The potent mitogen *Pasteurella multocida* toxin is highly resistant to proteolysis but becomes susceptible at lysosomal pH

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Abstract The susceptibility of the potent mitogen *Pasteurella multocida* toxin (PMT) to various proteases was investigated. PMT at a toxin to protease molar ratio of 1:1 was resistant to 8 of the 11 proteases tested after one hour. With longer incubation, PMT remained resistant to 7 proteases, and this correlated with a retention of biological activity, indicating that PMT might not require proteolytic cleavage at least until it bound to a cell receptor. Previous evidence had suggested that PMT is processed in the cell via an endosome or lysosome. We have shown that PMT became susceptible to proteolysis when the pH was lowered to 5 or below. This supports the previous suggestion that PMT is processed via a low pH compartment in the cell.

Key words: Mitogen; Toxin; *Pasteurella multocida*; Limited proteolysis

1. Introduction

Pasteurella multocida toxin (PMT) is the most potent mitogen identified for the Swiss murine 3T3 fibroblast cell line [1], and is the first intracellularly acting toxin that activates phosphatidyl inositol-specific phospholipase C (PI-PLC) [2]. The toxin is also mitogenic for the following established cell lines, Rat-1 [3], BALB/c 3T3, NIH 3T3, 3T6 and human fibroblasts [1]. The toxin has been cloned, sequenced and expressed in *Escherichia coli* [4–9]. PMT is encoded as a 146 kDa single-chain bacterial protein, which shares partial homology with the multinucleating toxins, cytotoxic necrotising factors types 1 and 2 (CNF-1, CNF-2), produced by some strains of *E. coli* [10,11]. Although PMT contains a putative ADP-ribosylation motif [6], recent data have shown that PMT is unlikely to ADP ribosylate its substrate [12,13]. There is evidence that PMT facilitates the coupling of a G protein to PI-PLC [14].

It is believed that PMT is internalised, probably via receptor mediated endocytosis (RME), and that binding to the cell membrane may involve a ganglioside-type receptor [15]. There is evidence that PMT may enter the cytosol from an acidic compartment (endosome or lysosome) after being internalised by RME, since weak bases such as methylamine, which increase intracellular pH, inhibited the mitogenic effects of PMT [1]. This indicates that low pH processing of PMT probably occurs to yield an active fragment. Many bacterial protein toxins require to be cleaved proteolytically before they become biologically active [16]. In this paper we analyse the susceptibility of PMT to various proteases and show that the toxin is highly resistant in its native state to proteolysis. We also show that PMT is susceptible to proteolytic cleavage after exposure to low pH which suggests a requirement for low pH processing.

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Abbreviations: PMT, *Pasteurella multocida* toxin; G protein, guanine nucleotide binding regulatory protein; CTAB, cetyltrimethylammonium bromide; RME, receptor mediated endocytosis.

2. Materials and methods

2.1. Reagents

Glu-c (EC 3.4.21.9), arg-c (EC 3.4.21.40), lys-c (EC 3.4.99.30), trypsin (EC 3.4.21.4) and asp-n (all sequencing grade), proteinase K (EC 3.4.21.14), subtilisin (EC 3.421.14), thermolysin (EC 3.4.24.2), chymotrypsin (EC 3.4.21.1), papain (EC 3.4.22.2) and pepsin (EC 3.4.23.1) were supplied by Sigma. All other chemicals (Analar grade) were obtained from BDH, Poole, UK, unless otherwise stated.

2.2. Electrophoresis

Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the mini Protean system (Bio-Rad, USA) according to published methods [17]. After electrophoresis gels were silver stained [18]. All buffers were made at 25°C.

2.3. Cell assay of PMT activity

The Swiss 3T3 mitogenicity assay was performed as described [1].

2.4. Purification of PMT

Purification of PMT was as described [19] with slight modifications [13].

2.5. Proteolysis of PMT

PMT and a protease at a 1:1 molar ratio were mixed and incubated at 37°C in 50 mM Tris-HCl, pH 8.2 for a limited time period. The reaction was stopped by the addition of trichloroacetic acid (7% (w/v), final concentration) before the addition of SDS-PAGE sample buffer. The pH was brought back to neutrality by the addition of 2 M Tris, pH 11 and the sample loaded on the gel (the sample was not boiled). Some digestions were in the presence of SDS (0.026% (w/v), final concentration) or cetyltrimethylammonium bromide (CTAB) (0.004% (w/v), final concentration). For the proteases subtilisin, proteinase K, trypsin, chymotrypsin and thermolysin the buffer contained 2 mM $CaCl_2$ (final concentration). In some protease experiments aldolase was added to the digestion mixture to show that the protease was active (aldolase to protease, 3:1 molar ratio). The buffer used for the low pH digestions was 50 mM $NH_4CH_3CO_2$ with CH_3CO_2H added until the desired pH was reached. pH 8 was obtained by adding 1 M NaOH to $NH_4CH_3CO_2$.

3. Results

3.1. PMT is highly resistant to proteolysis

Native PMT was not digested by 8 of the 11 proteases tested (Fig. 1A,B, lanes marked with (−), data not shown for chymotrypsin, papain and pepsin). To test whether the biological activity of native PMT was affected by this protease treatment, PMT was incubated with different proteases at 1:1 molar ratios for 7 hrs at 37°C. The mitogenic activity of these samples were

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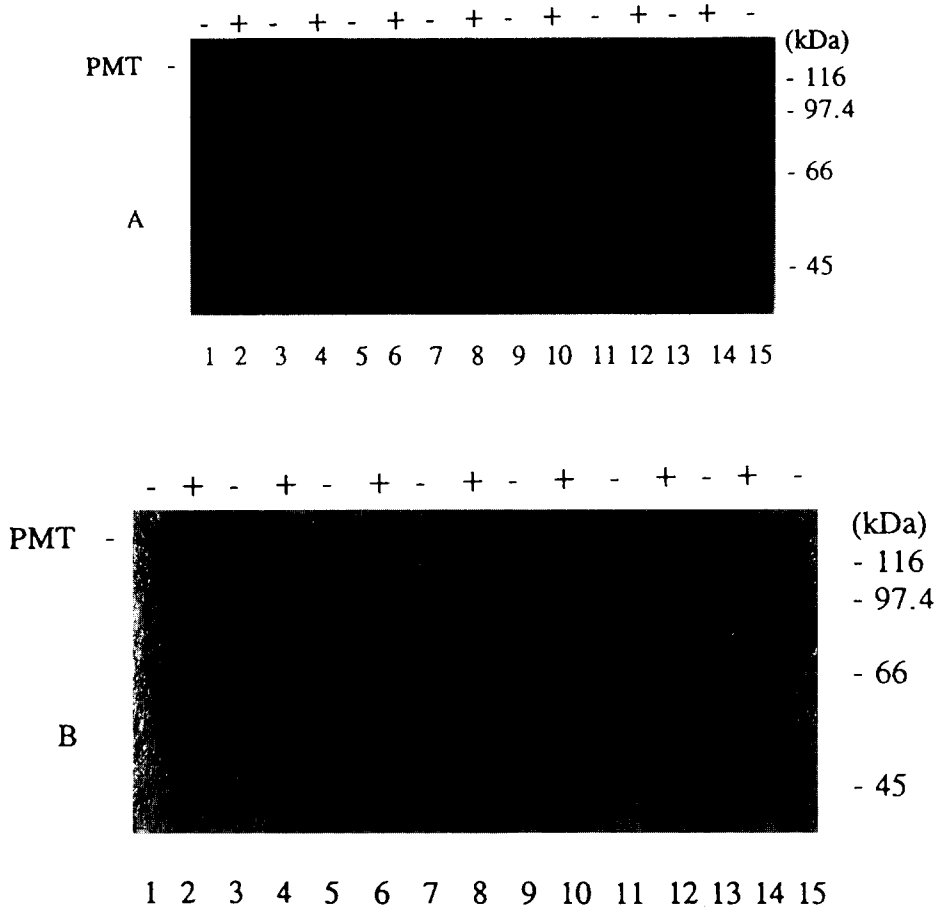

Fig. 1. Limited digestion of PMT by a variety of proteases. PMT was digested for 1 h at 37°C using different proteases at a PMT to protease, molar ratio of 1:1 in 50 mM Tris-HCl, pH 8. Gel A, lanes, 1 and 2, glu-c; 3 and 4, arg-c; 5 and 6, subtilisin; 7 and 8, lys-c; 9 and 10, asp-n; 11 and 12, proteinase K; 13 and 14, thermolysin; 15, PMT control. Gel B, lanes as A, except samples digested for 13 h at room temperature (27°C). Lanes marked with (+) indicate the presence or absence (−) of SDS (0.026% (w/v) final concentration) in the digestion buffer.

then assayed. PMT treated with glu-c, trypsin, chymotrypsin, thermolysin, papain, lys-c or arg-c were as biologically active as untreated PMT. Samples from these digests showed intact toxin bands on SDS-PAGE. The toxin samples treated with subtilisin, proteinase K and pepsin, which displayed greatly reduced biological activity, did not show an intact toxin band on SDS- PAGE (data not shown).

It had been reported that native PMT was digested by trypsin (PMT to trypsin 109:1 molar ratio) into three distinct polypeptide fragments [20]. We repeated this experiment, following the published experimental conditions, but found that PMT was resistant to proteolysis by trypsin (data not shown). Even at a PMT to trypsin molar ratio of 1:1, PMT was still highly resistant to proteolytic attack (Fig. 2, lanes 1 and 2). Several minor bands could be seen, but their molecular weights differed from those published [20]. Compared to other single chain bacterial protein toxins, PMT is highly resistant to proteolysis (Table 1).

3.2. *Charged detergents affect the susceptibility of PMT to proteolysis*

Since native PMT was resistant to many of the proteases, we decided to see if the structure of PMT would be affected by the addition of low concentrations of the anionic detergent SDS (Fig. 1A,B, lanes marked with (+)). Under these conditions PMT became susceptible to the action of all of the proteases, except arg-c, leading to the complete fragmentation of the toxin band into peptides. A reduction in the protease concentration resulted in the appearance of larger fragments of PMT and the

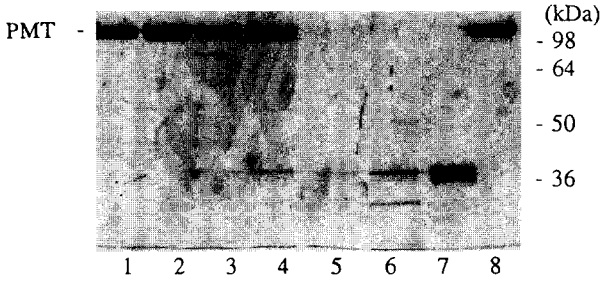

Fig. 2. Limited digestion of PMT by trypsin. PMT and aldolase (PMT to trypsin, 1:1 molar ratio; aldolase to trypsin 3:1 molar ratio) were incubated with trypsin for 1 h at 37°C in 50 mM Tris-HCl pH 8.2. Aldolase was added to show that trypsin was active. lane 1, PMT and trypsin; lane 2, as 1. except EDTA added (1 mM final concentration); lane 3, as 1. except aldolase added; lane 4, as 3. except EDTA added; lane 5, aldolase and trypsin; lane 6, as 5. except EDTA added; lane 7, aldolase control; lane 8, PMT only.

Fig. 3. Limited digestion of PMT by glu-c. PMT in 50 mM Tris-HCl, pH 8 containing SDS (0.026% w/v final concentration) was digested by glu-c (PMT to glu-c, molar ratio of 14:1) at 37°C for various times. At set times aliquot were taken out and the reaction stopped. Lane 1, 15 min; lane 2, 1 min; lane 3, 2 min; lane 4, 3 min; lane 5, 4 min; lane 6, 5 min; lane 7, 10 min; lane 8, 15 min; lane 9, PMT only; lane 10, glu-c only.

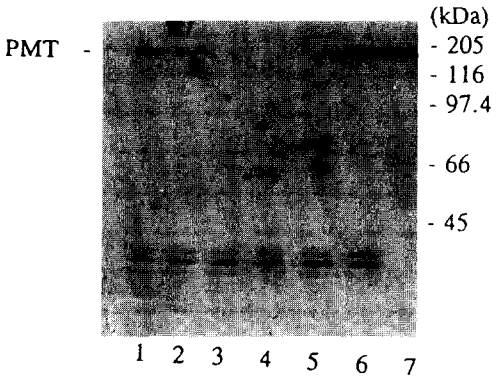

Fig. 4. Limited digestion of SDS treated PMT by glu-c. PMT, glu-c (1:1 molar ratio) and different concentrations of SDS were mixed and incubated at 37°C for 1 h. Lanes, 1, SDS concentration (w/v) 0.0052%; 2, 0.0104%; 3, 0.0156%; 4, 0.0208%; 5, 0.026%; 6, no SDS; 7, PMT only.

banding pattern also changed with the time of incubation (Fig. 3). After 15 min the toxin band was completely fragmented. Similar results were obtained with the specific protease lys-c, but not with non-specific proteases subtilisin and thermolysin, which completely fragmented the toxin in less than a minute (data not shown). Fig. 4 shows that an SDS concentration as low as 0.016% (w/v) was enough to permit complete digestion of PMT (similar results were obtained with the cationic detergent CTAB down to 0.004% (w/v), final concentration).

3.3. The effects of urea on proteolysis

PMT was completely cleaved by glu-c in the presence of urea at final concentrations of 4 M or 6 M (Fig. 5). At lower urea concentrations PMT was resistant to proteolytic attack.

3.4. The effect of low pH on the susceptibility of PMT to proteolysis

PMT at different pHs was incubated with glu-c, which is active over a wide pH range [27], to see if the toxin became susceptible to cleavage. At pH 5 or below, PMT was completely cleaved (Fig. 6). Above pH 5, PMT was resistance to cleavage even after a 1 h incubation. Aldolase, which was cleaved by glu-c over the pH range chosen, was coincubated with PMT to show that the protease was active (Fig. 6).

4. Discussion

For many bacterial toxins cleavage of a peptide bond is essential before they exert their biological effects on cells. The

Table 1
Properties of some common single-chain bacterial protein toxins

Toxin	Molecular weight (kDa)	Action of proteases in vivo (cellular processing)	Action of proteases in vitro (protease:toxin molar ratio)	References
Pseudomona exotoxin A	67	Cleaved into two fragments	Cleaved into three fragments (58, 45, 30 kDa) by trypsin (1:50) 45 min, 25°C pH 8.	21
Diphtheria toxin	58	24 kDa, 38 kDa	Trypsin (1:41) A: 24 kDa; B: 38 kDa, 10 min, 37°C, pH 8, 50 mM DTT.	22
Tetanus toxin	150	L: 50 kDa, H: 100 kDa	Papain (1:6) or trypsin (1:48) cleave the H chain into two fragments (98 and 52 kDa), 1 h, 25°C, pH 8.	23
Botulinum toxin A	150	L: 50 kDa; H: 100 kDa	Pepsin (1:2.5) 6 fragments, 1 h 25°C, pH 4 or 6; papain (1:73) chymotrypsin (1:82) and bromelain (1:105) cleave into two fragments (100 and 50 kDa), 4 has, 25°C, pH 6.	24
Bordetella bronchiseptica (DNT)	190	not known	Cleaved into two fragments (115 kDa, 75 kDa) by trypsin (1:84), 1 h, 25°C, pH 8.2.	25
Clostridium difficile Toxin A	250	not known	Resistant to trypsin (2:1) but cleaved by papain (2:1), 7 has, 25°C, pH 6.2.	26
Pasteurella multocida Toxin (PMT)	146	not known	Resistant to cleavage by trypsin, papain, chymotrypsin, thermolysin, subtilisin, glu-c, lys-c, arg-c. Partially resistant to asp-n and proteinase k. Protease: toxin 1:1 h, 37°C, pH 8.2.	

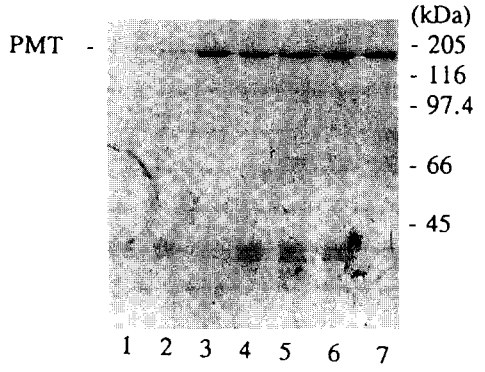

Fig. 5. Limited digestion of PMT in urea by glu-c. PMT and glu-c, at a 1:1 molar ratio, plus different concentrations of urea were mixed and digested at 37°C for 1 h in 50 mM Tris-HCl, pH 8. Lanes, 1, 6 M urea; 2, 4 M urea; 3, 2 M urea; 4, 1 M urea; 5, 0.5 M urea; 6, 0 M urea; lane 7, PMT only.

results show that PMT is resistant to a variety of specific and non-specific proteases and still displays full biological activity after this harsh treatment. The molar ratio of protease to toxin (1:1) used was high compared to that used to cleave other single-chain bacterial protein toxins, most of which are cleaved at low molar ratios into two or more fragments (see Table 1). Considering the size of PMT it seems remarkable that PMT is so resistant to cleavage. This indicates that PMT has a very compact tertiary structure, and suggests it must be in an intact form until it binds to a cell receptor.

There is confusion in the literature as to whether PMT is cleaved by trypsin. Our results show, using a wide range of experimental conditions, that PMT is resistant even at high PMT to trypsin molar ratios, and do not support the view that PMT can be completely cleaved into three distinct fragments [20]. Our results agree with other workers who have stated that PMT is resistant to trypsin [28,29].

The addition of ionic detergents, SDS or CTAB, made the toxin extremely sensitive to proteases, resulting in the cleavage of PMT into small peptides even at low protease to toxin ratios. Limited digestion with glu-c produced several distinct bands, possibly indicating domains that are partially resistant to unfolding by SDS. The susceptibility of PMT to proteolysis was shown to depend on the concentration of SDS. Limited digestion of urea treated PMT produced a cleavage pattern different from that obtained using SDS. The difference in these patterns is probably due to the dissimilar effects of these denaturants.

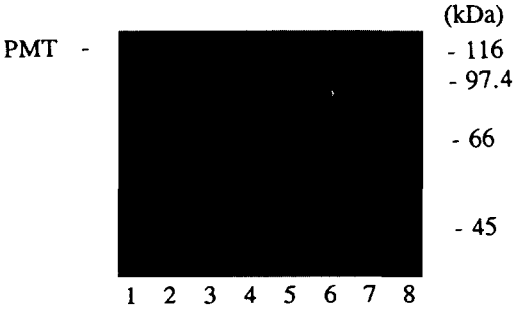

Fig. 6. Limited digestion of PMT and aldolase with glu-c at different pH values. PMT and aldolase were incubated together in 50 mM ammonium acetate at different pHs and digested with glu-c for 1 h at 37°C (PMT to glu-c 1:1 molar ratio; aldolase to glu-c 3:1 molar ratio). Lanes, 1, pH 4.5; 2, pH 5; 3, pH 5.3; 4, pH 6.7; 5, pH 8; 6, PMT only; 7, aldolase only; 8, glu-c only.

Several toxins require low pH processing to release their catalytic fragment into the cytosol [16], the classic example being diphtheria toxin (DT). The low pH in the endosome induces a conformational change in the B fragment of DT, simultaneously allowing translocation of the A fragment to the cytosol [30,31]. The low pH experiments reported here show that PMT was completely cleaved at pH 5 or lower, but not above this pH. It is therefore likely that a conformational change occurs within the toxin at pH 5 allowing proteolytic cleavage of PMT. This supports the suggestion that PMT, whose action is blocked by methylamine [1], is taken up by RME and trafficked to a low pH compartment where it is proteolytically activated.

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