

# Characteristics of the biologically active 35-kDa metalloprotease virulence factor from *Listeria monocytogenes*

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A. COFFEY, B. VAN DEN BURG, R. VELTMAN AND T. ABBE. 2000. *Listeria monocytogenes*, a facultative intracellular pathogen, synthesizes an extracellular protease which is responsible for the maturation of phosphatidylcholine phospholipase C (lecithinase), a virulence factor involved in cell-to-cell spread. This work describes the environmental parameters necessary for increased production of mature, 35-kDa active protease in strains of *L. monocytogenes*, and its detection using polyclonal antibodies raised against *Bacillus subtilis* neutral protease. High performance liquid affinity chromatography was exploited to isolate the biologically active form of the mature protease, which was then subjected to biochemical characterization using casein as a substrate. The protease is a zinc-dependent metalloprotease which degrades casein over a wide range of temperatures and pH values. It can also degrade actin, the most abundant protein in many eukaryotic cells. The *Listeria* protease was shown to exhibit a high thermal stability and a relatively narrow substrate specificity. A three-dimensional model built on the basis of the homology with thermolysin was used to understand the structural basis of these characteristics.

## INTRODUCTION

*Listeria monocytogenes* is a Gram-positive nonsporeforming facultative intracellular rod-shaped bacterium which is capable of causing serious infections in humans and animals. The tracing of recent epidemics of listeriosis to food contaminated with *L. monocytogenes* suggests that the natural route of infection is the gastrointestinal tract (Farber and Peterkin 1991; Falkow *et al.* 1992). The infectious process of the bacterium includes internalization (Lebrun *et al.* 1996; Parida *et al.* 1998), escape from the host phagosome (Goldfine *et al.* 1995), multiplication in the host cytoplasm (Marquis *et al.* 1993), and intracellular motility and cell-to-cell spread without an extracellular phase (Dabiri *et al.* 1990; Theriot *et al.* 1992;

Chakraborty *et al.* 1995; Friederich *et al.* 1995; Cossart and Lecuit 1998). Genetic determinants for virulence in *L. monocytogenes* are currently targets of considerable research efforts, which have recently been reviewed (Sheehan *et al.* 1994; Kuhn and Goebel 1995; Ireton and Cossart 1997; Cossart and Lecuit 1998). One virulence factor in the species is a protease, the deduced amino acid sequence of which exhibits similarities to proteases of the thermolysin family, a group of metalloproteases produced by *Bacillus* species and some Gram-negative bacteria, including *Serratia marcescens*, *Legionella pneumophila*, and *Pseudomonas aeruginosa* (Domann *et al.* 1991; Mengaud *et al.* 1991). The *Listeria* protease contains 510 amino acid residues with a typical N-terminal signal sequence and, like in all metalloproteases, there is a putative cleavage site for proteolytic maturation resulting in a 35-kDa mature protease. This protease is responsible for the extracellular processing of phosphatidylcholine phospholipase C (lecithinase), which has a role in lysis of the double membrane of the phagosome formed when *L. monocytogenes* spreads from cell to cell (Raveneau *et al.* 1992; Poy-

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art *et al.* 1993). Lecithinase is synthesized as a 33-kDa precursor and cleaved to yield an active 29-kDa enzyme (Kuhn and Goebel 1995). Transposon mutants in the *Mpl* gene are reduced in virulence, which has been attributed to the lack of proteolytic processing of the lecithinase (Poyart *et al.* 1993).

While amino acid sequence homology studies have led to the assumption that the protease is a zinc metalloprotease (Domann *et al.* 1991; Mengaud *et al.* 1991), little is known about the nature of this virulence factor on the basis of biochemical evidence. This paper firstly describes the detection of the mature form of protease in *L. monocytogenes* ScottA culture supernatants, and the growth conditions favouring enhanced production of this form. Then a high performance liquid affinity chromatography protocol which minimizes proteolytic autodigestion was exploited to purify the active mature protease. The enzyme was subsequently characterized using such substrates as casein and actin. A three-dimensional model of the protease based on thermolysin is described and used to interpret some of the observed characteristics of this virulence factor.

## MATERIALS AND METHODS

### Bacteria, media and chemicals

The *Listeria* species used were *L. innocua* II, *L. monocytogenes* ScottA and *L. monocytogenes* L028 (Coffey *et al.* 1996). These were routinely grown at 37 °C in brain heart infusion broth (BHI) (Difco Laboratories, Detroit, MI, USA), which included 1.5% (w/v) agar for solid medium. Chemically defined minimal medium of Premaratne *et al.* (1991) was supplemented with 0.01% (v/v) glycine to optimize growth, and with 1% (w/v) bacteriological agar (Oxoid, UK) for solid medium. All amino acids were obtained in the L configuration from Sigma Chemical Co., St. Louis, Mo. When required, the osmoprotectants betaine and carnitine (Sigma) were incorporated at a concentration of 1 mM (Beumer *et al.* 1994).

### Protein determination and SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and N-terminal sequencing was performed as described previously (Van den Burg *et al.* 1990), except that a Protean II or Mini-Protean II (Bio-Rad) electrophoresis system was employed. Protein was precipitated from *Listeria* culture supernatants using 8.0% (v/v) TCA. Protein samples were resuspended in sample buffer (125 mM Tris, pH 6.8, 2.5% (v/v) SDS, 25% (v/v) glycerol, 0.0025% (v/v) bromophenol blue, 1.25% (v/v)  $\beta$ -mercaptoethanol). For immunoblot reactions, rabbit anti-*Bacillus subtilis* neutral protease was used at a 1:1000 dilution. Formation of disulphide bonds was analysed by SDS-PAGE

under reducing vs. nonreducing conditions. Samples were incubated with SDS-PAGE sample buffer with and without  $\beta$ -mercaptoethanol (2 mM), and heated for 5 min at 100 °C, prior to electrophoresis.

### Protease purification

A high performance liquid affinity chromatography system, based on the affinity of bacitracin for the active site of neutral proteases, was exploited. Prior to coupling of bacitracin to the silica, the latter was activated by the addition of an epoxy group (Roy and Kundu 1979; van den Burg *et al.* 1989). Briefly, 100 g of silica C-gel-C 5300 with a pore diameter of 30 nm and a particle size range from 60 to 200  $\mu$ m (Promochem GmbH, Wesel, Germany) was treated with a 10% (w/v) solution of  $\gamma$ -glycidoxypropyltriethoxysilane (Polyscience Inc., Warrington, PA, USA) in toluene, with gentle stirring, for 15 h at 60 °C. The resulting epoxy-silica was filtered and washed extensively in acetone before drying under vacuum. 100 g of epoxy-silica was added to 10.5 g bacitracin (Sigma), suspended in 400 ml of 0.1 M Na<sub>2</sub>HPO<sub>4</sub> buffer, pH 8.15. After stirring for 5 d at room temperature, the silica was filtered from the suspension, washed with several changes of demineralized water, and air-dried. The column was cast by resuspending dried sorbent in 20 mM sodium acetate, 5 mM calcium chloride, 10% (v/v) isopropanol, pH 5.0. The column was employed for protease purification as described previously (van den Burg *et al.* 1989). The protease-containing fractions were verified by placing 10  $\mu$ l of each fraction on casein-agarose plates as described above. Samples which caused a clearing on the casein plates were pooled, desalted and lyophilized as described previously (van den Burg *et al.* 1990), resuspended in 50 mM Tris HCl, 5 mM CaCl<sub>2</sub>, 1 mM zinc, 50% (v/v) glycerol, resulting in a 30-fold concentration of the protease-containing fractions. Protein concentration was determined by the method of Bradford (1976).

### Detection of protease activity

Protease activity in *Listeria* cultures was assessed by plating on skim-milk agar supplemented with BHI (3.7% v/v) or 1.75% (w/v) NaCl, or both, and incubated at 37 °C for 2–5 d. Purified protease was detected by placing aliquots from the final eluent from the column on plates of 0.8% (w/v) casein (Sigma) dissolved in 40 mM Tris (pH 7.5) in 1% (w/v) agarose (Sigma). These were incubated overnight at 37 °C and clearing indicated protease activity in the aliquot. Quantitative determination of protease activity in the final preparations of protease was achieved by adding 2  $\mu$ l of purified protease to 1 ml of a 0.8% (w/v) casein solution containing 50 mM Tris (pH 7.5), 5 mM CaCl<sub>2</sub> and 0.08% (v/v) sodium azide. This solution was incubated for 2 h at 37 °C, after

which the reaction was stopped by addition of 1 ml of a solution containing 0.1 M trichloroacetic acid (TCA), 0.22 M sodium acetate and 1.886% (v/v) acetic acid. After mixing, the solution was kept at room temperature for 30 min and centrifuged for 12 min in a microcentrifuge. Absorbance at 275 nm indicates the relative concentration of TCA-soluble peptides resulting from proteolytic degradation of casein. Actin,  $\beta$ -casein, bovine serum albumin,  $\beta$ -lactoglobulin and lysozyme (Sigma) were compared as substrates to study enzyme specificity. One unit of protease was defined as the amount which generated an absorbance of 0.5 at 275 nm from TCA-soluble oligopeptides from  $\beta$ -casein after 1 h at 80 °C. Two units of protease per 1 ml reaction volume was routinely used in substrate assays.

### Protease inhibition assays

Protease inhibitors (Beynon and Boud 1989) were tested at the recommended concentrations for their effect on casein hydrolysis. The following inhibitors were examined: 1 mM EDTA, 1 mM EGTA, 2 mM 1-10-phenanthroline, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.1 mM phosphoramidon. Inhibition of protease was measured as a reduction in the relative concentration of TCA-soluble peptides resulting from proteolytic degradation of casein (0.8% w/v) when incubated in the presence of inhibitors with 2 units ml<sup>-1</sup> of metalloprotease. Inhibition of  $\beta$ -casein degradation was confirmed by SDS PAGE.

### Thermal stability determination

The thermal stability of the protease was determined by incubating purified protease in 50 mM sodium acetate pH 5.0, 5 mM CaCl<sub>2</sub> and 1% (v/v) isopropanol, for 30 min, at elevated temperatures, and determining the residual casein hydrolytic activity. Reference samples were incubated for 30 min at 0 °C. Thermal stability is quantified in terms of T50, which is defined as the temperature at which a 30-min incubation reduces the enzyme activity by half.

### Actin utilization by *L. monocytogenes* ScottA

Two milligrams of globular Actin (Sigma) was incubated in the presence and absence of 2 units of metalloprotease for 8 h at 80 °C in a volume of 200  $\mu$ l. Growth of *L. monocytogenes* ScottA at 30 °C in chemically defined medium lacking the essential amino acid valine and supplemented with degraded or undegraded actin was assessed over a 27-h period by measuring the OD at 620 nm using a microtitre reader (SLT Labinstruments, NL). OD readings were compared with those obtained from defined medium in the presence and absence of valine.

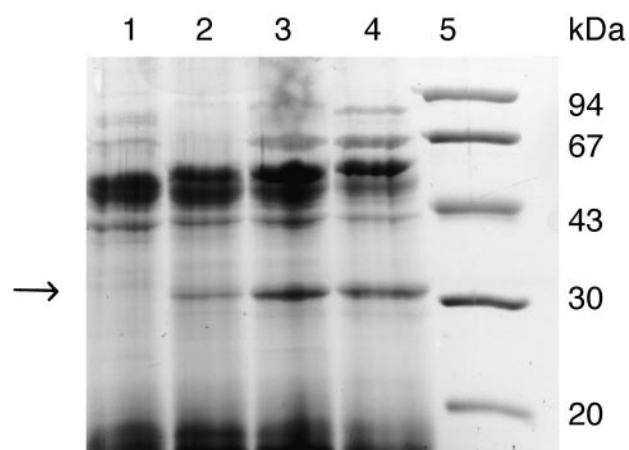
### Three-dimensional modelling

A three-dimensional model of the metalloprotease was constructed on the basis of the well defined thermolysin structure (Holmes and Matthews 1982) using the program WHAT IF (Vriend 1990). Structural characteristics of the *Listeria* protease model were analysed in detail using the various options of the WHAT IF program. The program for ribbon drawings is described by Carson (1987).

## RESULTS

### Application of growth conditions leading to enhanced production of mature *Listeria* protease

Since maturation of lecithinase in *L. monocytogenes* is dependent on the protease, lecithinase activity was used as an indirect indicator of active metalloprotease. Optimal environmental parameters for lecithinase production were determined previously by plating *L. monocytogenes* on BHI agar containing egg-yolk over a range of pH values, temperatures and salt (Coffey *et al.* 1996). These growth conditions, namely, BHI broth (pH 7.3) supplemented with 1.75% (w/v) NaCl at 37 °C, and also BHI broth (pH 7.3) without supplementary NaCl at 37 °C, were applied during growth of cultures used for detection of protease. Consistent detection of a 35-kDa mature form of the protease was achieved by growing cultures BHI (pH 7.3) in the absence of supplementary NaCl (Fig. 1) and this was confirmed by immunoblotting with polyclonal antibodies to *Bacillus subtilis* Npr. When the NaCl concentration was increased to 1.75% there

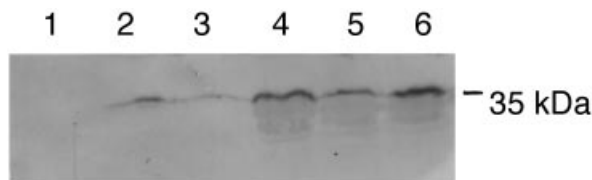


**Fig. 1** Comparison of extracellular proteins precipitated from BHI cultures of *L. innocua* II (Lane 1), *L. monocytogenes* ScottA grown in 0.5% (w/v) NaCl (lane 2) and in 1.75% (w/v) NaCl (lane 3), and *L. monocytogenes* L028 grown in 0.5% (w/v) NaCl (lane 4). Lane 5 shows molecular weight markers. The arrow indicates the position of the 35-kDa protease.

was a significant increase in intensity of the 35-kDa band (Fig. 1) and this was also borne out by immunodetection. Recognition of the *Listeria* protease by the *Bacillus subtilis* Npr antibodies is based on 47% amino acid sequence similarity between the two proteases (Mengaud *et al.* 1991). No antibody recognition was observed to the position of the pro-form (60 kDa approx) on the nitrocellulose filters (unpublished data). There was antibody recognition at the 45-kDa position but this is considered to be a false positive reaction, as it was equally evident in *Listeria innocua* II which does not produce the protease.

There was significant variation in the intensity of the 35-kDa band corresponding to the protease for different *L. monocytogenes* strains following precipitation of extracellular proteins from culture supernatants. For example, strain ScottA gave a weak 35-kDa band in the absence of supplementary NaCl, while strain LO28 exhibited a more intense band under the same conditions (Fig. 1). When strain ScottA was treated with 1.75% (w/v) NaCl, it gave a more intense band at the 35-kDa position and it also exhibited an additional band of 70 kDa (approx.) which was not evident with this strain in the absence of supplementary NaCl (Fig. 1). A 70-kDa band was also seen in *L. monocytogenes* L028 grown without additional NaCl. This latter strain also exhibits higher lecithinase activity than other strains of *Listeria monocytogenes* (Coffey *et al.* 1996). This variation between the three strains *L. innocua* II, *L. monocytogenes* ScottA and *L. monocytogenes* L028 was consistent over three separate experiments and was verified by immunodetection.

To rule out the influence of BHI constituents on this phenomenon, experiments were also performed in chemically defined medium. In this case, it was observed that higher levels of metalloprotease were present than those detected in BHI medium, and also that supplementary NaCl (1.0% w/v) increased the mature protease yield (Fig. 2). Detection of high levels of protease in a chemically defined medium in the



**Fig. 2** Immunodetection of 35-kDa protease in *Listeria* culture supernatants from BHI and DM supplemented with NaCl, using rabbit anti-Npr (*B. subtilis*) antiserum. *L. innocua* II grown in BHI with 0.5% (w/v) NaCl (lane 1), *L. monocytogenes* ScottA grown in BHI with 1.75% (w/v) NaCl (lane 2) and 0.5% (w/v) NaCl (lane 3), and *L. monocytogenes* ScottA grown in DM with 1.0% (w/v) NaCl (lane 4), 0% (w/v) NaCl (lane 5), and 2.0% (w/v) NaCl (lane 6).

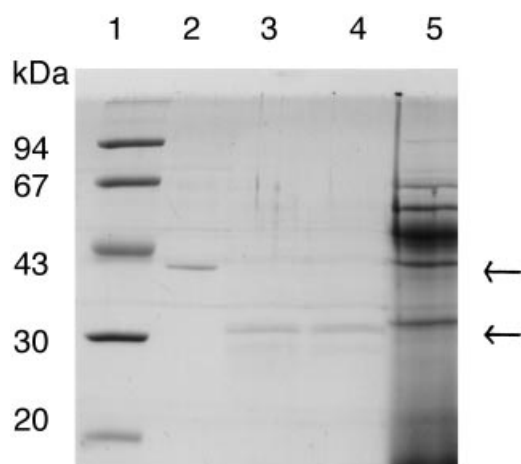
presence of 2.0% (w/v) NaCl was facilitated by the inclusion of the osmoprotectants betaine and carnitine during growth.

### Protease activity in *Listeria* cultures

*Listeria monocytogenes* strains were streaked on 10% (w/v) skim-milk agar and 10% skim-milk agar supplemented with BHI (3.7%), both medium with and without NaCl (1.75% w/v). No protease activity was evident on skim-milk agar. When the medium was supplemented with BHI, or BHI and NaCl, slight protease activity was visible in all strains but could not be assigned specifically to the protease under study since nonpathogenic species of *Listeria* also exhibited this activity. We assume that this proteolytic activity is due to intracellular peptidases released after cell lysis during the stationary phase of growth. Addition of NaCl to the BHI-skim-milk agar did not cause a detectable increase in proteolytic activity in these assays. The fact that distinct protease activity could not be detected in *L. monocytogenes* supernatants suggested that the concentration of secreted protease is very low.

### Protease purification from *L. monocytogenes* ScottA

*Listeria monocytogenes* ScottA was grown to early stationary phase in BHI medium in the presence of 1.75% NaCl (w/v) prior to application of culture supernatant to the affinity column. Application of fresh culture supernatant to the column and the exploitation of bacitracin, which has affinity for the active site of proteases of this class, both have the important benefit of allowing the harvesting of active protease. The elution process exploits the interaction of isopropanol with the active site, which mediates protease release from the bacitracin complex (van den Burg *et al.* 1989). After desalting and lyophilization, proteolytically active fractions were subjected to SDS PAGE. A band corresponding to the expected molecular mass (35 kDa) of the mature form of the *Listeria* protease was evident (Fig. 3). Lower molecular weight bands were also evident in the purified sample and these are believed to be protease breakdown products resulting from auto-digestion. A culture supernatant of *L. innocua* II, grown under identical conditions, was also applied to the affinity column and, as expected, protease could not be detected in the eluted fractions. Following purification of the protease from a *L. monocytogenes* ScottA supernatant, activity against casein could be readily detected in fractions by clearing on 0.8% (w/v) casein agarose plates (unpublished data). Purified *Listeria* protease was blotted to PVDF membranes and subjected to N-terminal sequence determination in order to locate the exact processing site. However, due to the presence of a blocked N-terminal amino acid, it was not possible to achieve this.



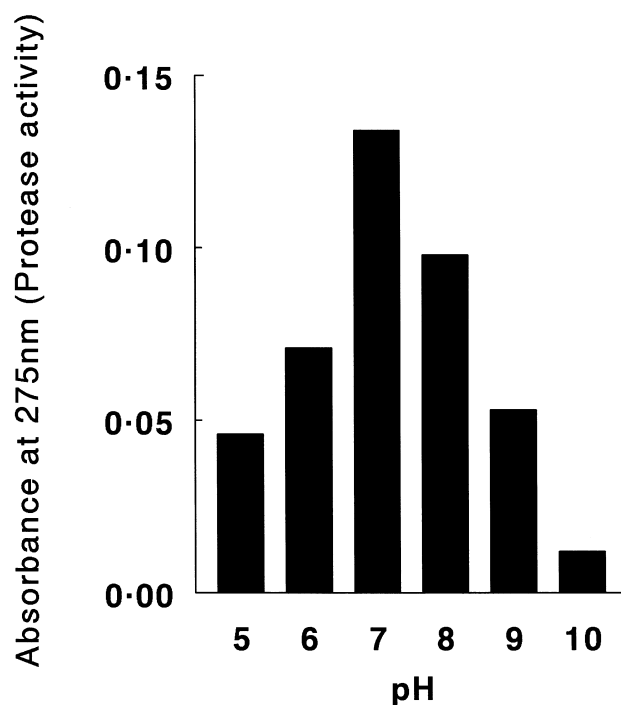
**Fig. 3** Purification of 35-kDa protease from *L. monocytogenes* ScottA. Molecular weight markers (lane 1), *B. subtilis* Npr (lane 2), purified *Listeria* protease (lanes 3 and 4), and culture supernatant proteins of *L. monocytogenes* ScottA grown in BHI with 1.75% (w/v) NaCl (lane 5).

#### Properties of the purified protease

Casein solution (0.8% w/v) was exploited for a more detailed examination of enzyme activity by the *Listeria* protease. The optimal pH for casein degradation was 7.0 but moderate activity was evident at pH 5.0 and pH 9.0 (Fig. 4). Casein degradation was moderate at 37 °C, still evident as low as 4 °C and reached an optimum at 80 °C (Fig. 5). The protease degraded casein extensively, as shown by SDS PAGE. Bands representing all forms of casein disappeared following incubation for 2 h at 37 °C in the presence of 1 unit of protease per ml (Fig. 6). Additionally,  $\beta$ -casein was used to examine degradation in more detail at 37 °C. During the first hour of digestion, this substrate was degraded to generate smaller peptides, of which the most prominent had masses of 23, 20, 18 and 13 kDa. Subsequently, these bands were further degraded (Fig. 6). Casein degradation was also monitored spectrophotometrically, and it was observed that during 2 h of proteolytic digestion, the increase in absorbance (275 nm) resulting from the generation of TCA-soluble peptides was linear (unpublished data).

#### Effect of inhibitors on activity of the purified protease

Various agents which are known to inhibit protease activity were assessed for their ability to inhibit casein degradation by the *Listeria* protease. The metal chelators (EDTA and 1-10-phenanthroline) were inhibitory, as was the calcium chelator (EGTA) and the thermolysin-like protease inhibitor (phosphoramidon). The cysteine/serine protease inhibitor (PMSF) had no effect (Fig. 6). SDS PAGE indicated that



**Fig. 4** Effect of pH on relative concentrations of TCA-soluble peptides resulting from proteolytic digestion of casein at 37 °C by the purified *Listeria* protease.

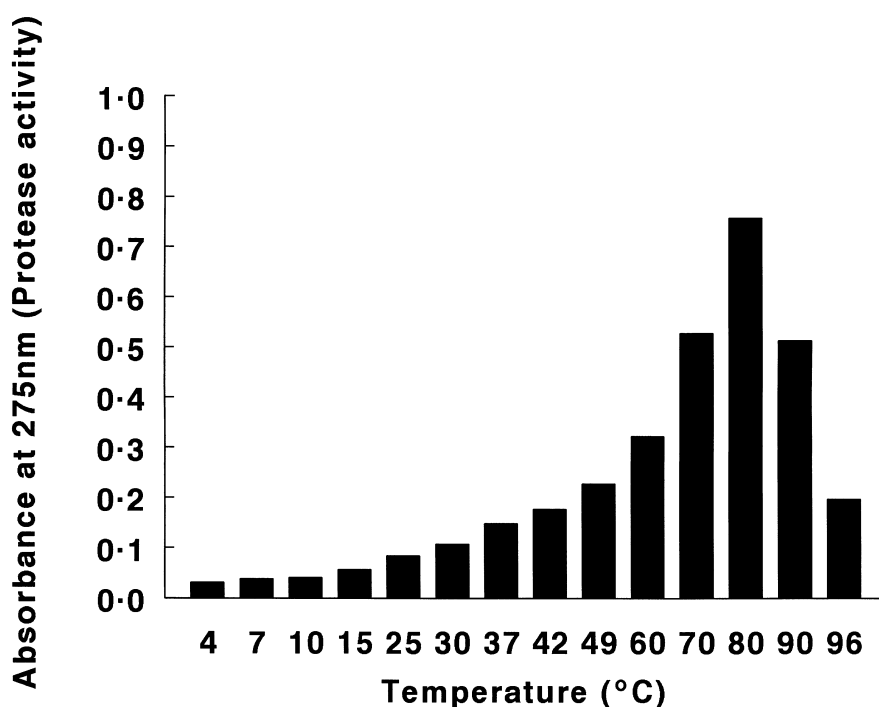
the addition of 0.5 and 1.0 mM  $ZnCl_2$  to  $\beta$ -casein digests containing 1-10-phenanthroline restored proteolytic activity. Enzyme activity was inhibited at higher zinc concentrations (unpublished data).

#### Substrate specificity of the purified *Listeria* protease

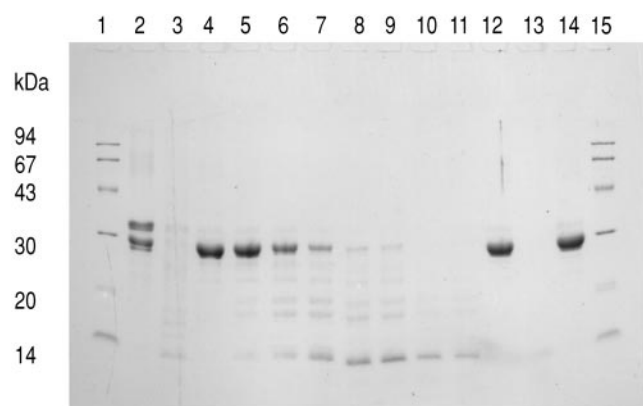
A variety of substrates were incubated in the presence of the protease to compare their susceptibility to degradation at 37 °C for 2 h. These included  $\beta$ -lactoglobulin, lysozyme, bovine serum albumin,  $\beta$ -casein and actin. In all cases, 1 mg of substrate was incubated at 37 °C for 2 h at pH 7.0 in the presence of 0.5 units of protease. Under these conditions, it was observed using SDS PAGE that degradation of  $\beta$ -lactoglobulin, lysozyme and bovine serum albumin did not occur. Casein was extensively degraded, and actin, a protein which surrounds *L. monocytogenes* during intracellular growth and cell-to-cell spread (Friederich *et al.* 1995), was degraded to at least 8 smaller visible fragments (Fig. 7). In control experiments where the protease was omitted, no degradation of actin was observed.

#### Actin utilization by *L. monocytogenes* ScottA

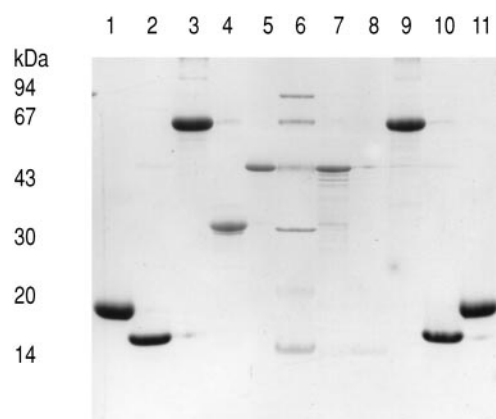
We have recently shown that a di- and tripeptide permease can supply *L. monocytogenes* with amino acids essential for



**Fig. 5** Effect of temperature on relative concentrations of TCA-soluble peptides resulting from proteolytic digestion of casein by the purified *Listeria* protease.



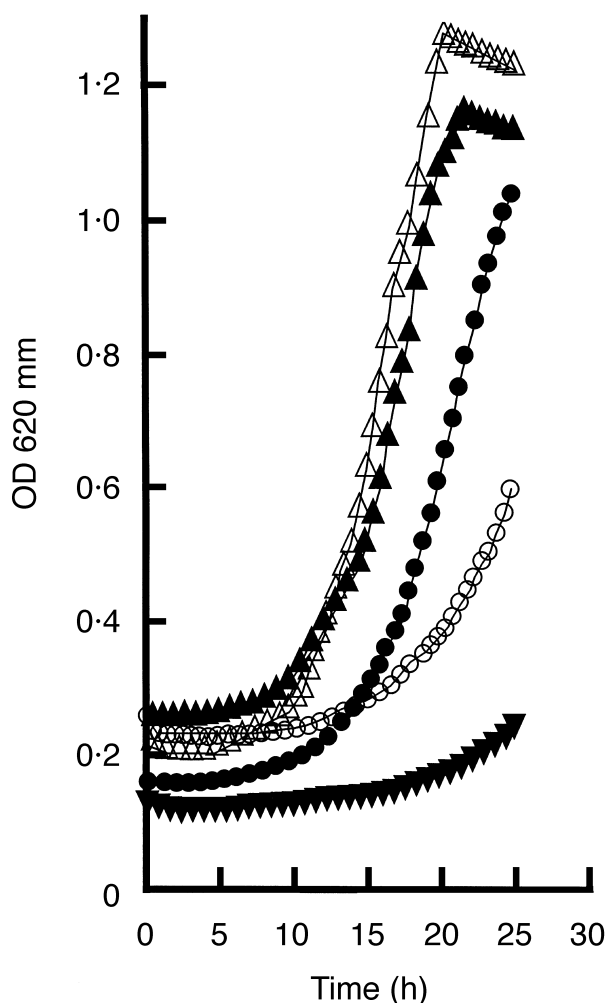
**Fig. 6** Analysis of casein degradation at 37°C by the purified *Listeria* protease. Molecular weight markers (lanes 1 and 15), sodium caseinate without and with protease (lanes 2 and 3, respectively). Lanes 4–11 show digestion of  $\beta$ -casein with purified protease at 10 min intervals, with the sample shown in lane 4 taken 5 min after addition of protease. Lane 12 shows the effect of 1-10-phenanthroline on proteolysis after 2 h, lane 13 PMSF (no effect) and lane 14 shows  $\beta$ -casein incubated in the absence of protease.



**Fig. 7** Examination of the proteolytic ability of the purified *Listeria* protease on different substrates:  $\beta$ -lactoglobulin (lane 1), lysozyme (lane 2), bovine serum albumin (lane 3)  $\beta$ -casein (lane 4) actin (lane 5); and 0.5 units of *Listeria* protease per mg of: actin (lane 7),  $\beta$ -casein (lane 8), bovine serum albumin (lane 9), lysozyme (lane 10),  $\beta$ -lactoglobulin (lane 11). Lane 6 shows molecular weight markers.

growth (Amezaga *et al.* 1995; Verheul *et al.* 1995). Furthermore, growth experiments revealed that *L. monocytogenes* can also use oligopeptides composed of four to eight amino acid residues as a nitrogen source (unpublished data). Therefore, growth experiments were performed to investigate whether

*Listeria* protease-degraded actin could support growth of *L. monocytogenes* ScottA in a chemically defined medium lacking the essential amino acid valine (DM-val). This medium did not support the growth of *L. monocytogenes* ScottA, whereas normal defined medium (DM) did (Fig. 8). Two milligrams



**Fig. 8** Growth of *L. monocytogenes* ScottA in chemically defined medium: DM (●), DM-val (▼), DM-val + undegraded actin (○), DM-val + degraded actin (▲), DM-val + degraded actin (duplicate) (△).

of actin was incubated in the presence and absence of 2 units of protease for 8 h at 80 °C at pH 7.0. SDS PAGE analysis revealed that actin was completely degraded by *Listeria* protease under these conditions (unpublished data). When degraded actin was incorporated into DM-val, growth was optimal. Incorporation of undegraded actin in DM-val did not sustain normal growth (Fig. 8).

#### Thermal stability of the purified metalloprotease

The observed optimum temperature for activity of the protease of 80 °C provoked an investigation into the thermal stability of this protease. A T50 value of 88 °C was obtained (unpublished data) indicating that this enzyme is the most

heat-stable natural thermolysin-like protease known to date (Eijsink *et al.* 1995).

#### DISCUSSION

The results presented indicate that, of the environmental parameters assessed, NaCl had a significant positive effect on expression of the protease associated with virulence in *Listeria monocytogenes*. This effect was previously perceived for lecithinase production (Coffey *et al.* 1996), an observation which correlates well with high levels of protease under these conditions, since lecithinase depends on the protease for maturation (Poyart *et al.* 1993). This may be due to a direct effect of the salt, enhancing maturation of the pro-form of the protease. Such a phenomenon was observed in the case of pro-subtilisin, where *in vitro* experiments showed that exposure to 200 mM sodium phosphate, compared to 20 mM, generated higher levels of mature subtilisin (Ikemura and Inouye 1988). The NaCl effect may also be indirect, by enhancing expression of PrfA, the positive regulator of virulence factors. The fact that NaCl has previously been shown to enhance listeriolysin activity in *L. monocytogenes* (Dallmier and Martin 1990) would support the latter view. Such an indirect effect of salt on these virulence factors may be explained by osmotic adaptation in the cytoplasm (Beumer *et al.* 1994; Amezaga *et al.* 1995) inducing increased expression of PrfA (Sheehan *et al.* 1994).

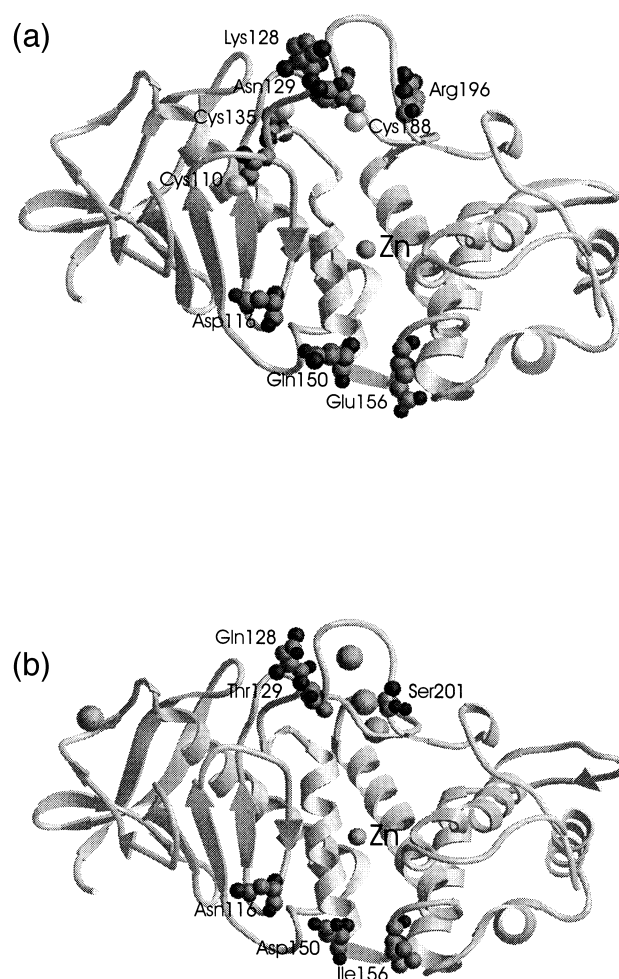
The observation that casein degradation specifically associated with the protease was not detectable *in vivo*, regardless of the strain used, strongly suggests that the enzyme is produced at very low levels by *L. monocytogenes*. Nevertheless, in the actual environment where this protease is required, in the immediate surrounding of the bacterium within a mammalian cell, the low level of production must be sufficient to mediate maturation of the secreted pro-form of lecithinase. It is not yet known what levels of NaCl are encountered in the cytoplasm of mammalian cells, or what factors, other than temperature (Leimeister-Wächter *et al.* 1992), could have a positive effect on virulence factors in this environment. It has been reported by Marquis *et al.* (1993) that certain amino acids may be limiting for *L. monocytogenes* in the cytoplasm (Piez and Eagle 1958). This may well cause increased production of protease in this environment, especially since our results indicate that higher levels of protease were obtained in a chemically defined medium than in a complex medium where peptides and amino acids are abundant. This indeed raises the question whether or not the protease could be responsible for actin degradation leading to generation of peptides and amino acids for intracytoplasmic growth of *L. monocytogenes*.

The capacity of the protease to degrade  $\beta$ -casein extensively indicates that the suggested cleavage site for the enzyme, between serine (51) and tryptophan (52) on pro-

lecithinase (Vazquez-Boland *et al.* 1992), is not the only possible cleavage site, as this sequence occurs only once in  $\beta$ -casein. Recognition by the protease is likely to depend on the overall conformation of the substrate, which in turn limits recognition and cleavage by the enzyme. Addition of 1 mM DTT to the reaction mixture did enhance degradation of  $\beta$ -casein, but such reducing conditions did not render the nondigestible substrates amenable to degradation. These observations on the activity of the enzyme are discussed later in relation to a three-dimensional model of the protease.

The observed inhibitory effect of 1-10-phenanthroline on the protease, coupled with the known high affinity which this agent has for zinc, and the observation that addition of supplementary  $ZnCl_2$  restored activity, provides biochemical evidence that this virulence factor is a zinc metalloprotease. Supplementation of 1-10-phenanthroline-treated protease with concentrations of zinc of 2 mM or greater had an inhibitory effect on activity, and this has also been observed for thermolysin (Holmquist and Vallee 1974). Indeed recently, an X-ray-derived structure of zinc-inhibited thermolysin showed that a second zinc atom is bound to the active site of the enzyme, thereby inhibiting enzymatic activity (Holland *et al.* 1995). This suggests structural similarities between the active sites of the two proteases. The protease sequence alignment data of Mengaud *et al.* (1991) and the properties described in this communication with regard to thermal stability and pH optimum indicates that the *Listeria* protease is a neutral protease of the thermolysin family. The high thermal stability value of 88 °C associated with this protease is intriguing, since the T50 value of neutral proteases normally correlates with a temperature where growth of the producer can occur (Fontana 1991). *L. monocytogenes* has a maximum growth temperature as low as 45 °C (Farber and Peterkin 1991). One could speculate that high thermal stability may be linked to a structural conformation which renders the protease resistant to degradation in the eukaryotic cytoplasm.

Inspection of the three-dimensional model built on the basis of the homology with thermolysin, for which a highly refined structure is available, indicates that the differences are mostly at solvent-exposed positions (data not shown). This indeed complicates an explanation of the observed high thermal stability of the *Listeria* protease since, in the case of other thermolysin-like proteases, it has been shown that surface-located structural elements determine the stability (Eijsink *et al.* 1995). None of the natural thermolysin-like proteases known to date contain disulphide bridges; however, these elements can contribute to stability. A disulphide introduced in the neutral protease from *Bacillus stearothermophilus* by site-directed mutagenesis was recently shown to increase the thermal stability significantly (Mansfield *et al.* 1995). SDS PAGE experiments indicate the presence of a disulphide bridge in the *Listeria* protease (unpublished data) and it can be anticipated that it contributes to the thermal stability



**Fig. 9** Ribbon drawing of the *Listeria* protease (A) and thermolysin (B). The side chains for residues in the substrate binding regions that differ between the two proteases, Asp116/Asn116, Lys128/Gln128, Asn129/Thr129, Gln150/Asp150, Glu156/Ile156, and Arg196/Ser201, for *Listeria* protease and thermolysin, respectively, are shown. Furthermore, locations of the three Cys residues, unique for *Listeria* protease, are indicated. The position of the catalytic zinc is indicated in both models, as are the calcium ions (large spheres) present in thermolysin

observed. In the model (Fig. 9), the distance between the side chains of residues Cys110 and Cys136 is such that disulphide formation, without major structural rearrangements, is feasible. It is clear from the proposed model that more charged amino acid residues are present at surface-located positions, compared to other thermolysin-like proteases. These charged residues could be involved in the formation of stabilizing salt bridges. However, the model is not distinct enough to predict the contribution of specific salt bridges to stability.

Comparison of the substrate binding regions in the active



site of thermolysin and the homologous regions in the *Listeria* protease active site indicates some differences in charge distribution and the sizes of the amino acids involved (Fig. 9). The presence of the more bulky side chains, e.g. Lys128, Asn129, Gln150 and arg196 in the *Listeria* protease vs. Gln128, Thr129, Asp150 and Ser201 in thermolysin, or the different charges of the residues presumed to be involved in substrate binding, e.g. Asp116, lys128, Asn129, Gln150, Glu156 and arg196 in the *Listeria* protease, and Asn116, Gln128, Thr129, asp150, Ile156 and ser201 in thermolysin, respectively, could affect the range of substrates that is able to bind in the active site of the *Listeria* protease. Its narrow substrate specificity may also have a role in the relatively high thermal stability, since it has been shown that thermolysin-like proteases are inactivated at higher temperatures by local unfolding followed by autoproteolytic degradation (Eijsink *et al.* 1995). In the case of the *Listeria* protease, fewer potential cleavage sites will be recognized after local unfolding at higher temperatures due to the narrower substrate specificity, as compared to the other 'broad-specificity' thermolysin-like proteases, and hence, fewer molecules will become irreversibly inactivated, resulting in a higher thermal stability.

The results presented in this communication provide evidence for a possible role of the *L. monocytogenes* protease, other than maturation of lecithinase. It is well documented that *L. monocytogenes* is unable to hydrolyse casein (Seeliger and Jones 1986); thus, it is interesting that it produces an extracellular protease which can extensively degrade this substrate. It is also interesting that the breakdown products of casein proteolysis can stimulate growth of *L. monocytogenes* in a chemically defined medium lacking essential amino acids (unpublished data). Significance may well be attached to the degradation of actin, which is relatively abundant in the immediate vicinity of *L. monocytogenes* during intracellular growth, and within the double-membraned phagosome during cell-to-cell spread. The fact that actin which was degraded by purified *Listeria* protease could significantly stimulate growth in a chemically defined medium suggests that this protein may, through the activity of the protease described, provide peptides or amino acids for intracellular proliferation and/or cell-to-cell spread of *L. monocytogenes*.

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