J Ind Microbiol Biotechnol (2005) 32: 355–363
DOI 10.1007/s10295-005-0002-2

ORIGINAL PAPER

Robin L. T. Churchill · Hung Lee · J. Christopher Hall

Rapid purification of recombinant listeriolysin O (LLO) from *Escherichia coli*

Received: 31 March 2005 / Accepted: 13 June 2005 / Published online: 29 July 2005

Abstract *Listeria monocytogenes* is an emerging foodborne pathogen that is responsible for about 28% of the food-related deaths in the United States. It causes meningitis, septicaemia and in pregnant women, abortions and stillbirths. It secretes the toxin listeriolysin O (LLO) that allows the bacteria to enter the cytoplasm of host cells, where they can replicate and cause further infection. The rapid and sensitive detection of LLO in food samples is a key to monitoring and prevention of listeriosis. To facilitate the development of an assay for the specific detection of LLO, a source of LLO is essential. We outline a method of producing a large amount of functional LLO by expressing the *hlyA* gene (encoding LLO) in *Escherichia coli* and purifying the recombinant LLO using a one-step purification method. Purification of the protein takes only about 4 h. We compared three different expression constructs for the production of the toxin, which tends to interact strongly with a number of column surfaces. The first construct, using an intein fusion system, could not be purified from the column. The second LLO construct contained an N-terminus His tag; it gave a yield of 3.5–8 mg l^{-1}. The third contained a C-terminus His tag; it gave a yield of 2.5 mg l^{-1} LLO. The purified LLO from the latter two constructs retained its activity at 4°C for over a year as determined by bovine red blood cell hemolysis assay. This paper provides a much-needed, high-yield, one-step purification method of recombinant LLO, and is the first to provide evidence of long-term stability of the toxin for further applications.

Keywords Foodborne pathogen · Hemolysis assay · Immobilized metal affinity chromatography · *Listeria monocytogenes* · Listeriolysin O · Protein purification

R. L. T. Churchill · H. Lee · J. C. Hall (✉)
Department of Environmental Biology, University of Guelph, N1G 2W1 Guelph, ON, Canada
E-mail: jchall@uoguelph.ca
Tel.: +1-519-8244120
Fax: +1-519-8370442

Introduction

Listeria monocytogenes is a foodborne pathogen found ubiquitously in the environment. It has been isolated from soil, plants, decaying vegetation [18], silage, water, and sewage, as well as the intestinal tracts of many birds and animals [2, 24]. *L. monocytogenes* is also commonly carried in the intestinal tracts of humans, cattle, sheep, pigs, and goats. *L. monocytogenes* has been found in raw or processed food samples including dairy products, meat, vegetables and seafood [12, 26]. Between 1979 and 1999 in the United States and Canada, there have been six major outbreaks of listeriosis. These were associated with eating such diverse foods as lettuce, carrots, commercially prepared coleslaw, pasteurized 2% milk (contaminated after pasteurization), chocolate milk, pâté, pork tongue in jelly, hotdogs, and soft cheeses made from raw milk [3, 5].

L. monocytogenes tends to affect people with lowered cell-mediated immunity, including neonates, the elderly and pregnant women. Human listeriosis is characterized by septicaemia, meningitis, and abortion in pregnant women. Although relatively few people become ill with *Listeria* infections, there is a 20% mortality rate for those that do [6].

Listeriolysin O (LLO), produced by *L. monocytogenes*, is a member of the cholesterol-dependent cytolysin (CDC) family of toxins. It is one of the main virulence factors in the pathogenesis of listeriosis, allowing the bacteria to escape from host-cell phagosomes to replicate in the cytoplasm. Mutants of *L. monocytogenes* lacking the *hlyA* gene (encoding LLO) or with mutated versions of the gene are generally non-virulent [3].

LLO is composed of 529 amino acids with a predicted MW of 58 kDa [15]. The *hlyA* gene is found within a cluster of virulence genes and its expression is regulated by PrfA, a global virulence transcriptional activator protein [15]. Within the host-cell phagolysosome, LLO binds as a monomer to the phagolysosome membrane, with subsequent oligomerization into large arc-or

ring-shaped structures that puncture the membrane [1], forming pores of about 20 nm in diameter [15]. This results in the escape of the *L. monocytogenes* into the cytoplasm of the host cell during infection [3].

It is desirable to have a large amount of purified LLO for use in the development of new immunoassays that can be used for the specific monitoring and detection of *L. monocytogenes* in foods. Several papers have described the purification of LLO from *L. monocytogenes* [7, 8, 19, 22, 30]. However, these methods are time consuming, requiring days for purification, and result in very low yields of LLO, taking as much as 10 l culture to purify 132 μg LLO [29], or at best, 3 l culture for 4.8 mg protein [4]. More recently, some researchers have purified truncated recombinant LLO, lacking the secretion signal, from *Escherichia coli* [9, 10]. Although the authors were able to produce 4.5 mg l^{-1} LLO, their methods were labour intensive and time consuming, requiring adsorption to hydroxyapatite, ammonium sulfate precipitation, and cation exchange chromatography [9].

The present study was undertaken to optimize the expression of LLO in *E. coli* by examining several expression systems and constructs, and focusing on the use of one-step purification methods in order to shorten purification time. We report here a 4 h purification method that allowed production of 3.5–8 mg l^{-1} LLO lacking the secretion signal or 2.5 mg l^{-1} LLO retaining the secretion signal. The purified recombinant LLO retained their red blood cell hemolytic activity when stored at 4°C for more than 12 months. To our knowledge, no other purified LLO construct has demonstrated stability for such a long period of time.

Materials and methods

Cloning and expression of LLO in pTWIN1

The *hlyA* gene encoding LLO was amplified from the pELis plasmid, kindly provided by Yvan Chapdelaine (National Research Council Canada, Ottawa, ON, Canada) using the Advantage-HF PCR kit (BD Biosciences, San José, CA, USA). For insertion into the pTWIN1 vector (New England Biolabs, Beverly, MA, USA), the following two oligonucleotide primers were used in the PCR: LLO-int-rev, 5′-ggtggt*catATG*AAAA AAATAATGCTAG-3′ and the LLO-int-for, 5′-ggtggtt *gctcttc*cgcaTTCGATTGGATTATC-3′. The oligonucleotides included *Nde*I and *Sap*I restriction sites at their respective 5′-ends to facilitate directional cloning into the vector. Non-coding regions of the oligonucleotides are in lower case while the restriction sites are in italics. All oligonucleotides were made at the Guelph Molecular Supercentre (Guelph, ON, Canada). PCR was performed by denaturing the pELis template DNA for 4 min at 94°C, then amplifying for 30 rounds of denaturation at 94°C, 30 s, annealing at 55°C, 30 s, and extension at 72°C, 1 min. This was followed by a final extension step of 72°C for 10 min. The resulting PCR product was run on a 1% agarose gel and purified by excision and DNA purification using the QIAquick Gel Extraction kit (QIAgen, Venlo, The Netherlands). The PCR product was digested with the restriction endonucleases mentioned above, and ligated into the pTWIN1 vector. This resulted in a fusion of the *hlyA* gene with the gene encoding a mini-intein and chitin-binding domain (CBD) that can be expressed as a single product. The constructs were electroporated into *E. coli* ER2566 cells. Positive clones were selected by colony PCR, sequenced and confirmed by western blot with an anti-CBD antibody. The construct was designated LLO-intein. Glycerol stocks of positive cultures were stored at −80°C.

Expression optimization was first performed in a small scale using 25-ml cultures with LB media (BD Biosciences) containing 75 μg ml^{-1} carbenicillin in 125-ml Erlenmeyer flasks. Single colonies were inoculated into 5 ml LB containing 75 μg ml^{-1} carbenicillin and shaken at 200 rpm overnight at 37°C in 50-ml Falcon tubes. The 25-ml optimization culture was inoculated with 1 ml of this inoculum culture. The culture was grown in 125-ml Erlenmeyer flasks at 37°C, 200 rpm to an A_{600} of 0.7 and induced for 2, 4, 6, 8, and 16 h with 0.5 or 1 mmol l^{-1} IPTG at 22 or 37°C. Optimal expression was found by growing the culture to an A_{600} of 0.7, then inducing the culture with 0.5 mmol l^{-1} IPTG, followed by expression at 37°C for 2 h. Cells were then pelletted by centrifugation at 5,000 *g* and frozen at −20°C until used for LLO purification.

For large scale LLO purification, 1-l cultures were grown in 4-l flasks with shaking at 200 rpm, 37°C, induced, and pelletted as described above. The cell pellet was resuspended in 30 ml Buffer B2 (20 mmol l^{-1} Na-HEPES, pH 7.0; 500 mmol l^{-1} NaCl; 1 mmol l^{-1} EDTA; 0.1% Tween-20) with 3.5 g alumina (Sigma, St. Louis, MO, USA). The cells were sonicated for three min in 15 s bursts. After sonication, the resulting slurry was centrifuged at 10,000 *g* for 30 min at 4°C to remove cell debris. The supernatant was passed over a 5-ml bed volume (1 cm diameter by 5 cm) of chitin column (New England Biolabs) that was previously equilibrated with the same buffer. The step was done at a rate of 0.5 ml min^{-1} to ensure binding of the LLO-CBD fusion protein to the column. The column was washed with 20 column volumes of buffer B3 (20 mmol l^{-1} HEPES, pH 8.5; 500 mmol l^{-1} NaCl; 1 mmol l^{-1} EDTA). Cleavage of the LLO from the intein fusion protein was initiated by washing with three column volumes of Buffer B3 plus 40 or 100 mmol l^{-1} dithiothreitol (DTT). The column was plugged and incubated with the DTT-containing buffer for 24 or 72 h at 4°C or for 24 h at 22–24°C. The protein was then eluted with Buffer B3 containing the same amount of DTT and 0.1% Tween-20. Elution was also performed with DTT containing Buffer B3 having an increased NaCl concentration of 500 mmol l^{-1} to ensure the LLO cleavage product would remain soluble.

The column was finally stripped for reuse with Stripping buffer 1 (SB1; 20 mmol l^{-1} Na-HEPES, pH 8.0; 500 mmol l^{-1} NaCl; 1% SDS) or SB2 (0.3 mol l^{-1} NaOH).

Cloning and expression of LLO in pQE31

Insertion of the *hlyA* gene into the pQE31 expression vector (QIAgen) was performed based on the work of Ito et al. [13]. The primers used were His-LLO back: 5′-cgat*ggatcc*tGATGCATCTGCATTCAATAAAG-3′ and His-LLO for: 5′-acgc*ctgcAG*TTCGATTGGATTATCTACACTATTAC-3′. PCR was performed as described above. After digestion of the pQE31 vector and PCR products with *Pst*I and *Bam*HI (italicized in the above primers), the products were ligated and electroporated into *E. coli* strain TG1 for screening purposes. Positive clones were selected by colony PCR using the above primers and confirmed by sequencing. One clone with the proper sequence for the *hlyA* gene, inserted in frame with the His tag at the N-terminus, was selected and transferred to *E. coli* strain SG13009 (QIAgen) for expression studies. The construct was designated His-LLO.

Optimization of LLO expression was performed as described above except that 0.3 mmol l^{-1} IPTG was also tested, and 25 μg ml^{-1} kanamycin was added to all cultures. Western blotting was conducted using the penta-His tag antibody (QIAgen) for detection to determine the optimal expression conditions. The optimal production of His-LLO from SG13009 cells containing the pQE31:His-LLO involved shaking the cultures at 200 rpm at 37°C until the A_{600} was 0.6. Induction was performed using 0.3 mmol l^{-1} IPTG, and shaking at 200 rpm at room temperature (22–24°C) for 4 h. For large-scale cultures, 1 l media was used with 5 ml inoculum from a small-scale culture. The cells were pelletted at 5,000 *g* and the pellets stored at −20°C until processed for LLO purification.

Cloning and expression of LLO in pQE70

The *hlyA* gene was inserted into the pQE70 vector (QIAgen) to have the protein with the His tag at the C-terminus. The two primers used to amplify the gene for insertion into the vector were LLO-SphI: 5′-ggtggt*gcATGC*AAAAAATAATGCTAGTTTTTATTACAC-3′, and LLO-BglII: 5′-ggtggt*AGATCT*TTCGATTGGATTATCTACTTTATTAC-3′. PCR was performed as described above. After insertion of the gene into the vector, the ligated product was transformed into *E. coli* strain SG13009. Colony PCR with *hlyA*-specific primers was used to check for the presence of the insert in several clones. One clone was randomly selected and the sequence of its insert determined to confirm successful insertion of the *hlyA* gene. Expression was confirmed using western blotting with the penta-His tag antibody (QIAgen). The construct was designated LLO-His. Initial assessment of protein expression followed the protocol described above for His-LLO.

Purification of the LLO expressed from pQE31 and pQE70

The two LLO constructs were purified by immobilized metal affinity chromatography (IMAC). Frozen pellets from 1-l induced cultures were resuspended in 30 ml IMAC A buffer (500 mmol l^{-1} NaCl; 10 mmol l^{-1} HEPES, pH 7.0) and sonicated for 3 min with 15 s bursts. The resulting cell fragments were centrifuged at 12,000 *g* in a Sorvall SS34 rotor at 4°C for 30 min. The volume of the clarified extract was measured and IMAC B buffer (500 mmol l^{-1} NaCl; 10 mmol l^{-1} HEPES, pH 7.0; 500 mmol l^{-1} imidazole) was added to a final imidazole concentration of 10 mmol l^{-1}.

The prepared sample was loaded onto a commercial 5-ml HiTrap Nickel Chelating column (Amersham Biosciences, Uppsala, Sweden) and washed with IMAC A buffer containing 50 then 75 mmol l^{-1} imidazole to remove any non-specifically bound proteins. The His-tagged LLO was eluted from the column with 250 mmol l^{-1} imidazole. Any aggregated protein was removed from the column by washing with 500 mmol l^{-1} imidazole. Fifty-two 1-ml fractions were collected during the elution procedure.

After elution with 250 mmol l^{-1} imidazole, fractions corresponding to those in the elution chromatogram with the highest A_{280} were subjected to SDS-PAGE analysis to determine which fractions contained pure His-LLO or LLO-His. Those five to seven fractions containing the highest concentrations of pure protein were combined and dialyzed against 2 l storage buffer (500 mmol l^{-1} NaCl; 10 mmol l^{-1} $NaPO_4$, pH 7.0; 0.5 mmol l^{-1} EDTA, 0.02% NaN_3) for 16 h at 4°C. Protein concentration was measured at 280 nm on an Ultraspec 3100 pro spectrophotometer (Amersham Biosciences), and the concentration of LLO was estimated using the molar extinction coefficient for LLO of 71,830 cm^{-1} (based on the primary sequence of LLO). The purified protein was stored at 4°C.

Red blood cell hemolysis assay

The hemolysis assays were done according to the method of McKeller [25] and Leimeister-Wächter and Chakrabarty [20] with a few modifications. Bovine blood was obtained from Better Beef (Guelph, ON, Canada) and stored at 4°C for a maximum of 3 days until use. The blood was centrifuged in a swinging bucket centrifuge at 2,200 *g* for 10 min at 4°C to pellet the red blood cells. The cells were washed twice with PBS, pH 7.0 to remove traces of serum and were centrifuged as described above. The red blood cells were then diluted in PBS so that if 100 μl of the blood cell suspension was lysed completely in 1 ml H_2O, the A_{541} would be 0.8.

The hemolysis assay was performed by adding 8 μg His-LLO or LLO-His and serially diluting the LLO twofold to 24 ρg of the protein in 1 ml total volumes. To each tube was added 20 mmol l^{-1} Cys as a reducing agent and 0.1% BSA. Each sample was mixed by inversion and 100 μl of the diluted blood was added. A positive lysis control in 1 ml H_2O and a negative lysis control in PBS treated as described above, but lacking any LLO, were also included. The samples were mixed by inverting again and incubated at 37°C for 1 h. At the end of the incubation period, the tubes were centrifuged for 2 min at 10,000 *g* in a bench top centrifuge and the supernatant was transferred to disposable cuvettes. The absorbance of the supernatant containing hemoglobin released from lysed red blood cells was measured at 541 nm. Each assay was performed at least three times and the results were plotted to determine the hemolytic units (HU) present. One HU is the amount of LLO required to lyse 50% of the RBCs in a sample.

Results and discussion

LLO acts as a secreted indicator of the presence of live *L. monocytogenes* in foods. As such it would be useful to have large amounts of the protein for the development of novel immunoassays to detect the pathogen. Several papers have described the purification of LLO from *L. monocytogenes* [7, 8, 19, 22, 30]. However, these methods are time consuming, requiring days for purification, and many had relatively low yields of LLO. The present study was undertaken to optimize the expression of LLO in *E. coli* by examining several expression systems and constructs, and focusing on the use of one-step purification methods to shorten purification time. In order to show that the recombinant LLO expressed in our system was equivalent to the native LLO, we confirmed the gene sequence, and compared the physical parameters generally used to characterize LLO - activation by thiol-reducing agents, pH dependence, and abrogation of activity by cholesterol.

Analysis of the construct sequences

The nucleotide sequences of the LLO expression constructs matched the sequence of LLO in GenBank listed under accession number M24199, and published by Mengaud et al. [27] Changes in the predicted protein sequences due to the cloning procedure are highlighted in Fig. 1. Expression using the IMPACT 1 system resulted in LLO fused to an intein tag and chitin-binding domain. On contact with a reducing agent such as DTT, the intein tag was cleaved to release the recombinant LLO without any extra amino acids.

The LLO-His construct incorporated two changes in the primary sequence. The changes included a point mutation near the N-terminus where Gln replaces Lys2, and the addition of two amino acids (RS) at the C-terminus, both derived from the multiple cloning sites in the vector. The His-LLO construct contained a few more changes in its sequence. In order to retain as much protein in the cells as possible, the secretion sequence at the N-terminus of the protein [27] was deleted. In addition, three amino acids (TDP) were incidentally inserted between the His-tag and the N-terminus and seven amino acids (LQPSLIS) were inserted at the C-terminus of the protein because of vector nucleotides incorporated during the insertion of the gene into the expression vector. The MRGS sequence before the His tag in His-LLO originated from the vector. These extra amino acids were not anticipated to have any effect on the activity of His-LLO. Furthermore, Giammarini et al. [9] showed that the removal of the secretion sequence does not affect the activity of LLO.

Expression and purification of LLO via the pTWIN1 vector

Once we confirmed that the sequences were correct and in frame, we proceeded to express and purify the recombinant LLO proteins from the clones. The LLO-intein-chitin-binding domain fusion product was well expressed after 2 and 4 h of induction with 0.5 mmol l^{-1} IPTG at 37°C. After 8 h of induction at 22, 30 or 37°C, the cells became difficult to pellet, with the pellets having a jelly-like consistency. Furthermore, the LLO toxin was not readily visible on a gel (data not shown). Goebel et al. [11] observed spontaneous hemolysis of the cells when trying to express recombinant listeriolysin in *E. coli* cells. This may explain why we (1) often had very slow growing cultures when expressing our recombinant listeriolysin, and (2) could not induce the cells for more than 4 h at 37°C without losing all the listeriolysin.

The recombinant full-length LLO-intein-chitin binding domain fusion product appeared to be hydrophobic and bound tightly to the chitin column. When elution buffer was passed over the column, nothing would elute. Altering the cleavage and elution conditions as described in the Materials and methods did not change this result. Only when the column was stripped with 0.3 mol l^{-1} NaOH was there any protein eluted, with about half in the fusion protein form (MW of 78 kDa) and half in the free LLO form (MW of 58 kDa) (data not shown). It has been our experience that all of the LLO constructs are relatively "sticky". There were problems with the full length LLO not eluting from Sephadex 75 and 200 columns, making purification difficult (unpublished observations). Many of the early purification procedures for LLO resulted in low yields [17, 28], likely because the protein was retained on the purification columns.

Expression and purification of LLO via the pQE vectors

Because of the lack of success in purifying the full length LLO using the IMPACT 1 system, we turned to a

```
LLO-I      MKKIMLVFITLILVSLPIAQQTEAKD ASAFNKENSIS.....  37
LLO-His    MQKIMLVFITLILVSLPIAQQTEAKD ASAFNKENSIS.....  37
His-LLO              MRGSHHHHHHTDPD ASAFNKENSIS.....  25
M24199     MKKIMLVFITLILVSLPIAQQTEAKD ASAFNKENSIS.....  37

LLO-I      LPLVKNRNISIWGTTLYPKYSNKVDNPIE          529
LLO-His    LPLVKNRNISIWGTTLYPKYSNKVDNPIERSHHHHHH  537
His-LLO    LPLVKNRNISIWGTTLYPKYSNKVDNPIELQPSLIS   524
M24199     LPLVKNRNISIWGTTLYPKYSNKVDNPIE          529
```

Fig. 1 Comparison of the partial sequences of the recombinant LLO constructs. The sequences of the recombinant LLO constructs were aligned and compared to the sequence of LLO from GenBank accession number M24199. Changes from the original sequence incorporated when the gene was inserted in the expression vector are *bolded and underlined*; the secretion signal of LLO is *boxed*. LLO-I, LLO produced from the IMPACT 1 intein system; LLO-His, LLO with a hexa-histindine tag on the C terminus; His-LLO, LLO with a hexa-histindine tag on the N-terminus; M24199, LLO sequence from GenBank Accession number M24199

different system that used a different type of column for purification. The pQE vectors (QIAgen) provide a His-tag that can be added on either end of the protein with only a few additional amino acids being included.

Figure 2 illustrates the time course of expression of His-LLO at 25 and 37°C. The main band produced on induction with IPTG was found near 60 kDa, close to the expected 58 kDa of LLO. The predicted MW based on the amino acid sequence is also close, 58.9 kDa. Induction with 0.3 mmol l^{-1} IPTG resulted in the greatest amounts of soluble protein. While there was good expression of His-LLO at 37°C, the product seemed to be susceptible to degradation as seen by the increasing number of bands recognized by the penta-His antibody below the main His-LLO band in the Western blot. By reducing the induction temperature to 22°C, the degradation of His-LLO was reduced. We saw the same trends with LLO-His. Leimeister-Wächter and Chakraborty [20] observed the same trend in their expression; they used pUC18:LLO and *E. coli* DH5α as the expression vector and strain, respectively. These authors observed greater hemolytic activity when the LLO was expressed at 30°C than at 37°C, although the actual expression levels, based on SDS-PAGE, were higher at 37°C.

After purification by IMAC (Fig. 3), we routinely recovered 3.5–8 mg l^{-1} of culture for the His-LLO and around 2.5 mg l^{-1} culture for the LLO-His. The entire purification procedure took about 4 h, and required no concentration steps. Final volumes of the purified protein were 5–7 ml. Compared to the procedures reported in other LLO purification papers (see Table 1), this represents one that saves time amounting to days and delivers significantly more protein per preparation than previously described [4, 7, 29, 30].

The first authors describing purification of secreted hemolytic fractions reported that the fractions resembled streptolysin O (SLO), having hemolytic properties, reactivity with anti-SLO antibodies, activation by thiol-reducing agents, and reduction of activity in the presence of cholesterol [16, 28], but the papers varied in the reported MW of the protein, ranging from <10 kDa [28] to 171 kDa [17]. Geoffrey et al. [7] were the first to purify and accurately determine the MW of the LLO from *L. monocytogenes* (Table 1). These researchers purified the protein using thiol-disulfide exchange affinity chromatography and multiple gel filtration and concentration steps. Like the other purified hemolysins, this protein expressed greater activity in the presence of reducing agents, was inhibited by the presence of cholesterol, and showed cross-reactivity with anti-streptolysin O antibodies. It also showed the now accepted optimal hemolytic activity peak at pH 5.5 [7]. Their purification method resulted in yields of only 0.6 mg protein from 27 l of activated carbon-pretreated growth media, using five different chromatographic columns and a number of ultracentrifugation steps for purification. The yield of LLO by their method was very low considering the volume of the starting material and the extensive labor and time involved [7]. Subsequent papers reported the use of various methods to purify the full-length native toxin from *L. monocytogenes*. These included the use of sulfopropyl-cation exchange chromatography and high performance liquid chromatography [22], batch adsorption to Q-Sepharose and column chromatography on a Mono-S HR5/5 column [4], ultrafiltration, multiple concentration steps, hydroxyapatite adsorption chromatography, and ion exchange chromatography [29], or DEAE-sephadex column chromatography [30]. These methods required between 3 [4] to 10 l [29] of culture and only 50 μg [22] to 4.8 mg [4] protein were purified; these methods required several days to complete.

Giammarini et al. [9, 10] increased the expression of a truncated LLO that lacked the secretion sequence by using an *E. coli* expression system, then purified the LLO on a hydroxyapatite column, concentrated it by ammonium sulfate precipitation, and further purified it using SP Sepharose cation-exchange chromatography. The authors were able to increase the recovery of LLO to 4.5 mg l^{-1} [9], but the columns used and requirement for SDS-PAGE gels after each chromatography step and hemolysis assays to confirm the presence of the protein added time and complexity to the procedure. Our

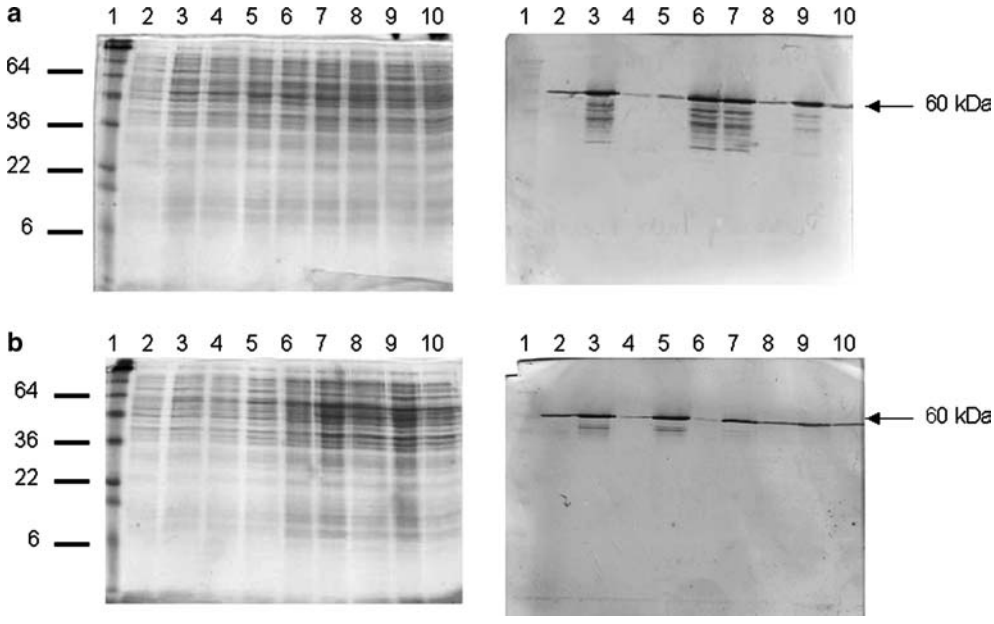

Fig. 2 Expression of His-LLO under several conditions as detected in cell extracts of *E. coli* carrying pQE31:His-LLO. **a** *Left panel* SDS-PAGE profile of proteins expressed at 37°C and *right panel* a Western blot probed with penta-His antibody (QIAgen). *Lane 1* MW markers, *lane 2* 0 h induction control, *lane 3* induced 2 h, *lane 4* uninduced 2 h, *lane 5* uninduced 4 h, *lane 6* induced 4 h, *lane 7* induced 8 h, *lane 8* uninduced 8 h, *lane 9* induced 16 h, *lane 10* uninduced 16 h. **b** SDS-PAGE profile of proteins expressed at 22°C. Lanes and conditions as in **(a)** except that lanes 5 and 6 are reversed

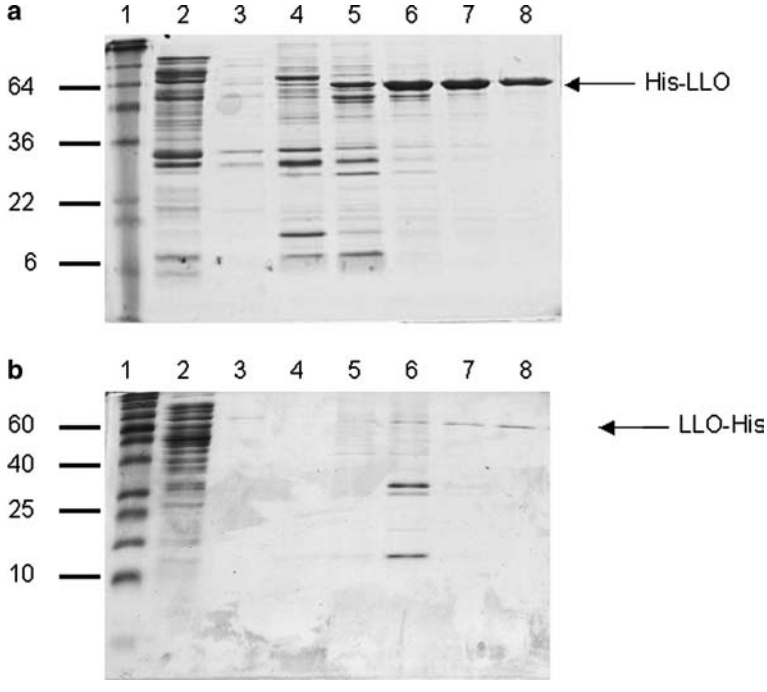

Fig. 3 **a** SDS-PAGE profile of the purification of His-LLO on a HiTrap Nickel Chelating column (Amersham Biosciences) using fast protein liquid chromatography. *Lane 1* MW markers, *lane 2* wash at 50 mM imidazole, *lane 3–4* wash at 75 mM imidazole, *lanes 5–8* elution with 250 mM imidazole. **b** Purification of LLO-His. *Lane 1* MW markers, *lane 2* wash at 75 mM imidazole, *lanes 3–7* elution with 250 mM imidazole

procedure required the SDS-PAGE step only after purification, and only the protein peak at 250 mmol l^{-1} imidazole contained the LLO constructs.

Our procedure offers the advantages that the purification of LLO occurs in one step, and the recovered protein required no further concentration. Our attempts at concentrating the protein with Amicon or Centricon filters resulted in a loss of 50–90% of the protein (unpublished observations). Our purification method requires no ammonium sulphate precipitation, and only one dialysis step to remove the imidazole that was used in the LLO elution step. The largest volume dealt with during the entire procedure was a 600 ml culture and the pellet was immediately resuspended in 30 ml sonication buffer. All of the protein was eluted in 5–7 ml from the IMAC column. An added advantage is that the 5-ml bed volume of the column was sufficient to adsorb all of the His-tagged protein and the small column was convenient to handle. The entire purification procedure took about 4 h.

The placement of the His tag at the C-terminus of LLO led to reduced levels of expression. The reason for this is not known. This is likely not due to the retention of the secretion signal on the LLO-His because we

Table 1 Comparison of methods to purify LLO

Source	Culture volume	Steps to purification[a]	Yield (mg l^{-1}) [Total yield (mg)]	Type of construct[b]	Reference
Media	27 l	1, 2, 1, 3, 1, 3, 2, 3	2.22×10^{-2} [6.0×10^{-1}]	N, F	[7]
Media	80 ml (approx)	4, 5, 6, 7	6.25×10^{-1} [5.0×10^{-2}]	N, F	[22]
Media	3 l	1, 8, 4, 7,	1.6 [4.8]	R (*L. innocua*), F	[4]
Media	10 l	4, 1, 6, 9, 1, 7, 1	1.32×10^{-2} [1.32×10^{-1}]	N, F	[29]
Media	6 l	1, 1, 7, 6, 10, 6	2.5×10^{-1} [1.5]	N, F	[30]
Cell extract	1 l	9, 11, 5, 7, 1	4.5 [4.5]	R (*E. coli*), T	[9]
Cell extract	600 ml	12	His-LLO: 3.5 – 8 [2.1 – 4.8] LLO-His: 2.5 [1.5]	His-LLO: R (*E. coli*), T LLO-His: R, F	This paper

[a] *1* ultrafiltration for concentration, *2* thiol-disulfide exchange affinity chromatography, *3* gel filtration, *4* filtration, *5* dialysis, *6* lyophilization, *7* Ion-exchange chromatography, *8* batch adsorption to Q-Sepharose *9* hydroxyapatite adsorption chromatography, *10* desalting *11* ammonium sulfate precipitation *12* immobilized metal affinity chromatography

[b] *N* Native, *R* Recombinant, *F* Full-length, *T* Truncated

Each of the former methods used to purify LLO involved multiple purification steps; most have required large volumes of culture; and most have low yields. The table highlights the ease of purification of LLO using our method and the high levels of LLO recovered from a small culture

confirmed that there was no secretion of the LLO into the media in either the LLO-His or His-LLO construct (data not shown). Furthermore, reduced expression is not likely due to size since the size difference contributed by the secretion signal is marginal.

Our findings on the lack of secretion of the LLO-His construct, which has the secretion signal sequence, agree with reports in the literature. For example, Leimeister-Wächter and Chakraborty [20] inserted full length LLO constructs into *E. coli* K12 strains. They noted the inability of the recombinant bacteria to express a hemolytic phenotype on blood agar plates, suggesting that the LLO was not being secreted or it was expressed extracellularly in non-functional form. They also demonstrated that the recombinant protein was being produced because there was hemolytic activity in the cell extracts of strains harbouring the expression plasmid [20]. Lety et al. [21] recently reported that modification of the signal sequence on LLO does not affect its secretion from *L. monocytogenes*. Although Giammarini et al. [9] removed the secretion signal on the LLO protein to maximize the retention of the LLO, they did not test the difference in expression if the sequence was present. The secretion signal may be specific to Gram positive bacteria, and is not recognized by *E. coli* expression systems.

Characterization and stability of recombinant LLO constructs

A red blood cell hemolysis assay was used to measure LLO activity. We first assessed the effect of pH since LLO is unique among the CDCs in having an optimum pH of 5.5 [7, 14]. Both His-LLO and LLO-His constructs were most active at pH 5.5 and 6.0, and less active at pH 7.0 and 8.0 (Table 2). At pH 7, activities of His-LLO and LLO-His decreased to 18 and 19%, respectively, of the activities at pH 5.5.

The response of LLO to pH seems to be dependent on the authors performing the study. Looking at only those papers that performed the assay in PBS, most researchers showed that the highest hemolytic activity occurs at pH 5.5 [7, 9, 30]. Walton et al. [30] included DTT as a reducing agent and reported a 92% decrease in hemolytic activity of LLO when pH was increased from 5.5 to 7 in the hemolysis assay. Geoffrey et al. [7] reported no hemolysis by LLO at pH 7.0, and Giammarini et al. [9] reported a reduction of RBC hemolysis by the LLO to 30% of pH 5.5 levels with little activity at pH values above 8 [9] . This latter result is similar to our results presented here.

Lety et al. [21] suggested that the removal of the secretion sequence from the LLO reduces the virulence of *L. monocytogenes*, while increasing the cytotoxicity. They postulated that the LLO produced is less sensitive to higher pH values, and is thus more active in the cytoplasm of the host cell (neutral pH) resulting in the LLO being more prone to injuring the host cell while in the cytoplasm. However, according to our results (Table 2), the presence of the secretion signal does not affect the pH dependence of LLO. The LLO-His construct, which contained the secretion signal, was no more sensitive to pH than His-LLO, which lacked the signal.

On review of the literature, the requirement for a reducing agent in the hemolysis assay is also somewhat variable. Some authors reported that the removal of the reducing agent, Cys [17] or DTT [30], from the hemolysis assay medium can result in the loss of LLO activity [17, 30]. In our study, the removal of Cys had little effect. In fact, less protein was required for 1 HU than if the assay was done at the same pH with Cys being present. Giammarini et al. [9] also showed that Cys was not required for activity of the hemolysin; none of their assays included the reducing agent. We are unsure why some constructs are less susceptible to the absence of the reducing agent. It may be related to the fact that the LLO constructs expressed in this paper and by Giammarini et al. [9] are recombinant proteins purified from *E. coli* rather than those purified *L. monocytogenes* secretions.

Table 2 Physical parameters affecting activity of LLO constructs

	pH 5.5	pH 6.0	pH 7.0	pH 8.0	No Cysteine[a]	10 μg Cholesterol[a]	50 μg Cholesterol[a]	Heat inactivated[a]
His-LLO	1.8 (0.0 ng)[b]	1 ng(0.0 ng)[b]	10.8(5.5 ng)	15.6(8.5 ng)	2.47 (0.06 ng)	225 ng – ND[c]	ND[c]	ND[c]
LLO-His	2.16 (0.75 ng)	1.40(0.0 ng)[b]	11.3 (5.8 ng)	7.7 (2.27 ng)	4.38 (3.11 ng)	ND[c]	ND[c]	

Each assay was performed three times and the standard deviation (SD) calculated
[a] Assays were performed at pH 7.0
[b] SD displayed is 0.0 because all assays at the accuracy level of these experiments had the same level of 50% hemolysis
[c] ND – not able to determine by this assay since more than 8 μg LLO was required to achieve 50% hemolysis
The data in the table express the quantity of His-LLO or LLO-His required for 50% hemolysis of bovine RBCs

Table 3 Stability studies with His-LLO and LLO-His at pH 7.0

	1 day	1 week	1 month	2 months	6 months	1 year
His-LLO	7.8 ng	ND	13.5 ng	16.8 ng	10 ng	4.05 ± 2.62 ng
LLO-His	13.8 ng	16 ng	12 ng	ND	24 ng	ND

Each assay performed three times and the standard deviation calculated if the results varied
ND not determined by hemolysis assay
Quantity of protein required for 50% hemolysis

Unlike the variability in other factors affecting the hemolysis assay, the presence of exogenous cholesterol in the assay always abrogates the activity of the LLO [7–9, 17, 29, 30]. In the host, LLO initially binds to cholesterol in the membrane before oligomerizing to form a pore. If cholesterol is present in the hemolysis assay, then this binding site is full, and the LLO cannot bind to the RBC, thus preventing lysis. In our study, addition of cholesterol readily reduced LLO activity. In the presence of 10 μg cholesterol, it took 15-fold more His-LLO to form 1 HU than in the equivalent assay in the absence of cholesterol (Table 2). With LLO-His, the amount of protein required to form 1 HU in the presence of cholesterol could not be determined under the assay conditions used because the amount would have been higher than 8 μg LLO-His. At 50 μg cholesterol, the amount of LLO required to form 1 HU could not be determined for either construct. In fact, no lysis was seen in any of the tubes, even with the highest amounts of LLO. These results agree with those reported in the literature using either native LLO or recombinant LLO [7–9, 17, 29, 30].

In terms of specific activity, the results of our LLO constructs seem to match the results found by other researchers. His-LLO and LLO-His had specific activities of 1.8 and 2.16×10^6 HU mg^{-1} respectively at pH 5.5; they had 1.0 and 1.13×10^5 HU mg^{-1} respectively at pH 7.0 (Table 2). These are similar to values reported for other LLO preparations which ranged from 2.6×10^5 HU mg^{-1} [30] to 10^6 HU mg^{-1} for both native LLO [4] and recombinant His-LLO [9].

The stability of the purified LLO constructs was tested over the course of storage for 1 year at 4°C using the hemolysis assays. When the protein was kept in PBS containing 150 mmol l^{-1} NaCl, it tended to precipitate. This problem was eliminated by increasing the NaCl concentration to 500 mmol l^{-1}. The hemolytic activity of the His-LLO remained unchanged after 1 year of storage at 4°C (Table 3), indicating that the protein is highly stable in solution given the correct storage conditions. The LLO-His construct showed similar stability over a 6 month period. To the best of our knowledge, the only other assessments of LLO stability are by Traub and Bauer [29], who showed that their purified native LLO was stable at −65°C for 6 months, and by Matar et al. [23], who showed that their LLO was stable at −20°C for 6 weeks, and at 4°C for 8 h.

In conclusion, our purification procedure offers the advantages of being a one-step process, while the recovered protein requires no concentration. Purification takes only 4 h plus desalting time. The constructs are stable since there is no loss of activity when stored for a year at 4°C. To our knowledge, this is the longest for which any purified LLO construct has been shown to be stable.

Acknowledgements Thanks to Yvan Chapdelaine of the National Research Council Canada for providing a vector containing the LLO gene. RLTC was the recipient of the Canadian Food Inspection Agency's President's Graduate Assistantship award. JCH was a recipient of the Canada Research Chair in Recombinant Antibody Technology. The authors were also funded by the Natural Sciences and Engineering Council of Canada and the Healthy Futures Program administered by the Ontario Ministry of Agriculture and Food.

References

1. Alouf JE (1999) Introduction to the family of the structurally related cholesterol-binding cytolysins (“sulfydryl-activated” toxins). In: Alouf JE, Freer JH (eds) The Comprehensive sourcebook of bacterial protein toxins. Academic, Toronto, pp 443–456
2. Archer DL (1990) *Listeria monocytogenes*: what is its ecological niche?. In: Miller AJ, Smith JL, Somkuti GA (eds) Foodborne listeriosis. Elsevier, New York, pp 5–8
3. Churchill RLT, Hall JC, Lee H (2005) Emerging bacterial foodborne pathogens and methods of detection. In: Hui YH,

Nip W-K, Leo MKN, Paliyath G, Sahlstrom S, Simpson BK (eds) Food biochemistry and food processing. Iowa State Press/ Blackwell Publishers (in press)
4. Darji A, Chakraborty T, Niebuhr K, Tsonis N, Wehland J, Weiss S (1995) Hyperexpression of listeriolysin in the non-pathogenic species *Listeria innocua* and high yield purification. J Biotechnol 43:205–212
5. Donnelly CW (2001) *Listeria monocytogenes*: a continuing challenge. Nutr Rev 59:183–194
6. Farber JM, Ross WH, Harwig J (1996) Health risk assessment of *Listeria monocytogenes* in Canada. Int J Food Microbiol 30:145–156
7. Geoffroy C, Gaillard J-L, Alouf JE, Berche P (1987) Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. Infect Immun 135:482–487
8. Geoffroy C, Gaillard J-L, Alouf JE, Berche P (1989) Production of thiol-dependent haemolysins by *Listeria monocytogenes* and related species. J Gen Microbiol 135:481–487
9. Giammarini C, Andreoni F, Amagliani G, Casiere A, Barocci S, Magnani M (2003) High-level expression of the *Listeria monocytogenes* listeriolysin O in *Escherichia coli* and preliminary characterization of the purified protein. Protein Expr Purif 28:78–85
10. Giammarini C, Andreoni F, Amagliani G, Casiere A, Barocci S, Magnani M (2004) Purification and characterization of a recombinant listeriolysin O expressed in *Escherichia coli* and possible diagnostic applications. J Biotechnol 109:13–20
11. Goebel W, Kathariou S, Kuhn M, Sokolovic Z, Kreft J, Köhler S, Funke D, Chakraborty T, Leimeister-Wächter M (1988) Hemolysin from *Listeria* - biochemistry, genetics and function. Infect 16:S149–S156
12. Gugnani HC (1999) Some emerging food and water borne pathogens. J Commun Dis 31:65–72
13. Ito Y, Kawamura I, Kohda C, Baba H, Kimoto T, Watanabe I, Nomura T, Mitsuyama M (2001) Difference in cholesterol-binding and cytolytic activities between listeriolysin O and seeligerolysin O: a possible role of alanine residue in tryptophan-rich undecapeptide. FEMS Microbiol Lett 203:185–189
14. Jacobs T, Cima-Cabal MD, Darji A, Méndez FJ, Vázquez F, Jacobs AAC, Shimada Y, Ohno-Iwashita Y, Weiss S, do los Toyos JR (1999) The conserved undecapeptide shared by thiol-activated cytolysins is involved in membrane binding. FEBS Lett 459:463–466
15. Jacobs T, Darji A, Weiss S, Chakraborty T (1999) Listeriolysin, the thiol-activated haemolysin of *Listeria monocytogenes*. In: Alouf JE (ed) The comprehensive sourcebook of bacterial protein toxins. Academic, Toronto, pp 511–521
16. Jenkins EM, Njoku-Obi AN, Adams EW (1964) Purification of the soluble hemolysins of *Listeria monocytogenes*. J Bacteriol 88:418–424
17. Jenkins EM, Watson BB (1971) Extracellular antigens from *Listeria monocytogenes* I. purification and resolution of hemolytic and lipolytic antigens from culture filtrates of *Listeria monocytogenes*. Infect Immun 3:589–594
18. Kathariou S (2002) *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J Food Prot 65:1811–1829
19. Kreft J, Funke D, Schlesinger R, Lottspeich F, Goebel W (1989) Purification and characterization of cytolysins from *Listeria monocytogenes* serovar 4b and *Listeria ivanovii*. Acta Microbiol Hung 36:189–192
20. Leimeister-Wächter M, Chakraborty T (1989) Detection of listeriolysin, the thiol-dependent hemolysin in *Listeria monocytogenes*, *Listeria ivanovii*, and *Listeria seeligeri*. Infect Immun 57:2350–2357
21. Lety M-A, Frehel C, Beretti J-L, Berche P, Charbit A (2003) Modification of the signal sequence cleavage site of listeriolysin O does not affect protein secretion but impairs the virulence of *Listeria monocytogenes*. Microbiol 149:1249–1255
22. Low JC, Davies RC, Donachie W (1992) Purification of listeriolysin O and development of an immunoassay for diagnosis of listeric infections in sheep. J Clin Microbiol 30:2705–2708
23. Matar GM, Bibb WF, Helsel L, Dewitt W, Swaminthan B (1992) Immunoaffinity purification, stabilization and comparative characterization of listeriolysin O from *Listeria monocytogenes* serotypes 1/2a and 4b. Res Microbiol 143:489–498
24. McCarthy SA (1990) *Listeria* in the environment. In: Miller AJ, Smith JL, Somkuti GA (eds) Foodborne listeriosis. Elsevier, New York, pp 25–30
25. McKellar RC (1992) Effect of reduced pH on secretion, stability and activity of *Listeria monocytogenes* listeriolysin O. J Food Safety 12:283–293
26. Meng J, Doyle MP (1997) Emerging issues in microbiological food safety. Annu Rev Nutr 17:255–275
27. Mengaud J, Vicente M-F, Chenevert J, Pereira JM, Geoffroy C, Gicquel-Sanzey B, Baquero F, Perez-Diaz J-C, Cossart P (1988) Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect Immun 56:766–772
28. Siddique IH, Fong Lin I, Chung RA (1974) Purification and characterization of hemolysin produced by *Listeria monocytogenes*. Am J Vet Res 35:289–296
29. Traub WH, Bauer D (1995) Simplified purification of *Listeria monocytogenes* listeriolysin O and preliminary application in the enzyme-linked immunosorbent assay (ELISA). Zentralbl Bakteriol 283:29–42
30. Walton CM, Wu CH, Wu GY (1999) A method for purification of listeriolysin O from a hypersecretor strain of *Listeria monocytogenes*. Protein Expr Purif 15:243–245