ENZYME ASSAYS FOR FOOD SCIENTISTS

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Part I THEORETICAL

1 Introduction

No apologia is needed for considering the food industry as a major influence in the use of enzymes, or for recognizing the importance of enzymes to the processing and preserving of foods. The importance of enzymes to food scientists is well attested by the frequent appearance of enzymological papers in food-oriented research journals. To help deal with this flood of information, several authors have written or edited books on the topic of food enzymology. Libraries in food science departments have many such books on their shelves. Some of the more familiar ones are the texts by Reed (1975), Whitaker (1972) and Schwimmer (1981) as well as the symposium proceedings edited by Whitaker (1974). In addition, every year or two sees the publication of many books addressing some aspect of enzymes and the food industry, for example those edited by Linko and Larinkari (1980), Birch, Blakebrough and Parker (1981), Godfrey and Reichelt (1983) and Kruger, Lineback and Stauffer (1987).

The basic discipline of enzymology is well covered in numerous textbooks (Dixon and Webb 1979), multi-volume sets (Barman 1969; Boyer 1970), and frequent updates by series such as *Methods in Enzymology* or *Advances in Enzymology*. Some of the articles in those volumes are applicable to food enzymes, although many of them tend to be more oriented towards work in medical areas. And of course, there are several research journals which frequently publish articles on the isolation and study of enzymes in foods.

Given this plethora of information, the obvious question is, What need is there for another book on food enzymes? The present volume is intended to lie somewhere between the broad coverage of a text such as Schwimmer's (1981) and the detail of an article in *Methods in Enzymology*. The central focus of this book is on accurate, reliable methods for measuring the amount of enzymes in foods and food-related systems. It is not meant to be a cookbook, although the later chapters present experimental details which will be helpful in designing and testing an assay for an unfamiliar enzyme. It is not an in-depth theoretical treatise; that has already been done, and is readily available. However, the discussions in the earlier chapters should make clear the kind of requirements applicable to a good enzyme assay,

and demonstrate the limitations of many assays commonly used in the food industry. Finally, it is not a handbook, although a number of tables of useful data are found in various chapters.

This book is written as a tool for the working scientist who has some background in enzymes but is not an in-depth expert enzymologist. The job demands that he/she does a competent, professional study on the properties and uses of an enzyme, but does not allow the luxury of several months browsing in the library and performing trial-and-error experiments. This book will answer most of the questions regarding experimental design and data analysis and interpretation which arise during the course of the study of a food enzyme. Hopefully it will be regarded as a working manual, and not just another reference book on the shelf.

STRATEGY OF AN ENZYME STUDY

A comprehensive study of a particular enzyme includes the following steps.

1. Identify the catalytic activity of interest. Develop an assay which will allow the quantification of that activity. At this stage, simplicity and speed are more important attributes of an assay than are sophistication and accuracy. For instance, the hydrolysis of p-nitrophenyl-acetate in a rack full of test tubes may be very useful for testing fractions from a chromatographic separation of esterase, although the accuracy may be no better than ± 20%.
2. Purify the enzyme. This may be only partial isolation, not the achievement of complete purity. However, it should be carried to the point at which there is only one enzyme present (not two or more with similar activities) and endogenous inhibitors or activators are removed or identified.
3. Characterize the enzymatic activity. This step deals with the kinetics of the enzyme. At this stage more care must be taken in choosing an assay which will give appropriate experimental data. In investigating different properties (e.g., the pH-dependence of activity and the rate of heat-denaturation under various conditions), different substrates and assay conditions may be needed. Other characterizing parameters might be specificity (rate of reaction with different substrates) and the effect of inhibitors and activators.
4. Determine the enzyme's chemical and physical properties. Once a pure (single molecular species) material is obtained, there are a number of factors to be found: composition (amino acids, sugars, metal ions, and any other prosthetic groups), molecular weight (quaternary structure, if any), amino acid sequence, secondary and tertiary structure. Of practical importance is the stability of the enzyme against denaturation under conditions which might be found in use.

5. Integrate the protein characteristics and enzymatic nature of the molecule. The amino acid residues which make up the active site are identified by chemical and kinetic studies. By crystallographic studies the orientation of the substrate at the active site is determined. If the source of the enzyme is amenable to gene engineering, it may be possible to tailor the amino acids around the active site to alter activity or specificity in ways which are technologically desirable.

Different readers are undoubtedly involved with enzymes at different points in this broad, overall scheme. A researcher for a company which produces enzymes will spend much time in the early stages of purification, and a fair amount of effort in understanding the factors which lead to stability (or instability) of the enzyme during use. An applications scientist using the enzyme in food processing is perhaps more interested in the ways that pH, inhibitors and activators affect the enzyme. A university graduate student might be concerned with the first step of developing a workable assay for an enzyme in a foodstuff, or with the last step, depending upon the interests and funding available in the department.

In choosing an enzyme assay, the first point to establish is the level of information which is needed. An assay for quickly screening hundreds of fractions from a chromatographic column needs speed and convenience, not accuracy. For following the rate of heat denaturation of an enzyme less speed but a higher degree of precision is required. In standardizing enzyme preparations for food processing applications moderately high accuracy is needed, and the question of substrate specificity comes into play. For investigating the effect of pH on enzyme action a sensitive, accurate assay is required which will allow the determination of V_{max} and K_M. A similar sort of sophisticated assay is needed to properly study inhibitors and activators of the enzyme.

The first question the researcher must ask is, What do I really need to know from the assay? and secondly, What kind of assay will give me that level of information? An assay will give one of four kinds of information: 1. the number of moles of catalytic center present; 2. the rate of product formation under given reaction conditions; 3. the extent of product formation under conditions of use; or 4. the amount of substrate present, inferred from the rate or amount of product formation under defined assay conditions.

The first kind of information is of most importance to researchers engaged in enzyme purification or to bioengineers who are trying to find optimum conditions for producing an enzyme. In fact, most assays as normally conducted yield this information only by inference. The assay result (v) is related to the molar amount of enzyme present (E_t) via the appropriate rate equation. If no other factors (inhibitors, activators, multiple enzymes) vary, then the proportionality factor is in fact relatively constant, and the

inference is correct. Balanced against this uncertainty is the fact that this assay is probably much faster and easier to perform than an assay which would give an unequivocal measure of enzyme molarity. The researcher is usually willing to trade off reliability for speed; the challenge is to always be aware of the compromise and its possible consequences.

The second kind of information represents the vast majority of assay results as performed by food enzymologists and product developers today. Such assays are often used for standardizing enzyme preparations. The difficulty is that they give a false sense of comparability between different enzymes. The problem is usually one of specificity; the assay substrate is different from the actual substrate to which the enzymes are applied. Thus "100,000 H.U. (hemoglobin units)" of each of three proteases may, upon application to bread doughs, have quite different effects because they are now hydrolyzing a different protein substrate (Petit 1974).

The third type of information, performance under practical applications condition, is of extreme importance and also difficult to obtain. For example, an assay to measure the effectiveness of a protease in reducing chill haze formation in beer is highly desirable. The substrate is ill defined (some mixture of malt proteins and polyphenols), the conditions for activity (refrigerator temperatures) are less than optimum for the enzyme, and the time to reach the endpoint (days to weeks) is greater than the patience of the enzymologist. Hence the tendency is to use some quick, familiar measure of protease activity, and correlate it with the ability to prevent chill haze. Again, the benefits of the quick assay are considered to outweigh the risks inherent in depending on a correlation. If the enzyme is being used as an immobilized enzyme, an accurate assay will require that the configuration of the applications equipment be reproduced, because such factors as flow rate, turbulence, and viscosity can have a major impact on the amount of product formed per unit time per unit of enzyme protein present.

The fourth kind of information requires the use of enzymes as analytical tools. They may be utilized to measure residual glucose in egg whites, lactic acid in a sourdough culture, ethanol in wine or beer, pectin in a fruit extract; the applications are legion. Often in developing the analytical method full use is not made of the insights available from enzyme kinetics. Rather, an existing assay method for the enzyme is turned on its head, and amounts of enzyme, cofactors (if any), temperature, time and pH are adjusted semi-empirically until answers obtained by the enzymatic method agree with answers obtained from another, more traditional analytical method. The resulting method is usually wasteful of biochemical reagents, and the inherent experimental error is determined from making many replicate determinations rather than from considerations of the theoretical basis for the reaction.

At this point a word about the units in which we express the results of enzyme assays is appropriate. For certain kinds of assays, e.g., the titration assay for serine proteases, the results are expressed in fundamental chemical units, moles per gram of protein. This corresponds to the first kind of information. Far more common are units correlating with the second kind of information, namely the rate, in moles per time unit, of appearance of product or disappearance of substrate. During the 1970s the Enzyme Commission established an International Unit (IU) as that amount of enzyme which causes the formation of 1 μmole of product per minute under defined assay conditions. Thus one may express enzyme activity as IU per some amount of enzyme preparation (mg protein, ml solution, etc.). More recently, in order to bring enzymology into line with the CGS system, enzyme catalytic activity is expressed as the katal, which is that amount of enzyme which leads to the formation of 1 mole of product per second. One katal is equivalent to 60×10^6 IU, or 1 IU equals 16.67 nanokatals (a more convenient magnitude for ordinary work). At this time, most activities such as specifications in biochemical supply house catalogs are still expressed in International Units. Finally, note should be made of the practice of defining enzyme units in terms of rate of change of the instrumental signal (e.g., 1 unit = a change of 0.001 Absorbance unit per second). This is often found where the instrument signal cannot be readily connected to the molecular events which comprise the enzyme action (turbidimetric measurement of lysozyme activity), in which case we just have to live with it. But too often the publication of work based on this sort of "enzyme unit" merely indicates laziness on the part of the researcher who has not gone the extra step of translating instrument signal into product molarity. This greatly decreases the value of the work to other scientists.

REFLECTIONS ON ENZYME ASSAY DESIGN

"Why do we never have time to do it right, but we always have time to do it over?" This wry comment on the pressures which we all feel has been copied and posted over the desks of many product development personnel, graduate students, and analytical service laboratory technicians. Unfortunately it is all too often apropos. In the beginning of an enzyme project an assay is put together which detects the activity and measures it during the steps of purification. The plan is that once the enzyme is purified the question of an accurate, powerful assay will be addressed and the shortcomings of the current one will be corrected. Other more pressing questions seem to intervene, and suddenly it is realized that several notebooks have been filled with data on studies of the properties of the enzyme, all based on this admittedly inadequate assay. If the assay system is changed at this point, much of that data will be irrelevant and the experiments will have to be

repeated, a task for which the researcher doesn't seem to have the time. So we do the best we can with the existing data, and write up a publication (a paper, a technical data sheet, a method for the analytical control laboratory) which is based on a weak assay, and hope the weaknesses don't compromise subsequent research based upon our work.

It is inefficient to try to develop a powerful, accurate assay for an enzyme which is available only as a minor component of a crude homogenate or extract. The presence of many other complicating factors (inhibitors, activators, stabilizing and destabilizing species) make the interpretation of results from a sophisticated kinetic experiment problematic at best. Nevertheless, even at the early stages, as purification is proceeding, monitored by the inelegant but rapid assay, thought should be given to the design of an ideal assay. As some small amounts of relatively pure enzyme become available this design may be tested and refined. The ultimate goal is that at the point where purification of the enzyme is routine and adequate amounts are available for studies of its catalytic characteristics, the assay will also be available which will enable those studies to be done in a scientifically elegant manner. Then notebooks #2, 3 . . . will contain data which will stand up under the scrutiny of your scientific peers, and will form a firm foundation for further work by other researchers.

As an analogy, consider the measurement of pH. No one would make a precise study of the effect of pH upon an enzyme reaction based on measurement of pH using Universal Indicator Paper. At the least a pH-meter which can accurately measure to the nearest 0.05 pH unit would be used, and perhaps an expanded-scale meter if the rest of the experiment warranted the expense. Nonetheless Universal Indicator Paper has a definite place in the biochemical laboratory; it is excellent for adjusting a homogenate to neutral pH, say 6.5 to 7.5, where the expanded-scale meter would definitely qualify as overkill. In the same way, the rough-and-ready assay is fine for choosing the time at which to terminate a biofermentation for production of an enzyme. But when a detailed study of the effect of certain inhibitors upon that enzyme is initiated, the accuracy of the assay needs to be an order of magnitude better.

This analogy points up the real nature of an enzyme assay: it is a tool. For the most part biochemists do not study the theory of pH meter operation or spectrophotometry or chromatography for their own sake. The purpose of many laboratory courses is to make sure that students learn enough theory to operate the tools properly, recognizing both their power and their limitations. The object of most enzymatic research is to learn more about the enzyme, not about the tools used in the research. Nevertheless, having more powerful tools (assays) available, and knowing how to use them to the utmost, enhances the possibilities in exploring enzyme properties. It is

a truism that for the most part advances in science during the last 50 years have been initiated by advances in the tools available for doing that science. On a personal note, when I began my graduate research 30 years ago I felt lucky to have a Coleman Jr. colorimeter at my disposal. Before I finished, the availability of a Beckman DU UV-VIS spectrophotometer broadened the scope of the project, and a continuous-recording UV-VIS at my first research laboratory made even more sophisticated research possible.

Modern instrumentation does nof decrease the work load; it enables us to obtain more penetrating insights with the same amount of work. The scientist who worked 60 hours per week before getting a personal computer still works 60 hours per week, but the nature of the output has changed due to the added analytical power of the computer. But if the PC is only used to add, subtract, multiply and divide, it is not much better than a simple pocket calculator. For many fixed-time assays the Coleman Jr. is equivalent to the most modern spectrophotometer. Taking full advantage of the capabilities inherent in the modern instrument requires an understanding of the kinetics underlying the experiments we would like to do, just as making good use of a PC requires some understanding and use of programming language. This book might be called a BASIC primer for enzyme assays; mastering and applying the contents will enable you to achieve improved insights into the nature of the enzyme you are working with, through better application of the fundamental tool of the enzymologist, the assay.

While a cursory glance at the literature concerned with food enzymes gives the impression that most of the studies would have benefitted from a better application of kinetic theory, a caveat must still be made. The justification for a new, more penetrating study of an enzyme should not simply be "Now we can do it." The aimless application of any tool without some goal in mind is a waste of resources. As stated in the next chapter, first decide what level of sophistication is required to meet the goal, then work at that level with neither compromise downwards nor overkill. Don't try to shave with an axe, but don't split firewood with a straight razor. Fit the tool to the task; choose an assay which gives the information you need.

2 Kinetics

The description of enzymatic characteristics involves kinetic analysis. In some cases this might be rather superficial, while in other instances many details of the rate of reaction will be cataloged. The researcher should decide what level of information is required before embarking on large-scale experimentation. The enzyme of interest might be an α-amylase for desizing fabric; the desired information might be no more than a surface response map of pH, temperature and enzyme quantity, finding the time to produce a desired level of modification. In this case a detailed analysis of kinetic rate parameters would not be justified. On the other hand, a study might be ordered to measure all the parameters which would allow the prediction of the optimum temperature and pH for operation of a glucose isomerase column. Now a rather more detailed experimental design is needed, to obtain rate constants for both the forward and reverse reactions, the influence of pH and temperature on these rate constants, and also the rate constant for heat-inactivation of enzyme in the presence of substrate and product. Such a project would obviously entail much more in the way of data-gathering and analysis than the former example; nevertheless, both studies are kinetic in nature, and the appropriate level of planning must be applied in order to obtain good results with reasonable expenditure of resources.

Determining rate constants in the first example would probably be overkill, using more time and money than is warranted for the application's needs. In the second example, using a response surface methodology approach might solve an immediate problem, but would not provide the insights and detailed information needed to quickly solve future problems, where some variation in the process is desired. As a general observation, the laboratory researcher and the applications engineer often find themselves arguing about how much effort to spend on characterizing the enzyme. If instead they work together to define how much information is needed and how it will be used, they will both be doing a better, more professional job.

Once the level of sophistication of information is established, the design of experiments to obtain that data requires knowledge and some insights into the kinetics of enzyme action. These chapters on kinetics, inhibitors, pH and temperature will present the requisite knowledge. The insight comes

only from experience in working with enzymes, both in the laboratory and in the plant. If more detailed discussion is wanted, there are many excellent texts which deal with enzyme kinetics (Cornish-Bowden 1976; Fromm 1975; Reiner 1969; Segel 1975).

THE MICHAELIS-MENTEN RATE EQUATION

One Enzyme, One Substrate Systems

Rapid Equilibrium Model. In early investigations of enzyme reactions it was found that the rate of product formation was a hyperbolic function of the concentration of substrate (Figure 2-1). At low substrate levels doubling the substrate concentration led to doubling the rate of the reaction, while at high substrate concentrations the rate became independent of substrate concentrations. The key to this behavior (transition from a rate which was first order in substrate to one which was zero order in substrate) was first proposed by V. Henri in 1902 and elaborated by L. Michaelis and M. L. Menten in 1913.

The basic concept is that an enzyme-catalyzed reaction involves two steps: 1. the reversible, rapid combination of enzyme (E) and substrate (S) to form a complex (ES); and 2. a somewhat slower breakdown of ES to give product (P) and regenerate free enzyme. The key idea is that E, S, and ES are in rapid equilibrium characterized by an equilibrium *dissociation* constant K_m. The rate of formation of P (i.e., v, the rate of the reaction) is directly proportional to the concentration of ES at any given time. The kinetic model is given as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad K_m = \frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_1} \tag{2-1a}$$

$$ES \xrightarrow{k_2} E + P \qquad v = \frac{dP}{dt} = k_2[ES] \tag{2-1b}$$

The basic Michaelis-Menten (M-M) equation is readily derived from this model by remembering that the total enzyme added, E_t is equal to $E + ES$, and defining a parameter V_{max}, the velocity at high $[S]$, as equal to $k_2[E_t]$. The derivation is shown in Appendix A, and the M-M equation is:

$$v = \frac{V_{max}[S]}{K_m + [S]} = \frac{dP}{dt} = \frac{k_2[E_t]}{k_{-1}/k_1 + [S]} \tag{2-2}$$

Steady-State Model. In about 1925 G. E. Briggs and J. B. S. Haldane pointed out that when enzyme and substrate are initially combined the only