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A novel surface autolysin of *Listeria monocytogenes* serotype 4b, IspC, contains a 23-residue N-terminal signal peptide being processed in *E. coli*

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Abstract

The 86-kDa protein IspC of 774 amino acids in *Listeria monocytogenes* serotype 4b has been recently identified as the target of humoral immune response to listerial infection and as a novel surface autolysin. A signal peptide is predicted at the N-terminal end of IspC, but no biochemical data has been shown to confirm the presence of the cleavage site of a signal peptidase. To address this and prepare sufficient amount of the protein for biochemical and structural characterization, we present a strategy for efficient expression and purification of IspC and analyze the purified protein by N-terminal sequencing and mass spectrometry. Expression of IspC in *Escherichia coli* using a pET30a-based expression construct was efficiently improved by incubating the culture at 37 °C for 2 h followed by 4 °C for 16–18 h. The recombinant product rIspC remained as a soluble form in the cellular extract and was purified to electrophorectic homogeneity by the combination of metal chelate affinity chromatography with cation-exchange chromatography. The IspC was shown to contain a 23-residue N-terminal signal peptide being processed between Thr 23 and Thr 24 in *E. coli*, resulting in an 84-kDa mature protein. The highly purified form of rIspC from this study, exhibiting both peptidoglycan hydrolase activity and immunogenicity as previously reported, would facilitate further biochemical, structural, and functional studies of this autolysin.
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Keywords: *Listeria monocytogenes*; Autolysin; Signal peptide; Peptidoglycan hydrolase

Bacterial autolysins are enzymes that catalyze the specific cleavage of covalent bonds in the cell wall peptidoglycan (murein) from the producing bacterial strains [32] potentially leading to bacteriolysis. They are ubiquitously found in both Gram-positive and Gram-negative bacteria [2,11]. Based on the specific bonds that they cleave, autolysins are classified into several types: *N*-acetylmuramidases, *N*-acetylglucosaminidases, *N*-acetylmuramyl-L-alanine amidases, endopeptidases, and transglycosylases [34]. Bacterial autolysins are involved or implicated in a variety of cellular functions including cell wall turnover, cell division, cell separation, chemotaxis, biofilm formation, genetic competence, protein secretion, antibiotic-induced lysis, sporulation, and formation of flagella [31,33], and in pathogenesis [3,7,13,14,25,35]. Multiple autolytic enzymes have been demonstrated and studied in a number of bacterial species [16,26,33], and the purpose of this functional redundancy (if there is any) is not clear. The data accumulated to date in the literature, however, appears to indicate that an individual autolysin in a bacterium performs its unique biological function(s) that is not necessarily overlapped by other autolysins of the same bacterial strains. Thus, for a bacterium of interest, it is necessary to identify and characterize all the autolysins in order to gain a comprehensive understanding of their biological functions and their roles in virulence.

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Listeria monocytogenes is a Gram-positive, facultatively anaerobic, intracellular bacterium that causes a severe food-borne disease (listeriosis) with clinical symptoms including septicemia, meningitis, and abortion, mainly affecting immunocompromised individuals, neonates, the elderly, and pregnant women [36]. The disease has a relatively high mortality rate of 20–30% [29,36]. Although 13 serotypes of *Listeria* are recognized, serotypes 4b, 1/2a, and 1/2b of *L. monocytogenes* are responsible for almost all human cases of listeriosis [9,23,37] with serotype 4b strains accounting for almost all major outbreaks and a large portion of sporadic cases, suggesting this serotype possesses a virulence potential highly specific to humans [10,18].

Several autolysins have been identified and characterized in *L. monocytogenes*, including P45 [30], P60 [39], NamA [20] or MurA [8], Ami [5,24], and Auto [6]. The murein-hydrolyzing activity of a flagellin FlaA from *L. monocytogenes* has been demonstrated [27], but its autolytic activity remains unknown. Recently we have identified and biochemically characterized a novel surface-localized autolysin from *L. monocytogenes* serotype 4b [38], an 86-kDa (deduced molecular mass) protein consisting of 774 amino acids known as the target (designated IspC) of humoral immune response to listerial infection [40]. A signal peptide containing the first 29 N-terminal residues was predicted by SignalP 3.0 using neural networks (http://www.cbs.dtu.dk/services/SignalP/); removal of it *in vivo* by a signal peptidase that cleavages between Leu 29 and Gln 30 would yield a mature protein of 82.598 kDa. Analysis of various truncated forms of IspC for cell wall hydrolyzing or binding activity has defined two separate functional domains: the N-terminal catalytic domain (aa 1–197) responsible for the hydrolytic activity and the C-terminal domain (aa 198–774) made up of seven GW (glycine–tryptophan dipeptide) modules responsible for anchoring the protein to the cell wall [38]. In this communication, we report efficient expression of the full-length IspC in *E. coli*, its purification by chromatographic methods combining metal affinity chromatography with cation-exchange chromatography, and analysis of the purified protein by N-terminal sequencing and mass spectrometry. Biochemical, structural, and functional analysis of the immunogenic autolysin IspC is now possible, since we have developed efficient preparation and purification methods for a sufficient quantity of the recombinant protein.

Materials and methods

Chemicals and reagents. Isopropyl-β-D-thiogalactoside (IPTG), phenylmethysulphonyl fluoride (PMSF), and kanamycin were obtained from Sigma (St. Louis, MO, USA). Ni–NTA superflow agarose and anti-histidine tag monoclonal antibody (anti-His mAb) were purchased from Qiagen (Santa Clarita, CA, USA); Horseradish peroxidase (HRP)-conjugated goat–anti-mouse IgG from Jackson ImmunoResearch Laboratories (West Grove, PA, USA); SP Sepharose Fast Flow from Amersham Biosciences (Baie d'Urfe, Quebec, Canada). All other chemicals and solvents were of commercially available analytical, HPLC or MS grade.

Induction of recombinant protein expression in E. coli. The construct pIspC previously created by inserting the *ispC* ORF into the *Nde*I and *Xho*I sites of pET30a [38] was used to produce the recombinant IspC (rIspC) in *E. coli* Rosetta (DE3)/pLysS (Novagen, Madison, WI, USA). The overnight culture was diluted 1:100 into 10 l fresh LB broth (250 ml per 1 l flask) supplemented with kanamycin (30 μg/ml), and subcultured at 37 °C with vigorous shaking until the cell growth reached an OD_{590} of 0.45 ± 0.05. IPTG (1 mM) was added to induce the expression of rIspC at 37 °C for 2 h and then maintained at 4 °C overnight. The cells were harvested by centrifugation at 16,900*g* at 4 °C for 10 min and frozen at −80 °C until use.

SDS–PAGE and Western blotting. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli [19], using a 4% stacking gel and a 12% resolving gel in a Bio-Rad minigel apparatus (Bio-Rad, Mississauga, Ontario, Canada). Following electrophoresis, the separated proteins were stained with either Coomassie brilliant blue or electrotransferred onto a nitrocellulose membrane using a Trans-Blot SD semi-dry transfer cell (Bio-Rad, Mississauga, Ontario, Canada) according to the manufacturer's instructions. The Western blot procedure for analysis of the target protein with anti-His mAb followed by horseradish peroxidase (HRP)-conjugated goat–anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, Penn.) was performed essentially as described [22].

Chromatographic procedures. The frozen cell pellets were resuspended in a minimum volume of phosphate-buffered saline (PBS, pH 7.2) containing 1 mM PMSF and lysed by passing through a French Press at 1500 lb/in.[2] The homogenates were spun at 27,000*g* at 4 ° C for 20 min. The supernatant, mixed with an equal volume of buffer A (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole), was applied at 1 ml/min to a column (1 × 1.5 cm) of Ni–NTA superflow (Qiagen) that had been pre-equilibrated with buffer A. The column was washed with 30 ml of buffer A, and the proteins were eluted in 1 ml per fraction with 30 ml of 250 mM imidazole in buffer B (25 mM NaH_2PO_4–NaOH, pH 8.0). The A_{280} peak fractions containing the target protein, judged by SDS–PAGE and Western blot analysis, were pooled and loaded at 1 ml/min into a column (1 × 2 cm) of SP Sepharose Fast Flow which had been pre-equilibrated with buffer C (10 mM phosphate buffer, pH 8.0, 5% glycerol). After washing with at least 10 times bed volume of buffer C, the rIspC was eluted in a sharp A_{280} peak following a linear 0–500 mM NaCl gradient in buffer C (30 ml; fraction size, 1 ml). The fractions containing rIspC were pooled, assessed for its purity by SDS–PAGE, and quantified by using the Bradford method [4] with bovine serum albumin (BSA) as a standard.

Estimation of protein extinction coefficient. The molar extinction coefficient (ε) of IspC at 280 nm was calculated from its amino acid sequence (i.e. the number of tryptophan, tyrosine, and cysteine residues within the protein) [38,40] according to the method of Gill and von Hippel [12].

N-terminal sequencing. The N-terminal sequence of rIspC was determined using Edman degradation chemistry. Following separation of the purified protein by SDS–PAGE and electrotransfer onto a PVDF membrane as described [21], the protein band was visualized by staining with 0.1% (w/v) Ponsceau S and excised for N-terminal sequencing performed at the Biotechnology Research Institute, National Research Council Canada (Montreal, Quebec, Canada).

Mass spectrometry. Electrospray ionization (ESI) mass spectrum was acquired on Waters Micromass Global Q-TOF mass spectrometer coupled to a Waters capillary HPLC (Milford, Massachusetts, USA). The mass spectrometer was calibrated with a mixture of proteins: [glu[1]]-fibrinopeptide B human, horse heart myoglobin, and chicken lysozyme(Sigma). The calibration for intact protein analysis was checked by running cytochrome *c*, which resulted in a mass accuracy of ±1 amu. The purified IspC at a concentration of 10 pmol/μl in water was loaded onto an Atlantis dC18 trap with a 50-μm, ID silica tubing in place of a capillary HPLC column. The autosampler parameters included sample loading flow rate of 15 μl/min for 3 min, which was then decreased to 5 μl/min for the remainder part of the run. The gradient was delivered at a flow rate of 7 μl/min which was split to a flow rate of through the column 300 nl/min

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