2. THE ENZYMES

2.1. Enzyme Nomenclature

Proteins that function as biological catalysts are called enzymes. Enzymes speed up specific metabolic reactions. Low contamination, low temperature and fast metabolism are only possible with enzymes. Enzymes are protein in nature. Their reactivity is specific and enzyme can act reversibly (i.e. can catalyze the reaction in both directions). Enzymes get denatured as well as their rate of action altered at high temperature and with change in pH. Enzymes are composed of C, H, O and N and also sulphur (S) may be present. They have one or more polypeptide chains – which are formed on the *70s* or *80s* ribosomes by the translation of mRNA during protein synthesis. The polypeptide chains are folded into a particular three-dimensional shape (H-bonds) which is essential for enzyme functionality. Different shape gives the enzyme special areas known as active sites. The compatible substrate molecules bind to the complementary active site.

An enzyme is a protein having catalytic properties. These catalytic properties are related to the amino acid sequence of protein chain and to the way the chain is folded up i.e., to the three dimensional structure of the protein. If the three-dimensional structure of an enzyme is changed, it will lose its catalytic activity. At high temperature, this three-dimensional structure of the enzyme will be changed. This is irreversible and leads to loss of enzyme activity. It is called heat inactivation or denaturation.By applying high enough temperature, all enzymes can be inactivated. However, there is big difference between

various enzymes as to the temperature they can withstand before this heat inactivation takes place.

2.1.1. ENZYME CLASSIFICATON AND NOMENCLATURE

The enzyme nomenclature and classification of enzymes by the reactions they catalyse is given below (Table 2.1) as per the recommendations of the Nomenclature Committee (NC) of the International Union of Biochemistry and Molecular Biology (IUBMB). The common names of all listed enzymes are depicted below, along with their EC numbers.

Table 2.1: Enzyme classification by the reactions they catalyze as per NC IUBMB.

EC number	Common names
EC 1	Oxidoreductases
EC 2	Transferases
EC 3	Hydrolases
EC 4	Lyases
EC 5	Isomerases
EC 6	Ligases

2.1.2. ENZYME SUBCLASSES

The detailed enzyme classification along with subclasses are enumerated in the Table 2.2 below. Each subclass is divided in several 'sections' which further may contain about 50 enzymes.

Table 2.2: Detailed enzyme classification in to subclasses.

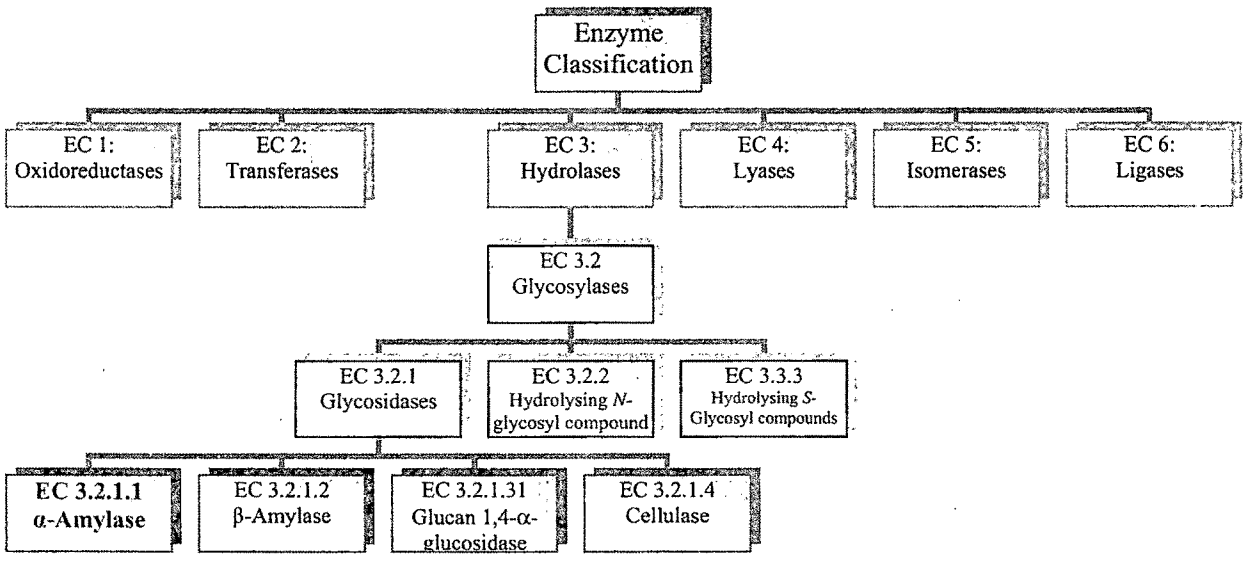
Subclass	Name
EC 1	**Oxidoreductases**
EC 1.1	Acting on the CH-OH group of donors
EC 1.2	Acting on the aldehyde or oxo group of donors
EC 1.3	Acting on the CH-CH group of donors
EC 1.4	Acting on the CH-NH_2 group of donors
EC 1.5	Acting on the CH-NH group of donors
EC 1.6	Acting on NADH or NADPH
EC 1.7	Acting on other nitrogenous compounds as donors
EC 1.8	Acting on a sulfur group of donors
EC 1.9	Acting on a heme group of donors
EC 1.10	Acting on diphenols and related substances as donors
EC 1.11	Acting on a peroxide as acceptor
EC 1.12	Acting on hydrogen as donor
EC 1.13	Acting on single donors with incorporation of molecular oxygen (oxygenases)
EC 1.14	Acting on paired donors, with incorporation or reduction of molecular oxygen
EC 1.15	Acting on superoxide radicals as acceptor
EC 1.16	Oxidising metal ions
EC 1.17	Acting on CH or CH_2 groups
EC 1.18	Acting on iron-sulfur proteins as donors
EC 1.19	Acting on reduced flavodoxin as donor
EC 1.20	Acting on phosphorus or arsenic in donors
EC 1.21	Acting on X-H and Y-H to form an X-Y bond
EC 1.97	Other oxidoreductases
EC 2	**Transferases**
EC 2.1	Transferring one-carbon groups
EC 2.2	Transferring aldehyde or ketonic groups
EC 2.3	Acyltransferases
EC 2.4	Glycosyltransferases
EC 2.5	Transferring alkyl or aryl groups, other than methyl groups
EC 2.6	Transferring nitrogenous groups
EC 2.7	Transferring phosphorus-containing groups
EC 2.8	Transferring sulfur-containing groups
EC 2.9	Transferring selenium-containing groups
EC 3	**Hydrolases**
EC 3.1	Acting on ester bonds
EC 3.2	Glycosylases
EC 3.3	Acting on ether bonds
EC 3.4	Acting on peptide bonds (peptidases)
EC 3.5	Acting on carbon-nitrogen bonds, other than peptide bonds
EC 3.6	Acting on acid anhydrides
EC 3.7	Acting on carbon-carbon bonds
EC 3.8	Acting on halide bonds
EC 3.9	Acting on phosphorus-nitrogen bonds
EC 3.10	Acting on sulfur-nitrogen bonds
EC 3.11	Acting on carbon-phosphorus bonds
EC 3.12	Acting on sulfur-sulfur bonds
EC 3.13	Acting on carbon-sulfur bonds

EC 4	**Lyases**
EC 4.1	Carbon-carbon lyases
EC 4.2	Carbon-oxygen lyases
EC 4.3	Carbon-nitrogen lyases
EC 4.4	Carbon-sulfur lyases
EC 4.5	Carbon-halide lyases
EC 4.6	Phosphorus-oxygen lyases
EC 4.99	Other lyases
EC 5	**Isomerases**
EC 5.1	Racemases and epimerases
EC 5.2	*cis-trans*-Isomerases
EC 5.3	Intramolecular isomerases
EC 5.4	Intramolecular transferases (mutases)
EC 5.5	Intramolecular lyases
EC 5.99	Other isomerases
EC 6	**Ligases**
EC 6.1	Forming carbon—oxygen bonds
EC 6.2	Forming carbon—sulfur bonds
EC 6.3	Forming carbon—nitrogen bonds
EC 6.4	Forming carbon—carbon bonds
EC 6.5	Forming phosphoric ester bonds
EC 6.6	Forming nitrogen—metal bonds

2.2. α-Amylase (EC 3.2.1.1)

α-Amylase (EC 3.2.1.1) is a digestive enzyme classified as a saccharidase (an enzyme that cleaves polysaccharides). It is mainly a constituent of pancreatic juice and saliva, needed for the breakdown of long-chain carbohydrates (such as starch) into smaller units. Amylase is also synthesized in the fruit of many plants during ripening, causing them to become sweeter, and also during the germination of cereal grains. Grain amylase is the key to the production of malt. There are two isoforms of amylase: pancreatic and salivary amylase. They behave differently on isoelectric focusing, and can also be separated in testing by using specific monoclonal antibodies. Ptyalin is the amylase found in saliva that breaks starch down into maltose and dextrin.

2.2.1. POSITION OF α-AMYLASE IN THE CLASSIFICATION TREE



2.2.2. α-AMYLASE (EC 3.2.1.1)

Common name: α-amylase (Fischer and Stein, 1960)

Systematic name: 1,4-α-D-glucan glucanohydrolase

Other name(s): glycogenase; α-amylase, alpha-amylase; endoamylase; Taka-amylase A

CAS registry number: 9000-90-2

Genetics: In humans, all amylase isoforms link to chromosome 1q21.

Reaction: Endohydrolysis of 1,4-α-D-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units (Schwimmer and Balls, 1949; Manners, 1962)

Comments: Acts on starch, glycogen and related polysaccharides and oligosaccharides in a random manner; reducing groups are liberated in the α-configuration. The term 'α' relates to the initial anomeric configuration of the free sugar group released and not to the configuration of the linkage hydrolysed.

Extinction coefficient $E_{280}^{1\%}$**:** =26 (Caldwell et al., 1952).

Inhibitors: Urea and other amide reagents (Toralballa and Eitingon, 1967).

Activators: Cl- is essential (O'Donnell et al., 1975).

Stabilizers: Calcium and chloride ions are necessary for stability.

Stability: Crystalline suspensions in sodium-calcium chloride are stable for several months refrigerated. Solutions in buffered sodium chloride, pH 7.0, are stable for months providing the protein concentration exceeds 0.1%.

2.2.3. STRUCTURES

Alpha amylase is classified as family 13 of the glycosyl hydrolases. The structure is an 8 stranded alpha/beta barrel containing the active site, interrupted by a ~70 amino acid

calcium-binding domain protruding between beta strand 3 and alpha helix 3, and a carboxyl-terminal Greek key beta-barrel domain (Groot et al., 1988; Gumucio et al., 1988; Brayer et al., 1995; Numao et al., 2002; Rydberg et al., 2002).

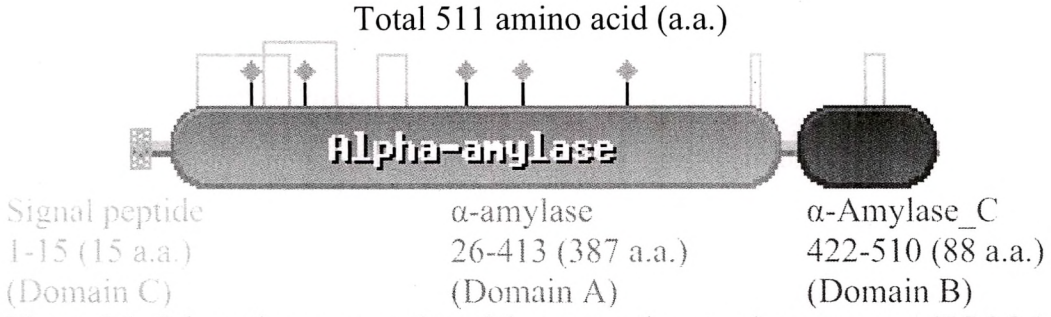


Figure 2.1: Schematic representation of the pancreatic α-amylase precursor (EC 3.2.1.1) (PA) (PDB code: AMYP_HUMAN; 1,4-α-D-glucan glucanohydrolase) structure (Qian et al., 1994b; Numao et al., 2002).

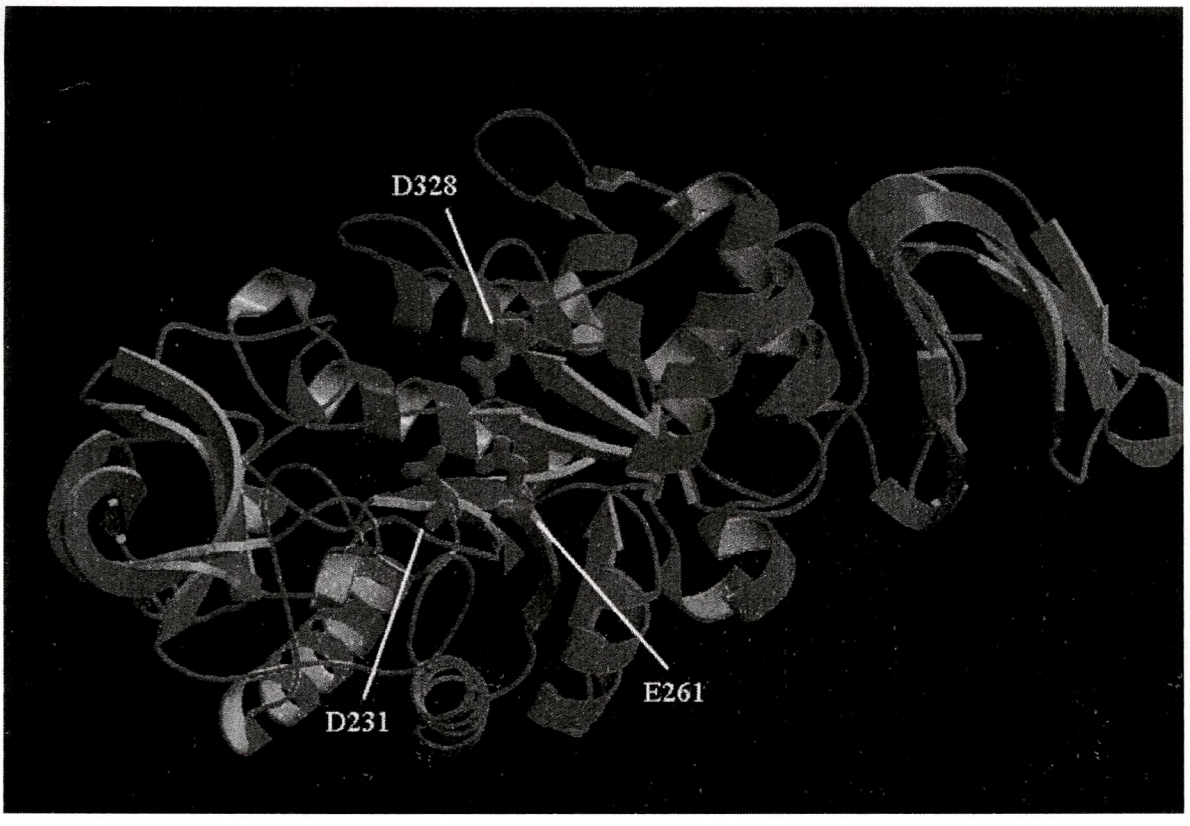


Figure 2.2: The BLA model of α-amylase. The three active site acids are shown in red. Green: domain A; magenta: domain B; cyan: domain C.

The α-amylases (EC 3.2.1.1) comprise a group of enzymes that hydrolyze the internal α-1–4 bonds in glucose polymers while retaining the configuration of the glucose anomeric carbon atom (Nielsen et al., 1999; Nielsen et al., 2001). They are enzymes with a wide range of substrate specificities, as well as both temperature and pH-activity optima. α-Amylases have several industrial applications. For example, they are used as additives in washing powders, or as catalysts in starch liquefaction, and they also improve the quality of dough (Guzman-Maldano and Predes-Lopez, 1995). X-ray crystallographic studies have shown that α-amylases have three domains. A central $(\alpha/\beta)_8$ TIM-barrel, called domain A, forms the core of the molecule, and contains the three active site residues (Figure 2.2) Asp231, Glu261 and Asp328 (BLA numbering). It has been suggested that Glu261 is the hydrogen donor in the reaction mechanism (Qian et al., 1994a; Knegtel et al., 1995), and it is well established that Asp231 is the nucleophile (McCarter and Withers, 1996). Domains B and C are located roughly at opposite sides of this TIM-barrel. Domain C constitutes the C-terminal part of the sequence and contains a Greek key motif. Domain B is a protrusion between the third strand and the third helix of the TIM-barrel (MacGregor, 1993) and forms an irregular β-like structure. The size and structure of domain B varies substantially among the various members of the α-amylase family. This domain is probably responsible for the differences in substrate specificity and stability among the α-amylases (Svensson, 1994). All α-amylase X-ray structures contain at least one conserved calcium ion, which is located at the interface between the A and B domains (Boel et al., 1990; Machius et al., 1995). One or more additional calcium ions have been found in several structures. It has been suggested that the role of the calcium ions is mainly structural (Larson et al., 1994; Gilles et al., 1996). Several α-amylases contain a chloride ion in the active site, which supposedly enhances the catalytic efficiency of the enzyme by elevating the pK_a of the hydrogen-donating residue in the

active site (see below (Larson et al., 1994; Ramasubbu et al., 1996)). Chloride ions have been found mainly in mammalian α-amylases, although recently a chloride ion has also been reported in a psycrophilic α-amylase from the *Alteromonas haloplanctis* bacterium (Aghajari et al., 1998; Machius et al., 1998). The α-amylase active site has been studied extensively, e.g. (Takase et al., 1992; Søgaard et al., 1993; Takase, 1993; Svensson, 1994; Declerck et al., 1995). Mutational studies show that all three acidic residues in the active site are crucial for activity, but the details of their roles in the catalytic mechanism are not yet entirely clear.

2.2.4. CATALYTIC MECHANISM

The proposed catalytic mechanism for retaining glycosyl hydrolases (Davies and Henrissat, 1995) consists of three steps (Figure 2.3). Step one is the protonation of the glycosidic oxygen by the proton donor (Glu261). This is followed by a nucleophilic attack on the C1 of the sugar residue in subsite-1 by Asp231 (nomenclature as described by Davies *et al.* (Davies et al., 1997)). After the aglycon part of the substrate leaves, a water molecule is activated, presumably by the now deprotonated Glu261. This water molecule hydrolyses the covalent bond between the nucleophile oxygen and the C1 of the sugar residue in subsite-1, thus completing the catalytic cycle. Asp328 plays no direct role in this catalytic mechanism, but is nevertheless known to be important for catalysis (Svensson, 1994). Asp328 is presumed to elevate the pK_a of Glu261 (Klein et al., 1992; Strokopytov et al., 1995). The residues Arg229, His327 and Asp100 are present within 5-Å of a titratable atom of the three acidic residues in the active site. This makes the active site a highly complex system of charged groups in close contact with each other. α-Amylase catalysis is thought to be limited by the deprotonation of the nucleophile at low

pH and by the protonation of the hydrogen donor at high pH (Qian et al., 1994a; Strokopytov et al., 1995). The rate limiting step at intermediate pH values is not known. This step has to be largely pH-independent since BLA has a pH–activity profile with a `flat top' at both 30°C and 70°C. The speed of release of the product is expected to be pH-independent, and we therefore speculate that the ratelimiting step is the product release at intermediate pH values.

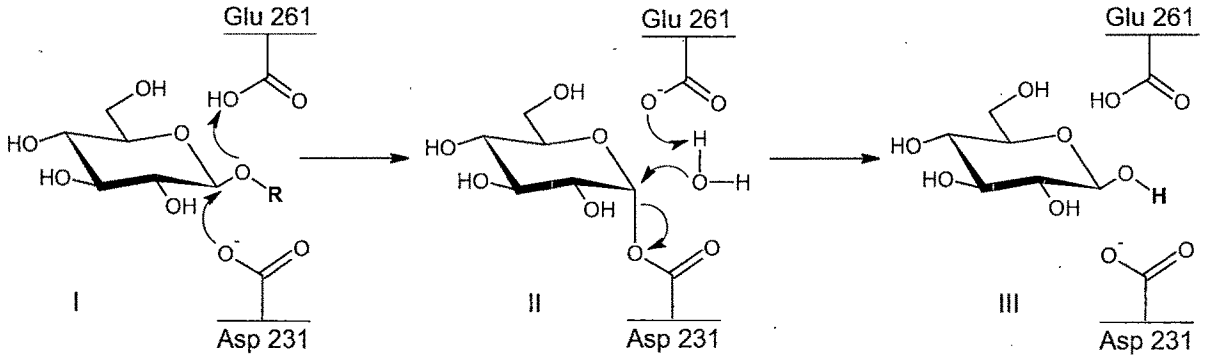


Figure 2.3: α-Amylase catalytic mechanism. I: Protonation of the glycosidic oxygen and attack on the glucose C1 by Asp 231. Departure of the reducing end of the substrate. II: Activation of a water molecule, cleavage of C1–Asp 231 covalent bond. III: Regeneration of initial protonation states.

2.2.5. CHARACTERIZATION

2.2.5.1. Fourier Transform Infra-Red Spectroscopy (FTIR)

IR transmission spectrum of α-amylase was obtained using a FTIR spectrophotometer (FTIR-8300, Shimadzu, Japan) and is shown in Figure 2.4. α-Amylase also showed the following characteristic peaks: one predominant band at 3190-3380 cm^{-1} (strong; s) due to N-H stretch of secondary N-substituted amides; 2980 cm^{-1} (weak; w) due to C-H stretch, medium bands at 1500-1600 cm^{-1} due to C---C, while 898 cm^{-1} (s) and 875 cm^{-1} (s) due to

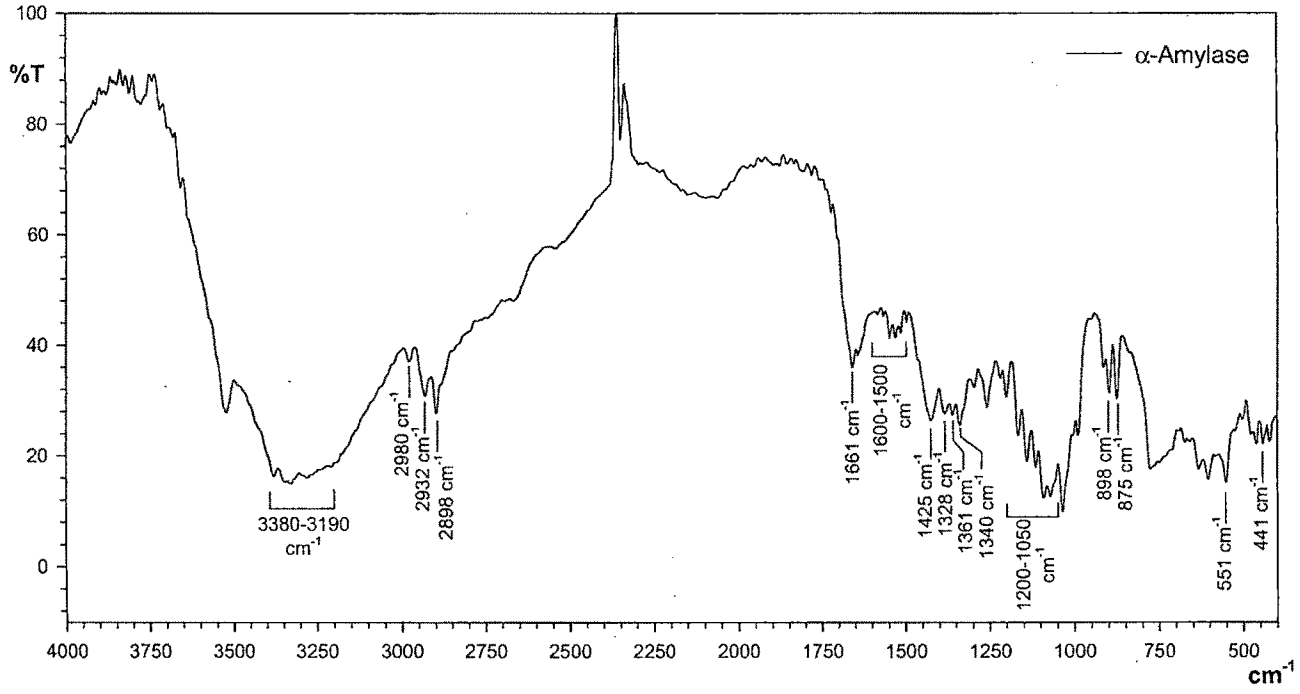


Figure 2.4: FTIR spectrum of α-amylase.

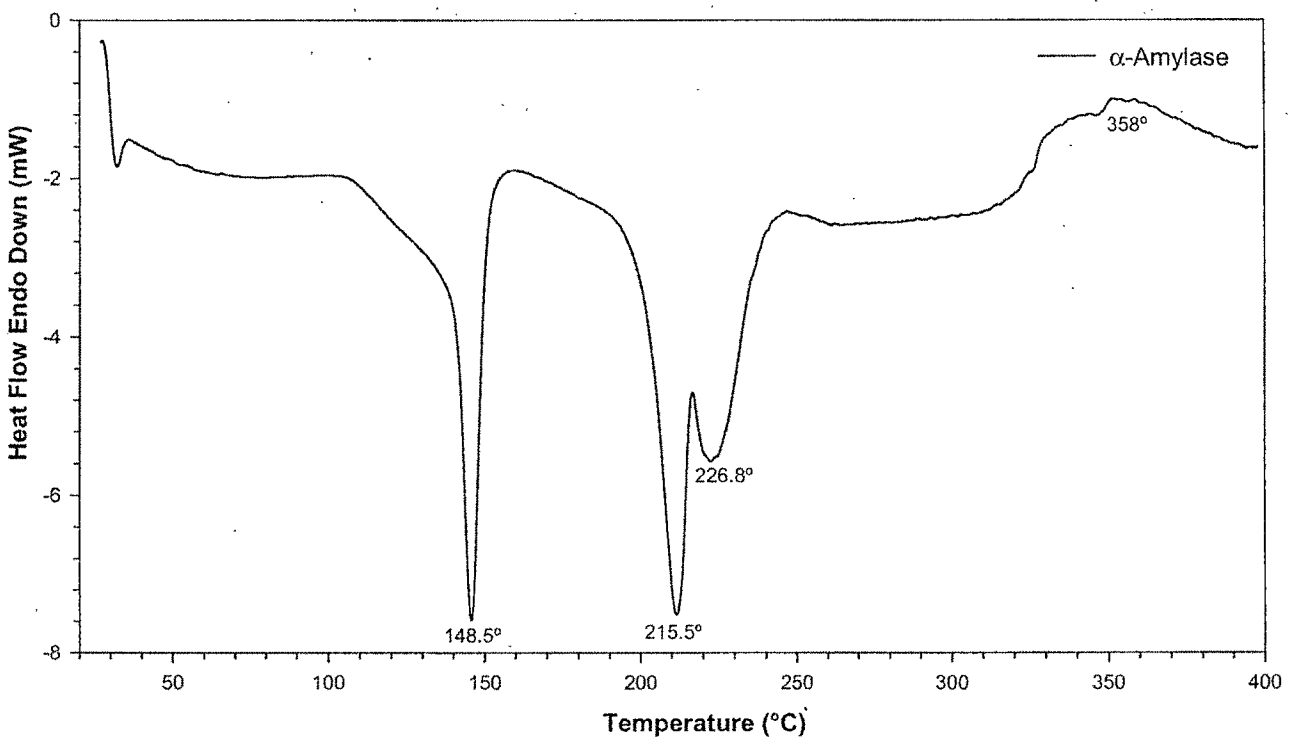


Figure 2.5: DSC thermogram of α-amylase.

p-substituted aromatic out of plane C-H deformation of aromatic residue of tryptophan or tyrosine; 2931 cm^{-1} (s) and 2898 cm^{-1} (s) due to C-H stretch, while 1425 cm^{-1} (s), 1382 cm^{-1} (medium; m), 1361 cm^{-1} (m), and 1340 cm^{-1} (m) due to C-H deformation of alkyl chain of amino acids; 1661 cm^{-1} due to C=O stretch of carboxyl anion and amide group; strong peaks between 1050-1200 cm^{-1}, weak bands at 550-600 cm^{-1}, and 400-450 cm^{-1} due to C-S stretch of sulfides and disulfides.

2.2.5.2. Differential Scanning Calorimetry (DSC)

DSC thermogram of α-amylase was obtained using an automatic thermal analyzer system (DSC-60, Shimadzu, Japan) and is shown in Figure 2.5. α-Amylase exhibited three endothermic peaks at 148.5°, 215.5°, and 226.8°C followed by a broad degradation exotherm at 358°C.

2.2.6. ESTIMATION OF α-AMYLASE

Method: That of (Bernfeld, 1951) wherein the reducing groups released from starch are measured by the reduction of 3,5-dinitrosalicylic acid. One unit releases from soluble starch one micromole of reducing groups (calculated as maltose) per minute at 25°C and pH 6.9 under the specified conditions.

Saccharogenic: This methodology measures the rate of appearance of reducing sugars (glucose, maltose). This methodology is invalid in the dog as dog serum contains maltase. Maltase is additive to the activity of amylase and will produce increased numbers of reducing sugars.

Amyloclastic: This method measures the hydrolysis of starch and the rate of its disappearance. Lipemic samples may show an inhibition of enzyme activity which can be overcome by dilution.

Turbidometric: Size of the starch substrate decreases with hydrolysis which reduces light scatter.

Chromogenic substrates: These use dyes bound to synthetic starch substrates, with the dye being released (and measured) once the substrate is hydrolyzed. Chromogenic substrate techniques are the current clinical amylase assay.

Amylase Production and Starch-Iodine Assay: The microorganisms were plated on the appropriate solid medium containing a 1% soluble starch to detect amylase production using (BC) as a control. After allowing them to grow to reasonable size colonies, the plates were then flooded with a 1:4 dilution of Lugal's iodine or Gram's iodine solution. Iodine forms a dark blue complex with starch. Amylase production is detected by the absence of blue color around the colonies indicating that starch has been hydrolyzed.

2-Cholor-4-Nitrophenyl Maltotrioside Reagent Assay: 2-chloro-4-nitrophenyl maltotrioside (CNP3) was used as a substrate in this assay. A yellow color is produced as the amylase breaks down the colorless CNP3 substrate. The assay was begun by adding 100 mL of the supernatant to 2.5 mL of the reagent and measuring the O.D. at 405 nm in the spectrophotometer with time.

Amylopectin Azure Reagent Assay: This assay consisted of the suspension of an insoluble blue starch, which amylase could degrade to release blue color into the solution. The assay consisted of 0.8 mL of the reagent substrate, along with 0.1 mL of supernatant or sterile medium, and 0.1 mL of 5X PBS. The O.D. at 595 nm was determined in the supernatant after pelleting the insoluble blue starch using a centrifuge. The amylase activity was recorded over time.

Reference Amylase Unit (RAU) Analytical Method: Bacterial amylase hydrolyzes starch into dextrins and maltose. Iodine is added to the hydrolysate at increasing times of incubation and the transmission of the color formed is measured spectrophotometrically at 620 nm. The time needed until the transmission is equal to that of the color reference, is a measure for the enzyme activity (2003).

Sandstedt, Kneen, and Blish (S.K.B): (Sandstedt et al., 1939) This procedure is used to accurately determine the α-amylase activity of enzyme preparations derived from *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus oryzae* and similar fungal derived enzymes. The assay is based on the time required to obtain a standard degree of hydrolysis. The degree of hydrolysis is measured by comparing the iodine color of the hydrolysate with the reference color standard.

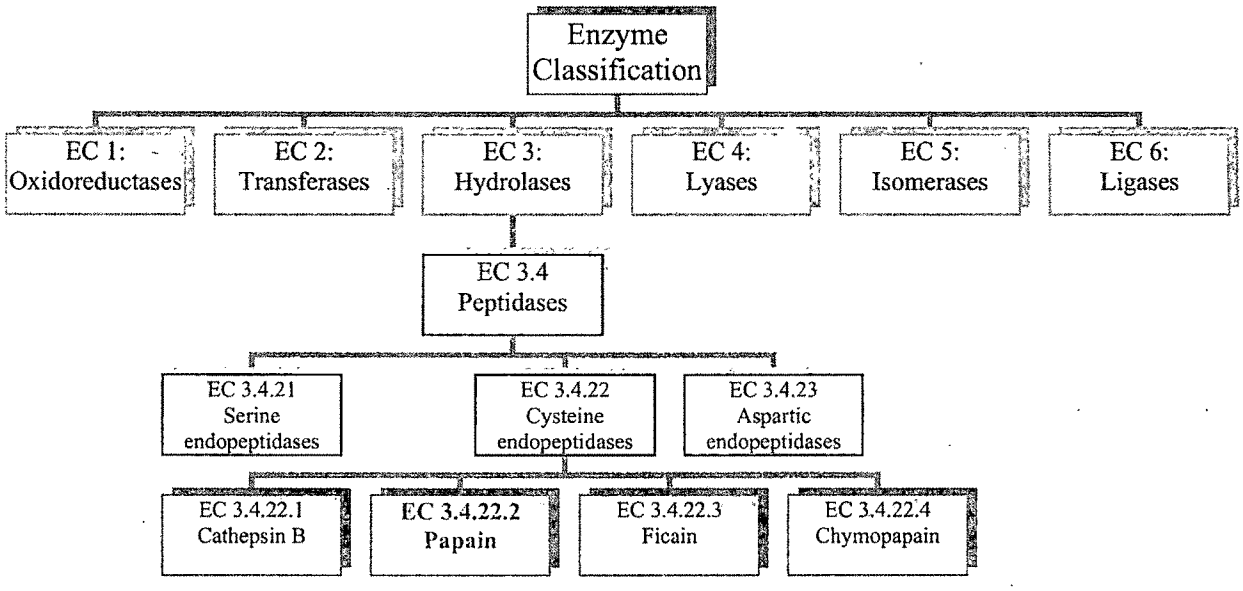
Starch-Gel Substrate: (Satoyama et al., 1998) The principles of the present method was based on the light absorbance change through a starch-agar gel made in a microplate well by α-amylase action. α-amylase activity in an extract from rice koji and Rhizopus culture liquid were successfully determined and glucoamylase was not affected to determine α - amylase activity as a result.

2.3. Papain (EC 3.4.22.2)

Cysteine proteases comprise a wide class of proteolytic enzymes in plants and animals that serve important functions such as the degradation of muscular protein, the processing of propeptides, prohormones, and zymogens, and the processing of foreign antigens for immunological responses (Serveau et al., 1994; Hilaire et al., 1999). Defects in the regulation of cysteine protease activity have also been reported in connection with several diseased states including osteoporosis, cancer metastasis, muscular dystrophy, viral replication, and parasitic infection (Harrison et al., 1997). Cysteine proteases are divided into about 20 families; the best known family being that of papain. The papain family contains peptidases with a wide range of activities including endopeptidases with broad or narrow specificities, amino peptidases, dipeptidylpeptidases, and peptidases containing both endo- and exo-peptidase activity. The papain superfamily of cysteine proteases includes a variety of enzymes with closely related amino acid sequences and overall folding structures (Schirmeister and Otto, 1997). Among them are vacuolar plant enzymes (e.g., papain) (Drenth et al., 1971), protozoen enzymes (e.g., cruzipain, falcipain) (Rosenthal et al., 1988), and mammalian lysosomal cathepsins (e.g., cathepsins B, L, H) and cytoplasmatic calpains (Rawlings and Barrett, 1994) (Murachi, 1983; Schirmeister, 1999). The subsite of papain, hence most cysteine proteases, is seven amino acids long and that there is a preference for an aromatic residue in the S_2 subsite (Schechter and Berger, 1967; Hilaire et al., 1999) (Thompson et al., 1986). Thiol groups of papain are readily oxidized in air to disulfides. To maintain these groups in the reduced state, another thiol such as cysteine, glutathione, mercaptoethanol, 2,3-dimercaptopropanol, or thioglycolate is often added (Cleland, 1964). The proteolytic

enzyme papain is a constituent of the latex from the green fruit of *Carica papaya*. The dried powdered whole latex is commonly referred to as papain. Pure papain is apparently as strong a proteinase as any of the well-known enzymes (Balls et al., 1940).

2.3.1. POSITION OF PAPAIN IN THE CLASSIFICATION TREE



2.3.2. PAPAIN (EC 3.4.22.2)

Recommended name: papain.

Other Name: papayotin, summetrin, velardon, papaine, Papaya peptidase I.

CAS registry number: 9001-73-4.

Class: Hydrolases, acting on peptide bonds (peptidases), Cysteine endopeptidases.

Reaction: Hydrolysis of proteins with broad specificity for peptide bonds, but preference for an amino acid bearing a large hydrophobic side chain at the P_2 position. Does not accept Val in P1'.

Comment: Type example of peptidase family C1 from latex of the papaya (*Carica papaya*) fruit.

Inhibitors: Inhibited by compound E-64 and proteins of the cystatin family.

Genes: PFA: PFI0135c

2.3.3. STRUCTURES

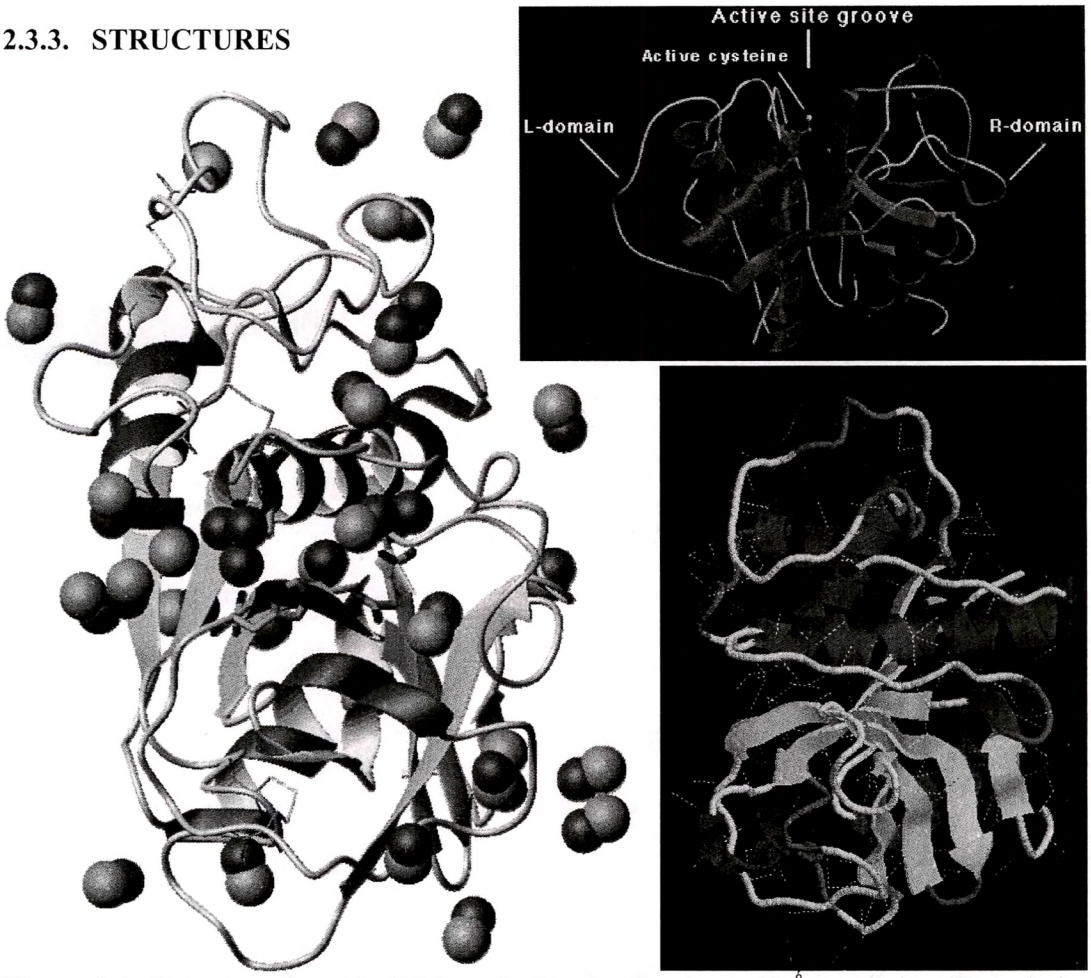


Figure 2.6: Structure of papain (PDB code: 9pap) refined at 1.65 Å resolution (Kamphuis et al., 1984; Kamphuis et al., 1985).

Eukaryotic serine and cysteine proteases are structurally distinct families of proteolytic enzymes that carry out analogous reactions(Higaki et al., 1989). Although the overall three-dimensional structures of the two classes of enzymes are very different, features of the serine protease catalytic triad (Ser195, His57, and Asp102) can also be observed in the cysteine proteases. The cysteine protease papain features an essential nucleophilic

thiol group of Cys25 and the imidazole of His159 whose Nδ1 nitrogen is within hydrogen-bonding distance of the thiol group. In addition, since the Nε2 nitrogen of His159 in papain can hydrogen bond to the side chain of Asn175 (Kamphuis et al., 1985; Baker and Drenth, 1987), Asn175 has been implicated as an essential active site residue in the Cys proteases (Brocklehurst, 1987). The three residues, Cys25, His159, and Asn175, constitute the putative catalytic triad of papain (Garavito et al., 1977).

2.3.4. CHARACTERIZATION

2.3.4.1. Fourier Transform Infra-Red Spectroscopy (FTIR)

FTIR spectrum of apain (Figure 2.7) showed various distinct peaks: one predominant band at 3225-3425 cm^{-1} (s) due to N-H stretch of secondary N-substituted amides; 2983 cm^{-1} (weak; w) due to C-H stretch, medium bands at 1500-1600 cm^{-1} due to C=C, while 868 cm^{-1} (s) and 850 cm^{-1} (s) due to *p*-substituted aromatic out of plane C-H deformation of aromatic residue of tryptophan or tyrosine; 2912 cm^{-1} (s) and 2941 cm^{-1} (s) due to C-H stretch, while 1429 cm^{-1} (m) and 1321 cm^{-1} (m) due to C-H deformation of alkyl chain of amino acids; 1661 cm^{-1} (w) due to C=O stretch of carboxylate anion and amide group; strong peaks between 1050-1150 cm^{-1}, weak bands at 525-600 cm^{-1}, and 472 cm^{-1} (s) due to C-S stretch of sulfides and disulfides.

2.3.4.2. Differential Scanning Calorimetry (DSC)

DSC thermogram of α-amylase was obtained using an automatic thermal analyzer system (DSC-60, Shimadzu, Japan) and is shown in Figure 2.8. papain exhibited two

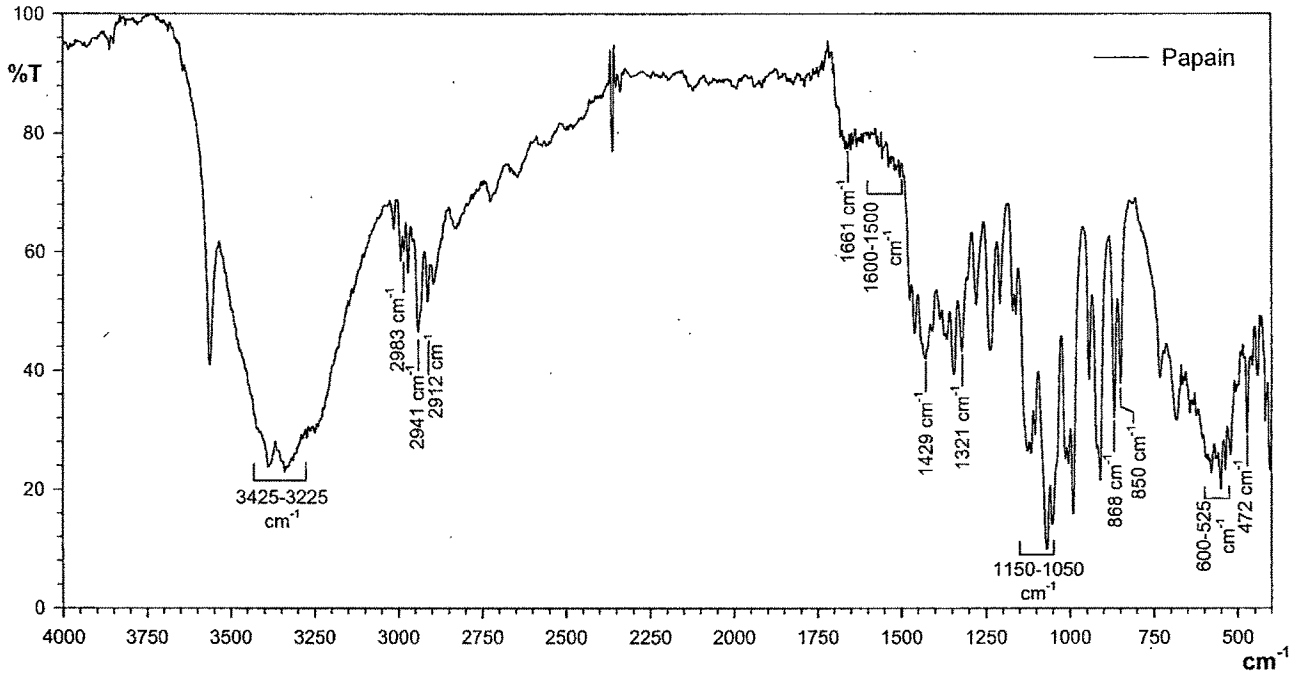


Figure 2.7: FTIR spectrum of papain.

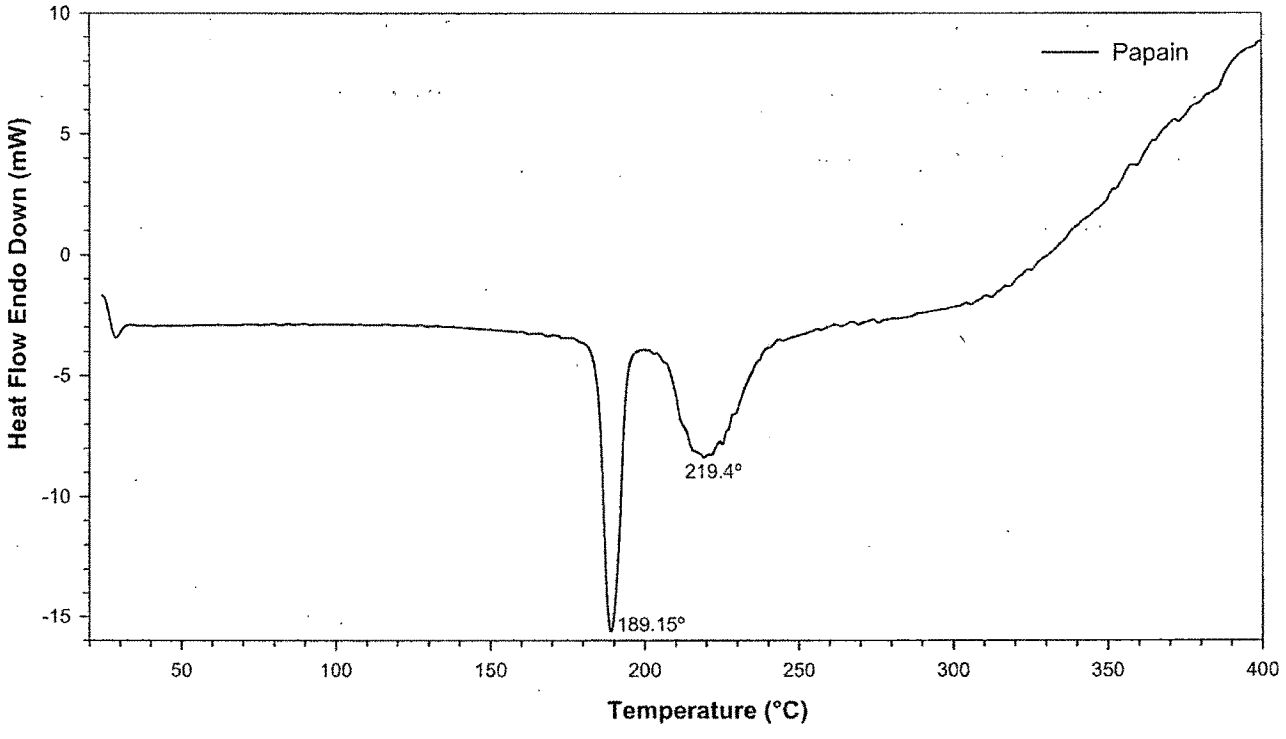


Figure 2.8: DSC thermogram of papain.

endothermic peaks at 189.15° and 219.4°C followed by a broad degradation exotherm after 350°C.

2.3.5. ESTIMATION OF PAPAIN

Nitrophenyl norleucinate digestion method: Williams and Lucas (1970) (Williams and Lucas, 1970) have synthesized the substrate, *p*-nitrophenyl-α-N-CB_z-D-norleucinate, a new active site titrator for papain, for a rigorous titration and estimate active site normalities accurately to less than 0.2 μM concentration. A low maximal deacylation rate constant allows titration of the papain over a pH range including the pH optimum for papain.

Casein or azocasein digestion method: Weerasinghe et al. (Weerasinghe et al., 1996) and An et al. (An et al., 1994) had analyzed the papain activity using casein or azocasein. A reaction mixture containing casein or azocasein, relevant buffer (0.1 M phosphate, pH 6.0, or 0.05 M Tris- HCl, pH 8.2), and water was preincubated at 37 °C for 5 min. The enzyme was then added, and its activity was measured at 37 °C for 5 min. The reaction was terminated by adding 0.2 mL of cold (4 °C) 50% (w/v) trichloroacetic acid (TCA). After allowing unhydrolyzed proteins to precipitate at 4 °C for 5 min, samples were centrifuged at 6000*g* for 5 min. For azocasein, TCA-soluble protein recovered from the supernatant was mixed with 10 N NaOH (800:60 v/v), and the absorbance of the azo compound at 428 nm was determined. For casein, TCA-soluble proteins were recovered in the supernatant, and its equivalent tyrosine content was determined by the Lowry assay (Lowry et al., 1951). Enzyme activity was expressed either as ΔA_{750} or U/mL. One unit of activity was defined as releasing 1 nmol of tyrosine/min.

Milk clotting methods: Balls and Hoover have detected papain by the milk clotting test (Balls and Hoover, 1937; Balls et al., 1940). The results of the milk test are expressed as milk clotting units. The unit was taken as the amount of enzyme required to clot 10 ml of a standard milk preparation from dried milk in 1 minute at 40°C. Units per milligram of a dry preparation are therefore equal to $1/(E \times t)$, where E represents the weight of the enzyme in milligrams, and t the clotting time in minutes.

Rate of casein digestion: The rate of digestion of casein at pH 5 -have been used for the assay of papain preparations (Balls and Lineweaver, 1939; Balls et al., 1940). The digestion of casein at pH 5 was determined by titration in alcohol. The enzyme was incubated for 20 minutes at 40°C. The increase over the zero time titration represents the proteolytic activity of the enzyme used. In order to make the results easily comparable, the titration difference (in ml of 0.1 N alkali) has been divided by the weight in milligrams of the enzyme sample that produced it.

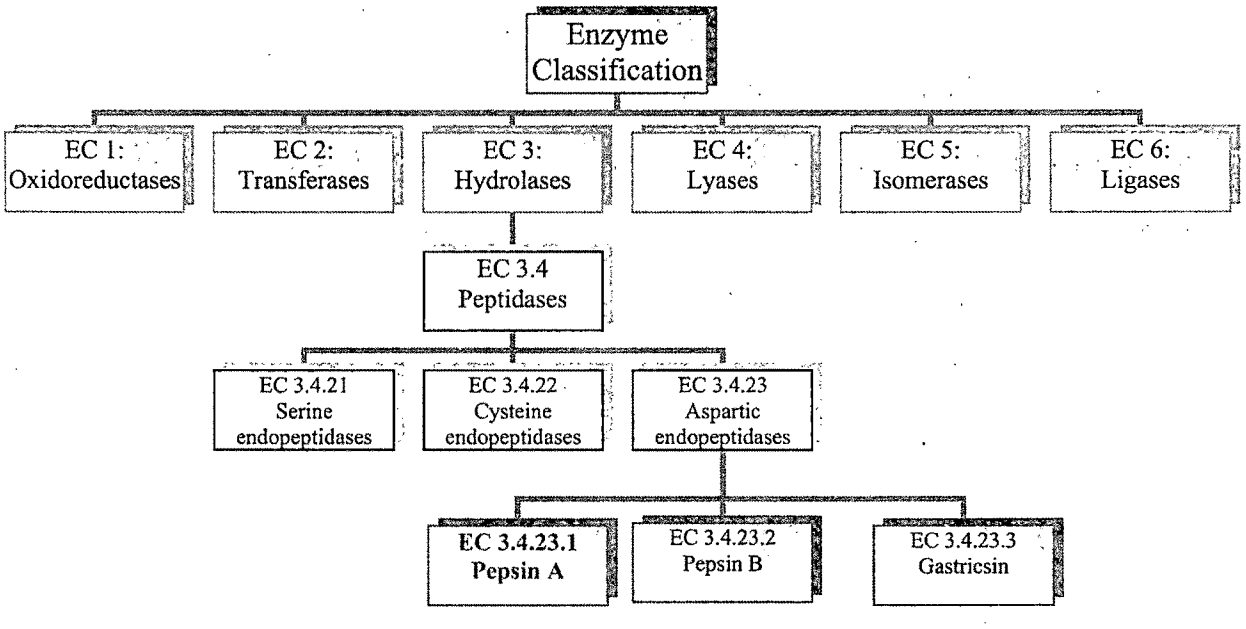
Egg albumin method: Rippetoe, J. R. (Rippetoe, 1912) had determined the digestive value of papain using coagulated egg albumin (prepared by boiling fresh hen-eggs in water for 15 minutes, cooling rapidly to room temperature by immersion in cold water, separating the whites and rubbing through a no. 44 sieve). Papain in acidified water was added to coagulated egg albumin in acidified water and digested for 4 hr at 51±1°C. The volumer of undissolved albumin was centrifuged and measured in volume.

2.4. Pepsin (EC 3.4.23.1)

Proteolytic enzymes are classified on the basis of their catalytic mechanisms. All proteases belong to one of four mechanistic classes and are either serine, cysteine, metallo or aspartyl proteases (Barrett, 1980). Enzymes belonging to the aspartic proteinase class (EC 3.4.23) deserve special note because of their involvement in physiologically important events including intracellular and extracellular protein catabolism (Asakura et al., 1997). Aspartyl proteases are found in a wide range of species, from vertebrates to lower eukaryotes and retroviruses (Hsu et al., 1977; Barrett and McDonald, 1980; Foltmann, 1981; Bedi et al., 1983; Kay, 1985; Katoh et al., 1987; Tang and Wong, 1987). Pepsin (EC 3.4.23.1) is a monomeric enzyme belonging to the aspartic proteinase family that is synthesized as a zymogen in the epithelial cells lining the stomach (Sielecki et al., 1990). They are all characterized by the presence of two aspartic acid residues at the active site and are inhibited by pepstatin, a pentapeptide naturally synthesized by strains of streptomyces (Umezawa et al., 1970). The best known source of aspartyl proteases is the stomach, which produces chymosin, gastricin, and pepsin. Animal proteases have been widely used in cheese manufacture. Bovine and porcine pepsins consist of a 326 amino acid residue chain with a molecular mass of 36 kDa, and the overall homology between them is more than 80%. (Tang et al., 1973; Foltmann and Pedreson, 1977) Nevertheless, they differ in their optimal pH, catalytic and proteolytic activities, and some substrate specificities (Fox, 1969; Fruton, 1976; Andren et al., 1983). Upon secretion into the acidic environment of the lumen, the zymogen (pepsinogen) undergoes a series of conformational changes accompanied by self-cleavage of the prosegment, resulting in the active, mature form of the enzyme (James and Sielecki, 1986; Bryksa et al., 2003).

Pepsinogen and its mature form, pepsin, differ in primary sequence by 44 amino acid residues at the N-terminus of the zymogen that are removed upon activation. The pepsin portion of the zymogen contains 327 amino acids (Sielecki et al., 1991), with a molecular mass of 36 kDa (Tang et al., 1973; Foltmann and Pedreson, 1977) and like all mammalian aspartic proteinases, it is a bi-lobal monomer, containing an approximate 2-fold axis of symmetry between two β-sheet domains (Rahuel et al., 1991). The prosegment covers the active site thereby restricting substrate access and rendering the zymogen inactive. Unlike its zymogen precursor, conformational changes of pepsin at neutral pH causes irreversible decreases in catalytic activity (Bohak, 1969; Lin et al., 1992).

2.4.1. POSITION OF PEPSIN IN THE CLASSIFICATION TREE



2.4.2. PEPSIN (EC 3.4.23.1)

Recommended Name: Pepsin A.

Other names: pepsin; lactated pepsin; pepsin fortior; fundus-pepsin; elixir lactate of pepsin; P I; lactated pepsin elixir; P II; pepsin R; pepsin D.

CAS registry number: 9001-75-6.

Reaction: Preferential cleavage: hydrophobic, preferably aromatic, residues in P1 and P1' positions. Cleaves Phe^{1}+Val, Gln^{4}+His, Glu^{13}+Ala, Ala^{14}+Leu, Leu^{15}+Tyr, Tyr^{16}+Leu, Gly^{23}+Phe, Phe^{24}+Phe and Phe^{25}+Tyr bonds in the B chain of insulin.

Comments: The predominant endopeptidase in the gastric juice of vertebrates, formed from pepsinogen A by limited proteolysis. Human pepsin A occurs in five molecular forms. Pig pepsin D is unphosphorylated pepsin A. Type example of peptidase family A1. Formerly EC 3.4.4.1

2.4.3. STRUCTURES

In the zymogen, residues 1p- 10p of the 44-residue prosegment form the first strand of a six-stranded β-sheet. The following hydrogen-bond pairs between strands 5 and 6 of the six-stranded β-sheet were deduced from the crystal structure of pepsinogen: Val2p-Leu167, Val4p-Val165, and Leu6p-Ser163 (Sielecki et al., 1991). Also, Val7p and Lys9p have a β-sheet interaction with Phe15 and Glu13, respectively. The N-terminal region of the prosegment plays an equivalent structural role to the N-terminus of the mature enzyme comprising the first strand of a six-stranded interdomain β-sheet (Sielecki et al., 1991). The remainder of the prosegment, together with the first 13 residues of the pepsin portion of pepsinogen (residues 9p-13), takes the shape of a flattened disk that fits into the substrate-binding cleft (James and Sielecki, 1986). Arg13p forms a hydrogen bond ion pair with Asp11, and similarly Arg8p and Glu13 interact, serving to tie down the N-terminus of the zymogen. A stability study of the individually expressed lobes of pepsin revealed that the N-terminal lobe was selectively denatured at pH 8.0 (Lin et al., 1993). Five residues, Asp11, Asp159, Glu4, Glu13, and Asp118, were implicated as potentially

important to the denaturation process due to electrostatic repulsion within the structure upon deprotonation of side-chain carboxyl groups (Lin et al., 1993). Tanaka and Yada (Tanaka and Yada, 2001) showed that denaturation of the N-terminal portion of pepsin initiated inactivation at neutral pH and that stabilizing the N-terminus in its native position inhibited the inactivation process (Tanaka and Yada, 2001).

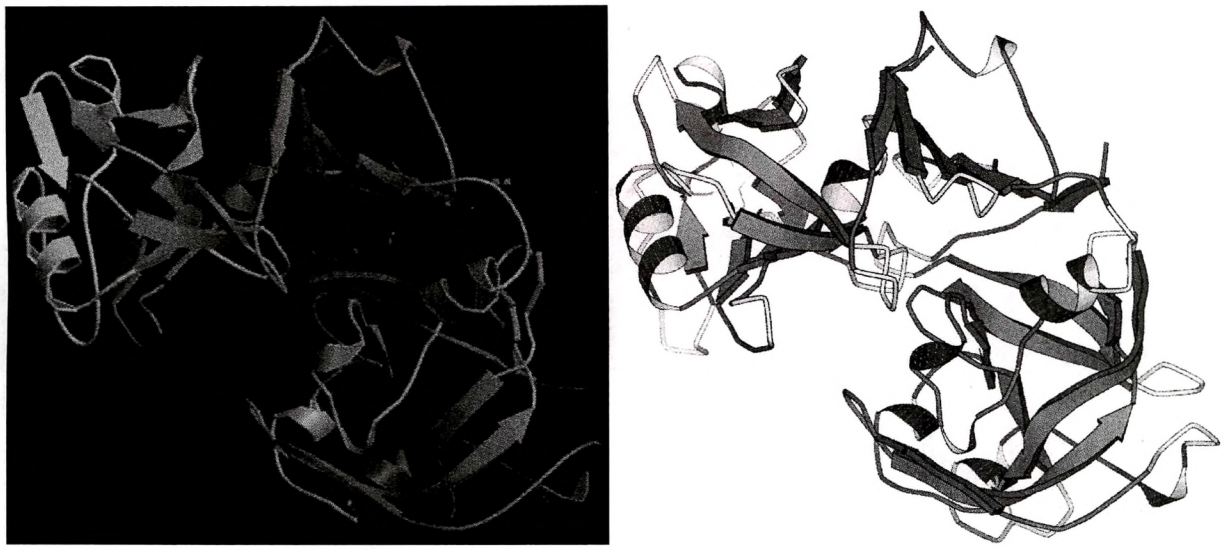


Figure 2.9: 3D structure of human (homo sapiens) pepsin 3a (PDB code: 1psn; 326 residues) refined at 2.20 Å resolution (Fujinaga et al., 1995).

2.4.4. CHARACTERIZATION

2.4.4.1. Fourier Transform Infra-Red Spectroscopy (FTIR)

IR transmission spectrum of pepsin was obtained using a FTIR spectrophotometer (FTIR-8300, Shimadzu, Japan) and is shown in Figure 2.10. Pepsin also showed the following characteristic peaks: one predominant band at 3220-3380 cm^{-1} (s) due to N-H stretch of secondary N-substituted amides; 2980 cm^{-1} (weak; w) due to C-H stretch, medium bands

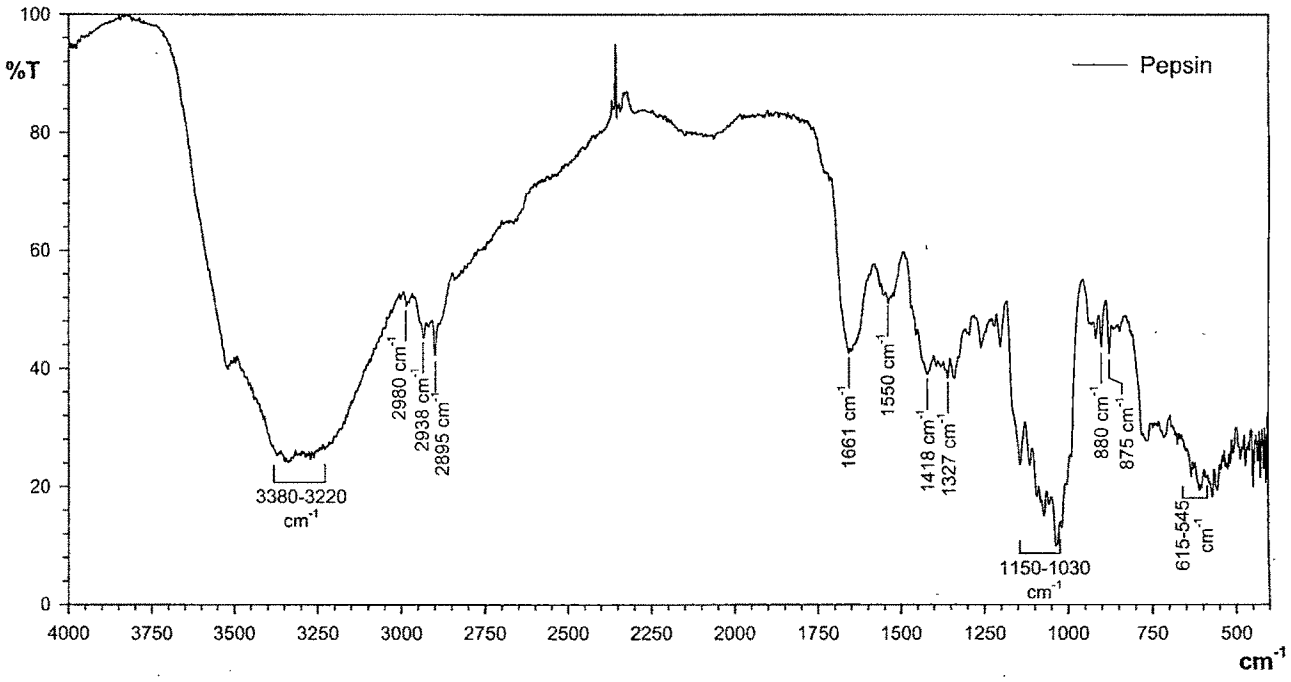


Figure 2.10: FTIR spectrum of pepsin.

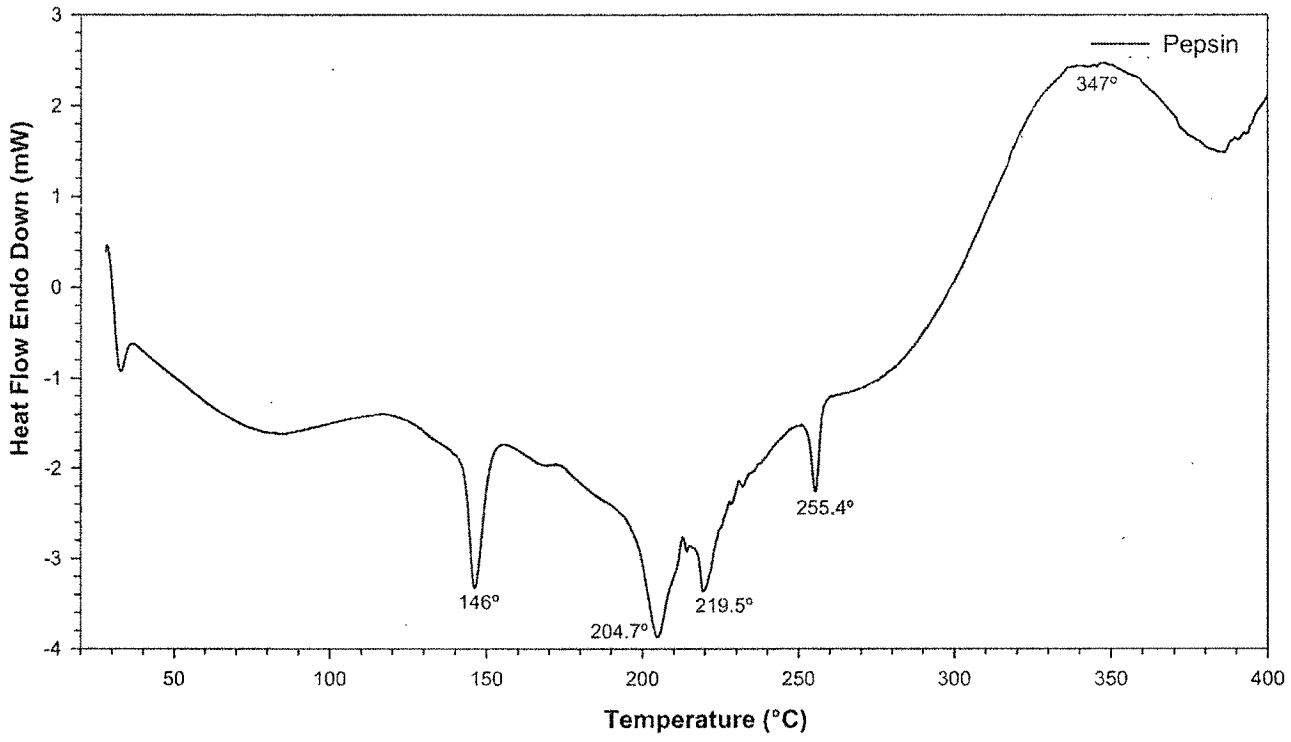


Figure 2.11: DSC thermogram of pepsin.

at 1550 cm^{-1} due to C⎓C, while 880 cm^{-1} (s) and 875 cm^{-1} (s) due to *p*-substituted aromatic out of plane C-H deformation of aromatic residue of tryptophan or tyrosine; 2895 cm^{-1} (s) and 2938 cm^{-1} (s) due to C-H stretch, while 1418 cm^{-1} (m), 1327 cm^{-1} (m), and 1337 cm^{-1} (m) due to C-H deformation of alkyl chain of amino acids (particularly of isopropyl group of leucine); 1662 cm^{-1} (w) due to C=O stretch of carboxylate anion and amide group; strong peaks between 1030-1150 cm^{-1}, weak bands at 545-615 cm^{-1}, and 400-450 cm^{-1} due to C-S stretch of sulfides and disulfides.

2.4.4.2. Differential Scanning Calorimetry (DSC)

DSC thermogram of pepsin was obtained using an automatic thermal analyzer system (DSC-60, Shimadzu, Japan) and is shown in Figure 2.11. Pepsin exhibited four endothermic peaks at 146°, 204.7°, 219.5°, and 255.4° followed by a broad degradation exotherm at 347°.

2.4.5. ESTIMATION OF PEPSIN

Heptapeptide digestion method: Gelb et al. (Gelb et al., 1985) had analyzed bovine pepsin in 40 mM formate, pH 4.0, by monitoring the decrease in absorbance at 300 nm accompanying the hydrolysis of a heptapeptide substrate (Lys-Pro-Ala-Glu-Phe(NO_2)-Arg-L; K_m=50 μM; k_{cat}= 100 s^{-1}).

Hemoglobin digestion method: A 10-50 μL portion of enzyme solution was added to 50 μL of 1% acid-denatured hemoglobin solution. The mixture was incubated at 37 °C, pH 3.3, for 60 min, and the reaction was terminated by the addition of 100 μL of 0.4 M

trichloro acetic acid (TCA). After centrifugation, the absorbance of the TCA-soluble fraction was measured at 280 nm. One unit of pepsin activity was the amount necessary to produce an increase in absorbance of 0.01 under the above conditions.

Milk-clotting assay: Enzyme solution containing about 0.3 mg pepsin was added to 0.2 ml of 3% (w/v) skim milk reaction mixture dissolved in 25 mM sodium phosphate buffer (pH 6.3), 20 mM $CaCl_2$. The reaction tubes were inclined and incubated at 37 °C for 120 min. The presence or absence of milk-clotting activity was judged by observing whether or not the incubation mixture showed sedimented coagulum formation.

κ-Casein digestion: Each 1.6 μg of pepsin was mixed with 8 µl of 100 mM glycine hydrochloride buffer (pH 2.0), 100 mM acetate buffer (pH 3.0) or 100 mM sodium phosphate buffer (pH 6.3) solution containing 80 μg of κ-casein. The mixtures were incubated at 37°C for 60 min and subjected to gel electrophoresis on 10% or 15% polyacrylamide gels in the presence of 0.1% SDS according to the method of Laemmli (Laemmli, 1970).

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