RAPID COMMUNICATIONS IN MASS SPECTROMETRY, VOL. **11**, 1749–1752 (1997)

# Assessing Enzyme Substrate Specificity Using Combinatorial Libraries and Electrospray Ionization-Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

**Maria Wigger[1,2], Joseph P. Nawrocki[1], Clifford H. Watson[1], John R. Eyler[1]* and Steven A. Benner[1]**

[1] Department of Chemistry, P.O. Box 117200, University of Florida, Gainesville, FL 32611-7200, USA
[2] Department of Chemistry, Swiss Federal Institute of Technology, CH-8092 Zurich, Switzerland

SPONSOR REFEREE: J. Amster, Department of Chemistry, University of Georgia, Athens, GA 30602-2556, USA

**A model experiment for the 'on-line' screening of substrate libraries by enzymes using combinatorial libraries in combination with electrospray ionization-Fourier transform ion cyclotron resonance (ESI-FTICR) mass spectrometry has been performed. The reaction between the electrophilic substrate 1-chloro-2,4-dinitrobenzene and components of a H-γ-Glu-Cys-Xxx-OH library, catalyzed by glutathione-S-transferase, has been monitored. It shows the feasibility of 'two-dimensional' screening of substrate libraries by ESI-FTICR mass spectrometry. © 1997 John Wiley & Sons, Ltd.**

*Received 2 September 1997; Accepted 2 September 1997*
*Rapid. Commun. Mass Spectrom.* **11**, 1749–1752 (1997)
No. of Figures: 3 No. of Tables: 0 No. of Refs: 21

Determining the substrate specificity of enzymes in biological systems is a major challenge in biological research, important in biotechnology, the design of drugs, and the assessment of physiological roles in biological systems.[1] Emerging combinatorial technology offers a powerful way to address this challenge. Although peptide,[2] oligonucleotide[3] and various small molecule[4–7] libraries have been successfully used to explore receptor–ligand interactions,[8,9] very few studies have been performed where components of libraries are enzymatically transformed as part of the combinatorial experiment.[10] This situation challenges the field of analytical chemistry to develop instrumentation for the high throughput screening of complex libraries. In response to these challenges, applications for sophisticated mass spectrometric instrumentation have been established that allow rapid, sensitive and high throughput analysis of complex libraries.[11,12]

Glutathione-S-transferases (GST) are intracellular, heterodimeric enzymes that play a crucial role in the detoxification of alkylating agents in the cell.[13,14] These enzymes catalyze the nucleophilic attack of glutathione(γ-glutamyl-cysteinyl-glycine) on a wide variety of hydrophobic electrophiles, including drugs and environmental pollutants, yielding conjugated or transformed metabolites that are less toxic and more easily excretable. Several electrophiles are used in *in vitro* assays as arbitrary electrophilic substrates to determine the activity of GSTs.[15]

Consistent with their physiological role, the structures of the electrophiles that GST accepts are quite diverse. Nevertheless, different isozymes of GST exist with different specificities for the electrophile. This is a common strategy in evolving a spectrum of detoxifying enzymes in higher organisms. Mammalian liver, for example, contains a set of isozymes of alcohol dehydrogenases that serve as detoxification enzymes; each is quite broad in terms of specificity, but with the collection of specificities presumably engineered to cover the spectrum of possible xenobiotic substances that might challenge the organism. The assessment of substrate specificity of enzymes by combinatorial libraries offers a new alternative for the fast discovery of natural substrates and inhibitors.

We report here the first example of the use of a powerful high resolution mass spectrometric method, electrospray ionization-Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR),[16] for determining substrate specificity in a 'two-dimensional' combinatorial experiment involving GST. We report that this technology permits combinatorial substrate libraries to be screened for substrate specificity in a simple experiment without tedious workup.

## EXPERIMENTAL

The experiments were performed at the University of Florida on a Bruker 47e external source FTICR mass spectrometer (Bruker Instruments Inc., Billerica, MA, USA) equipped with a shielded, horizontal, room-temperature, 15 cm inside diameter, 4.7 T superconducting magnet (Magnex Scientific Ltd., Abingdon, England). Ions produced externally to the magnet were guided into the 170 $mm^3$ cylindrical RF-shimmed Infinity™ analyzer cell[17] using electrostatic ion optics. The standard external source FTICR instrument has been previously described[18] and only relevant details are discussed further. The FTICR mass spectrometer was equipped with an external electrospray source which utilizes a hexapole ion guide (Analytica of Branford, Branford, CT, USA). The ESI source was modified in the laboratory to use a heated metal

*Correspondence to: J. R. Eyler, Department of Chemistry, P.O. Box 117200, University of Florida, Gainesville, FL 32611-7200, USA
Contract/grant sponsor: National Science Foundation
Contract/grant sponsor: Swiss Federal Government
Contract/grant sponsor: National High Magnetic Field Laboratory In-House Research Program

CCC 0951–4198/97/161749–04 $17.50

capillary similar in design to that reported by Ikonomov and Kebarle,[19] instead of a quartz capillary and counter-current drying gas.

The Bruker FTICR external source design has two stages of differential pumping to achieve a factor of $10^4$ pressure reduction between the source and the analyzer regions. The mass spectrometer was pumped by a series of cryopumps: an 800 L/s cryopump ($N_2$) pumped the FTICR external source housing, a 400 L/s cryopump evacuated the regions between the first and second conductance limits that contain the electrostatic transfer optics, and an additional 400 L/s cryopump was used to maintain high vacuum in the analyzer region. After leaving the ESI source, ions were accelerated by a 3 kV potential difference and focused through two conductance limits before being decelerated, accumulated and trapped in the FTICR analyzer cell.

A dual stage ESI source equipped with one 500 L/min mechanical pump on the first stage and a turbodrag pump on the second stage was used to reduce the pressure through the ESI source from atmospheric pressure to *ca*. $2 \times 10^{-3}$ mbar. Desolvation of the charged droplets occurred in a metal capillary heated to 120 °C and biased at *ca*. 80 volts relative to ground. The stainless steel capillary was 500 μm i.d. × 170 mm long with a capillary–needle distance of *ca*. 5 mm. The pressure after the hexapole conductance limit, in the normal FTICR external source housing, was $3.6 \times 10^{-6}$ mbar when pumped by the 800 L/s cryopump.

### Sample synthesis and preparation

*H–γ–Glu-Cys-Xxx-OH-library.* A library of H–γ–Glu-Cys-Xxx-OH tripeptides, where Xxx is any natural amino acid except Cys, Val, Thr, Pro, has been synthesized according to previously published work,[16] with the exception that a Fmoc-Xxx-OH mixture was directly coupled to the solid support under low racemization conditions (0.1 moleq 4-dimethylaminopyridine (DMAP), 3 h coupling time).

For the analysis 0.2 mg library were dissolved in 1 mL MeOH/$H_2O$/HOAc 49/49/2 and sprayed at a flow rate of 1 μL/min. For the screening experiment the peptide library was neutralized in degassed 0.1 M $NH_4HCO_3$ and lyophilized.

*Preparation of Glutathione-S-Transferase.* GST (0.8 mg, 84% Biuret, 83 units/mg protein, Sigma, St. Louis, MO, USA) from bovine liver was dissolved in $NH_4OAc$ buffer (200 μL, 0.1 M pH 6.5), placed in a MicroCon 10 (Amicon Inc., Beverly, MA, USA) and concentrated by centrifugation at 4 °C (12 000 rpm, 10–12 min). The protein was diluted 3 times in 100 μL buffer with subsequent concentration by centrifugation. The desalted protein was diluted in buffer (100 μL) and directly used.

*Substrate Specificity Assay.* Lyophilized H–γ–Glu-Cys-Xxx-OH library (1.8 mg) was redissolved in degassed $NH_4OAc$ buffer (200 μL, 0.1 M, pH 6.5). An aliquot of 1-chloro-2,4-dinitrobenzene solution (CDNB)[15] in ethanol (5 μL, 200 mM) was added, followed by a solution of freshly prepared enzyme (10 μL). After incubation at room temperature (10 min), the assay mixture was sprayed directly without further workup at low capillary voltages.

Glutathione was incubated under the above conditions with substrate and no enzyme to make sure that there is no background reactivity at this pH. Other negative controls were done with library and substrate as well as protein and substrate only. A positive control with commercially available glutathione (0.1 mg, Sigma), CDNB and enzyme under the above assay conditions was performed.

## RESULTS AND DISCUSSION

GST was chosen as a model enzyme to develop an experimental setup for a 'two-dimensional' substrate screening which is illustrated in Fig. 1. A library of glutathione analogs with the structure H–γ–Glu-Cys-Xxx-OH (Xxx being an amino acid) was synthesized. Not unexpectedly, the disadvantageous esterification of an amino acid mixture to a solid support yielded an unequal representation of less than the 19 expected tripeptides. Val, Thr and Pro did not couple to a detectable amount. Since this study served the purpose of showing the feasibility of a concept, the 'incompleteness' of the library did not disturb the task here.

Figure 2 (a) shows the ESI-FTICR mass spectrum of the crude library. The 'glutathione'-library was presented to the enzyme in the presence of CDNB. Since the enzyme was desalted and the reaction was performed in a volatile, quasi-physiological buffer, the enzymatic reaction could be monitored without further workup. After brief incubation, the result was analyzed by ESI-FTICR mass spectrometry.

As shown in Fig. 2 (b), the enzyme selected a single peptide from the library and catalyzed the reaction with CDNB to form a product ($[M_r + H]^+ = 474.1$) as illustrated in Fig. 1. The background peaks in the mass range of probable products (Fig. 2 (b)) are contaminants of the crude library and are identical to the ones in the crude library (Fig. 2 (a)). The use of 0.1 M $NH_4OAc$ as screening buffer caused an elevated level

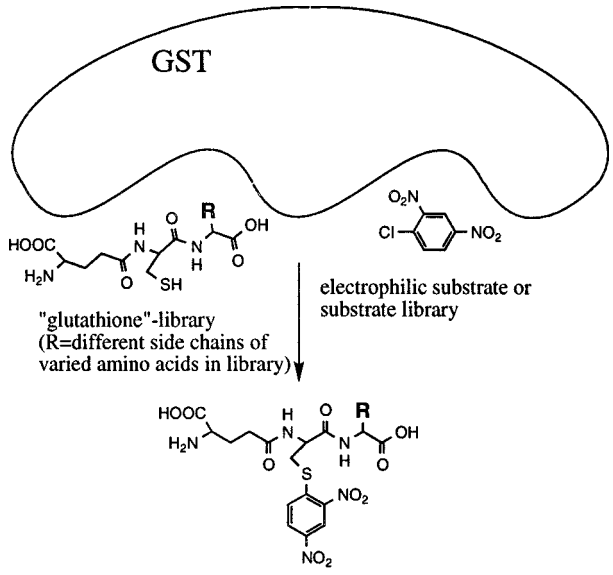


**Figure 1.** Schematic illustration of a 'two-dimensional' combinatorial screening experiment involving glutathione-S-transferase.

 

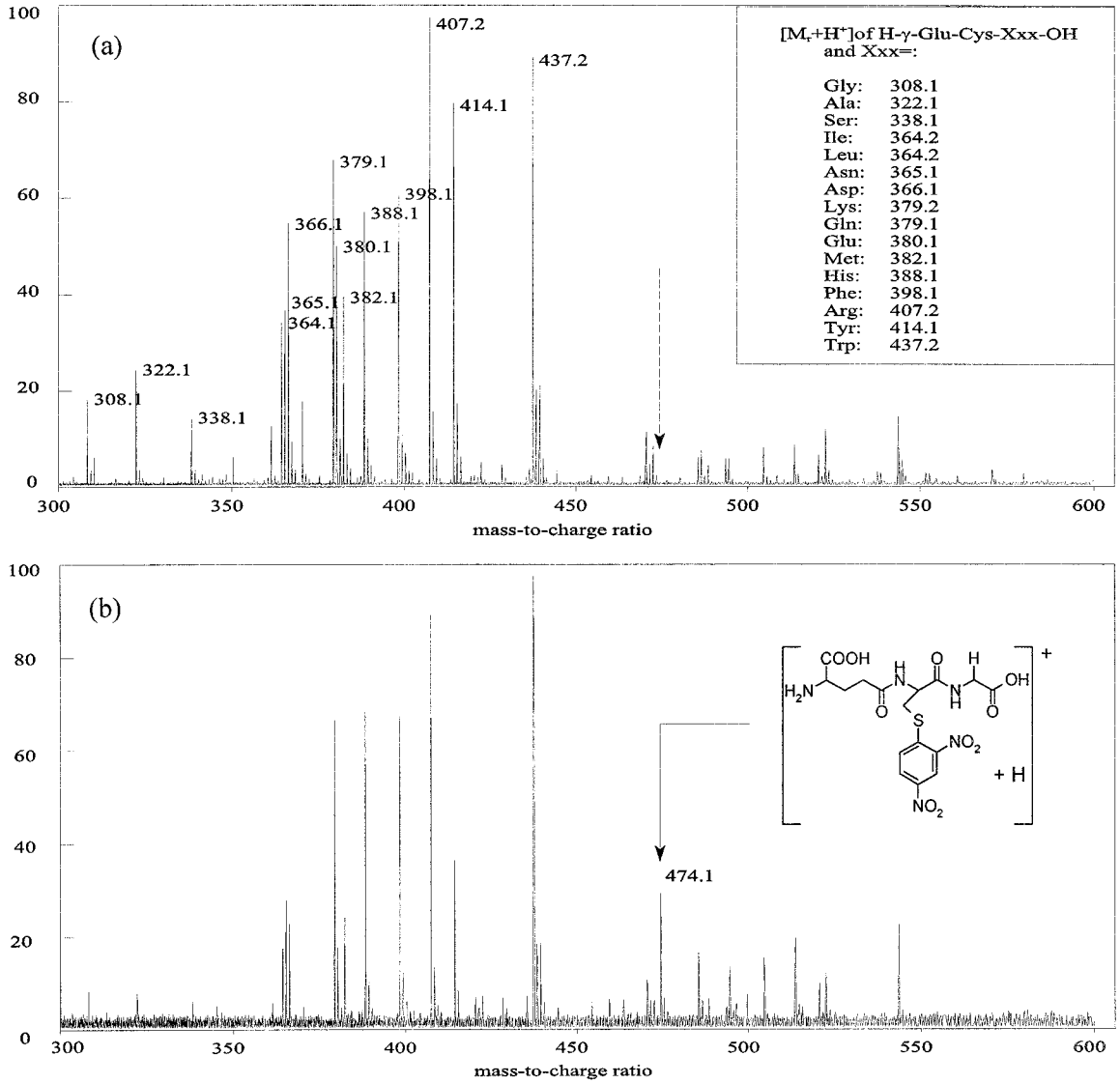


**Figure 2.** (a) ESI-FTICR broadband mass spectrum of the H-γ-Glu-Cys-Xxx-OH library. The arrow indicates where the product peak would be, if present. (b) The mass spectrum of the reaction mixture after 10 min incubation of the tripeptide library with electrophilic substrate (CDNB) in the presence of glutathione-S-transferase.

of noise and changed the intensities of some peaks slightly. Since the high resolution analysis by FTICR enables us to distinguish between peaks of the same nominal mass and different elemental composition, it would be possible to detect product peaks closely spaced to contaminant peaks, if they were present. The enzyme itself could not be detected at these high buffer salt concentrations. Nevertheless the purity and quality of the heterodimeric protein, consisting of two subunits with molecular masses of $M^1 = 25\ 702$ Da and $M^2 = 25\ 516$ Da, was confirmed by ESI-FTICRMS before the experiment was done (data not shown). A set of products was prepared by chemical reaction of the library with CDNB at high pH, proving that under the spraying conditions of this experiment all possible products were detected. Based on this standard, GST is $>95\%$ specific for the peptide where Xxx = Gly, the natural substrate glutathione. Were the structure of the product an issue, CAD analysis of the product peak would provide information to assign it. A negative control at pH 6.5 with glutathione, CDNB and no enzyme excluded the possibility that the observed reaction is due to a background reactivity of the thiolate under these conditions (Fig. 3 (a)). The addition of enzyme led to the expected product formation and reconfirmed the activity of the enzyme under these conditions (Fig. 3 (b)).

The experiment provides a simple demonstration of the feasibility of a 'real-time analysis' combinatorial experiment with GST. The approach is easily applied to a 'two-dimensional' combinatorial experiment, where two functionalized libraries targeting two substrate-binding pockets are presented to a protein. After selection of the best binding substrates of each library a bond is formed and the higher mass product is analyzed by ESI-FTICR mass spectrometry or by another suitable mass analyzer. This methodology enables us to assess more complex approaches, such as the self-assembly of functionalized ligand sublibraries in equilibrium as in a receptor-assisted combinatorial synthesis experiment[20] or in the virtual library concept[21] by ESI-FTICR mass spectrometry.

 

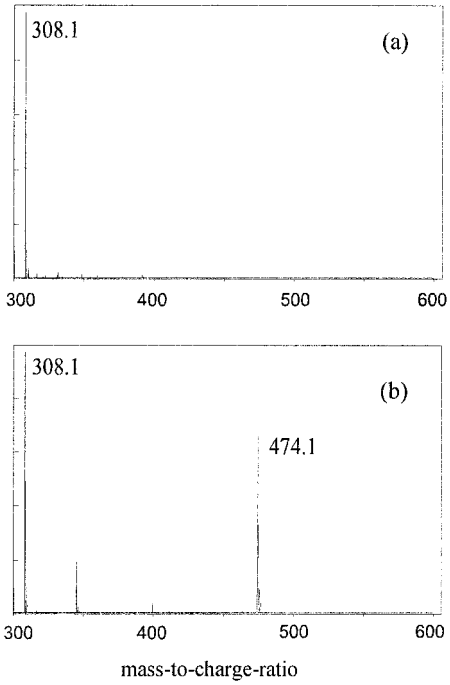


**Figure 3.** ESI-FTICR mass spectrum of glutathione after incubation with CDNB under assay conditions (a) without added enzyme, (b) with enzyme addition.

## CONCLUSIONS

Combining combinatorial chemistry technology with ESI-FTICR mass spectrometry has provided information about the substrate specificity of GST, an enzyme involved in cancer prevention and cell detoxification and having an interesting evolutionary history. The experiment does not require elaborate equipment and is extremely fast. No isolation of the binding substrates is required since they can be easily identified by their higher mass and analyzed by CAD. The special characteristics of FTICR mass spectrometry (e.g. ultra-high mass resolving power, high mass accuracy) should make it possible to extend this setup to libraries 1000 times more diverse than the library used in this model reaction.

## Acknowledgements

We are indebted to the NSF, the National High Magnetic Field Laboratory In-House Research Program and Swiss Federal Government for partial support of this work.

## REFERENCES

1. S. A. Benner and A. D. Ellington, *Bioorg. Chem. Front.* **1**, 1 (1990).
2. N. C. Wrighton, F. X. Farrell, R. Chang, A. K. Kashyap, F. P. Barbone, L. S. Mulcahy, D. L. Johnson, R. W. Barrett, L. K. Jolliffe and W. J. Dower, *Science* **273**, 458 (1996).
3. J. Nielsen, S. Brenner and K. D. Janda, *J. Am. Chem. Soc.* **115**, 9812 (1993).
4. K. Burgess, J. Ibarzo, D. S. Linthicum, D. H. Russell, H. Shin, A. Shitangkoon, R. Totani and A. J. Zhang, *J. Am. Chem. Soc.* **119**, 1556 (1997).
5. R. Wieboldt, J. Zweigenbaum and J. Henion, *Anal. Chem.* **69**, 1683 (1997).
6. D. Obrecht, C. Abrecht, A. Greider and J. M. Villalgordo, *Helv. Chimi. Acta* **80**, 65 (1997).
7. J. P. McDevitt and P. T. Lansbury, *J. Am. Chem. Soc.* **118**, 3818 (1996).
8. J. Jiracek, A. Yiotakis, B. Vincent, F. Checler and V. Dive, *J. Biol. Chem.* **271**, 19606 (1996).
9. D. Maclean, J. R. Schullek, M. M. Murphy, Z. J. Ni, E. M. Gordon and M. A. Gallop, *Proc. Nat. Acad. Sci. US* **94**, 2805 (1997).
10. Q. N. Wu, Q. N. Ma and K. S. Lam, *Biochem.* **33**, 14825 (1994).
11. Y. H. Chu, Y. M. Dunayevskiy, D. P. Kirby, P. Vouros and K. L. Karger, *J. Am. Chem. Soc.* **118**, 7827 (1996).
12. M. L. Nedved, S. Habibigoudarzi, B. Ganem and J. D. Henion, *Anal. Chem.* **68**, 4228 (1996).
13. C. S. Morrow and K. H. Cowan, *Cancer Cells* **2**, 15 (1990).
14. B. Mannervik and U. H. Danielson, *CRC Crit. Rev. Biochem.* **23**, 283 (1988).
15. W. H. Habig, M. J. Pabst and W. B. Jakoby, *J. Biol. Chem.* **249**, 7130 (1974).
16. J. P. Nawrocki, M. Wigger, C. H. Watson, T. W. Hayes, M. W. Senko, S. A. Benner and J. R. Eyler, *Rapid Commun. Mass Spectrom.* **10**, 1860 (1996).
17. P. Caravatti and M. Alleman, *Org. Mass Spectrom.* **26**, 514 (1991).
18. G. H. Kruppa, P. Caravatti, C. Radloff, S. Zürcher, f. Laukien, C. Watson and J. Wronka, in *Analytical Applications of Fourier Transform Ion Cyclotron Resonance Mass Spectrometry*, B. Asamoto (Ed.), VCH, New York, p. 107 (1991).
19. M. G. Ikonomou and P. Kebarle, *J. Am. Soc. Mass Spectrom.* **5**, 791 (1994).
20. T. Giger, M. Wigger, S. Audétat and S. A. Benner, *Synlett*, submitted (1997).
21. I. Huc and J. M. Lehn, *Proc. Nat. Acad. Sci. US* **94**, 2106 (1997).

 