JOURNAL OF BACTERIOLOGY, Mar. 2006, p. 2056–2062 Vol. 188, No. 6
0021-9193/06/$08.00+0 doi:10.1128/JB.188.6.2056–2062.2006

Specificity of a *Vibrio vulnificus* Aminopeptidase toward Kinins and Other Peptidyl Substrates

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Received 27 October 2005/Accepted 6 January 2006

Recently, phosphoglucose isomerase with a lysyl aminopeptidase (PGI-LysAP) activity was identified in *Vibrio vulnificus*. In this paper, we demonstrate the proteolytic cleavage of human-derived peptides by PGI-LysAP of *V. vulnificus* using three approaches: (i) a quantitative fluorescent ninhydrin assay for free lysine, (ii) matrix-assisted laser desorption ionization–two-stage time of flight mass spectrometry (MALDI-TOF-TOF), and (iii) Tricine gel electrophoresis. PGI-LysAP hydrolyzed bradykinin, Lys-bradykinin, Lys-(des-Arg^9)-bradykinin, neurokinin A, Met-Lys-bradykinin, histatin 8, and a myosin light chain fragment. We detected the proteolytic release of free L-lysine from peptide digests using a rapid, simple, sensitive, and quantitative fluorescent ninhydrin assay, and results were confirmed by MALDI-TOF-TOF. The use of the fluorescent ninhydrin assay to quantitatively detect free lysine hydrolyzed from peptides is the first application of its kind and serves as a paradigm for future studies. The visualization of peptide hydrolysis was accomplished by Tricine gel electrophoresis. Proteolytic processing of kinins alters their affinities toward specific cellular receptors and initiates signal transduction mechanisms responsible for inflammation, vasodilation, and enhanced vascular permeability. By applying novel approaches to determine the proteolytic potential of bacterial enzymes, we demonstrate that PGI-LysAP has broad exopeptidase activity which may enhance *V. vulnificus* invasiveness by altering peptides involved in signal transduction pathways.

Vibrio vulnificus is a naturally occurring marine bacterium indigenous to temperate and tropical estuarine environments. It has two routes of transmission: wound infections and the ingestion of contaminated seafood, especially raw oysters. During summer months, nearly 100% of the oysters harvested from the Gulf of Mexico contain *V. vulnificus* (9). Liver cirrhosis, hemochromatosis, diabetes, kidney disease, and immune disorders predispose individuals to *V. vulnificus* infection via the food-borne route (4, 24); however, even seemingly healthy individuals are susceptible to *V. vulnificus* through wound infections (24). Symptoms include fever, chills, hypotension, and the development of secondary bullous skin lesions. Septicemia is common, and death can occur within 24 h after contact with the pathogen (8, 24). Mortality rates for *V. vulnificus* infections are approximately 60% in the United States (15) but are somewhat higher in Asian countries (14, 25).

Over the past two decades, numerous virulence factors have been identified in *V. vulnificus* based on cell culture and animal models; however, the validity of these models in assessing virulence has come into question (7). A case in point involves *V. vulnificus* protease, a well-characterized metalloprotease (23). In the mouse model, injection of this protease causes skin necrosis; however, knockout of the gene in *V. vulnificus* does not attenuate the symptoms in *Vibrio*-infected laboratory animals (34).

Recently, we isolated and characterized a phosphoglucose isomerase (PGI) in *V. vulnificus* (30). This PGI exhibits not only isomerase activity but also lysyl aminopeptidase (LysAP) activity (30). Lysyl aminopeptidases are well-known processing enzymes which can cleave kinin peptides to elicit signal transduction cascades that are responsible for inflammation, vasodilation, enhanced vascular permeability, and other physiological responses (2, 29). Since PGI-LysAP activity occurs on the surface of the bacterium (30), it may be involved in *Vibrio* spread once the bacterium gains entry into the circulatory system or tissues of a susceptible animal host. In the absence of consistent results in studies of virulence in animal models, we evaluated the use of mass spectrometry, Tricine gel electrophoresis, and a fluorescent ninhydrin assay for free lysine to determine the proteolytic potential of PGI-LysAP toward human-derived peptides that are linked with bacterial invasiveness and other pathological conditions.

MATERIALS AND METHODS

Purification of PGI-LysAP. One hundred ml of tryptic soy broth (Becton, Dickinson and Co., Sparks, MD) supplemented with 0.5% NaCl (1% total NaCl) was inoculated with a colony of *Vibrio vulnificus* strain MLT364 (30) taken from an overnight culture grown on tryptic soy agar (Becton, Dickinson and Co.) containing 1% NaCl. The culture was incubated at 37°C overnight at 250 rpm and centrifuged at 800 × *g* for 15 min at 4°C, and the pellet was resuspended in 250 μl of 20 mM Tris-HCl, pH 9.0, frozen at −80°C, and thawed at room temperature. Chromatographic purification was performed as previously described (30). In essence, PGI-LysAP was purified by anion exchange perfusion chromatography and the fractions were screened for LysAP activity. The fractions giving the highest activity were combined and concentrated in an Amicon Centricon microconcentrator with a molecular mass cutoff of 50 kDa (Millipore Corp., Bedford, MA). The enzyme was desalted with 10 mM Tris-HCl, pH 8.0, further purified by aprotinin affinity column chromatography, concentrated as described above, and stored on ice until use (30).

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TABLE 1. Sources, masses, and amino acid sequences of seven peptides used in this study

Peptide	Source/catalog no.	FW (Da)[a]	Amino acid sequence
Bradykinin	Sigma/#B 3259[b]	1,060	RPPGFSPFR
Lys-bradykinin	Bachem/#H-2180[c]	1,189	KRPPGFSPFR
Lys-(des-Arg^9)-bradykinin	Bachem/#H-3122	1,032	KRPPGFSPF
Neurokinin A	Bachem/#H-3745	1,133	HKTDSFVGLM
Met-Lys-bradykinin	Bachem/#H-2190	1,320	MKRPPGFSPFR
Histatin 8	Sigma/#L 7404	1,562	KFHEKHHSHRGY
Myosin light chain fragment	Bachem/#H-3252	1,419	KKRAARATSNVFA-NH_2

[a] FW, formula weight expressed as average molecular mass.
[b] Sigma Chemical Co., St. Louis, MO.
[c] Bachem California, Inc., Torrance, CA.

Measurement of LysAP activity. The spectrophotometric measurement of LysAP activity was performed as previously described (29, 30) using the synthetic substrate L-lysyl-7-amino-4-methylcoumarin (L-Lys-AMC) (catalog no. AFC-008; MP Biomedicals, Aurora, IL). In essence, a 20 mM stock of L-Lys-AMC was prepared in dimethyl sulfoxide. Reaction mixtures contained 100 μl of chromatographic fraction or 1 μl of Centricon concentrate, 20 μM (1 μl) of stock L-Lys-AMC, and 20 mM Tris-HCl, pH 9.0, in a total volume of 1,000 μl. The LysAP activity was measured on an LS-50B luminescence spectrophotometer (Perkin Elmer, Shelton, CT) for 3 min at 21°C at an excitation wavelength of 380 nm (Ex 380) and an emission wavelength of 460 nm (Em 460). When L-Lys-AMC is used as the substrate, 1 unit of LysAP activity is defined here as the activity required to produce an increase of 1 fluorescent unit as measured at Ex 380 and Em 460 at pH 9.0 and at 21°C over 3 min.

Peptidyl substrates. The following peptidyl substrates were used in this study: bradykinin (BK); lysyl (Lys)-BK, also known as kallidin; Lys-(des-Arg^9)-BK, also known as des-Arg^{10}-kallidin; neurokinin A; Met-Lys-BK; myosin light chain fragment (residues 11 to 23); and histatin 8. The source, mass, and sequence of each peptide are listed in Table 1. These substrates were selected because all, except the BK, have a lysyl residue at or near the amino termini, and the kinin peptides mediate signal transduction pathways that enhance bacterial invasiveness (17–19, 21). Bradykinin was chosen to serve as a lysine-negative control. Stock solutions of the peptides were prepared by dilution to approximately 1 μg/μl in high-performance liquid chromatography (HPLC)-grade water, and aliquots were stored at −20°C.

Fluorescent ninhydrin assay for free L-lysine. For free L-lysine determinations by use of ninhydrin, substrate digestions were performed in reaction mixtures containing 20 μl of 20 mM Tris-HCl (pH 9.0), 1,000 units of PGI-LysAP, and approximately 20 μg of BK, Lys-BK, Lys-(des-Arg^9)-BK, neurokinin A, Met-Lys-BK, myosin light chain fragment, and histatin 8. The digestions were performed at 37°C for 2 h. Negative controls consisted of the same buffers, substrates, and digestion conditions but without enzyme. To perform the assay for free lysine, ninhydrin (Sigma) was diluted to 2% in HPLC-grade water. The potential substrates and their corresponding digests were added to 50-ml screw-cap polypropylene tubes containing 1.5 ml of the 2% ninhydrin solution and enough 20 mM NaH_2PO_4 buffer, pH 5.84, to bring the total volume to 25 ml. The buffer was made with HPLC-grade water. Each tube was placed in boiling water, with the lid loose, for 10 min and then cooled to room temperature. Blanks consisting of 2% ninhydrin and buffer were also prepared and boiled. The fluorescence of 1 ml of each cooled solution was determined at Ex 380 and Em 468 on an LS-50B luminescence spectrophotometer. Readings from blanks were subtracted from those for the test samples. Spectrophotometric readings were determined in eight replicates. A quantitative assessment of free lysine was made by comparison of the fluorescence intensities of the test samples with those obtained from standard curves prepared with 0.0, 0.1, 0.5, 1.0, 5.0, and 10.0 μg of L-lysine (Sigma)/25 ml of reaction mix. This range is well within the limits of the assay, which were reported to be from 0 to 48 μg lysine per 25 ml reaction mix, with a detection limit of 0.005 μg/ml (37).

Mass spectrometry. Substrate digestions and the negative controls were prepared as described above (see "Fluorescent ninhydrin assay for free L-lysine"). Peptides and their associated PGI-LysAP digests were extracted using C_{18} ZipTip pipette tips (Millipore), washed with HPLC-grade water containing 0.1% trifluoroacetic acid (TFA), reextracted with acetonitrile-water (50:50) containing 0.1% TFA, and mixed with a recrystallized α-cyano-4-hydroxy-cinnamic acid matrix solution (5 mg/ml; acetonitrile-water [50:50] containing 0.1% TFA) to a final concentration between 100 fmol and 1 pmol/μl. Approximately 0.6 to 0.7 μl of the peptide-matrix solution was spotted onto target plates. Mass spectra of the peptides were acquired by matrix-assisted laser desorption/ionization–two-stage time of flight mass spectrometry (MALDI-TOF-TOF) on a 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems, Framingham, MA) in the positive reflection mode with a 200-Hz neodymium-yttrium aluminum garnet 355-nm laser. Spectra were obtained by averaging 1,000 acquired spectra in the mass spectrometric mode. Conversion of time of flight to mass for the protonated monoisotopic ions, $(M + H)^+$, was based on the calibration of the instrument with a peptide standard calibration kit (Applied Biosystems) that contained the following peptides: des-Arg^1-bradykinin (*m/z* 904.4681), angiotensin I (*m/z* 1,296.6853), Glu^1-fibrinopeptide B (*m/z* 1,570.6774), adrenocorticotropin hormone (ACTH) clips 1 to 17 (*m/z* 2,903.0867), ACTH clips 18 to 39 (*m/z* 2,465.1989), and ACTH clips 7 to 38 (*m/z* 3,657.9294), where *m/z* is the mass-to-charge ratio. The loss of amino-terminal residues was determined as the difference between the masses of the intact peptides and the masses of the peptide fragments obtained after digestion with PGI-LysAP. Results were compared with corresponding peptide masses that were calculated using the ExPASy Proteomics Server of the Swiss Institute of Bioinformatics, located at http://ca.expasy.org/tools/peptide-mass.html.

Comparative SDS-PAGE on Tricine gels. Chromatographically purified *V. vulnificus* PGI-LysAP was diluted with buffer (20 mM Tris-HCl, pH 9.0) to 500 units per μl (30). Digestions were performed by combining up to 20 μg of each substrate, 1,000 units of PGI-LysAP, and 15 μl of 20 mM Tris-HCl, pH 9.0, and digesting for 2 h at 37°C. Negative controls consisting of PGI-LysAP combined with buffer but without the addition of substrate were also incubated for 2 h at 37°C. Reactions were stopped by boiling to near dryness followed by the addition of 15 μl of Tricine sample buffer (200 mM Tris-HCl [pH 6.8], 40% glycerol, 2% sodium dodecyl sulfate [SDS], and HPLC-grade H_2O) and boiling for an additional 3 to 5 min with the lids open to reduce the volume to less than 15 μl, which is the volume that will fit into the well of a 15-well gel. We modified the Tricine sample buffer formulation of Bio-Rad to exclude the Coomassie blue G-250, since it migrated at approximately the same rate as smaller (~1,000-Da) peptides and interfered with their visualization on the gels. The hydrolysis of the amino-terminal residue(s) from peptidyl substrates by PGI-LysAP was evaluated by comparative SDS-polyacrylamide gel electrophoresis (PAGE) on 16.5% Tricine Minigels (Bio-Rad Laboratories, Hercules, CA) under reducing (β-mercaptoethanol) conditions. The negative controls and the PGI-LysAP-digested samples were electrophoresed side by side in Tris/Tricine/SDS running buffer (Bio-Rad) at 80 V for 1 h. Peptides were stained for 5 min with Coomassie G-250, briefly destained, and digitally scanned, and the migrations of the bands for the undigested samples and the PGI-LysAP-digested samples were compared.

Kinetics of Lys-BK digestion. Lys-BK was used to evaluate the rate of free L-lysine production. The effects of digestion duration were determined by combining 100 μg of stock Lys-BK (at 25 μg/μl stock) with 20 μl of 20 mM Tris-HCl, pH 9.0, and 1,000 units of chromatically purified PGI-LysAP, centrifuging for 2 s, and incubating at 37°C for 0 to 2 h at 30-min intervals. After each period, reactions were stopped by placing the tubes in boiling water for 5 min and then at 4°C. Free lysine was directly determined by use of the fluorescent ninhydrin reaction and indirectly determined by MALDI-TOF-TOF of the hydrolyzed Lys-BK (BK product). Substrate cleavage was also evaluated on Tricine gels after the digestion of 50 μg of Lys-BK with 1,000 units of enzyme in 10 μl of 20 mM Tris-HCl, pH 9.0, for 0 to 2 h at 15-min intervals. The positive control, consisting of substrate and buffer only, was incubated for 2 h at 37°C before boiling.

RESULTS AND DISCUSSION

PGI-LysAP hydrolyzes human peptidyl substrates. Using MALDI-TOF-TOF, we determined the loss of amino-terminal

TABLE 2. Peptides identified by mass spectrometry before and after digestion with PGI-LysAP from *Vibrio vulnificus*

Peptide	Peptide sequence	$(M + H)^+$	
		Observed[a]	Calculated[b]
Negative control[c]	None (no ions detected between 800 and 4,000 Da)		
Bradykinin	RPPGFSPFR	1,060.606	1,060.5686
Digest	RPPGFSPFR	1,060.629	1,060.5686
	PPGFSPFR[d]	**904.528**	**904.4675**
Lys-bradykinin	KRPPGFSPFR	1,188.669	1,188.6636
Digest	KRPPGFSPFR	1,188.679	1,188.6636
	RPPGFSPFR	**1,060.603**	**1,060.5686**
	PPGFSPFR	**904.504**	**904.4675**
Lys-(des-Arg^9)-bradykinin	KRPPGFSPF	1,032.571	1,032.5625
Digest	KRPPGFSPF	1,032.578	1,032.5625
	RPPGFSPF	**904.485**	**904.4675**
Neurokinin A	HKTNSFVGLM	1,133.596	1,133.5772
Digest	HKTNSFVGLM	1,133.592	1,133.5772
	KTNSFVGLM	**996.537**	**996.5183**
	TNSFVGLM	**868.431**	**868.4233**
Met-Lys-bradykinin	MKRPPGFSPFR	1,319.726	1,319.7041
Digest	MKRPPGFSPFR	1,319.733	1,319.7041
	KRPPGFSPFR	ND[e]	1,188.6636
	RPPGFSPFR	**1,060.590**	**1,060.5686**
	PPGFSPFR	**904.486**	**904.4675**
Myosin light chain fragment	KKRAARATSNVFA-NH_2	1,418.852	1,418.8344
Digest	KKRAARATSNVFA-NH_2	1,418.859	1,418.8344
	KRAARATSNVFA-NH_2	**1,290.766**	**1,290.7394**
	RAARATSNVFA-NH_2	**1,162.672**	**1,162.6445**
	AARATSNVFA-NH_2	**1,006.562**	**1,006.5434**
	ARATSNVFA-NH_2	**935.525**	**935.5063**
	RATSNVFA-NH_2	**864.481**	**864.4691**
Histatin 8	KFHEKHHSHRGY	1,562.768	1,562.7723
Digest	KFHEKHHSHRGY	1,562.745	1,562.7723
	FHEKHHSHRGY	**1,434.665**	**1,434.6774**
	HEKHHSHRGY	**1,287.600**	**1,287.6089**
	EKHHSHRGY	**1,150.541**	**1,150.5500**

[a] Observed mass of the protonated peptide ion as determined by mass spectrometry.
[b] Calculated mass of indicated peptides determined with the ExPASy Proteomics Server of the Swiss Institute of Bioinfomatics located at http://ca.expasy.org/tools/peptide-mass.html.
[c] Negative control containing enzyme (PGI-LysAP) and buffer but no substrate.
[d] Hydrolyzed products detected by MALDI-TOF-TOF and corresponding mass spectrometry values are indicated in boldface.
[e] ND, none detected.

residues based on the molecular masses of the products found in the digests. Results indicate the sequential, enzymatic hydrolysis of the peptide bonds associated with amino-terminal residues including, but not limited to, lysine. Table 2 shows the peptides detected in the digests, which in each case included some undigested peptide and one or more hydrolyzed products (bold). Clearly, Arg is cleaved from BK, while Lys and Arg are cleaved, one at a time, from Lys-BK. The amino-terminal lysyl residues were also hydrolyzed from Lys-(des-Arg^9)-BK, the myosin light chain fragment, and histatin 8 (Table 2). Phe and His were then cleaved from histatin 8. A second Lys followed sequentially by an Arg and two Ala residues were cleaved from the myosin light chain fragment. This demonstrates that PGI-LysAP from *V. vulnificus* not only has a lysyl aminopeptidase function but also is an exopeptidase with broad substrate specificity. Serial cleavage was also seen for neurokinin A, where His was first hydrolyzed and the Lys was subsequently hydrolyzed (Table 2). For Met-Lys-BK, PGI-LysAP cleaved the Met-Lys together; there was no Lys-BK detected. Further hydrolysis was observed with the removal of the Arg residue (Table 2).

TABLE 3. Free lysine released from kinins and other peptides after digestion with PGI-LysAP[a]

Peptide digest	Mean L-lysine detected (ng)[b]
Bradykinin	ND
Lys-bradykinin	1,390 ± 40
Lys-(des-Arg^9)-bradykinin	2,070 ± 30
Neurokinin A	1,970 ± 20
Met-Lys-bradykinin	ND
Histatin 8	700 ± 50
Myosin light chain fragment	2,690 ± 70

[a] Free lysine released from 20 μg of kinins and other peptides after digestion with 1,000 units of PGI-LysAP for 2 h at 37°C ($n = 8 \pm$ SD).
[b] ND, none detected.

Quantitative detection of free lysine using a fluorescent ninhydrin reaction. A standard curve for free L-lysine was determined using a fluorescent ninhydrin assay. By plotting the mass in μg of free L-lysine against fluorescence at 468 nm, we obtained a perfect linear relationship ($R^2 = 1.000$) within a range of 0 to 10 μg of free L-lysine. For test samples, the quantitative detection of free lysine was performed with the peptides after digestion with PGI-LysAP. Spectrophotometric measurements for the peptides alone served as blanks, and these values were subtracted from the measurements obtained for the substrate digests. Results are shown in Table 3. Free lysine was produced in all the digests, except Met-Lys-BK and the BK-negative control (Table 3). Digests of Lys-BK and Lys-(des-Arg^9)-BK produced 1,390 and 2,070 ng of free lysine, respectively (Table 3). Neurokinin A hydrolysis was initially not expected due to a His residue preceding the Lys residue on the amino terminus. Nevertheless, the digestion of neurokinin A produced 1,970 ng of free lysine (Table 3), which supports the mass spectrophotometric data indicating that the amino-terminal His and Arg were sequentially hydrolyzed (Table 2). In contrast to the digestion of neurokinin A, Met-Lys-BK did not produce free lysine. This supports the MALDI findings showing that PGI-LysAP can act as an exopeptidase by cleaving the Met and Lys as a unit. By definition, an exopeptidase can cleave a single residue or two residues at one time from the terminal ends of proteins and peptides. The most lysine detected (2,690 ng) was from the digestion of the myosin light chain fragment (Table 3). This was expected, since it contained two lysyl residues at the amino terminus which, according to mass spectrometry (Table 2), were sequentially cleaved. Our results support the findings of Wang and Ji (37), who showed that ninhydrin could readily detect free lysine but not other primary amino acids or organic compounds. The fluorescent ninhydrin reaction was rapid, simple, inexpensive, sensitive, and quantitative, making it ideal to evaluate the presence of lysine cleaved from potential peptidyl substrates. To our knowledge, this assay is underutilized, since a search of Science Citation Index for papers referencing the 1988 paper by Wang and Ji (37) revealed no hits. We believe that our use of the fluorescent ninhydrin assay is the first application of its kind to quantitatively assess the enzymatic degradation or activation of peptides and that this application serves as a paradigm for future studies.

Electrophoretic confirmation of peptide cleavage. Tricine gel electrophoresis was performed on 20 μg of undigested and digested peptides without the use of dyes in the sample buffer.

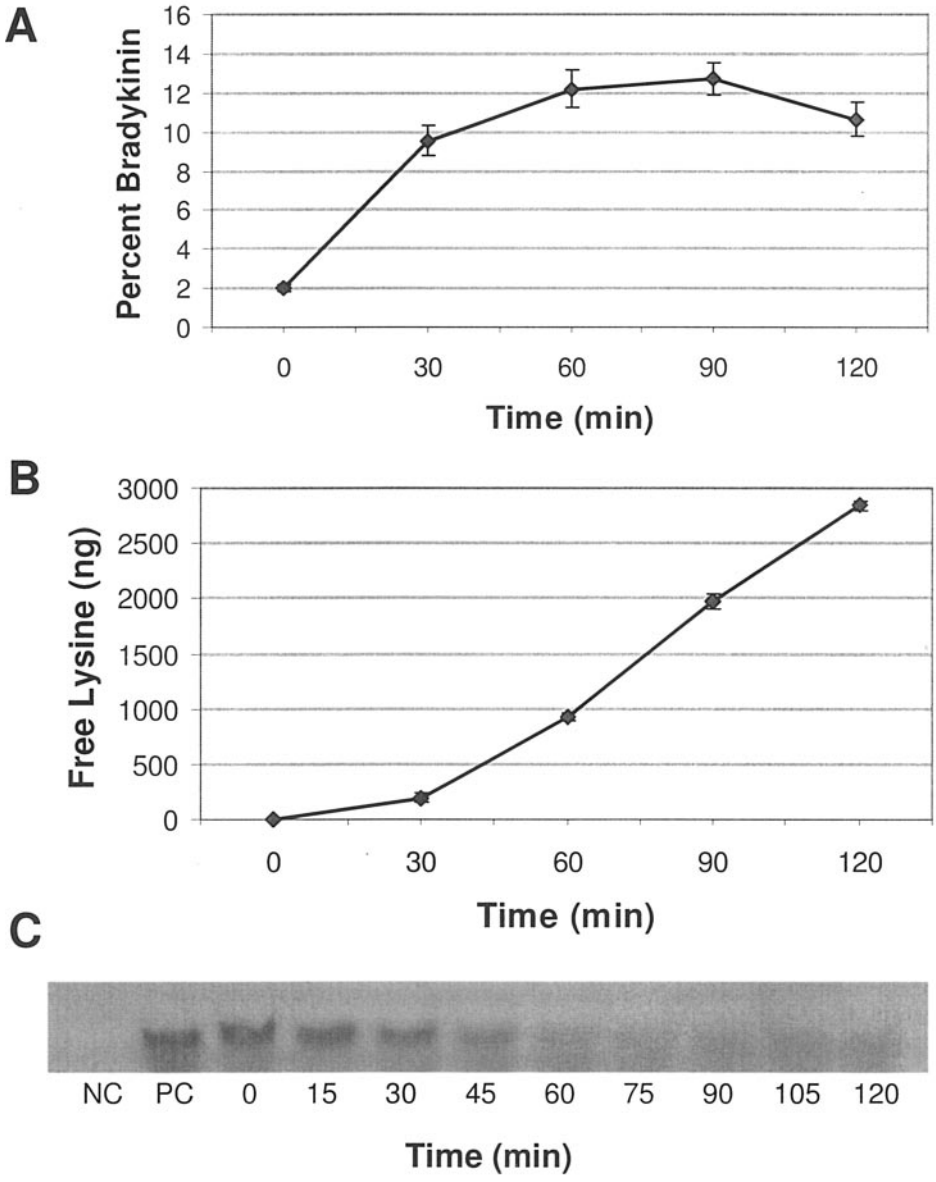

FIG. 1. Bradykinin and free lysine production from Lys-BK digested with PGI-LysAP from *Vibrio vulnificus*. (A) Mass spectrophotometric determination of the percent BK present after PGI-LysAP digestion of Lys-BK with 1,000 units of PGI-LysAP for up to 2 h at 37°C. Bradykinin is represented by the ion intensity of product detected at *m/z* 1,060. Each point represents the mean of six readings ± standard deviation. (B) Graph showing the release of free lysine from 100 μg of Lys-BK after digestion at 37°C with 1,000 units of PGI-LysAP for up to 2 h as determined using a fluorescent ninhydrin assay. Each point represents the mean of eight readings ± standard deviation. (C) Results from SDS-PAGE of 50 μg of Lys-BK and its cleavage products on a Tricine gel after digestion at 37°C with 1,000 units of PGI-LysAP for various periods. The negative control (NC) lane was inoculated with enzyme but no substrate, while the positive control (PC) lane was inoculated with substrate but no enzyme.

After digestion with 1,000 units of PGI-LysAP for 2 h at 37°C, the peptides were substantially cleaved and no bands were visible; in contrast, bands were visible for the undigested controls. The loss in peptide mass by the cleavage of Lys and other residues resulted in a mixture of peptide fragments which, due to their extremely low masses and inability to capture sufficient Coomassie stain, were below the level of detection. The minimum mass typically detected on Tricine gels is around 1,100 Da; however, by eliminating dye in the sample buffer, we were able to visualize smaller peptides, including Lys-(des-Arg^9)-BK, which has a mass of only 1,032 Da. The absence of visible bands from the digests supports the MALDI-TOF-TOF data (Table 2), which indicate cleavage and, at times, multiple, sequential cleavage of residues from the peptides.

The kinetics of Lys-BK digestion. Lys-BK has an $(M + H)^+$ ion at an *m/z* of 1,188, and the enzymatic removal of the lysyl residue produces BK with an *m/z* of 1,060. We used mass spectrometry to determine the percentage of BK present in a Lys-BK digest at 30-min intervals for 2 h by comparing the ion intensities at the corresponding *m/z*. By plotting the percentage of the digestion product at *m/z* 1,060 against the digestion duration (Fig. 1A), one sees a rapid increase in BK production

between 0 and 30 min, a tapering off between 30 and 90 min, and an apparent reduction from 90 to 120 min. Both the tapering off and the reduction of product at *m*/*z* 1,060 can be accounted for by the continued, sequential processing of BK by the cleavage of Arg from the amino-terminal end of the peptide, which effectively reduces the amount of product at *m*/*z* 1,060. This is consistent with the digestion data shown in Table 2 for Lys-BK, where Lys is first removed, followed by the Arg. About 2% of the Lys was cleaved at 0 min, due to the lag period required for setting up the digests and subsequently inactivating the enzyme by boiling.

We also determined the kinetics of free lysine production as a function of digestion time. Figure 1B shows a nearly linear increase in free lysine production between 60 and 120 min. Free lysine production is consistent with the mass spectrometry data (Table 2). The hydrolysis of 50 μg of Lys-BK is also apparent on an electrophoretic gel (Fig. 1C). With no digestion (0 min), the band produced on the Tricine gel was relatively strong, but the band diminished in intensity as the digestion time increased. The positive control differed from the other samples in that it did not contain any enzyme, only substrate in buffer. Consequently, its migration proceeded unhampered, unlike what was seen for the digests, where the presence of proteins in the enzyme preparation may have slightly impeded band migration, thus accounting for a slight shift in the apparent mass between the positive control and 0-min digests. With increasing digestion periods, bands became more diffuse and broader as lower-molecular-mass peptides were produced (Fig. 1C). Enzymatic removal of residues beyond the amino-terminal lysyl residue was clearly indicated by mass spectrometry (Table 2 and Fig. 1A).

Comparative analysis of enzyme specificity. In a previous study using synthetic peptidyl substrates covalently bound to AMC, we showed that PGI from *V. vulnificus* preferentially cleaved Lys residues (30). The relative cleavage preferences were as follows: 100% for L-Lys, 19% for L-Arg, and 10% and 4% for cleavage on the carboxyl side of Z-Phe-Pro-Arg and Z-Phe-Arg, respectively (30). There was no cleavage on the carboxyl side of the lysyl residues in D-Val-Leu-Lys-, D-Ala-Leu-Lys-, and Z-Ala-Ala-Lys-AMC. Consequently, we named PGI with this strong lysyl aminopeptidase activity PGI-LysAP. In the present study, we demonstrated by mass spectrometry that PGI-LysAP serves as an exopeptidase, cleaving not only Lys residues but also Arg, His, Ala, and Phe from the amino-terminal ends of human peptides and Met and Lys residues as a unit from Met-Lys-BK. Together, the results indicate that PGI-LysAP is a lysyl aminopeptidase but has relatively broad exopeptidase activity.

Kinin activation of signal transduction pathways. Bacterial proteases have been well documented to serve as universal triggers of kinin generation, which, in turn, enhances vascular permeability and inflammatory events associated with microbial infections (16–18, 20, 22). We clearly demonstrated the processing of kinins by PGI-LysAP cleavage of Lys-BK and Met-Lys-BK. Bradykinin-related peptides are well known for binding to two G protein-coupled, seven-transmembrane, helical receptors known as the B1 and B2 receptors (2, 5, 27). These receptors are on the surface of a variety of cell types, where they trigger signal transduction events leading to a host of physiological responses (2, 5, 27). Proteolytic processing of BK alters the affinity of the ligand and its receptor. Bradykinin generated as a result of bacterial infections induces pain, edema, vasodilation, hypotension, and shock (16, 20). Bradykinins have also been shown to enhance the invasiveness of *V. vulnificus* by facilitating the transvascular translocation of the bacterium (17, 19, 21). The extent of peptide processing by PGI-LysAP is likely related to the amount of enzyme present, the duration of contact with the enzyme, the temperature and pH of the reaction, and other physiological conditions; therefore, the digestion products detected in this study may represent only a small sampling of those actually produced in an infected individual. The extent of PGI-LysAP's proteolytic cleavage may be limited to the amino-terminal lysyl residue for transient peptides in the general circulation, since they may be in only brief contact with the enzyme, which is on the surface of the vibrios (30). Localized and more-focused proteolytic processing of peptides would be expected in tissues infected by *V. vulnificus*. Tissue involvement leads to necrotizing fasciitis, the appearance of bullous skin lesions, and general inflammatory reactions—conditions common to *V. vulnificus* infection.

Neurokinin A is a tachykinin with broad tissue distribution and function (26, 33, 36). In skin and immune cells, neurokinin A exerts a physiological role in modulating cell proliferation, cytokine production, antigen presentation, and inflammation of the epidermal and dermal layers (32). Tachykinins in general are substantially involved in inflammatory reactions (3, 10, 32), vasodilation (6, 27), and the regulation of local blood flow (6). Wallengren (36) found high levels of neuropeptides in bullae associated with inflammatory skin disorders.

Currently, three G protein-coupled receptors are recognized for the tachykinins: neurokinin (NK)-1, NK-2, and NK-3 (28). Neurokinin A is a decapeptide with high affinity for NK-2 receptors that are present on the surface of many cells where ligand-receptor binding elicits second messenger signaling cascades (1). Other tachykinins, such as substance P, preferentially bind to receptor NK-1, while neurokinin B has strong affinity for NK-3 (28). In the present study, we observed the sequential cleavage of amino-terminal His and Lys from neurokinin A (Table 2). Neurokinin-degrading enzymes are known to participate in the modulation of skin inflammation, wound healing, and skin immune responses (32). Residues constituting the C-terminal hexapeptide are essential for receptor binding (28). Regoli et al. (28) demonstrated that for rabbit pulmonary artery and rat portal vein, tissues that have high sensitivity, selectivity, and specificity for binding neurokinin A, the binding of neurokinin A to NK-2 receptors doubled with the removal of the first three amino-terminal residues. Our studies demonstrated that PGI-LysAP cleaved at least two amino-terminal residues from neurokinin A, which would likely alter the affinity of human neurokinin A to NK-2 receptors but would not disrupt the essential C-terminal hexapeptide sequence required for receptor binding (28). Clearly, PGI-LysAP's proteolytic processing of neurokinin A may enhance receptor binding and elicit inflammatory and vasodilatory reactions, which would help explain the symptoms of vibriosis and provide clues to explain the rapid invasiveness of *V. vulnificus*. A better understanding of the mechanisms of receptor binding by peptides and their proteolytic digests is crucial to unraveling the mysteries of bacterial invasiveness.

Processing of histatin 8 and myosin light chain kinase. The enzymatic cleavage of lysine from histatin 8 and from a myosin light chain kinase fragment was also evident (Table 2). Histatins are salivary peptides known for their antimicrobial properties (11, 35). The hydrolysis and possible inactivation of histatin 8 by *Vibrio* PGI-LysAP may be a bacterial defense mechanism unique to *Vibrionaceae* family members. The myosin light chain fragment is a peptide analog consisting of residues 11 to 23 from the amino-terminal end of the myosin light chain. This region is an effective substrate for smooth muscle myosin light chain kinase and was shown to inhibit the phosphorylation of the calmodulin-binding region (12, 13). As previously mentioned, the myosin light chain fragment produced the greatest amount of free lysine (Table 3) due to the release of the two amino-terminal lysyl residues. This peptide was also sequentially cleaved five times (Table 2), demonstrating the extent to which PGI-LysAP can serve as a hydrolytic enzyme.

Other virulence enhancers are required for *V. vulnificus* infection. In a survey for PGI-LysAP in other members of the *Vibrionaceae* family and in non-*Vibrionaceae* bacterial pathogens, we detected PGI-LysAP in all 11 species of *Vibrionaceae* examined but not in any of 14 non-*Vibrionaceae* pathogens (31). We also detected PGI-LysAP in strains of virulent and avirulent *V. vulnificus*, which were determined as such by mouse 50% lethal dose assays (30). The presence of PGI-LysAP in all *Vibrionaceae* family members, whether human pathogens or not, suggests that other virulence enhancers or predisposing conditions must be present for *V. vulnificus* to infect an individual via the gastrointestinal route. Some of the putative virulence factors that have been identified over the years using cell culture and animal models (7) may indeed be required for *Vibrio* to gain entry via the gastrointestinal route and to permit the bacterium to evade host defenses. Vibrios that are introduced through wound infections could forgo the need for certain virulence factors, since their introduction into the tissues or bloodstream is achieved through physical means (trauma) rather than by biological actions. This may explain why healthy individuals are susceptible to *V. vulnificus* wound infections but not to infection through the gastrointestinal route.

Cellular complexities in understanding virulence mechanisms. Within the extracellular milieu of mammalian systems are countless proteins, peptides, and degradation products which have important biological functions in the normal physiology of the individual cell as well as in the entire organism. Disruption of the normal metabolism of proteins and peptides by invading bacteria and their enzymes alters the levels of these compounds, affecting the biological, chemical, mechanical, and structural integrity of the organism. Sorting out the complex interactions between proteins and peptides, their catabolytes and inhibitors, and potential cellular receptors represents a daunting task. Through the enzymatic alteration of bradykinin and related peptides, this study demonstrates a few of the many potential virulence mechanisms that may be available to enhance bacterial invasiveness. As we gain insights into the functions of proteolytic enzymes in bacterial infections, it seems likely that new, non-antibiotic-related interventions directed toward blocking the enzymes' activities or the development of new receptor antagonists may provide the key for preventing or controlling morbidity and mortality from *V. vulnificus* and other pathogens.

ACKNOWLEDGMENTS

We thank Mingxin Guo, Delaware State University, for translating into English the paper by Wang and Ji (37) on the fluorescent ninhydrin reaction. We also acknowledge the excellent technical assistance of Michael Watson, USDA, ARS (Dover, DE), and Laurie Fortis, USDA, ARS (Wyndmoor, PA).

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