Chapter 3

LITERATURE SURVEY

3.1 DIGESTION AND THE ROLE OF ENZYMES [1]

3.1.1 Digestion

We all need a regular supply of nutrients in our diet. Unfortunately, the nutrients in food are not ready for use by our cells. Without processing by the digestive system, food would be of no more use to us than a lump of coal in the gas tank of a car. Cells perform metabolic reactions that provide energy for the synthesis of ATP. These reactions require two essential ingredients: (a) oxygen and (b) organic molecules that can be broken down by intracellular enzymes. The respiratory system, working with the cardiovascular system, provides the necessary oxygen. The digestive system, working with the cardiovascular and lymphatic systems, provides the organic molecules. In effect, the digestive system provides both the fuel that keeps all the body's cells running and the building blocks needed for cell growth and repair.

The digestive system consists of a muscular tube, the digestive tract, and various accessory organs. The oral cavity (mouth), pharynx, esophagus, stomach, small intestine, and large intestine make up the digestive tract. Accessory digestive organs include the teeth, tongue, and various glandular organs, such as the salivary glands, liver, and pancreas, that secrete into ducts emptying into the digestive tract. Food enters the digestive tract and passes along its length. On the way, the secretions of the glandular organs, which contain water, enzymes, buffers, and other components, assist in preparing organic and inorganic nutrients for absorption across the epithelium of the digestive tract.

Digestive functions can be considered to be a series of integrated steps:

1. Ingestion occurs when materials enter the digestive tract via the mouth. Ingestion is an active process involving conscious choice and decision making.
2. Mechanical processing is physical manipulation and distortion that makes materials easier to propel along the digestive tract and increases the surface area for enzymatic attack. Mechanical processing may or may not be required before ingestion; liquids can swallow immediately but must process most solids first. Tearing and crushing with the teeth, followed by squashing and compaction by the tongue, are examples of preliminary mechanical

processing. Swirling, mixing, and churning motions of the stomach and intestines provide mechanical processing after ingestion.

3. Digestion refers to the chemical breakdown of food into small organic fragments suitable for absorption by the digestive epithelium. Simple molecules in food, such as glucose, can be absorbed intact, but epithelial cells have no way to deal with molecules the size and complexity of proteins, polysaccharides, or triglycerides. These molecules must be disassembled by digestive enzymes prior to absorption. For example, the starches in a potato are of no value until enzymes have broken them down to simple sugars that can absorb and distribute to cells.

4. Secretion is the release of water, acids, enzymes, buffers, and salts by the epithelium of the digestive tract and by glandular organs.

5. Absorption is the movement of organic substrates, electrolytes (inorganic ions), vitamins, and water across the digestive epithelium and into the interstitial fluid of the digestive tract.

6. Excretion is the elimination of waste products from the body. The digestive tract and glandular organs secrete waste products in secretions discharged into the lumen of the tract. Most of these waste products, after mixing with the indigestible residue of the digestive process, will leave the body. The ejection of materials from the digestive tract, a process called defecation, or egestion, eliminates materials as feces.

3.1.2 Digestion and absorption in the oral cavity

Saliva initiates the digestion of complex carbohydrates before the material is swallowed. The enzyme involved is salivary amylase, which is also known as ptyalin or alpha-amylase. Although the digestive process begins in the oral cavity, it is not completed there, and no absorption of nutrients occurs across the lining of the oral cavity. Food remains in the mouth only for short time, and probably not more than 5 % of all the starches that are eaten will have become hydrolyzed by the time the food is swallowed [2].

3.1.3 Digestion and absorption in the stomach

The stomach performs four major functions: a) the bulk storage of ingested food, b) the mechanical breakdown of ingested food, c) the disruption of chemical bonds in food material through the action of acids and enzymes, and

d) the production of intrinsic factor, a glycoprotein whose presence in the digestive tract is required for the absorption of vitamin B_{12}. The mixing of ingested substances with the secretions of the glands of the stomach produces a viscous, highly acidic, soupy mixture of partially digested food. This material is called chyme.

The stomach performs preliminary digestion of proteins by pepsin and, for a variable period, permits the digestion of carbohydrates and lipids by salivary amylase and lingual lipase. Until the pH throughout the material in the stomach falls below 4.5, the salivary amylase and lingual lipase continue to digest carbohydrates and lipids in the meal. These enzymes generally remain active 1-2 hours after a meal [2].

As the stomach contents become more fluid and the pH approaches 2.0, pepsin activity increases and protein disassembly begins. Protein digestion is not completed in the stomach, because time is limited and pepsin attacks only specific types of peptide bonds, not all peptide bonds. However, there is generally enough time for pepsin to break down complex proteins into smaller peptide and polypeptide chains before the chyme enters the duodenum. Although digestion does occur in the stomach, there is no nutrient absorption there because (a) the epithelial cells are covered by a blanket of alkaline mucus and are not directly exposed to the chyme, (b) the epithelial cells lack the specialized transport mechanisms of cells that line the small intestine, (c) the gastric lining is relatively impermeable to water, and (d) digestion has not proceeded to completion by the time chyme leaves the stomach. At this stage, most carbohydrates, lipids, and proteins are only partially broken down.

3.1.4 Digestion and absorption in the small intestine

Stomach is a holding tank where food is saturated with gastric juices and exposed to stomach acids and the digestive effects of pepsin. These are preliminary steps, for most of the important digestive and absorptive functions occur in small intestine, where the products of digestion are absorbed. The mucosa of the small intestine produces only a few of the enzymes involved. The pancreas provides digestive enzymes as well as buffers that assist in the neutralization of acidic chyme. The liver and gallbladder

provide bile, a solution that contains additional buffers and bile salts, compounds that facilitate the digestion and absorption of lipids.

The specific pancreatic enzymes involved include the following:

- Pancreatic alpha-amylase is a carbohydrase, an enzyme that breaks down certain starches. Pancreatic alpha amylase is almost identical to salivary amylase.
- Pancreatic lipase breaks down certain complex lipids, releasing fatty acids and other products that can be easily absorbed.
- Nucleases break down nucleic acids.
- Proteolytic enzymes break certain proteins apart. The proteolytic enzymes of the pancreas include proteases and peptidases; proteases break apart large protein complexes, whereas peptidases break small peptide chains into individual amino acids. Proteolytic enzymes account for about 70 percent of the total pancreatic enzyme production.

A typical meal contains a mixture of carbohydrates, proteins, lipids, water, electrolytes, and vitamins. Digestive system handles each of those components differently. Large organic molecules must be broken down through digestion before absorption can occur. Water, electrolytes, and vitamins can be absorbed without preliminary processing, but special transport mechanisms are commonly involved.

Food contains large organic molecules, many of them insoluble. The digestive system first breaks down the physical structure of the ingested material and then proceeds to disassemble the component molecules into smaller fragments. This disassembly eliminates any antigenic properties, making the fragments suitable for absorption. The molecules released into the bloodstream will be absorbed by cells and either (a) broken down to provide energy for the synthesis of ATP or (b) used to synthesize carbohydrates, proteins, and lipids.

Most ingested organic materials are complex chains of simpler molecules. In a typical dietary carbohydrate, the basic molecules are simple sugars. In a protein, the building blocks are amino acids, and in lipids, they are generally

fatty acids. Digestive enzymes break the bonds between the component molecules in a process called hydrolysis.

The classes of digestive enzymes differ with respect to their specific targets. Carbohydrases break the bonds between sugars, proteases split the linkages between amino acids, and lipases separate the fatty acids from glycerides. Specific enzymes in each class may be even more selective, breaking bonds between specific molecules. For example, a particular carbohydrase might break bonds between glucose molecules but not those between glucose and another simple sugar.

The enzymes involved in digestion are localized in two different sites. The enzymes secreted by the salivary glands, tongue, stomach, and pancreas are mixed into the ingested material as it passes along the digestive tract. These enzymes break down large proteins, lipids, and carbohydrates into smaller fragments, which in turn must typically be broken down further before absorption can occur. The final enzymatic steps involve brush border enzymes, which are attached to the exposed surfaces of microvilli.

Figure 3.1 summarizes information concerning the digestive fates of carbohydrates, lipids, and proteins, and Table 3.1 reviews the major digestive enzymes and their functions.

Figure 3.1 Digestive fates of carbohydrates, lipids, and proteins

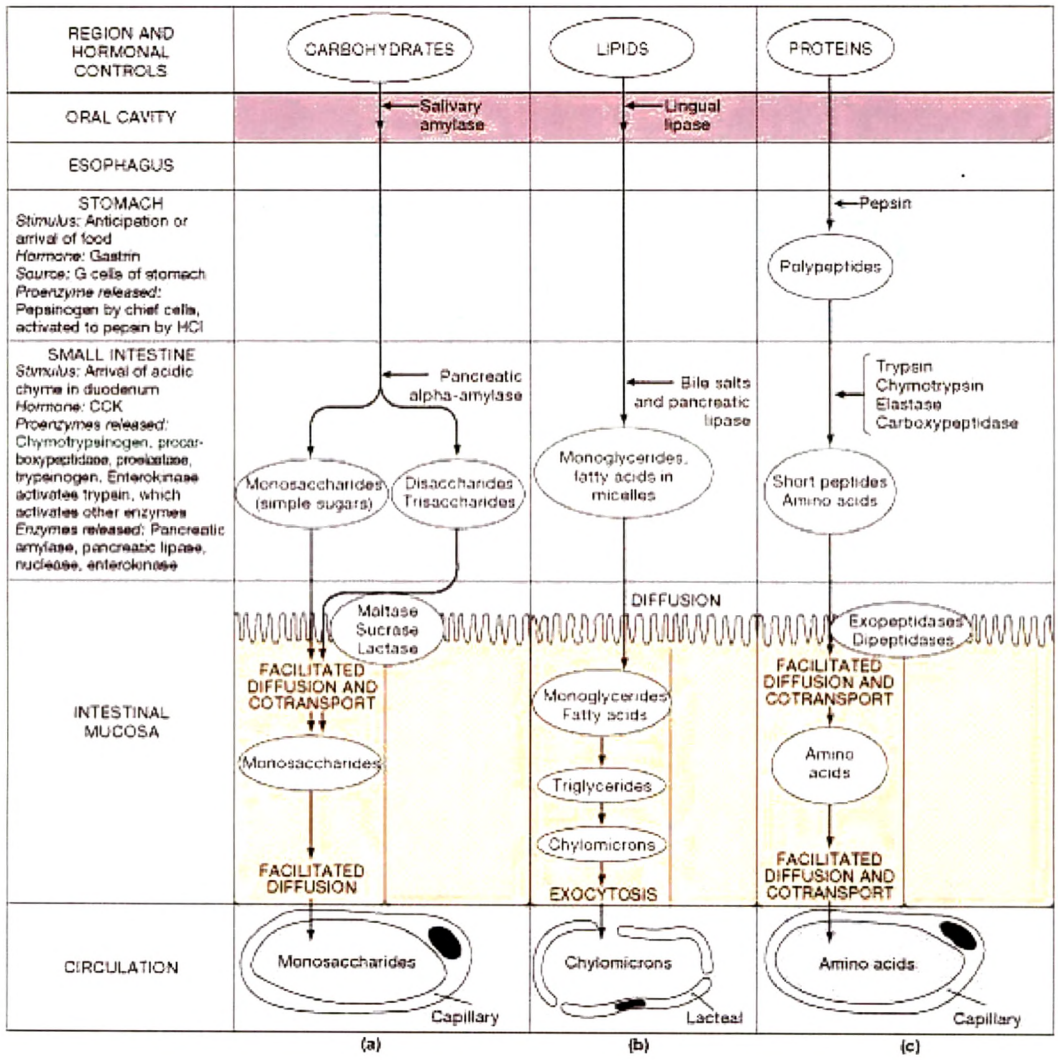


Table 3.1 Digestive enzymes and their functions

Enzyme (Proenzyme)	Source	Optimal pH	Target and Action	Products	Remarks
Carboxypeptidase (Procarboxypeptidase)	Pancreas	7-8	Proteins, polypeptides	Short-chain peptides	Activated by trypsin and amino acids
Chymotrypsin (Chymotrypsinogen)	Pancreas	7-8	Proteins, polypeptides	Short-chain peptides	Activated by trypsin
Dipeptidases	Brush border of small intestine	7-8	Dipeptides	Amino acids	
Elastase (Proelastase)	Pancreas	7-8	Elastin	Short-chain peptides	Activated by trypsin
Enterokinase	Brush border and lumen of small intestine	7-8	Trypsinogen	Trypsin	Reaches lumen through disintegration of shed epithelial cells
Exopeptidases	Brush border of small intestine	7-8	Dipeptides, tripeptides	Amino acids	Found in membrane surface of microvilli
Lingual lipase	Glands of tongue	6.7-7.5	Triglycerides	Fatty acids and monoglycerides	
Maltase, sucrase, lactase	Brush border of small intestine	7-8	Maltose, sucrose, lactose	Monosaccharides	Found in membrane surface of microvilli
Nuclease	Pancreas	7-8	Nucleic acids	Nitrogenous bases	Includes ribonuclease for RNA and deoxyribonuclease for DNA
Pancreatic alpha-amylase	Pancreas	6.7-7.5	Breaks bonds between monomers in complex carbohydrates	Disaccharides and trisaccarides	
Pancreatic lipase	Pancreas	7-8	Triglycerides	Fatty acids and monoglycerides	Bile salts must be present for efficient action
Pepsin (Pepsinogen)	Chief cells of stomach	1.5-2.0	Breaks bonds between amino acids in proteins	Short-chain polypeptides	Secreted as proenzyme, pepsinogen; activated by H^+ in stomach acid
Rennin	Stomach	3.5-4.0	Coagulates milk proteins		Secreted only in infants
Salivary amylase	Salivary glands	6.7-7.5	Breaks bonds between monomers in complex carbohydrates	Disaccharides and trisaccharides	
Trypsin (Trypsinogen)	Pancreas	7-8	Proteins, polypeptides	Short-chain peptides	Proenzyme activated by enterokinase; activates other pancreatic proteases

3.1.5 Carbohydrate digestion and absorption

The digestion of complex carbohydrates (simple polysaccharides and starches) proceeds in two steps. One step involves carbohydrases produced by the salivary glands and pancreas; the other involves brush border enzymes.

3.1.5.1 Salivary and pancreatic enzymes

The digestion of complex carbohydrates involves two enzymes—salivary amylase and pancreatic alpha-amylase (Figure 3.1a), that function effectively at a pH of 6.7-7.5. Carbohydrate digestion begins in the mouth during mastication, by the action of salivary amylase from the parotid and submandibular salivary glands. Salivary amylase breaks down starches (complex carbohydrates) into smaller fragments, producing a mixture composed primarily of disaccharides (two sugars) and trisaccharides (three sugars). Salivary amylase continues to digest the starches and glycogen in the meal for 1-2 hours before stomach acids render it inactive. Because the enzymatic content of saliva is not high, only a small amount of digestion occurs over this period. In the duodenum, the remaining complex carbohydrates are broken down by the action of pancreatic alpha-amylase. Any disaccharides or trisaccharides produced, as well as any already present in the food, are ignored by both salivary and pancreatic amylases. Additional hydrolysis does not occur until these molecules contact the intestinal mucosa.

3.1.5.2 Brush border enzymes

Prior to absorption, disaccharides and trisaccharides are fragmented into monosaccharides (simple sugars) by brush border enzymes of the intestinal microvilli. Maltase splits bonds between the two glucose molecules of the disaccharide maltose. Sucrase breaks the disaccharide sucrose into glucose and fructose, another six-carbon sugar. Lactase releases a molecule of glucose and one of galactose from the hydrolysis of the disaccharide lactose. Lactose is the primary carbohydrate in milk, so by breaking down lactose, lactase provides essential services throughout infancy and early childhood. If the intestinal mucosa stops producing lactase by adolescence, the individual becomes lactose-intolerant. After ingesting milk and other dairy products, individuals who are lactose-intolerant can have a variety of unpleasant digestive problems.

3.1.5.3 Absorption of monosaccharides

The intestinal epithelium then absorbs the monosaccharides by facilitated diffusion and cotransport mechanisms. There are three major differences between facilitated diffusion and cotransport:

1. Facilitated diffusion moves only one molecule or ion through the cell membrane; cotransport moves more than one molecule or ion through the membrane at the same time. In cotransport, the transported materials move in the same direction: down the concentration gradient for at least one of the transported substances.
2. Facilitated diffusion does not require ATP. Although cotransport by itself does not consume ATP, the cell must often expend ATP to preserve homeostasis. For example, the process may introduce sodium ions that must later be pumped out of the cell.
3. Facilitated diffusion will not occur if there is an opposing concentration gradient for the particular molecule or ion. Cotransport can occur despite an opposing concentration gradient for one of the transported substances. For example, cells lining the small intestine will continue to absorb glucose when glucose concentrations inside the cells are much higher than they are in the intestinal contents. The cotransport system responsible for the uptake of glucose also brings sodium ions into the cell. This passive process resembles facilitated diffusion except that both a sodium ion and a glucose molecule must bind to the carrier protein before they can move into the cell. Glucose cotransport is an example of sodium-linked cotransport. Comparable cotransport mechanisms exist for other simple sugars and for some amino acids. Although these mechanisms deliver valuable nutrients to the cytoplasm, they also bring in sodium ions that must be ejected by the sodium-potassium exchange pump. The simple sugars entering the cell diffuse by the cytoplasm and reach the interstitial fluid by facilitated diffusion at the base of the cell. Once in the interstitial fluid, these monosaccharides diffuse into the capillaries of the villus for eventual transport to the liver in the hepatic portal vein.

3.1.6 Protein digestion and absorption

Proteins have very complex structures, so protein digestion is both complex and time-consuming. The first problem to overcome is the disruption of the

three-dimensional organization of the food so that proteolytic enzymes can attack individual proteins. This step involves mechanical processing in the oral cavity, through mastication, and chemical processing in the stomach, through the action of hydrochloric acid. Exposure of the bolus to a strongly acidic environment breaks down plant cell walls and the connective tissues in animal products and has the extra benefit of killing most pathogenic microorganisms.

The acidic contents of the stomach also provide the proper environment for the activity of pepsin, the proteolytic enzyme secreted by chief cells of the stomach (Figure 3.1c). Pepsin, which works effectively at a pH of 1.5-2.0, breaks peptide bonds within a polypeptide chain. A protease with this kind of activity is called an endopeptidase. For instance, pepsin might take a polypeptide 500 amino acids long and break it into two smaller polypeptides, one containing 200 amino acids and the other 300. In the few hours chyme spends in the stomach, pepsin has time to reduce the relatively huge proteins (10,000- 100,000 amino acids in length) of the chyme into smaller polypeptide fragments.

When the chyme enters the duodenum, enterokinase produced in the small intestine triggers the conversion of trypsinogen to trypsin, and the pH is adjusted to 7-8. Pancreatic proteases can now begin working. Trypsin, chymotrypsin, and elastase are endopeptidases that, like pepsin, break peptide bonds within a polypeptide. Each enzyme has a different specialty. For example, trypsin breaks peptide bonds involving the amino acids arginine or lysine, whereas chymotrypsin targets peptide bonds involving tyrosine or phenylalanine. Carboxypeptidase is called an exopeptidase, because it simply chops off the last amino acid of a polypeptide chain, ignoring the identities of the amino acids involved. Thus, while the endopeptidases are generating peptide fragments of varying length, carboxypeptidase is producing free amino acids.

3.1.6.1 Absorption of amino acids

The epithelial surfaces of the small intestine contain exopeptidases and dipeptidases that break short peptide chains into individual amino acids.

(Dipeptidases break apart dipeptides.) These amino acids, as well as those produced by the pancreatic enzymes, are absorbed through both facilitated diffusion and cotransport mechanisms. After diffusing to the opposite end of the cell, the amino acids are released into the interstitial fluids by facilitated diffusion and cotransport. Once within the interstitial fluids, the amino acids diffuse into intestinal capillaries for transport to the liver within the hepatic portal vein.

3.2 DIGESTIVE DISORDERS

3.2.1 Poor digestion

Several factors are known to contribute to the development of digestive problems. Poor eating habits, such as inadequate chewing of food, "eating on the run," or eating late in the day, can result in inadequate enzyme production. Certain dietary choices, such as excessive consumption of alcohol, refined carbohydrates and fat, as well as a high meat and cooked food diets with few raw, enzyme-rich foods may also place stress on the body's digestive capacity. In addition, problems with digestion can occur simply as a result of aging [3]. It is fairly common for elderly individuals to experience both a decrease in hydrochloric acid production as well as a general decline in digestive enzyme secretion [3]. With an increasing aging population burdened with unhealthful diets and stressful life styles, it is likely that healthcare professionals will see more and more patients developing digestive problems. Currently, it is estimated that 58 % of the population suffers from some type of digestive disorder [4]. A lack of optimal digestive function associated with enzyme inadequacy may lead to malabsorption and a host of related conditions.

3.2.2 Naturally occurring digestive enzymes

Perhaps one largely overlooked factor that may contribute to problems with digestion may be the lack of raw, uncooked foods present in the average diet. Raw foods contain many enzymes that may help facilitate digestion. When they are eaten, mastication breaks down cell membranes allowing the release and activation of the naturally occurring enzymes present in the food. These enzymes may play a beneficial role by initiating the breakdown of the food and giving the body a head start on the digestive process [5, 6]. Unfortunately, in this part of the world we tend to eat mostly cooked foods. When a food is cooked at 118° F or greater the natural enzymes are destroyed. Even though cooking destroys these helpful, naturally occurring enzymes, it may not be reasonable to suggest that a total raw foods diet is appropriate. For food safety reasons alone, this practice would not be advisable. Furthermore, some foods such as carrots and broccoli provide adequate nutrient value when cooked and may actually cause fewer gastrointestinal symptoms in patients who have difficulty digesting cellulose [5, 6]. Inclusion of appropriate raw foods

combined with enzyme replacement may be the most balanced and rational way to support those with digestive enzyme insufficiencies.

3.2.3 Enzyme replacement

In addition to consuming adequate levels of raw foods, a common approach to supporting the patient with digestive enzyme inadequacy is oral enzyme replacement. This entails providing enzyme supplements, whether they are animal or nonanimal derived, in the quantity necessary to maintain adequate digestive capacity and facilitate absorption of essential nutrients. Oral enzyme replacement has proven to be helpful over many years of use [7-11]. In addition to the well known benefits associated with enzyme replacement, it has been suggested that oral supplementation with enzymes may have a sparing effect on the body's own digestive enzymes [5, 12].

3.2.4 Advantages of non-animal derived enzymes

Oral supplementation with non-animal derived enzymes, such as microbial enzymes those manufactured by a fermentation process of *Aspergillus*, for example is associated with a number of inherent uniquenesses. They offer effective digestive support that works synergistically with, or as an alternative to, animal derived enzymes and are free of some of the inherent limitations of conventional pancreatic enzyme supplements [6]. They provide a broad spectrum of digestive enzymes — protease, lipase, amylase, lactase, maltase, invertase, and even cellulase for aiding cellulose hydrolysis. One of the most functionally valuable attributes related to microbial enzymes is that they appear to possess unusually high stability and activity throughout a wide range of pH conditions (from a pH of 2-10) [8]. This enables them to be more consistently active and functional for a longer distance as they are transported through the digestive tract. It is important to note that these enzymes, although microbially derived, contain no microbial residue. Modern filtration technology allows for a clean and pure product, consisting strictly of enzymes.

3.2.5 Gastrointestinal symptoms

Insufficient amounts of digestive enzymes can cause or exacerbate abnormal digestive conditions, such as maldigestion, food allergies or sensitivities,

intestinal fermentation, putrefaction and peroxidation, and the phenomenon known as intestinal hyperpermiability, or "leaky gut." Some research suggests that microbial enzymes may support healthy digestion. They have been the subject of various studies evaluating their effects on lactose intolerance, pancreatic exocrine deficiency, steatorrhea, celiac disease and a variety of other digestive disorders [5, 8, 9, 13-15]. Indigestion is the term used to describe pain and discomfort in the upper abdomen or chest that can develop after a meal. The medical term for it is dyspepsia. Sometimes a burning feeling is felt in the chest, and this is known as heartburn. People of all ages (including children) and both sexes are affected by indigestion. Most people have suffered from indigestion after a large meal or after excessive alcoholic consumption at some time, especially after a holiday meal or party. Up to 20% of people suffer from heartburn at least once a week. Indigestion may be a symptom of a disorder in the stomach or the intestines, or it may be a disorder in itself. Symptoms can include Gas (flatulence), Abdominal pain, rumbling noises in stomach and/or intestines, a bloated feeling, belching (burping), loss of appetite, nausea, vomiting and a burning sensation after eating. Heartburn, a burning pain caused by reflux (regurgitation) of the stomach's contents back up the esophagus. The medical term for the condition is gastroesophageal reflux (GORD). The pain is normally felt in the center of the chest or behind the sternum (breastbone). If severe, it can be hard to distinguish from a heart attack.

Pancreatin is indicated as a pancreatic enzyme supplement and replacement therapy in conditions where pancreatic enzymes are either absent or deficient, resulting in inadequate fat and carbohydrate digestion. Such conditions are usually due to chronic pancreatitis, pancreatectomy, cystic fibrosis, gastrointestinal bypass surgery and ductal obstruction from neoplasm of the pancreas or common bile duct.

Amylase digests not only carbohydrates but also dead white blood cells (pus). Deficiencies of amylase causes abscesses (inflamed areas with pus but not bacteria). In case of toothache, there is a chance of abscess, which is not cured by antibiotics. Amylase is involved in anti-inflammatory reactions such as those caused by the release of histamine and similar substances. The

inflammatory response usually occurs in organs which are in contact with the outside world, i.e., the lungs and skin. These include skin problems such as psoriasis, eczema, hives, insect bites, allergic bee and bug stings, atopic dermatitis, and all types of herpes. Some lung problems including asthma and emphysema may require amylase plus other enzyme formulas depending on the particular condition [16].

3.3 PROFILE OF FUNGAL ALPHA AMYLASE

3.3.1 History

The first commercial enzyme from a fungus *Aspergillus oryzae* was Taka-diastase produced by Dr. Jokichi Takamine in 1894. Since then, major enzyme manufacturers continued production of fungal diastase by a unique tray culture fermentation process using *Aspergillus oryzae* which is cultured on wheat bran and the produced enzyme is extracted with water, purified with alcohol and diluted with suitable diluents and made them available for agriculture, food, beverages, animal feed and pharmaceutical industries.

3.3.2 Appearance in pharmacopoeia [17, 18]

Indian, Brazil, Japan, Portuguese, France

3.3.3 Synonyms [17, 18]

Amylase, Amilasa, Diastase, Glucogenase, Mastin, ptyalin

3.3.4 Chemical name and CAS registry number [19]

Amylase [9000-92-4]

3.3.5 Composition

Fungal alpha amylase is a glycoprotein having 476 amino acid residues in single polypeptide chain. A value of 51,000 for the molecular weight of *Aspergillus oryzae* has been obtained by sedimentation, diffusion, and partial specific volume measurements [20] and of 52,500 from end-group analyses [21].

3.3.6 Functional category

Digestive enzyme [22]

3.3.7 Dose

60 mg to 300 mg daily [17].

3.3.8 Uses and administration [18]

The term amylases refer to an enzyme catalyzing the hydrolysis of α–1, 4-glucosidic linkage of polysaccharides such as starch, glycogen or their degradation products. Amylases may be classified accordingly to the manner in which the glucosidic bond is attacked. Endoamylases attack the α–1, 4-glucosidic linkage at random. Alpha amylases are the only types of endoamylases known and yield dextrins, oligosaccharides and monosaccharides. The more common alpha amylases include those isolated from human saliva, mammalian pancreas, *Bacillus Subtilis, Aspergillus oryzae* and barley malt. Exoamylases attack α–1, 4- glucosidic linkage only from nonreducing outer polysaccharide chain ends. They include beta amylases and glucoamylases (amylo-glycosidase or gamma-amylases) and are of vegetable or microbial origin. Beta amylase yield beta-limit dextrins and maltose and glucoamylases yield glucose.

Amylases are used in the production and predigested starchy foods and in the treatment of amylaceous dyspepsia. It is also used for the conversion of starch to fermentable sugars in the brewing and fermentation industries. Amylases form various sources have been used as ingredient of preparations of digestive enzymes and have been given by mouth for its supposed activity in reducing respiratory tract inflammation and local swelling and odema.

3.3.9 Description

Cream to light brown-coloured powder; almost odourless or with faint characteristic odour; hygroscopic [22].

3.3.10 Pharmacopoeial specification

Table 3.2 Pharmacopoeial specifications for fungal alpha amylase

Test	IP 1996
Identification	-
Characters	+
Microbial limits	–
Loss on drying	< 5.0 %
Assay (anhydrous basis)	NLT 800 units

3.3.11 Typical properties

Solubility: Almost entirely soluble in water, insoluble in alcohol [17].

Loss on drying: Not more than 5.0 %, determined on 1 g by drying in an oven at 105°C for 1 hour [22].

3.3.12 Stability and storage conditions

Amylase solution has maximum activity at pH 5.2 and is inactivated at pH 1.7. It shows stability over pH range 6 to 9 [17, 23, 24]. Store in tightly-closed containers in a cool, dry place [22].

3.3.13 Incompatibilities

Incompatible with acids, alkalies, alum, iron salts and tannins.

3.3.14 Safety

Hypersensitivity reactions have been reported [18].

3.4 FUNGAL ALPHA AMYLASE STRUCTURE

Structure: Alpha amylase (taka amylase) [25]

Source: *Aspergillus oryzae*

Resolution: 2.10Å. **R-factor:** 0.198.

SWISS-PROT code: AMYA_ASPOR

Enzyme classification: 3.2.1.1

Molecule(s) in Protein Data Bank (PDB) file 6taa: contains

Protein: 476 residues

2 Metal ions: Ca ions

239 water molecules.

The fungal alpha amylase consist of three domains called A, B and C. Domain A is a TIM-barrel [$(\alpha/\beta)_8$-barrel],which is interrupted by an irregular β-domain (domain B) inserted between the third β-strand and the third α-helix of the TIM-barrel. Domain C is a Greek Key motif which is located approximately on the opposite side of the TIM-barrel with respect to domain B. The active site is situated in a cleft at the interface between domains A and B [26].

Figure 3.2 Secondary structure of fungal alpha amylase

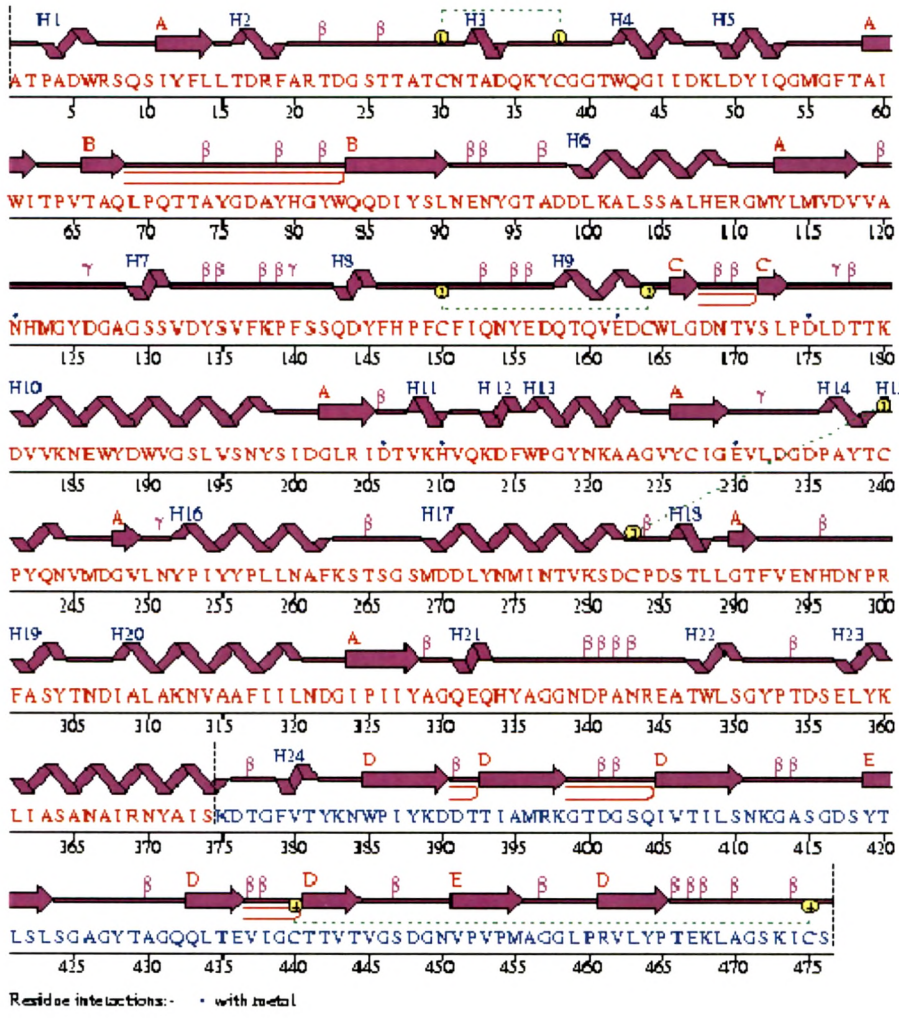


Figure 3.3a PROMOTIF summary: Secondary structure of fungal alpha amylase

Number of residues is: 476

Percentage of strand is: 18.1 Percentage of alpha helix is: 26.5 Percentage of 3,10 helix is: 6.5

SHEETS

Number strands	Name	Type	Barrel	Topology
8	A	PAR	N	-1X-1X-1X-1X-1X 7X -1X
2	B	ANTI	N	-1
2	C	ANTI	N	1
6	D	ANTI	N	1 1 2X 1 -2X
2	E	ANTI	N	1

STRANDS

Location	Sheet
11 - 14	A
59 - 62	A
66 - 68	B
84 - 90	B
113 - 118	A
166 - 167	C
172 - 173	C
202 - 205	A
226 - 229	A
248 - 249	A
290 - 291	A
324 - 328	A
385 - 390	D
393 - 398	D
405 - 410	D

STRANDS

Location	Sheet
419 - 423	E
433 - 436	D
441 - 444	D
451 - 455	E
461 - 465	D

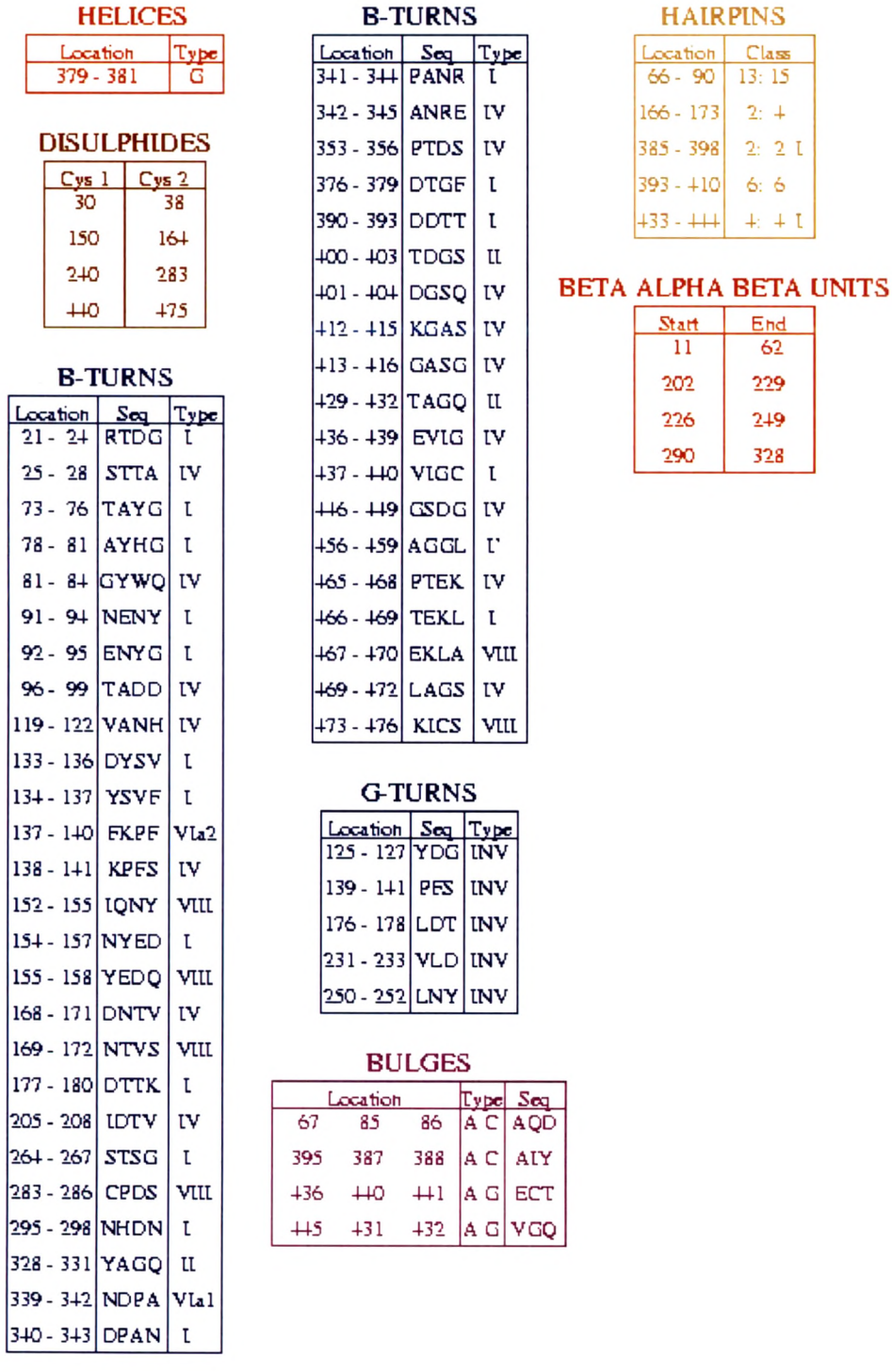
HELICES

Location	Type
3 - 6	H
16 - 19	H
32 - 34	G
42 - 46	H
49 - 53	H
99 - 109	H
129 - 131	G
143 - 145	G

HELICES

Location	Type
158 - 163	H
181 - 198	H
208 - 210	G
213 - 215	G
216 - 223	H
236 - 239	H
240 - 244	G
252 - 262	H
269 - 282	H
286 - 288	G
301 - 304	H
308 - 320	H
331 - 333	G
347 - 350	G
357 - 375	H

Figure 3.3b PROMOTIF summary: Secondary structure of fungal alpha amylase



HELICES

Location	Type
379 - 381	G

DISULPHIDES

Cys 1	Cys 2
30	38
150	164
240	283
440	475

B-TURNS

Location	Seq	Type
21 - 24	RTDG	I
25 - 28	STTA	IV
73 - 76	TAYG	I
78 - 81	AYHG	I
81 - 84	GYWQ	IV
91 - 94	NENY	I
92 - 95	ENYG	I
96 - 99	TADD	IV
119 - 122	VANH	IV
133 - 136	DYSV	I
134 - 137	YSVF	I
137 - 140	FKPF	VIa2
138 - 141	KPFS	IV
152 - 155	IQNY	VIII
154 - 157	NYED	I
155 - 158	YEDQ	VIII
168 - 171	DNTV	IV
169 - 172	NTVS	VIII
177 - 180	DTTK	I
205 - 208	IDTV	IV
264 - 267	STSG	I
283 - 286	CPDS	VIII
295 - 298	NHDN	I
328 - 331	YAGQ	II
339 - 342	NDPA	VIa1
340 - 343	DPAN	I

B-TURNS

Location	Seq	Type
341 - 344	PANR	I
342 - 345	ANRE	IV
353 - 356	PTDS	IV
376 - 379	DTGF	I
390 - 393	DDTT	I
400 - 403	TDGS	II
401 - 404	DGSQ	IV
412 - 415	KGAS	IV
413 - 416	GASG	IV
429 - 432	TAGQ	II
436 - 439	EVIG	IV
437 - 440	VIGC	I
446 - 449	GSDG	IV
456 - 459	AGGL	I'
465 - 468	PTEK	IV
466 - 469	TEKL	I
467 - 470	EKLA	VIII
469 - 472	LAGS	IV
473 - 476	KICS	VIII

G-TURNS

Location	Seq	Type
125 - 127	YDG	INV
139 - 141	PFS	INV
176 - 178	LDT	INV
231 - 233	VLD	INV
250 - 252	LNY	INV

BULGES

Location			Type	Seq
67	85	86	A C	AQD
395	387	388	A C	AIY
436	440	441	A G	ECT
445	431	432	A G	VGQ

HAIRPINS

Location	Class
66 - 90	13: 15
166 - 173	2: 4
385 - 398	2: 2 1
393 - 410	6: 6
433 - 444	4: 4 1

BETA ALPHA BETA UNITS

Start	End
11	62
202	229
226	249
290	328

3.5 Mechanism of enzyme action [27]

Many types of amylases are found throughout the animal, vegetable and microbial kingdoms. They have evolved along different pathways to enable the organism to convert insoluble starch (or glycogen) into low molecular weight, water soluble dextrins and sugars. Alpha amylases are dextrinogenic and can attack the interior of starch molecules. The products retain the alpha anomeric configuration. Beta amylases act only at the non-reducing chain ends and liberate only beta maltose. Both alpha and beta amylases exhibit multiple (repetitive) attack, that is, after the initial catalytic cleavage; the enzyme may remain attached to the substrate and lead to several more cleavages before dissociation of the enzyme-substrate complex. Amylases have extended substrate binding sites, in the range 4-9 glucose units. This enables the enzyme to stress the substrate and lower the activation energy for hydrolysis. Similarly the enzyme exerts torsion on the glucose unit at the catalytic site, inducing a transition state conformation (oxycarbonium ion). Alpha and beta amylases differ in the stereospecific hydration of the oxycarbonium ion, in the sequence of liberation of the right-hand vs. the left-hand product, and the direction of motion of the retained substrate to give multiple attacks.

3.6 Fungal alpha amylase active site

MacGregor [28] evaluated two computerized methods of predicting protein secondary structure from amino acid sequences by using them on the alpha-amylase of *Aspergillus oryzae*, for which the three-dimensional structure has been determined. The methods were used, with amino acid alignments, to predict the structures of other alpha-amylases. It was found that all alpha-amylases of known amino acid sequence have the same basic structure, a barrel of eight parallel stretches of extended chain surrounded by eight helices. Strong similarities were found in those areas of the proteins believed to bind an essential calcium ion and at that part of the active site that catalyzes bond hydrolysis in the substrates. The active site, as a whole, was formed mainly of amino acids situated on loops joining extended chain to the adjacent helix. Variations in the length and amino acid sequence of these loops, from one alpha-amylase to another, provide the differences in binding

the substrates believed to account for the known variations in action pattern of alpha-amylases of different biological origins.

Matsuura et al. [29] prepared complete molecular model of Taka-amylase and showed a possible productive binding mode between substrate and enzyme by fitting of an amylose chain in the catalytic site of the enzyme. On the basis of the difference Fourier analysis and the model fitting study, glutamic acid (Glu230) and aspartic acid (Asp297), which are located at the bottom of the cleft, were concluded to be the catalytic residues, serving as the general acid and base, respectively.

Catalytic Site Atlas (CSA) version 2.0.10 entry [30] for Taka-amylase (6taa) showed His 122, Arg 204, Asp 206, Glu 230, His 296 & Asp 297 as catalytic residues.

The enzymes whose structure has been determined by X-ray crystallography consistently possess a (beta/alpha) 8 barrel supersecondary structure. The active site is always located at the C-terminal end of the 3rd and 4th beta-strands of the central beta-barrel. For these reasons, the catalytic mechanism of the hydrolysis of starch in the family enzymes can be considered as essentially the same. Furthermore in the three-dimensional structure, there exist several sub-domains in addition to the main domain, which are characteristic in the polypeptide chain foldings and relative positions with respect to the main domain. The role of the sub-domains is not yet fully understood. However, for enzymes capable of raw-starch binding, the sub-domain at the C-terminal end has been found to be responsible for this activity. The differences in the substrate specificities are related to the structures of the sub-domains that flank the main domain, forming altogether the active cleft.

The roles of the three catalytic residues are discussed here according to the results obtained by the author's group on the *Pseudomonas stutzeri* maltotetraose-forming alpha-amylase (G4-amylase), in which the complexes between mutant enzymes and the substrate have been analyzed [31, 32] . These mechanisms can be generalized in the alpha-amylase family enzymes.

1. Glu219 (Glu230: Taka-amylase)

This residue is always conserved in the family enzymes (conserved region 3). However, there are no other residues conserved near this residue, making it the sole conservative. The carboxylate of this residue is believed to have a high pKa value caused by the environment, making it possible to easily liberate a proton on binding the substrate, followed by its addition to the glucosidic bond leading to cleavage (acid catalyst).

2. Asp193 (Asp206: Taka-amylase)

X-ray structure analyses revealed that one carboxylic oxygen of this residue resides very close to the atom C-1 of the cleaving end glucose residue (position -1), suggesting that this residue is involved in the intermediate formation of the catalytic reaction (base catalyst or nucleophile). It is still under dispute whether the true intermediate takes a carbonium ion or undergoes covalent bonding with the carboxyl oxygen of this residue. Recently a paper has been published which describes the structure of a covalent adduct between sugar and the caboxyl oxygen [33]. However, it may not be valid proof of what was the true intermediate. The author of this article believes that the true intermediate may rather take a somewhat intermediate form between two possible forms or take both forms cousecutively in the reaction time coordinates.

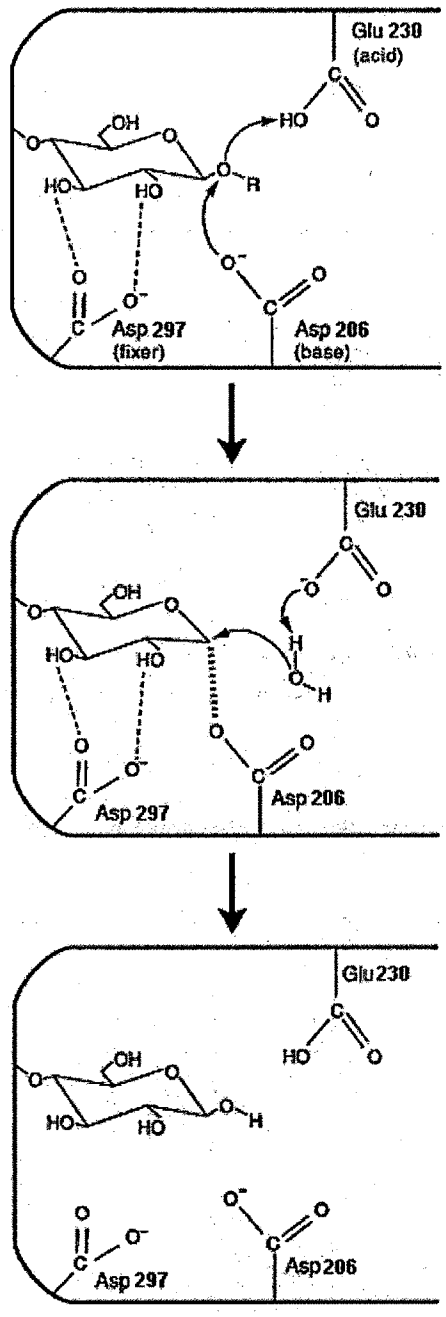
3. Asp294 (Asp297: Taka-amylase)

Recently the author's group has clarified the role of this residue by detailed X-ray structure analyses of the complexes between maltotetraose and five mutants of G4-amylase. These studies showed that the ring distortion of the glucose residue at position -1 which is consistently observed when this residue is aspartic acid, disappeared when it was altered to asparagine, adopting an approximate regular chair conformation. Furthermore these studies put forward evidences that the maltotetraose binds weakly in the case of asparagine. From these results, we can conclude that the role of this residue is to strengthen the substrate binding, giving rise to distortion of the sugar ring leading to the easy cleavage of the glucoside bond (fixer for catalysis) [32].

A schematic picture showing a catalytic mechanism involving three residues is shown in Figure 3.4.

Some enzymes require no chemical groups for activity other than their amino acid residues. Others require an additional chemical component called cofactor either one or more inorganic ions, such as Fe 2+, Mg2+, Mn2+, Zn2+ and others ions. Enzyme molecules which include metals are called metalloenzymes. Metals, whether tightly bound to the enzyme or taken up from solution along with the substrate, can participate in catalysis in several ways. Ionic interactions between an enzyme-bound metal and a substrate can help orient the substrate for reaction or stabilize charged reaction transition states [34].

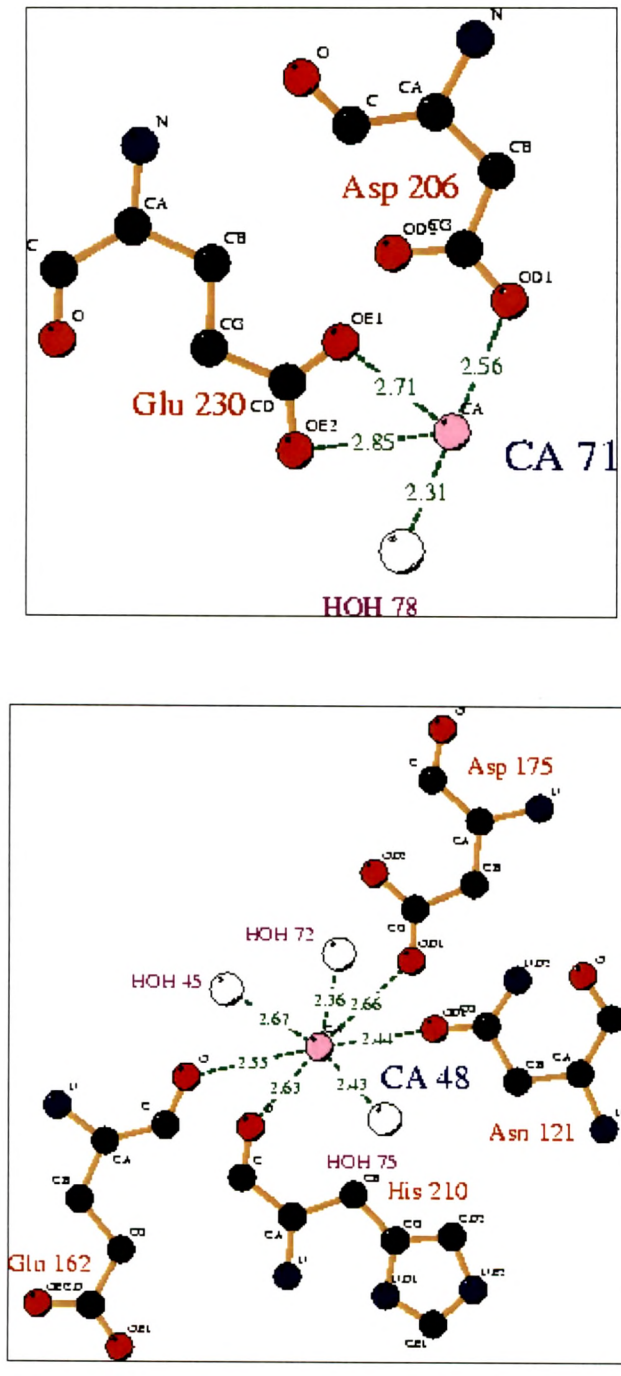
Figure 3.4 Catalytic mechanism of fungal alpha amylase involving three residues.



Boel et al. [35] carried out X-ray diffraction analysis (at 2.1-A resolution) of an alpha-amylase from Aspergillus allowed a detailed description of the stereochemistry of the calcium-binding sites. The primary site (which is essential in maintaining proper folding around the active site) contains a tightly bound Ca2+ with an unusually high number of eight ligands (O delta 1 and O delta 2 of Asp175, O delta of Asn121, main-chain carbonyl oxygens of Glu162 and Glu210, and three water molecules). A secondary binding site was identified at the bottom of the substrate binding cleft; it involves the residues presumed to play a catalytic role (Asp206 and Glu230). This explains the inhibitory effect of calcium observed at higher concentrations.

LIGPLOT [25] of metal's interactions with Taka-amylase is shown in Figure 3.5.

Figure 3.5 LIGPLOT of metal's interactions with Taka-amylase



3.7 Inhibitors

Ali et al. [36] studied the effect of 8 growth regulators at concentrations of 1,000, 5,000 and 10,000 ppm on the activity of fungal alpha-amylase was studied. Indol acetic acid (IAA) and naphthalene acetic acid (NAA) inhibited alpha-amylase activity by 2% and 7% at 1,000 ppm. The other 6 growth regulators, indol butyric acid (IBA), gibberellic acid, cumarin, cycocel (CCC), atonik-G and kylar, did not inhibit but stimulated alpha-amylase activity (0 to 9%) at 1,000 ppm. All growth regulators studied inhibited alpha-amylase activity at 5,000 and 10,000 ppm concentration except kylar. The effect of organic acids and formaldehyde at 0.01, 0.005, and 0.001 M was studied. Acetic acid stimulated alpha-amylase at all concentrations, but formic acid, oxalic acid, lactic acid and citric acid inhibited alpha-amylase activity by 91, 100, 100 and 79%, respectively, at a concentration of 0.01 M, while by 31, 100, 15 and 20%, respectively, at 0.005 M. Formaldehyde induced 7, 3 and 2% inhibition at 0.01, 0.005 and 0.001 M, respectively. At 0.01 M either sorbitol or fructose inhibited alpha-amylase by 8%, Maltose 7%, sucrose 6%, phenol, glucose and galactose each by 5%, ethanol, glycerol, arabinose and sodium benzoate each by 4%, isopropanol and mannitol 1%, but methanol and ammonium citrate dibasic did not inhibit alpha-amylase. The results indicate that $CuCl_2$, $SnCl_2$, $AgNO_3$ and $Fe_2(SO_4)_3$ were the strongest inhibitors, followed by $Cd(C_2H_3O_2)$, $HgCl_2$, Na_2-EDTA, Na_2HPO_4 in decreasing order. NaCl, NaBr and $MnSO_4$ did not inhibit alpha-amylase at concentrations from 10 mM to 0.01 mM.

3.8 Estimation methods

Somogyi [37], who first quantified amylase activity by measuring the time required to hydrolyze starch, in a carefully standardized substrate solution. A simple assay to measure this time takes advantage of differently colored products generated by the reaction between iodine and the saccharides depending of their degree of degradation. After mixing the amylase-containing samples with a standardized starch solution, the reaction is monitored by removing portions of the mixture at timed intervals and adding these to aliquots of an iodine solution. As long as starch is present, a blue-purplish color will develop. As the incubation proceeds, the color will change from blue to blue-purple, to red-purple and then to reddish-brown. If the solution remains yellow, all the starch has been hydrolyzed to glucose and maltose and

the assay must be repeated. The reaction is considered to have reached its endpoint when samples produce a reddish-brown color with iodine. The time required to reach the endpoint is a function of alpha-amylase activity expressed in Somogyi units (one Somogyi Unit is defined as the amount of amylase required to produce the equivalent of 1 mg of glucose in free aldehyde groups in 30 minutes at 40°C. (Somogyi Units/dL may be converted to International Units (μmol $minute^{-1}$ L^{-1}) by multiplying by 1.85.) The best estimate of amylase activity can be made using samples diluted to around 3 - 6 Somogyi Units/dL, so that the assay reaches the endpoint after 3 - 6 minutes.

AACC method [38] is absolutely specific for alpha-amylase. The substrate mixture contains the defined oligosaccharide "nonreducing end-blocked *p*-nitrophenyl maltoheptaoside" (BPNPG7) in the presence of excess levels of a thermostable alpha-glucosidase (which has no action on the native substrate due to the presence of the "blocking group"). On hydrolysis of the oligosaccharide by *endo*-acting alpha-amylase, the excess quantities of alpha-glucosidase in the mixture give instantaneous and quantitative hydrolysis of the *p*-nitrophenyl maltosaccharide fragment to glucose and free *p*-nitrophenol. The absorbance at 400 nm is measured, and this is a direct measure of the level of alpha-amylase in the sample analyzed. The assay can be used over the pH range 5.2-7.0 and at temperatures of up to 60°C. The optimal pH for cereal and fungal alpha-amylases is 5.4 and for bacterial alpha-amylase is 6.5.

Davis [39] gave a simpler method to study enzymes using agar. The substrate starch was dissolved in this agar gel and enzyme solution was added to small wells in the gel. The enzyme was diffused out through the gel and created a starchless area around the well. Iodine stain was used to cause starch to turn a dark purple. They concluded that clear zones which were not purple were the areas that the enzyme had digested the starch to sugar. This technique made it simple to test many samples for activity or to determine the amount of activity a specific enzyme might have. In addition, it was easily modified to test various variables that might affect enzyme activity.

Bernfeld [40] developed the method wherein the reducing groups released from starch were measured by the reduction of 3, 5-dinitrosalicylic acid. Different enzyme dilutions were added into a series of numbered test tubes including blank and then incubated the tubes at 25°C for 3-4 minutes to achieve temperature equilibration. At timed intervals, starch solution at 25 °C were added and incubated for exactly 3 minutes. Then at timed intervals, dinitrosalicylic acid color reagent was added to each tube and incubated all tubes in a boiling water bath for 5 minutes. The tubes were cooled to room temperature and read A_{540} versus blank. From standard curve of maltose, micromoles of maltose released were calculated. Activity was calculated by following formula

$$\text{Units/mg} = \frac{\text{micromoles maltose released}}{\text{mg enzyme in reaction mixture X 3 min}}$$

3.9 PROFILE OF CYCLODEXTRINS [41]

3.9.1 Nonproprietary names

BP: Betadex

PhEur: Betadexum

USPNF: Betadex

Beta-cyclodextrin is the only cyclodextrin to be currently described in a pharmacopoeia.

3.9.2 Synonyms

Cyclodextrin: *Carvitron*; cyclic oligosaccharide; cycloamylose; cycloglucan; *Encapsin*; Schardinger dextrin

α-cyclodextrin: alfadex; alpha-cycloamylose; alpha-cyclodextrin; alpha-dextrin; *Cavamax W6 Pharma*, cyclohexa-amylose; cyclomaltohexose.

β-cyclodextrin: beta-cycloamylose; beta-dextrin; *Cavamax W7 Pharma*; cycloheptaamylose; cycloheptaglucan; cyclomaltoheptose; *Kleptose*.

γ-cyclodextrin: *Cavamax W8 Pharma*; cyclooctaamylose; gamma cyclodextrin.

3.9.3 Chemical name and CAS registry number

α-cyclodextrin (10016-20-3)

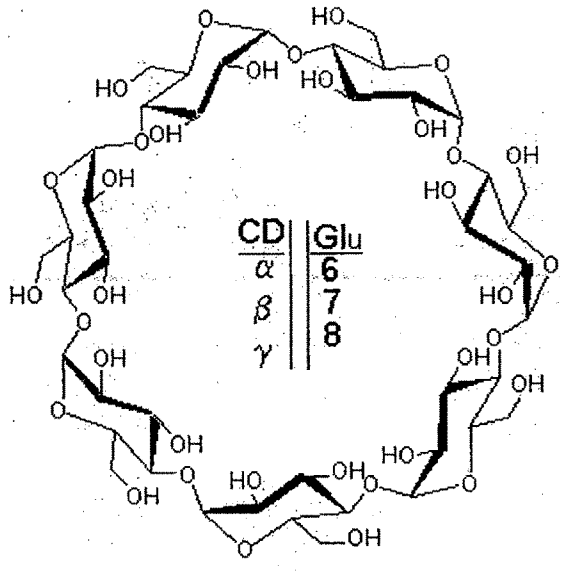
β-cyclodextrin (7585-39-9)

γ-cyclodextrin (17465-86-0)

3.9.4 Empirical formula		Molecular weight
α-cyclodextrin	$C_{36}H_{60}O_{30}$	972
β-cyclodextrin	$C_{42}H_{70}O_{35}$	1135
γ-cyclodextrin	$C_{48}H_{80}O_{40}$	1297

3.9.5 Structural formula

Figure 3.6 Structure of cyclodextrin



3.9.6 Functional category

Solubilizing agent; stabilizing agent

3.9.7 Application in pharmaceutical formulation or technology

Cyclodextrins are crystalline, nonhygroscopic, cyclic oligosaccharides derived from starch. Among the most commonly used forms are α-, β-, and γ-cyclodextrin, which have respectively 6, 7, and 8 glucose units.

Cyclodextrins are 'bucketlike' or 'conelike' toroid molecules, with rigid structure and a central cavity, the size of which varies according to the cyclodextrin type. The internal surface of the cavity is hydrophobic and the outside of the torus is hydrophilic; this is due to the arrangement of hydroxyl groups within the molecule. This arrangement permits the cyclodextrin to accommodate a guest molecule within the cavity, forming an inclusion complex.

Cyclodextrins may be used to form inclusion complexes with a variety of drug molecules, resulting primarily in improvements to dissolution and

bioavailability owing to enhanced solubility and improved chemical and physical stability.

Cyclodextrin inclusion complexes have also been used to mask the unpleasant taste of active materials and to convert a liquid substance into a solid material.

β-cyclodextrin is the most commonly used cyclodextrin, although it is the least soluble. It is the least expensive cyclodextrin; it is commercially available from a number of sources, and it is able to form inclusion complexes with number of molecules of pharmaceutical interest. However, β-cyclodextrin is nephrotoxic and should not be used in parenteral formulations.

β-cyclodextrin is considered to be nontoxic when administered orally, and is primarily used in tablet and capsule formulations. α-cyclodextrin is used mainly in parenteral formulations; however, as it has the smallest cavity of the cyclodextrins it can form inclusion complexes with only relatively few, small-sized molecules. In contrast, γ-cyclodextrin has the largest cavity and can be used to form inclusion complexes with large molecules; it has low toxicity and enhanced water solubility.

In oral tablet formulations, β-cyclodextrin may be used in both wet-granulation and direct- compression processes. The physical properties of β-cyclodextrin vary from manufacturer to manufacturer. However, β-cyclodextrin tends to possess poor flow properties and requires a lubricant, such as 0.1 % w/w magnesium stearate, when it is directly compressed. Cyclodextrins have also been used in the formulations of eye drops [42], solutions [43, 44], suppositories [45, 46] and cosmetics [47, 48].

3.9.8 Description

Cyclodextrins are cyclic oligosaccharides containing at least six D-(+) - glucopyranose units attached by α-(1→ 4) glucoside bonds. The three natural cyclodextrins, α, β, and γ, differ in their ring size and solubility. They contain 6, 7, or 8 glucose units, respectively.

Cyclodextrins occur as white, practically odourless, fine crystalline powders, having a slightly sweet taste. Some cyclodextrin derivatives occur as amorphous powders. The PhEur 2002 lists α-cyclodextrin and γ- cyclodextrin as potential impurities in β-cyclodextrin.

3.9.9 Pharmacopoeial specifications

Table 3.3 Pharmacopoeial specifications for β-cyclodextrin

Test	PhEur 2002 (suppl 4.3)	USPNF 20
Identification	+	+
Characters	+	–
Colour and clarity of solution	+	+
pH	5.0 – 8.0	–
Specific rotation	+ 160 to 164°	+ 160 to 164°
Microbial limits	–	≤ 1000/g[a]
Sulfated ash	≤ 0.1 %	≤ 0.1 %
Heavy metals	≤ 10 ppm	≤ 5 ppm
Light absorbing impurities	+	–
Loss on drying	≤ 16.0 %	≤ 14.0 %
Related substances	+	–
Residual solvents	+	–
Reducing sugars	≤ 0.2 %	≤ 1.0 %
Organic volatile impurities	–	+
Assay (anhydrous basis)	98.0-101.0 %	98.0-101.0 %

(a) Tests for *Salmonella* and *Escherichia coli* are negative.

3.9.10 Typical properties

Compressibility: 21.0 -44.0 % for β-cyclodextrin.

Density (bulk):

α-cyclodextrin: 0.526 g/cm^3

β-cyclodextrin: 0.523 g/cm^3

γ-cyclodextrin : 0.568 g/cm^3

Density (tapped):

α-cyclodextrin: 0.685 g/cm³

β-cyclodextrin: 0.754 g/cm³

γ-cyclodextrin : 0.684 g/cm³

Melting point:

α-cyclodextrin: 250-260°C

β-cyclodextrin: 255-265°C

γ-cyclodextrin : 240-245°C

Moisture content:

α-cyclodextrin: 10.2 % w/w

β-cyclodextrin: 13.0-15.0 % w/w

γ-cyclodextrin : 8-18 % w/w

Particle size distribution:

β-cyclodextrin: 7.0- 45.0 μm

Physical characteristics:

Figure 3.7 Dimensions of cyclodextrins

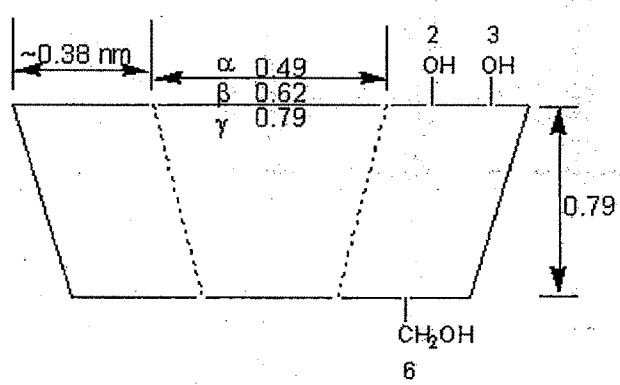


Table 3.4 Physical characteristics of cyclodextrins

Characteristic	Cyclodextrins		
	α	β	γ
Cavity diameter (A°)	4.7-5.3	6.0-6.5	7.5-8.3
Height of torus (A°)	7.9	7.9	7.9
Diameter of periphery (A°)	14.6	15.4	17.5
Approximate volume of cavity ($A°^3$)	174	262	472
Approximate cavity volume per mol cyclodextrin (mL)	104	157	256
Approximate cavity volume per g cyclodextrin (mL)	0.1	0.14	0.20

Note: 1 A° = 0.1 nm.

Solubility:

α-cyclodextrin: soluble 1 in 7 parts of water at 20°C. 1 in 3 at 50°C

β-cyclodextrin: soluble 1 in 200 parts of propylene glycol, 1 in 50 of water at 20°C, 1 in 20 at 50°C; practically insoluble in acetone, ethanol (95%), and methylene chloride.

γ-cyclodextrin : soluble in 1 in 4.4 parts of water at 20°C, 1 in 2 at 45°C

Specific rotation $(\alpha)^{25}_{D}$:

α-cyclodextrin: + 150.5°

β-cyclodextrin: + 162.0°

γ-cyclodextrin : + 177.4°

Surface tension (at 25°C):

α-cyclodextrin: 71mN/m (71 dynes/cm)

β-cyclodextrin: 71mN/m (71 dynes/cm)

γ-cyclodextrin : 71mN/m (71 dynes/cm)

3.9.11 Stability and storage conditions

β-cyclodextrin and other cyclodextrins are stable in the solid state if protected from high humidity. Cyclodextrin should be stored in tightly sealed container, in a cool, dry place.

3.9.12 Incompatibilities

The activities of some antimicrobial preservatives in aqueous solution can be reduced in the presence of hydroxypropyl - β-cyclodextrin.

3.9.13 Method of manufacture

Cyclodextrins are manufactures by the enzymatic degradation of starch using specialized bacteria. β-cyclodextrin is produced by the action of the enzyme cyclodextrin glucosyltranferase upon starch or a starch hydrolysate.

3.9.14 Safety

Cyclodextrins are starch derivatives and are used in oral and parenteral pharmaceutical formulations. They are also used in topical and ophthalmic formulations.

Cyclodextrins are also used in cosmetics and food products and are generally regarded as essentially nontoxic and nonirritant materials. However, when administered parenterally, β-cyclodextrin is not metabolized but accumulates in the kidneys as insoluble cholesterol complexes, resulting in severe nephrotoxicity. Other cyclodextrins such as 2-hydroxypropyl- β-cyclodextrin, have been the subject of extensive toxicological studies; they are not associated with nephrotoxicity and are reported to be safe for use in parenteral formulations.

Cyclodextrin administered orally is metabolized by microflora in the colon, forming the metabolites maltodextrin, maltose, and glucose, which are themselves further metabolized before finally excreted as carbon dioxide and water. Although a study published in 1957 suggested that orally administered cyclodextrins were highly toxic , more recent animal toxicity studies in rats and dogs have shown this not to be the case, and cyclodextrins are now approved for use in food products and orally administered pharmaceuticals in number of countries.

Cyclodextrins are not irritant to the skin and eyes, or upon inhalation. There is also no evidence to suggest that cyclodextrins are mutagenic or teratogenic.

α-Cyclodextrin:
LD_{50} (rat, IP): 1.0 g/kg
LD_{50} (rat, IV): 0.79 g/kg

β-cyclodextrin:

LD_{50} (mouse, IP): 0.33 g/kg

LD_{50} (mouse, SC): 0.41 g/kg

LD_{50} (rat, IP): 0.36 g/kg

LD_{50} (rat, IV): 1.0 g/kg

LD_{50} (rat, oral): 18.8 g/kg

LD_{50} (rat, SC): 3.7 g/kg

γ-cyclodextrin :

LD_{50} (rat, IP): 4.6 g/kg

LD_{50} (rat, IV): 4.0 g/kg

LD_{50} (rat, oral): 8.0 g/kg

3.9.15 Regulatory status

Included in oral and rectal pharmaceutical formulations licensed in Europe, Japan and the USA.

3.10 PROFILE OF CELLULOSE ACETATE PHTHALATE [49, 50]

3.10.1 Nonproprietary names

BP: Cellacefate

JP: Cellulose acetate phthalate

PhEur: Cellulosi acetas phthalas

USPNF: Cellacefate

3.10.2 Synonyms

Acetyl phthalyl cellulose; *Aquacoat cPD*; CAP; cellacephate; cellulose acetate benzene-1,2-dicarboxylate; cellulose acetate hydrogen 1,2-benzenedicarboxylate; cellulose acetate hydrogen phthalate; cellulose acetate monophthalate; cellulose acetophthalate; cellulose acetylphthalate.

3.10.3 Chemical name and CAS registry number

Cellulose, acetate, 1, 2-benzenedicarboxylate (9004-38-0)

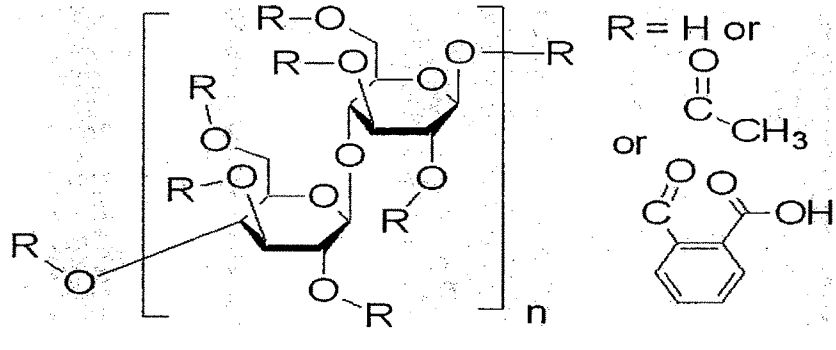
3.10.4 Empirical formula

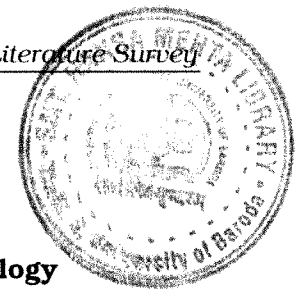
Cellulose acetate phthalate is cellulose in which about half the hydroxyl groups are acetylated, and about a quarter are esterified with one of two acid groups being phthalic acid; where the remaining acid group is free.

3.10.5 Structural formula

The PhEur 2002 and USPNF 20 describe cellulose acetate phthalate as a reaction product of phthalic anhydride and a partial acetate ester of cellulose containing 21.5 – 26 % of acetyl (C_2H_3O) groups, and 30-36 % of phthalyl (o-carboxybenzoyl, $C_8H_5O_3$) groups.

Figure 3.8 Structure of cellulose acetate phthalate





3.10.6 Functional category

Coating agent

3.10.7 Application in pharmaceutical formulation or technology

Cellulose acetate phthalate (CAP) is used as an enteric film coating material, or as a matrix binder for tablets and capsules [51-56]. Such coatings resist prolonged contact with the strongly acidic gastric fluid, but dissolves in the mildly acidic or neutral intestinal environment.

Cellulose acetate phthalate is commonly applied to solid dosage forms either by coating from organic or aqueous solvent systems or by direct compression. Concentrations generally used are 0.5 -9.0 % of the core weight. The addition of plasticizers improves the water resistance of this coating material, and formulations using such plasticizers are more effective than when Cellulose acetate phthalate is used alone.

Cellulose acetate phthalate is compatible with many plasticizers, including acetylated monoglyceride, butyl phthalybutyl glycolate; dibutyl tartrate; diethyl phthalate; dimethyl phthalate; ethyl phthalylethyl glycolate; glycerin; propylene glycol; triacetin; triacetin citrate; and tripropionin. It is also used in combination with other coating agents such as ethyl cellulose, in drug controlled-release preparations.

Therapeutically, cellulose acetate phthalate has recently been reported to exhibit experimental microbicidal activity against sexually transmitted disease pathogens, such as the HIV-1 retrovirus [57-58].

3.10.8 Description

Cellulose acetate phthalate is a hygroscopic, white to off-white, free-flowing powder, granule, or flake. It is tasteless and odourless, or might have a slight odour of acetic acid.

3.10.9 Pharmacopoeial specifications

Table 3.5 Pharmacopoeial specifications for cellulose acetate phthalate

Test	JP2001	PhEur 2002 (suppl 4.3)	USPNF 20	IP 1996
Identification	+	+	+	+
Characters	+	+	_	+
Free acid	≤ 3.0 %	≤ 3.0 %	≤ 3.0 %	≤ 3.0 %
Heavy metals	≤ 10 ppm	≤ 10 ppm	≤ 0.001 %	≤ 20 ppm
Organic volatile impurities	_	_	+	-
Phthaloyl groups	_	+	+	-
Residue on ignition	≤ 0.1 %	≤ 0.1 %	≤ 0.1 %	≤ 0.1 %
Viscosity (15 % w/v/soln)	45-90 mPa s	45-90 mPa s	45-90 mPa s	50-90 mPa s
Water	≤ 5.0 %	≤ 5.0 %	≤ 5.0 %	≤ 5.0 %
Assay	+	+	+	+
Acetyl groups	21.5-26.0 %	21.5-26.0 %	21.5-26.0 %	17.0-26.0 %
Carboxybenzoyl groups	30.0- 40.0%	30.0- 36.0%	30.0- 36.0%	30.0- 40.0%

3.10.10 Typical properties

Density (bulk): 0.260 g/cm3

Density (tapped): 0.266 g/cm3

Melting point: 192°C, Glass transition temperature is 160-170°C.

Moisture content: 2.2 %. Cellulose acetate phthalate is hygroscopic and precautions are necessary to avoid excessive absorption of moisture.

Solubility: practically insoluble in water, alcohols, and chlorinated and nonchlorinated hydrocarbons. Soluble in a number of ketones, esters, ether alcohols, cyclic ethers, and in certain solvent mixtures. It can be soluble in certain buffered aqueous solutions as low as pH 6.0. Cellulose acetate phthalate has a solubility of ≤ 10 % w/w in a wide range of solvents and solvent mixtures.

Viscosity (dynamic): a 15 % w/w solution in acetone with a moisture content of 0.4 % has a viscosity of 50 – 90 mPa s. This is a good coating solution with a honey-like consistency, but the viscosity is influenced by the purity of the solvent.

3.10.11 Stability and storage conditions

Slow hydrolysis of cellulose acetate phthalate will occur under prolonged adverse conditions such as high temperatures and high humidity, with a resultant increase in free acid content, viscosity, and odour of acetic acid. However, cellulose acetate phthalate is a stable if stored in a well-closed container in a cool, dry place.

3.10.12 Incompatibilities

Cellulose acetate phthalate is incompatible with ferrous sulfate, ferric chloride, silver nitrate, sodium citrate, aluminum sulfate, mercuric chloride, barium nitrate, basic lead acetate, and strong oxidizing agents such as strong alkalis and acids.

3.10.13 Method of manufacture

Cellulose acetate phthalate is produced by reacting the partial acetate ester of cellulose with phthalic anhydride in the presence of a tertiary organic base such as pyridine, or a strong acid such as sulfuric acid.

3.10.14 Safety

Cellulose acetate phthalate is widely used in oral pharmaceutical products and is generally regarded as a nontoxic material, free of adverse effects. Results of long-term feeding in rats and dogs have indicated a low oral toxicity. Rats survived daily feedings of up to 30 % in the diet for up to 1 year without showing a depression in growth. Dogs fed 16 g daily for 1 year remained normal.

3.10.15 Regulatory status

Included in the FDA Inactive Ingredients Guide (oral tablets). Included in nonparenteral medicines licensed in the UK.

3.11 LITERATURE REVIEW ON DIFFERENT APPROACHES FOR AMYLASE ENZYME FORMULATION

3.11.1 Formulation with refolding agents

Sierks et al. [59] has described a method for increasing the hydrolytic activity of a starch hydrolase comprising reacting a starch hydrolase with a substrate in an aqueous reaction solution comprising a cosolvent of about 0.001 to 80% (w/v) of an ethylene glycol or polyethylene glycol. The substrate was selected from the group consisting of maltose, isomaltose, maltodextrin and pullulan and starch hydrolase was selected from the group consisting of glucoamylase, alpha-amylase, beta-amylase, amylo-1,6-alpha-glucosidase, isomaltase, maltotriase, maltase, alpha-glucosidase, cyclodextrinase, pullulanase, branching enzyme and glucanotransferase. They invented that when PEG was included in a starch hydrolase enzymatic reaction solution, the catalytic rate of the starch hydrolase was increased.

Eliana DBC [60] described a simpler strategy to prevent aggregation by interfering with intermolecular hydrophobic interactions was to use additives, small molecules that were relatively inexpensive and easy to remove once refolding goes to completion. A variety of additives had been tested for their ability to prevent aggregation. They acted by stabilizing the native state, by preferentially destabilizing incorrectly folded molecules, by increasing the solubility of folding intermediates, or by increasing the solubility of the unfolded state. In general, these additives did not seem to accelerate the rate of folding, but they did inhibit the unwanted aggregation reaction. Additives that had been shown to promote higher refolding yields are listed in Table 3.5.

Table 3.6 In vitro folding aids

Additive	**Protein**
Non-denaturing concentrations of chaotropic agents	
GdmCl	*P. fluorescens* lipase
	Hen egg-white lysozyme
	Carbonic anhydrase II
	Interferon-β-polypeptides
Urea	Porcine growth hormone
	Hen egg-white lysozyme
	IGF-I
	Interferon- β -polypeptides
L-arginine	*P. fluorescens* lipase
	Fab fragments
	Hen egg-white lysozyme
	α-gluocosidase
Salts	
Ammonium sulphate	Hen egg-white lysozyme
Sugars	
Glycerol	*P. fluorescens* lipase
	Hen egg-white lysozyme
	IGF-I
Sucrose	IGF-I
Glucose	Hen egg-white lysozyme
N-acetyl glucosamine	Hen egg-white lysozyme
Sarcosine	Hen egg-white lysozyme
Detergents and surfactants	
Chaps	TGF- β -like proteins
	Carbonic anhydrase II
Tween	Human growth hormone
SDS	Interferon- β -polypeptides
Sarkosyl	RNA polymerase q factor
Sodium lauorsylsarcosine	Single chain Fv fragment
Dodecyl maltoside	Class II MHC
Triton X-100	Carbonic anhydrase II
Polyethylene glycol	Carbonic anhydrase II
Octaethylene glycol monolauryl	Carbonic anhydrase II
Phospholipids	Hen egg-white lysozyme
	TGF- β -like proteins
Sulphobetaines	Hen egg-white lysozyme
	β -D-galactosidase
Short chain alcohols	
n-pentanol	Carbonic anhydrase II
n-hexanol	Carbonic anhydrase II
cyclohexanol	Carbonic anhydrase II

3.11.2 Formulation with cofactors

Liliana et al. [61] studied the effect of calcium ions on alpha amylase activity used for apple juice processing. It was also found that the addition of calcium chloride (1 g/L) after thermal treatment in media containing citrate reactivated the enzyme treated at 60 and 65 °C.

Tessier et al. [62] showed the increased thermostability of purified human pancreatic alpha-amylase by combination of human serum albumin and calcium ions. They used pancreatic fluid from a patient with a post operative pancreatic fistula to isolate human alpha-amylase by means of acarbose affinity chromatography. They measured amylase thermostability in 4 solutions: (a) EDTA-dialyzed; (b) dialyzed solution plus 0.15 mmol/l (1.0 g/dl) human serum albumin; (c) dialyzed solution plus 0.25 mmol/l (1.0 mg/dl) calcium ions; and (d) dialyzed solution with both human serum albumin and calcium ions. They measured amylase activity at predetermined times in samples heated to 60°C. Thermostability was characterized by t1/2, the time to 50% initial amylase enzyme activity. They found that t1/2 in the dialyzed solution was 0.75 ± 0.19 min which rose to 1.62 ± 0.34 min with added human serum albumin, and to 8.24 ± 0.13 min with added calcium ions. They shown the combination of human serum albumin and calcium ions resulted in a synergistic increase of t1/2 to 180 ± 26 min.

Violet et al. [63] studied the irreversible thermal inactivation of Bacillus licheniformis alpha-amylase. They found two-step behavior in the irreversible denaturation process. They suggested that the deactivation mechanism involved the existence of a temperature-dependent intermediate form. They have shown that Ca^{2+} was necessary for the structural integrity of alpha-amylase. They reported the key role of Ca^{2+} in the alpha-amylase thermostability. It was found that the stabilizing effect of Ca^{2+} was reflected by the decrease of the denaturation constants of both the native and the intermediate forms. They found that below 75°C, in the presence of 5 mM-$CaCl_2$, alpha-amylase was completely thermostable. It was observed that neither other metal ions nor substrate had a positive effect on enzyme thermostability.

Saboury [64] studied the interaction of alpha amylase from Bacillus amyloliquefaciens (BAA) with divalent calcium and cobalt cations by equilibrium dialysis, isothermal titration microcalorimetry, UV spectrophotometry and temperature scanning spectrophotometry methods at 27°C in Tris buffer solution at pH 7.5. It was found that the binding of calcium stabilized the enzyme against surfactant and thermal denaturation. He suggested that the binding of calcium prevented the spontaneous decrease in biological activity of alpha-amylase. He concluded that the divalent calcium act as stabilizer for Bacillus amyloliquefaciens.

3.11.3 Formulation with protective coating

Bilton [65] presented the inventions of orally-administrable compositions which comprise effective amounts of pepsin, a hydrochloric acid source, a source of pancreatic enzymes, a proteolytic enzyme of plant origin and a choleretic agent. The proteolytic enzymes and pancreatic enzymes were adhered to sugar beads which were further coated by mixture of carnauba wax and stearic acid in a ratio of about 1:1-5, so as to render them resistant to gastric acid, whereas the other components were formulated so as to be readily released and/or solubilized in the stomach. Thus pepsin and an acid source were available to enhance the digestive action of the stomach juices, bile salts were made available to aid in gut fat absorption, and pancreatic and proteolytic enzymes were provided in an active form to the small intestine to augment the digestion of fats, proteins and starches. These components combined with minor amounts of suitable inert adjuvants and tabletted, or encapsulated in powdered form.

Sipos [66] developed improved enteric coated digestive enzyme-containing compositions which are capable of withstanding hours of exposure to gastric fluids while protecting the biological activity of the enzymes and thereafter releasing the digestive enzymes in their biologically active state within 5 to 30 minutes after being exposed to intestinal fluids, these compositions comprising (a) an enzyme concentrate in (b) a binder system comprising at least about 0.5 wt. %, preferably about 1 to about 10 wt. % (based on the weight of the binder system plus enzymes) of (i) a binder, preferably selected from the group consisting of polyvinylpyrrolidone, microcrystalline cellulose (Avicel), cellulose acetate phthalate, methylcellulose and alginic acid, and

preferably (ii) from about 0.1 to about 10 wt. % of a stabilizer, preferably selected from the group consisting of calcium carbonate, polyvinylpyrrolidone, cellulose acetate phthalate, methylcellulose, alginic acid, starch and modified starches, e.g., carboxymethyl starch (Primojel); and (c) from about 0.1% to about 30 wt. %, based on the weight of the total composite (enzyme plus binder system plus disintegrant) of a disintegrant, preferably selected from the group consisting of citric acid, sodium carbonate, sodium bicarbonate, calcium carbonate and other suitable carbonates, alginic acid, starch and modified starches, e.g., carboxymethyl starch (Primojel) were prepared by a process in which the presence of water was avoided and which included the step of blending enzyme, binder and disintegrant in the presence of a selected inert solvent as well as the subsequent coating of the resulting enzyme/binder/disintegrant composite with from about 2.5% to about 10% by weight, based on the weight of the enzyme/binder/disintegrant composite, of a gastric juice insoluble, intestinal juice soluble, non-porous, pharmaceutically acceptable enteric coating polymer.

Dale et al. [67] produced a granular enzyme composition having reduced tendency to form dust and leave a residue, and improved stability and delayed release characteristics. The composition had a core, optionally coated with a vinyl polymer, a layer containing an enzyme and a vinyl polymer and optionally a plasticizer or anti-agglomeration agent, and an outer coating containing a polymer and optionally a low residue pigment and/or lubricant. Preferably, the core was a salt or sugar nonpareil, the vinyl polymer coating the core was polyvinyl alcohol and most preferably partially hydrolyzed polyvinyl alcohol, the vinyl polymer in the enzyme layer was polyvinyl pyrrolidone, and the polymer of the outer coating was polyvinyl pyrrolidone, polyvinyl alcohol which might be partially hydrolyzed, polyethylene glycol or mixtures thereof such as a mixture of polyvinyl pyrrolidone and polyvinyl alcohol or a mixture of polyvinyl pyrrolidone and polyethylene glycol. Preferably, the pigment was titanium dioxide and the lubricant was a nonionic or anionic surfactant such as a linear primary alcohol of a 9-15 carbon atom chain length alkane or alkene or an ethoxylate or ethoxysulfate derivative thereof. The enzymes tried were protease, amylase, lipase, cellulase, xylanase, oxidase, peroxidase or mixtures thereof. The composition produced by spray coating in a fluidized bed a solution of the enzyme and vinyl polymer onto the

core to form the enzyme layer, and then spray coating in a fluidized bed a solution of polymer on the enzyme layer to form the outer coating.

Fulberth et al. [68] described a process for the preparation of high dose pancreatic enzyme products in tablet form. A water-based insulating layer consisting of primary covering syrup prepared from sugar, gelatin and starch and mixture of talc and calcium carbonate was used as the primary covering powder and then final lacquer such as cellulose acetate phthalate, hydroxyl propyl methyl cellulose phthalate, polyvinyl acetate phthalate, and copolymers which have anionic properties and were based on methacrylic acid and methacrylic esters (Eudragit RL or S) in organic solvents were applied to the core which contains the active compounds. The product developed was stable at 40°C up to six months and the test for resistance to gastric juice complied with the requirements of the European Pharmacopoeia.

Santus [69] developed a method to protect bioactive compounds such as peptides, polypeptides, enzymes, vitamins and microorganisms from inactivation in the gastric tract as well as in foods. The method comprised of preparation of microgranules by mixing the bioactive material with at least one excipients such as lactose, dibasic calcium phosphate, povidone and thereafter adding water and subsequent drying to form microgranules having size 50 -400 μm. These microgranules were enteric coated with coating polymer like cellulose acetate phthalate, Eudragit S, and Eudragit L. The microgranules were suspended in common foodstuffs such as milk and milk products and fruit juices.

Sipos [70] developed gastric acid-resistant polymer-coated, buffered digestive enzymes compositions, process for their preparations and methods for treating digestive disorders, pancreatic enzyme insufficiency, impaired liver function, and cystic fibrosis for regulating the absorption of dietary iron and cholesterol, and for dissolving gallstones by administering the compositions to a mammal in need of such treatment. The enzyme were selected from the group consisting of pancreatic proteases, pancreatic lipases, pancreatic nucleases and pancreatic amylases and developed microencapsules by addition of buffering agent such as anhydrous sodium carbonate, sodium

bicarbonate, potassium carbonate, ammonium carbonate, tromethamine, di(tris(hydoxymethyl)aminomethane) carbonate, tris-glycine, di-arginine; and adhesive polymer such as hydroxypropyl cellulose, polyvinylpyrrolidone, cellulose acetate phthalate, methyl cellulose and propylene glycol alginate using marumerizer. Thereafter the microspheres containing enzymes were enteric coated with acid resistant polymer.

Becker et al. [71] prepared granules in fluid bed coater by spraying the aqueous solution of alpha amylase and corn starch on sucrose crystals. The coated particles were then coated with aqueous solution of magnesium sulfate heptahydrate. Thereafter these particles coated with methylcellulose solution. This coated enzyme granules enhanced storage stability.

Pabst [72] developed a baby formula having enzymes added to imitate the effect of those present in normal breast milk, aiding digestion of protein, carbohydrate (simple and complex sugars), and lipid. The enzymes were either of procaryotic or eucaryotic origin, isolated from fermentation broth or tissue, or expressed from recombinant gene sequences. The enzymes were provided in a form for addition to the formula prior to feeding the infant or at the time of feeding. In the preferred form, the enzymes were provided in a form that was stable to storage in the formula, but active when the formula reaches the portion of the gastrointestinal tract where the formula would normally be digested. In the most preferred embodiment, the enzymes were provided in a matrix with an enteric coating that releases the enzyme in the upper portion of the intestine. Depending on the formulation, proteases, carbohydrate degrading enzymes such as alpha-amylase, lactase, fructase, and sucrase, or lipases, were added to the formulation.

3.11.4 Formulation by immobilization technique

Ulbrich [73] studied alpha-Amylases from B. licheniformis and A. oryzae, which differ in their thermal stability, with respect to their resistance to chemical denaturants as guanidine hydrochloride, urea, aliphatic alcohols, and formaldehyde. Moreover, the thermal and chemical stabilities of both enzymes were compared after their covalent binding to gamma-aminopropyl silica. The thermolabile alpha-amylase (A. oryzae) as well as the thermostable alpha-amylase (B. licheniformis) were remarkably stabilized by immobilization.

Soluble and immobilized alpha-amylases from B. licheniformis were more resistant to guanidine hydrochloride, urea, methanol, ethanol, propranol, and t-butanol than the alpha-amylases from A. oryzae. An exception was given by the effect of formaldehyde, which resulted in chemical modifications of the protein molecule. Enzymes from B. licheniformis were more sensitive against formaldehyde than those from A. oryzae. As in thermal inactivation, immobilization protected alpha-amylase from A. oryzae against denaturation by guanidine hydrochloride and urea, but failed in the denaturation by alcohols. Therefore, stabilizing effects in immobilized enzymes were dependent on the kind of denaturant.

Aksoy et al. [74] prepared Poly (methyl methacrylate-acrylic acid) microspheres and the acid groups were activated by using either carbodiimide (CDI) or thionyl chloride ($SOCl_2$). Alpha-amylase was covalently bound on these activated microspheres. The properties of the immobilized enzyme were investigated and compared with those of the free enzyme. The relative activities were found to be 80.4 and 67.5% for carbodiimide and thionyl chloride bound enzymes, respectively. Maximum activities were obtained at lower pH and higher temperatures upon immobilization compared to free enzyme. No change in Vmax and approximately 12-fold increase in K(m) were observed for immobilized enzymes. The enzyme activities, after storage for 1 month, were found to be 24.5 and 52.5% of the initial activity values for CDI and $SOCl_2$ activated matrices, respectively. On the other hand the free enzyme lost its activity completely in 20 days. Immobilization, storage stability and repeated use capability experiments carried out in the presence of Ca^{2+} ions demonstrated higher stability, such as $SOCl_2$ immobilized enzyme retained 83.7% and CDI immobilized enzyme retained 90.3% of the original activity of the enzyme. The immobilized enzymes that were used 20 times in 3 days in repeated batch experiments demonstrated that, in the absence of Ca^{2+} ions about 75% and in the presence of Ca^{2+} ions greater than 90% of the original enzyme activity was retained.

Carvalho et al. [75] immobilized alpha-amylase on polyamide 11 which shown higher specific activity and retention of activity than the derivatives employing polyacrylamide and polyethyleneterephthalate as supports. Polyamide 11 and

polyethyleneterephthalate alpha-amylase derivatives exhibited a higher extent of multiple attacks on starch than the water-soluble enzyme whereas the polyacrylamide derivative presented less. The polyamide 11 alpha-amylase derivative acted on amylose-azure in the same way as the water-soluble alpha-amylase.

Roig et al [76] developed the covalent immobilization of alpha-amylase on new isocyanate, acid chloride and carboxylic acid-activated plastic supports showed the viability of such supports for immobilizing enzymes, especially those reacting with 1,6-diaminohexane and glutaraldehyde for producing side arms. The operational stability of immobilized alpha-amylase could be extended by crosslinking the enzyme or by extending the support's side arm. Inactive immobilized alpha-amylase were unfolded and then refolded at elevated temperature, these supports were found to be essential in increasing the stability of the enzyme during refolding. The pH curves for the immobilized enzyme were in general found not to be shifted from the soluble enzyme's pH optimum, although one isocyanate plastic support derivative shifted the pH activity profile of alpha-amylase to a higher range by 1.5 pH units, probably due to reaction between the enzyme and the free anhydride groups existing on the support's surface. In all cases, the immobilized enzyme's temperature activity profiles were shifted to a lower temperature range when compared to the soluble enzyme. The immobilized alpha-amylase Michaelis constants increased and the maximum rates and specific activities decreased when compared to the soluble enzyme kinetic parameters.

Raviyan et al. [77] determined the suitability as a time-temperature indicator for dielectric pasteurization processes, the thermal stability (50°-75°C) of *Aspergillus oryzae* alpha-amylase immobilized in polyacrylamide gel in phosphate buffer, mashed potatoes, and minced shrimp was examined. Changing the cross-linking agent concentration from 3.3 to 5.3% and adding 2% salt did not markedly affect the thermal stability of the immobilized alpha-amylase. Thermal inactivation was first order, and immobilization generally improved the thermal stability of alpha-amylase.

Sardar et al. [78] performed the noncovalent immobilization of enzymes such as alpha-amylase, beta-glucosidase, trypsin, and alkaline phosphatase by adsorption on the water-soluble polymer eudragit S-100. The strength of the binding with enzymes in some cases was critically dependent upon the initial polymer concentration used during binding. In all the cases tried, a moderate increase in polymer concentration ensured adequate immobilization of enzymes. The immobilized enzymes retained different activities: 87, 59, 49, and 24% for beta-glucosidase, alpha-amylase, trypsin, and alkaline phosphatase, respectively. The K_m value of immobilized enzyme was the same as that of native enzyme for beta-glucosidase and alpha-amylase whereas the K_m value decreased in the case of trypsin upon immobilization. The immobilized trypsin showed improved stability to autolysis at 35°C whereas immobilization resulted in a decrease in the thermal stability of alpha-amylase at 50°C. No significant changes were observed in pH optimum of the enzymes upon immobilization. U.V. and fluorescence emission spectra of immobilized trypsin reflected the conformational changes which enzymes undergo upon adsorption on the polymer.

3.11.5 Formulation with cyclodextrin

Gellman et al. [79] presented a method for refolding an enzyme from a misfolded configuration to a second native and active configuration. They suggested the method which comprised of adding a linear alkyl detergent to a misfolded carbonic anhydrase B previously denatured by heat or by chemical means to form an enzyme-detergent complex, and then the enzyme-detergent complex was contacted with a cyclodextrin to allow the enzyme to assume a second active configuration. They concluded that the addition of beta-cyclodextrin to the denatured protein-detergent complex solution after cooling, caused reactivation of the enzyme with a yield of 81% recovered activity.

A common obstacle to proper renaturation of an unfolded protein is aggregation, an intermolecular side reaction of immense importance in biotechnology and in the pathogenesis of several neurodegenerative diseases. Sharma et al. [80] evaluated the effect of cyclodextrin chemistry on aggregation and refolding of carbonic anhydrase. Size-exclusion HPLC showed that cyclodextrins inhibited aggregate formation without interfering with the

correct renaturation of carbonic anhydrase. PAGE of refolded enzyme provided further evidence of inhibition of folding-related aggregation by natural and chemically modified cyclodextrins. They suggested that the amount of aggregate formed and recovery of active enzyme was dependent on cavity size, the nature of the chemical substituents found on the rims of the sugar molecule seems to play a more important role in cyclodextrin-assisted refolding of carbonic anhydrase. Neutral or cationic cyclodextrins with small cavities were found to be better folding aids than anionic cyclodextrins with larger cavity.

Karuppiah et al. [81, 82] suggested that aggregation of proteins is a frequent occurrence during their transition from random coil to native structure. They studied the influence of cyclodextrins in the refolding of carbonic anhydrase under aggregating conditions. Cyclodextrin prevented formation of protein aggregates during renaturation of carbonic anhydrase. In addition, over 90% of active enzyme was recovered even at protein concentrations as high as 67 μM. They concluded that the enhanced protein reactivation by cyclodextrins might be due to their ability to bind to hydrophobic sites in protein folding intermediate(s) followed by their subsequent removal as the protein refolds.

Hora et al. [83] provided the method for the solubilization and/or stabilization of polypeptides, especially proteins, using cyclodextrin selected from the group consisting of hydroxypropyl, hydroxyethyl, glucosyl, maltosyl and maltotriosyl derivatives of beta- and gamma-cyclodextrin. They described method for solubilized and stabilized lyophilized formulations of Interleukin-2, tumor necrosis factor or TNF, macrophage colony stimulating factor or m-CSF, insulin and human growth hormone with addition of cyclodextrin.

Branchu et al. [84] tested the use of sucrose and hydroxypropyl-beta-cyclodextrin as stabilizing excipients in the spray- drying of a model protein, beta-galactosidase. The solutions were processed using a Buchi 190 cocurrent Mini Spray Dryer at an outlet temperature of 61 ± 2 ° C. The powders were redissolved and analyzed for catalytic activity, aggregation, chemical decomposition, and thermal susceptibility as observed by high-resolution calorimetry. Spray-drying significantly inactivated beta- galactosidase. Spray-drying beta-galactosidase in the presence of sucrose did not prevent

inactivation. However, after spray-drying beta-galactosidase in the presence of HP-beta-CD, or HP-beta-CD and sucrose, full catalytic activity was exhibited on reconstitution. Furthermore, the reconstituted product was unchanged in terms of molecular weight, charge, and thermal stability. These findings were consistent with a hypothesis that the change responsible for inactivation of beta-galactosidase was mainly a monomolecular, noncovalent change, that is, the formation of incorrect structures that arose from surface denaturation. They demonstrated that cyclodextrins can be useful stabilizing excipients in the preparation of spray-dried protein pharmaceuticals.

Yazdanparast et al. [85] studied alpha-cyclodextrin (alpha-CD) and Ca^{2+} ion, two important folding agents in reactivation and the refolding processes of denatured enzyme solutions. Bacillus sp. alpha-amylase was extensively denatured in 6M guanidinium chloride (GdmCl) solution overnight. Under suitable renaturation conditions, 20-30% more activity was recovered in the presence of various concentrations of alpha-CD (0-100 mM). Similarly, 25-30% more activity was recovered under the influence of different concentrations of Ca^{2+} ion (0-100 mM). Regardless of these data, both alpha-CD and Ca^{2+} at 100 mM concentrations were separately capable of preventing the extent of aggregate formation by 86% and 77%, respectively. They clearly indicated that the effect of alpha-CD and/or Ca^{2+} ion on the process of aggregation and refolding (activity recovery) are not synchronized: both agents mostly act as anti-aggregatory agents than as refolding aids.

Daniel et al. [86] investigated the interactions between human growth hormone and different cyclodextrins at low pH. They observed that human growth hormone when treated at pH 2.5 in 1 M NaCl formed amorphous aggregates, which in presence of 25 to 50 mM of various beta-cyclodextrin derivatives inhibited the aggregation.

3.11.6 Formulation by entrapment

Gehrke et al. [87] developed a method of loading a drug into a crosslinked polymer network and protecting the drug from the effects of inactivation. Alpha amylase from *B.Subtilis* was loaded in to dextran gel and the gel was made by from dextran in a 10% solution and crosslinked with 0.14 g divinyl surfone /g dry dextran. Loaded gels were dried in a desiccator jar at room

temperature. The dried gel was equilibrated with buffer containing PEG and KCl. Release of amylase were carried out from this gel and about 100 % retention was seen. This PEG-KCl loaded gel was seen to stabilize the amylase against heat denaturation.

Galzigna et al. [88] prepared liposome by incorporating alpha-amylase (EC 3.2.1.1) from hog pancreas into artificial phosphatidylcholine and phosphatidylserine. To kinetically follow the enzyme-catalyzed hydrolysis of amylose the liposome-bound amylase was incubated in a medium containing amylose-iodine substrate. The reaction was studied at different concentrations of amylose and at different ionic strengths. Activation of amylase incorporated into liposomes by chloride ions varies with the type of phospholipid utilized to prepare the liposomes. When amylase bound to negative charged liposomes was used, a sigmoidal relationship between the reaction velocity and substrate concentration was found. Incorporation into liposomes protects amylase from heat inactivation.

Gajjar et al. [89] reported the observations of the activity of two hydrolyzing enzymes-protease and alpha-amylase entrapped inside the reversed micelles formed by surfactants in hexane, benzene, and cyclohexane. The surfactants chosen for this study were: Tween 80, a nonionic surfactant, cetyl pyridinium chloride, a cationic surfactant, and two anionic surfactants, sodium lauryl sulfate and Aerosol OT. Tween 80 enhances the activity of both protease and alpha-amylase. Sodium lauryl sulfate and Aerosol OT, which are ionic surfactants, enhanced the activity of protease, but inhibited the activity of alpha-amylase. Cetyl pyridinium chloride, however, enhanced the activity of alpha-amylase, but inhibited the activity of protease. Enhanced activity was generally several folds greater in comparison to the activity observed in the usual aqueous system in the absence of reversed micelles. It has also been observed that the enhanced activity of the enzymes entrapped inside the reversed micelles remains preserved for a much longer period of time in comparison to the activity in the usual aqueous systems. These observations, which support the view that with proper choice of surfactant and the organic solvent, reversed micelles act like a microreactor that provides a favorable aqueous micro-environment for enzyme activity, have biotechnological overtones.

3.12 MOLECULAR MODELING

Molecular modeling is the science or art of representing molecular structures numerically and simulating their behavior with the equations of quantum and classical physics and it is one of the fastest growing fields in science. Molecular modeling has become a valuable and essential tool to medicinal chemists in the drug design process. The modeling techniques are widespread in their uses and have become more and more important in widely varying fields of chemistry, ranging from small organic molecules to proteins, polymers, inorganic solids, and liquids. Molecular modeling varies from building, visualizing and comparing molecules to performing complicated and time demanding calculations on rather big molecular systems. As with all models, however, the intuition and training of the chemist is necessary to interpret the results appropriately. Comparison to experimental data, where available, is important to guide both laboratory and computational work.

3.12.1 Visualization

Visualization is often the first step to inspect a structure. Through different display methods, e.g., ribbons, molecular surface, cartoon, and lines, structure visualization provides a convenient way to study spatial relationships of atoms, residues, secondary structures, domains, and subunits. Commercial packages for protein modeling, such Insight-II [90], SYBYL [91], and LOOK [92], typically include visualization tools with extensive features. Users can also find popular public domain visualization tools, such as VMD [93] and RasMol [94]. Several tools are best known for their unique strengths for particular visualization aspects. Molscript [95], which produces illustrative graphs in postcript format with high quality, is widely used by researchers in their publications. CHIME [96] shows protein graphics inside Web browsers. TOPS [97] can automatically generate protein topology cartoons, using circles and triangles to depict the arrangement of α-helices and β-strands. GRASP [98] can show protein surface color-coded with electrostatic potential or geometry properties.

Various classes of computers are required for molecular modeling. For chemical information systems the choice of a computer is generally larger, and many packages run on VAX, IBM, or PRIME machines. Currently, the

molecular modeling community is using equipment from manufacturers such as Digital, IBM, Sun, Hewlett-Packard and Silicon Graphics running with the UNIX operating system.

3.12.2 Molecular modeling strategies

Currently, two major modeling strategies are used for the conception of new drugs. They are:

i) *Direct drug design:* In the direct approach, the three-dimensional features of the known receptor site are determined from X-ray crystallography to design a lead molecule. In direct design, the receptor site geometry is known; the problem is to find a molecule that satisfies some geometric constraints and is also a good chemical match. After finding good candidates according to these criteria, a docking step with energy minimization can be used to predict binding strength.

ii) *Indirect drug design:* The indirect drug design approach involves comparative analysis of structural features of known active and inactive molecules that are complementary with a hypothetical receptor site. If the site geometry is not known, as is often the case, the designer must base the design on other ligand molecules that bind well to the site.

3.12.3 Geometry analysis

Geometry analysis of a given protein structure provides further information related to the conformation and energetics, as well as the quality of a structure model. There are two types of geometry analysis. One is based on the geometrical relationship between atoms. For example, DSSP [99] is a program that assigns protein secondary structure based on the geometrical features of the hydrogen bonds on protein backbones; HBPLUS [100] determines a hydrogen bond according to the atomic distances and angles. Another type of geometry analysis is based on solvent-accessible surface [101] and molecular surface [102]. The two types of surfaces are defined through an imaginary spherical probe (as a model for a water molecule) with a typical radius of 1.4 Å rolling on the protein structure while maintaining contact with the van der Waals surface of the protein. The trace of the probe center is the solvent-accessible surface, while the inward-facing surface of the probe sphere as it rolls over the protein is the molecular surface. Solvent-accessible

surface area can be calculated using NACCESS [103] or ASC [104]. The Molecular Surface Package [105] can compute the molecular surface area and volume. One can use hydrophobic and hydrophilic surface areas to derive semi-empirical energetics [106-108], such as solvation energy, entropy, and free energy in protein folding or binding. Another application of protein surface is domain partitioning, which cuts a protein structure into several compact domains measured by their surface area and volume. The Protein Domain Server [109] can be used for domain partitioning. In addition, the DALI domain library [110] and the 3Dee database [111] provide the domain definitions for the structures in the PDB [112].

Geometry analysis can also be employed to check the quality of a protein structure model. Various errors can be generated when building a structure model, including (a) bad backbone conformations, e.g., artificial cis peptide bonds; (b) poor stereochemistry, e.g., unwanted D-amino residues; and (c) unfavorable inter-residue packing. These errors can be detected using programs such as WHATIF [113] and PROCHECK [114]. The overall quality of a model can be further assessed by PROVE [115], which checks the departures of the assessed structure from the standard atomic volumes in high quality experimental structures.

3.12.4 Structure comparison and structure family

The 3D structures of proteins are better conserved during evolution than their sequences. Two proteins can share a similar structural fold even when their sequences are not similar, and in some cases not homologous. The relationship between proteins having similar folds is clearly revealed through structure-structure comparison, which often provides more reliable information about the relationship between proteins than sequence-sequence comparison alone. Several structure comparison tools are available, e.g., VAST [116], SARF [117], and ProSup [118]. A popular tool for comparing a query protein structure against all the structures in the PDB is the DALI server [119]. When new structures are solved, researchers often submit them to the DALI server to find structural neighbors and their alignments. The results may reveal biologically interesting similarities that are not detectable by sequence comparison.

The relationship between the proteins in a structure database can be classified at different hierarchical levels according to structural and evolutionary relationships. A widely used classification includes family, superfamily, and fold [120]. Proteins clustered into a family are clearly evolutionarily related with a significant sequence identity between the members. Different families whose structural and functional features suggest a common evolutionary origin are placed together in a superfamily. Different superfamilies are categorized into a fold if they have the same major secondary structures in the same arrangement and with the same topological connections. The structural similarities between different superfamilies in the same fold may arise just from the protein energetics favoring certain packing arrangements instead of a common evolutionary origin. Most protein structure classification tools follow the concepts similar to family, superfamily, and fold, but differ due to detailed classification criteria and different structure-structure comparison methods. CATH [121] is a hierarchical classification of protein domain structures. CE [122] provides structural neighbors of the PDB entries with structure-structure alignments and 3D superpositions. FSSP [123] features fold tree, sequence neighbors, and multiple structure alignments. SCOP uses augmented manual classification with the hierarchical levels of class, fold, superfamily, and family of close homologs [120]. Among them, SCOP provides more function related information. However, SCOP is not updated as frequently as others due to the manual work involved, while FSSP and CATH follow the PDB updates closely.

3.12.5 Molecular dynamics, quantum mechanics, and electrostatics

Most protein functions are achieved through a dynamic process. A well established method to study a dynamic process of protein is molecular dynamics simulation [124, 125], which has been applied to proteins for more than two decades [126-128]. A molecular dynamics simulation uses a given structure for the initial coordinates. Each atom is modeled as a particle with a certain mass and a partial charge. The force fields, which describe atomic interactions such as bond energy, van der Waals energy, and Coulomb energy, are based on empirical functions with analytical forms. Several sets of energy function parameters have been developed, including CHARMM [129], GROMOS [130], and AMBER [131]. After assigning random initial velocities to the

atoms of the protein according to the Boltzman distribution for a given temperature, the dynamics governed by the Newton's Law are carried out using numerical integrations with a time step of about one femtosecond ($1X10^{-15}$ second). Many molecular dynamics simulation programs are available, such as CHARMM, GROMOS, and AMBER, TINKER [132], XPLOR [133] and NAMD [134]. A molecular dynamics simulation can be used to study small conformational change and energetics such as free energy differences between two protein states. A limitation of molecular dynamics simulation is that the time scale it can model (up to several nanoseconds for a sizable protein) is shorter than many interesting dynamic processes in protein (at a time scale of several seconds or longer). Active research is going to reach longer time scales through algorithm developments [135, 136], parallel implementations [137, 138], and special protocols to artificially accelerate a dynamic process [139, 140].

Classical molecular dynamics simulation alone cannot describe the quantum mechanical processes, such as electronically excited states, spectroscopic transitions, and chemical reactions in which bonds are altered. The modeling tools for quantum mechanical calculations, such as GAUSSIAN [141], GAMESS [142], and Q-Chem [143], are designed to tackle these problems. They can also be used to obtain atomic partial charges and parameters of energy functions for molecular dynamics simulation. However, the quantum mechanics calculation is very time consuming to simulate a whole protein. A good approach is to combine a quantum mechanical treatment for a small part of a system with a molecular dynamics simulation procedure to the rest [144-146]. This allows the description of processes which cannot be represented by a molecular dynamics potential.

Another weakness of classical molecular dynamics simulation is the description of solvation effects, such as solvation energy and electrostatics. Although molecular dynamics simulation can add explicit water molecules around a protein, it is often insufficient to describe solvation effects due to the lack of description for electronic polarization and the limited time scale it can simulate. A better way to calculate solvation effects is to use continuum electrostatics [147, 148] governed by the Poisson-Boltzman equation, where the water is modeled by continuum media with a dielectric constant of about 80.

A widely used program is DelPhi [149], which uses finite difference method to solve the Poisson-Boltzmann equation.

3.12.6 Prediction of 3D structure

Predicting the 3D structure of a protein from its amino acid sequence using computational methods becomes more and more practical due to the development of new methods. Many non-trivial structure predictions [150-152] produced prior to the experimental structure determinations turned out to be fairly accurate. Most notably, the success of protein structure prediction has been demonstrated in the community-wide experiments in the Critical Assessment of Techniques for Protein Structure Prediction (CASP) [153]. In this contest, there are two types of tertiary structure predictions, i.e., *ab initio* methods which predict a protein structure based on physi-chemical principles directly, and template-based methods, which use known protein structures as templates. Template-based methods include homology or comparative modeling, and fold recognition via threading. The coverage of protein sequences by template-based methods (about 50-70% now) is expanding as more and more structures are solved.

3.12.7 *Ab Initio* prediction

An *ab initio* protein structure prediction derives a structure model through the optimization of an energy function which describes the physical properties or statistical preferences of amino acids. *Ab initio* tertiary structure prediction from sequence has proven to be extremely difficult even after tremendous effort for decades [154-158]. *Ab initio* prediction programs require long computing time, and the prediction results are generally unreliable. However, some recent developments using hierarchic approaches, which first build local structures and then assemble them into a global structure, seem to provide new hope for generating low resolution structures. Once local structures are more or less defined, assembling them requires a significantly smaller computational search space. The optimization process is typically carried out using genetic algorithms [159] or Monte Carlo simulations [156]. Local structures can be built through a search based on empirically derived data about preferred torsion angles in secondary structure elements as done by the program LINUS [160]. The "mini-threading" method [161] may be a more efficient

way to build local structures. Mini-threading methods obtain the matches between short structure segments of template and the query sequence for building local structures. Some success of mini-threading has been demonstrated in CASP-3 [153]. However, *ab initio* prediction programs are typically unavailable to the general research community.

3.12.8 Docking (Molecular interactions)

Modeling the interaction of a drug with its receptor is a complex problem. Many forces are involved in the intermolecular association: hydrophobic, dispersion, or van der Waals, hydrogen bonding, and electrostatic. The major force for binding appears to be hydrophobic interactions, but the specificity of the binding appears to be controlled by hydrogen bonding and electrostatic interactions. Modeling the intermolecular interactions in a ligand-protein complex is difficult because there are so many degrees of freedom and insufficient knowledge of the effect of solvent on the binding association. The process of docking a ligand to a binding site tries to mimic the natural course of interaction of the ligand and its receptor via a lowest energy pathway. There are simple methods for docking rigid ligands with rigid receptors and flexible ligands with rigid receptors, but general methods of docking conformationally flexible ligands and receptors are problematic.

Protein docking determines a bound structure complex formed from two proteins or a protein and a substrate, starting with two separate unbound structures. When the conformational changes of each structure upon binding are assumed to be insignificant (so called "rigid binding"), one can often use shape complementarity to find tight match between the surfaces of the two structures [162, 163]. In addition to the geometric fitness, the energetics across the binding interface can be also considered [164, 165]. A widely used docking program is DOCK [162]. Prediction of rigid binding often finds the experimental binding conformation ranked among the top of the candidate list. When a small ligand is flexible and the binding protein is rigid, the search problem to find an optimal solution can still be manageable, although the results tend to be less reliable than the rigid docking. AutoDock [166] is a program to predict the bound conformations between flexible ligands and rigid proteins. When the larger structure in the binding complex undergoes a significant

conformational change upon binding, e.g., in some protein-protein interactions, the structure flexibility makes the induced docking problem as difficult as the *ab initio* structure prediction. Current docking techniques are typically unable to identify the bound structure in this case.

3.12.9 Experimental *vs.* Computational approaches

Experimental approaches and computational methods complement each other in protein science. Modern experimental techniques rely more and more on computing. There are many computer tools that assist experimental measurement or interpret experimental data, for example, tools to help determine X-ray crystallographic structures. Many experimentalists use computational tools routinely to study proteins. On the other hand, most results from computational tools are predictions and subject to further experimental verification. A user should always keep in mind the general quality and the confidence level of the predictions when using them to draw any conclusion. Usually it is rewarding to try different tools available. The consensus and variations among different predictions may provide a clue about whether the predictions are reliable or not. Whenever any experimental information is available, a user should incorporate the information in the tools or at least use the information to verify the output results.

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