FLUOROMETRIC AND ELECTROCHEMICAL fMLP HPLC ANALYSIS

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Abstract

Formyl-methionyl peptides are potent chemotactic and inflammatory agents produced by bacteria. Formyl-methionyl-leucyl-phenylalanine (fMLP) is the best characterized of these chemotactic peptides. It is the most abundant of the formyl peptides produced by *E.coli.*

The present paper describes a HPLC fluorometric and electrochemical analytical method. The site of both chemical and electrochemical oxidation was determined to be the thiol in methionine. Substitution or alteration of the amino acids around leucine enhanced fMLP's fluorescent properties. This method, when used in conjunction with HPLC-UV detection, can indicate the presence of compounds that interfere with the analysis of fMLP from environmental samples. All environmental samples extracted and chromatogramed for oxidized and reduced fMLP were found to contain substances that interfered with quantitation.

Introduction

N-formylmethionyl peptides are involved in bacterial protein synthesis. The major n-formyl peptide produced by E-coli is n-formylmethionyl-leucine-phenylalanine (fMLP, 1). These peptides are potent biological agonists that are chemotactic , cause cell activation (2), mediate airway smooth muscle contraction and induce arachidonic acid metabolism (3, 4). The methionine has been reported to be subject to oxidation by granulocyte myleoperoxidases (5). The product of this oxidization is thought to be methionine sulfoxide. Oxidation of fMLP attenuates its biological activity (6).

It has been suggested by Burrel R., and Rylander R. (7, 8) that fMLP may be involved in organic dust induced pulmonary diseases (ODTS). We, and others, have reported the presence of both fMLP and oxidized fMLP (ox-fMLP) in bulk environmental samples using reverse phase high performance liquid chromatographic - ultraviolet (HPLC-UV) analysis (9, 10). Environmental agricultural dust that have been associated with respiratory diseases are complex mixtures containing bacteria, fungi and their products. The contribution of formyl-peptides to the pathological sequelae caused by these organic dust exposures is unknown. Environmental analytical techniques are needed to explore such a relationship.

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The present paper describes a new fluorometric (FLUOR) and electrochemical (EC) HPLC technique for the analysis of fMLP . This technique, when used in parallel with a UV detector can indicate the presence of co-eluting substances that may interfere with environmental sample analysis. A variety of environmental extracts, evaluated by this method, had both fMLP and ox-fMLP co-eluting interferences that preclude their quantitation by simple HPLC-UV methods.

Materials and Methods

All peptides were purchased from Sigma Chemical (St. Louis, MO.). Chromatographic solvents were obtained from Fisher Scientific (Pittsburgh, PA.). The HPLC system consisted of a Schimadzo Scientific Instruments, Inc. (Columbia, MD.). LPI-6B interface module controller, SIL-6B auto injector, 2 LC-600 pumps, SPD-6AV UV-viable spectrophotometric detector, RF-551 spectrofluorometer and a Coulochem II electrochemical detector (ESA, Bedford, MA).

Chromatography

First Method: Samples and standards were dissolved in methanol and injected (10μl) onto a 250 x 4.6mm, 5µm, C18 column (Whatman Inc., Clifton, NJ) and eluted off at 1 ml/min with a 40/60 acetonitrile/NaH_2 PO_4, pH 2.7 mobile phase. The UV detector was set at 195nm. Fluorescence was measured with excitation/emission = 200nm/280nm. The EC detector contained two cells. The first cell was set at 600mV and the second (detection) cell was at 850mV.

Second Method: One hundred seventy five microliter of samples or fMLP standards (in methanol) were derivitized with 100 µg/ml 1-pyrenyldiazomethane (PDAM, Molecular Probes, Eugene, OR) for 1hr at 45ºC, and then filtered through a 0.45µm filter. The anilities were eluted from the previously mentioned column using a gradient starting at 40%/60% 0.05M H_3PO_4, pH 3.7/acetonitrile (ACN). The ACN concentration was increased linearly to 65% over 20 minutes and then to 70% by 70 minutes. PDAM binds to carboxylic acid groups in fMLP and produces a strong fluorescence at λ excitation/emission = 340nm/395nm. The PDAM-fMLP was also measured using a UV detector set at 210nm, and the EC detector with the previously stated settings.

Sample and Standard Preparation

fMLP was oxidized by incubation in 0.75% H_2O_2/methanol (25mg/4ml) for 12 hr. at room temperature. The solvent was removed under nitrogen and the ox-fMLP stored at -20ºC until use. Standards were kept dry until the day of analysis at which time they were reconstituted in methanol. Samples were extracted with methanol (100mg/ml) for 4 hr. at room temperature. The extracts were then filtered through a 0.22µm Millex-GV filter (Millipore, Bedford, MA), evaporated to dryness under nitrogen and stored at -

20°C until analysis. Samples were reconstituted in methanol immediately prior to analysis.

Results

Optimum detector conditions were determined using fMLP. Figure 1 is a plot of the potential applied across the EC detector cell vs the response as measured by area under the curve (AUC) of the resultant peak. A potential of 600mV was used for the initial (interference) cell as fMLP was not oxidized at this voltage. Eight hundred fifty millivolt was used as the optimum detector potential. Maximal fluorescent wavelengths were determined using HPLC stop flow spectral analysis.

The oxidation kinetics of fMLP was examined (Figure 2). Reduced fMLP could be detected in the oxidation reaction mixture for up to 10 hrs. The ox-fMLP was found to be stable for the entire 14.5 hrs. All subsequent oxidation reactions were run for 12 hrs. for convenience and to ensure complete oxidation of the standards.

Several peptides were examined to evaluate the effect of specific amino acid substitutions and alteration of the formyl group on oxidation by H_2O_2, fluorescence and electrochemical activity (Table 1). These peptides included both oxidized and reduced methionyl-leucyl-phenylalanine (MLP), formyl-methionyl-leucyl-tyrosine (fMLT), acetyl-methionyl-leucyl-phenylalanine (aMLP) and formyl n-leucyl-leucyl-phenylalanine (fNLP). All fMLP analogues tested could be separated by the chromatographic technique described in the methods section (figure 3a&b). Formyl n-leucyl-LP (fNLP) was not subject to oxidation and was not EC active suggesting that the methionine residue was the site of electrochemical and H2O2 oxidation. Substituting tyrosine (fMLT) for the phenylalanine enhanced the peptides EC activity. Ox-fMLT EC activity was reduced by the amount of EC activity found in fMLP. This suggests that the EC activity of hydroxyl group found on the tyrosine was additive to that produced by the methionine. Chemical or EC oxidation of the methionyl group enhanced fluorescence. Amino acid substitution around the leucine also enhanced the fluorescence. The formyl group had little effect on either EC or FLUOR activity.

All detector responses for fMLP were linear in the range tested (Figures 4a & b). The fluorescent properties of fMLP were relatively weak. Oxidation of fMLP caused an enhancement of it's fluorescence (Figure 4b). Loss of EC activity was noted upon oxidation of fMLP. Our lowest standard used was 6.25µg/ml (62.5ng injected).

Several environmental samples that had bacterial contamination were extracted and chromatogramed (Table 2). All extracts produced peaks that eluted at the same point as ox-fMLP. Only one of the paper dust extracts had a fMLP co-eluting peak. The range of the ratios of absorbance to EC and FLUOR are reported in Table 2 as well as the predicted ratios based on fMLP and ox-fMLP standards. Hay, sorghum and saw mill dust ox-fMLP co-eluting peaks all had EC activity. The paper dusts' ox-fMLP peaks did not have EC activity but their fluorescence was more than 2X predicted. The paper dusts' co-eluting fMLP peaks had both very high EC and FLOUR activity (>300X and >100X predicted, respectively). This data suggest that all of the environmental co-eluting fMLP and ox-fMLP peaks contained interfering substances.

Conjugation efficiency with pdam was 43% using cotton dust sample with a known amount of fMLP added. Extraction efficiency from these samples was greater than 95%. No fMLP peak was found from cotton dust samples after extraction, conjugation with pdam and HPLC analysis (data not shown).

Discussion and Conclusion

HPLC-UV analysis of fMLP and ox-fMLP is a useful technique to monitor aerosol exposures in animal studies (11) and to examine the oxidation of fMLP in cellular and cell free systems. We and others have reported the presence of fMLP and ox-fMLP co-eluting peaks from a variety of environmental samples including cotton dust and sorghum (9, 10). The presence of confounding interferences in these analyses had not been evaluated in the past. The selectivity of the chromatographic methods employed lay almost entirely in the separation by reverse phase columns. Electrochemical and fluorescent properties of fMLP and ox-fMLP were explored in the present paper as a tool to evaluate the authenticity to quantify these peptides from environmental dust extracts.

Both the oxidized and reduced forms of fMLP, when excited at λ=200nm, fluoresced at λ = 280nm. This fluorescence was relatively weak, providing no appreciable increase in sensitivity. Only the reduced form of fMLP was electrochemically active. Our data from analysis of fMLP analogues confirms that the site of both chemical and electrochemical oxidation is the methionine. Any manipulation of amino acids around the leucine also seemed to enhance the peptides fluorescence.

The new method using the HPLC with UV/EC/FLUOR detection described herein was used to examine several environmental samples. Both EC and FLUOR are more selective than UV. The authenticity of the fMLP HPLC peaks, when all three detectors are used concomitantly, can be evaluated. The ratios of EC/UV and FLUOR/UV from both samples and standards were compared and indicated the presence of co-eluting interfering substances in all the environmental bulk extracts tested by both methods mentioned earlier.

It is reasonable to expect fMLP in dust contaminated with bacteria such as *E. coli*, because fMLP is cleaved during post-transcriptional processing. The present study;

however, suggest that HPLC, even when coupled with EC and FLUOR detectors, may not be able to accurately measure the quantity of fMLP in environmental dust.

References

Marasco, W.A., Phan, S.H., Krutzch, J., Showell, H.J., Feltner, D.E., Nairn, R., Becker, E.L., and Ward, P.A. (1984). J. Biol. Chem. 259: 5430-5439.

Korchak, H.M., Wilkenfeld, C., Rich, A.M., Radin, A.R., Vienne, K., and Rutherford, L.E. (1984). J. Biol. Chem. 259: 7439-7445.

Shore SA, Stimler-Gerard NP, Smith E, and Drazen JM. (1987). J. Appl. Physiol. 63:2450-2459.

Souhrada M., Showell HJ, and Souhrada JF. (1987). Am. Rev. Resp. Dis. 135:557-561.

Tsan Min-Fu, and Denison RC. (1981). J. of Immunology. 126, 4, 1387-1389.

Dyke KV, Castranova V., Van Dyke CJ, Ma JHK, Michaux K., Mollison KW., and Carter, GW. (1984). Inflammation 8: 87-99.

Burrell R., Rylander R., (1983). Proc. Seventh Beltwide Cotton Dust Res. Conf., National Cotton Council, Memphis. pp. 34-35.

Burrell R., Rylander R., (1985). J. Toxicol. Env. Hlth. 16: 607-613.

Fedan J.S., Castranova V., Ma J.H.K., Mo C.G., and Frazer D.G.(1990).Cotton Dust- Proc. 14th Cotton Dust Res. Conf. 105-106.

Siegel P.D., Ronk E.A., Clark P.R., Shahan T.A., and Castranova V. (1994). J. Toxicol. Env. Hlth. 42: 275-288.

Frazer DG, Jones W, Siegel PD, Barger MW, Masters B, Chandler C, Vincent D, Robinson VA,and Castranova V. (1992). Proc. 16th Cotton Dust Res. Conf., LN Domelsmith, RR Jacobs, PJ Wakelyn (eds), Memphis, TN, pp 266-270.

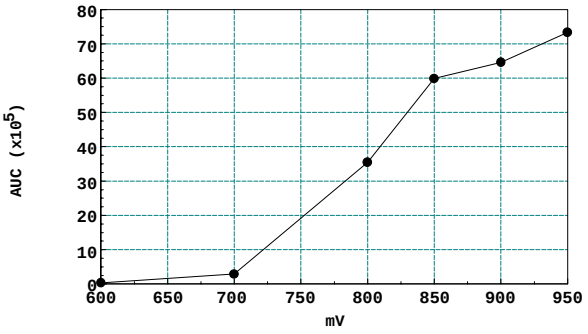


Figure 1. fMLP electrochemical potential-response curve. Mobile phase = 40/60 acetonitrile/0.1M NaH_2PO_4, pH 2.7.

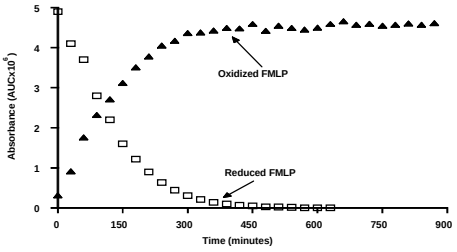
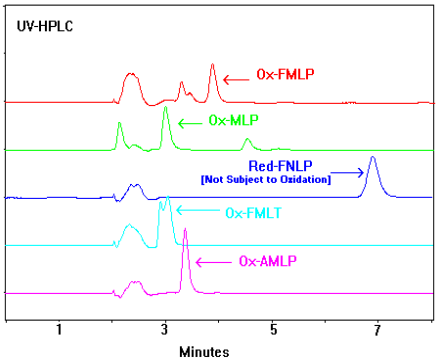


Figure 2. Oxidation time course of fMLP. Twenty five milligrams fMLP were inclubated at room temperature in 4mls of 0.75% H202/MeOH

Table 1 fMLP structure-fluorescence/electrochemical analysis

	K'[1]	ABSORB-ANCE[2]	FLUORE-SCENCE[2]	ELECTRO-CHEMICAL[2]
Red FMLP	1.62	0.94	0.18	4.47
Red MLP	1.05	0.83	0.16	9.58
Red FNLP	2.38	1.37	2.20	NEA[4]
Red FMLT	0.76	1.07	1.6	40.67
Re AMLP	1.71	0.96	0.27	3.39
Ox. FMLP	0.86	0.68	1.93	NEA
Ox. MLP	1.19	0.21	1.83	NEA
Ox. FNLP[3]	-------	--------	--------	--------
Ox. FMLT	0.48	1.54	2.08	33.44
Ox. AMLP	0.67	0.97	1.76	NEA

[1]K' = Capacity Factor
[2]Area Under the Curve x 10^6
[3]FNLP not subject to oxidation.
[4]NEA = Not Electrochemically Active



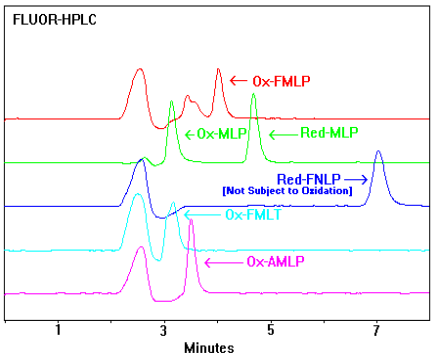


Figure 3a
Chromatographic Tracing of Oxidized Peptides

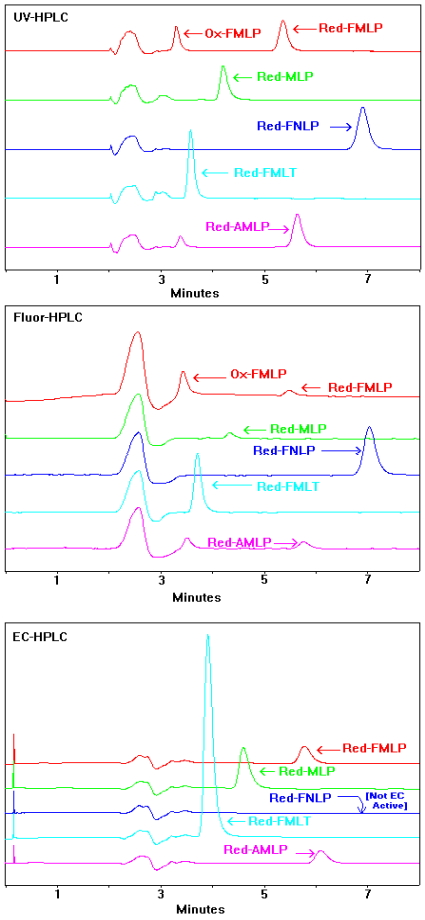
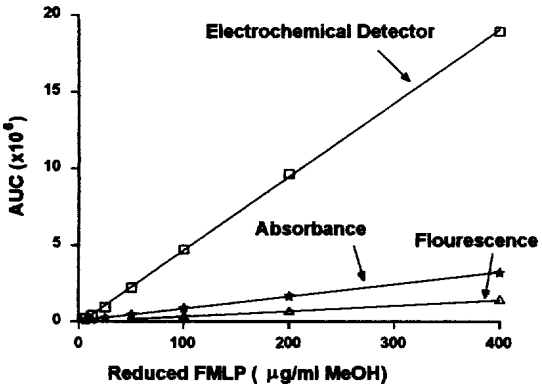


Figure 3b
Chromatographic Tracing of reduced Peptides

(A)



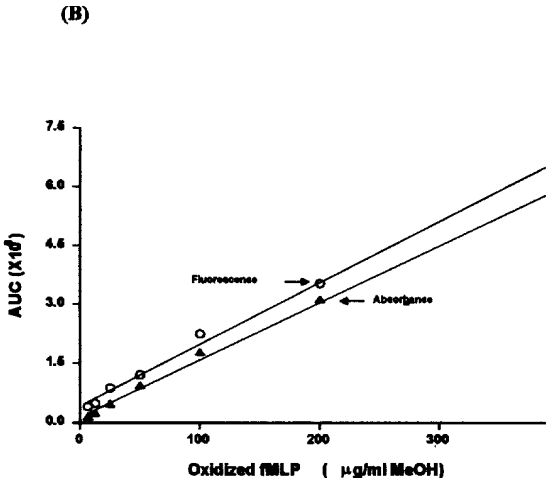


Figure 4a&b Typical standard curves for (a) fMLP and (b) Ox -fMLP generated using HPLC-EC, Flour and UV detectors. All standard curves were linear with $r \geq 0.997$.

TABLE 2. Chromatographic Evaluation of Environmental Samples

SAMPLE	n	FMLP Coeluting peaks	EC/ABS (RATIO)	FLUOR/ABS (RATIO)
Standard FMLP	7	Reduced	4.98	0.38
Standard Oxidized FMLP	7	Oxidized	0	2.2
Paper Dust	2	Reduced	125-128	22.8-26.2
Paper Dust	2	Oxidized	0	5.0-5.5
Hay	1	Oxidized	32-34	2.9
Sorghum:				
Red Milo	6	Oxidized	35.6-70	0
White Milo	4	Oxidized	41-60	0-2.3
Saw Mill Dust	5	Oxidized	8.9-32.2	0.5-5.3

* Number's represent range of measured ratio's.
EC: first cell = 600, second (detection) cell = 850.
Fluorescence: $\lambda_{\text{excitation/emission}} = 200/280$.