Nardilysin Cleaves Peptides at Monobasic Sites†

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ABSTRACT: Nardilysin (N-arginine dibasic convertase, EC 3.4.24.61) was first identified on the basis of its ability to cleave peptides containing an arginine dibasic pair, i.e., Arg−Arg or Arg−Lys. However, it was observed that an aromatic residue adjacent to the dibasic pair (i.e., Phe−Arg−Lys) could alter the cleavage site. In this study we determined whether nardilysin can cleave peptides at a single basic residue. Nardilysin cleaves β-endorphin at the monobasic site, Phe^{17}−Lys^{18}, with a k_{cat}/K_m of 2×10^8 M^{-1} min^{-1}. This can be compared to a k_{cat}/K_m of 8.5×10^8 M^{-1} min^{-1} for cleavage between a dibasic pair in dynorphin B-13. Nardilysin also cleaves calcitonin at His−Arg and somatostatin-14 at Cys−Lys. We examined the hydrolysis of fluorogenic peptides based on the β-endorphin 12−24 sequence, Abz-T-P-L-V-T-L-X_1-X_2-N-A-I-I-K-Q-EDDnp. Nardilysin hydrolyzes the peptides when X_1-X_2 = F−K, F−R, W−K, M−K, Y−K, and L−K. The kinetics of cleavage at F−K and F−R are similar; however, K−F is not hydrolyzed. Nardilysin cleaves at two monobasic sites M−K and F−R of the kallidin model peptide Abz-MISLMKRPPGFSPFRSSRI-NH_2, releasing desArg^{10} kallidin (KRPPGFSPF). However, nardilysin does not release desArg^{10} kallidin from the physiological precursor low molecular weight kininogen. These studies extend the range of potential substrates for nardilysin and further substantiate that nardilysin is a true peptidase.

Nardilysin (N-arginine dibasic convertase, EC 3.4.24.61), is a member of the M16 family of metallopeptidases, possessing an inverted active site zinc binding motif HXXEH relative to the more common HEXXH motif (*1*). Nardilysin is unique among other metallopeptidases in that it contains an acidic domain located between the N-terminus and the active site zinc motif. This domain is comprised of 43 (human) to 59 (mouse) glutamate and aspartate residues within a 76 amino acid stretch. Recent studies indicate that this acidic domain is a regulatory domain binding polyamines as well as forming heteroligomers with other proteins (*2*−*4*; Chow et al., manuscript in preparation). As nardilysin is highly expressed in the testes (*5*), it has been proposed to be involved in the later steps of sperm maturation and transformation (*6*). Additionally, nardilysin has recently been found to be located at the cell surface (*7*), where it acts as a receptor for heparin-binding EGF-like growth factor (*8*). It appears that nardilysin exhibits multiple physiological functions dependent in part on its subcellular localization.

Although the physiological substrates of nardilysin have yet to be identified, the enzyme cleaves a number of bioactive peptides in vitro including dynorphin A, somatosatin-28, α-neoendorphin, and atrial natriuretic factor (*2*, *5*). The specificity of the enzyme was initially reported as being directed at the N-terminus of an arginine residue within paired basic residues (*5*). Thus, the enzyme cleaves between Arg−Arg in dynorphin A (*2*) and on the amino side of arginine of the Arg−Lys pair in somatosatin-28 (*5*). However, the enzyme also cleaves between Arg−Lys in α-neoendorphin. Using synthetic peptide substrates, we previously reported that the residue adjacent to the Arg in the sequence X−Arg−Lys has a significant influence as to whether cleavage occurs between the dibasic pair or at the amino side of the arginine residue (*9*). For example, if X were an aromatic amino acid, cleavage occurred exclusively at the X−Arg bond. In contrast, if X were Ile or Val, cleavage occurred exclusively between the Arg−Lys bond. With other amino acids, cleavage occurred at both positions to varying extents.

Since aromatic amino acids produced cleavage on the amino side of the first basic residue in a dibasic pair, we have extended this observation to determine whether nardilysin can cleave peptides containing only a single basic residue. The results of this study show that this is indeed the case with both synthetic and physiologically active peptides.

EXPERIMENTAL PROCEDURES

Abz/EDDnp-containing peptides were synthesized as previously described (*10*) using a Shimadzu PSSM 8 automated solid-phase peptide synthesizer employing Fmoc methodology. The Nova Syn TGR resin (NovaBiochem, San

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Diego, CA) was used and all peptide couplings were performed with O-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate/1-hydroxybenzotriazole/HOBt using *N*-methyl morpholine as base. Porcine calcitonin was purchased from Peninsula Labs Inc., dynorphin B-13 and human β-endorphin were purchased from Bachem, and porcine somatostatin-14 was from American Peptide Co. Human low molecular weight kininogen and porcrine pancreas kallikrein were purchased from Calbiochem.

Separation of Peptides and Cleavage Products. The synthetic Abz/EDDnp containing peptides were purified by reverse phase chromatography on a Vydac C_4 column with detection of the peptides by absorbance at 214 nm and, in some cases, by simultaneously monitoring absorbance at both 214 and 357 nm. The peptides were separated by a linear gradient from 0.1% trifluoroacetic acid in 95% water/5% acetonitrile to 0.1% trifluoroacetic acid in 50% water/50% acetonitrile.

The same system was used to separate reaction products from both synthetic and physiological peptides. In the case of calcitonin and somatostatin-14, the peptides were reduced and carboxymethylated prior to HPLC analysis. Peptides or their reaction products were collected manually, freeze-dried, and identified by mass spectrometry using a Kratos Kompact SEQ MALDI-TOF mass spectrometer operating in a linear mode. These analyses were performed at the University of Kentucky Mass Spectrometry Facility. The matrix was α-cyano-4-hydroxy-cinnamic acid. In some cases products from one reaction identified by mass spectrometry were used as standards to identify other reaction products by comparison of their HPLC retention times.

Enzyme Purification. Recombinant mouse nardilysin was purified as previously described (*4*). In short, the recombinant enzyme was expressed as an N-terminal hexahistidine containing fusion protein in Sf-9 cells and purified on a nickle-nitrilotriacetic acid column (Qiagen). The hexahistidine affinity tag was removed by cleavage at the linker region with TEV protease (Gibco-BRL). The enzyme was judged to be homogeneous by SDS−PAGE, Figure 1.

Fluorometric Assay of Nardilysin. Cleavage of the flourogenic peptides by nardilysin was measured by following the increase in fluorescence that occurred upon peptide bond cleavage. Data from the fluorescence spectrophotometer were collected either by computer or by a strip chart recorder. An excitation wavelength of 319 nm and an emission wavelength of 419 nm were used. Reaction mixtures (400 μL) contained 50 mM potassium phosphate buffer, pH 7.0, and variable concentrations of the fluorogenic peptide substrate. The reaction was initiated by the addition of enzyme. The concentration of the fluorogenic peptides was determined from the total fluorescence change produced by trypsin and by measuring peptide absorbance at 357 nm (*11*). Kinetic constants were calculated using the computer programs of Cleland (*12*).

RESULTS

To determine whether nardilysin might cleave a physiological peptide lacking an arginine containing dibasic pair, we examined the cleavage of β-endorphin by purified recombinant mouse nardilysin. As shown in Figure 2, recombinant nardilysin indeed cleaved β-endorphin initially at a single site producing two products that were identified by mass spectrometry as Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe and Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Gln. Upon prolonged incubation, the C-terminal product was further cleaved at the $Tyr^{27}-Lys^{28}$ bond. Thus nardilysin cleaved β-endorphin between an aromatic and basic residue, rather than at an arginine containing dibasic pair. Using β-endorphin as an alternate substrate inhibitor of Abz-GGFIRRVGQ-EDDnp hydrolysis, we determined a K_m of 1.4 μM. A k_{cat} of 302 min^{-1} was determined by measuring the hydrolysis of β-endorphin under saturating conditions (30 μM) by following substrate disappearance by HPLC.

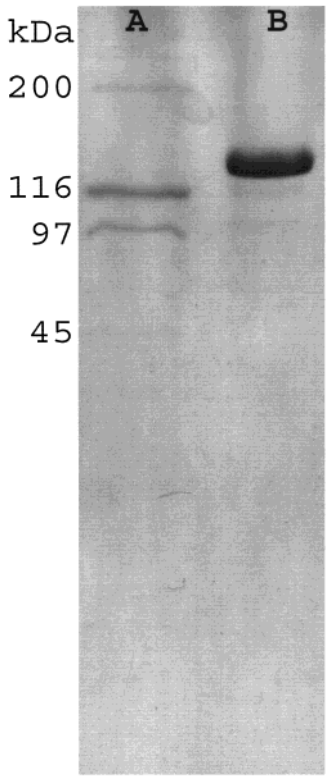

FIGURE 1: Purity of recombinant nardilysin. Recombinant nardilysin, purified as described in Experimental Procedures, was analyzed by SDS−PAGE on a 10% gel. Lane A: molecular weight markers. Lane B: 3 μg of purified recombinant nardilysin.

To further examine the ability of nardilysin to cleave at a single basic residue, a series of peptides corresponding to residues 12−24 of β-endorphin was synthesized. Each contained an N-terminal 2-aminobenzoyl moiety (Abz) and a C-terminal Gln-ethylenediamine-2,4-dinitrophenol group (Gln-EDDnp). The residues at positions 18 and 19 (X_1 and X_2) that correspond to the Phe−Lys cleavage site of β-endorphin were varied.

The kinetic constants for each of these peptides as substrates for nardilysin are shown in Table 1, as well as the sites of cleavage. The latter was determined by separating the reaction products by HPLC and determining their identity either by mass spectrometry or by comparison to authentic standards. A typical example with Abz-T-P-L-V-T-L-F-K-N-A-I-I-K-Q-EDDnp as substrate is shown in Figure 3. It can be seen in Table 1 that in addition to cleaving at dibasic

[1] Abbreviations: Abz, 2-aminobenzoyl; EDDnp, ethylenediamine-2,4-dinitrophenyl; Fmoc, 9-fluorenylmethoxycarbonyl; MALDI-TOF, matrix assisted laser desorption ionization-time of flight.

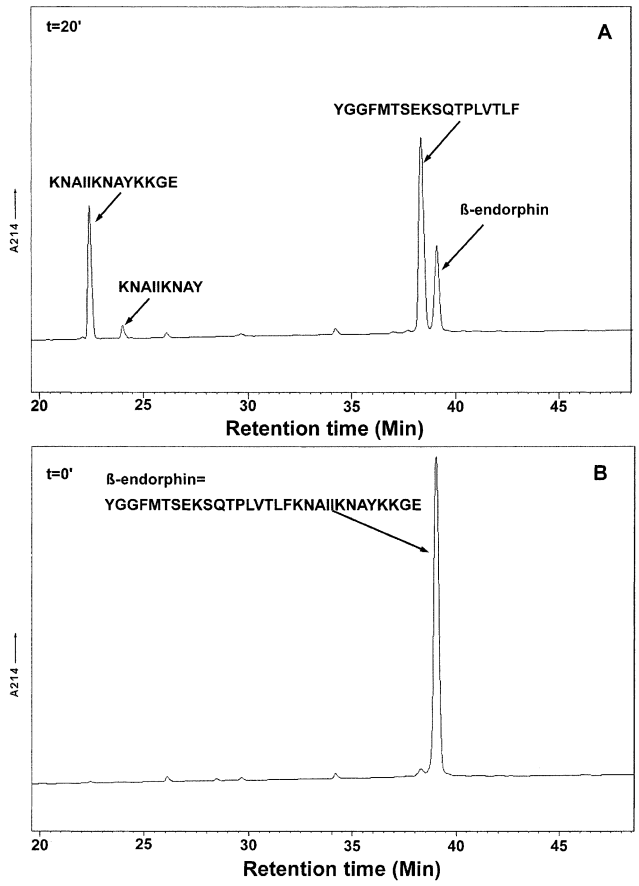

FIGURE 2: HPLC chromatogram of the hydrolysis products generated from β-endorphin. (A) Reaction after 20 min of incubation. (B) Reaction at zero time. Reaction mixtures containing 30 μM β-endorphin in 25 mM potassium phosphate buffer, pH 7, were incubated with 50 ng nardilysin at 37 °C.

Table 1: Kinetics of Hydrolysis of Abz-T-P-L-V-T-L-X_1-X_2-N-A-I-I-K-Q-EDDnp[a]

X_1-X_2	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\,min^{-1} \times 10^{-6}$)	cleavage site
R−K	41.6 ± 0.3	0.54 ± 0.01	77.0 ± 1.2	R↓K
K−K	29.9 ± 1.6	1.17 ± 0.1	25.6 ± 1.4	K↓K
F−K	37.7 ± 7.6	7.7 ± 2.1	4.9 ± 0.4	F↓K
F−R	76.0 ± 10.8	17.1 ± 2.8	4.4 ± 0.16	F↓R
W−K	27.3 ± 1.5	8.4 ± 0.6	3.3 ± 0.1	W↓K
M−K	27.3 ± 0.8	13.5 ± 0.2	2.0 ± 0.04	M↓K
Y−K	46.8 ± 1.4	26.6 ± 1.0	1.8 ± 0.002	Y↓K
L−K	13.0 ± 8.7[b]	46.6 ± 34.5[b]	0.28 ± 0.02	L↓K
I−K	<0.4[c]	–	–	–
V−K	<0.6[d]	–	–	–
F−F	<0.3[e]	–	–	–
K−F	<0.04[f]	–	–	–

[a] Kinetic constants are reported with their standard errors. A molecular weight of 130 kDa was used to calculate k_{cat}. [b] The insolubility of this peptide limited the range in which it could be tested, resulting in large standard errors in the kinetic constants. [c] Rate observed at 10 μM substrate using 1 μg of enzyme. [d] Rate observed at 30 μM substrate using 1 μg of enzyme. [e] Rate observed at 5 μM substrate using 1 μg of enzyme. [f] Rate observed at 25 μM substrate using 1 μg of enzyme.

residues (X_1-X_2 = R−K or K−K), the enzyme also cleaves between an aromatic residue and a basic residue, and the basic residue can be either an arginine (X_1-X_2 = F−R) or a lysine (X_1-X_2 = F−K or W−K). The enzyme also cleaves between a methionine or leucine and a basic residue (X_1-X_2 = M−K or L−K), but cleavage is not observed when isoleucine or valine are amino terminal to the basic residue. The absence of cleavage when phenylalanine occupied both the X_1-X_2 position suggests the requirement for a basic residue. Additionally, the absence of cleavage of the Lys−Phe sequence shows that the basic residue must occupy the P_1' position as defined by Schechter and Berger (*13*).

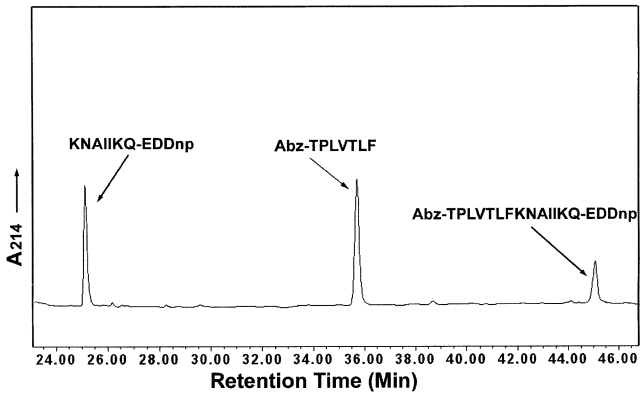

FIGURE 3: Identification of products generated from Abz-T-P-L-V-T-L-F-K-N-A-I-I-K-Q-EDDnp. Reaction mixtures containing 20 μM Abz-GGFHRRHGQ-EDDnp in 50 mM potassium phosphate buffer, pH 7.0, were reacted with nardilysin and products analyzed by gradient HPLC as in Experimental Procedures.

We next examined two other physiological peptides that contain an aromatic residue followed by a basic residue, namely, somatostatin-14 that contains the Trp^8-Lys^9 sequence and calcitonin that contains the $Trp^{13}-Arg^{14}$ sequence. We compared the cleavage of these peptides to β-endorphin and dynorphin B-13, the latter containing an Arg−Arg dibasic pair as a known cleavage site. The K_m for each of these peptides was determined by using them as an alternate substrate inhibitor of Abz-GGFIRRVGQ-EDDnp hydrolysis, while k_{cat} was determined by measuring hydrolysis by HPLC under saturating substrate concentrations. As shown in Table 2 and Figure 4, each of these peptides is cleaved by nardilysin, although not necessarily at the anticipated sites. Somatostatin-14, which is a cyclic peptide containing a disulfide bridge between Cys^3 and Cys^{14}, was cleaved at the Cys^3-Lys^4 bond, the Phe^7-Trp^8 bond, and the Phe^6-Phe^7 bond. The cleavage ratios between these bonds are 72.2%, 20.2%, and 7.6%, respectively. In contrast, reduced somatostain-14 was cleaved exclusively at the Cys^3-Lys^4 bond. Calcitonin, which is also a cyclic peptide containing a disulfide bridge between Cys^1 and Cys^7, was cleaved at the $His^{20}-Arg^{21}$ bond, Figure 5. In this case the cleavage site of reduced calcitonin was the same (data not shown).

The ability of nardilysin to cleave a peptide bond between Phe−Arg and Phe−Lys resembles peptide cleavage by human tissue kallikrein (*14*). We thus determined whether nardilysin could release kinin from a synthetic peptide that contains the C-terminal sequence of human kininogen (*15*). As shown in Table 2, nardilysin cleaved this peptide (Abz-MISLMKRPPGFSPFRSSRI-NH_2) at both the M−K and F−R bonds, releasing the peptide KRPPGFSPF known as $desArg^{10}$-kallidin. The HPLC and mass spectral analysis showed that $desArg^{10}$-kallidin and Abz-MISLM were the major products, indicating the intermediates Abz-MISLMKRPPGFSPF and KRPPGFSPFRSSRI-NH_2 did not accumulate and were readily converted to $desArg^{10}$-kallidin.

Table 2: Kinetics of Hydrolysis of Physiological Peptides Containing a Single Basic Residue[a]

peptide	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} $min^{-1} \times 10^{-7}$)	cleavage site
dynorphin B-13 (15 μM) (YGGFLRRQFKVVT)	1689	2.0 ± 0.1	84.5	Arg^6−Arg^7
β-endorphin (30 μM)[b] (YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE)	302	1.4 ± 0.1	21.6	Phe^{18}−Lys^{19} (Tyr^{27}−Lys^{28})
somatostatin −14 (28 μM)[c] (AGCKNFFWKTFTSC)	47	8.0 ± 0.7	0.6	Cys^3−Lys^4, Phe^6−Phe^7*, and Phe^7−Trp^8*
reduced-somatostatin −14 (28 μM)[c]	57	0.5 ± 0.01	11.3	Cys^3−Lys^4
calcitonin (28 μM) (CSNLSTCVLSAYWRNLNNFHRFSGMGFGPETP)	109	1.7 ± 0.1	64.1	His^{20}−Arg^{21}
Abz-MISLMKRPPGFSPFRSSRI−NH_2	376 ± 11	4.1 ± 0.1	9.2 ± 0.1	M−K and F−R
Abz-MISLMKRPQ-EDDnp	569 ± 11	3.5 ± 0.3	16.3 ± 0.3	M−K
Abz-GFSPFRSSRQ-EDDnp	461 ± 11	1.9 ± 0.1	24.1 ± 0.9	F−R and (S−R, 11%)

[a] K_m values were determined by using the peptide as an alternate substrate inhibitor of Abz-GGFIRRVGQ-EDDnp, at 2.2 μM (K_m = 2.2 μM). [b] Cleavage at the Tyr^{27}−Lys^{28} bond occurred within the β-endorphin 19−31 C-terminal fragment, as judged by both the disappearance of β-endorphin 19−31 and the isolation of the β-endorphin 19−27 fragment. [c] The three somatostatin cleavage sites were all primary cleavage sites, as judged by the isolation of somatostatin 8−14, somatostatin 1−7, somatostatin 9−14, and somatostatin 4−14. No intermediate fragments were detected. *, 72% of the somatostain was cleaved at Cys^3−Lys^4, 20% at Phe^6−Phe^7, and 7% at Phe^7−Trp^8.

To determine whether nardilysin could hydrolyze kininogen, the enzyme was incubated with human low molecular weight kininogen for 2 h at 37 °C at molar ratios of 1/10, 1/50, and 1/100. The reaction mixture was then examined for products by MALDI-TOF mass spectrometry. As a positive control, porcine pancreatic kallikrein was used to cleave kininogen for the same time period of ratios of 1/100, 1/500, and 1/1000. With kallikrein a positive ion product ($MH^+ = 1189$) corresponding to Lys-bradykinin (KRPPGFSPFR, calcd $MH^+ = 1188.7$) was detected at molar enzyme-to-substrate ratios of 1:100 and 1:500. For nardilysin, no positive ion corresponding to either Lys-Bradykinin or desArg^{10}-kallidin was detected even at a 1:10 enzyme to substrate molar ratio.

DISCUSSION

Nardilysin was originally identified as an enzyme whose specificity is directed at cleaving peptides on the amino side of arginine residues within a dibasic pair (*5*). However, it was recently shown that the residues adjacent to the dibasic pair, particularly the P_2 residue, influenced the cleavage site. Thus, within the peptide Abz-GGFXRKVGQ-EDDnp, where X represents variable residues, cleavage occurred exclusively on the amino side of arginine (X↓RK) when X was Phe or Tyr. In contrast, when X was Ile or Val, cleavage occurred exclusively between the dibasic pair (XR↓K) (*9*).

In this study we have further studied the specificity of nardilysin by examining its ability to cleave peptides that contain only a single basic residue. The results presented in Table 1 of this study show that nardilysin is indeed capable of cleaving peptides at a single basic residue if the adjacent (P_1) residue is a hydrophobic residue. Little difference was observed when the basic residue was arginine or lysine. Cleavage between an F−K bond exhibited just a 2-fold lower k_{cat} then for cleavage between an F−R bond, while cleavage at F−K exhibited a 2-fold lower K_m. Thus, k_{cat}/K_m was essentially the same when the basic residue was arginine or lysine. Cleavages at these single basic residues exhibited very similar k_{cat} values to cleavages between the "traditional" dibasic pair, although K_m values were 15−30-fold higher. This suggests that the P1 site, although accommodating an aromatic residue in place of a basic residue, does so with less favorable binding interactions. Substrate binding appears to fit a lock-and-key model in that the binding energy derived from this interaction does not appear to be used for catalysis. It would appear from the data in Table 1 that the hydrophobic residue cannot occupy the S_1' site as the sequences K−F and F−F are not cleaved.

That cleavage can occur between a hydrophobic residue and a basic residue in a physiological peptide is seen when β-endorphin was examined as a substrate. β-Endorphin is cleaved between Phe^{18}−Lys^{19} with a k_{cat}/K_m value of 2.16×10^8 min^{-1} M^{-1} that is similar to the k_{cat}/K_m values of 8.45×10^8 min^{-1} M^{-1} obtained with dynorphin B-13, a good substrate. Somatostatin-14, containing a disulfide bond, is also cleaved at a single basic residue, although in this case cysteine occupies the P1 site. In addition cleavage was also observed between two hydrophobic residues, Phe−Trp. This is a surprising finding, as cleavage between Trp−Lys was anticipated. This suggests that the specificity of nardilysin is even more complex including two aromatic residues. That conformation plays a role in determining the cleavage site is seen when the cystine residue in somatostatin-14 is reduced. Cleavage then occurs exclusively between Cys and Lys. Reducing somatostatin had little effect on k_{cat} but it lowered the K_m 16-fold. This suggests that steric effects lowered the affinity of the enzyme for somatostatin-14 and somehow contributed to the cleavage at Phe−Trp. On the basis of k_{cat}/K_m (0.06×10^8 min^{-1} M^{-1}) somatostatin-14 is a relatively poor substrate. Calcitonin, which contains a potential cleavage site at Trp−Arg, was actually cleaved at the His−Arg bond with a k_{cat}/K_m of 0.6×10^8 min^{-1} M^{-1}. Cleavage at a His−Arg bond is consistent with the previous observation that ~10% of the cleavage of the synthetic substrate Abz-GGFHRKVGQ-EDDnp occurred at the His−Arg bond (*9*).

The release of desArg^{10}-kallidin from the model peptide Abz-MISLMKRPPGFSPFRSSRI-NH_2 requires cleavages at two monobasic containing sites, M−K and F−R. The cleavage at these bonds once again demonstrates that nardilysin is able to hydrolyze a peptide bond between a

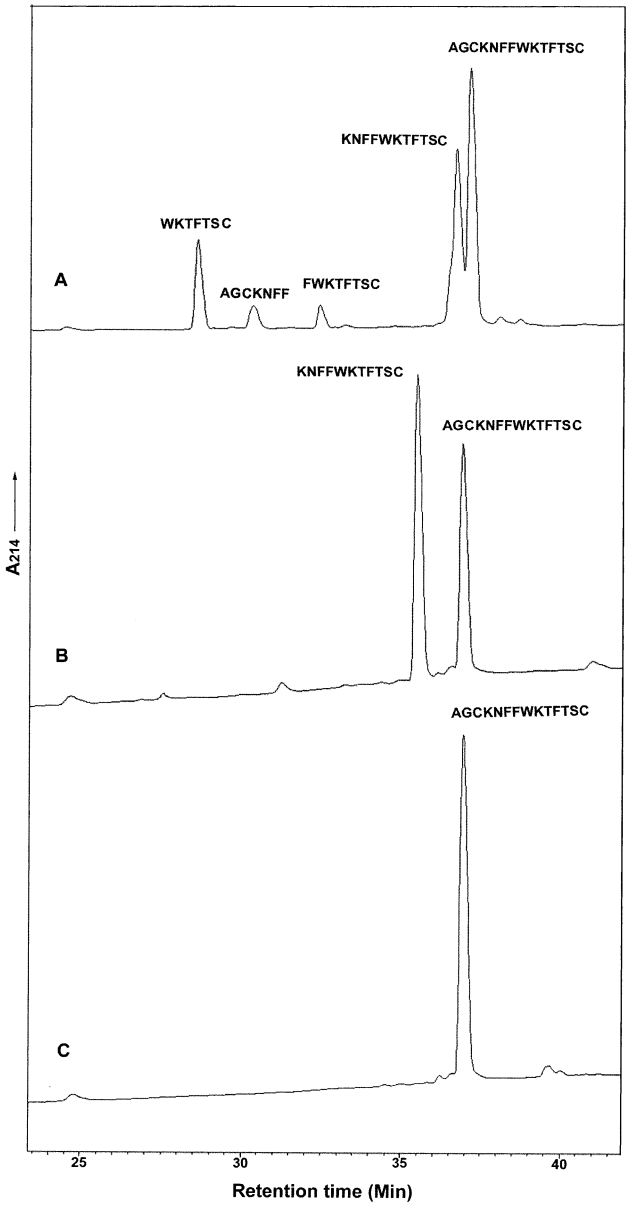

FIGURE 4: Identification of products generated from oxidized (A) and reduced (B) porcine somatostatin-14. Reaction mixtures containing 28 μM peptide in 25 mM potassium phosphate buffer, pH 7, were incubated with 100 ng nardilysin at 37 °C. In the case of reduced somatostatin-14, 1 mM DTT was included in the reaction mixture. Prior to HPLC analysis, the reaction mixtures were reduced and carboxymethylated. (A) Reaction products generated from somatostatin-14 after 20 min of reaction. (B) Reaction products generated from somatostatin-14 reduced with DTT after 20 min of reaction. (C) Reaction mixture for reduced somatostatin-14 at time zero.

bulky or aromatic residue and a basic amino acid. For peptides containing dibasic residues, nardilysin can cleave either at the amino side of a dibasic pair or between the dibasic residues depending on the amino acids adjacent to the scissile bond particularly at the P2 position. It is thus anticipated that nardilysin would prefer to cleave the K−R to the M−K bond (*9*). However, nardilysin cleaved the peptide Abz-MISLMKRPQ-EDDnp exclusively at M−K and not at K−R (Table 2). It is possible that the proline residue at the P2′ position may have affected the cleavage site.

desArg^{10}-kallidin is a physiological ligand for the B_1 kinin receptor which responds to tissue injury. It has been

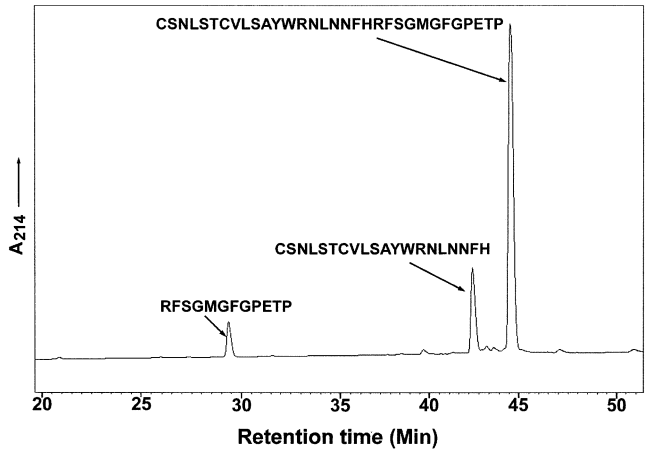

FIGURE 5: Identification of products generated from porcine calcitonin. Reaction mixtures containing 28 μM peptide in 25 mM potassium. Prior to HPLC analysis, the reaction mixture was reduced and carboxymethylated.

established that the release of desArg^{10}-kallidin from the human low molecular weight kininogen is via the kallikrein-kinin system (*16*, *17*). It is shown in the present study that nardilysin releases desArg^{10}-kallidin from a synthetic 19-mer precursor peptide, but does not act on low molecular weight kininogen. This further substantiates that nardilysin is a true peptidase. Determination of the structure of the peptidase neurolysin (*18*) showed that the substrate size was limited by a deep channel that prevents access of large molecules to the active site. It would appear a similar situation may occur in nardilysin.

The finding that nardilysin efficiently cleaves β-endorphin at a monobasic site yielding β-endorphin 1−18 and its C-terminal product β-endorphin may have physiological significance. It is know that β-endorphin metabolites, particularly β-endorphin 1−17, are active in modulating immune system response (*19*−*21*); thus the products of β-endorphin produced by nardilysin may be physiologically active or serve as a precursor for physiologically active metabolites.

Taken together, these studies extend the substrate specificity of nardilysin beyond that of cleaving at an arginine residue within a dibasic pair. The specificity of the enzyme is more complex than previously thought (*5*) and has now been extended to include cleavage between a hydrophobic residue and a basic residue, and even between two aromatic residues. These studies further define nardilysin as a true peptidase, excluding large peptides and proteins as substrates.

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