HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN ENZYMATIC ANALYSIS

Applications to the Assay of Enzymatic Activity

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Preface

The importance of the introduction of high performance liquid chromatography (hplc) to studies in the life sciences is now widely recognized. Since its introduction, this method has been rapidly accepted by biochemists and more recently by biologists and clinicians. Such rapid acceptance should not be surprising, since advances in separation and analysis have usually been readily assimilated.

It is the ability of hplc to accomplish separations completely and rapidly that led to its original application to problems in the life sciences, particularly those related to purification. An analysis of the literature revealed that this technique was used primarily for the purification of small molecules, macromolecules such as peptides and proteins, and more recently antibodies. This application to purification has all but dominated the use of the method, and there has been a plethora of books, symposia, and conferences on the use of hplc for these purposes. However, it was only a matter of time before others began to look beyond and to explore the possibilities that result from the capacity to make separations quickly and efficiently.

What emerged from these early studies was the idea that hplc might be used as a method for the analysis of enzymatic activities rather than its traditional use as a tool for separation. This change in emphasis is particularly attractive to those who wish to make use of the activity of an enzyme as an indicator of cellular function, a determinant of a given stage of differentiation (or dedifferentiation), or even as a measure of gene function. In the past, because contaminating activities led to conflicting results, tedious purification of the enzyme was often necessary

to clarify the results of ambiguous activity determinations brought about in part as a consequence of methods that measure only one component of the reaction mixture. Such ambiguous results will occur much less often with the hplc method, since its ability to separate quickly a group of related compounds allows for the assay of one activity in the presence of several others. Thus, the advantage of analyzing an activity after only a minimal amount of purification is inherent to the hplc technique.

This book describes the hplc method and explains and illustrates its use. Each chapter deals with a different aspect of the method, beginning with an overview and ending with a detailed summary. Throughout, an attempt has been made to focus on questions related to the assay of the activity of an enzyme rather than its purification. More detailed discussions on the theory of hplc and on its use for purification, particularly for the purification of proteins, will be found in the references at the end of each chapter.

No task of this magnitude can be completed without the guidance, inspiration, and help of many others. I wish to thank Jessica Hodge Jahngen, who introduced me to the possibilities and potential of hplc. She and E. G. Jahngen have provided much of the work in this volume from my laboratory. More recently, data and assistance have been provided by Jane Hadjimichael, whose persistence and insistence in the final stages helped it all come together. A special note of appreciation and thanks to Vickey Shockley for organizing and typing the manuscript together with Pamala Vachon, and to Sherry Perrie for the original artwork.

My appreciation is also extended to my colleagues Edward J. Kollar and James A. Yaeger for editing the early drafts of the manuscript. More recently, editing assistance has been provided by Cynthia Beeman, Mina Mina, and David Richards. Finally, I wish to thank Phyllis Brown for encouraging me to complete this task in the way I thought best and Stanley Kudzin for his interest in the subject and for providing the opportunity to write the book. Also, this work could not have been completed without the contributions of numerous investigators who consented to have their work cited here. My thanks to them as well.

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Contents

Chapter One

Application of HPLC to the Assay of Enzymatic Activities

Overview

In this chapter the anatomy of an enzyme assay will be described. The focus will be on the significance of separation and detection in the assay procedure. A classification of the methods used in the assay of enzymatic activities will be developed using the separation step as the criterion for the grouping. Having placed the high performance liquid chromatography (hplc) method within this classification, the question of when to use it will be examined, and some strategies developed for its use discussed. Those parts of the enzyme assay that will be affected by the selection of hplc as the method of choice will be analyzed.

1.1 INTRODUCTION

Increasingly, investigators in the life sciences have expressed interest in the application of hplc to the assay of enzymatic activities. Some of their reasons for considering hplc for this purpose are that it provides a method to enhance the separation of reaction components, allows extensive and complete analysis of the components in the reaction mixture during the reaction, can employ sensitive detectors, and 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 has been designed to explore and answer these questions.

In Section 1.2 the anatomy of the enzymatic assay is presented, and from a dissection of its components it is possible to obtain an appreciation of how hplc can be used. In Section 1.3, a classification of enzyme methods is developed that allows the advantages and limitations of the hplc method to be presented fairly. In Section 1.4, criteria are developed for the selection of hplc as an assay system. Wherever possible, these points will be 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 enzyme. The reaction mixture usually contains such components as the buffer used to establish the correct pH, the substrate, and any cofactors such as metals that may be required for catalysis. Preparation of the reaction mixtures involves mixing these ingredients in a reaction vessel such as a test tube or, for some assay methods, a cuvette. In some cases the reaction mixture is brought to the required temperature prior to initiation of the reaction. The enzyme must also be prepared. This, however, is a more complex topic and will be discussed in detail in a later chapter.

In most cases, the second step in the assay is *initiation* and *incubation*. A reaction can be initiated by the addition of the enzyme preparation to the substrate in the reaction mixture or vice versa. This step is considered the start of the reaction, and all subsequent time points are related to this time.

Many reactions require *termination*, which is the step that brings about the cessation of catalysis and thus stops the reaction. Termination may be achieved in several ways, all of which usually involve inactivation of the enzyme.

Termination is often followed by *separation* of the components in the reaction mixture. Most often separation involves isolating the substrate from the reaction product.

Detection, the fifth step, refers to that process by which the amount of

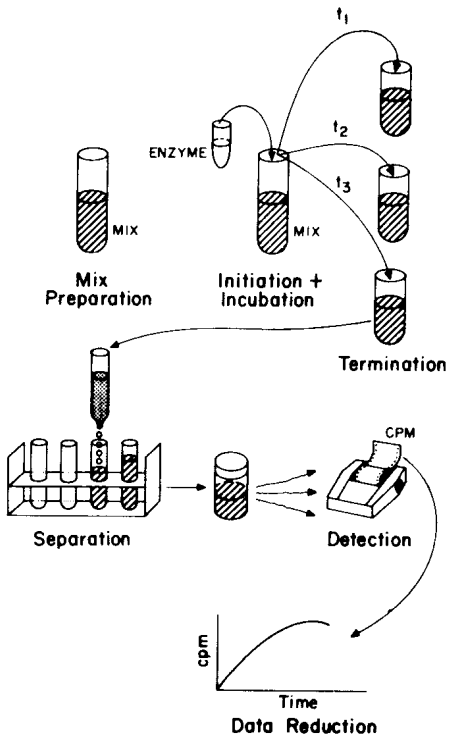

Figure 1.1 Schematic of a representative enzymatic assay to illustrate its several components. The reaction mixture is prepared (Mix Preparation) and the reaction can be started (Initiation) by the addition of the enzyme. During the reaction (Incubation), samples are removed at intervals labeled t_1, t_2, and t_3, and the reaction is stopped (Termination) by inactivating the enzyme. The incubation mixture is fractionated (in the illustration a traditional chromatographic column is being used), and the product is isolated from the substrate (Separation). In the assay illustrated, a radiochemical has been 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 (Detection). The progress of the reaction is given by the amount of radioactive product recovered (Data Reduction).

product formed by the enzyme during a specific incubation interval is determined.

Finally, the last step in an assay involves *reduction* of the data. This step includes all procedures in which the data are analyzed and graphed to determine initial rates as well as kinetic constants.

Table 1.1 Classification System For Enzymatic Assay Methods

Assay Method	Characteristics	Example
Continuous	Separation of substrate(s) from product(s) not required	$4NP \rightarrow 4N + P_i$ colorless yellow
Coupled	Separation not required for detection	$PEP + ADP \rightarrow$ $\text{pyruvate} + ATP$ $\text{pyruvate} + NADH \rightarrow$ $\text{lactate} + NAD$
Discontinuous	System for separation of substrate(s) from product(s) required for detection	$ATP + AA \xrightarrow{Enz}$ $Enz-AA-AMP + PP_i$ $Enz-AA-AMP + tRNA \rightarrow$ $tRNA-AA + AMP$

Not all steps are involved in all assay methods, and in some methods one or more of the steps might be complex. The introduction of hplc as an enzymatic assay method has improved the separation and detection steps primarily, although its use may also affect the preparation and termination steps.

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 (1) continuous, (2) coupled, and (3) 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-nitro phenyl phosphate (4NP), a colorless compound, to

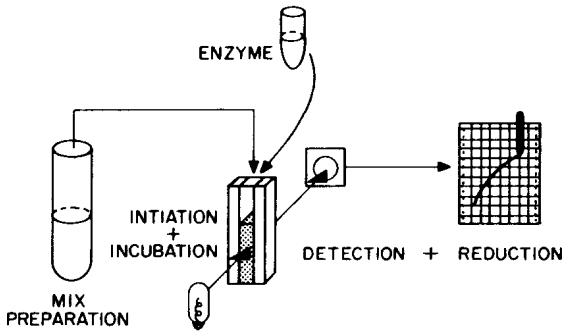

Figure 1.2 The assay of an enzymatic activity by the continuous assay method. In the illustration, the reaction mixture is transferred to a cuvette, which is shown in place in the light path of the spectrometer. The addition of the enzyme directly to the cuvette initiates the reaction. Product formation 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.

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 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, the reaction of interest, such as A→B and the second, the reaction that converts B to C and might be referred to an an *indicator reaction*, not only because it uses the product of the first reaction, that is, B, as a substrate, but also because the 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

$$\text{Phosphoenolypyruvate (PEP)} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP}$$

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:

$$\text{Pyruvate} + \text{NADH} \rightarrow \text{lactate} + \text{NAD}$$

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

In the discontinuous method, product must be separated from the substrate in order to measure activity. 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 are 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 of an amino acid, usually radioactive (as indicated by the asterisk in the reaction), to tRNA as follows:

$$\text{ATP} + {}^*\text{AA} + \text{Enz} \longrightarrow \text{Enz-AMP-}{}^*\text{AA} + \text{PP}_i \quad (1)$$

$$\text{Enz-AMP-}{}^*\text{AA} + \text{RNA} \longrightarrow \text{RNA-}{}^*\text{AA} + \text{AMP} \quad (2)$$

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 of an acid such as trichloroacetic acid (TCA) to the sample, which also serves to terminate the