Purification and amino acid sequencing of naturally occurring N-formyl-methionyl oligopeptides from *Escherichia coli*

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Summary. A novel purification procedure for N-formyl methionyl oligopeptides from bacterial culture supernatants has been developed. C-terminal carboxypeptidase microsequencing of purified peptides from *E. coli* supernatants enabled identification of 6 naturally occurring formyl-oligopeptides.
Key words. Formyl peptides; microsequencing; *E. coli.*

Synthetic hydrophobic N-formylated oligopeptides induce neutrophil leucocyte chemotactic, metabolic and secretory activities via a specific formyl-peptide receptor. The peptide f-met-leu-phe (FMLP) was found to possess the greatest activity among a group of synthetic formyl oligopeptides, with an ED_{50} for chemotaxis of 7×10^{-11} M and for lysosomal enzyme secretion of 2.6×10^{-10} M [1]. Marasco et al.[2] demonstrated that FMLP was a naturally occurring peptide in cell free supernatants of *E. coli* cultures. In total, Marasco identified 5 distinct formyl methionyl peptides, though the structures of the remaining 4 compounds were not elucidated.

Chadwick et al.[3] demonstrated that bioactive hydrophobic factors were present in the culture filtrates of several species of intestinal bacteria and in intestinal fluids obtained by in vivo rectal dialysis. The bioactivity was highly susceptible to exhaustive carboxypeptidase digestion but relatively unaffected by aminopeptidase digestion. These results suggested that secretion of N-terminally blocked peptides into culture supernatants may be a ubiquitous phenomenon amongst such bacteria and that the intestinal lumen was a potential source of these potent inflammatory peptides.

Formyl methionyl peptides probably arise from amino-terminal processing of nascent bacterial proteins[4]. Structural analysis of secreted formyl peptides is difficult because of the low concentrations of these factors present in culture filtrates and previously[2] very large volumes of culture media were used. In this paper we report a novel purification procedure for bioactive N-formyl peptides from 7 l of *E. coli* culture filtrate.

Materials and methods

Purification of bioactive peptides. E. coli (strain K12) was cultured overnight in 7 l of minimal medium to a density of 10^{10} cells/ml. The supernatants were clarified by centrifugation at 8,000 × g and filtered through 0.45 µm and 0.22 µm Millipore filters. The medium was then acidified to pH 2.8 with HCl and pumped (300 ml/h) through a 4 cm × 4.5 mm (internal dimension) C18 lichroprep (Merck) column for removal of hydrophobic material. The material which eluted with acetonitrile was freeze dried, redissolved in 0.1 M ammonium acetate (pH 5) and chromatographed by gel filtration on a Sephadex G15 (30 cm × 1 cm flow rate 6 ml/h). The column was precalibrated using blue dextran, f-met-leu-phe and tryptophan. Fractions containing peptides were bioassayed and then passed through a C18 Seppak (Waters), for desalting. The acetonitrile elute was lyophilized, redissolved in 2.0 ml of 0.01 % TFA and then fractionated by reverse-phase HPLC using a previously described method[3]. A portion of each fraction was assayed for bioactivity measuring beta-glucuronidase release by human neutrophils[1].

Sequencing of bioactive factors. To remove contaminating non N-terminal blocked peptides each fraction was digested overnight (37 °C) with 100 µl of amino-peptidase (0.5 U Boehringer Mannheim). The samples were rechromatographed individually by HPLC reverse phase (see above). The 2-ml fractions were freeze dried and reassayed for bioactivity. Bioactive fractions were pooled and the sample dissolved in a minimal volume of 0.05 M phosphate buffer, pH 6.1. Sequence analysis was generally performed as follows; 3.3 µl of carboxypeptidase Y solution (Boehringer Mannheim) was added to 145 µl of the fraction. The amount of enzyme added was optimised using synthetic f-met-leu-phe and was sufficient to generate equimolar release of Phe and Leu from 100 pmols substrate, over a 60-min period. The incubations were performed at 28 °C. At various time intervals after the addition of carboxypeptidase, 25-µl aliquots of the incubation mixture were removed and placed into 15 µl of 0.81 M potassium borate buffer pH 10.6 at 90 °C (5 min) to inactivate the enzyme. Amino acid products were analysed using o-phthaldialdehyde (OPA) derivatisation and reverse phase chromatography[5].

Determination N-terminal formylmethionine. Determination of formylmethionine by OPA derivatisation and amino acid analysis was not possible because of the N-terminal acyl group. The presence of formylmethionine in the fractions was verified after generating the samples for sequence analysis. A portion of the 60-min carboxypeptidase digest was acidified with 0.1 N HCl and then extracted 3 times with an equal volume of ethyl acetate. The pooled organic phases were lyophilised, and taken up in a small volume (50 µl) of 50 mM phosphate buffer, pH 7.5 containing 10 mM sodium azide. Half of

this material was subjected to overnight digestion using a formylmethionine deformylase[6]. The background methionine was determined in the remaining portion of the ethyl acetate extract.

Results

Multiple peaks of bioactivity (lysosomal enzyme release from human neutrophil leucocytes) were observed throughout the molecular weight range (200–1500) (fig. 1).

Figure 2 shows the bioactivity profile of fractions obtained after C18 reverse phase chromatography. Prior to sequencing each fraction was exhaustively digested with amino-peptidase and then re-chromatographed individually. Figure 2 illustrates the bioactivity assay of fraction 25 after such a procedure.

C-terminal sequence analysis with carboxypeptidase Y demonstrated successive release of amino acids to equimolar levels in fractions 22, 25, 26, 28, 29, 30 and 36. The carboxypeptidase digestion profile of peptides in fractions 25, 28, 30 and 36 are shown in figure 3a, b, c and d respectively. Two of these fractions contained two peptides, the sequences being identified by equimolar plateau of component amino acids at two different levels. In other fractions, no successive release of amino acids was observed indicating that the concentrations of bioactive peptides in these fractions were too low or that the factors were not peptides. The structures identified and their respective fractions are listed in the table.

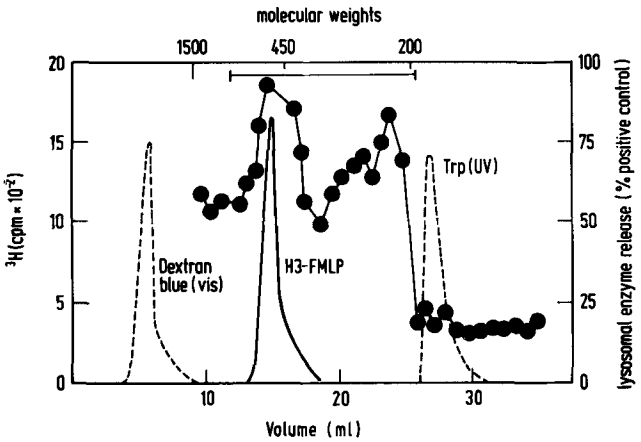

Figure 1. Gel filtration chromatography of *E. coli* bioactive factors. Hydrophobic factors concentrated from *E. coli* culture supernatant were fractionated using Sephadex G15. The column was previously calibrated using blue dextran, [^{3}H] FMLP, and tryptophan. After chromatography of the *E. coli* extract, the fractions indicated (see bars) were assayed for bioactivity measuring beta-glucuronidase release by human neutrophils[1] and pooled for further analysis.

Amino acid sequences of 6 bioactive peptides from *E. coli* culture supernatants

Sequence obtained	HPLC number
f-met-ser-leu	22
f-met-leu-phe	25–26
f-met-met-ile-ala	28
f-met-phe-leu	28–30
f-met-gly-met-ile	30
f-met-val-phe-ile-leu-leu	36

Sequencing was performed by carboxypeptidase Y digestion of purified bioactive peptides. N-terminal formyl methionine confirmed after deformylase digestion of completed carboxypeptidase digest.

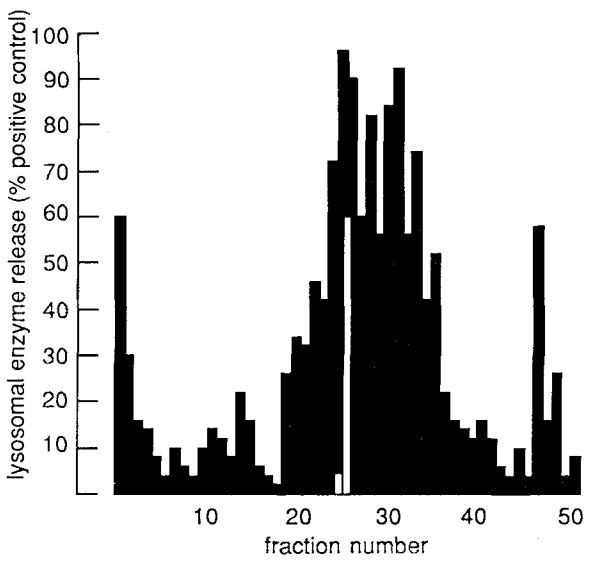

Figure 2. Reverse phase chromatography of *E. coli* bioactive factors. *E. coli* peptides were rechromatographed by reverse phase HPLC. The fractions were assayed for bioactivity (shaded region). Prior to sequencing, each fraction was digested by aminopeptidase and rechromatographed to recover N-terminally blocked bioactive peptides. The rechromatography of fraction 25 after digestion is shown (unshaded region).

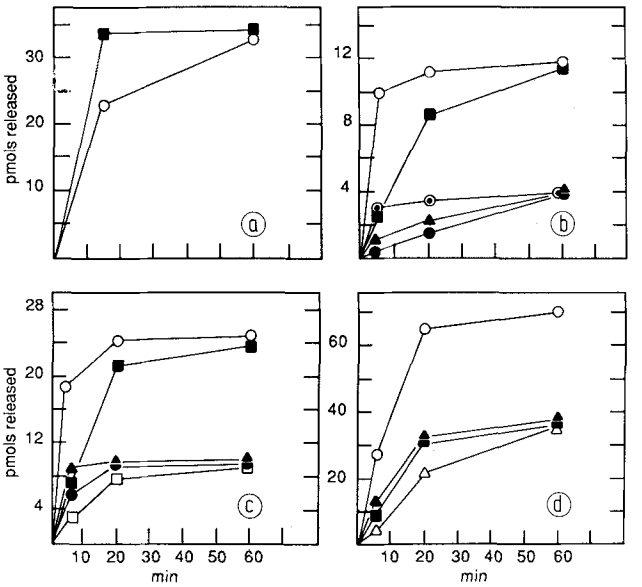

Figure 3. Generation of sequential amino acids by carboxypeptidase Y microsequencing of purified bioactive peptides from *E.coli*. The HPLC fractions demonstrated are (a) 25; (b) 28; (c) 30; (d) 36. Individual amino acids are represented as follows: ■, Phe; ○, Leu; ▲, Ile; ●, Met; □, Gly; △, Val; ⊙, Ala.

Discussion

FMLP has previously been detected in culture filtrates of *E. coli*[3]. In addition, these authors detected 4 other formyl peptides although the structures were not determined. We have confirmed the presence of FMLP in *E. coli* culture fluid (fig. 3a). In addition to FMLP, two other tripeptides (f-met-ser-leu, f-met-phe-leu), two tetrapeptides (f-met-met-ile-ala, f-met-gly-met-ile) and a hexapeptide (f-met-val-phe-ile-leu-leu) were demonstrated (table).

The structures presented may represent only some among many formyl peptides secreted by *E. coli*. Some fractions which elicited lysosomal enzyme release by human leucocytes did not generate sequential amino acid release on carboxypeptidase Y digestion. The concentrations of these putative peptides may be insufficient to yield detectable amino acids by OPA derivatisation. Alternatively, peptides containing amino acids which are not detectable by OPA derivatisation (e.g. cysteine and proline) may trigger the PMN response. However the structure/function specificity of the formyl peptide receptor suggest that this is probably not so[1].

There is accumulating evidence suggesting that low molecular weight pro-inflammatory N-formyl-methionyl oligopeptides could play a role in intestinal inflammatory disorders. Intestinal bacteria produce such peptides in vitro and bioactive peptides have been demonstrated in colonic fluid obtained by in vivo dialysis techniques[3]. In experimental animals both colonic infusion and rectal administration of millimolar concentrations of FMLP resulted in experimental colitis[7,8]. While healthy rat colon shows only very limited permeability to luminal formyl-peptides, experimental damage to the colonic mucosal barrier markedly increases peptide absorption[9]. Absorbed formyl-peptide is excreted by the liver into bile undergoing entero-hepatic circulation, providing a pathophysiological basis for the association of hepatobiliary and intestinal inflammation[9,10]. Determining the structures and properties of naturally occurring N-formyl oligopeptides is one aspect of defining their possible role in inflammatory disorders of the gut and extra-intestinal sites.

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In vivo degradation of noradrenaline by MAO A in locus coeruleus of the rat

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Summary. Depletion of noradrenaline in locus coeruleus neurons after reserpinization was prevented by clorgyline, a selective inhibitor of MAO A, but not by deprenyl, a selective inhibitor of MAO B. Only MAO A is therefore responsible for the degradation of homoneuronal noradrenaline in locus coeruleus nerve cells.
Key words. MAO A; MAO B; locus coeruleus; rat; noradrenaline histofluorescence; reserpine; deprenyl; clorgyline.

Different forms of MAO (monoamine oxidase – monoamine: oxygen oxidoreductase; EC 1.4.3.4) are present in the central nervous system. They are classified as mitochondrial enzymes MAO A and MAO B and the cytosolic MAO. The latter differs from MAO forms A and B by its resistance to inhibition by clorgyline and deprenyl[1–3]. In the present investigation, special attention was paid to the possible functional role of the above-mentioned forms of MAO in the noradrenergic neurons of the locus coeruleus (LC). The LC differs from other nuclei of the rat brain by showing the highest activity of MAO[4]. Intensively stained MAO-positive neurons were shown in the LC by an improved histochemical method[5]. Previous reports on the localization of molecular forms of MAO (A and B) in the LC were based on histochemical, immunohistochemical and microgasometric methods. Histochemical staining demonstrated the presence of MAO A in neurons and MAO B in the nerve endings