COMMUNICATION

ChemComm
www.rsc.org/chemcomm

Probing lipase/esterase libraries for lipid A hydrolases—discovery of biocatalysts for the detoxification of bacterially-expressed recombinant protein†

Jung-Mo Ahn, Paul Wentworth, Jr.* and Kim D. Janda*

Department of Chemistry, The Scripps Research Institute and the Skaggs Institute for Chemical Biology, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA. E-mail: kdjanda@scripps.edu; Fax: +1 858 784 2590

Received (in Cambridge, UK) 10th October 2003, Accepted 19th November 2003
First published as an Advance Article on the web 19th January 2004

In our ongoing efforts to develop new methods for lipopolysaccharide (LPS) detoxification, we have screened lipase/esterase libraries for the ability to deacylate the 2′- and 3′-fatty acid chains from lipid A: the most active esterases were successfully employed to inactivate LPSs in a crude concentrated cell supernatant of *E. Coli* containing a recombinant single chain antibody (scFv).

Recombinant protein expression in Gram-negative bacteria is a standard technique that underpins much of 21st century biotechnology. An unwanted side-effect of this technology however, is contamination of the expressed protein with lipopolysaccharide (LPS). LPS, also known as endotoxin, is a major component of the outer membrane of Gram-negative bacteria.[1] LPS is a potent immunostimulant and exerts powerful effects in higher organisms.[2] Exposure to endotoxin results in many adverse effects, in the most severe cases leading to sepsis[3] which is a common syndrome, especially in hospital intensive care units, where the mortality rate is high.[4] Thus, in cases where recombinant proteins are expressed for applications *in vivo*, rigorous purification of the LPS-contaminated protein is a necessity prior to administration. Typical purification approaches include addition of endotoxin-neutralizing proteins and synthetic peptides.[5–14] Whilst such approaches have shown promise, each method has its limitations with no procedure being general. The most common approach to this problem is the use of endotoxin-selective affinity sorbents,[15] however this approach is limited because in cases where LPS complexes to the expressed protein, the extent of endotoxin removal can be highly variable.

As part of an ongoing strategy that is aimed at developing new strategies for the catalytic destruction of LPS,[16] we report the discovery of recombinant lipase/esterase enzymes that destroy lipid A and inactivate endotoxin. We further show that when these enzymes are added to *E. coli*-expressed and endotoxin-saturated protein solutions, endotoxin amounts are reduced to levels acceptable for *in vivo* administration.

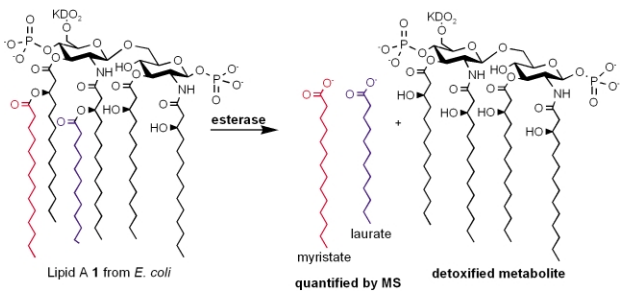
Endotoxin consists of two distinct structural features, a heteropolysaccharide chain and a lipid A moiety. Lipid A (**1**) (the *E. coli* structural form is shown in Scheme 1) is established as the main toxic determinant of LPS, stimulating host macrophages to secrete increased amounts of various cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β and interleukin-6.[17] Raetz[18] has shown that the biological effects of *E. coli* lipid A **1** require the presence of several key structural features: both phosphate groups, the glucosamine disaccharide, and all the fatty acyl chains, especially the 2′-lauroyl and 3′-myristoyl acyloxy residues. In fact, the deacylated form of lipid A, after removal of the 2′-lauroyl and 3′-myristoyl acyloxy residues, is pathologically inactive. Munford[19] has shown that within human neutrophils, the main phagocytic cell of bacterial defence, there is an enzyme termed acyloxyacyl hydrolase that catalyzes the hydrolysis of the fatty acid chains of lipopolysaccharides, leading to reduced toxicity.

We therefore envisaged that within recombinant lipase/esterase libraries, novel catalysts with acyloxyacyl hydrolase-like activity may be found (Scheme 1). To allow for high-throughput screening of the esterase/lipase library we had first to develop a routine and robust assay to monitor lipid A destruction. Traditional analytical techniques such as HPLC with UV detection were not applicable in this case due to the lack of a suitable chromophore within the lipid A molecule. Furthermore, we chose not to modify the structure of lipid A by adding a chromophoric group because of concerns that enzymes that utilize such modified lipid A structures as substrates may not have the same activity against native lipid A. We found that negative-ion electrospray MS could be used to quantify mixtures of lauric and myristic acid (over a broad range of concentrations 50–500 μM). Palmitic acid (100 μM) was used as an internal standard. Thus this in-line LC/MS assay, that allowed accurate quantification of laurate and myristate, the presumed products of enzyme-mediated lipid A hydrolysis, was our primary read-out during esterase library screening.†

A number of commercially available esterases/lipases with origins from a range of species, were screened for deacylase activity against *E. coli* serotype (O111:B4) lipid A (250 μM) in phosphate-buffered saline (PBS, pH 7.4) at 37 °C (Fig. 1). Significant oxyacyl hydrolase activity of lipid A was observed with two members of the CloneZyme Library, mutant esterases derived from thermophilic bacteria (ESL04 and ESL06). Interestingly, the MS analysis confirmed that each enzyme has different specificity in terms of the side-chain of the lipid A that it hydrolyzed. ESL04 catalyzes the specific hydrolysis of the 2′-lauroyl side chain, whereas ESL06 preferentially hydrolyzes the 3′-myristoyl chain.

These two esterases were then investigated for their ability to inactivate endotoxin derived from *E. coli* (O111:B4). Each esterase (0.1 mg mL^{-1}) was incubated with endotoxin (2000 EU mL^{-1}) in PBS (pH 7.4) at 37 °C. Endotoxin activity was determined, in a time-dependent manner, using the Limulus Amebocyte Lysate (LAL) assay (Fig. 2). While both enzymes were active in the destruction of endotoxin, ESL04 was by far the most effective having an initial rate of destruction of ~4000 EU min^{-1} mg^{-1}.

Encouraged by the rapid inactivation of endotoxin by ESL04, the purification of a crude concentrated cell supernatant of *E. coli*



Scheme 1 Theorized detoxification of lipid A **1** by hydrolase enzymes with deacylating activity. The released 2′-lauroyl and 3′-myristoyl fatty acids are quantified by an MS assay. KDO = 3-deoxy-D-*manno*-octulosonate.

† Electronic supplementary information (ESI) available: details of the LC/MS assay to determine the concentrations of lauric and myristic acid, the scFv expression and purification of proteins. See http://www.rsc.org/suppdata/cc/b3/b312662e/

DOI: 10.1039/b312662e

This journal is © The Royal Society of Chemistry 2004

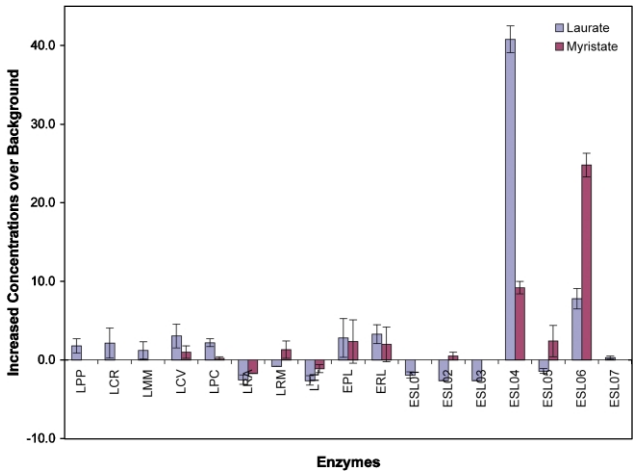


Fig. 1 Screening of lipases and esterases by the quantitative LC/MS method: lipases from porcine pancreas (LPP), *Candida rugosa* (LCR), *Mucor meihei* (LMM), *Chromobacterium viscosum* (LCV), *Pseudomonas cepacia* (LPC), *Rhizopus arrhizus* (LRA), *Rhizomucor miehei* (LRM), and *Thermomyces lanuginosus* (LTL), esterases from porcine liver (EPL) and rabbit liver (ERL), and seven esterases from CloneZyme Library (ESL01–07). The activity of these enzymes (0.75 mg mL^{-1}) was screened by mixing with a solution of lipid A from *E. coli* (0111:B4, 250 μM) in PBS (pH 7.4), and the resulting solutions were incubated at 37 °C for 72 h, before they were diluted with a solution containing palmitate (200 μM) and the concentrations of laurate and myristate determined.

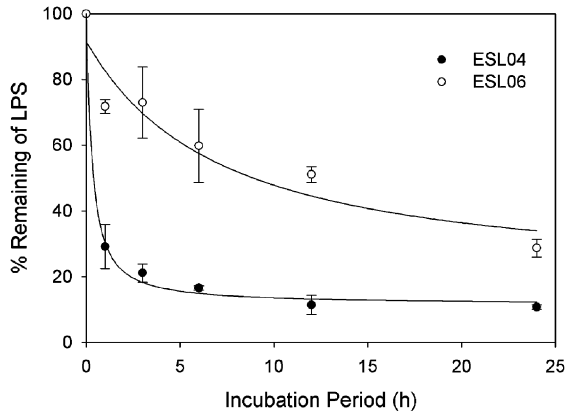


Fig. 2 Time-dependent study of the inactivation of endotoxin from *E. coli* by the esterases ESL04 and ESL06. Each point is the mean value ± SEM of at least duplicate measurements.

(O111:B4)-expressed recombinant His-tagged single-chain variable domain fragment (scFv) antibody against cocaine[20] was attempted.† ESL04 (0.1 mg mL^{-1}) was added to the concentrated cell supernatant containing scFv (~ 5–10 mg mL^{-1}) with endotoxin levels of ~ 10 200 EU mL^{-1}. After only 1 h of incubation the endotoxin levels were reduced to 440 EU mL^{-1} (clearance factor of 23). At 12 h the endotoxin levels were 150 EU mL^{-1} (clearance factor of 68). The resultant His-tagged scFv could then be routinely purified to homogeneity using a standard IMAC (immobilized metal affinity chromatography) procedure. This high clearance factor achieved by ESL04 emphasizes the potential utility of an enzymatic approach for the routine purification of protein preparations expressed in Gram-negative bacteria.

In summary, we have discovered that certain bacterial esterases can catalyze the hydrolysis of the 2′-lauroyl and 3′-myristoyl chains of lipid A. In the course of the study, we have developed a routine and robust quantitative MS method to determine the concentration of these analytes without structural modification of the Lipid A molecule. A commercially available esterase was found that destroys endotoxins from *E. Coli* and effectively purifies endotoxin-saturated bacterially-expressed antibody scFv to a level suitable for *in vivo* administration. We are now investigating the *in vivo* potential of enzymes to protect against the effects of administered lipid A. These endotoxin-inactivating enzymes are envisaged as being successfully applied in the future in the treatment of sepsis and in the purification of therapeutically important proteins contaminated with endotoxin.

Notes and references

1 C. R. H. Raetz, *Annu. Rev. Biochem.*, 1990, **59**, 129.
2 E. T. Rietschel, H. Brade, O. Holst, L. Brade, S. Mueller-Loennies, U. Mamat, U. Zaehringer, F. Beckmann, U. Seydel, K. Brandenburg, A. J. Ulmer, T. Mattern, H. Heine, J. Schletter, H. Loppnow, U. Schonbeck, H. D. Flad, S. Hauschildt, U. F. Schade, F. di Padova, S. Kusumoto and R. R. Schumann, *Curr. Top. Microbiol. Immunol.*, 1996, **216**, 39.
3 G. D. Martich, A. J. Boujoukos and A. F. Suffredini, *Immunobiol.*, 1993, **187**, 403.
4 J. L. Vincent, *Curr. Top. Microbiol. Immunol.*, 1996, **216**, 1.
5 C. M. Galanos, M. A. Freudenberg, F. Jay, D. Nerkar, K. Veleva, H. Brade and W. Strittmatter, *Rev. Infect. Dis.*, 1984, **6**, 546.
6 E. J. Ziegler, C. J. Fisher, Jr., C. L. Sprung, R. C. Straube, J. C. Sadoff, G. E. Foulke, C. H. Wortel, M. P. Fink, R. P. Dellinger, N. N. H. Teng, I. E. Allen, H. J. Berger, G. L. Knatterud, A. F. LoBuglio and C. R. Smith, *N. Engl. J. Med.*, 1991, **324**, 429.
7 B. Beutler, I. W. Milsark and A. C. Cerami, *Science*, 1985, **229**, 869.
8 J. C. Mathison, E. Wolfson and R. J. Ulevitch, *J. Clin. Invest.*, 1988, **81**, 1925.
9 P. Elsbach, *J. Leukocyte Biol.*, 1998, **64**, 14.
10 M. N. Marra, C. G. Wilde, J. E. Griffith, J. L. Snable and R. W. Scott, *J. Immunol.*, 1990, **144**, 662.
11 T. Morita, S. Ohtsubo, T. Nakamura, S. Tanaka, S. Iwanaga, K. Ohashi and M. Niwa, *J. Biochem.*, 1985, **97**, 1611.
12 R. I. Roth, D. Su, A. H. Child, N. R. Wainwright and J. Levin, *J. Infect. Dis.*, 1998, **177**, 388.
13 A. Rustici, M. Velucchi, R. Faggioni, M. Sironi, P. Ghezzi, S. Quataert, B. Green and M. Porro, *Science*, 1993, **259**, 361.
14 S. Dankesreiter, A. Hoess, J. Schneider-Mergener, H. Wagner and T. Miethke, *J. Immunol.*, 2000, **164**, 4804.
15 F. B. Anspach, *J. Biochem. Biophys. Methods*, 2001, **49**, 665.
16 L. H. Jones, L. J. Altobell III, M. T. MacDonald, N. A. Boyle, P. Wentworth, Jr., R. A. Lerner and K. D. Janda, *Angew. Chem., Int. Ed.*, 2002, **41**, 4241.
17 N. Quereshi and K. Takayama, in *The Bacteria*, eds. B. H. Iglewski and V. L. Clark, Academic Press, New York, 1990, Vol. 11, p. 319.
18 C. R. H. Raetz, in *Escherichia coli and Salmonella: Cellular and Molecular Biology, vol. 1*, ed. F. C. Neidhart, American Society of Microbiology, Washington DC, 1996, p. 1035.
19 R. S. Munford and C. L. Hall, *Science*, 1986, **234**, 203.
20 M. R. A. Carrera, J. A. Ashley, P. Wirsching, G. F. Koob and K. D. Janda, *Proc. Nat. Acad. Sci. U.S.A.*, 2001, **98**, 1988.