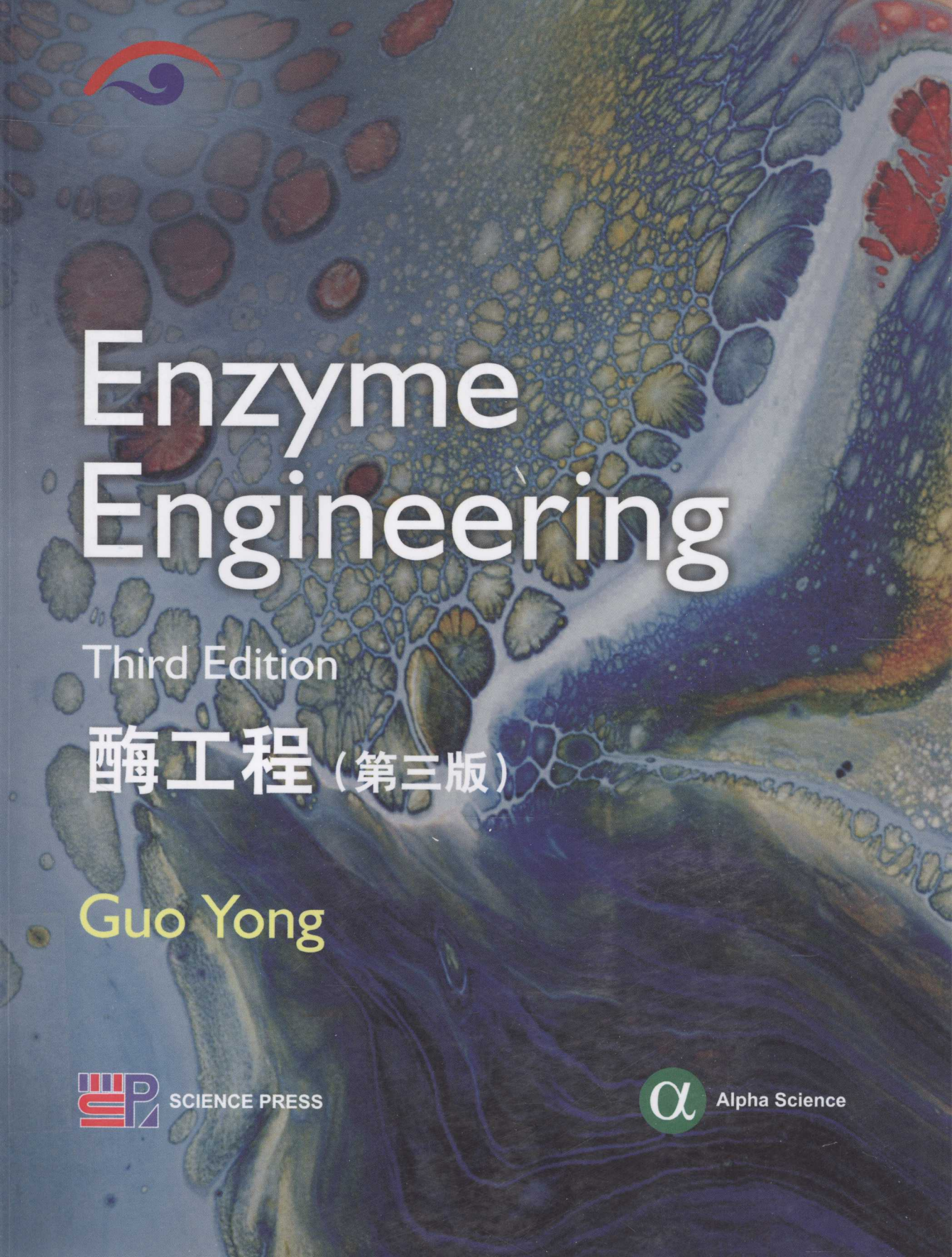

Enzyme Engineering

Third Edition

酶工程（第三版）

Guo Yong

SCIENCE PRESS

Alpha Science

Enzyme Engineering

Third Edition

Guo Yong

Science Press

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Translation Committee

Preface

Enzyme engineering is one of the main parts of biotechnology. Enzyme engineering has been developing quickly since 1970s; it has got great achievements in the theoretical and application researches and has been playing an important role on the development of science, technology and economy in the world. Enzyme engineering will develop even more quickly and play a much more important role.

Enzyme engineering is a technical process of enzyme production and its applications. The main tasks of enzyme engineering are obtaining the required enzymes through design and artificial operation, improving the enzyme catalytic characters with enzyme improvement technologies, and making full use of enzyme catalytic function.

This book was translated from Chinese "*Enzyme Engineering*" (Third Edition) published in 2009 by Science Press, Beijing, China. The contents of the book include 10 chapters which are; chapter 1: introduction; chapter 2: Enzyme production by microbial fermentation; chapter 3: Enzyme production by animal and plant cell culture; chapter 4: Extraction, isolation and purification of enzymes; chapter 5: Enzyme molecular modifications; chapter 6: Immobilization of Enzymes, Cells and Protoplasts; chapter 7: Enzyme catalysis in non-aqueous phases; chapter 8: Enzyme directed evolutions; chapter 9: Enzyme reactors and chapter 10: Applications of enzymes. There is a review and thinking section given at the of each chapter. Chapter 1, chapter 3 and chapter 7 were translated by Prof. GUO Yong; chapter 2 and chapter 6 were translated by Dr. ZHENG Suiping; chapter 4 and chapter 10 were translated by Prof. LIN Ying; chapter 5 was translated by Dr. HAN Shuangyan; chapter 8 was translated by Dr. WANG Bin and; chapter 9 was translated by Dr. ZHU Mingjun.

The book could be used as a text book for the undergraduate or graduate students specializing in biotechnology, bioengineering, biochemical engineering, enzyme engineering, fermentation engineering, and bioscience and so on. It also could be used at conferences for teachers, scientists, engineers and technicians working in the related fields.

During the writing and the translating of this book, I received a lot of intellectual and insightful assistance from numerous colleagues and friends. I wish to acknowledge their invaluable help. I also wish to acknowledge the pleasant cooperation and assistance provided by the members of the translation committee and the editors involved in the completion of this book.

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Chapter 1

Introduction

Enzymes are biological macromolecules with bio-catalytic functions. Natural enzymes can be divided into two types, protein enzymes (proteozymes) and RNA enzymes (ribozymes), according to the difference of major component with catalytic function in the molecules.

The cells of every kind of animal, plant and microorganism may synthesize many kinds of enzymes in suitable conditions, and so, people may produce required enzymes with suitable cells in the bioreactor under control conditions.

Enzymes may catalyze all kinds of biochemical reactions under suitable conditions. Enzymatic catalysis possesses great efficiency, high degree of specificity and mild action conditions, so that, enzymes can be widely used in the fields of medicine, food, industry, agriculture, environmental protection, energy, biotechnology research and so on.

To solve the weak points of enzymes which appear in enzyme application process e.g., inefficiency and instability, several kinds of enzymes improving technologies have been developed to improve the enzyme characters for the demands of application.

Enzyme engineering is the technical process for enzyme production and application.

Enzyme production is the technical process to obtain required enzymes through several technologies that include the production of enzymes by microbial fermentation, the production of enzymes by animal and plant cell culture, enzyme extraction, separation and purification.

Enzyme improvement is the technical process to improve the enzyme catalytic characters through several technologies that include enzyme molecule modification, enzyme immobilization, enzyme catalysis in non-aqueous phase, and enzyme directed evolution.

Enzyme application is the technical process to get required substances, to remove unwanted substances and to obtain required information with catalysis of enzymes.

The main contents of enzyme engineering include enzyme production by microbial fermentation; enzyme production by animal and plant cell culture; enzyme extraction, separation and purification; enzyme molecule modification; immobilization of enzymes, cells and protoplasts; enzyme non-aqueous catalysis; enzyme directed evolution; enzyme reactors and enzyme application.

The main tasks of enzyme engineering are obtaining the required enzymes through design and artificial operation, improving the enzyme catalytic characters through enzyme improvement technologies, and making full use of enzyme catalytic functions.

1.1 Conception of Enzyme and Its Development

The catalytic reactions of enzymes were used by our ancestors thousands of years ago to produce foods and medicines. Historical documents show that people in ancient China had mastered the techniques to produce wines 4000 years ago in Xiayu period; to manufacture foods of jam and malt sugar 3000 years ago in Zhou dynasty; to treat illness of indigestion with "Qu" 2500 years ago in Chunqiu Zhanguo period. The Chinese character "Mei" is the Chinese name of enzyme which means "Jiumu" (wine's mother) in the dictionary "*Kangxi Zidian*" published in 1716.

However, the real modern recognition of the action and nature of enzymes has been since the thirties of the 19th century. The recognition has been a continuous development and a step by step progress.

Payen and Persoz in 1833 isolated a substance that could catalyze starch to hydrolyze into glucose from the alcohol extracts of malts, named it "diastase" and pointed out its thermo instability, preliminary indication of the essence of enzymes.

Pasteur and others researched the alcohol fermentation by yeast in the middle of 19 century; they believed that there was something in living cells which could ferment sugar into alcohol. Kuhne in 1877 coined the name "enzyme", which means "in yeast".

Buchner brothers in 1897 showed that a cell-free juice from yeast was capable of fermenting sugar with the production of alcohol. The fact indicated that enzymes can catalyze the intracellular reactions as well as extracellular reactions under suitable conditions. After that, many scientists studied widely the nature and reaction of enzymes.

Henri in 1902 proposed the intermediate theory that the first step of enzyme catalysis reaction is the formation of intermediate complex between the enzyme and its substrate, then the complex transforms to products, and releases the enzyme.

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightleftharpoons} E+P$$

Michaelis and Menten in 1913 derived a general rate equation according to the intermediate theory, which is now known as the Michaelis-Menten equation.

$$V=\frac{V_m[S]}{K_m+[S]}$$

People recognized that "enzymes are catalysts (substances with bio-catalytic function) produced by living cells" in the about 100 years period between the thirties of 19 century and twenties of 20 century, but the components of enzymes remained unclear. Willstatter who was a chemist determined the highly purified catalase with high activity in 1920. The results showed that protein was not found, a wrong recognition that enzymes were not proteins, was proposed as the determining techniques were not good enough.

Sumner in 1926 isolated urease in a crystalline from Jack beans, and proved it possessing the properties of proteins. After that, researches on a lot of enzymes showed that the chemical nature of enzymes was proteins, and the conception that "enzymes are proteins with bio-catalytic function" was well known during the 50 years and more period between 1926 and 1982.

Jacob and Monod in 1960 proposed the operon theory which expounds the general regulation mechanism of enzyme biosynthesis.

Cech in 1982 discovered that the precursor of 26S rRNA in *Tetrahymena* cells possesses the function of self-splicing. The precursor of RNA with 6400 nucleotides contains one intron (intervening sequence, IVS) and two exons; the precursor cleavages the IVS and links up the two exons to form the mature RNA in the mature process called splicing. The splicing process does not need any protein but requires guanosine or 5′-GMP. Cech named the process as self-splicing, and recognized that the RNA possessed catalysis function, and named the RNA with catalysis activity as ribozyme.

Altman in 1983 discovered that the M1 RNA which is the RNA component of RNase P possessed the activity of RNase, can catalyze the tRNA precursor to cleavage some fragment of nucleotides, and to form the mature tRNA. At the same time, the C5 protein which is the component of the RNase P doesn't possesse the activity of enzyme.

The discovery that RNA possessed catalytic activity changed the conception of enzymes, and is considered one of most heartening discoveries in the near twenty years and more. For this, Cech and Altman shared the Nobel Prize in 1989.

More and more of new ribozymes have been discovered since 1980's. The known ribozymes possessed the functions of self-splicing; self-cleavage; and catalyzing the reaction of other molecules. The substrates of ribozymes include RNAs, DNAs, carbohydrates, amino acids esters, and so on. The research results indicated that ribozymes have a stereo structure and active center; possess unique catalytic mechanism and high specificity of substrate and the kinetics accord with the Michaelis and Menten equation. Ribozymes possess all the natures of biocatalysts, and are a kind of enzymes constituted of RNA. The new conception that "enzymes are biological macromolecules with bio-catalytic function" is suggested. There are two kinds of enzymes, protein enzymes (proteozymes) and RNA enzymes (ribozymes), in nature according to the diffluence of major component with catalytic function in the molecules. The major component of proteozymes is proteins, and the major component of ribozymes is nucleic acid (RNA).

1.2 The Characteristics of Enzyme Action

Enzymes are biocatalysts. Compared to the non-biocatalysts, enzymes possess the remarkable characters of great specificity, high efficiency and mild action conditions.

1.2.1 Enzymes action exhibit a high degree of specificity

The specificity is the most important character of enzymes and the main difference between

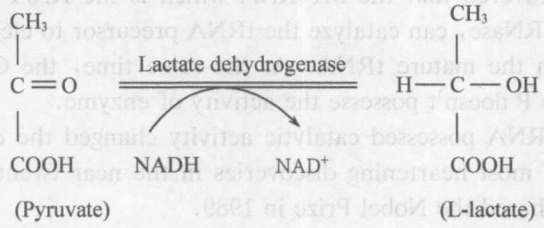
enzyme and non-enzyme catalysts. The order metabolite law in cells is dependent on the specificity of enzymes. Enzyme specificity is the fundamental of application of enzymes in every field.

The specificity of enzymes is that an enzyme may only catalyze one type of reaction specific for a substrate or one kind of substrates with similar structure.

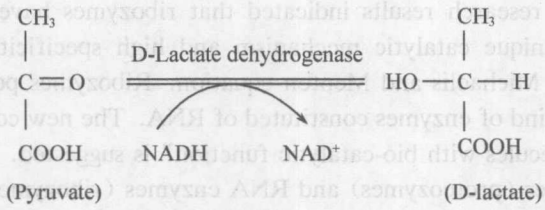
Enzyme specificity can be divided into absolute specificity (strict specificity) and relative specificity (somewhat broader specificity) depending on degree of strictness.

1.2.1.1 Absolute specificity

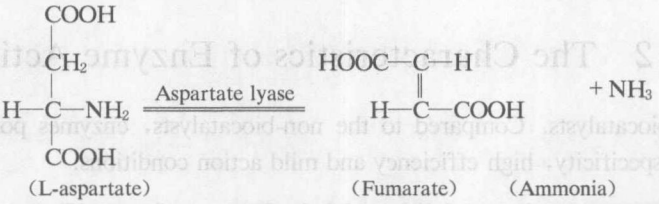
Absolute specificity is that an enzyme can only catalyze one type of reaction specific for one substrate. Enzymes can only act an isomer while substrate of enzyme action has unsymmetrical carbon atom; this absolute specificity is called the stereo isomeric specificity. For example, lactate dehydrogenase (EC1.1.1.7) catalyzes the dehydrogenation of pyruvate to form L-lactate.



D-lactate dehydrogenase (EC 1.1.1.28) may only catalyze the dehydrogenation of pyruvate to form D-lactate.



Other sample is aspartate lyase (EC 4.3.1.1) which may only catalyze the deamination specific for the substrate of L-aspartate to form fumarate, and its reversible reaction.



Neither maleate which is the *cis* stereoisomer of fumarate, nor D-aspartate is a substrate.

Ribozymes also possess the absolute specificity. For example, the self-splicing ribozymes, precursor of 26S rRNA in *Tetrahymena* cells and other self-splicing ribozymes, may only catalyze the splicing reaction specific for the substrate of self-molecules, other molecules are not substrates.

Another sample is the L-19 IVS, which is a RNA enzyme containing 395 nucleotides, may catalyze the reaction specific for the substrates of GGCCUCUAAAAA and guanine (G) to form the products of GGCCUCU and GAAAAA. The oligonucleotides GGCCUGUAAAAA and GGCCGCUAAAAA and so on are not substrates.

1.2.1.2 Relative specificity

Relative specificity is that an enzyme may catalyze one type of reaction specific for one kind of substrates with similar structure.

Relative specificity can be divided into bond specificity and group specificity.

The enzymes with bond specificity may catalyze the reactions specific for the same bands of their substrates. For example, esterases catalyze the hydrolysis specific for the ester bonds of esters to form an acid and an alcohol.

$$\underset{\text{(Ester)}}{R-\overset{\overset{O}{\|}}{C}-O-R'} + \underset{\text{(Water)}}{H_2O} \xrightarrow{\text{Esterase}} \underset{\text{(Acid)}}{R-COOH} + \underset{\text{(Alcohol)}}{R'-OH}$$

The enzymes with group specificity may catalyze the reactions specific for the same functional groups of their substrates. For example, trypsin (EC3.4.31.4) selectively catalyzes the hydrolysis of the peptide bonds formed by lysinyl or arginyl groups, all compounds containing lysinyl or arginyl groups, e.g., amides, esters, peptides, proteins, can be catalyzed.

Another sample is ribozyme M1 RNA (the RNA component of RNase P), which catalyzes the mature of tRNA precursor, requires the 3′-terminal of substrate RNA is a tRNA, the sequence and the length of 5′-terminal are not required, the products of this reaction are a mature tRNA and a oligonucleotide.

1.2.2 Enzyme action possesses high efficiency

Another character of enzyme action is that the efficiency of enzyme action is extremely high; the turnover numbers (the molecule numbers that an enzyme molecule may catalyze the transformation of substrate per minute) are about 10^3/min; for example, the turnover number of β-galactosidase is 12.5×10^3/min, and the turnover number of carbonic anhydrase is high at 3.6×10^7/min.

Efficiency of enzymatic catalysis is 10^7-10^{13} times higher than non-enzymatic catalysis. For example, hydrogen peroxide (H_2O_2) can be catalyzed by iron ion or hydrogen peroxidase to form oxygen and water ($2H_2O_2 = 2H_2O + O_2$). 1mol of iron ion may catalyze the decomposition reaction of 10^{-5}mol of substrate, but 1mol of hydrogen peroxidase may catalyze the decomposition reaction of 10^5mol of substrate.

That enzymes possess high efficiency can be seen by the fact that an enzyme lowers the activation energy required for a reaction.

The substrate molecules require sufficient energy to form the activated molecules. The reaction can proceed when the collisions between the activated molecules, and products can be formed. The activation energy is the free energy needed for the conversion of 1mol of molecules to the activated molecules; its unit is J/mol. As shown in Fig. 1.1, the activation energy is different for enzyme reaction and non-enzyme reaction.

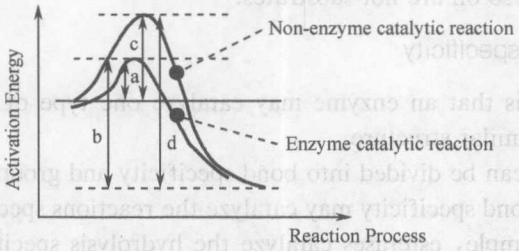


Fig. 1.1 Activation energy required for enzyme catalytic reaction and non-enzyme catalytic reaction

a. Activation energy required for forward enzyme catalytic reaction;
b. Activation energy required for reverse enzyme catalytic reaction;
c. Activation energy required for forward non-enzyme catalytic reaction;
d. Activation energy required for reverse non-enzyme catalytic reaction

The activation energy for enzyme catalytic reaction is much less than non-enzyme catalytic reaction, as shown in Fig. 1.1. For example, in the hydrolysis reaction of hydrogen peroxide (H_2O_2) to form oxygen and water, activation energy is 75.24kJ/mol for the uncatalyzed reaction, 48.94kJ/mol for the catalyzed reaction with Palladium as catalyst, 8.36kJ/mol for the reaction with hydrogen peroxidase as catalyst respectively.

1.2.3 Enzymes action under mild conditions

The more remarkable difference between an enzyme catalytic reaction and a non-enzyme catalytic reaction is that an enzyme catalytic reaction can take place under mild conditions. Enzyme catalytic reactions usually carry on in the conditions of normal temperature, atmospheric pressure, and near neutral pH. On the contrary, the non-enzyme catalytic reactions require the conditions of high temperature, high pressure, and extreme pH. So application of enzymes as catalysts is beneficial to save energy, to reduce investment in equipment, and to optimize environment and working conditions.

Why may enzymes catalyze under mild conditions? One reason is that the energy of activation for enzyme catalysis is lower. Another reason is that enzymes are biological macromolecules, and most of the enzymes may inactivate and lose their catalytic function under high temperature, high pressure, and extreme pH.

1.3 Factors that Affecting Enzyme Action

Enzyme catalysis is affected by several factors, e.g., concentration of substrates, concentration of enzymes, temperature, pH, concentration of activators, and concentration of inhibitors. Environment conditions have to be controlled in enzyme application processes for enzyme to bring out their catalytic functions.

1.3.1 Effect of concentration of substrates

Concentration of substrates is a major factor affecting enzyme catalysis. The relationship between reaction rates and substrates concentration is shown in Fig. 1.2.

As shown in Fig. 1.2, enzyme reaction rate is in direct proportion to the substrate concentration while substrate concentration is lower. The reaction rate is not in direct proportion to the substrate concentration and progressively reaches balance when the substrate concentration comes up to a certain amount.

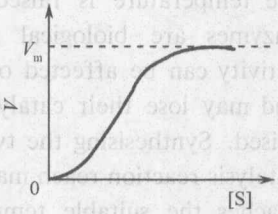


Fig. 1.2 Relationship between reaction rate and concentration of substrate

Many people researched it for interpreting this observation. Michaelis and Menten in 1913 proposed famous Michaelis and Menten rate equation on the basis of the research results of their predecessors.

$$V = \frac{V_m S}{K_m + S}$$

Where, V is the velocity of reaction; S is the substrate concentration;

V_m is the maxim velocity of the reaction;

K_m is the Michaelis constant which is equal to the substrate concentration (moles per liter) that results in one-half the numerical maximum velocity.

This equation is the basic kinetic equation for enzyme reaction and shows the relationship between the velocity of reaction and substrate concentration.

Sometimes the velocity of reaction decrease when the substrate concentration is in excess. This is caused by the inhibition of high concentration of substrates.

1.3.2 Effect of concentration of enzymes

The velocity of enzyme reaction is in direct proportion to the concentration of enzymes under the condition of substrate concentration being sufficient, as shown in Fig. 1.3.

The relationship of velocity of reaction and enzyme concentration is shown as the following.

$$V = k\,[\mathrm{E}]$$

Where, V is the velocity of enzyme reaction;

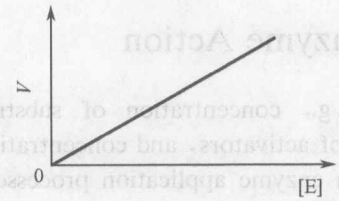


Fig. 1.3 Relationship between velocity of reaction and enzyme concentration

k is the reaction constant;
[E] is the concentration of enzymes.

1.3.3 Effect of temperature

Every enzyme reaction possesses a suitable temperature scope and an optimal temperature. Enzymes may catalyze reactions on their suitable temperature scope; the velocity of enzyme reaction reaches maximum velocity at the optimal temperature, as shown in Fig. 1.4.

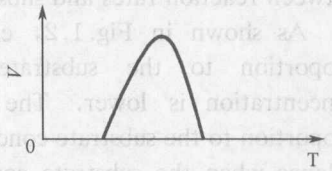


Fig. 1.4 Relationships between velocity of reaction and temperature

The velocity of chemical reaction increases 1-2 times as the temperature is raised to 10℃. On the other hand, enzymes are biological macromolecules and so enzyme activity can be affected or they may even become inactive and may lose their catalytic activity as the temperature is raised. Synthesising the two results, the velocity of enzyme catalysis reaction reach maximum velocity as the temperature reaches the suitable temperature which is called optimal temperature. The velocity progressively decreases as the temperature goes beyond the optimal temperature; enzymes usually becomes inactive and lose their activity as the temperature becomes higher than 60℃. However, there are some enzymes which possess higher thermostability; for example, *Taq* polymerase, widely used in polymerase chain reaction(PCR) may stably catalyze at 95℃; thermostable α-amylase still possesses its catalytic function at a temperature of 90℃ and more. Addition of enzyme substrates or stabilizers may increase the stability of enzymes.

1.3.4 Effect of pH

The pH value of the reaction media has a bearing on enzymes catalysis. All enzymes have their suitable pH scope and an optimal pH.

Enzymes may display their catalytic activity in suitable pH scope. The velocity of an enzyme reaction reaches maximum velocity at the optimal pH. As shown in Fig. 1.5, enzymes may become inactive and lose their activity if the pH higher or lower than the suitable pH scope. Therefore, pH conditions must be controlled in enzyme reaction processes.

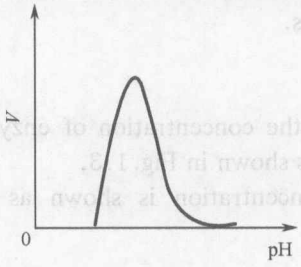


Fig. 1.5 Relationship between velocity of reaction and pH

Why is the enzyme catalysis affected by pH? The major reason is that the dissociation state of groups in the enzyme molecules and substrate molecules is changed, which influences the conformation of enzyme molecules, and the combined capacity of enzyme and substrate, and the catalytic function.

The stereo structure of enzyme molecules is changed in the extreme pH conditions, which causes the enzyme inactivation and loss of its activity.

1.3.5 Effect of inhibitors

The substances which may decrease or cause loss of the catalytic activity of enzymes are called inhibitors of enzymes.

Some inhibitors are the normal metabolites of cells, which may affect the metabolic regulation in cells as some kind of enzyme inhibitors. For example, tryptophan inhibits the catalytic activity of the enzyme (*O*-amino benzoic acid synthetase) that catalyzed the first step reaction in the way of tryptophan synthesis, thus regulating the biosynthesis of tryptophan. Most of the inhibitors are exogenous substances. Major exogenous inhibitors are inorganic ions, small molecular organics and proteins, etc. For example, several heavy metal ions e.g., silver ion (Ag^+), mercury ion (Hg^{2+}), lead ion (Pb^{2+}) exhibit the inhibition of many enzymes; ascorbic acid (vitamin C) inhibits the activity of sucrase; trypsin inhibitor inhibits the activity of trypsase, etc. Some inhibitors are a kind of drugs with important value for application. For example, trypsin inhibitor treats pancreatitis; choline esterase treats blood vessel disease and so on.

The activity of enzymes decreases or even fully list under the effect of inhibitors, and thus, they influence the catalytic function of enzymes.

Inhibitors can be divided into two groups: reversible inhibitors and irreversible inhibitors.

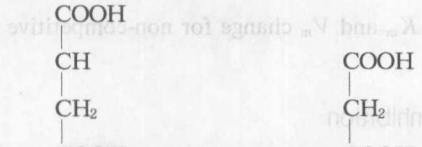
When irreversible inhibitors combine with the enzyme, the inhibitors are difficult to remove, and thus the enzyme activity cannot be returned.

The combination of reversible inhibitors and enzyme is reversible, and so enzyme activity can be returned when the inhibitors are removed. The reversible inhibition can be divided into three types: competitive inhibition, non-competitive inhibition, and uncompetitive inhibition, according to the mechanism of reversible inhibition.

1.3.5.1 Competitive inhibition

Competitive inhibition is caused by the inhibitors that compete with substrate to combine with enzyme molecule.

The structure of a competitive inhibitor is similar to that of the substrate; when the inhibitor combines with enzyme, substrate molecule cannot combine with the enzyme, and that inhibits the enzyme catalysis. For example, malonic acid which is structurally similar to succinic acid may compete for the binding site of succinate dehydrogenase which can only catalyze the dehydrogenation of succinic acid. Therefore, malonic acid is a competitive inhibitor of succinate dehydrogenase.



(Succinic acid) (Malonic acid)

The actual results of competitive inhibition depend on the concentration of competitive inhibitor, the concentration of substrate and the affinity between the substrate and enzyme. The amount of inhibition decreases along with increasing the concentration of substrate.

The character of competitive inhibition is that the maximum velocity of enzyme reaction V_m is constant, but the Michaelis constant K_m increases along with the increasing of inhibitors concentration as shown in Fig. 1.6.

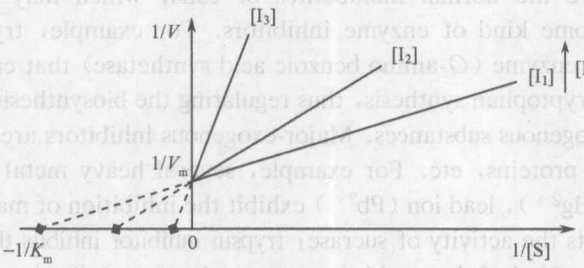


Fig. 1.6 The K_m and V_m change for competitive inhibition

1.3.5.2 Non-competitive inhibition

Noncompetitive inhibition is the inhibition in which inhibitor and substrate combine with the diffluent sites of enzyme molecules respectively, and thus cause the decrease of enzyme activity.

There is no relationship between the molecular structure of inhibitors and substrate, as the combined locus of non-competitive inhibitors is other than the active site of enzymes. Increasing the concentration of substrate cannot reverse the non-competitive inhibition.

The character of non-competitive inhibition is that the maximum velocity of reaction V_m decreases, but the Michaelis constant K_m has no change along with the increasing of inhibitors concentration as shown in Fig. 1.7.

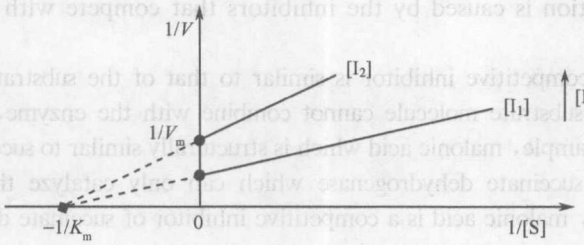


Fig. 1.7 The K_m and V_m change for non-competitive inhibition

1.3.5.3 Uncompetitive inhibition

The inhibition caused by the inhibitors combining with the intermediate complex when