MCs have been found in adversed myocardial remodeling of animal models as well as human heart tissues with coronary spasm, idiopathic dilated, ischemic cardiomyopathy and at the inflammatory sites in other diseases [6–12].

Based on the content of neutral proteases in secretary granules, two subtypes of MCs have been recognized in human tissues: tryptase-positive MCs ($MC_T$) and $MC_{TC}$, which is positive for tryptase in combination with chymase, CPA and cathepsin G [1,13]. A single α-form of chymase has been found in human MCs [14]. Whereas human tissues contain several types of CPA (CPA1 to CPA6) that have diverse functions ranging from catabolism to protein maturation [15–18]. CPA1 and CPA2 are pancreatic exopeptidases. CPA3 (originally called MC-CPA) has only been detected in MC and MC-like cell lines [17]. CPA4, previously reported as CPA3 by Huang et al. [16] was renamed by a gene nomenclature committee in the order of their discovery. Both CPA4 and CPA5 have not been well studied. CPA6 is broadly expressed in many tissues and has been linked to Duane syndrome [19,20].

Various studies show the existence of additional angiotensin substrates upstream of Ang I and increased expression of angiotensin processing enzymes in human cardiovascular and car-diometabolic diseased tissues [21–26]. The existence of an extended form of Ang I, the dodecapeptide angiotensin-(1–12), which serves as a primary substrate for Ang II formation, and the co-existence of hydrolytic proteases in human atrial appendage tissue radically altered our understanding of Ang II production [22]. Our research clearly established that chymase is primarily responsible for direct Ang II generation from the alternate substrate Ang-(1–12) in addition to Ang I in human and rodents [22–24,27,28]. Compared to Ang I, an increased level of Ang-(1–12) was detected in rat tissues [29]. Another study shows the expression of Ang-(1–12) was higher in spontaneously hypertensive rat heart and kidney tissues compared to corresponding normotensive Wistar-Kyoto rats [30].

MC proteases (chymase and CPA) are stored in their fully active form but they have no functional effects as long as they are confined within the MC. Activation and degranulation of MCs lead to the massive release of proteases into the extracellular environment, which might have a major impact on MC-driven cardiovascular disease development and progression [31,32]. Since CPA is also released along with chymase from the MC into the extracellular environment after activation/degranulation, we investigated the individual and

combined effects of CPA and rhChymase in the processing of human Ang-(1–12) (hAng-(1–12)) substrate. Our findings show that CPA and rhChymase hydrolyze the hAng-(1–12) substrate differently. Compared to CPA, rhChymase has a higher substrate affinity and catalytic efficiency for hAng-(1–12) to generate Ang II.

## 2. Materials and Methods

### 2.1. Reagents

All custom-made angiotensin peptides (hAng-(1–12), Ang I, Ang-(1–9), Ang II, and Ang-(1–7); purity >98%) were purchased from GenScript USA Inc. (Piscataway, NJ). CPA and recombinant human chymase (rhChymase) enzymes were purchased from Sigma-Aldrich Co. (St. Louis, MO). $^{125}$I was purchased from Perki-nElmer Life and Analytical Sciences, Inc. (Waltham, Massachusetts). All other chemicals used in this study were of analytical grade and were obtained from Sigma (St. Louis, MO) and Fisher Scientific (Atlanta, GA).

### 2.2. Radiolabeling of hAng-(1–12) peptide and HPLC purification

hAng-(1–12) peptide {DRVYIHPFHLVI} was radiolabeled with $^{125}$I at the Tyrosine 4th position using oxidant chloramine-T and purified on a C18 column by reverse-phase high performance liquid chromatography (HPLC), as previously described [23].

### 2.3. Hydrolysis of $^{125}$I-hAng-(1–12) substrate by CPA and rhChymase enzymes

Metabolic products of $^{125}$I-hAng-(1–12) by CPA and rhChymase enzymes were analyzed by HPLC. Briefly, in a 200 μL reaction volume highly purified radiolabeled human $^{125}$I-Ang-(1–12) substrate (~50 fmoles, specific activity 3900 cpm/fmol, purity ≥98%) was incubated with CPA (0.325 μg/mL), rhChymase (0.325 μg/mL) or a combination of CPA + rhChymase (1:1 or 1:⅓ ratio) in 50 mM Tris-HCl buffer solution containing 150 mM NaCl (pH 8.0) for 15 min (5 min for combination experiments) at 37 °C. The enzymatic re-actions were stopped by adding an equal volume of 1% phosphoric acid and centrifuged at 28,000 *g* for 10 min. The $^{125}$I-hAng-(1–12) products were separated by HPLC on a C18 column using a linear gradient from 10% to 50% mobile phase B at a flow rate of 0.35 mL/min at 32 °C. The solvent system consisted of 0.1% phosphoric acid (mobile phase A) and 80% acetonitrile/0.1% phosphoric acid (mobile phase B). The eluted $^{125}$I-hAng-(1–12) products were monitored by an in-line flow-through gamma detector (BioScan Inc., Washington, DC). Products were identified by comparison of retention times of synthetic ($^{125}$I) standard Ang peptides and the data were analyzed with Shimadzu LCSolution (Kyoto, Japan) acquisition software.

### 2.4. $K_m$ and $V_{max}$ of CPA and rhChymase for hAng-(1–12) substrate

To determine the $K_m$ and $V_{max}$ of cardiac CPA and rhChymase enzymes for hAng-(1–12) substrate, CPA (0.325 μg/mL) or rhChymase (0.325 μg/mL) were incubated with increasing concentrations (0–300 μM) of non-radiolabeled hAng-(1–12) substrate in 200 μL of 50 mM Tris-HCl buffer solution containing 150 mM NaCl (pH 8.0) at 37 °C for 20 min. The hydrolytic products generated from the hAng-(1–12) substrate by CPA and rhChymase were separated by HPLC connected to UV-detector as described above and eluted fractions were monitored as the absorbance at 215 nm. The concentrations of hAng-(1–12) products were

determined using a standard curve of angiotensin synthetic peptides. The $K_m$ and $V_{max}$ of CPA and rhChymase for hAng-(1–12) substrate were calculated using the Michaelis-Menten equation.

### 2.5. Statistical analysis

Experiments were repeated three or more times. Data were analyzed using GraphPad Prism 7.0 software (San Diego, CA) and are presented as mean ± SEM.

## 3. Results

As shown in Fig. 1, a highly pure (purity ≥98%) radiolabeled $^{125}$I hAng-(1–12) substrate was used in CPA and rhChymase-mediated hydrolysis studies. The purity of $^{125}$I-hAng-(1–12) was routinely checked on the HPLC to make sure that the radiolabeled substrate was not degraded at the time it was used for enzymatic hydrolysis.

Fig. 2 illustrates the HPLC chromatogram of the hydrolytic products generated from $^{125}$I-hAng-(1–12) substrate by CPA or rhChymase. We found that CPA sequentially metabolized the $^{125}$I-hAng-(1–12) into Ang-(1–9) (53% (major product)), Ang II (22%) and Ang-(1–7) (11%) (Fig. 2A). rhChymase directly metabolized the $^{125}$I-hAng-(1–12) substrate into Ang II (89%) and no further hydrolysis of Ang II was detected (Fig. 2B). Ang II (68%) was the major product generated from hAng-(1–12) when the substrate was incubated with both CPA + rhChymase (1:1 ratio) for 5 min (Fig. 3A). In addition to Ang II, a small amount of Ang-(1–9) and Ang-(1–7) products (11% and 10%, respectively) were also detected in the reaction mixture. When CPA and rhChymase were incubated with hAng-(1–12) substrate at 1:⅓ ratio for 5 min, Ang II was still a major product (65%) (Fig. 3B). Ang-(1–9) and Ang-(1–7) production from $^{125}$I-hAng-(1–12) amounted to only 27% and 7%, respectively (Fig. 3B). To determine Ang-(1–9) hydrolysis by rhChymase, human $^{125}$I-hAng-(1–12) substrate was first incubated with CPA (0.325 μg/mL) for 5 min, next the CPA activity was stopped by adding 50 μM of benzylsuccinate [19], and then this reaction mixture was further incubated with rhChymase (0.325 μg/mL) for an additional 5 min. In these experiments, we found that the hydrolytic product of CPA “Ang-(1–9)” was not metabolized by rhChymase (data not shown).

Kinetic analysis ($K_m$, $V_{max}$ and catalytic efficiency) of CPA and rhChymase enzymes for hAng-(1–12) substrate was also determined. A representative curve showing the hydrolytic products generated by CPA and rhChymase with increasing concentrations of hAng-(1–12) substrate is shown in Fig. 3C. The $K_m$ and $V_{max}$ were 150 ± 5 μM and 384 ± 23 nM/min/mg of CPA/hAng-(1–12) and 40 ± 9 μM and 116 ± 20 nM/min/mg of rhChymase/hAng-(1–12) reactions, respectively (Table 1). The catalytic efficiency (the ratio of $V_{max}/K_m$ ratio) was higher for rhChymase/hAng-(1–12) (2.97 ± 0.1) compared to CPA/hAng-(1–12) (2.56 ± 0.1).

## 4. Discussion

The discovery of an Ang I upstream precursor (Ang-(1–12)), which serves as an alternate substrate for biologically active Ang II formation in the human heart, radically altered our

understanding of Ang II production [22]. Our laboratory has done pioneer work in unraveling the complexity of the biotransformation pathways that account for the formation of the Ang II hormone from angiotensinogen [24–26,33–37]. Although chymase-mediated Ang II formation from Ang I has a long-standing history in humans [38,39], the clinical importance of the Ang II-forming pathways from hAng-(1–12)/MC proteases axis is largely unknown. To date, the specific role of MC CPA in hAng-(1–12) substrate hydrolysis has not been demonstrated. In this study, we investigated the individual and combined effects of two MC proteases to hydrolyze the hAng-(1–12) substrate.

Incubation of CPA with hAng-(1–12) substrate for 15 min at 37 °C, yielded the generation of Ang-(1–9), Ang II and Ang-(1–7) and no further hydrolysis was detected. rhChymase rapidly generates Ang II from hAng-(1–12) and no further hydrolysis was detected. In the presence of both CPA and rhChymase (1:1 and 1:⅓ ratio), we found that hAng-(1–12) was rapidly metabolized into Ang II within 5 min. In both conditions, relatively small amounts of Ang-(1–9) and Ang-(1–7) products were generated. Although hAng-(1–12) is rapidly metabolized into Ang II directly, our current investigation shows that CPA-mediated Ang-(1–9) production has negligible substrate affinity for rhChymase enzyme to generate Ang II. These findings clearly indicate that hAng-(1–12) is primarily hydrolyzed by chymase into Ang II. Furthermore, the enzyme kinetic results confirm the specificity and primacy of chymase over CPA to generate directly Ang II product from hAng-(1–12) substrate.

The classical view of the biochemical pathways for the formation of biologically active angiotensin peptides continues to undergo significant revision as new data uncovers the existence of important alternate non-renin dependent mechanisms of Ang II formation from the novel dodecapeptide Ang-(1–12) by MC proteases. In contrast to rodents, humans only have the α-form of chymase (a chymotryptic serine endopeptidase) stored in MC secretory granules in large amounts [14]. The MC CPA resembles bovine pancreatic CPA in cleaving C-terminal aromatic and aliphatic amino acid residues [2]. CPAs cleave newly exposed C-terminal residues after endopeptidase cleavage by chymase, thereby sequentially degrading common proteins and peptides substrates [40,41]. Our recent findings suggest that the CPA has a low affinity to cleave the newly exposed C-terminal amino acid of Ang II generated from hAng-(1–12) hydrolysis by rhChymase. Once Ang-(1–9) is cleaved by CPA from hAng-(1–12), chymase has negligible substrate affinity to hydrolyze the $Phe^{8}$-$His^{9}$ bond of the Ang-(1–9) to generate Ang II product (Fig. 4).

The hydrolytic potential of an enzyme depends on the amino acid sequence of the peptides/proteins at the cleavage site. In Table 2, we document the characteristic differences between chymase and CPA enzymes. CPA1 and CPA2 isolated from rat mesenteric arterial bed perfusate (identical with their pancreatic counterparts), hydrolyze rat Ang-(1–12) substrate differently [42]. CPA1 has negligible affinity to hydrolyze the C-terminal amino acid of the rat Ang-(1–12) sequence. However, CPA2 rapidly hydrolyzes the rat Ang-(1–12) into Ang I. Further hydrolysis of Ang I into Ang-(1–9) by CPA2 is negligible. The amino acids sequence of the rat and hAng-(1–12) are different (rat sequence, $Asp^{1}$-$Arg^{2}$-$Val^{3}$-$Tyr^{4}$-$Ile^{5}$-$His^{6}$-$Pro^{7}$-$Phe^{8}$-$His^{9}$-$Leu^{10}$-$Leu^{11}$-$Tyr^{12}$ and human sequence, $Asp^{1}$-$Arg^{2}$-$Val^{3}$-$Tyr^{4}$-$Ile^{5}$-$His^{6}$-$Pro^{7}$-$Phe^{8}$-$His^{9}$-$Leu^{10}$-$Val^{11}$-$Ile^{12}$).In humans, -$Val^{11}$-$Ile^{12}$ is present at the C-terminal end, whereas in the rat, -$Leu^{11}$-$Tyr^{12}$ exists at the C-terminus [24]. Both hAng-(1–12) and

Ang I substrates have high affinity for human cardiac chymase to generate Ang II. We showed for the first time that rat cardiac chymase has a much higher substrate affinity for rat Ang-(1–12) substrate compared to Ang I to generate Ang II [33]. Our current findings show that CPA hydrolyzes the hAng-(1–12) substrate into Ang-(1–9), Ang II and Ang-(1–7) products. No intermediate products (Ang-(1–11) and Ang I) were detected in the reaction mixture suggesting that CPA sequentially cleaves the hAng-(1–12) C-terminal three peptide bonds ($Val^{11}$-$Ile^{12}$, $Leu^{10}$-$Val^{11}$ and $His^{9}$-$Leu^{10}$) rapidly. Once Ang-(1–9) is generated (after the cleavage of C-terminal $His^{9}$-$Leu^{10}$ bond of Ang I) by CPA, the formation of Ang II and Ang-(1–7) is markedly delayed, indicating that CPA has less substrate affinity for Ang-(1–9) to generate Ang II, and an even further decrease for Ang II to generate Ang-(1–7) ($Phe^{8}$-$His^{9}$ and $Pro^{7}$-$Phe^{8}$ bonds, respectively).

CPA3 (the MC CPA) shares significant homology with the other CPA subfamilies. CPA3 resembles the pancreatic CPA1 in cleaving the C-terminal end of aromatic (Phe, Tyr and Trp) and aliphatic (Ala, Leu, Ile and Val) amino acids. CPA3 functions together with endo-peptidases (chymases and tryptases) secreted from mast cells to degrade proteins and peptides, including Ang I [41,43]. CPA3 may be involved in host defense against certain parasites, snake venom toxins, and the vasoconstricting peptide endothelin 1 [44–46]. CPA3 is upregulated, making it a potential diagnostic parameter in allergic inflammation and/or autoimmune disease models [47–50]. CPA3 mRNA was not detected in normal human tissues (including lung, heart, and kidney) but its expression could be induced in disease [16]. Our studies clearly show that both chymase and Ang-(1–12) substrate were predominantly expressed intracellularly in human atrial cardiac myocytes obtained from diseased patients [22]. The expression and precise role of CPA in diseased hearts remains to be established.

Overall, our study suggests that Ang II generation from hAng-(1–12) substrate was primarily mediated by chymase rather than CPA. Our studies also suggest that selective inhibition of chymase may provide greater benefit in the management of adverse cardiac remodeling than the MC CPA therapeutic approaches.

## Acknowledgment

This work was supported by grant from the National Heart, Blood, Lung Institute of the National Institutes of Health (P01 HL-051952).

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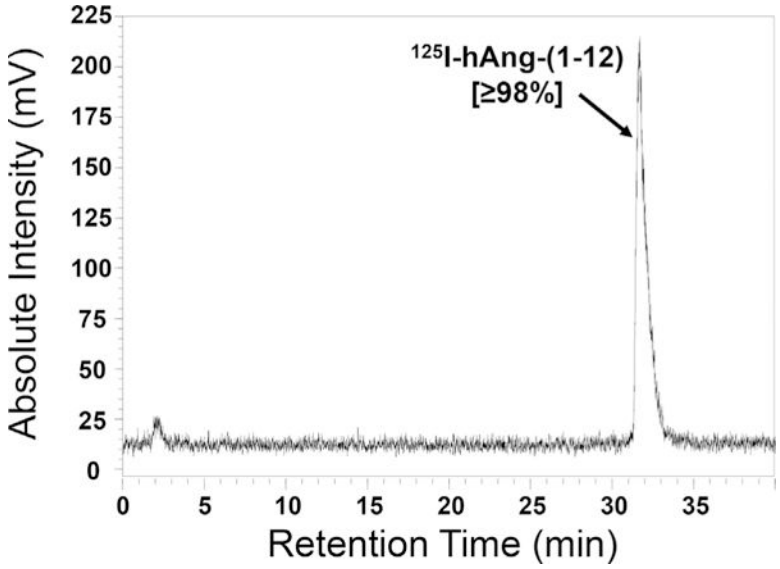


**Fig. 1.**

HPLC Chromatogram of Purified $^{125}$I-hAng-(1–12). The HPLC chromatogram shows the $^{125}$I-hAng-(1–12) substrate was highly pure (purity ≥98%).

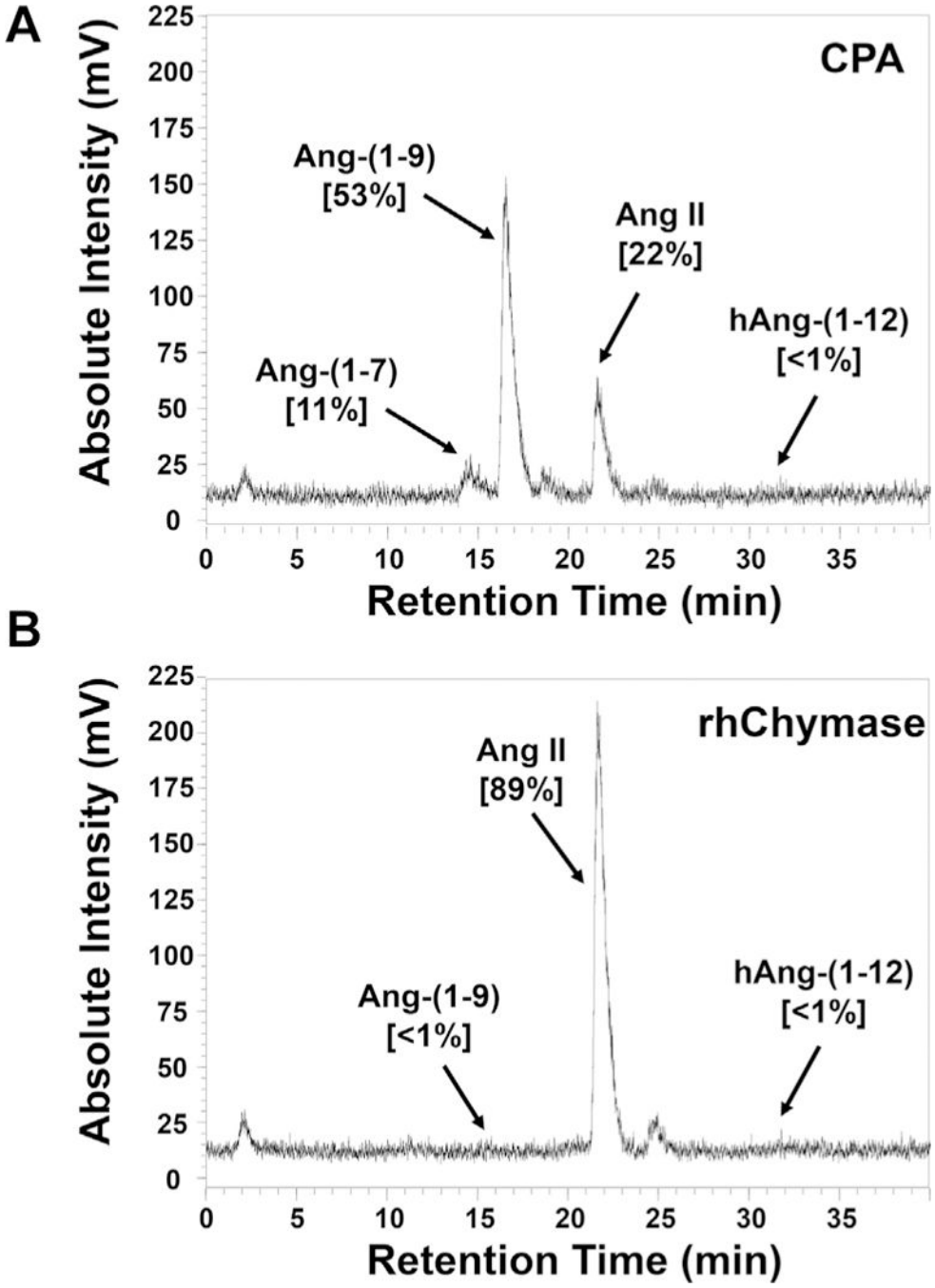


**Fig. 2.**

Hydrolysis of $^{125}$I-hAng-(1–12) by CPA or rhChymase alone. The HPLC chromatogram shows the $^{125}$I-Ang products generated from $^{125}$I-hAng-(1–12) substrate by (A) CPA or (B) rhChymase. Results are representative of three or more separate experiments.

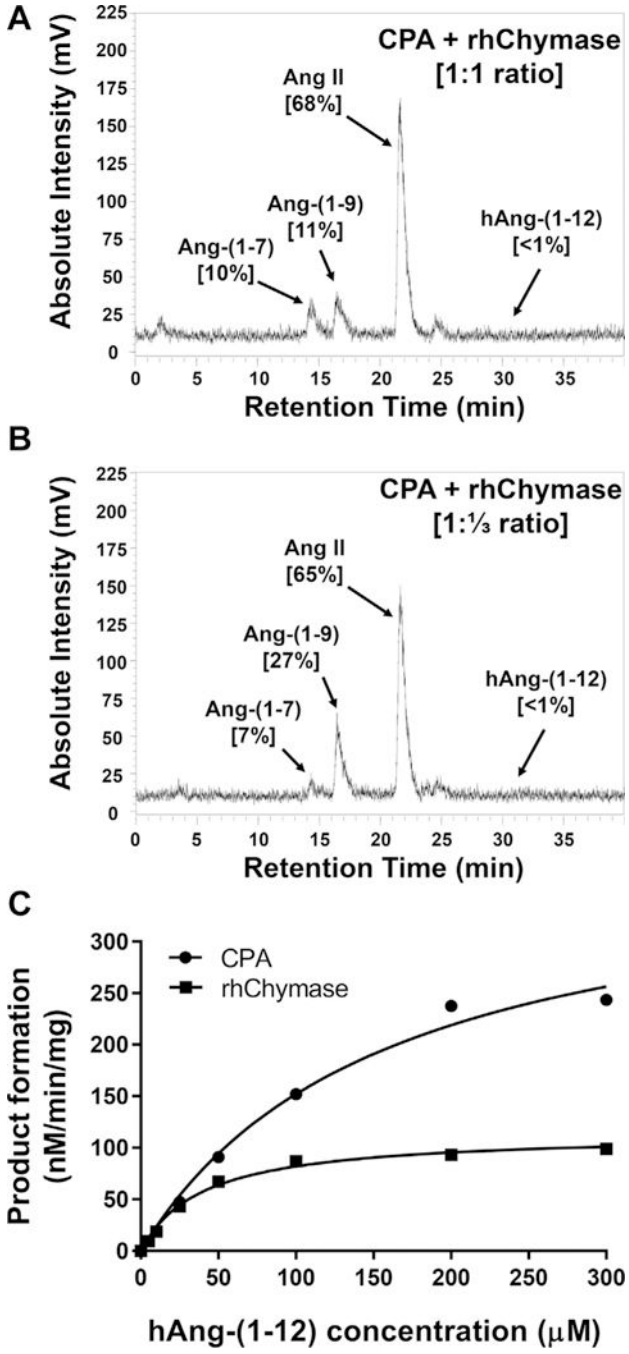


**Fig. 3.**

Hydrolysis of $^{125}$I-hAng-(1–12) by CPA + rhChymase (1:1 or 1:⅓ ratio). The HPLC chromatogram shows the $^{125}$I-Ang products generated from $^{125}$I-hAng-(1–12) substrate by a combination of CPA + rhChymase (A) 1:1 ratio or (B) 1:⅓ ratio. (C) Kinetics ($K_m$ and $V_{max}$) of CPA and rhChymase for hAng-(1–12) substrate. Results are representative of three or more separate experiments described in the Materials and Methods.

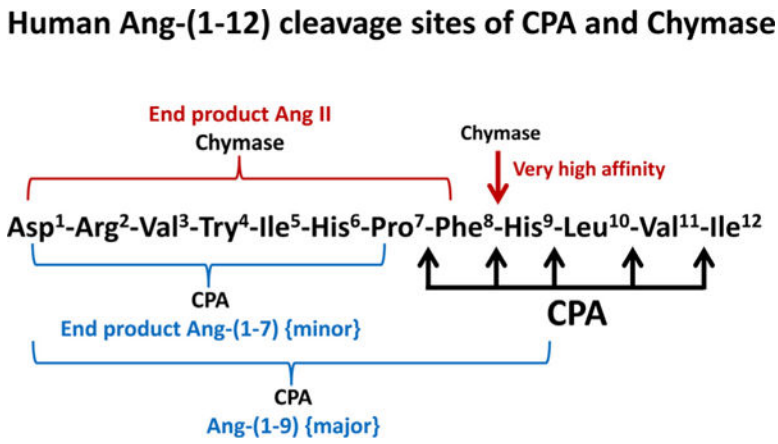


**Fig. 4.**

Human Ang-(1–12) Cleavage Sites of CPA and rhChymase. CPA sequentially cleaves the C-terminal end amino acids (Ile, Val, Leu, His and Phe) of hAng-(1–12). Chymase is an endopeptidase that cleaves one $Phe^{8}$-$His^{9}$ bond of the hAng-(1–12) substrate.

**Table 1**

Enzyme Kinetics ($K_m$, $V_{max}$ and $V_{max}/K_m$) of CPA and rhChymase for hAng-(1–12) Substrate.

| Enzyme Kinetics | CPA | rhChymase |
|---|---|---|
| $K_m$ (μM) | 150 ± 5 | 40 ± 9 |
| $V_{max}$ (nM/min/mg) | 384 ± 23 | 116 ± 20 |
| $V_{max}/K_m$ ratio (Catalytic efficiency) | 2.56 ± 0.1 | 2.97 ± 0.1 |

Results are representative of three or more separate experiments. Details of the kinetic analysis were described in Materials and Methods.

**Table 2**

Characteristics of human chymase and CPA.

| Chymase | CPA |
|---|---|
| - Single α-form of chymase is present in human MC. | - Six different isoforms of CPA (CPA-1 to CPA-6) is present in human tissues. |
| - Belongs to serine endopeptidase of family S1. | - Belongs to Zinc-metallocarboxypeptidase family. |
| - High affinity to hydrolyze Phe-His bond. | - Sequentially hydrolyzes aliphatic amino acids and aromatic amino acids from C-terminus. Low affinity for His. |
| - α-Chymase has high affinity for both Ang I and hAng-(1–12) substrates to directly generate Ang II. | - High affinity for hAng-(1–12)/Ang I to generate Ang-(1–9) sequentially. |
| - α-Chymase has low/negligible affinity to hydrolyze Ang-(1–9) into Ang II. | - Low affinity to hydrolyze Ang-(1–9) into Ang II, and subsequently Ang II into Ang-(1−7). |
| - Enzyme content in human adult foreskin (4.5 μg/$10^6$ MC). | - Enzyme content in adult foreskin (16 μg/$10^6$ MC). |