Communications to the Editor

A Cross-Reactive, Class-Selective Enzymatic Array Assay

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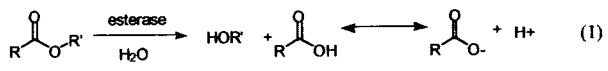
Both natural and designed sensors use two general approaches to sensing. The first approach employs the exquisite specificity found in most ligand−receptor interactions, wherein a receptor possesses complementary structural and electronic features to interact with its ligand, while precluding interaction with closely related molecules. The second approach is unique to the olfactory system[1] and utilizes promiscuous receptors with the ability to bind many molecular species. In the second system, recognition is accomplished by using the patterns of response over many different receptors. Artificial systems using this approach are dubbed "electronic noses" and use nonspecific sensors in a cross-reactive array format.

A cross-reactive sensor array is generated from a group of sensors that react to a broad range of analytes, where each analyte elicits a response from multiple sensors.[2] An advantage to the cross-reactive sensor array is that fewer sensors are needed to distinguish a wide variety of analytes because a pattern recognition program can differentiate many combinations of responses.[3] In this paper, we describe a third approach that combines these two general approaches to sensing, by employing enzymes that all catalyze the same type of reaction but that have different and somewhat overlapping specificities. In this way, we are able to restrict the specificity of the sensor array to a certain class of substrates. We utilize the cross-reactivity of these enzymes in combination with a pattern-recognition scheme to identify the specific molecule present.

Enzymes catalyze reactions with intrinsic specificity and selectivity. In their recognition of substrates, many enzymes are selective; for example, L-glutamate oxidase oxidizes only L-glutamate. Other enzymes are class-selective, such as L-amino acid oxidase, which catalyzes the oxidation of a range of L-amino acids with varying kinetics. The incorporation of class-selective esterases into an enzymatic array assay format exploits the esterase's inherent cross-reactive nature.

Esterases catalyze the hydrolysis of esters to carboxylic acids (eq 1). In the assay reported here, a fluorescent pH indicator,



(1)

fluorescein, is added to the reaction mixture to measure the change in acidity resulting from the hydrolysis reaction in a 96-well microtiter plate format. The fluorescence response is monitored over time to give a temporal pH-induced fluorescence pattern.

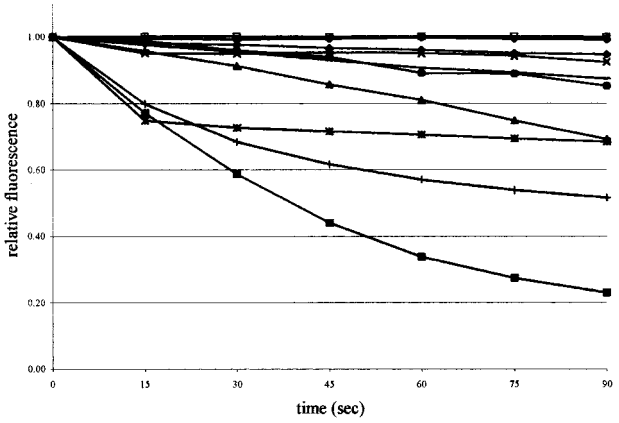


Figure 1. Kinetic traces of nine esterases and PA. Legend: (■) ach; (+) cholesterol; (×) hog; (∗) horse; (▲) porcine; (●) rabbit; (−) fungi; (◇) bacteria-1; (◆) bacteria-2; and (□) blank.

Esterases are utilized for the initial demonstration of the enzymatic array assay because they are commercially available, relatively stable, and react with a wide range of esters. Esterases have been used to test ester chirality[4] and as catalysts for efficiently hydrolyzing a variety of esters.[5]

The microtiter plate assay contains nine esterases in different columns and the ester analytes in the different rows. Each esterase-catalyzed hydrolysis reaction is addressed individually to monitor the kinetics by scanning each well independently. Using a microtiter plate reader (Molecular Devices), the reaction is monitored for 90 s after an initial 10-s agitation. The 90-s exposure time is necessary to measure the changes in pH relative to a blank. The microtiter plate format provides a rapid and reproducible system to measure the hydrolysis reactions.

Nine lyophilized esterases[6] (Sigma and Fluka) were used as received and were chosen on the basis of their availability and wide range of specific activities. Twenty-three esters, ranging from simple aliphatic esters to multi-functional chiral esters, were chosen as analytes.[7] The esters vary in the placement of functional groups close to the reaction center and include representatives ranging from methyl, ethyl, and propyl esters, as well as acetates.

The reaction volume of a microtiter plate well is 105 μL, and comprises 29 μM analyte from a substrate working solution[8] and 0.4−30 μg of esterase in phosphate buffer solution (pH 7.4), similar to work done by Kazlauskas.[4] The analyte concentration

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(6) Esterases are rabbit liver, porcine liver, horse liver, hog liver, *Mucor miehei* (fungi), *Bacillus* sp. (bacteria-1), *Bacillus* th. (bacteria-2) [all [EC 3.1.1.1]]; acetylcholine esterase from *Electrophorus electricus* [EC 3.1.1.7]; and cholesterol esterase from hog pancreas [EC 3.1.1.13].

(7) Esters are ethyl propionate (EP), ethyl benzoate (EB), ethyl valerate (EV), ethyl acetate (EA), ethyl butyrate (BA), propyl butyrate (PB), isopropyl nicotinate (IN), isopropyl acetate (IA), methyl 2-methyl butyrate (MMBU), methyl butyrate (MBU), methyl benzoate (MB), methyl 2-methyl glycidate (MMG), methyl nicotinate (MNI), methyl 6-methyl nicotinate (MMNI), methyl cyclohexane carboxylate (MC), L-alanine methyl ester (LM), D-alanine methyl ester (DM), *tert*-butyl acetate (TA), hexyl acetate (HA), 2-naphthyl acetate (NA), acetylcholine chloride (AC), phenyl acetate (PA), and propyl acetate (PRA).

(8) Substrate working solution: 0.42 mL of 100 mM substrate in CH_3CN.; 0.47 mL CH_3CN.; 0.6 mL of a 250 nM fluorescein dye solution in 10 mM PBS buffer, pH 7.4; and 10.51 mL 0.01 mM PBS, pH 7.4. All solvents and substrates were purchased from Aldrich, Sigma, and Fluka Chemical Companies and used as received.

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is chosen to be at least one-fifth of the Michaelis constant (K_m) for the esterases.

The differing hydrolytic susceptibility of the esters to the esterases results in reactivity rate patterns, which are used to distinguish the esters. Among all of the esters, phenyl acetate (PA) is hydrolyzed the fastest. As seen in Figure 1, each esterase reacts with PA at a varying rate. These patterns of reactivity provide a means to distinguish PA from other substrates.

Of the nine esterases examined, acetylcholine esterase hydrolyzes all 23 esters. Rabbit esterase reacts with all of the esters except the simple aliphatic esters. To exemplify the discriminating ability of the current system, D-alanine (DM) and L-alanine (LM) methyl esters were included as analytes. DM and LM are both hydrolyzed by acetylcholine esterase and bacteria 1 esterase, while bacteria 2 esterase hydrolyzes only LM and not DM. These differences in reactivities provide a "fingerprint" of each ester.

The esterase array is tested for its reproducibility. The hydrolysis reaction slopes of three esters, PA, methyl butyrate (MB), and ethyl butyrate (BA), are measured initially and after 3 months. The initial slope range and standard deviation for the three esters with acetylcholine esterase were PA $(-1.4 \pm 0.2) \times 10^6$, MB $(-1.3 \pm 0.01) \times 10^5$, and BA $(-2.9 \pm 1.6) \times 10^5$ respectively; after three months the slopes were PA -1.5×10^6, MB -1.3×10^5, and BA -3.2×10^5. The slopes, therefore, lie within the initial ranges after 3 months.

The hydrolysis reaction initial slopes are used as input for principal component analysis (PCA).[9] PCA is a computational method of reducing high-dimensional raw data into lower-dimensional graphical representations of data's variations. The individual interactions are analyzed and separated into clusters for which the tightness of the clusters indicates the array's ability to distinguish the analytes.[10] By combining the response patterns of all nine esterases for the 23 analytes from four independent assays, a confusion matrix is compiled from the PCA data. The confusion matrix compares the calculated versus actual ester identity and is 90% correct using 98% of the data's variance.

As seen in Table 1, four out of the 23 esters are misidentified at least once. For three of the four misidentified esters, no clear structural basis exists to cause the esters to be misclassified in PCA; however, their hydrolysis reaction slopes are similar. The fourth ester, BA is misidentified four times—twice as ethyl acetate (EA) and once each as MB and methyl 2-methyl butyrate (MMB). The completely incorrect assignment of BA is based on structural similarities as twice it was misidentified as an ethyl ester and twice as a methyl ester of butyrate.

Table 1. Confusion Matrix Results: Column Number Indicates Number Incorrect; Abbreviation in Parenthesis Indicates the PCA Identification of the Incorrect Ester

0	1	2	3	4
EA; PA; MB; IN; MNI; EV; MMG; MBU; AC; IA; PB; EB; MC; EP; DM; LM; MMNI; HA; MMBU	NA (IA)	PRA (DM(2)); TB (EV; LM)		BA (EA(2); MMBU; MBU)

Initial binary mixture analysis was completed to determine whether binary ester mixtures contain a linear component corresponding to the individual esters. Concentration runs of four esters, PA, ethyl valerate (EV), methyl nicotinate (MNI), and methyl 6-methyl nicotinate (MMNI), were run with the nine esterases. The rates of the esterase reactions were plotted on a Lineweaver–Burk plot to determine the K_m and V_{max}. The individual rates were used to identify binary mixtures of the four esters. For example, an equal volume mixture of PA/MMNI gave a reaction rate of 1.1×10^9 M s^{-1} for bacteria esterase 1, while the reaction rates of the individual components added to 1.3×10^9 M s^{-1}. These results suggest that it should be possible to employ linear discriminant analysis for binary mixture identification of other esters, where the distance between classes is maximized and the distance within the classes is minimized, because the concentrations of the esters are well below the K_m for the esterases.[2]

We have demonstrated the ability to use the inherent cross-reactivity of esterases to distinguish approximately 20 individual analytes as well as several binary mixtures. The extension to more complex mixtures would be difficult, as it would require extensive additional training. On the other hand, the ability to distinguish this diverse group of ester analytes using a limited suite of sensing materials demonstrates the utility of the approach and could prove useful for identifying unknown samples of limited complexity.

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