

# The metalloproteolytic activity of the anthrax lethal factor is substrate-inhibited

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*Running title:* Substrate inhibition of the anthrax lethal factor

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*Abbreviations:* EF, anthrax edema factor; LF, anthrax lethal factor; MAPKK, mitogen-activated protein kinase kinase; PA, protective antigen; *p*NA, *p*-nitroaniline; AMC, 7-amido-4-methylcumarine.

## **Abstract**

**The anthrax lethal factor (LF) is a Zn<sup>2+</sup>-endopeptidase specific for mitogen-activated protein kinase kinases (MAPKKs), which are cleaved within their *N*-termini. Here, the proteolytic activity of LF has been investigated using novel chromogenic MAPKK-derived peptide substrates, which allowed us to determine the kinetic parameters of the reaction. LF displayed maximal proteolytic activity at the pH and temperature values of the cell cytosol, which is its site of action. LF undergoes substrate inhibition, in keeping with the non-productive binding geometry of the MAPPK-2 *N*-terminus to LF.**

*Keywords:* Anthrax lethal factor; peptide chromogenic substrates; total substrate inhibition; peptide inhibitors.

## Introduction

Toxigenic strains of *Bacillus anthracis* secrete three proteins: the protective antigen (PA, 87.2 kDa), the edema factor (EF, 88.8 kDa), and the lethal factor (LF, 90.2) [1-3]. PA derives its name from the fact that immunization with it confers a protective immunity to vaccinated animals [4-5]. PA binds to a rather ubiquitous plasma membrane type I protein encoded by the tumor endothelial marker gene 8 [6-7] and to human capillary morphogenesis protein 2 [8]. PA is then proteolytically processed on the cell surface by furin and membrane furin-like peptidases, to a 63 kDa form (PA<sub>63</sub>) which oligomerizes and binds LF or EF [3]. The lethal toxin (PA + LF) and the edema toxin (PA + EF) are then endocytosed by a lipid raft-mediated clathrin-dependent process [9]. Then, PA<sub>63</sub> undergoes an acidic triggered conformational rearrangement [10] that mediates the transfer of EF or LF from the lumen of a late endocytic compartment to the cytoplasm [11].

EF is a Ca<sup>2+</sup>- and calmodulin-dependent adenylate cyclase that increases cytosolic cAMP, altering water homeostasis and the elaborate balance of intracellular signaling pathways. EF impairs neutrophil function(s) and it is believed to be responsible for the edema found in cutaneous anthrax [12].

LF displays metalloproteolytic activity directed toward the *N*-terminus of mitogen-activated protein kinase kinases (MAPKKs) [13-16]. The active site zinc ion is coordinated tetrahedrally to the domain 4 of LF (residues 551-777) by a water molecule and three side chains (i.e. H686, H690, and E735), in a structural arrangement shared by metalloproteases of the thermolysin family [17]. LF has a deep and long (~40 Å long) groove contiguous to the active site center which binds peptide substrates and peptide inhibitors [17-18]. The groove has an overall negative electrostatic potential containing clusters of E/D as well as Q/N residues. The determination of the sites of proteolysis of various MAPKK isoforms led to the identification of a consensus motif for the cleavage site: positively charged residues are

located at positions P<sub>7</sub> to P<sub>4</sub> and hydrophobic residues at P<sub>2</sub> and P<sub>1</sub>' ; in addition, an aliphatic hydrophobic residue is present at position P<sub>1</sub> (Fig. 1)<sup>1</sup> [15].

The lethal toxin metalloproteolytic activity is cytotoxic to macrophages [19] and there is evidence that it plays a major role in the development of systemic anthrax, a rapid and often fatal disease of several vertebrates including humans [20]. It is worth noting that LF active site mutants are non toxic [21] and membrane permeable metalloprotease inhibitors prevent the LF cytotoxic activity on cultured macrophages [18].

Here, we present a detailed analysis of the LF catalyzed hydrolysis of chromogenic substrates designed on the basis of the consensus motif of MAPKK *N*-termini and of the inhibition caused by the substrates themselves. These findings are relevant to the design of novel inhibitors of LF and for the understanding of the macrophage toxicity of LF.

## Experimental Procedures

### *LF and peptide chromogenic substrate synthesis*

Recombinant LF was prepared as previously reported and the LF concentration was determined by absorbance at 280 nm ( $E^{0.1\%}_{1\text{ cm}} = 0.798$ ) [15]. Small aliquots were frozen in liquid nitrogen and stored at -80°C. Peptides from the Leu P<sub>2</sub> residue to the *N*-terminal amino acid (Table 1) were synthesized by Fmoc (9-fluorenyl methyloxy-carbonyl)-solid phase peptide synthesis [22] on a 4-hydroxymethyl-phenoxyethylcopolystyrene-1% divinylbenzene resin (Perkin Elmer) using a peptide synthesizer (Model 431-A, Applied Biosystems) according to the manufacturer's protocol. Fmoc-amino acids were purchased from Bachem (Germany). The proper *C*-terminal P<sub>1</sub> amino acid residue (Pro or Arg; Table 1) derivatized with *p*-nitroaniline (*p*NA) or with 7-amido-4-methylcoumarin (AMC) (Bachem)

was coupled to the peptides by a condensation reaction in dimethylformamide with HATU (*N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide) (Millipore) as coupling reagent and 2,4,6-trimethylpyridine (Merk) as base [23]. The peptide chromogenic substrates were purified by reverse phase HPLC Jupiter C18, 5  $\mu$ m, 150  $\times$  4.6 mm (Phenomenex) using a linear gradient of CH<sub>3</sub>CN/water including 0.1% TFA. The racemic peptide mixture was well resolved with this method and the peptide containing L-leucine was retained. The molecular weight of the various peptides was checked with a KRATOS MALDI-TOF MS (Shimadzu) and it agreed well in all cases with the theoretical value.

The concentration of peptide chromogenic substrates derivatized with *p*NA was determined by measuring their absorbance at 342 nm ( $\epsilon = 8270 \text{ M}^{-1} \text{ cm}^{-1}$ ). The concentration of the peptide chromogenic substrate derivatized with AMC was determined using a standard curve of the emission fluorescence at 460 nm ( $\lambda_{\text{exc}} = 360 \text{ nm}$ ) from known concentrations of AMC (Fluka). Then, the emission fluorescence of the solution containing the completely cleaved AMC-derivatized substrates by LF was determined.

#### *Determination of the LF catalytic properties*

The LF catalyzed hydrolysis of peptide chromogenic substrates listed in Table 1 was measured between pH 4.5 and 8.5 (25 mM phosphate buffer) and between 23.0°C and 43.0°C; NaCl was present with concentrations ranging from 15 to 100 mM. The LF concentration ranged between  $1.0 \times 10^{-9} \text{ M}$  and  $3.0 \times 10^{-8} \text{ M}$ . Substrate concentration ranged between  $3.0 \times 10^{-6} \text{ M}$  to  $1.0 \times 10^{-3} \text{ M}$ . The LF catalyzed hydrolysis of the *p*NA-derivatized substrates (i.e. the *p*NA release) was monitored spectrophotometrically at 405 nm ( $\epsilon = 9920 \text{ M}^{-1} \text{ cm}^{-1}$ ) [24]. The LF catalyzed hydrolysis of AcRRRRVLR-AMC (i.e. the AMC release) was monitored spectrofluorometrically at 460 nm ( $\lambda_{\text{exc}} = 360 \text{ nm}$ ) [25].



On the basis of the knowledge of the LF:MAPKK recognition properties [15], the LF peptide chromogenic substrates listed in Table 1 were designed and synthesized. These substrates contain at their C-terminus the *p*-nitroaniline (*p*NA) or the 7-amido-4-methylcoumarin (AMC) group, which are released upon the hydrolysis reaction catalyzed by LF. Product (i.e. *p*NA and AMC) concentration is easily determined spectrophotometrically at 405 nm and spectrofluorometrically at 460 nm ( $\lambda_{\text{exc}} = 360$  nm), respectively. Under all the experimental conditions, product formation (i.e. *p*NA and AMC release from peptide chromogenic substrates) catalyzed by LF was monitored for 5 - 10 min and it was linear over the assay time. Moreover, over the whole range explored ( $1.0 \times 10^{-9}$  M to  $3.0 \times 10^{-8}$  M), the initial velocity (i.e.  $v_i$ ) was strictly linear with the LF concentration. As expected, no substrate hydrolysis was detected in the presence of apo-LF (data not shown).

As shown in Figure 2, LF undergoes total substrate inhibition, indicating that the substrate may bind to the enzyme with different geometries, the substrate bound in the non-productive binding mode(s) impairs LF action. Total substrate inhibition is in keeping with the observation that the LF:MAPKK-2 substrate adduct is the first example of a protease in complex with its uncleaved substrate [17]. It is worth noting that although the closest main-chain approach to the  $\text{Zn}^{2+}$  ion is the scissile bond following MAPKK-2 P10 (i.e. the  $\text{P}_1\text{-P}_1'$  bond), it is about 6 Å from the zinc-bound water. Modelling studies have shown that a rotation about a main-chain dihedral angle at K6 (i.e.  $\text{P}_5$ ) would allow P10 (i.e.  $\text{P}_1$ ) to swing down into the  $\text{S}_1$  specificity subsite to generate the productive cleavage complex. The substrate productive conformation requires an abrupt  $90^\circ$  turn in the peptide chain (favoured by the P10 residue) at the  $\text{P}_1$  position (Fig. 1) [17].

The analysis of the data shown in Figure 2, according to Eqn 1, provided the values of  $K_m$ ,  $k_{\text{cat}}$ , and  $k_{\text{cat}}/K_m$  for the LF catalyzed hydrolysis of peptide chromogenic substrates, as well

as of  $K_i$  for enzyme inhibition by the *p*NA- and AMC-derivatized substrates (Table 1). The turnover number of LF shows a limited variation (from 2 to 6 molecules of substrate hydrolysed per second by one molecule of LF) upon changing the length of peptide substrates and the number of R residues, whose addition at the *N*-terminus renders peptides permeable to the plasma membrane of cells [18]. This figure can be compared with that of the prototype metalloproteinase, thermolysin, whose turnover number is comprised in the range 6 to 16 substrate molecules hydrolysed per enzyme molecule [28].

The values of  $K_m$  and  $K_i$  for the LF catalyzed hydrolysis of peptide chromogenic substrates containing 6 to 9 residues are very favorable. Moreover, the  $k_{cat}/K_m$  values indicate that the LF substrates here reported are very specific. Furthermore, peptides containing more R residues are cleaved more efficiently. The  $K_m$  values decrease (i.e. the LF:substrate affinity increases) by adding positively charged R residues at the *N*-terminal region of the peptide substrates (Table 1). Though one cannot disregard the effect of increasing length, this finding can be taken as an additional evidence of the importance of electrostatic interactions in LF-substrate recognition. This is in agreement with the decrease of the initial velocity (i.e.  $v_i$ ) for the LF catalyzed hydrolysis of the peptide chromogenic substrate AcMLARRRPVLP-*p*NA on increasing the ionic strength (i.e. the NaCl concentration) (Fig. 3). It should be noted that the negatively charged residues E336, E334, D387, and D394 located in the elongated cleft-shaped recognition site of LF (Fig. 1) are in such a position within the active site as to suggest that they interact directly with the substrate *N*-terminal positively charged R residues thus playing a major role in the binding and the positioning of the substrate [17].

As shown in Table 1, values of  $k_{cat}$  for the LF catalyzed hydrolysis of peptide chromogenic substrates are essentially substrate-independent (Table 1). This may reflect a common rate limiting step in catalysis. As shown in Figure 3, the maximum initial velocity (i.e. maximum  $v_i$ ) for the LF catalyzed hydrolysis of AcMLARRRPVLP-*p*NA occurs at pH



7.4 and 37°C, corresponding to pH and temperature values of the cytosol of mammalian cells, which is the site of action of LF.

It should be noted that values of  $k_{cat}$  for the LF catalyzed hydrolysis of the peptide substrates conjugated with *p*NA or with AMC, which differ considerably in size, are almost identical (Table 1). This is in agreement with the previous findings [15,17] that the main determinants of the substrate binding into the active site cleft of LF are located on the *N*-terminus side. The AMC-derivatized substrate AcRRRRVLR-AMC offers a much higher sensitivity than the *p*NA-derivatized substrates allowing one to follow the proteolytic activity of minute amounts of LF (about one hundred fold less enzyme is needed). The hydrolysis of both types of substrates can be monitored with very simple apparatuses and in a wide range of experimental conditions, including high throughput assays.

## Conclusions

This is the first detailed study of the kinetic parameters of the metalloproteolytic activity of LF *in vitro* obtained with ad hoc designed chromogenic substrates whose hydrolysis can be monitored with very simple and widely available techniques. It describes the substrate inhibition of LF hydrolytic activity. The subtle modulation of LF activity by total substrate inhibition is in keeping with the non-productive binding mode of MAPKK-2 *N*-terminus to LF [17] and could be relevant for the enzyme action *in vivo*. In fact, LF cleaves several isoforms of MAPKK present within the cytosol of cells. Given the different structural properties of these cellular substrates and their different sub-cellular locations [29,30], it is very likely that they are cleaved with different velocities, and that there is a non-uniform LF-induced cleavage of the MAPKK isoforms. Such heterogeneity of cleavage of MAPKK

isoforms could additionally vary among cells giving rise to different cellular outcomes of the intoxication with LF. Indeed, the effect of LF varies with cell types and conditions. Upon exposure to PA+LF, macrophages lyse in culture [19,31], but die from apoptosis if they are primed with lipopolysaccharides or tumor necrosis factor- $\alpha$  [32,33]. On the other hand, other cells are resistant though their MAPKK-3 is cleaved by LF [15,34,35]. A quantitative analysis of the differential LF cleavage of MAPKK isoforms within cells is not a simple goal to achieve, but it appears to be essential for the molecular understanding of the *in vivo* action of LF.

## **Acknowledgements**

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## Footnotes

Footnote to page 4:

<sup>1</sup>The specificity subsites surrounding the catalytic center of LF have been identified as  $S_n \dots S_1, S_1' \dots S_n'$ . The amino acid residues forming the recognition site of substrates and inhibitors of LF have been labeled as  $P_n \dots P_1-P_1' \dots P_n'$ .  $P_1-P_1'$  is the (potentially) scissile reactive site peptide bond of the substrate (or the inhibitor) [15,17].

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**Table 1.** Catalytic and inhibitory properties of LF.<sup>a</sup>

Peptide substrates													$k_{cat}$ (s <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat}/K_m$ (sec <sup>-1</sup> $\mu$ M <sup>-1</sup> )	$K_i$ ( $\mu$ M)						
										P <sub>8</sub>	P <sub>7</sub>	P <sub>6</sub>	P <sub>5</sub>	P <sub>4</sub>	P <sub>3</sub>	P <sub>2</sub>	P <sub>1</sub>	P <sub>1</sub> '				
											AcR	R	R	R	V	L	R	<i>p</i> NA	5.0 $\pm$ 0.3	9.5 $\pm$ 1.4	0.5 $\pm$ 0.1	190 $\pm$ 28
											AcR	R	R	R	V	L	R	AMC	5.5 $\pm$ 1.3	82 $\pm$ 29	0.1 $\pm$ 0.04	170 $\pm$ 60
						AcM	L	A	R	R	R	R	P	V	L	P	<i>p</i> NA <sup>b</sup>	4.5 $\pm$ 0.4	30 $\pm$ 2	0.15 $\pm$ 0.02	600 $\pm$ 220	
						AcM	L	A	R	R	R	R	P	V	L	R	<i>p</i> NA <sup>b</sup>	3.9 $\pm$ 0.1	13 $\pm$ 0.5	0.3 $\pm$ 0.02	260 $\pm$ 70	
AcG	Y	$\beta$ A	R	R	R	A	R	R	R	R	R	R	V	L	R	R	<i>p</i> NA <sup>b</sup>	3.7 $\pm$ 0.1	1.8 $\pm$ 0.2	2.0 $\pm$ 0.6	36 $\pm$ 7	
AcG	Y	$\beta$ A	R	R	R	R	R	R	R	R	R	R	V	L	R	R	<i>p</i> NA <sup>b</sup>	6.5 $\pm$ 0.5	1.5 $\pm$ 0.2	4.3 $\pm$ 0.9	30 $\pm$ 5	

<sup>a</sup> Ac, acetylic group; *p*NA, *p*-nitroaniline;  $\beta$ A,  $\beta$ -alanine; AMC, 7-amino-4-methylcoumarin. All data were obtained at pH 7.4 and 25.0°C.

<sup>b</sup> Preliminary results have been reported in ref. [18].

## Figure legends

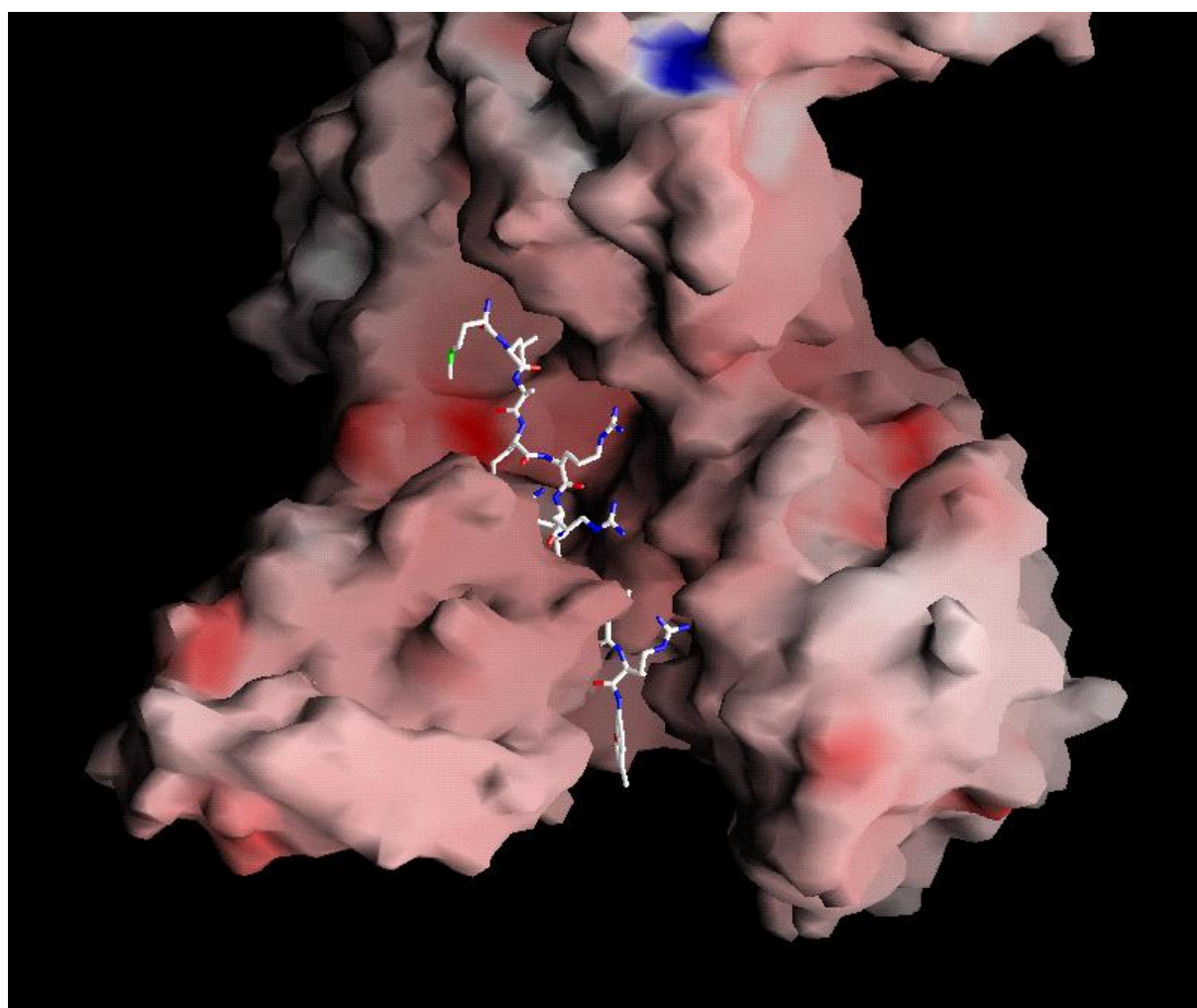
Fig. 1. Structural basis for LF:MAPKK recognition. Binding mode of the peptide chromogenic substrate MLARRRRVL-AMC to the recognition cleft of LF [17] (top panel). Amino acid sequences of the *N*-terminal region of MAPKKs cleaved by LF [15] (bottom panel). For further details, see text.

Fig. 2. Effect of the substrate concentration on the LF catalyzed hydrolysis of AcRRRRVLR-*p*NA and of AcRRRRVLR-AMC, at pH 7.4, 25.0°C, and [NaCl] =  $1.5 \times 10^{-2}$  M. Solid lines, calculated according to Eqn 1; values of  $K_m$ ,  $k_{cat}$ ,  $k_{cat}/K_m$ , and  $K_i$  given in Table 1 were obtained with an iterative non-linear least square curve fitting procedure. For further details, see text.

Fig. 3. Effect of the NaCl concentration, pH, and temperature on the initial velocity (i.e.  $v_i$ ) of the LF catalyzed hydrolysis of AcMLARRRPVLP-*p*NA. Data shown in panel A were obtained at pH 7.4 and 25°C. Data shown in panel B were obtained at 25°C and [NaCl] =  $1.5 \times 10^{-2}$  M. Data shown in panel C were obtained at pH 7.4 and [NaCl] =  $1.5 \times 10^{-2}$  M. Under all the experimental conditions the AcMLARRRPVLP-*p*NA concentration was 10  $\mu$ M. For further details, see text.



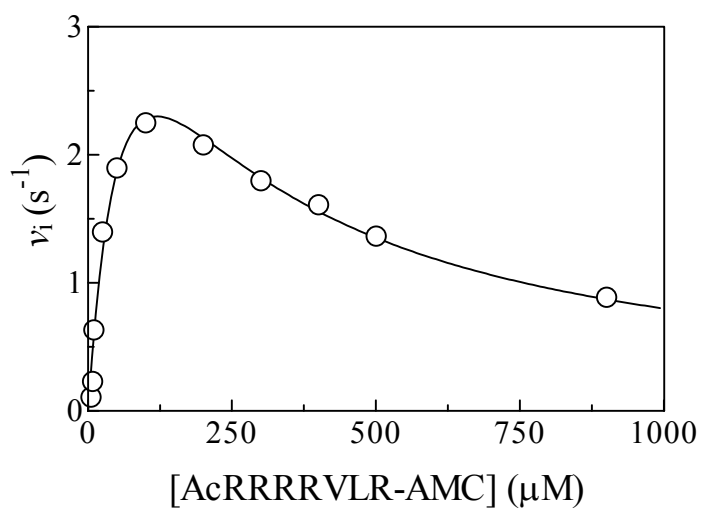
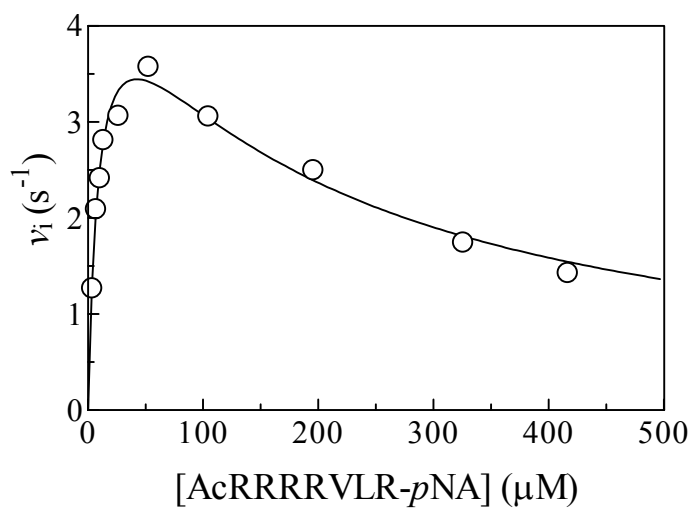
Figure 1



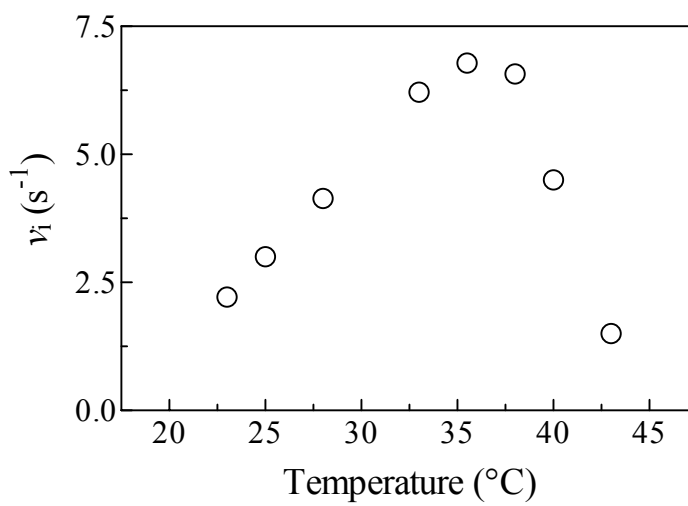
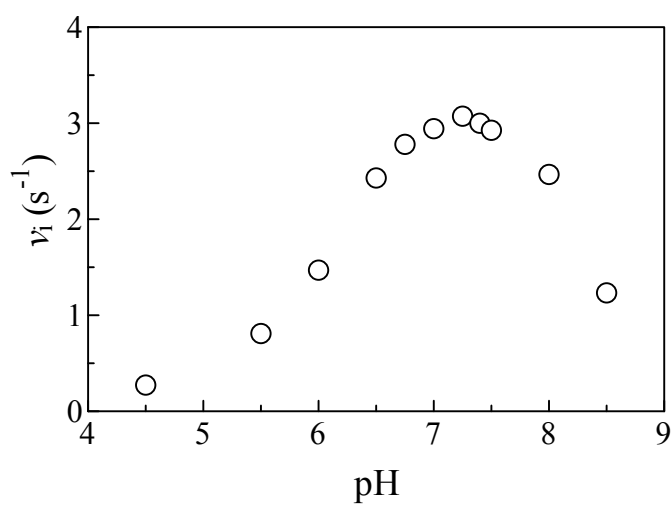
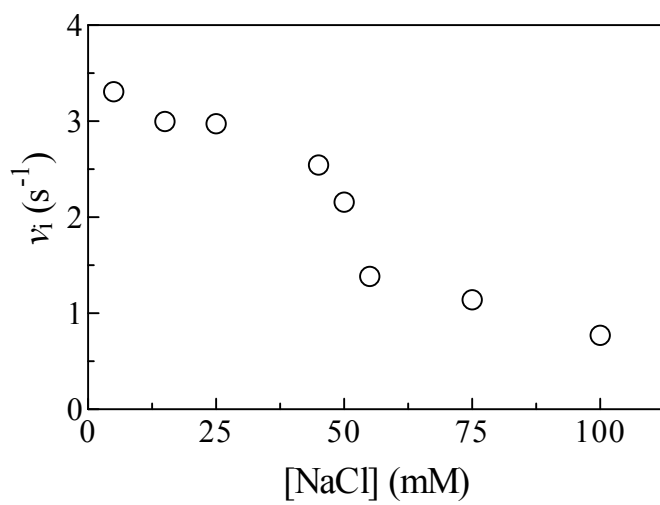
Substrate site	P <sub>8</sub>	P <sub>7</sub>	P <sub>6</sub>	P <sub>5</sub>	P <sub>4</sub>	P <sub>3</sub>	P <sub>2</sub>	P <sub>1</sub>	P <sub>1</sub> '	P <sub>2</sub> '	P <sub>3</sub> '	P <sub>4</sub> '	P <sub>5</sub> '	P <sub>6</sub> '	P <sub>7</sub> '	P <sub>8</sub> '
MAPKK-1 (P <sup>8</sup> -I <sup>9</sup> )	M	P	K	K	K	P	T	P	I	Q	L	N	P	A	P	D
MAPKK-2 (P <sup>10</sup> -A <sup>11</sup> )	A	R	R	K	P	V	L	P	A	L	T	I	N	P	T	I
MAPKK-3b (R <sup>26</sup> -I <sup>27</sup> )	S	K	R	K	K	D	L	R	I	S	C	M	S	K	P	P
MAPKK-6b (K <sup>14</sup> -I <sup>15</sup> )	K	K	R	N	P	G	L	K	I	P	K	E	A	F	E	Q
MAPKK-4 (K <sup>45</sup> -L <sup>46</sup> )	Q	G	K	R	K	A	L	K	L	N	F	A	N	P	P	F
MAPKK-4 (R <sup>58</sup> -F <sup>59</sup> )	P	P	F	K	S	T	A	R	F	T	L	N	P	N	P	T
MAPKK-7 (Q <sup>44</sup> -L <sup>45</sup> )	Q	R	P	R	P	T	L	Q	L	P	L	A	N	D	G	G
MAPKK-7 (G <sup>76</sup> -L <sup>77</sup> )	A	R	P	R	H	M	L	G	L	P	S	T	L	F	T	P
Consensus motif <sup>a</sup>		+	+	+	+		h		h							

<sup>a</sup> +, positively charged residue; h. hydrophobic residue.

Figure 2



**Figure 3**



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