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REVIEW

Enzyme assays ☆

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Abstract
The essential requirements for enzyme assays are described and frequently occurring errors and pitfalls as well as their avoidance are discussed. The main factors, which must be considered for assaying enzymes, are temperature, pH, ionic strength and the proper concentrations of the essential components like substrates and enzymes. Standardization of these parameters would be desirable, but the diversity of the features of different enzymes prevents unification of assay conditions. Nevertheless, many enzymes, especially those from mammalian sources, possess a pH optimum near the physiological pH of 7.5, and the body temperature of about 37 °C can serve as assay temperature, although because of experimental reasons frequently 25 °C is preferred. But in many cases the particular features of the individual enzyme dictate special assay conditions, which can deviate considerably from recommended conditions.
In addition, exact values for the concentrations of assay components such as substrates and enzymes cannot be given, unless general rules depending on the relative degree of saturation can be stated. Rules for performing the enzyme assay, appropriate handling, methodical aspects, preparation of assay mixtures and blanks, choice of the assay time, are discussed and suggestions to avoid frequent and trivial errors are given. Particularities of more complex enzyme assays, including reversible reactions and coupled tests are considered.
Finally the treatment of experimental data to estimate the enzyme activity is described. The procedure for determining the initial enzyme velocity and its transformation into defined enzyme units as well as suggestions for documentation of the results are presented.

Contents

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Introduction

Enzyme assays are performed to serve two different purposes: (i) to identify a special enzyme, to prove its presence or absence in a distinct specimen, like an organism or a tissue and (ii) to determine the amount of the enzyme in the sample. While for the first, the qualitative approach, a clear positive or negative result is sufficient, the second, the quantitative approach must deliver data as exact as possible. A great advantage of enzymes is that they can be identified by their catalysed reactions, in contrast to the other components of the cell, like functional proteins or nucleic acids, which must be determined by direct detection. During the enzyme reaction product accumulates in amounts exceeding by far the intrinsic enzyme concentration. However, the conclusion from the product formed back to the amount of enzyme in the sample comprises various difficulties and pitfalls.

Procedures for enzyme assays are documented or cited in various standard books (Methods in Enzymology; Advances in Enzymology and Related Areas of Molecular Biology; Methods of Enzymatic Analysis (Bergmeyer, 1983); Springer Handbook of Enzymes (Schomburg, 2009); Practical Enzymology (Bisswanger, 2011) and databases (ExPASy database; Brenda database), but even accurate observance gives no guarantee of an unequivocal outcome. The same assays performed independently under obviously identical conditions may yield quite different results. In fact, the enzyme activity depends on manifold factors and general understanding of the particular features of enzymes is required, which cannot be described in all details in protocols for special enzyme assays. The most important aspects to be considered for enzyme assays are the subject of this article.

It was the merit of Leonor Michaelis and Maud Menten (Michaelis and Menten, 1913) to realize that the enzyme activity depends decisively on defined conditions with respect to temperature, pH, nature and strength of ions and enzyme assays can reliably only be compared, if such conditions are strictly regarded. Considering these conditions, it may appear a simple task to define general rules valid for all enzyme assays, but such an endeavour will fail because of the great diversity of enzymes and their features. Enzymes display their highest activity at their respective optimum conditions, deviations from the optimum cause a reduction of the activity, depending on the degree of the deviation. Moderate deviations produce only small activity decreases which can be tolerated (Figure 1), and so the physiological conditions prevailing in the cell may be taken as standards for at least of the mammalian enzymes. However, assay procedures are usually adapted directly to the features of the individual enzyme and not to obey general standards. Enzymes are sensitive substances present in small amounts and their activity in the cell can often be detected only at their optimum conditions. Various enzyme reactions require special conditions, e.g. if the thermodynamic equilibrium is unfavourable. Other enzymes, especially from extremophilic organism are only active under conditions completely different from the physiological range.

For enzyme assays it must be considered that enzymes reactions depend on more factors than pH, temperature and ionic strength.[2] Of great importance are the actual concentrations of all assay components. Further influences of compounds not directly involved in the reaction may occur, e.g. interactions of ions, especially metal ions, hydrophobic substances or detergents with the protein surface,[3]

[2]The dependence on pressure is usually not considered, because of the resistance to high pressure of proteins compared with the relatively weak fluctuations of atmospheric pressure.

[3]In this article enzymes are regarded to consist of protein, but the considerations are also valid for other enzyme classes, like ribozymes and artificial enzymes.

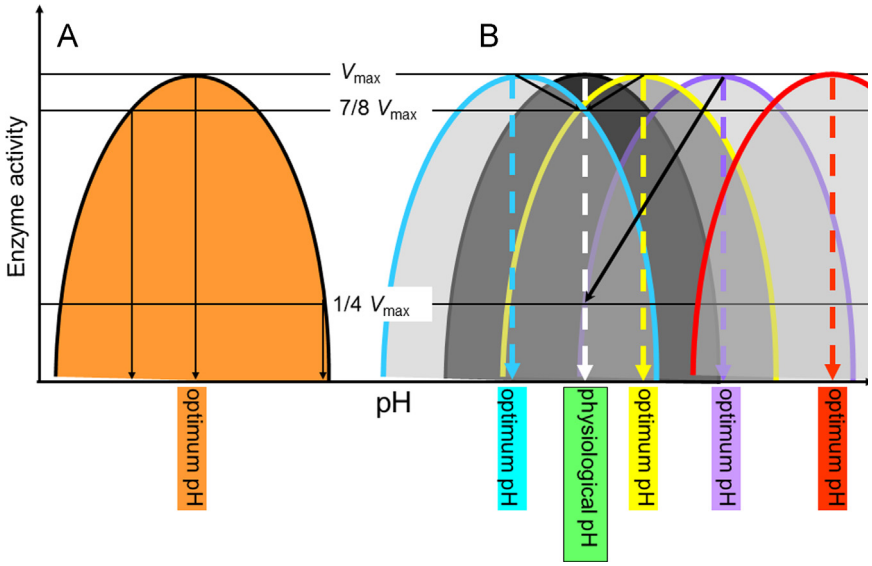

Figure 1 Difficulties to define general standards for enzyme assays with the example of the pH dependency. (A) Schematic pH curve with the highest activity (V_{max}) at the optimum. The arrows left and right from the optimum show that the enzyme activity can be determined also at a pH outside the optimum, however, but smaller values must then be accepted (the arbitrarily chosen ratios of V_{max} should symbolize the degree of decrease). (B) Enzymes differ in their pH optima and not every enzyme has its pH optimum activity just at the physiological pH (black curve). But accepting decreased activity, a greater number of enzymes can be measured at one standardized pH (blue and yellow curves), while for other enzymes considerable reductions occur (pink curve), they will be tested preferentially at their own pH optimum. Enzymes whose pH optima range completely outside the physiological range (red curve) appear inactive there and must be tested at their own pH optimum.

either stabilizing, e.g. as counter ions, or destabilizing. For example, enzyme reactions dependent on ATP need Mg^{2+} as essential counter ions. If only ATP without Mg^{2+} is added to the assay mixture even in sufficient concentration, it can become limiting, especially if complexing compounds, like inorganic phosphates or EDTA are present.

Essential conditions for enzyme assays

General considerations

Although detailed descriptions of enzyme assays can be found in the relevant literature (Methods in Enzymology; Advances in Enzymology and Related Areas of Molecular Biology), Methods of Enzymatic Analysis (Bergmeyer, 1983), Springer Handbook of Enzymes (Schomburg, 2009), Practical Enzymology (Bisswanger, 2011), and (ExPASy database; Brenda database), it is often necessary to modify the procedure, e.g. to adapt it to the special features of an individual enzyme or to differing instrumentation. In particular situations a new assay must be developed, for a newly discovered enzyme, for example. For all such cases, but even when performing standard procedures, it is important to consider the general rules valid for all enzyme assays.

The predominant rule is the clear and easy mode of observation of the enzyme reaction. Common to all enzyme-catalysed reactions is the fact that a substrate becomes converted into a product and thus the aim of any assay is to observe the time-dependent formation of the product. To achieve this, a procedure must be found to identify the product. Since formation of product is directly connected with the disappearance of substrate, its decline is an adequate measure of the reaction. In cases where two or more products are formed, or two or more substrate molecules are involved in the reaction, the determination of only one component is sufficient.[4] Obviously the easiest detectable reaction component will be chosen.

A simple but important condition is that substrate and product must differ in the observed feature. The product may be very well detectable by a distinct method, but if the substrate shows a similar signal with equal intensity, no turnover can be observed at all. Often both components show a small difference of otherwise similar large basic signals, especially when only small molecular modifications occur, as with many isomerase reactions (Figure 2). Such changes may be principally detectable, but are usually difficult to quantify, because large signals are mostly subject to strong scattering, so that the small change produced by the enzyme reaction becomes lost within this noise. In such cases the signal to noise ratio must be analysed (Figure 2, right). As a rule the intensity of the signal displayed by the reaction must exceed the noise at least by a factor of two. This is a general problem, since any method is to a more or less extent subject to scatter. Scattering can have various origins, some, e.g. instability of the instruments or measurements in turbid solutions like cell homogenates, cannot be avoided, while others, like contaminations, turbidity caused by weakly soluble substances, soiling, dust or air bubbles can at least be reduced by careful handling. Scattering is also lowest if only the observed component (substrate or product) produces the signal (e.g. an absorption), while the other components

[4]The stoichiometric ratio must be considered, e.g. if two equal substrate molecules produce only one product, like the formation of an oxygen molecule from two oxygen atoms.

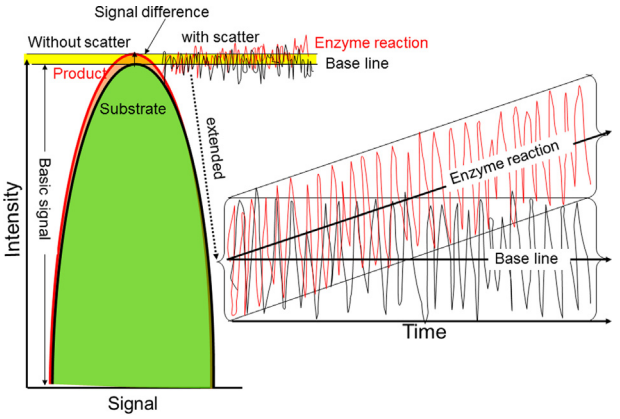

Figure 2 Difficulties to observe an enzyme reaction, when both substrate and product show a similar large signal with only a small difference between them (left side). Vigorous scattering of the large signal superposes the weak increase produced by the enzyme reaction. Right side: signal-to-noise ratio: for strongly scattering data the intensity of the signal, i.e. the enzyme reaction, must exceed the basic noise at least by a factor of two.

show no signal (no absorption) in the observed range, so that the reaction starts actually at zero and any change in the signal indicates the ongoing reaction.

Methods for observing the enzyme reaction

In the simplest case an enzyme reaction can be observed by the appearance (or disappearance) of a coloured compound, so that it can be even observed by eye. The advantage is not just to avoid the use of an instrument; rather the reaction can immediately and directly be controlled, excluding any operating error. Such a procedure, however, will yield no accurate and reproducible data and therefore an appropriate instrument, a colorimeter or a photometer, must be applied to determine the colour intensity. Various types are available and because of their broad applicability also for determination of proteins, nucleic acids and metabolites such an instrument should belong to the standard equipment of any biochemical laboratory. Spectrophotometers covering also the invisible UV range, where practically all substances show absorption, extend the observation range considerably. Due to the relative easy handling and the low susceptibility against disturbances photometric assays are applied as far as possible (Cantor and Schimmel, 1980; Chance, 1991; Harris and Bashford, 1987).

If an enzyme reaction cannot be observed photometrically, other optical methods may be used. Fluorimetry is more sensitive than absorbance measurements (about hundredfold), but only a few enzymatic substrates or products emit fluorescence, such as NADH and some artificial substrate analogues. Spectrofluorimeters are more complicated to handle and there exist more sources for errors, therefore fluorimetric assays are unusual, and a deeper experience is needed (Cantor and Schimmel, 1980; Harris and Bashford, 1987; Guibault, 1990; Lakowicz, 1999; Dewey, 1991). Similar arguments hold for CD and ORD measurements, which are valuable techniques for the observation of asymmetric compounds, like sugars (Cantor and Schimmel, 1980; Chance, 1991; Adler et al., 1973). Enzymatic degradation of particles, like starch, can be observed by turbidimetry (Bock, 1980), while luminometry is applied for ATP dependent reactions (Campbell, 1989; DeLuca and McElroy, 1978). Besides optical methods, electrochemical methods are in use, especially pH determinations for reactions proceeding with pH changes, like the liberation of acids by lipase or choline esterase. Since pH changes influence severely enzyme activity, a pH stat connected with an auto-burette is used, which keeps the pH constant by adding a neutralizing solution, its amount being a direct measure of the proceeding reaction (Taylor, 1985).

The methods mentioned so far allow the continuous, time-dependent following of the enzyme reaction (*continuous assay*). This is important for the determination of the reaction velocity and for evaluating the enzyme activity. Moreover, it permits the detection of erroneous influences and artifactual disturbances and especially the control of the reaction course (*progress curve*). As will be discussed below, a catalysed reaction must initially follow a linear relationship, from which its velocity is derived. Due to depletion of substrates during the later progression the reaction slows down and finally ceases. Therefore it is important that for determination of the velocity only the linear part of the progress curve is taken, but if it is not possible to observe the complete progress curve, it cannot be confidently excluded, that calculation of the velocity includes also the non-linear part of the progress curve and aberrant results will be obtained (Figure 3). This holds for all cases, where no direct signal for the conversion of substrate or product can be found. To determine the velocity the reaction must be stopped after a defined time and the amount of product formed or substrate converted must be analysed thereafter by a subsequent chemical indicator reaction or a separation method, like HPLC (*stopped assay*). Instead of a continuous progress curve these methods provide only one single point and the

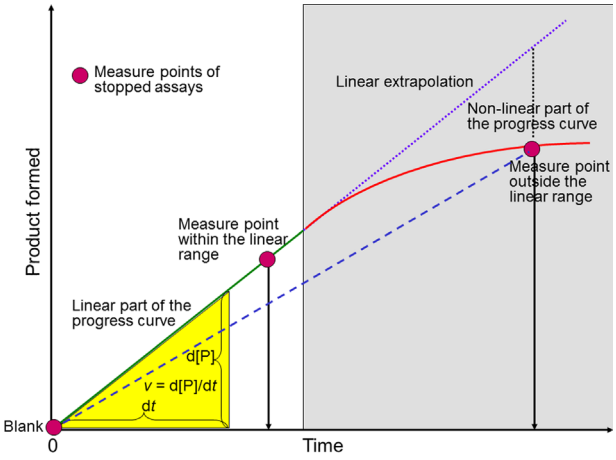

Figure 3 Progress curve of a typical enzyme reaction. The velocity is obtained from the slope of the linear part of the curve, referred to a distinct time unit (1 min or 1 s). Stopped assays provide only one measure point; the velocity is derived from the slope of a line connecting this point with the blank at the start of the reaction. Correct results will only be obtained, if the measure time lies within the linear part of the progress curve. If it extends outside into the non-linear part erroneous data will be obtained.

velocity must be calculated from the slope of a line connecting this point with the blank before starting the reaction. Such a procedure gives no guarantee that the measurement occurs indeed within the linear part of the progress curve and therefore control measurements at different reaction times must be undertaken to establish this fact. These procedures are laborious and especially for quantitative measurements continuous assays, if any possible, are preferred, while stopped assays are equally useful for qualitative determinations, where only the presence of the enzyme activity should be detected.

Influence of the pH on enzyme assays

The activity of enzymes depends strictly on the pH in the assay mixture. The activities of most enzymes follow a bell-shaped curve, increasing from zero in the strong acid region up to a maximum value, and decreasing to zero to the strong alkaline region (Figure 4). Two different effects are responsible for this behaviour: (i) the state of protonation of functional groups of amino acids and cofactors involved in the catalytic reaction and (ii) the native, three-dimensional protein structure of the enzyme. While protonation is a reversible process, damaging of the protein structure is mostly irreversible. In the simplest case protonation of one functional group promotes the catalytic activity, while protonation of another essential group breaks it down. In this case two conventional titration curves, an increasing and a decreasing one, form the bell-shaped curve. The inflexion points of the curves at half-maximum velocity ($V_{max}/2$) indicate the pK_a-value approximately, i.e. the pH at which the respective group is just half dissociated. The pK_a-values can help to identify the functional group, but it must be regarded, that pK_a-values of amino acids integrated into the protein structure can be changed by up to ± 2 pH units. More complex catalytic centres consist of several ionizable groups and the pH optimum curve becomes a superposition of various titration curves.

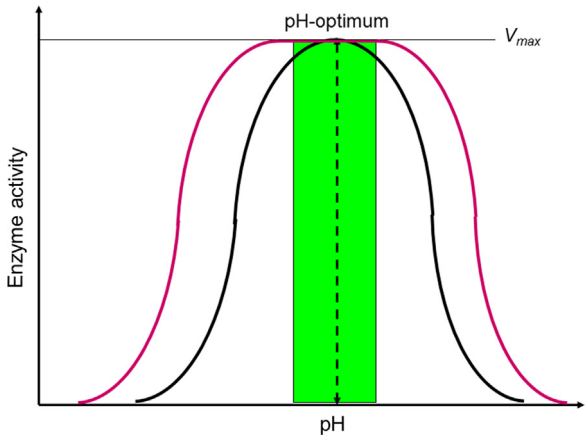

Figure 4 pH optimum curve for the activity of an enzyme (black). The pH of the maximum is the pH optimum; the inflection points indicate the respective pK_a values. The green area shows the physiological range. The red line shows the broader pH stability curve of the enzyme.

The pH-value of the maximum of the pH-activity curve is the pH optimum. Since here the enzyme exhibits its highest activity (V_{max}), it is usually chosen as standard pH for the assay of this enzyme. The pH optimum of many enzymes is within the physiological range (about pH 7.5), not in any case accurately at this pH, but frequently between pH 7–8. Since the optimum curve has a broader maximum, the physiological pH can be taken in such cases without considerable reduction of the enzyme activity (Figure 4).

The pH optima of some enzymes, however, are far away from the usual physiological range. A prominent example is pepsin, the protease of the stomach, with a pH optimum of 2, the optimum of the acid phosphatase is at pH 5.7, that of the alkaline phosphatase at pH 10.5 (Brenda database). Such enzymes must be tested at their own optima. Sometimes particular conditions recommend an assay pH different from the pH optimum. The activity optimum of alcohol dehydrogenase is just at the physiological pH (7.5) and there it can easily be tested with acetaldehyde and NADH as substrates. However, manipulating the toxic and volatile acetaldehyde, and starting the reaction with the strongly absorbing NADH; is inconvenient. Due to reversibility of the reaction, the enzyme can likewise be tested with ethanol and NAD (which do not absorb in this range) as substrates, but the equilibrium is already on this side, disfavouring the formation of acetaldehyde and NADH. The reaction, however, can be forced in the opposite direction by applying an alkaline pH of 9.0, which causes deprivation of H^+ ions (Bergmeyer, 1983).

Normally the enzyme is fairly stable at its own pH optimum, and so this is recommended not only for testing, but also for storage. This is also of some importance for the performance of enzyme assays, since addition of an aliquot of the enzyme stock solution to the assay mixture will not affect the assay pH. Sometimes, however, the stock solution of the enzyme possesses a different pH, like trypsin, which should be stored at a strong acid pH of 3.0 albeit its alkaline pH optimum of 9.5, in order to suppress autolysis (unlike most other enzymes, trypsin tolerates this extreme pH) (Bisswanger, 2011). In such cases care must be taken that the added aliquot does not modify the pH of the assay mixture, a circumstance, which must be considered for any addition, if its pH deviates from that of the assay mixture.

While the enzyme is stable within the range of its pH optimum, more extreme pH values in both directions attack its tertiary structure in an irreversible manner. This process is time-dependent and depends on the effective pH, the further it deviates from the optimum pH, the faster the inactivation. In strong acid (<3) as well as at strong basic (>11) pH inactivation occurs practically at once, therefore contacts of the enzyme with such pH values, even for short time, and must strictly be avoided (with the exception of special enzymes resistant to such conditions, like trypsin). A pH stability curve shows the dependence of the stability of the respective enzyme on the pH (Figure 4). It is similar in its shape, but broader than the bell-shaped pH curve.

Buffers and ions

Buffers serve to adjust and stabilize the desired pH during the enzyme assay. They consist of a weak acid and a strong basic component. The relationship between the pH and the

buffer components is described by the Henderson-Hasselbalch equation:

$$pH = pK_a - \log [HAc]/[Ac^-]$$

HAc and Ac^- is the acid in the non-dissociated and the dissociated form, respectively, $pH = -\log[H^+]$ is the negative logarithm of the proton concentration, $pK_a = -\log K_a$, the negative logarithm of K_a, the dissociation constant of the buffer components. The pK_a value indicates the pH, where the buffer components are just half dissociated; at this point the buffer possesses its highest buffer capacity. It is accepted that the capacity of buffers comprises a range from one pH unit below to one pH unit above the pK_a value (a more strict rule allows only a deviation of ± 0.5). Lists of commonly applied buffers with their respective pK_a values are given in the standard literature (Bisswanger, 2011; Cooper, 1977; Tipton and Dixon, 1979; Stoll and Blanchard, 1990; Perrin and Dempsey, 1979), where a suitable buffer system for covering the pH optimum of a special enzyme can be found. Prepared buffer solutions and reference standard buffers are available from various suppliers. Besides the appropriate pH range, for buffers two further criteria must be considered, the ionic strength and concentration, and the nature of buffer components.

The more concentrated a buffer system, the higher its capacity to stabilize the pH. However, most enzymes accept only moderate ionic strength, commonly between 0.05 and 0.2 M, only halophilic and thermophilic enzymes prefer higher concentrations up to 1 M (Vieille and Zeikus, 2001; Rainey and Oren, 2006; Gerday, 2007). On the other hand, low ionic strength destabilizes the protein structure. It must be further taken into account that each component of the assay mixture, like substrates, cofactors, and additives like stabilizing factors (e.g. enzymes are frequently stored in concentrated ammonium sulphate solutions) contributes to the overall concentration. Moreover each addition can influence the adjusted pH, for example when a component (substrate, cofactor, or effector) is added in an acid or alkaline form without previous neutralisation. While the buffer neutralizes low amounts, this need not be the case with higher amounts. Since any deviation from the pH optimum reduces obligatorily the enzyme activity, such an effect can easily be misinterpreted as enzyme inhibition: the more of the particular component is added, the lower the enzyme activity.

The enzyme reaction itself can cause pH shifts and consequently a continuous decrease of the activity, e.g. if an acid or alkaline component becomes released during a cleavage reaction, like the liberation of fatty acids by lipase. In such cases only short initial reactions should be measured under continuous control of the actual pH in the solution. Alternatively, the pH can be kept constant applying a pH stat with an auto-burette, containing a neutralizing solution. The amount of this solution required for stabilizing the pH is a direct measure of the reaction rate (Taylor, 1985).

Ions influence the enzyme activity both by means of their ionic strength and by their nature. The activity of a distinct enzyme can considerably differ when tested in two distinct buffer systems, even if they share the same pH and concentration. Various reasons are responsible for this behaviour. In some cases components of the buffer, like mono- or divalent metal ions influence directly the catalytic process, if required as essential cofactors, or by displacing the intrinsic factors. Complexing agents, like diphosphate (even monophosphate has a weak complexing capacity) can sequester essential ions, e.g. from ATP-dependent reactions, which require Mg^{2+} as counterions. Since ATP and not Mg^{2+} is the reacting component, such effects can easily be overlooked.

Components of the buffer may have stabilizing or destabilizing influences on the protein structure. Destabilizing effects are incidentally ascribed to the frequently used Tris buffer (tris(hydroxymethyl)aminomethane). Especially recommended are the *biological buffers* or Good buffers, like MOPS (3-(*N*-morpholino)propanesulfonic acid), HEPES (*N*-(2-hydroxyethyl) piperazine-*N*′-ethanesulfonic acid), or TES (*N*-tris(hydroxymethyl)methyl-2-aminoethansufonic acid) (Good and Izawa, 1972; Good et al., 1966; Ferguson and Good, 1980). With the restriction of weak complexing capacity monophosphate buffers with potassium or sodium as counter ions are broadly applicable.

As already mentioned above, the capacity range of buffers is narrow, comprising two pH units at best. If a broader range is required, e.g. for analysing the pH dependence of an enzyme, several buffer systems may be combined. This is, however, an unsatisfactory procedure, due to the varying activities of the enzymes in different buffers. In such cases universal buffers, like the Teorell-Stenhagen and the Britton-Robinson buffer, consisting of more than two components and covering a broad pH range, should be used (Bisswanger, 2011; Teorell and Stenhagen, 1939).

Finally it must be considered that dissociation of compounds and, consequently, also of buffers, depends strongly on the temperature. Therefore the pH changes with the temperature and for exact pH specification the prevailing temperature must be indicated. Usually 20 °C is used as standard temperature for buffers and the pK_a values refer to this temperature.

Solvents

According to the cellular milieu water is the standard solvent for enzyme assays. Only for special cases, like enzymes connected with the membrane, e.g. lipases, apolar organic solvents are used, while such solvents will denature most enzymes. However, for some enzyme assays organic solvents cannot be completely avoided, e.g. when an essential component, like a substrate, is sparingly soluble in water. It must be dissolved in higher concentration in an organic, water-miscible solvent, like ethanol, DMSO or acetone. An aliquot of this solution is added to the assay mixture, where it should remain dissolved in its final concentration. To keep the concentration of the organic solvent in the assay mixture as small as possible the volume of the aliquot should be rather small. In such cases the problem arises that smaller volumes require a higher concentration of the component in the organic solvent and it may immediately precipitate upon addition to the aqueous assay mixture. To prevent precipitation either the final concentration of the weakly soluble compound in the assay mixture must be kept rather low, or the fraction of the organic solvent in water must be higher to mediate solubility. So the ratio of the organic solvent in the assay mixture is directly connected with the concentration of the weakly soluble compound and sometimes lower concentrations than effectively required must be accepted. Further it has to be

considered that solubility depends strongly on temperature, e.g. the compound can be just soluble at the assay temperature, but may precipitate if the assay mixture is kept in the cold before testing. Even if the ratio of the organic solvent in the assay mixture is not so high to denature the enzyme, it can influence its activity. Therefore, to compare the results of different assays, the volume of the organic solvent added to the assay mixture must always be kept constant, even if the concentration of the weakly soluble substrate is reduced.

Dependence of the enzyme activity on the temperature

The temperature dependence of the activity of enzymes resembles in some respect the pH dependence: increasing with rising temperature, passing a maximum, followed by a decrease. Therefore this behaviour is frequently described as *temperature optimum*, although an optimum temperature for the enzyme activity does not necessarily exist at all. Indeed, two counter-acting processes are responsible for this behaviour (Figure 5). The velocity of any chemical reaction increases with temperature, according to an empirical rule two to three times every 10 °C. This holds also for enzyme reactions and only boiling of water limits this progression. On the other hand the three-dimensional structure of enzymes is thermo-sensitive and becomes destabilized at high temperature causing denaturation. This process opposes the acceleration of the reaction velocity and is responsible for its decline at high temperature. The progression of denaturation depends both on the actual temperature and on time, the higher the temperature, the faster denaturation. Therefore, no fixed temperature can be given for the maximum enzyme activity; rather it depends on the pre-treatment of the enzyme. If the enzyme is immediately tested at a moderate denaturation temperature, its activity will be considerably higher than if it is kept at the same temperature for a longer time before starting the assay. Such a situation can easily arise if a certain time is needed to prepare and start the assay, while the enzyme is already present in the thermostatted assay mixture. During this time denaturation already proceeds and since such preparation times are not always equal, the loss of the enzyme activity will also vary (Figure 5).

For assay temperatures specified in the assay protocols usually such facts are taken into account, but with special applications, e.g. enzymes that have not yet been investigated, it should be ensured that the assay temperature is within the stability range. Some enzymes (e.g. alcohol dehydrogenase) denature slowly even at the physiological temperature (37 °C). In the living organism components of the cell, especially the high protein concentration, act as stabilizers, but even there the lifetime of enzymes is limited and they are steadily supplemented by *de novo* synthesis (Hinkson and Elias, 2011). To establish the appropriate assay temperature for a distinct enzyme, the temperature dependence of its activity must be analysed. Plotted in the Arrhenius diagram (Figure 5B) a straight line should be obtained in the lower temperature range indicating the area of enzyme stability, while beginning denaturation in the higher temperature range causes a deviation of the straight line. The assay temperature must be within the linear range, although the enzyme possesses there not its maximum activity.

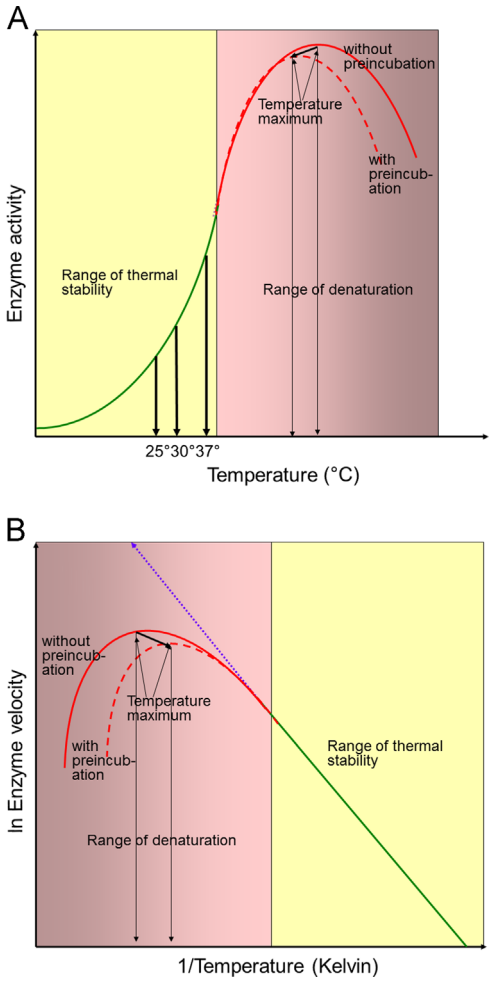

Figure 5 Typical dependence of the enzyme activity on the temperature. (A) Direct plotting and (B) Arrhenius diagram. The green lines represent the range of the increase of the reaction velocity with the temperature; its continuation (dotted violet line) is interrupted by progressive inactivation (red lines). Inactivation is forced by pre-incubation of the enzyme at the high temperature, causing a decrease and shift of the temperature maximum to the lower range (black arrows). In (A) the three most commonly used assay temperatures are indicated.

From these considerations it becomes clear that a general standard temperature for all enzyme assays cannot be defined. For the majority of assays, especially for mammalian enzymes, three distinct temperatures are in use. The physiological temperature, 37 °C, matches directly the natural condition of the enzyme and, compared with the other two assay temperatures, the enzyme develops there its highest activity, i.e. the lowest enzyme amounts are required (Figure 5A). However, this temperature is nearest to the denaturation range, and it requires efficient thermostatting. Since the assay mixture is usually stored at low temperature, a considerable time of several minutes to warm up the assay is needed. The attainment of the proper temperature should be controlled, but to save time, especially with larger test series, the experimenter may be tempted to shorten the thermostatting time and the

reaction will in fact proceed with reduced activity. To save time a separate thermostatting device is recommended, where one sample can already be pre-thermostatted while measuring the actual sample.

Performing the assay at room temperature may eliminate the problem of thermostatting. Room temperature, however, is not constant; it varies not only between different laboratories, but changes also in the same room upon opening or closing windows and doors, radiation of sunlight, or defective air conditioning. Therefore a slightly elevated temperature, 25 °C, is used. Here thermostatting is not very crucial, the accurate temperature will be attained within a short time and even insufficient thermostatting cause only slight aberrations of the results. Compared with tests at the physiological temperature, however, the activity is evidently lower and thus significantly more enzymes is needed to obtain comparable velocities (Figure 5A). Nevertheless, due to the easier manipulation and more robust data most protocols suggest 25 °C as assay temperature. This is convenient for simple and routine assays as long as enough enzyme material is available, while for more thorough investigations of enzymes the physiological temperature should be preferred.

The third of the frequently used temperatures, 30 °C, is a compromise between the other two. It is closer to the physiological temperature but easier to achieve, the enzyme is more active than at 25 °C, and thermal denaturation must not be feared.

In special cases none of these three temperatures can be employed. Enzymes from thermophilic organisms, growing at temperatures up to and even above the boiling point of water, show very low activities at moderate temperatures and should preferentially be tested at the growth temperature of their organism (Vieille and Zeikus, 2001; Rainey and Oren, 2006; Gerday, 2007). But even with such enzymes thermal denaturation must be considered. Several thermophilic enzymes show indeed a remarkable stability at high temperatures, while others are unstable in pure preparations and obviously need the stabilizing capacity of cellular components. Tests at high temperatures are more complicated, not only due to more difficult thermostatting. Other components of the assay mixture may become unstable and oxidation processes are accelerated.

Dependence of enzyme assays on substrates and cofactors

Besides the enzyme itself, substrates, co-substrates and cofactors[5] are the most important components of the enzyme assay. Their state, their purity and stability is of particular importance and highest demands have to be made for these substances. With respect to the substrates a significant aspect must be considered. Usually it is taken that the enzyme has a defined substrate according to its physiological function, as lactate dehydrogenase oxidizing lactate to pyruvate, or fumarase forming malate from fumarate. However, the substrate is not clearly defined in every case. Many enzymes show broad specificity, accepting also substances structurally related to the physiological substrate, like alcohol dehydrogenase, which reacts with various alcohols. The same holds for cofactors. Divalent cations are essential cofactors for many catalytic reactions and they can often be substituted by other divalent cations. An interesting example is glucose isomerase, a microbial enzyme. Its physiological substrate is xylose, which becomes isomerized to xylulose with Mn^{2+} acting as essential cofactor. Due to its capacity to isomerize also glucose to the more valuable sugar fructose, the enzyme gained great interest in biotechnology. This non-physiological reaction proceeds more efficient with Co^{2+} than with Mn^{2+}. So the change of the substrate causes also a change of the cofactor (Antrim et al., 1979; Lehmacher and Bisswanger, 1990). In other cases the physiological substrate is replaced by an artificial, synthetic substrate, e.g. if the physiological substrate is unstable or, as in the case of proteases, if the (protein) substrate is not well defined, rather the single peptide bond within the protein must be regarded as the genuine substrate.

If the enzyme accepts different substrates, the question arises which substrate should be used for the enzyme assay? Due to the varying catalytic efficiency, results obtained for the same enzyme, but with different substrates, will hardly be comparable. The efficiency of a substrate is determined by its K_m value, the lower this value the better the substrate. Usually the most efficient substrate may be taken, but also other aspects must be considered, like the availability, stability, solubility and the accessibility to a detection method. Sometimes natural substrates are modified to facilitate the detection. So it is not always the physiological substrate which is applied for the enzyme assay, but it is obvious that for comparison of the results the same substrate must always be used.

Practical considerations

Preparation of the assay mixture

For simple enzyme assays, like some proteases, only the enzyme and the substrate in a buffered solution are needed. But most assays require various components, two to three substrates, cofactors, activators, and reagents for stabilization or prevention from deactivating processes, like oxidation or proteolysis. These components can be added step by step to the assay until, with the last addition, the reaction starts. Such a procedure is not only laborious and time consuming, especially for extensive test series; it is also not very accurate. Pipetting is usually the severest source of error and, therefore, pipetting steps should be reduced as far as possible. Especially pipetting of small volumes proceeds with higher uncertainty than of larger volumes.

[5]One must distinguish between different notions: prosthetic groups are non-protein components required for the catalytic process, which are bound in non-dissociable, mostly covalent mode to the enzyme (e.g. lipoic acid); therefore they must not be separately supplemented to the enzyme assay and are not considered in this article. Cofactors are non-protein dissociable groups indispensable for the catalytic process, like essential metal ions and coenzymes; they must be included in the enzyme assay, and are accordingly considered here. Coenzymes are dissociable non-protein components, which become converted during the enzyme reaction (e.g. NAD); they are considered not separately, but taken as cofactors.

Therefore it is advantageous to prepare a larger quantity, an *assay mixture* for the whole test series instead of executing each assay sample separately. The assay mixture should contain all necessary components in their final concentrations, with the exception of one, which is added finally to the individual assay sample to start the reaction. If, for example, 5 components of 2 μl must be added step by step to an assay sample of 1 ml, 500 pipetting steps are required for 100 tests, while only 5 pipetting steps of 0.2 ml are required to prepare 100 ml assay mixture. Besides time saving the accuracy increases significantly, as the scatter of the data will considerably be reduced, because all samples (with the sole exception of the last component to be added to start the reaction) possess exactly the same composition. This opens, however, the risk, that an error of one single step, e.g. wrong pipetting, obligatorily affects all assays, while by direct pipetting only the one sample, where the error happens, will be concerned. Nevertheless, the risk is minor, since preparation of a large quantity with few single steps can (and *should*) be done with great care, while such care cannot be given to any of the separate assays.

The required components are preferentially added to the assay mixture from concentrated stock solutions. They can be prepared in a larger quantity and frozen for storage. Immediately before usage they will be thawed and the portions not consumed can be frozen again. Since sensitive substances, like NADH, do not stand repeated freezing and thawing, such solutions may be divided into small portions, each sufficient for one test series, and frozen separately. Reagents which are not stable in solution at all must be prepared directly before usage. Some solutions, like buffers and inorganic salts, are principally stable at room temperature, but for long-term storage to avoid microbial contamination they should also be frozen.

Care must be taken that all components of the assay mixture are compatible with one another. Any reaction, like oxidation, reduction, precipitation or complexing (e.g. EDTA), must be excluded, disturbing components should be added directly to the assay, and if possible they may be used to start the reaction. Substances existing in acid or alkaline form must be neutralized before addition. In the assay mixture all components must be present already in their final concentration, considering, however, the volume change caused by the addition of the starting component. Assay mixtures should be prepared always freshly and kept at low temperature (ice), only the sample directly prepared for the assay must be thermostatted. After finishing the test series the assay mixture should be discarded and not stored for a longer time.

A further question concerns the component to be used for starting the enzyme assay. In principle all substances essential for the catalytic reaction, like substrates or cofactors may be candidates, but usually the enzyme as the catalyst is preferred. Its limited stability in dilute solution and possible interactions with components of the assay mixture makes the enzyme the most suitable as the starter component. In some cases, however, the substrate is preferred, e.g. if it is unstable in aqueous solution and must be added immediately before the reaction. Some enzymes need an activation phase, e.g. by interaction with a cofactor. They must be preincubated with this factor or with the whole assay mixture, and another component must initiate the reaction.

Pretreatment of the enzyme

Various modes are applied to store enzymes, frozen in solution, as crystal suspension, as precipitate or lyophilized. For performing the enzyme assay a stock solution must be prepared from the storage form. Since enzymes are more stable in the condensed protein milieu of the cell, the stock solution should be concentrated, but the enzyme must be completely dissolved. A buffer, preferentially with the same pH as the assay mixture, should be used. Even under such conditions the enzyme may not be stable and its activity can decrease considerable during an experimental period of some hours. Various reasons can cause a loss of activity, like oxidative processes, poisoning of thiol groups, both often assisted by metal ions, or degradation by contaminating proteases. Elevated temperature promotes such processes. Therefore enzyme solutions should be kept cool, preferentially on ice. Thiol reagents, like mercaptoethanol, dithioerythritol or dithiothreitol protect from oxidative processes. High concentrations of inert proteins, like bovine serum albumin, have a general stabilizing effect and protease inhibitors, like phenylmethanesulfonylfluoride, leupeptin and macroglobulin protect against degradation (Umezawa, 1976; Sottrup-Jensen, 1989). EDTA traps divalent metal ions and serves as inhibitor of metallo-proteases, but it also sequesters essential ions from the enzyme, e.g. in ATP dependent reactions, which need Mg^{2+} as counterions and thus EDTA reduces the effective ATP concentration. Cofactors and substrates protect enzymes against poisoning of their catalytic sites. It must in principle be considered, that, together with the enzyme, such protective reagents get into the assay solution and may influence the reaction. The stock solution of the enzyme should be prepared freshly for the actual test series and not stored for longer time.

Performing the enzyme assay

To carry out an enzyme assay an aliquot of the assay mixture, e.g. 1 ml, will be transferred into an observation vessel, e.g. a photometric cuvette. The vessel should be connected with a thermostatting device to achieve rapid warming up. When the assay temperature is reached, the reaction is started by adding the lacking component, e.g. the enzyme. The volume of this last addition should be considered, e.g. if the starter solution comprises 20 μl, only 0.98 ml of the assay mixture is needed to obtain a final assay volume of 1 ml. Mixing is a very crucial task, because the reaction starts immediately after addition, and during a slow mixing and manipulation procedure, e.g. to turn on the instrument, the reaction already proceeds and valuable information may get lost. Therefore mixing must be fast and intense to ensure homogeneous distribution, but any disturbances, like inclusion of air bubbles or dust particles must be avoided. Direct pouring of the solution from the pipette tip into the assay mixture and stirring with the tip is not advisable, since parts of the solution adhering to the outside surface of the tip will get into the assay and modify the concentration. Disposable stirring sticks are available; the aliquot can be placed on their tip before stirring.

Recording of the reaction should start immediately after the last addition and mixing. The reaction should proceed

within an appropriate time (between 1 and 5 min), not too fast and not too slow. During this time an intense, easily detectable signal should arise. If possible (dependent on the detection method used) the complete time course (*progress curve*) of the reaction should be documented; otherwise the reaction is stopped and the signal is measured after a distinct time. For enzyme-catalysed reactions the velocity is directly proportional to the enzyme amount. This rule allows adapting the velocity to the conditions of recording. While for enzyme assays the concentrations of all other components are determined, the amount of enzyme can be varied in order to obtain an optimum reaction course (see next section).

Concentration of the assay components

The concentration of all substrates and cofactors directly involved in the enzyme reaction should be saturating, so that no component will be rate limiting. The question is, what does "saturating" mean? Binding of these components to the enzyme obeys a hyperbolic saturation function according to the Michaelis-Menten equation (Michaelis and Menten, 1913; Bisswanger, 2008), i.e. the degree of binding is not directly proportional to the concentration of the component, rather occupation of the binding sites occurs more efficiently at lower concentrations, while with progressive occupation increasing amounts of the component are required. Complete saturation can only be attained with infinite high amounts of the component (Figure 6). Thus, in the strict sense, saturation cannot be realized at all. To circumvent this dilemma saturation is understood as almost complete saturation. But what does "almost" mean? A measure for the binding affinity according to Michaelis-Menten equation is the Michaelis constant K_m. This value indicates the concentration of the compound at half saturation. It may be assumed that subsequent addition of the same amount should saturate the residual 50% binding sites, but in fact this share can only occupy 16.7% of the free sites (since the enzyme velocity is directly related to the degree of saturation, the ratio of occupied sites determines the velocity). Even a fivefold concentration of the K_m value saturates the enzyme only to 83% leaving 17% still unoccupied and 9% free sites are still present at 10 fold K_m. To occupy 99% a 100-fold surplus is required. This can be taken as "practical saturating", assuming the still 1% unoccupied sites to be within experimental error.

From these considerations it becomes obvious, that not a general value for the concentration of the components can be given. Rather each component must be supplemented according to its particular K_m value, e.g. for a K_m value of 1 mM a saturating concentration of 0.1 M should be taken. Such high concentrations cannot be achieved in every case, especially for barely soluble substances. Moreover, high concentrations can influence the enzyme activity in an unspecific manner; sometimes the particular component acts directly as an inhibitor of the enzyme reaction (e.g. substrate inhibition). A further aspect is demonstrated with the example of NADH. Its absorbance at 340 nm serves as signal in the optical assay. Its K_m with alcohol dehydrogenase is 0.11 mM, so 11 mM should be taken in the assay for saturation (Wagner et al., 1984). At this concentration the absorption will be 69, far above

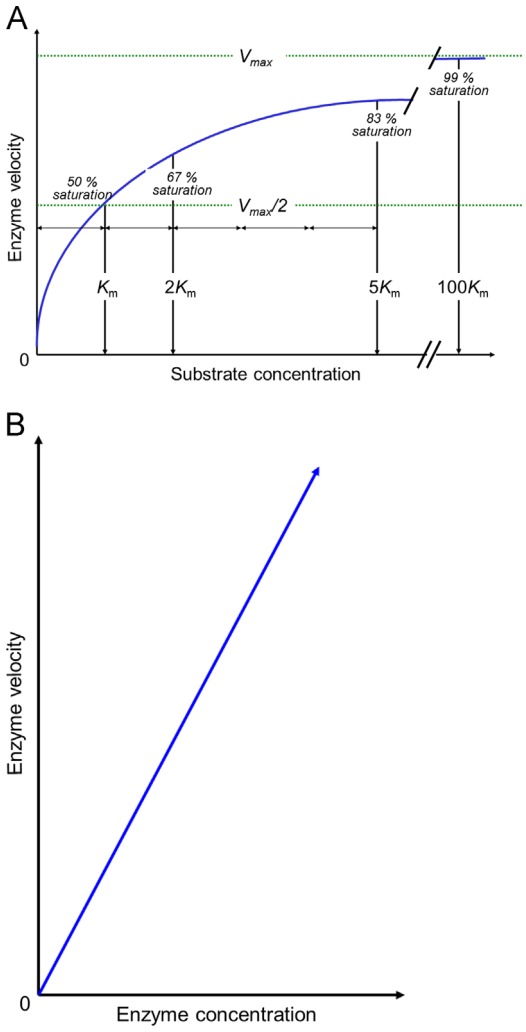

Figure 6 Saturation function for substrates and cofactors according to the Michaelis-Menten equation (A). The K_m value represents the substrate concentration at half saturation, i.e. half maximum velocity. Concentrations of substrate in the range 2-5K_m are much too small to approach saturation, rather 100K_m is required. (B) Dependence of the reaction velocity on the enzyme concentration.

the accessible detection range, which should not exceed essentially a value of 1. To remain within this limit the assay concentration of NADH should not be higher than 0.2 mM, less than 2K_m. Such conditions enforce a deviation from the rules, which must be considered in the calculation of the enzyme activity. Because of the difficulties with high concentrations various reports suggest generally 10K_m for saturation, though it deviates considerably from true saturation.

Components not directly involved in the enzyme reaction, like antioxidants or proteolysis inhibitors, are included in concentrations required for their efficiency.

Concentration of the enzyme and observation time

Unlike the other components involved in the enzyme reaction the amount of the enzyme should be as low as possible, only *catalytic* amounts are necessary, a condition meeting the fact that enzymes are usually rare and valuable

substances. The fundamental Michaelis-Menten equation is derived on the assumption of minor, even negligible enzyme amounts (Bisswanger, 2008). In practice the lower limit for the enzyme is determined by experimental reasons, the amount must at least be sufficient to observe the reaction. The reaction velocity is directly proportional to the enzyme concentration showing a linear dependence, in contrast to the hyperbolic dependence on substrates and cofactors (Figure 6B). Therefore, the reaction velocity can be regulated by varying the amount of enzyme, adding more if the reaction proceeds too slowly, and less if it is too fast. In general too low amounts of the enzyme are less a problem than too high amounts. The latter convert the substrate instantly, already during the mixing and starting procedure and, at the worst, the reaction will already be finished at the onset of recording and no reaction can be observed. In such a case inexperienced experimenter add even more enzyme, supposing a too low enzyme activity. Often a distinct enzyme amount is indicated in the assay protocol; it can also be calculated, as described in the following section. However, since the activity of enzyme preparations does not remain constant, but depends on different conditions, like mode and time of storage, preliminary tests for the control of the actual enzyme activity are strongly recommended.

Directly related with the enzyme amount is the observation time. Although defined time periods (seconds or minutes) are specified for calculation of the enzyme activity, there exists no general rule for the time observing the reaction, only that it must be within the area of the initial linear progression of the velocity, while the following non-linear phase will yield erroneous results. It may be supposed, that the initial phase should be rather short, but this is not indispensable. If in a special assay the linear initial phase lasts for only 10 s, this will be a barely observable period for the conventional assay methods. However, tenfold reduction of the enzyme amount will expand the linear period to 100 s, a hundredfold reduction even to about 17 min, a fairly long time for observation. But, on the other hand to obtain the same intensity for the signal the long observation time of 17 min, instead of 10 s, must be accepted. The reaction proceeds very slowly and, finally, with very low enzyme amounts the signal will not be detectable at all. To intensify the signal the sensitivity of the detection method can be increased, but only within a distinct range, until the basic noise of the method exceeds the signal intensity (Figure 2). Therefore a suitable combination of enzyme amount and observation time should be tried out; longer observation times save enzyme, but are time consuming.

Computer-controlled instruments like spectrophotometers usually have available programs calculating the enzyme velocity immediately after the assay. This is convenient, but should not be used uncritically. The trace of the progress curve should be displayed on the screen and its fitting with the calculated regression line checked, because the programme does not distinguish between random scatter and systematic deviation, and will include in the calculation the non-linear part of the progress curve, if it is within the observation range, as well as any systematic and erroneous deviations.

The reaction time for stopped assays is usually indicated in the protocol and it must be assumed that this time is indeed within the range of the initial velocity. One must, however, be aware that any modification of the protocol, like higher enzyme activities, reduced substrate concentrations or change of the assay temperature, can cause the stop time to fall outside the permitted range. In such cases the linear progression of the reaction should be checked by performing several assays varying the stop time.

Blank and zero adjustment

Any enzyme assay requires a blank. For stopped assays the blank value is obligatory to determine the velocity from the difference between the stopped value and the blank, while with continuous assays the velocity is calculated from the slope of progress curve. This can be done without a blank value, but even here a blank is needed to adjust the instrument to zero, otherwise the reaction may fall outside the observation range of the system. Usually the assay mixture without the starting component is taken as blank, but care must be taken that the starting component does not change the blank. Otherwise another component must be taken to initiate the reaction. When the signal of the substrate is higher than that of the product, as is the case for dehydrogenase reactions with NADH as substrate, the signal will decline into the negative area. This is no principal problem, but if the system is adjusted to zero before starting, the reaction will run out of the observation range. In such cases the instrument should be adjusted to a higher value before starting, or the assay mixture without the substrate should be taken as a blank.

It must be established that the blank remains constant during the measuring period. Sometimes, however, the blank show a considerable drift, which may influence the reaction course, and thus the result of the assay. Often the drift progresses in a constant linear (positive or negative) manner. Such drift may be caused by the instability of the instrument, e.g. warming up of photometric lamps and a longer accommodation time for the instrument will eliminate the problem. But also spontaneous side reactions, oxidative processes, instability of a component, incipient turbidity or other processes in the assay mixture can be responsible for the drift. In such cases its origin should be identified and as far as possibly eliminated, because such reactions will change the assay mixture, especially if it is kept for a longer time during an extensive test series. If the origin of the disturbance cannot be eliminated, the drift must be considered for the calculation of the enzyme velocity. Supposing the effect to be constant and reproducible under defined conditions, the velocity can be corrected by a constant drift value. If the drift is not constant, but appears to be more arbitrary, reliable measurements will not be possible. Contaminations, soiling or air bubbles can produce such effects and may be eliminated by careful manipulation; otherwise the assay system should be changed.

Reversibility of enzyme reactions

In principle any chemical reaction, and thus also any enzyme reaction, is reversible, and may be observed both from the substrate as well as from the product side. However, reactions releasing energy (*exergonic reactions*, e.g. cleavage

reactions) strongly favour one direction (*quasi-irreversible reactions*), while energy-consuming (*endergonic*) reactions are grossly disfavoured. Consequently, enzyme assays use normally the favoured direction. Enzyme reactions that do not show a strictly favoured direction (*reversible reactions*) like dehydrogenases or isomerases can be tested from both sides. Usually the direction easier to achieve will be preferred, e.g. better stability and availability of substrates as well as instrumental aspects.

An important advantage of quasi-irreversible reactions is the fact that the substrate will be completely converted to product, while reversible reactions convert the substrate to product only until the equilibrium is reached, at the end of the reaction both substrate and product remain in the assay solution in a constant ratio. For example, the equilibrium for the isomerase reaction between glucose to fructose is nearly at 50%, and thus at the end of the reaction both sugars will be present in comparable concentrations, irrespective of whether the reaction started from glucose or from fructose as substrate (Antrim et al., 1979; Lehmacher and Bisswanger, 1990). The alcohol dehydrogenase reaction with ethanol and NAD as substrates is more convenient than the back reaction with the toxic and volatile acetaldehyde and the expensive and less stable NADH. Moreover it is easier to observe a reaction starting from zero with an increasing absorption, instead to start with the high absorbing NADH. Unfortunately, the equilibrium favours the back reaction. However, with a trick the reaction can be forced in the desired direction, trapping the released protons at high pH and the acetaldehyde by a subsequent reaction with semicarbazide (Bergmeyer, 1983).

For enzyme assays complete conversion of the substrate to product is preferred. Analysis of the product is easier in the absence of substrate and also the linear initial velocity is longer.

Coupled enzyme assays

Difficult detectable enzyme reactions are frequently coupled with easily observable reactions, preferentially NAD(P)H dependent dehydrogenases. An example is the hexokinase reaction (1) connected with the glucose-6-phosphate dehydrogenase (2):

$$\text{Glucose} + \text{ATP} \rightarrow \text{glucose-5-phosphate} + \text{ADP} \quad (1)$$

$$\text{Glucose-6-phosphate} + \text{NADP}^+ \rightarrow \text{gluconate-6-phosphate} + \text{NADPH} + \text{H}^+ \quad (2)$$

The second, the *indicator* reaction can easily be detected by the absorption increase at 340 nm. The conditions for coupled enzyme assays are comparable to assays with single reactions, but some special aspects must be regarded. Optimum conditions cannot be achieved simultaneously for both enzymes. As the first reaction is the one to be determined, the indicator reaction should never become limiting. Its enzyme must be present in excess, while for the first enzyme the rule of very low, catalytic amounts still holds. So the test enzyme more than the indicator enzyme determines the assay conditions.

Unlike single reactions, coupled assays show a lag phase until the linear steady state phase is reached, where formation and conversion of the intermediate becomes constant. The duration of the initial lag phase depends on the observance of the conditions for the coupled assay, the better the conditions are fulfilled, i.e. the less the indicator reaction becomes rate limiting, the shorter the lag (Bergmeyer, 1983, 1977).

Substrate determination

Enzyme assays are used also to determine the concentration of substrates in samples. The high specificity of enzymes allows the determination of a distinct substrate within a crude sample, like cell homogenates. Here it is not the initial phase of the reaction that is of importance, rather the reaction must come to its end, and from the difference between the start and the end point the amount of product formed, and, thus, the amount of substrate in the sample is calculated. Therefore it must be checked that the reaction becomes completely finished and higher enzyme amounts are needed to accelerate the reaction. The other conditions, concerning temperature, pH, ionic strength and the concentration of the other components should be as defined for the enzyme assay. Components involved in the catalytic reactions, like cosubstrates and cofactors, must in any case be present in higher amounts than the expected concentration of the substrate to be determined, otherwise the limiting compound would be determined (Bergmeyer, 1983, 1977).

Evaluation of enzyme assays

Determination of the enzyme velocity

The enzyme activity must be evaluated from the signal provided by the respective analysis method, like absorption or relative fluorescence. The intensity of this signal is a measure for the concentration of the observed substrate or product. In photometric assays the concentration can directly be calculated from the signal intensity applying an absorption coefficient. If such a factor is not available (with fluorescence a comparable factor does not exist at all), a calibration curve with varying amounts of the respective compound must be prepared under assay conditions. The first value of this curve should be a blank without the compound in question. From this zero value the curve should increase linearly with increasing concentrations, and, at higher concentrations, the curve may deviate from linearity. Only the linear part of the curve should be taken for the calculation. Also the signal intensity of the enzyme assay should range within this linear part.

From the slope of the linear part of the progress curve the enzyme velocity is obtained as the amount of substrate (product) converted (formed) during a time unit (Figure 3). At first a part of the progress curve long enough to get reliable results is taken. A reaction time sufficiently long to obtain a clear slope must be chosen, especially in the presence of remarkable scattering. Computer controlled instruments provide a regression analysis; otherwise a straight line is drawn through the scattering trace displaying the immediate reaction course. The increase (or decrease) of the slope within the time unit (1 s or 1 min), calculated

Table 1 Definitions for enzyme assays.

Name	Definition	Notation	Dimension	Conversion
Enzyme units (measure of enzyme activity)	Enzyme amount converting 1 mol substrate/s	katal (kat)	mol/s	1 kat=60,000,000 IU 1 nkat=0.06 IU
	Enzyme amount converting 1 μmol substrate/min	International unit (IU)	μmol/min	1 IU=0.0000000167 kat 1 IU=0.0167 nkat
Volume activity	Enzyme units per volume unit	katal/volume IU/volume	kat/L IU/mL	
Specific enzyme activity	Enzyme units per protein; volume activity/protein concentration	katal/protein IU/protein	kat/kg IU/mg	
Enzyme velocity	Turnover per time unit	v	mol/s μmol/min	
Maximum velocity	Turnover per time unit at saturating conditions of substrates and cofactors under standard conditions	V_{max}	mol/s μmol/min	
Turnover number (catalytic constant)	Maximum velocity divided by the enzyme concentration	$k_{cat}=V_{max}/[E]_0$	s^{-1}	
Michaelis constant	Substrate concentration for half-maximal velocity	$K_m=(k_{-1}+k_{cat})/k_1$	M	

for the converted substrate (mol or μmol) yields the reaction velocity v in mol per s or μmol per min. Such velocity values serve for further calculation of the enzyme activity. They can be used to investigate the features of the enzyme in question, varying different conditions, like the concentrations of substrates or cofactors, the pH, temperature, or behaviour with effectors or metal ions. Only if optimum conditions prevail, as discussed in the previous sections, i.e. substrate and cofactor saturation, standard pH temperature and ionic strength, the relevant value can be taken as maximum velocity (V_{max}) to determine the enzyme activity (Table 1). From the maximum velocity the turnover number or catalytic constant $k_{cat}=V_{max}/[E]_0$ can be derived. It is the maximum velocity divided by the enzyme concentration corresponding to a first order rate constant (s^{-1}). To get this the enzyme concentration in molar dimensions must be known (Bisswanger, 2008).

Stopped assays provide usually only one measure value after stopping the reaction. A straight line, connecting this value with the blank value at time zero yields the slope from which the velocity can be calculated in the same manner as described for the continuous assay. Compared with continuous progress curves single determinations are subject to greater uncertainty. Repeated measurements under identical conditions are required and treated according to statistical rules.

Enzyme units

The enzyme activity is generally determined as substrate converted respectively product formed per time unit. According to the present valid SI system the concentration should be in mol and the time unit is s. Correspondingly the enzyme unit 1 katal (1 kat) is defined as the amount of enzyme converting 1 mol substrate respectively forming 1 mol product/s. Besides the katal the *International Unit* (IU) continues to be in common use, in fact more than the katal, e.g. most suppliers still offer their enzyme preparations in IU; 1 IU is defined as the enzyme amount converting 1 μmol substrate (forming the 1 μmol product)/min (International Union of Pure and Applied Chemistry, 1981; Nomenclature Committee of the International Union of Biochemistry (NC-IUB), 1982)

Comparing the two definitions allows us to understand the unpopularity of the katal. This should be demonstrated with the example of lactate dehydrogenase reacting with pyruvate and NADH as substrates. 1 IU enzyme converts 1 μmol NADH per min, corresponding to an absorption decrease of 6.3. This value is too high for photometric determination; rather an absorption decrease within the range of 0.1/min will be feasible. To achieve this about 0.016 IU of LDH should be added to a single assay. Preparing a stock solution of lactate dehydrogenase with just 1 IU/ml and adding 0.02 ml from it to 0.98 ml of the assay mixture, the absorption decrease per min will be 0.126, just within the expected range. In comparison, 1 kat lactate dehydrogenase produces an absorption change of 6,300,000/s. Since one second is too short for measuring, the absorption decrease within 1 min would be 378,000,000, far away from any reality. To obtain an absorption decrease of 0.1/min, 0.00000000026 kat lactate dehydrogenase is needed. A common lactate dehydrogenase preparation contains about 500 IU/mg protein, 1 IU-2 μg. 1 kat=60,000,000 IU, corresponding to 120 kg lactate dehydrogenase, a completely unrealistic quantity. Obviously calculation with katal is somewhat difficult. However, the problem can be avoided by using nanokatal (nkat) for calculation, 1 nkat=0.06 IU, 1 IU=16.67 nkat.

There are also enzyme units in use that differ from both definitions with respect to the time unit (e.g. 1 h) and the amount of substrate. As far as possible such units should be adapted to katal or IU to enable comparison with other reports. This is in principle possible with respect to the time unit, but it is not always easy to define accurately the substrate concentration, e.g. with enzymes degrading

macromolecules like proteins or starch. Such substrates vary in their molecular mass and, in the strict sense, not the macromolecule itself but the binding to be cleaved is the real substrate. Correspondingly the Anson units for proteases are defined according to the colour intensity of the assay instead of a molarity (Peterson, 1979).

Enzyme units serve to quantify the amount of an enzyme. The amount of the enzyme is not defined by its mass (protein) rather by its function. This is reasonable, because the catalytic potential and not the protein is the essential feature of the enzyme. Even enzymes comparable in their purity can differ considerably in their activities; a partially inactivated enzyme cannot be discriminated from an active one only by protein analysis. The purity of an enzyme is usually expressed by the *specific enzyme activity*, i.e. the enzyme units divided by the protein content of the respective enzyme preparation. The higher the value the purer the enzyme, lower values indicate either impurities or partial inactivation of the enzyme.

Estimation of the required enzyme amount

Enzyme units can serve to evaluate the amount of enzyme required for a distinct enzyme assay. As already mentioned, for theoretical reasons the enzyme concentration should be as low as possible, the detection limit determining the lowest amount. From this statement it becomes already clear that the actual enzyme amount depends on the sensitivity of the detection method, and no general advice can be given. However, for a distinct method and its detection range, the required enzyme amount can be estimated. This will be demonstrated with the example of the UV/visible spectroscopy. The authentic absorption range is between 0 and 1, while for higher absorptions the Lambert–Beer law is no longer valid. To determine the initial velocity of an enzyme reaction, e.g. of a dehydrogenase, an absorption range of 0.1 is sufficient, and higher absorptions will easily exceed the linear phase of the progress curve. So an enzyme amount producing an absorption difference of 0.1/min will be convenient. The absorption coefficient of NADH at 340 is 6300 $M^{-1}\,cm^{-1}$, 1 μmol NADH per ml has an absorption of 6.3; 0.016 μmol NADH/ml show an absorption of 0.1. To convert 0.016 μmol NADH/min in 1 ml assay mixture 0.016 IU respectively 0.27 nkat enzyme are required.

Conclusions

Due to the divergent features of enzymes a general standardization of enzyme assays is not possible, rather special rules can be given as follows:

1. *pH*: Preferentially the pH of the pH optimum of the respective enzyme is chosen, as far as possible at or near the physiological pH (∼7.5).
2. *Buffers and ionic strength*: To stabilize the pH, buffers are used, and their pK_a value should correspond to the pH optimum of the enzyme assay. Buffer concentrations of about 0.1 M are suitable for most enzyme assays, some (halo- and thermophilic) enzymes require a considerably higher ionic strength.
3. *Temperature*: One of three favoured temperatures should be chosen:
   - 25 °C, the most frequently used one, easy to maintain, but giving relatively low enzyme activities.
   - 30 °C, a compromise between 25 °C and the physiological temperature, especially for temperature sensitive enzymes.
   - 37 °C, the physiological temperature, relatively high enzyme activity, but more difficult to maintain.

   Different temperatures are needed for special cases (e.g. thermophilic enzymes).
4. *Concentrations of substrates, and cofactors*: should be saturating, as far as possible $100K_m$, but at least $10K_m$.
5. *Concentration of the enzyme*: as low as possible, but enough to observe the progressing reaction.
6. *Concentrations of additives*: (stabilizers, antioxidants, thiol reagents, protease inhibitors, complexing reagents) as required for efficiency. Generally all assay components must be compatible with one another, increase of ionic strength and influence on the pH of the assay must be taken into account.
7. Conditions of the particular enzyme assay must accurately be specified in the protocol.

Conflict of interest statement

The author has no conflict of interest.

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