CHAPTER II

BACKGROUND AND LITERATURE REVIEW

Silks are generally defined as protein polymers that are spun into fibers by some lepidoptera larvae such as silkworms, spiders, scorpions, mites and flies. Each of these different silks has a different amino acid composition and exhibits different mechanical properties. The most extensively characterized silks are from the domesticated silkworm, *Bombyx mori*, and from spider (*Nephila clavipes* and *Araneus diadematus*). However, the silkworm's fibre is more commonly produced commercially. There are two main types of the silkworms: mulberry silk *Bombyx mori*, also called 'cultivated silk' (as shown in Figure 2.1), and 'wild silk' of which Tussah silk, produced by *Antheraea pernyi*, native to China and India, is the most important representative. Cultivated silk is different from Tussah silk in that the silkworm larvae are cultivated in provided habitats and fed with mulberry leaves whereas the Tussah silkworms is fed almost only on oak leaves. The cultivated silks also provide finer and softer filaments.

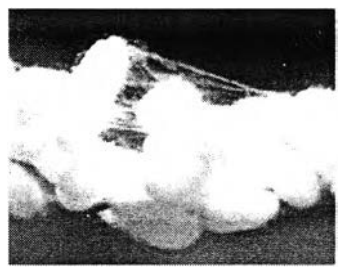

Figure 2.1 The *Bombyx mori* cocoons[1]

[1]Source: http://www.pclaunch.com /~kayton/Silkworms /lifecycle.htm.

2.1 Silk waste

In silk industry, tons of silk waste are produced annually which arise from damaged cocoons or from cocoons that are difficult to unreel, together with waste fibre from the spinning processes. Silk waste has the physical and chemical characteristic properties similar to silk cocoon, that is, it contains fibroin and sericin proteins. Recovery of these useful protein and amino acids products from silk waste offers a wide spectrum of application in various fields.

2.2 Structure of silk

2.2.1 Silk fibre

The principle components of silkworm *Bombyx mori* are fibroin protein (about 75-83% by weight), sericin protein (17-25%), cere or waxes (about 0.4-0.8%), and hydrocarbon (about 1.2-1.6%) [Lee et al., 2005]. A microscopic view of silk filament shown in Figure 2.2 demonstrates the structure of the silk fibre which consists of inner fibroin filaments that are surrounded by a cementing layer of sericin.

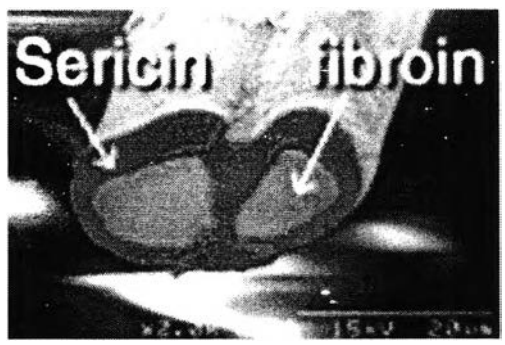

Figure 2.2 Structure of the raw silk fibre [1]

[1]Source: http://www.thejamushop.com /Beauty_silk.htm.

2.2.2 Silk proteins

Silk serincin and fibroin proteins which are the major components of silk take various forms which are described below:

Primary structure of silk

The primary structure of silk is a linear sequence of amino acids: Ser-Gly-(Ala-Gly)$_n$ (Figure 2.3). This sequence occurs in repetitive crystallizable blocks which are separated by the amorphous or less repetitive sequences of amino acids with bulkier side chains that do not crystallize.

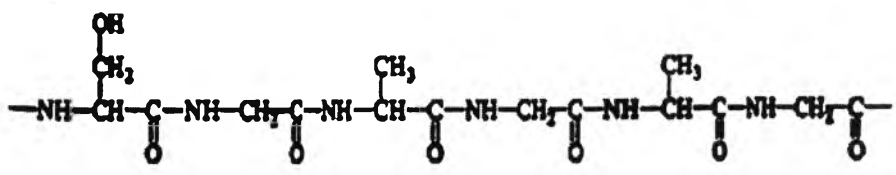

Figure 2.3 Structure of crystalline-encoding region of silk: Ser-Gly-(Ala-Gly)$_n$

Li et al. (2003) reported that the amorphous domain of silk fibroin derived from *Bombyx mori* has the following amino-acid sequence: Thr-Gly-Ser-Ser-Gly-Phe-Gly-Pro-Tyr-Val-Ala-Asp-Gly-Gly-Tyr-Ser-Arg-Arg-Glu-Gly-Tyr-Glu-Tyr-Ala-Trp-Ser-Ser-Lys-Ser-Asp-Phe-Glu-Thr.

Secondary structure of silk

In general, there are three secondary molecular conformations of silk protein:

1. Random coils are small regions of peptide backbone that can form small loops which, often contain glycine. The conformation of silk fibroin in aqueous solution is a good representative of this structure.

2. Silk I, or α-helix conformation, is the structure in which polypeptide chains arrange themselves into helical segments. This occurs when the carbonyl (C = O) of each amino acid is H-bonded to the amide (N-H) of the adjacent amino acid as shown as in Figure 2.4.

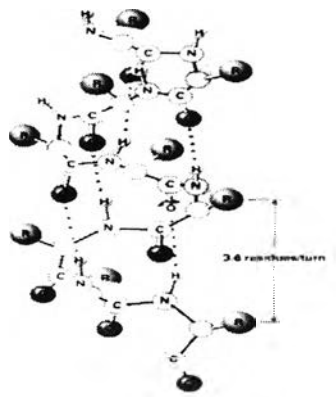

Figure 2.4 α-helix[1]

[1]Source:. Kmabiotech.Co.Ltd, 2005.

3. Silk II is the common β-sheet conformation found in the silk fibroin.

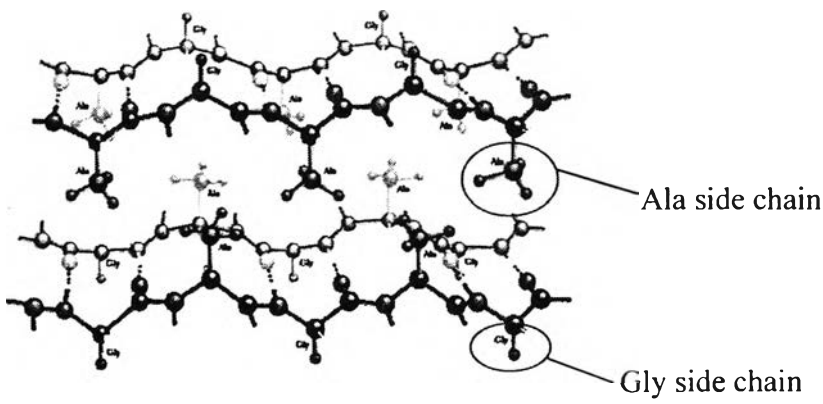

Figure 2.5 Silk β-sheet[1]

[1]Source:. Kmabiotech.Co.Ltd, 2005.

It is characterized by a β-pleated sheet secondary structure with extended polypeptide chains in which the carbonyl oxygens and amide hydrogens are at near right angles to the long axis of the chain. Hydrogen bonds form between the carbonyl oxygens and amide hydrogens of neighboring chains, forming a pleated structure along the backbone of the peptide chain. The chain-chain interactions in silks include extensive hydrogen bonding (intra- and inter-chain) as well as van der Waals interactions for stacked sheets due to the predominance of short side chain amino acids such as glycine, alanine, and serine as shown as in Figure 2.5. The alternating Ala side chains ($-CH_3$), extending perpendicularly from the top two sheets, nestle in the gaps left by the Gly side chains (-H). These small, non-polar side chains allow the sheets to pack closely together, stabilized by hydrophobic forces.

2.3 Sericin

As was shown earlier, silk sericin constitutes the sticky layers that envelops the fibroin fiber and ensures the cohesion of the cocoon by gluing silk threads. It accounts for approximately 25 % of the weight of the raw silk. It is a complex mixture of 5–6 polypeptides widely differing in molecular weights, ranging from about 10 to over 300 kDa. Sericin is made of 18 amino acids most of which have strongly polar side groups such as hydroxyl, carboxyl, and amino groups. Table 2.1 shows different proportions of amino acids in sericin. The major amino acids are serine, aspartic acid, and glycine, constitute approximately 31.9%, 13.8 %, and 12.7% of sericin, respectively.

Sericin takes on a "globular" shape, that is, it is a mixture of secondary structures with specific domains of α-helix, β-sheet, and random coil that form complex conformations rendering the important functions in catalysis and molecular recognition.

Table 2.1 Amino acid compositions of silk sericins (residues/1000 residues) [Robson, 1985.]

Amino acids	*B.mori* cocoon	Tussah *A. pernyi* cocoon
Non-polar Amino acids		
Glycine	127.0	149.9
Alanine	55.1	27.8
Valine	26.8	11.9
Leucine	7.2	9.9
Isoleucine	5.5	8.0
Proline	5.7	19.1
Phenylalanine	4.3	6.0
Tryptophan	-	-
Oxy amino acid		
Serine	319.7	226.3
Threonine	82.5	149.6
Tyrosine	34.0	49.2
Acidic amino acid		
Aspartic acid	138.4	122.5
Glutamic acid	58.0	67.4
Basic amino acid		
Lysine	32.6	14.7
Arginine	28.6	54.5
Histidine	13.0	25.0
Sulfur-complex Amino acid		
Methionine	0.5	1.3
(Cysteine)	1.4	1.8

When subjected to physical, chemical, or enzymological processes, sericin degrades into sericin peptides or hydrolysate. Some of the resulting sericin peptides

have lower molecular weight of less than 20 kDa (commonly less than 5 kDa). These sericin peptides are water soluble and have excellent moisture absorption and release as well as important biological activities such as antioxidation, tyrosinase activity inhibition [Kato et al., 1998], and pharmacological functions such as anticoagulation, anti-cancer activities, cryoprotection [Kazuhisa et al., 2001], and digestion promotion [Sasaki et al., 2000]. Sericin of higher molecular weight between 20 and 300 kDa is soluble in boiling water but poorly soluble in cold water. These sericin proteins can be applied in many fields such as degradable biomaterials, biomedical materials, functional biomembrane materials, and functional fibers, fabrics, and articles.

2.4 Fibroin

Unlike sericin, fibroin is the principal water insoluble "fibrous" protein (i.e. 78 % of the weight of raw silk) consisting of three polypeptides: (1) a 350 kDa heavy chain (High-molecular weight, "H-chain", (2) a 26 kDa light chain (Low-molecular weight "L-chain", and (3) 25 kDa silk glycoprotein or fibrohexamerin (fhx), originally named P25 [Inoue et al., 2000], containing asparagin-linked oligosaccharide chains. The elementary unit of silk fibroin consists of a 6:6:1 molar ratio of the H-chain:L-chain:fibrohexamerin, the molecules of the L-chain is linked to the H-chain with a disulfide bond by noncovalent interactions. The P25 plays an important role in maintaining integrity of the complex. The amino acid compositions of silk fibroin are shown in Table 2.2. The major ones are Gly, Ala, and Ser (3:2:1 molar ratio), which comprise to 44.6%, 29.4 %, and 12.1%, respectively, of the total amino acids in silk fibroin.

Fibroin chains are generally aligned along the axis of silk fiber, by a close network of interchain hydrogen bonds, with adjacent -(ala–gly)$_n$- sequences forming the well known β-sheet crystals [Takahashi et al., 1991]. It is the repetitive structure in fibrous proteins that imparts significant influence on the noncatalytic macroscopic properties of silk fiber such as mechanical support and molecular recognition.

Table 2.2 Amino acid compositions of silk fibroins (residues/1000 residues) [Robson, 1985.]

Amino acids	*B.mori* fibre	Tussah *A. pernyi* fibre
Non-polar Amino acids		
Glycine	446.0	265.0
Alanine	294.0	441.0
Valine	22.0	7.0
Leucine	5.3	8.0
Isoleucine	6.6	-
Phenylalanine	6.3	6.0
Proline	3.6	3.0
Tryptophan	1.1	11.0
Oxy amino acid		
Serine	121.0	118.0
Threonine	9.1	1.0
Tyrosine	51.7	49.0
Acidic amino acid		
Aspartic acid	13.0	47.0
Glutamic acid	10.2	8.0
Basic amino acid		
Lysine	3.2	1.0
Arginine	4.7	26.0
Histidine	1.4	8.0
Sulfur-complex Amino acid		
Methionine	1.0	-
(Cysteine)	2.0	-
	Gly > Ala	Gly < Ala

The comparison of mechanical properties of common silk were shown in Table 2.3. The average tensile strength of *B. mori* silk (without sericin) is approximately 610-700 MPa, and Yong's modulus is approximately 15-17 GPa. This demonstrates that silk fibre is very strong and elastic, making it useful in textile and biomaterials applications.

Table 2.3 Comparison of mechanical properties of common silk to several types of biomaterial fibers and tissues commonly used today [Altman et al., 2003].

Material	UTS (MPa)	Modulus (GPa)	% Strain at break
B. *mori* silk (w/ sericin)[a]	500	5–12	19
B. *mori* silk (w/o sericin)[b]	610–690	15–17	4–16
B. *mori* silk[c]	740	10	20
Spider silk[d]	875–972	11–13	17–18
Collagen[e]	0.9–7.4	0.0018–0.046	24–68
Collagen X-linked[f]	47–72	0.4–0.8	12–16
PLA[g]	28–50	1.2–3.0	2–6
Tendon (comprised of mainly collagen)	150	1.5	12
Bone	160	20	3
Kevlar (49 fiber)	3600	130	2.7
Synthetic Rubber	50	0.001	850

[a] *Bombyx mori* silkworm silk—determined from bave (multithread fibers naturally produced)

[b] *Bombyx mori* silkworm silk—determined from single brins (individual fibroin filaments following extraction of sericin).

[c] *Bombyx mori* silkworm silk—average calculated from data in Gulrajani, M.L,1996.

[d] *Nephila clavipes* silk produced naturally and through controlled silking.

[e] Rat-tail collagen Type I extruded fibers tested after stretching from 0% to 50%.

[f] Rat-tail collagen dehydrothermally cross-linked and tested after stretching from 0% to 50%.

[g] Polylactic acid with molecular weights ranging from 50,000 to 300,000.

2.5 Application of silk protein

2.5.1 Application of sericin

In the conventional processing of silk fiber in textile industry, sericin must be removed through the process called "degumming" process. Although sericin is traditionally considered waste of silk industry and is mostly discarded, it is becoming more and more useful in various applications such as those summarized in Table 2.4. The size of the sericin molecules ranges from 20 to over 300 kDa., depending on factors such as temperature, pH, and process time used during the degumming process.

Table 2.4 The rages of size of sericin peptide for application [Zhang, 2002]

Size of the sericin molecules	Application for uses
Lower molecular weight sericin peptides (≤ 20 kDa) (sericin hydrolysates)	Cosmetics including skincare Haircare products Health product Medications
High-molecular weight sericin peptides (≥ 20 kDa)	Medical biomaterials Degradable biomaterials Compound polymers Functional biomembranes Hydrogels Functional fibers and fabrics.

2.5.1.1 Application of sericin in cosmetics and medications

Because 80% of the sericin amino acids has hydrophilic lateral group, about 1/3 of which is serine which has water absorption of 50 times high than that of glycerin, it can readily permeate and be absorbed by the skin, providing moisture that very effectively nurture the skin. Sericin is also a natural antioxidant, thus can be used as an anti-aging agent in skin care products. When used in hair care products, it provides nice luster, and makes the hair spring. The anti-oxidant and anti-microbial activities of sericin have also been employed to restrain food spoilage especially in rich grease food, such as cream and milk products.

2.5.1.2 Application of sericin in biodegradable materials

The biodegradable polymers can be produced by blending sericin with other resins. The reacting of aqueous sericin solution or sericin powder and polyol solution (a mixture of polyol, a foaming agent, a foam-shaping agent, a catalyst, and a fire retardant), to generates heat and gas that produces polymer materials with foam characteristics, rigid urethane foam. The synthetic resin pumice including the numerous cells is completed by molding rigid urethane. For example, polyurethane foam can be produced reacting sericin solution with a comprising a polyol and polyisocyanate. The resulting foam have excellent moisture absorbing and desorbing properties with two- to five-fold greater rates than that produced without sericin, and has excellent mechanical and thermal properties.

2.5.1.3 Application of sericin in membrane materials

Sericin can be used to make membranes for separation processes. However, pure sericin is not easily made into membranes, but membranes of sericin cross-linked, blended, or copolymerized with other substances are made more readily. Because sericin contains a large amount of amino acids with neutral polar functional groups, sericin-containing membranes are quite hydrophilic.

To have been reported that the membrane made by mixing hydrochloric acid, a formaldehyde cross-linking agent, and aqueous sericin could effectively separate alcohol from the mixture of water and alcohol and could be reused. Yoshikawa et al.

(2001) suggested that 20 kDa sericin could be mixed with agar or agarose to form porous agar/agarose-sericin film that absorb water, and can be used as a good membrane for separate ether–alcohol mixtures, especially mixtures of MTBE and methanol.

2.5.1.4 Application of sericin for functional biomaterials

The sericin protein can be formed into a thin film attached to another matrix to enhance the functionality of coated surfaces. An example of this is the sericin-coated film used on the surfaces of refrigeration equipment to promote antifrosting action. Furthermore, sericin can be coated on surfaces of various materials to increase the durability, improve weatherability, provide good permeability, or provide materials that do not warp on drying.

A blended hydrogel made of sericin and polyvinyl alcohol (PVA) of 91 kDa molecular weight using dimethyl urea as the cross-linking agent [Nakamura and Koga, 2001] has good thermal and mechanical properties, excellent moisture adsorbing and desorbing properties, as well as elasticity and durability, making it useful as a functional film.

2.5.1.5 Application of sercin for medical biomaterials

Kazuhisa et al. (2001) have found that the sericin-rich repetitive sequence in silk sericin and natural sericin hydrolysate can protect both cells and proteins from freezing stresses. Their results indicated that sericin and sericin hydrolysates have important cryoprotective activity which would be valuable in numerous applications.

Furthermore, it was discovered that the films made of sericin has an excellent oxygen permeability and is similar to human cornea in its functional properties.

Minoura et al. (1995) investigated the attachment and growth of animal cells on films which were made of sericin and fibroin composite membrane. They found that the film membrane composed of around 90% sericin or pure component proteins (i.e., fibroin or sericin) permitted cell attachment and growth comparable to that on collagen, and can be used as a substitute for collagen.

Silk protein can be made into a biomaterial with anticoagulant properties by a sulfonation treatment of sericin and fibroin [Tamada, 2004]. Such anticoagulant is a

potential substitute for heparin, which can be used to treat surfaces of medical devices. The sulfonated silk protein anticoagulant has been claimed to interfere with the attachment of the human immunodeficiency virus to immunocytes.

2.5.1.6 Application of sercin for functional fibers, fabrics, and articles

The functional properties of some synthetic fibers can be improved by coating it with natural macromolecules such as chitin, chitosan, fibroin, and sericin. At present, the synthetic fibers such as polyamine fiber (6-nylon) and polyolefin fiber have been modified chemically with sericin. An example, polyester fiber can be modified with sericin by cross-linking with glyceryl polyglycidyl ether and diethylene triamine. Sarovat et al. (2003) studied the enhancement of air filter properties by coating the filter with 10-20% silk sericin from different species of waste cocoon and found that the coated surface of sericin on nylon fiber and PET fiber resulted in smooth fiber whose antibacterial efficiency was increased when concentration of sericin was increased.

Some useful animal and plant natural fibers have also been subjected to treatment with sericin. Zhang (2003) has also reported the sericin-coated on hydrophilic fibers (thermoplastic fiber, "rayon" and a cellulose fiber, "cotton") can prevent abrasive skin injuries and did not cause skin rash.

The sericin powder, whose particles are smaller than 20 μm in diameter can be blended with a compound rubber (e.g., butadiene or olefin rubber) and thermoplastics (e.g., vinyl acetate resin), and the mixture can be made into an artificial leather product. The blending of hydrolyzed sericin (5–50 kDa molecular weight, 0.01–10.0% w/w) in rubber can produce a product with reduced irritability to skin than native rubber. This modified rubber can be made into articles such as rubber gloves, bicycle handle grips, and handles for various sport equipment.

2.5.2 Application of silk fibroin

Fibroin is widely used in various fields of life science such as in separation processes, clinical diagnostic assays, cosmetics, biomaterials, natural polymers, matrix for enzyme immobilization, hair care production, health food production, and drug delivery system.

2.5.2.1 Application of fibroin for functional fibre and fibrics

Silk fibre is extremely strong. A filament of silk is stronger than an equal diameter filament of steel. Normally, the dry silk fibre has a tenacity varying from 2.4 to 5.1 grams per denier. The wet strength of the fibre is about 80-85 % of the dry strength [Lee et al., 2005].

Lee et al. (2005) investigated the mechanical and thermal properties of novel short silk fibre (*Bombix mori*) reinforced polybutylene succinate (PBS) biocomposites that have been fabricated with varying fibre contents using a compression molding method. The results showed that the mechanical properties were improved with increasing the short fibre content in the composites upto a fibre loading of 50 wt%. The thermal stability of silk/PBS biocomposites is likely to be intermediate between the PBS matrix and the silk fibre depending on the short fibre content.

2.5.2.2 Application of fibroin for membrane materials

Fibroin can be used to make membranes for separation processes. Transparent membranes of silk fibroin have been processed with liquid silk. The processing of blend solution of two different fibroins or one fibroin with cellulose, polyvinyl alcohol, polyurethane, cellulose acetate, chitosan, sodium polyglutamate, polyethyleneglycol, sodium alginate, and S-carboxymethyl keratin have also been used. Toshima et al. (2002) suggested that *Bombyx mori* yolk protein (225 kDa), of a spherical shape and size of 20 nm in diameter incorporated smoothly into a phospholipids bilayer membrane because of interaction between silk fibroin and membranes .

The insolubilized silk fibroin membrane could be used to preferentially remove water from a mixture of water and alcohol. Chen et al. (1999) investigated that a novel natural silk fibroin-chitosan membrane (about 30 μm in thickness) can be used for pervaporation of mixture of alcohol–water. They found that the silk membrane exhibited the best performance when silk content was 20 wt % and the glutaraldehyde crosslinking agent content was 0.5 mol %. Their result showed that the membrane was water selective and the separation factor of ethanol–water mixture

could be improved compared to pure chitosan membrane, when silk fibroin content in the membrane was no more than 40 wt %.

2.5.2.3 Application of fibroin for functional biomaterials

The protein-based materials such as silk protein polymer can be processed into excellent film sponge, fiber, coating. The silk protein polymer must first be dissolved in a solvent, in which formic acid was found to be a good solvent for this purpose. Um et al. (2003) investigated the casting of aqueous silk fibroin in formic acid onto polystyrene plate. They found that the β-sheet crystallization of silk fibroin molecules was occurred by the elimination of formic acid upon drying. Furthermore, the silk fibroin molecules in formic acid solution were stable and the crystallization of silk fibroin was attributed to compact molecular shape of silk fibroin in formic acid. Other different solvents had been used and the types of solvents used depend on the implication of the desired biomaterials

2.5.2.4 Application of fibroin for medical biomaterials

There are several uses of silk fibroin as medical biomaterials, such as, a porous tissue matrix, a carrier for controlled-released medicine, due to its unique physicochemical properties such as minimal inflammatory reaction and good water vapor permeability. The silk proteins are also applicable as contact lens materials due to their high oxygen permeability, biocompatibility and optical properties. Mori (2000) investigated the permeability coefficient of the silk fibroin membranes compared to hydrogel membranes including 2-hydroxyethyl methacrylate and synthetic polypeptide membranes. And found that the silk fibroin membrane compared favorably with polypeptide membranes in respect of the oxygen permeability at low water content, which is commonly used as soft contact lens material.

2.5.2.5 Application of fibroin for production of cosmetics and medication

The silk powder is an excellent natural moisture-adjusting factor because of its hydrophilic group existed in its molecular structure. A thin layer of silk powder adhered to the skin surface can absorb or release the moisture along with the change

of temperature and humidity of skin surface, to prevent and cure the dermatosis caused by moisture or dry conditions in certain degree, and to keep the skin in hygiene. Because of its special structure of decreasing the reflective light, the silk powder, applied to the cosmetics on the micro powder, can reflect the soft color naturally. Compared with talcum powder or titanium white powder, the silk powder have the excellent character of air permeability which can be applied to those powder beauty cosmetics, such as the facial powder or rouge, and etc.

The silk microspheres powder has also been developed for sustained drug release. For example, Hino et al. (2003) have prepared silk microspheres by spray-drying an aqueous solution of scoured silk containing theophylline as a model drug and a small amount of ethanol. The microparticles were found to be amorphous which recrystallized as its secondary structure changed to β-sheet conformation by the exposure of the microspheres to an atmosphere of 89% RH for 3 h.

2.5.2.6 Application of fibroin for tissue engineering

Silk fibroin offers an application in the design of matrix scaffold used in tissue engineering. The mechanical performance and biological interactions are the major factors for the success in this field.

Presently, research and development of silk fibroin for application in tissue engineering is of growing interest. Inoue et al. (1998) investigated the use of silk fibroin film in culturing various types of animal cells (SE1116, human colon adenocarcinoma; KB, human mouth epidermoid carcinoma; Colo201, human colon adenocarcinoma; QG56, human lung carcinoma) in vitro. They demonstrated that the film of derived from fibroin and collagen supported equivalent cell growth after 5 days , and resulted in equivalent rates of carcinoembryonic antigen (CEA) production.

Minoura et al. (1995) compared the ability of fibroin and sericin films, with collagen film. The results showed that fibroin and collagen films were equivalent in their ability to support the attachment, physiological morphology, and growth of L-929 fibroblast cells and were superior to the sericin film.

2.6 Silk protein processing

Depending on the application of the silk protein, the process required differ quite considerably. For fabric industry, sericin layers must be removed to obtain purified fibroin fibre. This process is called degumming, which is at the heart of wet processing of raw silk. Figure 2.6 shows the raw silk fibres prior degumming process. This figure shows seven striations each of an average diameter of 50–60 μm, physically held together with sericin.

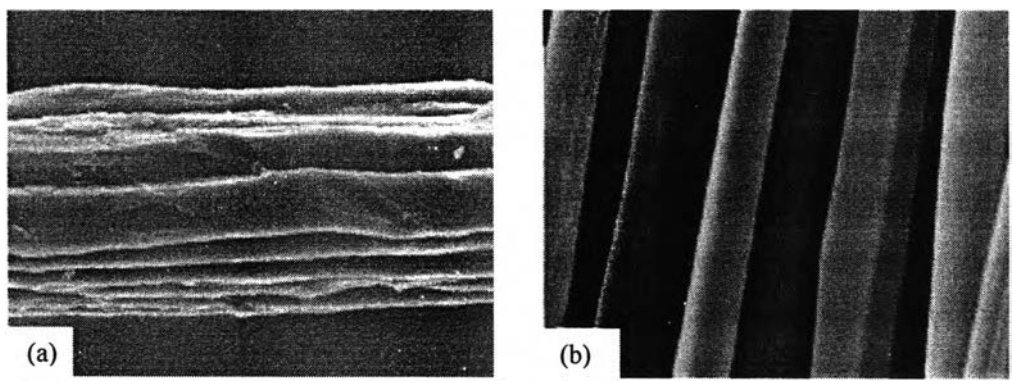

Figure. 2.6 A scanning electron micrograph of (a) a raw silk thread consisted of multiple filaments, showing the lengthwise striations, [Lee et al., 2005], (b) fibroin fiber [Altman et al., 2002].

After the degumming process, fibroin filaments (brins) separate into individual filaments giving different fibre geometry from the raw silk (i.e. a finer and more lustrous fibre) as shown in Figure 2.6 b. This fibrobin fibre could be futher processed into soluble form or power depending on the application of the final product. Some of the key processes for silk fibre and protein production are highlighted here.

2.6.1 Degumming methods

The mechanism of sericin removal in chemical degumming is a combination of various effects such as: dispersion/solubilization and hydrolysis of the different sericin polypeptides [Freddi et al., 2003]. The degumming processes are classified into five methods as follows:

2.6.1.1 Degumming with hot water

As mentioned earlier, silk sericin can easily solubilize in boiling water. Water slightly above the boiling condition (~115 °C) is therefore used for sericin removal. However, the process carries a risk of the fibroin being damaged when the time of treatment is prolonged. Another disadvantage of this process is that this process gives incomplete degumming. Sometimes soap or synthetic detergent must be added to improve the degumming effect. Therefore, this process is very difficult to control and now it is not used in the silk industry [Gulrajani, 1996].

2.6.1.2 Degumming with soap

Marseilles soap, an olive oil soap, is an outstanding soap for degumming. For example, this process may be carried out using 10-20 g/l soap at 92-98 °C for 2-4 hours with adjusted pH to 10.2-10.5 in order to react effectively upon the sericin. As a result of the high temperature, this process tends to attack both sericin and fibroin because of the sensitive nature of fibroin itself and the chemical similarity of fibroin and sericin. Moreover, the most important requirement of the process is a soft water to avoid the formation of a calcium soap.

2.6.1.3 Degumming with synthetic detergent

Synthetic detergents play a crucial role in this process because they have some advantages over soap such as reduction of the treatment time (e.g. 30-40 min at 98 °C) and less fibre damage.

To have reported that the degumming process using 2.5 g/l non-ionic synthetic detergent pH 11.5 for 30 minutes gave a most efficient degumming effect. The disadvantage of this process is that it is carried out at high temperature (e.g. at 98 °C) and high pH (e.g. at pH 11.5). The temperature, time of treatment, and the amount of detergent used must be properly controlled to avoid damage to the fibroin.

2.6.1.4 Degumming with acid

Some acids such as sulphuric, hydrochloric, tartaric, and citric acids can be used as degumming agents. The problem of this process is that it does not receive much attention in the silk industry because of the idea that degumming in alkaline solution is safer for fibroin than in acid solution. It has been found that the complete degumming of silk with strong mineral acids (e.g. sulphuric and hydrochloric acids) cannot be achieved without harm to the fibroin.

2.6.1.5 Degumming with enzymes

Alternatively, degumming can be achieved enzymatically to completely remove sericin, while retaining tensile properties. Because enzymes generally appear in living cells, they work at atmospheric pressure and in mild conditions (e.g. at 40 °C, pH 8.0). Hydrolytic enzymes commonly used in the process of silk industry are cellulase , trypsin, and papain [Duran, 2000].

Trypsin, papain, and bacterial enzymes are the main types of enzymes for silk degumming. These enzymes are called 'proteases' because they degrade proteins by hydrolysis of the -CO-NH- linkage [Freddi et al., 2003] and their degradation products are polypeptides, peptides, and other substances. The proteolytic enzyme action is shown in Figure 2.7.

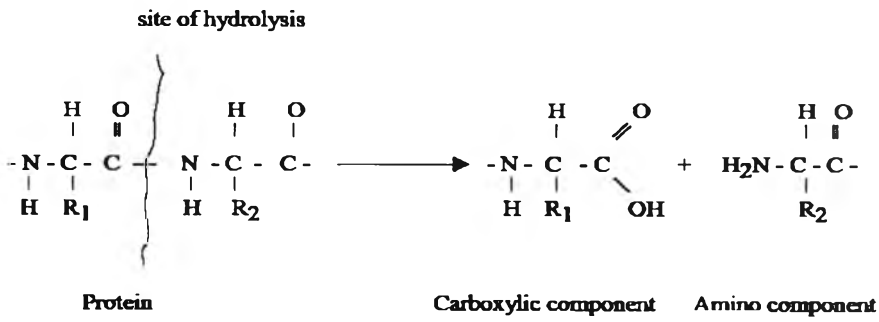

Figure 2.7 Proteolytic enzyme action on protein.

1. Trypsin

Secreted from the pancreas, trypsin is a serine protease which is most active in the pH range between 7 and 9 at 37 °C. It reacts with peptide bonds between the carboxylic acid group of lysine or arginine and the amino group of the adjacent amino acid residue (Figure 2.8) [Duran, 2000].

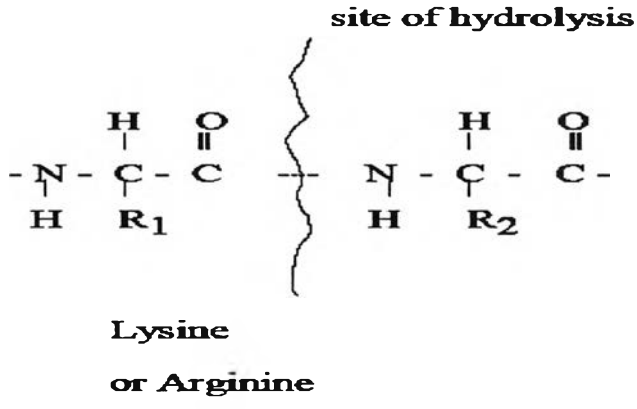

Figure 2.8 Specific site of protein hydrolysis by trypsin.

Fibroin may be less affected by trypsin due to its lower proportion of lysine and arginine residues.

2. Papain

Papain enzyme, derived from papyrus latex, is a sulphydryl enzyme which is most active in the range of the pH between 5.0 and 7.5 at 70-90 °C. Before the degumming process, papain must be activated by a sulphydryl reagent. Activated papain attacks the peptide bonds between the carboxylic acid group of lysine or arginine and the adjacent amino acid residue. Minor cleavage occurs at the carboxylic acid group of histidine and also of glycine, glutamic acid, glutamine, leucine, and tyrosine residues [Gulrajani, 1996].

3. Bacterial enzymes

A bacterial enzyme, alkalase, has been found to be effective in hydrolysing sericin. This bacterial enzyme was claimed to be more effective than trypsin and papain in that it will completely decompose sericin in 1 h at 60 °C, at pH of 9.0 [Gulrajani, 1996].

In general, enzymatic hydrolysis of sericin took place by selective peptide bond cleavage. Chemical properties of soluble sericin peptides changed as a function of the kind of enzyme and treatment time used. Via enzymatic hydrolysis, complete sericin removal was achieved, while the quality of silk goods in terms of handle, appearance and tensile properties is maintained, due to the lower extent of chemical and physical stresses to which silk is subjected to during enzymatic processing, as compared to the traditional chemical process.

However, the process of degumming by enzymatic, has some economic disadvantages in that it needs some pre-treatment processes, since the gum must be swollen before the enzyme bath [Duran, 2000] and it is a very slow reaction compared to alkaline soap degumming .

2.6.2 Processing silk fibroin

After degumming, sericin is removed, resulting in rather purified silk fibroin fibre which can either be weaved into silk fabric or further processed into silk fibroin solution used in other applications above mentioned. In addition, fibroin protein solution can be further process into micro or nano particles which could either be directly added into cosmetics and pharmaceutical products and used as vehicle for drug delivery, or can be redissolved in a solvent and casted into various functional films or biomaterials

Like degumming, conversion of fibroin into soluble protein is the combination of various effects such as: dispersion/solubilization and hydrolysis of the different fibroin polypeptides. Hydrolysis could again be carried out by use of acid, alkali, or enzyme. As is the case of sericin, acid hydrolysis of fibroin tends to be more damaging to fibre than alkaline hydrolysis. Acid hydrolysis occurs at nearly all the peptide linkages in the chain while alkali hydrolysis firstly attacks at the end of the peptide chains [Sadov et al., 1978]. Concentrated sulphuric acid and hydrochloric acid completely dissolve the fibre, nitric acid colours silk yellow, and dilute acids do not attack the fibre under mild conditions. Alkalis such as soap, borax, and ammonia normally dissolve sericin but they also attack fibroin when the treatment time at boiling point is prolonged. Both acid and alkali hydrolysis however are less preferred compared with hydrolysis with enzymes due to its severe conditions (high temperature and acid-base conditions) which possible denature the protein products

and the toxicity of the acid and alkali agents used. However, the high cost of enzymes so far limits the development of industrial processes.

Silk fibroin can also be dissolved in solvents. Nowadays, there are several solvents used to prepare silk fibroin solution. The most common solvent systems used are aqueous 9.0-9.5 M LiBr, mixtures of $CaCl_2/H_2O$/EtOH (1:8:2 mole ratio) [Yeo et al., 2003], $Ca(NO_3)_2$-MeOH, or aqueous LiBr-EtOH-H_2O, depending on its implications [Putthanarat et al., 2002]. However, a disadvantage of dissolving silk fibroin in these solvents is the impurities such as salt and toxic solvents associated with this method particularly when the protein is to be applied to biomedical and pharmaceutical products, thus they must be removed. Solvent could generally be evaporated out of the solution while salt is removed by dialysis or gel chromatography. These steps are however time consuming, other new benign techniques for silk protein production are thus investigated.

2.7 Reactions in sub and supercritical water

Water near the critical point (T=374.2 °C and P=221 bar) has been tested as environmentally friendly reaction media and is attractive due to its unique feature. When water is isobarically heated at a pressure greater than its critical pressure, several properties: e.g. density (ρ), diffusivity, dielectric strength (ε), and ion dissociation product (K_w), undergo dramatic changes in passing through the critical temperature region [Akiya and savage, 2002]. Static dielectric constant ϵ of water changes from 78.5 at ambient temperature and pressure to 5.9 at 673.2 K and 30 MPa [Goto et al., 2004]. Ion product of water K_w changes specifically around the critical point .The value of K_w at 25 MPa increases with temperature up to 10^{-11} at 523.2 K and then decreases to 10^{-19} at 673.2 K and 10^{-22} at 773.2 K, due to the large change of ϵ and K_w with temperature and pressure, the reaction field changes from ionic reaction to radical reaction. In the higher-temperature regions, components mix uniformly at arbitrary composition. Thus, when water is supercritical, hydrocarbons and water can mix uniformly to form homogeneous phase. In general, sub- or supercritical water has been exploited in two major types of reactions: oxidation and hydrolysis

2.7.1 Oxidation in supercritical water

Water oxidation is mostly carried out in supercritical water conditions. Supercritical water (SCW), defined as water at temperatures and pressures above its critical point, it has unique solvation properties making it an attractive medium for oxidation reactions. High destruction efficiencies of this process have been established for wide varieties of aqueous organic waste and hazardous materials.

In most practical processes under development, aqueous waste is brought into contact with an oxidant typically air or oxygen at temperatures between 400 and 650°C and at pressures above 250 bar. Under these conditions, the resulting water/oxygen/organic mixture exists as a single fluid phase (dense fluid), and oxidation is initiated spontaneously. Organics in the waste stream are oxidized to carbon dioxide (CO_2) and water (H_2O) with conversions in excess of 99.999% in residence times of about 1 min or less. Since supercritical water oxidation (SCWO) temperatures are significantly lower than those reached in incineration, little NO_x is produced. Instead, fuel-bound nitrogen atoms are converted to N_2 and N_2O. Heteroatoms such as chlorine, sulfur, and phosphorous are converted to their corresponding inorganic acids (e.g. HCl, H_2SO_4, H_3PO_4), which can be neutralized to their respective salts.

There have been many studies on the global reaction rates of SCWO for various compounds most of which adopted the following equation based on the global law [Erdogan K, 1994]

$$\frac{d[S]}{dt} = -k[S]^a[O_2]^b \qquad \text{eq. (1)}$$

where k, a, and b are rate constant and reaction orders with respect to the substrate and the oxygen oxidant, respectively. The global rate constant k can be explained by Arrhenius-type temperature as in equation (2).

$$k = k_o \exp\left(\frac{-E_a}{RT}\right) \qquad \text{eq. (2)}$$

where k_0 is the pre-exponential, E_a is the activation energy, T is temperature, and R the gas constant.

The reaction order for most substrates is close to unity, whereas that for oxygen varies from zero to 0.5 and the reaction order with respect to oxygen in wet

oxidation is almost unity which suggests that the dependence of the decomposition rate on the oxygen concentration is smaller at supercritical conditions.

An example of the application of equation (1) and equation (2) to water oxidation is given here for the oxidation of ethyl alcohol in supercritical water investigated by Schanzenbacher et al. (2002). The reaction was run in a plug-flow reactor, operated at temperatures ranges from 433 to 494 °C and a fixed pressure of 246 bar, for the residence times ranged from 2 to 12 s. The major liquid-phase oxidation products were acetaldehyde and formaldehyde, while acetic acid was identified in small quantities occasionally, and carbon monoxide, carbon dioxide, methane and ethylene were the products found in the gas phase effluent. The oxidation reaction was initially assumed first order in ethanol and zero order in oxygen, which gives the calculated activation energy of 163.9±3.3 kJ mol^{-1} and the pre-exponential factor was 1011.1±4.5 to a 95% confidence level. The apparent induction time was observed to vary 0.5 to 3.8 s, and it decreased as temperature increased. To investigate the dependence of the reaction rate on oxygen concentration, the fuel equivalence ratio was varied from 0.5 to 1.9. Experiments were conducted at the residence times range between 2.5 and 3.0 s at 470 °C and 246 bar. The reaction orders for ethanol and oxygen were 1.34±0.11 and 0.55±0.19, respectively, and the resulting pre-exponential factor was 1017.23±1.65 and the activation energy was 213.9±18.3 kJ mol^{-1}

2.7.2 Hydrolysis Reactions

In milder condition of near critical or subcritical water, hydrolysis rather than oxidation takes place. This occurs again without any acid or base catalyst due to the increase in the concentration of $[H^+]$ and $[OH^-]$ of water at elevated temperatures. Recently, various noncatalytic hydrolysis with subcritical water of cellulose, saccharides, fish meat, and silk has been reported.

2.7.2.1 Hydrolysis of cellulose (Glucolysis)

Generally, hydrolysis of cellulose is carried out with acids or alkaline catalyst at low temperature (100-120 °C). Sasaki et al. (1998 and 2000) proposed a new method to hydrolyze cellulose rapidly in supercritical water (SCW) to recover useful

products such as glucose, fructose and oligomers (cellobiose, cellotriose, cellotetraose, and etc.). Cellulose decomposition experiments were conducted with a flow type reactor in the range of water temperature between 290 and 400 °C at 25 MPa, with cellulose-water slurries of 10% wt. The solvated products were analyzed by H-NMR and FAB-MS for the identification of the constituents. Quantitative analysis was done by HPLC with refractive index detector and a total organic carbon analyzer.

The kinetics study explaning the difference in the product distributions among the reaction conditions was carried out. The first-order rate constant, kυ, evaluated from the conversion of cellulose was plotted as log k against reaction temperatrue for the temperature range between 290 to 400 °C (Figure 2.9) showed a straight line Arrhenius plot. From the plot, it can be seen that above 350 °C, the reaction rate dramatically increased.

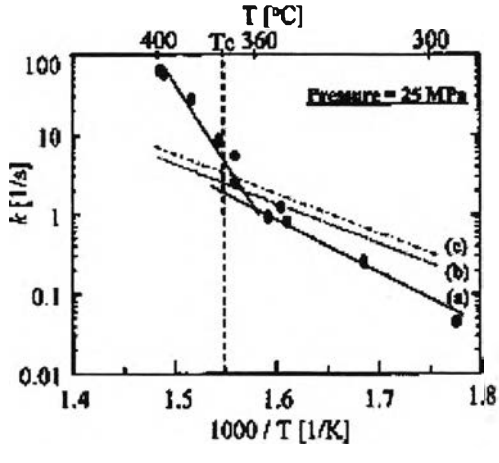

Figure 2.9 Arrhenius plot of decomposition rate constanats of cellulose, cellobiose, and glucose in subcritical and supercritical water at 25 MP:, (a) Cellulose, (b) Cellobiose (c) Glucose [Saaki et al., 2000]

The reason for the drastic change of cellulose hydrolysis rate was that at lower temperature, heterogeneous hydrolysis takes place on the cellulose crystal surface, while at higher temperatures, cellulose dissolution increases, resulting higher reaction rate.

2.7.2.2 Hydrolysis of saccharides

Several studies follow that of Sasaki et al (1998) involves hydrolysis of disaccharides to form monosaccharides. Oomori et al. (2004) suggested that the susceptibility of a disaccharide to hydrolysis in subcritical water largely depended on the constituent monosaccharides and the type of glucosidic bond. The hydrolysis process of disaccharides consisting of two glucose residues or of glucose and fructose or galactose residues in subcritical water was measured using a tubular reactor at 10 MPa and 180–260 °C.The authors found that Threhalose with α-1,1-glucosylglucoside was the most resistant to the hydrolysis, while sucrose with β-2,1-glucosylfructoside was the most easily hydrolyzed among the tested disaccharides. The relationship between the fraction of remaining disaccharide and the residence time was expressed by the Weibull equation (as shown as in equation 3) for every disaccharide at any temperature [Khajavi et al., 2005]:

$$\frac{C}{C_0} = \exp[-(k\tau)^n] \qquad \text{Eq..(3)}$$

where C and C_0 represent the glucose concentration in the efluent and feed reservoir, respectively, τ is the residence time, k is the rate constant, the reverse of which is called the scale constant, and n is the shape constant. The temperature dependence of the rate constant was expressed by the Arrhenius equation for every disaccharide.

2.7.2.3 Hydrolysis of protein

Amino acids have wide uses and applications in pharmaceuticals, food products, animal nutrition, and cosmetic industries, and can be produced conventionally via acid, alkali hydrolysis. Amino acids formation from the hydrolysis in near critical water however could be safer than those from acids or alkali.

Yoshida et al. (1999) studied the hydrolysis of fish meat for the production of organic and amino acids under subcritical condition without oxidants, in a closed batch reactor (stainless tube volume 7 cm^3) in the rang of temperature between 473 and 673 K and various time 1 to 30 min. The analysis of organic acids and amino

acids was carried out with HPLC. The result showed that Lactic acid produced from hydrolysis of raw fish meat (about 0.03 g/g-dry meat) was stable up to the reaction temperature of 513 K (3.35 MPa). Pyroglutamic acids was produced with a yield of 0.095 kg/kg of dry meat by 30 min reaction at 553 K (6.42 MPa). Amino acids such as cystine, alanine, glycine, and leucine were produced in the temperature range 513-623 K with a maximum peak at 542 K in 5 min and oil portion of the product contained useful fatty acids such as eicosapentanoic acid (EPA) and docosahexianoic acid (DHA). However, because the system consists of wide range of impurities, it was difficult to examine the accurate reaction mechanism.

Kang and Chun (2004) investigated the hydrolysis of silk fibroin protein into to amino acids in near-critical water. The reaction was carried out in a novel semi-batch reactor for quick injection of solid materials into a reactor at pre-existing hot, compressed water and in a batch reactor. It was found that high-molecular amino acids such as serine (Ser), aspartic acid (Asp), and other complex amino acids was obtained initially in significant amount, and gradually decreased as reaction time and temperature increased as a result of thermal decomposition (Figure 2.10).

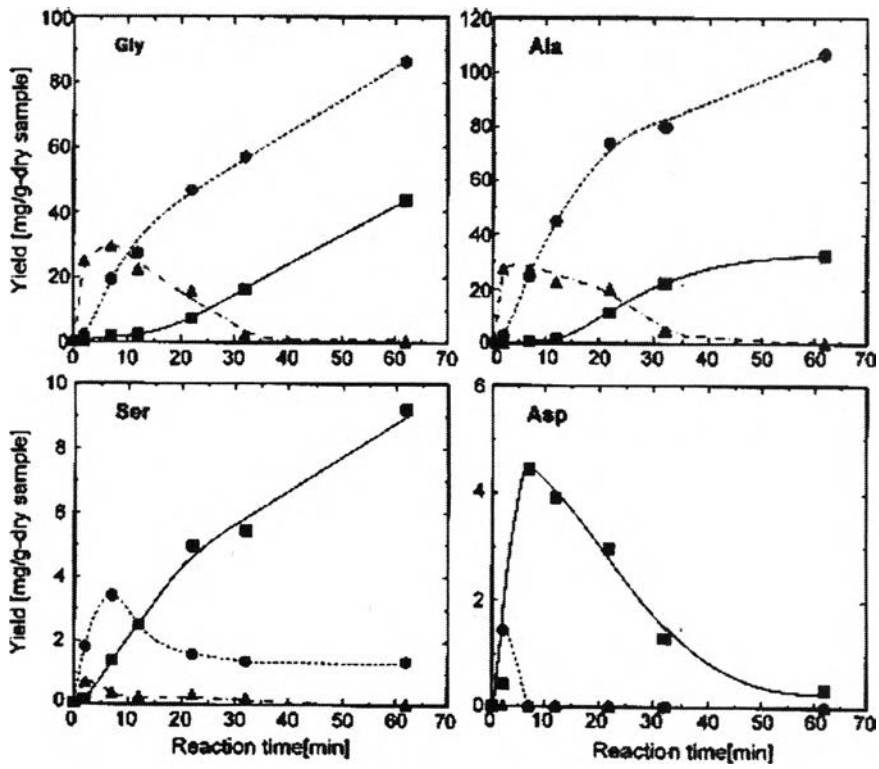

Figure 2.10 Behavior of Gly, Ala, Ser, and Asp formation from hydrolysis of silk fibroin under hydrothermal conditions [■ 473 K (1.4 MPa), ● 523 K (4 MPa), ▲ 573 K (9 MPa)] [Kang and Chan, 2004].

It is likely that simple, low-molecular amino acids such as glycine (Gly) and alanine (Ala) are formed by decomposition of Ser and Asp. At temperature higher than 523 K, values of K_w are relatively low as shown in Figure 2.11, it was likely that Gly and Ala decomposed to form other organic compounds such as organic acids.

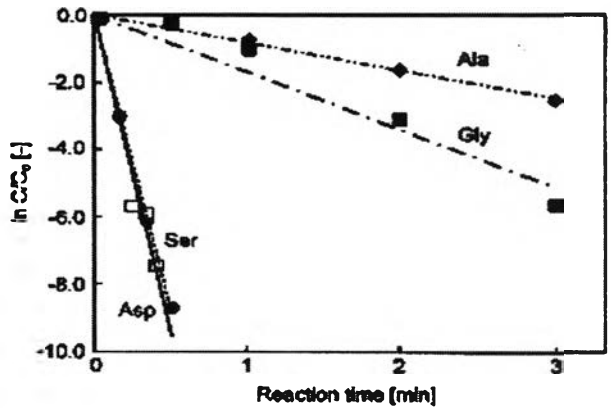

Figure 2.11 First-order plot of decomposition rate of Gly, Ala, Ser, and Asp in subcritical water (573 K, 20 MPa) [Kang and Chan, 2004].

The effects of various additives were determined and there was no significant effect on the yield of amino acids observed with the addition of oleic acid. However, the presence of NaOH and formic acid (FA), both in 5 mol% aqueous solution, had significant effect on the yield. With the increase either in alkalinity or acidity, the production of amino acids was enhanced in either acidic or basic conditions.

These results clearly demonstrate that hydrolysis in subcritical water is a promising technology application in various fields.