CHAPTER 1

Application of HPLC to the Assay of Enzymatic Activities

OVERVIEW

This chapter describes the anatomy of an enzyme assay, focusing on the significance of separation and detection in the assay procedure. A classification of the methods used in the assay of enzymatic activities is developed, using the separation step as the criterion for the grouping. Having placed the high performance liquid chromatography (HPLC) method within this classification, we then examine the question of when to use it and discuss some strategies developed for its use. The chapter also identifies and comments on the parts of the enzyme assay that will be affected by the selection of HPLC as the method of analysis.

1.1 INTRODUCTION

Increasingly, investigators in the life sciences have expressed interest in the application of HPLC to the assay of enzymatic activities. This method not only provides a method to enhance the separation of reaction components, it also allows extensive and complete analysis of the components in the reaction mixture during the reaction. In addition, it can employ sensitive detectors, and it can be used for purification.

A number of questions must first be addressed, however, concerning the biochemical reaction catalyzed by the enzyme, the assay conditions normally used for this enzyme, and the enzyme itself. This chapter explores and answers these questions.

Section 1.2 presents the anatomy of the enzymatic assay, and from a dissection of its components, it is possible to obtain an appreciation of how HPLC can be used. Section 1.3 develops a classification of enzyme methods that allows the advantages and limitations of the HPLC method to be presented fairly. Section 1.4 is devoted to criteria for the selection of HPLC as an assay method.
system. Wherever possible, these points are illustrated with examples taken from work carried out in the author's laboratory.

1.2 ANATOMY OF AN ENZYME ASSAY

The assay of an enzymatic activity is composed of several discrete steps or events (Fig. 1.1). The first is preparation of both the reaction mixture and the

![Figure 1.1](image-url)

**Figure 1.1** Schematic of a representative enzymatic assay. The reaction mixture is prepared (Mix Preparation) and the reaction can be started (Initiation) by the addition of the enzyme. During the reaction (Incubation), samples are removed at intervals labeled $t_1$, $t_2$, and $t_3$, and the reaction is stopped (Termination) by inactivating the enzyme. The incubation mixture is fractionated (the illustration shows a traditional chromatographic column), and the product is isolated from the substrate (Separation). In this assay, a radiochemical was used as the substrate and therefore the amount of product that formed is determined by its collection, the addition of scintillation fluid, and the measurement of radioactivity by scintillation counting (cpm: Detection). The progress of the reaction is given by the amount of radioactive product recovered (Data Reduction).
1.3 CLASSIFICATION OF ENZYMATIC ASSAY METHODS

The methods in use for the assay of enzymatic activities may be divided into three groups. These will be referred to as the continuous, coupled, and discontinuous methods (see Table 1.1).

1.3.1 Continuous Methods

Continuous methods do not require a separation step prior to detection. For assays using this method, the substrate and product must differ in some property such that either one may be measured directly in the incubation solution. For example, the activity of an enzyme catalyzes the conversion of 4-nitrophenyl phosphate (4NP), a colorless compound, to 4-nitrophenol, which is yellow and has an absorption maximum at 510 nm. Since the substrate does not absorb in this region of the spectrum, the reaction can be carried out...
TABLE 1.1 Classification System for Enzymatic Assay Methods

<table>
<thead>
<tr>
<th>Assay Methods</th>
<th>Characteristics</th>
<th>Example</th>
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<tbody>
<tr>
<td>Continuous</td>
<td>Separation of substrate(s) from product(s) not required</td>
<td>4NP → 4N + P&lt;sub&gt;i&lt;/sub&gt; colorless yellow</td>
</tr>
<tr>
<td>Coupled</td>
<td>Separation not required for detection</td>
<td>PEP + ADP → pyruvate + ATP pyruvate + NADH → lactate + NAD</td>
</tr>
<tr>
<td>Discontinuous</td>
<td>System for separation of substrate(s) from product(s) required for detection</td>
<td>ATP + AA → Enz - AA - AMP + PP&lt;sub&gt;i&lt;/sub&gt; Enz - AA - AMP + tRNA → tRNA - AA - AMP</td>
</tr>
</tbody>
</table>

directly in a cuvette (Fig. 1.2), and the amount of product formed may be determined continuously by measuring the change in optical density with time at this wavelength.

1.3.2 Coupled Method

In the second category of assays, the coupled assay method, activity is measured indirectly. In this method two reactions are involved. The first is the reaction of interest, such as A → B, second, the reaction that converts B to C, might be referred to an indicator reaction, not only because it uses the product of the first reaction (i.e., B) as a substrate, but also because the

Figure 1.2 The assay of an enzymatic activity by the continuous assay method. In the illustration, the reaction mixture is transferred to a cuvette, which is shown in place in the light path of the spectrometer. The addition of the enzyme directly to the cuvette initiates the reaction. Product formation results in a change in absorbance, which is monitored continuously by the detector. This change signals a deflection on a recorder. Note that product formation requires neither termination of the reaction nor separation of the substrate from the product.
1.3 CLASSIFICATION OF ENZYMATIC ASSAY METHODS

formation of C may be assayed by a continuous method—that is, without a separation step. In this way, the two reactions are coupled, the product of the first reaction, B, acting as the substrate for the second reaction.

For example, pyruvate kinase may be assayed by such a method. This enzyme catalyzes the reaction

\[
\text{Phosphoenolpyruvate (PEP) + ADP} \rightarrow \text{pyruvate} + \text{ATP}
\]

This, of course, is the reaction of interest that cannot be assayed directly by the continuous method. However, when a second enzyme, a dehydrogenase, such as lactate dehydrogenase, is added as the indicator together with pyruvate and NADH to the reaction mixture, a second reaction occurs and NAD forms in the cuvette as follows:

\[
\text{Pyruvate} + \text{NADN} \rightarrow \text{lactate} + \text{NAD}
\]

The formation of NAD may be followed in a continuous manner by the decrease in absorbance at 340 nm, and therefore the progress of the kinase reaction of interest may be followed through this coupling of the formation of pyruvate to the formation of NAD.

1.3.3 Discontinuous Method

The discontinuous method measures activity by separating the product from the substrate. Assays characteristic of this group usually require two steps, since separation often does not include detection. Thus, first, the substrate and the product are separated, and usually the amount of product formed is measured. Assays that use radiochemical substrates are included in this group, since radiochemical detectors are unable to differentiate between the radiolabel of the substrate and that of the product. Examples of enzymes whose assay methods fall into this category are legion, and these approaches characterized by a separation step.

As an illustration, consider the assay to measure the activity of the tRNA synthetases. These enzymes catalyze the covalent attachment to tRNA of an amino acid, usually radioactive as follows:

\[
\text{ATP + }^*\text{AA + Enz} \rightarrow \text{Enz-AMP-}^*\text{AA + PP}_i \tag{1}
\]

\[
\text{Enz-AMP-}^*\text{AA + RNA} \rightarrow \text{RNA-}^*\text{AA + AMP} \tag{2}
\]

(By convention, radioactivity is indicated by an asterisk preceding one labeled substance, here the amino acid AA.)

The activity is usually followed by measuring the amount of RNA-*AA, the product of reaction (2) formed during the incubation. Since the radiochemical detector cannot differentiate the free radioactive amino acid
used as the substrate from that bound covalently to the RNA, the free and the bound amino acids must be separated prior to the detection or quantitation step.

This separation step requires first the addition to the sample of an acid such as trichloroacetic acid (TCA), which also serves to terminate the enzymatic reaction. However, since TCA also precipitates the RNA and any radioactive amino acid covalently linked to it, the reaction product RNA-*AA will be precipitated as well. And since the precipitate can be separated from the soluble components by a sample filtration step, the separation of the bound from the free amino acid can be accomplished. As illustrated in Figure 1.3, the reaction product, which is trapped on the filter as a precipitate, can be detected by transferring the filter to a scintillation counter for quantitation and, of course, measuring the amount of product formed. Since assays of this design usually focus on one component at a time, no information is obtained about the amount of ATP, AMP, PP_i, or free amino acid during the course of the reaction.

1.3.4 HPLC as a Discontinuous Method

Within the framework of the scheme just described, the HPLC method would be classified as discontinuous, since a separation step is part of the procedure. However, because termination can be accomplished by injecting the sample directly onto the column, the HPLC detection is usually “on-line,” that is, carried out continuously with separation. Thus, the separation and detection steps merge into a single operation, which for all practical considerations means that it is a “continuous” method.

In addition, unlike many other discontinuous assays that focus on only one of the components of the reaction, the HPLC assay offers the potential to monitor several. For example, consider adenosine kinase, the enzyme that uses two substrates and forms two products according to the reaction Ado + ATP → AMP + ADP. Since HPLC can readily separate all four compounds (see Fig. 1.4), and all four compounds can be detected at 254 nm, it is apparent that with the HPLC method, the level of each component can be monitored during the course of the reaction, providing a complete analysis of each “time point.”

Having a complete analysis of the contents of the reaction vessel during the incubation can be helpful in another way: It provides information on what is not present as well; and since most other assay methods are designed to detect only one component, it is often difficult to account for a result that occurs unexpectedly during a study. For example, consider the results obtained during the purification of the enzyme E-1, which catalyzes the conversion of substrate A to product B. Consider also that the method used to follow activity measures only the amount of B in the incubation mixture. As illustrated in panel I of Figure 1.5, when the activity E-1 is assayed in the crude sample, the formation of substantial product (B) is observed (graph line 1).
1.3 CLASSIFICATION OF ENZYMATIC ASSAY METHODS

Figure 1.3 The assay of an enzymatic activity by the discontinuous assay method. In assays of this type, the reaction mixture is prepared, and usually the reaction is started by the addition of the enzyme. Samples are removed at intervals $t_1$, $t_2$, $t_3$, and the reaction is terminated by transferring the sample to a solution that inactivates the enzyme. Here a radioactive substrate is converted to radioactive product that is precipitable in trichloroacetic acid, (TCA), while the substrate remains soluble in the acid. Thus two components can be separated by filtration. The product is shown being collected on the filter while the unreacted substrate flows through into the filtrate. The amount of radioactivity trapped on the filter is determined by scintillation counting. When these data are graphed as a function of sample time (i.e., $t_1$, $t_2$, $t_3$, they provide the kinetics of product formation.

Imagine now that the enzyme sample is purified further and the purified enzyme is assayed for the same activity E-1 by the same method. However, in this case, following the addition of the substrate (A), the formation of product (B) during the course of the reaction is greatly reduced (graph line 2). While this result might indicate a true loss of E-1 activity, it might also be a result of an increase in the activity of E-2, a second enzyme that catalyzes the degradation of B to C (Fig. 1.5, panel II). In the absence of data on the level of the substrate (A) during the course of these reactions, the second possibility cannot be excluded.
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Figure 1.4 Separation of substrates and products of an adenosine kinase reaction on ion-paired reversed-phase HPLC. The separation was carried out on a prepacked C$_{18}$ (μBondapak) column with a mobile phase of 65 mM potassium phosphate (pH 3.7) containing 1 mM tetrabutylammonium phosphate and 5% methanol. The column was eluted isocratically, and the detection was at 254 nm. Four relative elution positions (elution times) are shown.

To test this second possibility will require an assay for E-2, which in turn will call for a method for the measurement of the amount of C. Therefore, a reaction mixture optimized for E-2 will have to be prepared, and determinations of C formation in both the crude and purified samples will have to be carried out. If the data obtained in these determinations appeared as shown in panel II of Figure 1.5, these results would show that in the crude sample E-2 activity was indeed lower than that observed in the purified sample, thus indicating that the purification of E-2 could account for the loss of activity of E-1 during the purification. This example clearly shows that an assay method that measures only the levels of a single compound such as the product may provide very limited results.

In contrast, with a well-designed HPLC assay for activity E-1, capable of separating A, B, and C, the levels of each may be obtained from an analysis of a single sample from the reaction mixture of both the crude and purified samples (panel III of Fig. 1.5). In fact, the data obtained provide information not only on what compounds are present but also on what are not. The availability of such negative information can provide the “data” leading to the exclusion of alternative explanations that had been proposed to explain unexpected results.
Figure 1.5  Comparison of the advantage of the HPLC assay method to traditional methods of assay for following the activity of an enzyme during purification. (I) The assay of the hypothetical enzyme, called E-1, which catalyzes the conversion of the substrate A to the product B. In this traditional method, an assay would be developed to follow the production of the product B. Note that in the crude extract E-1 produced B at the rate shown by line (1). However, after purification, E-1 produced B at a much slower rate, as indicated by line (2). To understand the reduction in rate, a second hypothetical enzyme was proposed that would convert B, the product of E-1, to a new product C. However, another traditional assay was used, and only the formation of C was measured. (II) E-2 activity was measured in both fractions, and while activity was found in both the crude and purified fractions, the rate of C formation in the purified fraction shown by line (4) was significantly greater than in the crude as shown by line (3). The activity of the enzymes E-1 and E-2 is shown being measured by the HPLC method. This assay was developed to separate A, B, and C simultaneously, and therefore it was possible to measure the activity of both E-1 and E-2 simultaneously. The results of assays carried out on both the crude and the purified preparations are shown. The plots at the right display the difference in levels of A, B, and C during the course of the incubation with both the crude and purified preparations.
1.4 CRITERIA FOR THE SELECTION OF AN ASSAY METHOD

The HPLC assay may not always be the procedure of choice, and several points should be considered before deciding. These points are summarized in Table 1.2.

1.4.1 Separation and Detection of Components

To utilize HPLC for an enzymatic assay requires first a system for separating the components. This involves the selection of a solid phase (the column packing), a mobile phase (the column eluent), and a method of elution from the solid phase by the mobile phase. Two procedures are generally used for elution: the isocratic and the gradient methods. In the isocratic method the mobile phase composition remains constant throughout elution, while in the gradient method the mobile phase varies in some parameter and in some fixed manner (e.g., a linear increase in salt concentration during elution). In addition, the HPLC method requires a monitor for the detection of the product. While a variety of detectors are available for monitoring various properties of molecules, a number of compounds cannot be detected on-line at present.

1.4.2 The Reaction Mixture

It is important to determine early on whether the reaction conditions previously developed for the assay of a given activity can be adapted for use with HPLC assay. For example, is the reaction mixture of sufficient volume to permit the withdrawal of multiple samples? For assays carried out in volumes of a few microliters, it is virtually impossible to withdraw samples of sufficient volume for analysis on the HPLC system. Thus, unless dilutions can be made after sampling, HPLC analysis must be ruled out in such cases.

Other factors should be considered as well. These include whether the reaction mixture contains any components that might make using HPLC

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<tr>
<th>TABLE 1.2 Questions to Be Considered Prior to the Selection of HPLC for the Assay of an Enzymatic Activity</th>
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<tbody>
<tr>
<td>1. Separation and detection</td>
</tr>
<tr>
<td>Must product be separated from substrate for analysis?</td>
</tr>
<tr>
<td>Are detectors available?</td>
</tr>
<tr>
<td>2. The reaction mixture</td>
</tr>
<tr>
<td>Are there limits to the total volume of the incubation mixture?</td>
</tr>
<tr>
<td>Are cofactors such as metals a problem?</td>
</tr>
<tr>
<td>How will the reaction be terminated?</td>
</tr>
<tr>
<td>3. The enzyme</td>
</tr>
<tr>
<td>Is the enzyme pure, or will contaminating activities be present and affect product levels?</td>
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difficult. Such components include metals, which often hinder the interpretation of chromatograms. While the problem of metals can be solved easily by the addition of chelators, for other problems the solution may be more complex. For reactions that must be terminated prior to injection, for example, the termination process itself often alters the incubation mixture. Termination by acids (e.g., trichloroacetic, perchloric) reduces the pH of the sample. Since differences between the pH of the sample and the mobile phase can produce discrepancies in chromatographic profiles, any reduction in pH brought about by the termination may cause problems in interpretation. Also, termination increases the possibility of producing a precipitate, which will have to be removed before the sample can be injected into the column, to prevent clogging of the system. While not a difficult task, the removal of precipitates does introduce an additional step into the assay procedure.

1.4.3 The Enzyme Sample

A final point to be considered in the use of HPLC as an assay procedure is the enzyme itself. Will the activity be a pure enzyme? Will it be part of a rather crude cell-free extract? Or will it be present in a fermentation broth? In the latter two cases, the presence of contaminating activities must be considered. While as mentioned above, these activities, by affecting the recovery of the product or even by affecting substrate levels during the course of the reaction, could easily cause problems with other assay procedures, they are not a problem for the HPLC assay method. Thus, HPLC should be considered first when activity in some crude extracts is to be assayed.

1.5 SUMMARY AND CONCLUSIONS

The assay of the activity of an enzyme can be subdivided into several steps: formation of a reaction mixture, preparation of an enzyme sample, combination of the two to initiate the reaction, incubation of the reaction, termination of catalysis, separation of components, their detection, and finally, reduction or processing of the data.

Not all assays require a separation step, and this fact may be used to develop a classification scheme for assay methods. Assays that require no separation have been grouped under the heading “continuous assay methods,” while “discontinuous methods” incorporate those that do.

The need to use a discontinuous assay method does not automatically mean that the HPLC method is the procedure of choice. For HPLC to be suitable, it must be possible to separate the components, and some method for detection and quantitation must be available. Next, neither the ingredients in the reaction mixture nor those used to terminate the reaction should produce problems for the separation and detection. Finally, the enzyme itself should be considered.
Excess protein can contaminate columns, and extraneous enzymes can cause problems in quantitation.

**GENERAL REFERENCES**

*Reviews of liquid chromatography*


*Classification of enzyme assay methods*
