Food and Chemical Toxicology 50 (2012) 3886–3891

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

# Characterization of Heat-Labile toxin-subunit B from *Escherichia coli* by liquid chromatography–electrospray ionization-mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

I. Sospedra [a,*], C. De Simone [b], J.M. Soriano [a], J. Mañes [a], P. Ferranti [b], A. Ritieni [c]

[a] Department of Preventive Medicine and Public Health, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain
[b] Department of Food Science, University of Naples Federico II, Via Universitá 100, I-80005 Portici, Naples, Italy
[c] Department of Pharmaceutical Chemistry and Toxicology, University of Naples Federico II, Via D. Montesano 49, 80100, Napoli, Italy

ARTICLE INFO

*Article history:*
Received 10 July 2012
Accepted 7 August 2012
Available online 16 August 2012

*Keywords:*
*Escherichia coli*
Heat labile toxin
B subunit
Mass spectrometry

ABSTRACT

The possibilities of characterizing the heat-labile enterotoxin (LT) of enterotoxigenic *Escherichia coli* (ETEC) by liquid chromatography electrospray mass spectrometry (LC/ESI-MS) and matrix-assisted laser desorption with time-of-flight mass spectrometry (MALDI-TOF-MS) were investigated. The B subunit from recombinant *E. coli* (expression in *Pichia pastoris)* can be detected by LC/ESI-MS expressed in *P.* pastoris and the charge envelope signals can be observed; LC/ESI-MS and MALDI-TOF-MS analysis allowed the acquisition of labile toxin subunit B (LTB) molecular weight and preliminary structural characterization of LTB toxin. MALDI-TOF analysis after reduction and alkylation of the protein evidenced the presence of one disulfide bond in the structure of the protein. Confirmatory analysis was carried out by detection of most of the tryptic fragments of the B subunit by MALDI-TOF-MS, obtaining total coverage of the protein sequence. Possible biovariations in the toxin can mostly be determined by sequencing, where an increase of molecular mass in the N-terminal side of the protein was identified. This modification may be due to an O-GlcNAc-1-phosphorylation.



## 1. Introduction

*Escherichia coli* is a Gram-negative bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). *E. coli* are not always confined to the intestine, and their ability to survive for brief periods outside the body makes them an ideal indicator organism to test environmental samples for fecal contamination (Feng et al., 2002). Most *E. coli* strains are harmless, but some of them can cause gastroenteritis, urinary tract infections and neonatal meningitis (Vogt and Dippold, 2005). Food poisoning caused by *E. coli* is usually caused by eating unwashed vegetables or undercooked meat (Sheen and Hwang, 2010). Severity of the illness varies considerably; it can be lethal, particularly to young children, the elderly or the immunocompromised, but is more often mild. Toxigenic *E. coli* strains that cause infections in human and domestic animals have been classified into different categories, including enterotoxigenic *E. coli* (ETEC) (Nataro and Kaper, 1998), which is the most important pathogen of diarrhea in infants, children, and adults, accounting for 280 million episodes and more than 400,000 deaths annually and nowadays diarrheal disease remains a leading global health problem (WHO, 2005). ETEC is endemic in many developing countries and is frequently encountered by tourists, members of the military or other visitors (Coster et al., 2007; Aranda-Michel and Gianella, 1999). In addition to traveler's diarrhea, ETEC can cause disease symptoms clinically indistinguishable from cholera caused by *Vibrio cholerae* (Vicente et al., 2005). These diseases can be extremely debilitating and may be fatal in the absence of treatment (Spangler, 1992). ETEC produces two types of enterotoxins that cause diseases in man and various domestic animals: low weight, heat-stable enterotoxin (ST), and high weight, heat-labile enterotoxin (LT). Heat-stable enterotoxin (ST) is considered an important cause of diarrhea in pigs but is rarely associated with humans (Handl and Flock, 1992). LT is the major virulent factor of ETEC (Holmgren and Svennerholm, 1992). The severe losses of water and electrolytes which occur during infection appear to be caused by this toxin whose action is mediated by stimulation of adenylate cyclase activity in the epithelial cells of the small intestine. In this respect the heat-labile *E. coli* enterotoxin shows some characteristics of *V. cholerae* enterotoxin, LT is also very similar in sequence and structure to cholera toxin (CT) (Sharp et al., 1973). Both toxins consist of a B pentamer with five identical B subunits and a catalytic A subunit (Mudrak and Kuehn, 2010). LTA and LTB are composed of 236 and 103 amino acids and have molecular masses of approximately 27–28 and 11.6 kDa, respectively (Spicer and Noble,

* Corresponding author. Tel.: +34 963544958; fax: +34 963544954.
*E-mail address:* m.isabel.sospedra@uv.es (I. Sospedra).

0278-6915/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2012.08.014

1982; Dallas and Falkow, 1980). LT of enterotoxigenic *E. coli* and CT of *V. cholera* are representative of the heteromultimeric AB toxins produced by a number of bacterial pathogens; these toxins are the most effective enterocyte-targeting molecules (Williams et al., 1999). The *E. coli* heat-labile enterotoxin B subunit (LTB) and cholera toxin B subunit (CTB) were shown to function as effective carriers and adjuvants for genetically linked foreign proteins (Bagdasarian et al., 1999) and were found be immunogenic in mice (Yu and Langridge, 2001) and humans (Tacket et al., 1998). Although LTB and CTB showed only 20% difference in their nucleotide and amino acid sequences, they can be distinguished by their individual biochemical and immunological properties. Heat treatment of the toxin breaks down the pentameric LTB ring into monomers and releasess LTA. The enzymatically active A subunit is responsible for the toxicity and the B subunit binds the receptor and facilitates the entry of the A domain into cells of the intestinal epithelium, and disruption of the holotoxin thereby prevents intoxication of host cells (Mudrak and Kuehn, 2010).

Although LT and CT have many features in common, they are clearly distinct molecules with biochemical and immunologic differences which make them unique (Dickinson and Clements, 1996). In addition, LT has an unusual affinity for carbohydrate-containing matrices (Clements et al., 1980). LT binds not only to agarose in columns used for purification but also, more importantly, to other biological molecules containing galactose, including glycoproteins and lipopolysaccharides. LT is a key factor associated with the non-invasive secretory diarrhea caused by ETEC strains either in humans or domestic animals. The results of numerous investigations have shown that LTB is a promising candidate to be a vaccine antigen against LT-producing ETEC (Kim et al., 2007). For this reason it would be interesting to find a sensitive, specific, fast and simple method for the characterization of LTB. The development of an effective vaccine for ETEC would have a significant global impact on reducing morbidity due to diarrheal episodes in both developed and developing countries. Such a vaccine must be inexpensive and easy to manufacture, be able to generate long lasting protection against ETEC be easy to administer, have minimal side effects and be effective in children (Ranallo et al., 2005). To achieve this objective is necessary to generate a protective immune response against ETEC strains. *E. coli* heat-labile enterotoxin B subunit (LTB) has been regarded as one of the most powerful mucosal immunogen and mucosal adjuvant, and elicits a strong immune response to co-administered antigens (Pizza et al., 2001). The non-toxic LTB has been found to be a potent adjuvant, so an accurate characterization and knowledge about its structure and possible sequence modifications are important to design a suitable vaccine with good performance. Moreover, application of proteomics techniques can be a useful tool for high molecular biopolymers identification. It seems evident that the purification and characterization of LT is necessary to fully evaluate its role in disease and to study the antitoxic immunity, and this is expected to contribute to the development of rapid in vitro tests for the recognition of *E. coli* enteropathogens. Most of rapid in vitro tests for the recognition of *E. coli* enteropathogens available are immunoaffinity-based methods and, although they are rapid and sensitive, rely on the antibody-antigen interaction. So, if another molecule reacts with the binding site, the assay may generate a false positive or false negative signals. Where positives are observed, a confirmatory method that can make a direct measurement of the analyte is needed. The development of methods as electrospray ionization and MALDI coupled to mass spectrometry has been a great advantage for the analysis of high molecular biopolymers such as proteins. As well as these methods provides good sensitivity and specificity determination of accurate molecular masses, can be a useful instrument for advances in the identification and confirmation of *E. coli* toxins and quality control of potential vaccines.

The CTB has been isolated in a pure state and has been subjected to extensive physical and chemical characterization, whereas the LT from *E. coli* has resisted efforts aimed at purification and characterization because of its structural complexity, inclusion forms, heat-lability, and low stability (Ma et al., 2010; Amin and Hirst, 1994; Jacks et al., 1973). The methods that have been successfully used for the study of the pathophysiological effect of CTB were, therefore, applied to elucidate the biological activities of the LT itself (Evans et al., 1972).

Until now, a few mass spectrometric investigations of the B subunit of cholera toxin have been reported, and this article reports the first mass spectrometric proteomic investigation and characterization of the LTB. A study on biochemical properties of the B-chain has been performed employing liquid chromatography electrospray mass spectrometry (LC/ESI-MS) and matrix assisted laser desorption with time of flight mass spectrometry (MALDI-TOF-MS) is proposed.

## 2. Materials and methods

### 2.1. Reagents

All chemicals were of the highest purity commercially available and were used without further purification. HPLC grade $H_2O$, $CH_3CN$, MeOH, $NH_4HCO_3$, and analytical-grade formic acid (FA) were purchased from Carlo Erba (Milan, Italy). Sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (CHCA), iodoacetamide, dithiothreitol (DTT), Ethylenediaminetetraacetic acid (EDTA), guanidine, trifluoroacetic acid (TFA) and Tris–HCl were obtained from Sigma Aldrich (St Louis, MO, USA). Modified trypsin sequencing grade was from Promega (Madison, WI, USA). Centriprep® cartridges having a 3KDa cut-off membrane and ZipTip™ were obtained from Millipore (Bedford, MA, USA). Bakerbond SPE™ C18 500 mg/3 ml was purchased from J.T. Baker.

LT B subunit from *E. coli* (recombinant, expressed in *Pichia pastoris*) was purchased as lyophilized powder with a purity of >90% (SDS–PAGE), according to Sigma Aldrich manufacturer. The enterotoxin was dissolved in water to prepare a 1 mg mL$^{-1}$ stock solution, which was maintained in frozen form until used.

### 2.2. Liquid Chromatography/Electrospray Ionization–Mass Spectrometry (LC/ESI-MS)

LC/ESI-MS was carried out on a single quadrupole instrument (HP1100-MSD, Agilent Technologies, Santa Clara, CA, USA) using C18 columns (Vydac, Hesperia, CA, USA; 2.1 × 250 mm, 3 μm). The eluents were 0.1% (v/v) TFA in HPLC-grade water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B). LTB was separated at a constant flow-rate of 0.2 ml min$^{-1}$, with a gradient of solvent A in the following proportions (v/v): started at 95% A for 15 min and with a linear gradient of 95–40% A in 75 min, then changed to 100% A in 5 min and in the next 10 min arrived at the initial conditions. The LTB protein stock solution was diluted in solvent A to a final concentration of 0.5 μg mL$^{-1}$. In LC–ESI-MS analysis, external calibration was carried out performing automated instrument tuning by Chemstation software (Agilent Technologies) and using a peptide mixture in the $m/z$ range 1–2500 provided by the instrument Manufacturer (Agilent). A mass accuracy of ±0.25 $m/z$ within the calibrated mass range in scan was obtained.

### 2.3. Maldi-Tof-Ms

MALDI-TOF-MS experiments were carried out on a Voyager DEPRO mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with delay extraction technology and N2 laser at 337 nm. Mass spectra were acquired both in positive linear or in reflector mode and 10 mg mL$^{-1}$ SA and CHCA both dissolved in $H_2O$/ACN/TFA (50/50/0.1;v/v/v), were used as matrices for the analysis of proteins and peptides, respectively. MALDI-TOF analysis of intact protein was obtained in linear positive ion mode over the $m/z$ range 8000–14,400 and was averaged from about 150 laser shots. In MALDI analysis, manual external calibration for intact proteins was performed by acquiring separate spectra of a protein mixture containing Insulin (m/z 5734), *E. coli* thioredoxin ($m/z$ 11674) and myoglobin ($m/z$ 16952). For peptide analysis, peptide calibrant mixtures in the range 500–6000 $m/z$, provided by the Manufacturer (Perseptive Biosystems) were used.

For the analysis of the whole protein, 0.5 μl of LTB stock solution were loaded on the stainless steel target together with 0.5 μl of SA.

The mixtures of LTB tryptic peptides were subjected to a desalting/concentration step with Zip-Tip C18 microcolumns prior to analysis by MALDI-TOF. Spectra were obtained in reflectron positive ion mode over an $m/z$ range 400–7000 and were averaged from about 250 laser shots. Identification of the protein fragments was carried out with MASCOT software from Matrix Science (http://www.matrixscience.com).