CHAPTER II
LITERATURE REVIEW

2.1 Information about amylase

Amylases are enzymes that catalyze the hydrolysis of α-1,4-glucosidic linkages in polysaccharides such as starch and glycogen into oligosaccharides. Groups of amylases were classified based on their modes of action into three groups (Figure 2.1).

2.1.1 Endoamylase

Endoamylase, which is termed as α-amylase and systematic name as α-1,4-glucanohydrolase (EC 3.2.1.1), can cleave α-1,4 glycosidic bonds in amylose, amylopectin and related polysaccharides such as glycogen. The product of hydrolysis is oligosaccharide with varying chain lengths and has an α-configuration at C_1 of the reducing glucose unit, hence it was named the α-amylase. The α-amylase hydrolyzes the bonds located on the inner regions of the substrate, resulting in a rapid decrease of the viscosity of starch solution as well as a decrease in iodine staining power.

2.1.2 Exoamylase

Exoamylase, which is termed as β-amylase, can cleave α-1, 4 glycosidic bonds in amylose, amylopectin and glycogen from non-reducing end by successive removal of maltose/glucose in the stepwise manner. The products of hydrolysis are maltose and limit dextrins, both having the β-configuration at the C1 of the reducing glucose unit due to inversion of the product. In contrast to the action of endoamylase, this results in a slow decrease in the viscosity and iodine staining power of starch.

2.1.3 Debranching enzymes

The branch points containing 1, 6 glycosidic are resistant to be attacked by α- and β-amylases, resulting in α/β limit dextrins, respectively. Glucoamylase can also attack α-1,6 glycosidic linkages, but the reaction proceeds slowly when compared to pullulanase action. Glucoamylase is an inverting exo-acting

starch hydrolase that releases β-glucose from the nonreducing ends of starch and related substrates (Sauer et al., 2000).

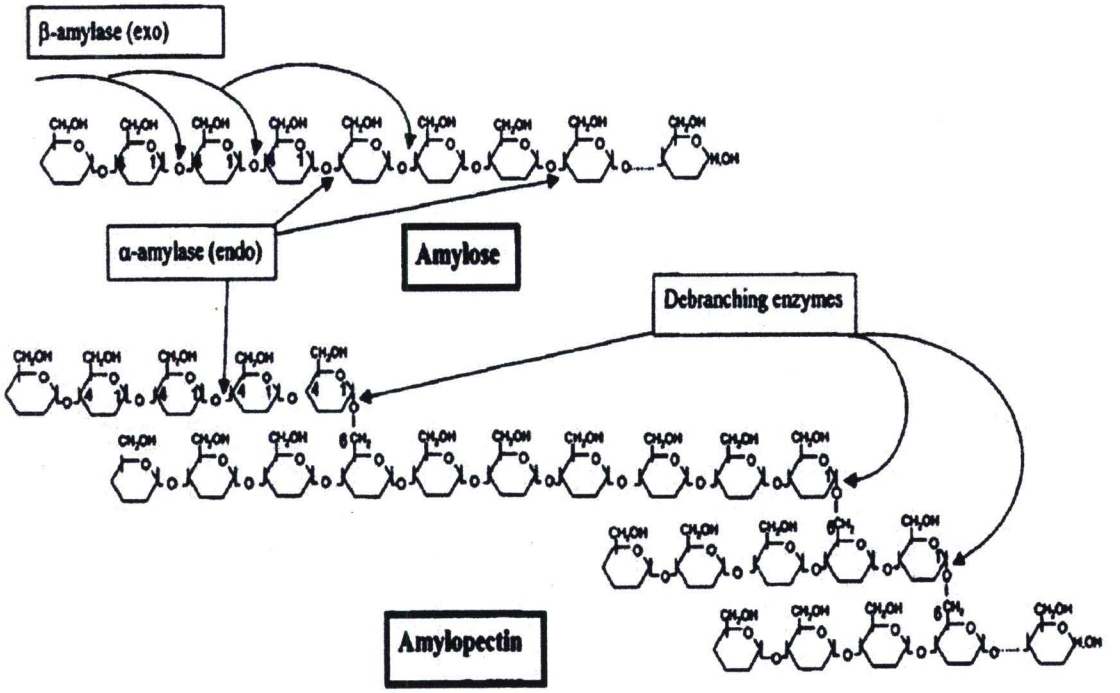

Figure 2.1 Hydrolysis position on polysaccharide molecules of hydrolase family. (Tester et al., 2004)

2.2 Amylase source (Brena et al., 1996)

α-amylase is found in several animal organs such as pancreas (human, pig, dog, horse) and salivary glands. Moreover, the α-amylase is found in higher plants as well, especially in germinated seeds of cereal grains such as malted barley, oats and wheat and in micro-organisms such as molds (*Aspergillus oryzae*) and bacteria (*Bacillus subtillis*, var. *amyloliquefaciens*, *B. mesentericus*, *B. polymixa*, etc). In addition, the occurrence of α-amylase has also been reported in avocados and mangoes (Edna et al.,1978; Fuchs et al., 1980).

β-amylase is found in some species of higher plants, particularly in seeds and germinated cereal grains (barley, rye, oats, wheat, sorghum, rice) and in soybean, maize, sweet potato tubers, tap roots of alfalfa, tuber of taros and mustard seedlings. β-amylases are also present in some bacteria such as *Bacillus* (*B.*) *megaterium*, *B. circulans*, *B. cereus*, *B. polymixa*, *Pseudomonas* sp. and *Streptomyces* sp. In higher

plants, β-amylase exists in sweet potato tubers, grains of various cereal species such as barley, wheat, rice and soybean. β-amylase may play a role in the mobilization of starch during seed germination or the sprouting of tubers in starch-enriched organs. γ-amylase or glucoamylase can be found in general microorganisms.

2.3 The properties of amylase

The presence of metal ions, pH and temperature depends on the specific action of α- and β-amylase, which dependent differs markedly according to the origin of the enzyme. Generally, α-amylases obtained from higher plants are very stable in the pH range 5.5-8.0. Optimal active pH and temperature of kiwi fruit is 6.0 and 50 °C, respectively. For cereal α-amylases, optimal active pH is between 4.5 and 6.0; irreversible inactivation can possibly occur below pH 4.5 (Table 2.1). Optimal active pH for β-amylase is generally between 4.0 and 6.0. The enzymes are relatively stable in the acidic range above pH 3.6 (Table 2.2). α-amylase can be inactivated by calcium chelating agent such as EDTA. Excess of calcium ions can reverse the inhibition caused by EDTA. α-amylase contains bound calcium ions for full activity. In addition, excess calcium does not produce an increase in activity, but favours maximal enzymatic stability. Calcium ions increase the stability of α-amylase in malt, pancreas and other sources; on the other hand, decrease that of β-amylase in barley. Certain salt ions such as iodides, fluorides and heavy metals, can irreversibly inactivate amylases. Reversible inactivation of β-amylase is caused by some oxidants such as peroxides and iodine (Brena et al., 1996).

Table 2.1 The properties of α-amylase

Source	MW ($\times 10^{-3}$)	Optimum pH	Optimum temperature (°C)	Stability pH range
Barley		5.5	47.5	5.5-8.0
Barley (malted)	45 and 59	4.7-5.5	48-55	5.0-8.0
Broad bean	45	5.6	45	5.5-8.0
Oats	45	4.7	47	5.0-7.5
Rye	45	5.0	47	5.0-7.5
Sorghum (malted)	50	4.5-5.0	>40	
Soybean	45	6.0	55	5.5-8.0
Wheat	45	5.0	49	5.0-7.5
Kiwi		6.0	50	
Antheraea mylitta	58	9.5		
Hog pancreas	45	6.9[b]		7.0-8.5
Human pancreas		6.9[b]		4.8-11.0
Human saliva	55	6.9[b]		4.8-11.0
B. subtillis		5.5-6.0	70	
B. licheniformis		7.0	90	
B. amylo-liquefaciens	58	5.5	50-70	6.0-12.0
B. circulans	45	7.0	50	6.0-9.0
A. niger	58	4.0-5.0		2.2-7.0
A. niger	61	5.0-6.0		5.0-8.5
A. oryzae		5.0	40	6.0-8.0
Chalara paradoxa	80	5.5	45	5.5-7.5

[b]Chloride-activated enzyme (Brena et al., 1996)

Table 2.2 The properties of β-amylase

Source	MW ($\times 10^{-3}$)	Optimum pH	Optimum temperature (°C)	Stability pH range	pI
Sweet potato	197	4.5-5.0	60	5.5-8.0	4.77
Soybean	61.7	6.0	45	5.0-10.0	5.0-5.9
Wheat	64.2	5.2		4.5-9.2	6.0
Malt		5.2		4.5-8.0	6.0
Mustard	58	5.8-6.2		3.0-8.0	4.6-4.8
Pea	56	6.0			4.35
Barley	56	5.2	>50	4.5-8.0	6.0
Ichoimo	60	6.0	55	4.4-8.5	5.0-5.2
Sorghum	20	5.0-5.5	30-40	4.3-8.5	
Taro tubers[a]	66 and 67	5.0	60		
Mize	65				4.0
Kiwi		6.0	35		
B. megaterium No. 32	67	6.5		5.0-7.5	9.1
B. polymxa NCIB 8158	67	6.8	37	6.4-7.2	
B. polymixa N 72	44	7.0-8.0	45	4.0-9.0	8.35
B. cereus (var. mycoides)	35	7.0	50	5.0-10.0	
Pseudomonas sp. BQ06	37	6.5-7.4	45-55	6.5-8.0	

[a]Two forms. (Brena et al., 1996)

2.4 Structure and mechanism of amylase

2.4.1 α-amylase

α-amylase is a calcium-containing enzyme with a single polypeptide chain and small proteins with a molecular weight of 20-55 kDa. The four highly conserved regions, especially in the catalytic and substrate binding regions in 11 different α-amylases are usually found. The residues Asp-206, Glu-230 and Asp-297 were identified as the catalytic residues in Taka amylase-A, *B. subtilis*, *B. stearothermophillus* and barley. The catalytic site has cleft Topology most likely endohydrolase. They have been an important amino acid with 2 residues, Asp, which is nucleophile or base and Glu, which is acid or proton donor (Muralikrishna and Nirmala, 2005).

In general, α-amylase is comprised three distinct domains. A large "A" domain with a typical barrel shaped $(\beta/\alpha)_8$ super structure which is important for catalysis reaction. A "B" domain, inserted between the third β sheet and the following α helix of the $(\beta/\alpha)_8$ super structure that attached to the "A" domain by disulphide bond. A "B" domain is probably responsible for the difference in substrate specificity and stability among the α-amylase. A "C" domain with a β sheet structure linked to the A domain by a simple polypeptide chain and constitutes the C-terminal part of the sequence. Its functional role is not yet to be established. X-ray structure of α-amylase contains at least one conserved calcium ion. This is located on the interface between the A and B domain which suggests that the role of calcium ions can stabilize the three-dimensional structure as allosteric activator. X-ray crystal structure has been described in the case of amylases from *Aspergillus oryzae* (Taka amylase 3.0 and 2.1 A°) porcine pancreatic amylase (2.1 A°) and barley malt α-amylase isoforms Amy-2 (68 A°× 53 A° × 36 A°) (Muralikrishna and Nirmala, 2005). Ribbon diagram of barley Amy-2 is shown in Figure. 2.2.

Figure 2.2 Ribbon diagram of barley α-amylase (Kadziola et al., 1994)

The active site of amylase from *Aspergillus oryzae* has been localized in the cleft of the $(\beta/\alpha)_8$ barrel domain with Asp 206, Glu 230, Asp 297 residues playing the catalytic role; whereas His 122 and His 296 might bind to glucosyl residues of substrate.

In an SN1 type of reaction (Figure 2.3a), step one is initiated when the proton of an un-ionized carboxyl group attacks the glucosidic oxygen. Consequently, the reducing end fragment of the substrate, H-O-R, leaves from the catalytic center. A carboxylate group promotes the formation and stability of the carbonium ion intermediate. Because the hydroxyl ion is the carbonium ion intermediate, the hydroxyl ion of water nucleophilic can attack the carbonium ion intermediate to complete the reaction. In an SN2 type of reaction (Figure 2.3b), a carboxylate group attacks C-1 as a nucleophile, and then, a covalent bond is formed between the remaining glycosyl residue and the catalytic nucleophile via the transition state with substantial carbonium ion character. The formation of the glycosyl-enzyme complex involves the first anomer inversion, the Walden inversion. Then, C-1 from the reversed side of the covalent bond of the glycosyl-enzyme complex finalizes the

reaction via the transition state. As a result, the anomeric configuration of the substrate is retained in the product by the nucleophilic double displacement mechanism (Kuriki and Imanaka, 1999).

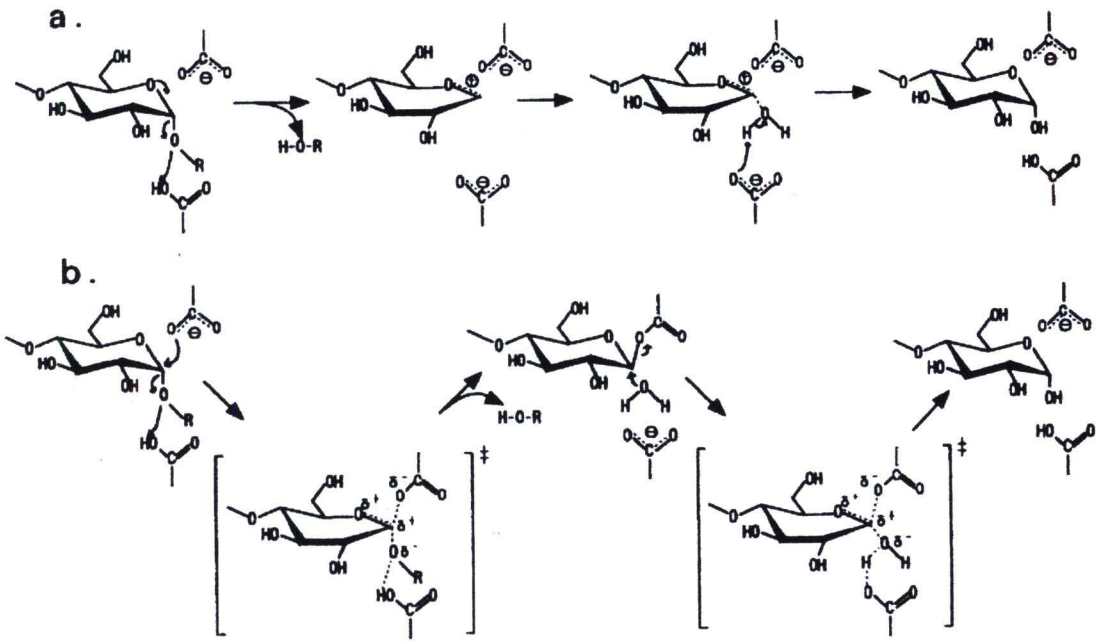

Figure 2.3 Possible catalytic mechanism of α-amylase. (a) S_N1 reaction via carbonium ion intermediate. (b) S_N2 reaction via formation of glycosyl-enzyme complex though a covalent bond. (Kuriki and Imanaka, 1999)

2.4.2 β-amylase

Three-dimensional structure and the catalytic site of enzyme mostly were analyzed by crystallization which was reported in enzymes obtaining from soybean, sweet potato, barley, and *B. cereus*. The experiment showed that β-amylase has 3 parts of structure or 3 domains which are similar to α-amylase. The differences in details of domain, tertiary structure of enzyme of plant and bacteria, are very similar although primary structure is very different. The 3 parts consist of the first part which is the main structure $(\beta/\alpha)_8$, there have been different in amino acid sequence from α-amylase, found in N terminal to the middle of polymer and nearly to C terminal, this part is deep nook 18 Å and catalytic site of the enzyme. The second part is lobed; there is a small size which formed by 3 long lobes (L3, L4, and L5) that

extended from β3, β4, and β5. The third part is the terminal of C part which there is long lobe, the amino acid length is about 50 residues for α8 or H8 (Figure 2.4).

The α-amylase from soybean contains the two active site residues: Glu 186 and Glu 380; Glu 186 is general acid or proton donor and Glu 380 is general base. The substrate binding site contains 20 residues such as Val 99, Tyr 192, Trp 198, Phe 200, Met 346 and Leu 383 (Piumsuk, 2008).

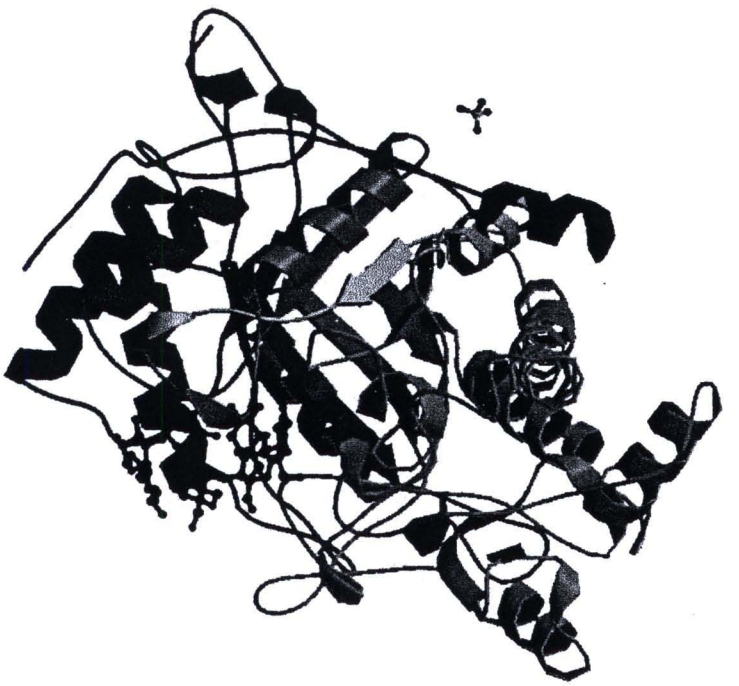

Figure 2.4 Crystal structure of recombinant soybean beta-amylase complexed with beta-cyclodextrin.

β-amylase is not able to transfer glycosyl to any water donor. This enzyme has no tranglucosylation activity which is the one different property from α-amylase. These enzymes are composed of sulfhydryl group which is important for enzymatic activity that there is no needed coenzymes and cofactors such as metal ion for catalytic reaction. Most plant enzymes have several isoforms. For example, sweet potato composed of 4 subunits (tetramer) while other plant enzymes are single polypeptide. The catalytic mechanism hydrolyzed glucosidic bond with alternative configuration at first carbon which has 2 reaction mechanisms: 1) nucleophilic displacement mechanism (Figure 2.5) which is single displacement reaction initiating with adding proton to glycoside oxygen with COOH group, which functions as the general acid. Such acid causes maltosyl C-O bond to become weak and causes the reducing end of the substrate. Then hydroxyl ion of water which performs like nucleophile, binds to the front side (beta side) of the substrate molecule with helping force from COO^- of the other groups of the enzyme that performs as a general base. Accordingly, this causes the product has carbon alternative configuration. 2) Oxocarbonium ion mechanism, initial reaction from adding proton from COOH of enzyme to glycoside oxygen causes the different intermediate of oxocarbonium ion of water in the next step. In case of β-amylase, water which is binding to intermediate from back side causes alternative configuration product, β-type (Piumsuk, 2008).

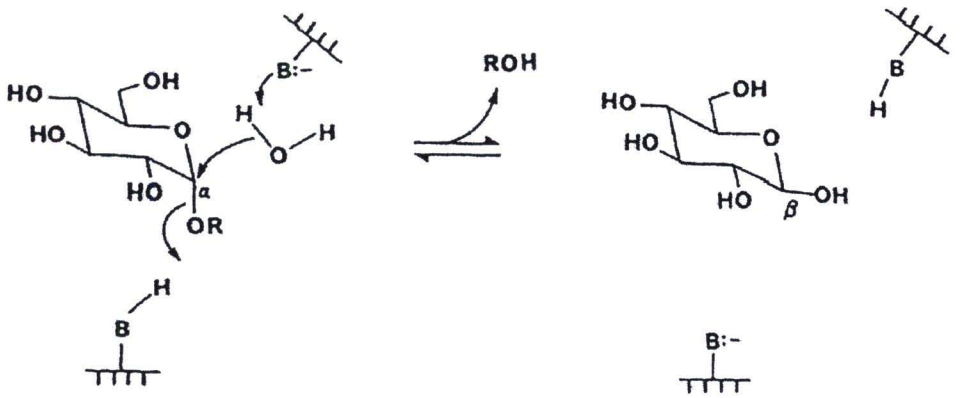

Figure 2.5 The catalytic mechanism of β-amylase (single displacement reaction) (Piumsuk, 2008).

2.4.3 γ-amylase or glucoamylase

The important quality of catalytic reaction accelerates hydrolysis of starch, it is able to hydrolyze starch into 2 bonds as α-1,4 and α-1,6-glycosidic linkage by hydrolyzing at the end terminal polymer for 1 unit from nonreducing end which the product is only the glucose. The catalytic mechanism of glucoamylase is similar to that of β-amylase.

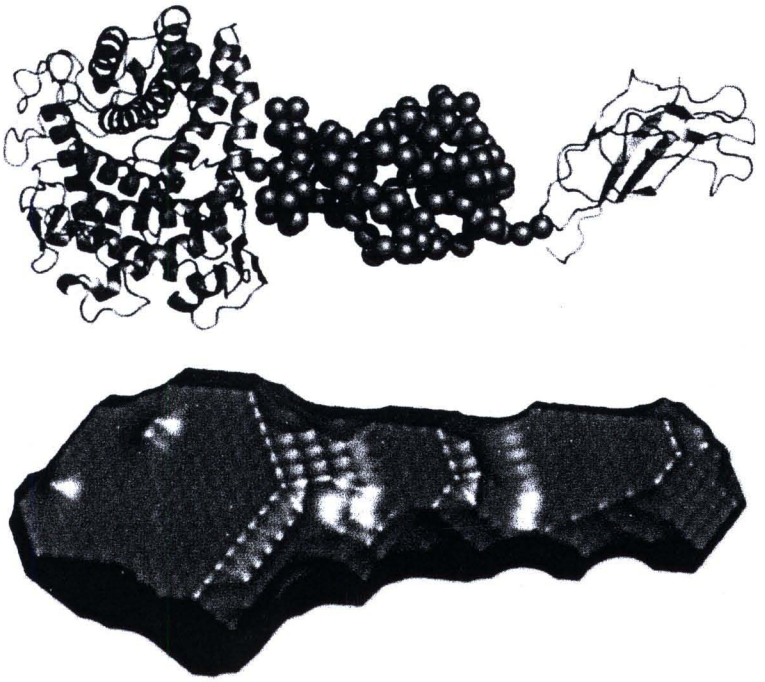

Figure 2.6 The structure model of *Aspergillus niger* glucoamylase A = Catalytic Domain; B = Linker; C = Starch-Binding Domain (Alexander et al., 1992)

Pongsawat (2008) studied enzymes from *A. awamori*, and *A. niger*, the main structure consists of 3 parts (Figure 2.6), one of them is similar to β-amylase. There has been a different point as follows: 1) Catalytic domain site at N-terminal. 2) Raw starch binding site at C terminal. 3) There is between 1 and 2 part, called highly glycosylated linker, the catalytic site has topology similar to a pocket as same as β-amylase which consists of $(\alpha/\alpha)_6$ barrel structure.

2.5 **Application of amylases** (Aiyer, 2005; Souza and Perola, 2010)

The history of the industrial production of enzyme dated back to 1894 by Dr. Jhokichi Takamine, who initiating the process of digestion enzyme preparation by wheat bran koji culture of *Aspergillus oryzae*. In 1959, α-amylase and glucoamylase were used in the manufacturing production of dextrose powder and dextrose crystals from starch. Since then, amylase had been being used for multipurpose utilization, such as starch processing industry, bread and baking industries, starch liquefaction and saccharification, textile, paper and detergent industry etc. Some of the appealing applications of amylase are exemplified here as a noteworthy guideline to see how wide the practical application of amylase can be:

2.5.1 Starch conversion

Amylase is used for starch hydrolysis in the starch liquefaction process, which converts starch into fructose and glucose syrups. The enzymatic conversion of all starch includes: saccharification, which involves the production of glucose and maltose via further hydrolysis; gelatinization, which involves the dissolution of starch granules, then forming a viscous suspension; liquefaction, involving partial hydrolysis and loss in viscosity.

2.5.2 Fuel alcohol production

In liquefaction and saccharification process, starch is converted into sugar by using amylase, followed by fermentation. Then, using an ethanol fermenting microorganism, for example, yeasts; sugar is eventually transformed into ethanol.

2.5.3 Textile industry

Amylase is used in desizing process. Starch paste is applied for warping in textile weaving in order to provide strength to the textile. It also prevents the loss of string by cutting, friction and generation of static electricity on the string. After that, starch on cloth is eliminated by the application of α-amylase by hydrolysis, resulting in dextrins, which is water-dissolving compound and the cloth goes to scouring and dyeing.

2.5.4 Detergent industry

Amylase is used to degrade the residues of starchy foods such as chocolate, custard, etc. to smaller oligosaccharides for laundry and automatic dishwashing. Removal of starch is important in providing a whiteness benefit because starch can be an attractant for many types of particular solids.

2.5.5 Food industry

Amylase is extensively employed in several manufacturers of baking, brewing, production of cake, fruit juices and starch syrups. For baking industry, these enzymes are added to dough of bread so as to degrade the starch in the flour into the smaller dextrin. Then, it is subsequently fermented by yeasts. In addition, it generates additional sugar in the dough, which improves the taste, crust colour and toasting qualities of the bread.

In beer and fruit juice industry, amylase is also used for the clarification, or for pretreatment of animal feed to improve the digestibility of fiber.

2.5.6 Paper industry

Starch of coated paper, which is modified by amylase in the pulp and paper industry, coating treatment serves to make the surface of paper sufficiently smooth and strong, and to improve the writing quality of the paper. In the application, amylase can be altered properly because the viscosity of the natural starch is too high for paper sizing in continuous processes.

2.5.7 Treatment of starch processing waste water

Starch waste causes pollution problems. Biotechnological treatment of food processing waste can produce valuable products such as microbial biomass protein and also purifies the effluent.

2.6 Information about Durian

The durian, one of the most popular and famous seasonal fruits in South East Asia, is the important commercial fruit of Thailand. Over three centuries ago, durian had been cultivated in Thailand and was introduced to central Thailand in 1787 from the southeast region of Myanmar and the southern Thailand by Malaysia.

2.6.1 Botanical characteristics of durian (Suhadrabandhu and Ketsa, 2001)

The durian is a tropical fruit tree in the order Malvales, family Bombacaceae. The economically valuable species is *Durio zibethinus* Murr. Botanists have studied the botanical structure of durian and summarized as follows;

2.6.1.1 Tree size and form

Durian trees have a life span between 80 and 150 years. They have a trunk diameter from 50 to 120 cm. The tree has a columnar, buttressed, low-branched trunk and a dense crown. The bark is dark reddish-brown, peeling off from many deep longitudinal spits. The branches grow from the main trunk in every direction. The larger branches are covered with numerous lenticels. The secondary branches are grooved with reddish pubescences (Figure 2.7).

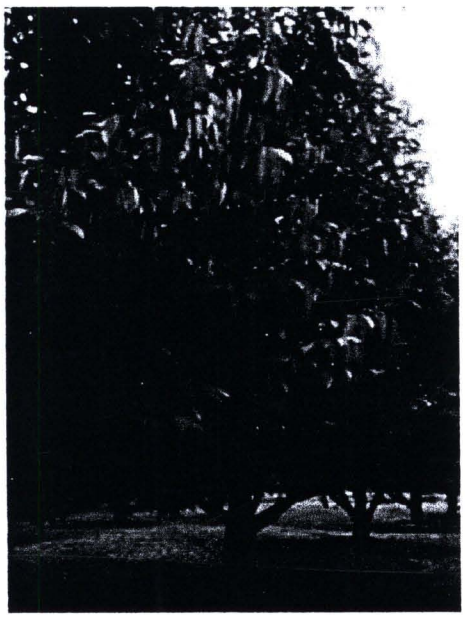

Figure 2.7 Durian tree

2.6.1.2 Leaf structure

The leaves are simple, alternately elliptical and lanceolate, 10 to 20 cm long and 4 to 6 cm wide. The petioles are thick, about 1.5 to 3.0 cm long and 0.15 to 0.35 cm wide. The leaf blade is shiny, smooth and dark green on the upper surface but the lower surface is densely covered with silver or gold scales and a layer of stellate hairs (Figure 2.8)

Figure 2.8 Durian leaf

2.6.1.3 Flower structure

Durian flowers, which are complete or perfect flowers – meaning each flower has both stamen and pistil, appear in clusters on the branches or trunk of the tree and are possible to have 50 flowers per cluster. Inflorescences are from fascicles of corymbs of 3 to 50 flowers. The epicalyx, the outermost covering of the flower, is entirely closed initially, and later, splits into two to four segments. The five petals are white; light yellow or cream and longer than the calyx. The ovary shape is ovoid to oblong, densely covered with silvery scales, containing four to five cells (Figure 2.9).

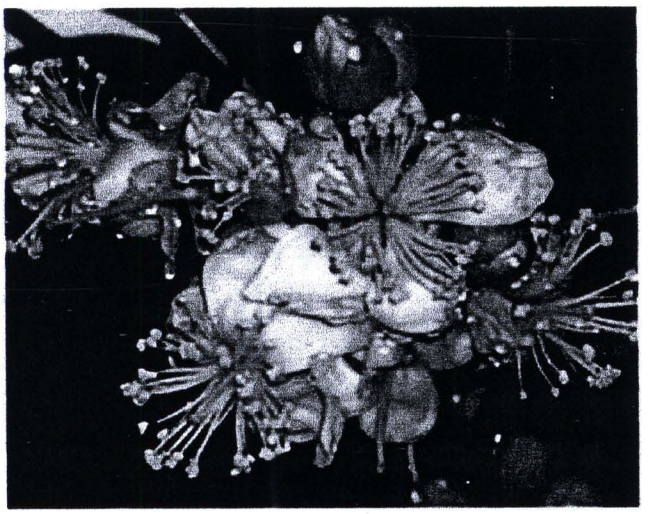

Figure 2.9 Durian flower

2.6.1.4 Fruit

The durian fruit, which emerged on the underside of branches, is formed from one flower and one ovary. Shape of fruit, which exceeds 3 kg in weight, is somewhat round to oblong, commonly 13 to 16 cm wide and 15 to 25 cm long. The color varies from olive-green to yellow. The spines, which are slightly angled and abruptly taper to a very hard, sharp point, are abound, 1.5 cm long and 1.9 cm wide at the base. The colour of aril is cream or yellow-coloured aril of custard-like consistency. The aril, edible portion of fruit, varies in colour, texture and thickness between different varieties (Figure 2.10).

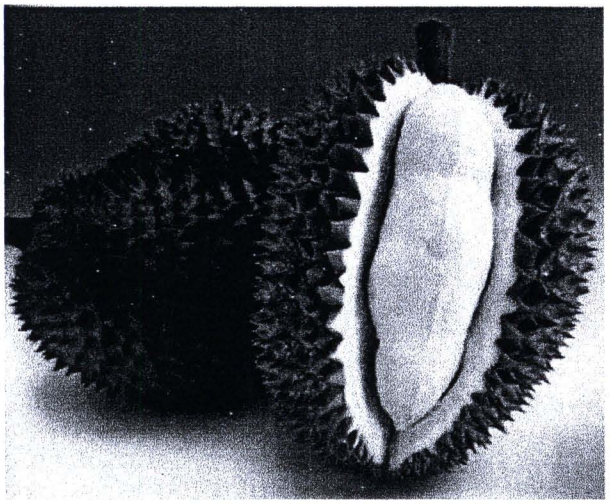

Figure 2.10 Durian fruit

2.6.2 Durian in Thailand

In Thailand, Durian is a very favorite fruit of most of the Thai people. The 72.1% of the Thais who sampled the fruit liked to consume durian, they preferred Mon Thong cultivar because of the sweet and buttery taste with soft and fine texture, rather than its mild odor (Somsri, 2007). Production of commercial scale durian rather concentrated in the central region, and some parts of the north, the east and the south. The east and the south were main arable areas for durian agriculture. The provinces with the highest production are Chantaburi, Rayong and Chumphon (Figure 2.11). However, some areas in the northeast, such as Uttaradit, Nakhaon Phanom, Si Sa Ket, etc., showed the capacity of growing durian as well (Somsri, 2007).

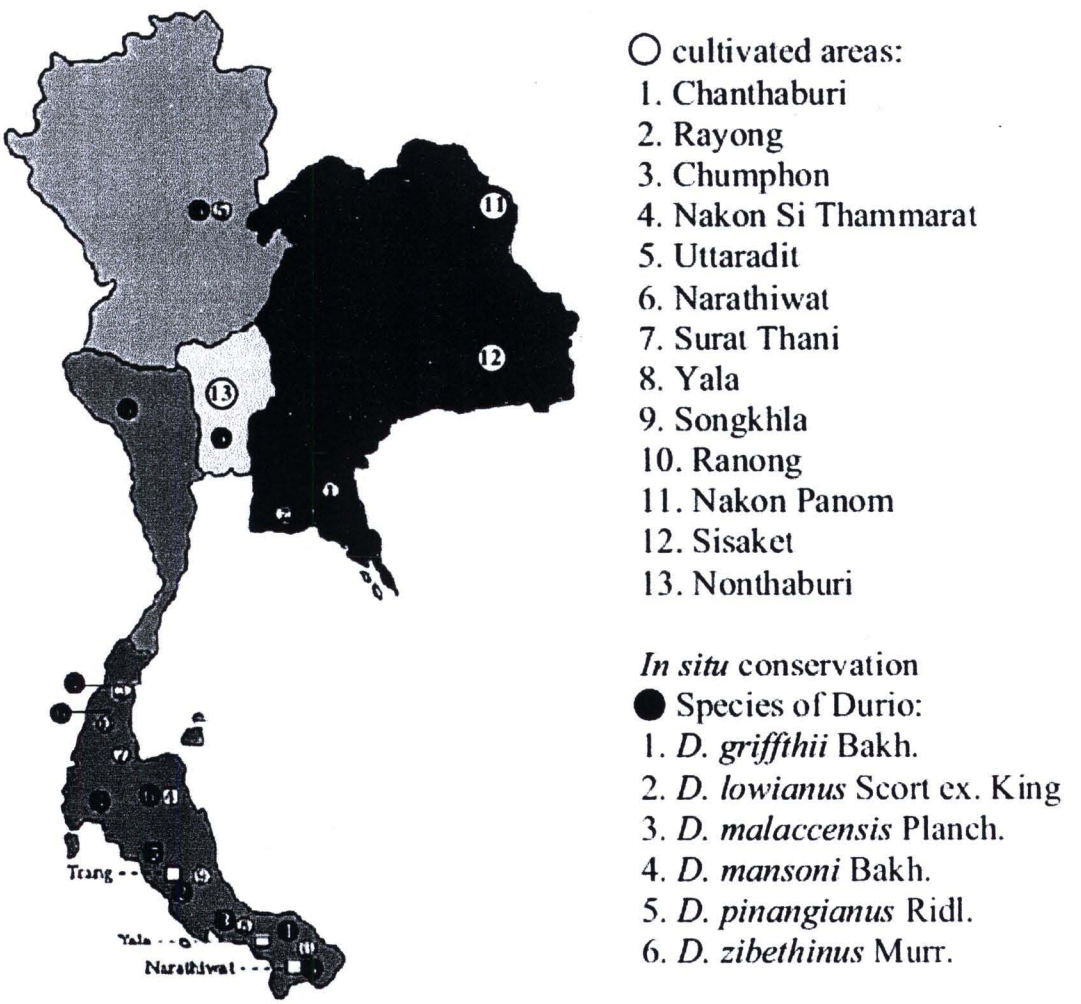

Figure 2.11 The Durian Suitable Growing Locations (Somsri, 2007).

2.6.3 Cultivars of durian in Thailand (Suhadrabandhu and Ketsa, 2001)

Bhusiri (1987) suggested a method of classification based on: 1) the appaearance of the fruit itself, as in Mon Thong which means golden pillow, i.e. the fruit is shaped like a pillow; 2) the colour or texture of the flesh or pulp, as in Kam Pan Daeng which means reddish-yellow pulp; and 3) the preson or agency who selected the cultivar, as in the Maetao cultivar which was named after Mr Kop Maetao. Using these criteria, Bhusiri proposed eight categories as follows:

1) Chanee group
2) Kop group
3) Kan Yao group
4) Mon Thong group
5) Thong Yoi group
6) Kam Pan group
7) Luang group
8) Miscellaneous group

The popular durian cultivars commercial grown in the east, south, north and northeast of Thailand are: Kan Yao, Chani, Mon Thong, Kradum Thong, Kop Phikul, Kop Ta Kham, Kop Mae Thao, Phuang Mani and Nok Yip (Figure 2.12).

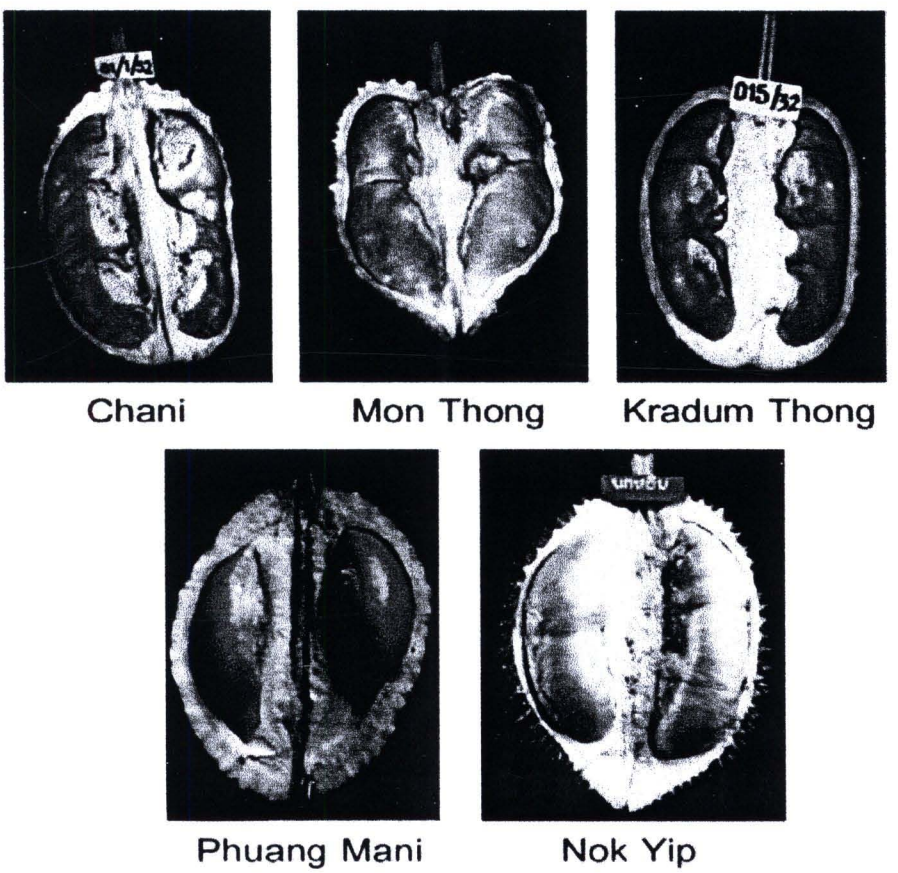

Figure 2.12 Some of commercial cultivars in Thailand (Somsri, 2007).

Table 2.3 Nutrient Composition per 100 g Edible Portion of Durian

Durian Nutrients	Units	Value per 100 grams	Number of Data Points
Proximates			
Water	g	64.99	2
Calories in Durian-Energy	kcal	147	2
Energy	kJ	615	0
Protein	g	1.47	2
Total lipid (fat)	g	5.33	2
Ash	g	1.12	2
Carbohydrate, by difference	g	27.09	0
Fiber, total dietary	g	3.8	2
Minerals			
Calcium, Ca	mg	6	2
Iron, Fe	mg	0.43	2
Magnesium, Mg	mg	30	2
Phosphorus, P	mg	39	2
Potassium, K	mg	436	2
Sodium, Na	mg	2	1
Zinc, Zn	mg	0.28	2
Copper, Cu	mg	0.207	2
Manganese, Mn	mg	0.325	2
Vitamin			
Vitamin C, total ascorbic acid	mg	19.7	2
Thiamin	mg	0.374	2
Riboflavin	mg	0.200	2
Niacin	mg	1.074	2
Pantothenic acid	mg	0.230	2
Vitamin B-6	mg	0.316	2
Folate, total	mg	36	2
Folate, food	mg	36	2
Vitamin B-12	mg	0.00	0
Vitamin A, RAE	Mcg_RAE	2	2
Retinol	mcg	0	0
Carotene, beta	mcg	23	2
Carotene, alpha	mcg	6	2
Cryptoxanthin, beta	mcg	0	2
Vitamin A, IU	IU	44	2
Durian Nutrition Facts: Lipids			
Cholesterol	mg	0	0

(National Nutrient Database for Starndard Reference, 2009)

2.6.4 Changing during ripening of durian (Subhadrabandhu and Ketsa, 2001)

Durian is a climacteric fruit. Starch contents in durian decrease concomitantly with a rapid increase in total sugars and soluble solids as ripening advances. This decrease in starch is typical of post-harvest carbohydrate changes in the climacteric fruit. The aril of ripened durian is very sweet, with a rich taste unlike any other fruits because the total sugar contents are rather high at 21% to 26%. The aril of ripening durian contains 2% to 5% reducing sugar, depending on the cultivars, Chanee has more reducing sugar than Mon Thong, but less non-reducing sugar (sucrose). Mon Thong aril contains non-reducing sugar (sucrose), contributing 70% to 80% of the total sugars. Sucrose is an important sugar contributing to the sweet taste of ripening durian.

In ripening stage, durian aril changes from light to dark yellow, depending on the cultivars, which increase in β-carotene synthesis in the aril during ripening.

The firmness of the aril rapidly decreases during ripening and is accompanied by a rapid increase in water soluble pectin. Polygalacturonase and pectinesterase activities increase as the aril softens in the ripening durian. In unripening, polygalacturonase activity is very low, but as the durian ripens, increases more dramatically than the pectinesterase activity which is already quite high in the unripe fruit. β-galacturonase is also very high in the unripe aril and the levels slightly increase as the fruit ripens. Cellulase activity is equally high in the unripe aril, but remains constant as the aril softens. This suggests that the integrated action of polygalacturonase and pectinesterase is required for durian aril to ripen and that both β-galacturonase and cellulase are not involved in this process.

The distinctive durian aroma, which indicates the ripeness of the fruit, can be detected before the respiratory peak. The odour has two distinguishable components: one strong and onion-like, the other more delicate and fruit-like. If the fruit is kept in an enclosed space, the third component, better described as a stink, can then be detected. Thiols and thioethers are responsible for the onion-like odour and esters for fruit-like odour. The chief thiols are in order of importance propanethiol, ethanethiol and methanethiol. The ester compounds included 17 components. One

ester predominated over all thiols and had a pleasant fruity smell; this was identified as ethyl 2-methybutyrate.

2.7 Role of amylase to starch mobilization in fruits (Seymour et al., 1993 and Hulme, 1971)

In many fruits, the breakdown of starch to glucose, fructose or sucrose, is a characteristic ripening event. There are several enzymes in plant tissue capable of metabolizing starch of which pathways involved are shown in Figure 2.13. α-amylase (EC 3.2.1.1) hydrolyses the α(1-4) linkages of amylose at random to produce a mixture of glucose and maltose. In contrast, β-amylase (EC 3.2.1.2) attacks only the penultimate linkage and thus releases only maltose. After that, glucosidase (EC 3.2.1.20) can hydrolyze the maltose to glucose. Starch phosphorylase (EC 2.4.1.1) hydrolyses the terminal α(1-4) linkage to give glucose-1-phosphate, which can be converted to glucose-6-phosphate by the action of glucose phosphate mutase (EC 2.7.5.5).

The main starch degrading enzyme in fruit is α- and β-amylase and starch phosphorylase, which have been identified. In the pulp of unripe banana, starch form 20-25% of the fresh weight. During the ripening, the peel tissue also contains starch, about 3% fresh weight. During ripening this starch is degraded rapidly and the sugars sucrose, glucose and fructose accumulate; traces of maltose may also be present. In the banana pulp, sucrose is the predominant sugar, at least at the start of ripening and its formation precedes the accumulation of glucose and fructose. Starch degrading enzyme isolated from banana are shown in Table 2.4 , with α-amylase, β-amylase and α1,6-glucosidase (EC 3.2.1.11) activity having all been reported to increase in ripening bananas. However, it has been noted that starch phosphorylase activity is present in both unripe and ripe banana.

Table 2.4 Starch degrading enzymes isolated from bananas.

Enzyme	Mode of action
α-amylase (EC 3.2.1.1)	Endo, acting on α 1-4 glucose linkages
β-amylase (EC 3.2.1.2)	Exo, acting α 1-4 glucose linkages at non-reducing end of substrate
α-1,6-glucosidase (EC 3.2.1.11)	Attack α 1-6 glucose linkages of amylopectin
Phosphorylase (EC 2.4.1.21)	Attack the non-reducing end of polymer producing glucose-1-phosphate

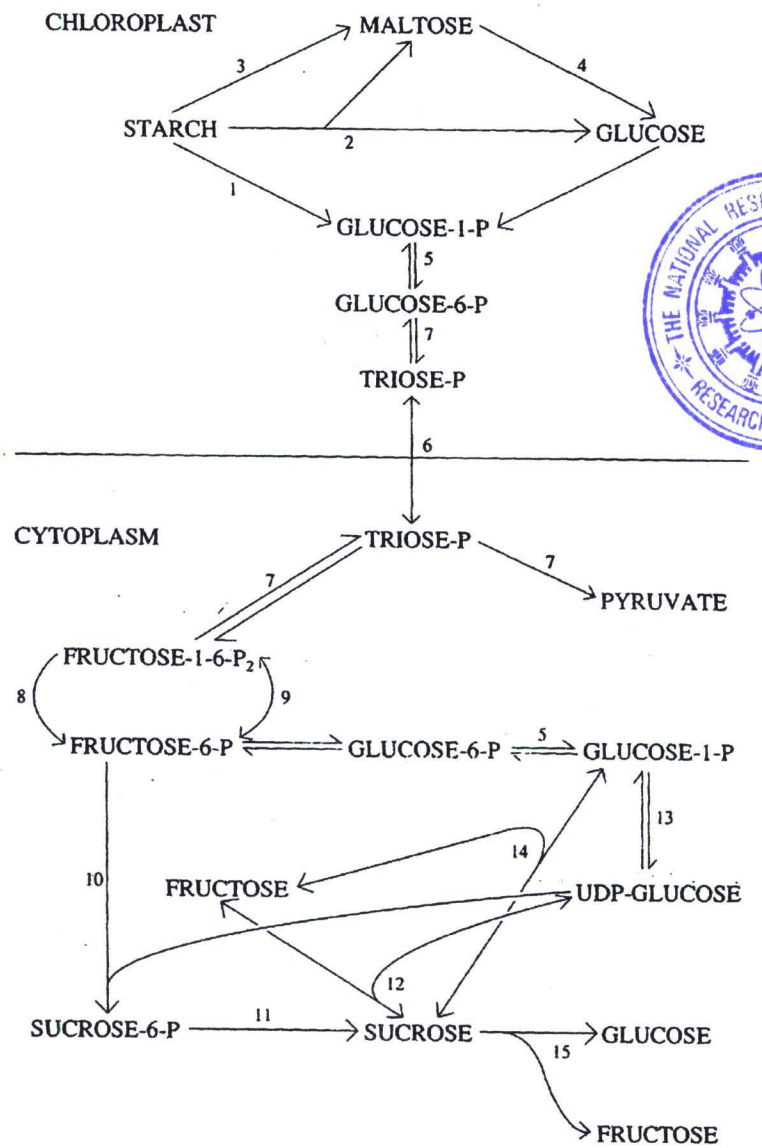

Figure 2.13 The pathway of starch degradation and sucrose synthesis in fruit (Seymour et al., 1993)

1. Starch phosphorylase (EC 2.4.1.1)
2. α-amylase (EC 3.2.1.1)
3. β-amylase (EC 3.2.1.2)
4. α-glucosidase (EC 3.2.1.20)
5. Glucose phosphate mutase (EC 2.7.5.5)
6. Triose phosphate/phosphate transporter.

It is possible that in some cases, export of starch degradation products from the chloroplast may occur via the transport of hexose phosphates.

7. Glycolysis/gluconeogenesis
8. Fructose bisphosphatase (EC 3.1.3.11)
9. Phosphofructophosphotransferase (EC 2.7.1.90)
10. Sucrose phosphate synthetase (EC 2.4.2.14)
11. Sucrose phosphate phosphatase (EC 3.1.3.24)
12. Sucrose synthase (EC 2.4.1.13)
13. UDP-glucose pyrophosphorylase (EC 2.7.7.9)
14. Sucrose phosphorylase (EC 2.4.1.7)
15. Invertase (EC 3.2.1.26)

2.8 The research about amylase in plant

Edna et al. (1978) studied starch content and amylase activity in avocado fruit pulp. The results showed that amylase activity was higher in young than in mature fruit. After harvest, amylase activity started to rise with the onset of the respiratory climacteric. Additionally, along with the increase in amylase activity, there was a substantial reduction in the starch content of the fruit as well (Figure 2.14).

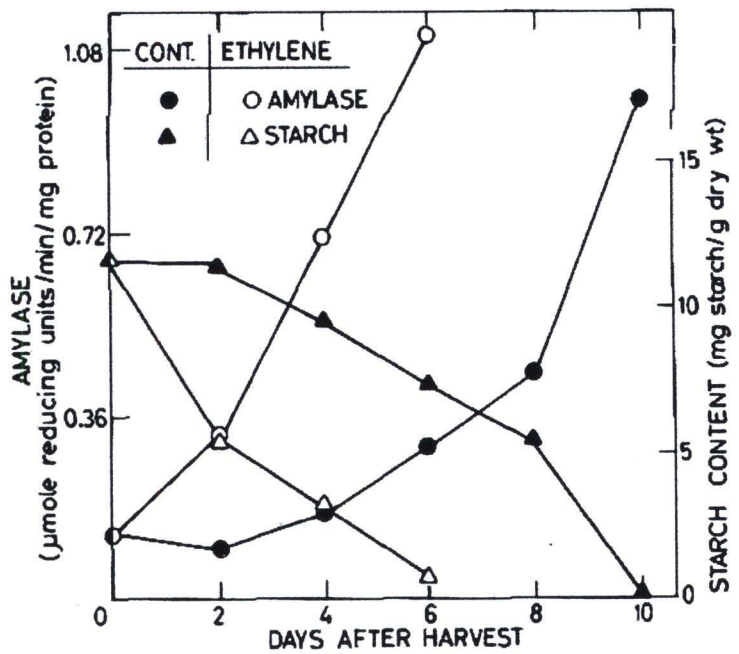

Figure 2.14 Effect of postharvest ethylene treatment on amylase activity and starch content in mature avocado fruit pulp.

Fuchs et al. (1980) studied changes in amylase activity, starch and sugar contents in mango fruit pulp. The results showed that amylase activity in the fruit pulp increased with the development of the fruit, parallelling to the increase in fruit weight (Figure 2.15 A) In addition, during mango ripening, the starch was being hydrolyzed and at the same time, amylase activity was changing (Figure 2.15 B). For, over-ripe fruit, total sugars contents decreased (Figure 2.15 C) because the fruit was using up reducing sugar for its respiration and for other energy consuming ripening processes, while no more starch which was probably the main origin of this reducing sugar left in fruit.

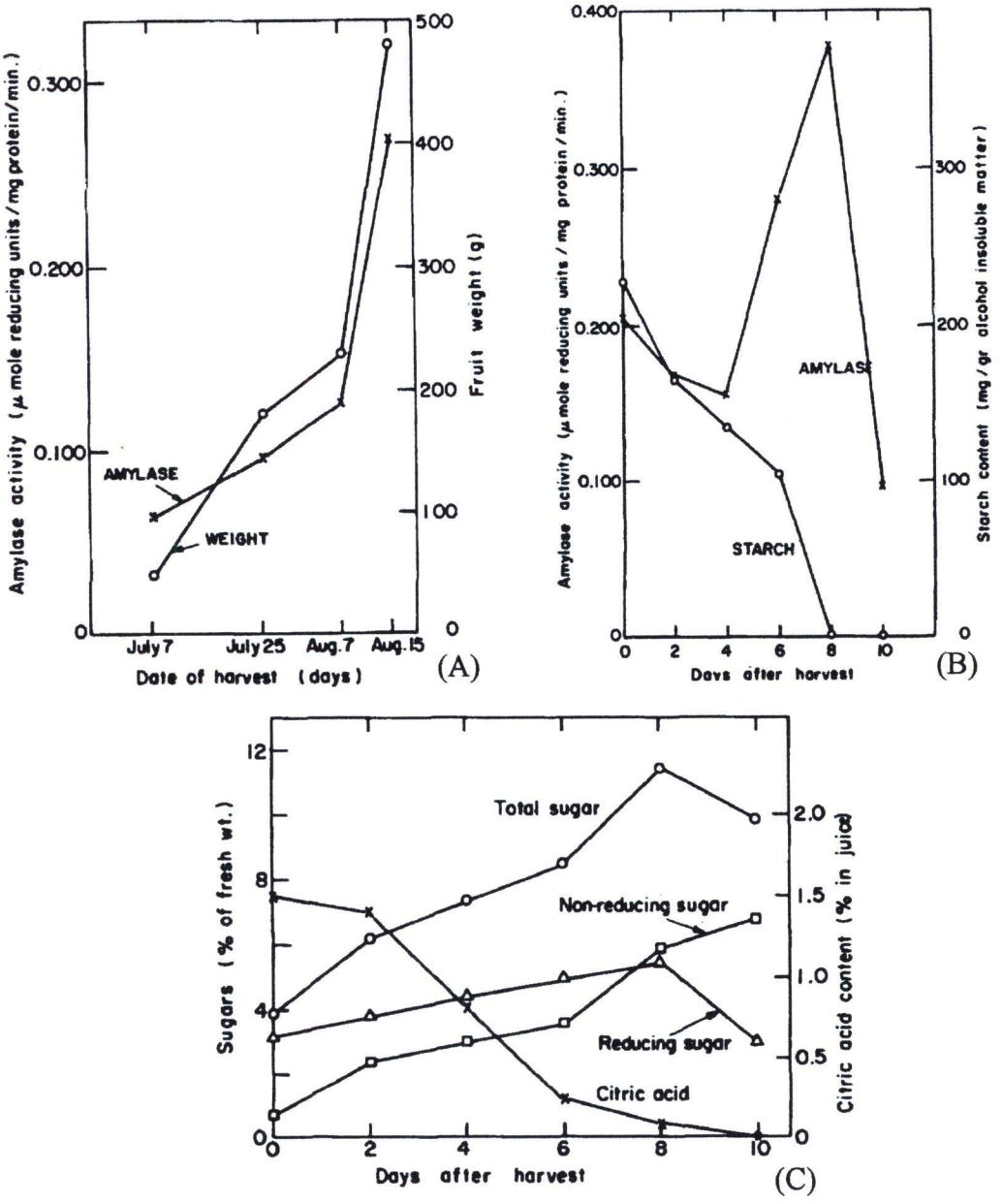

Figure 2.15 (A) = Changes in amylase activity and fresh fruit weight in developing mango fruits (B) = Changes in starch content and amylase activity during ripening of mango fruit (C) = Changes in acid and sugar contents during mango fruit ripening

Noman et al. (2003) purified α-amylase from post-harvest *Pachyrhizus erosus* L. tuber by DEAE- and CM-cellulose columns. The result showed that purified amylase had a molecular weight 40 kDa, 110 fold of purification and 22.8% of yield. The enzyme was of α-type as it lost total activity in the presence of EDTA and had

optimal activity at pH 7.3 and 37 °C. The enzyme was strongly inhibited by Cu^{2+}, Fe^{2+} and Zn^{2+}, moderately by Li^{2+}, Hg^{+} and Cd^{2+} and slightly by Ag^{+}, Mg^{2+} and K^{+}.

Kanwal et al. (2004) purified and characterized α-amylase from apples (*Malus pumila*). The results showed the activity of 5.025 U/ml and specific activity 38.95 U/ml with 20-fold purification. Molecular weight was determined by Sephadex G-150 column that was 51, 180 Dalton. Amylase showed a pH optimum of 6.8, temperature 37 °C, K_m value of 2.0×10^{-3} g/ml

Tripathai et al. (2007) study correlation of biochemical properties and tertiary structure of α-amylase from mungbean (*Vigna radiata*). The result showed that α-amylase has been purified 600-fold, final specific activity of 437 U/mg and molecular weight 46 kDa. The optimum pH was 5.6 and high specific activity for starch as substrate. Furthermore, addition of EDTA (10 mM) caused irreversible loss of activity. The purified enzyme was inhibited in a non-competitive manner by heavy metal ions such as mercury. Besides, barley α-amylase Amy1 and Amy2 was used as templates to study tertiary structure of mungbean α-amylase. The outcomes showed a very similar structure as expected from the high sequence identity. The model showed that α-amylase had no sugar binding site, it has a methionine, instead. Moreover, instead of two tryptophans, it has Val227 and Lys278, which are the conserved residues, important for proper folding and conformational stability.

Amylase from germinated soybeans seed (*Glycine max*) (Kumari et al., 2010) has been purified 400-fold with a final specific activity of 384 units/mg. The molecular mass determined to be 84 kDa by MALDI-TOF and gel filtration on Superdex-200 (FPLC). The enzyme exhibited maximum activity at pH 5.5 and pI value of 4.85. The energy of activation was determined to be 6.09 kcal/mol in the temperature range 25-85 °C. Apparent Michaelis constant ($K_{m(app)}$) for starch was 0.71 mg/ml and turnover number (k_{cat}) was 280 s^{-1} in 50 mM sodium acetate buffer, pH 5.5. Thermal inactivation studied at 85 °C showed the first-order kinetics with rate constant (k) equaling to 0.0063 min^{-1}. Interestingly, cereal α-amylase has gained importance due to their compatibility for biotechnological application. Wide availability and easy purification protocol make soybean as an attractive alternative for plant α-amylase.