

Behavior of Hydrothermal Decomposition of Silk Fibroin to Amino Acids in Near-Critical Water

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Abstract—The behavior of protein decomposition to amino acids in near-critical water is elucidated by using silk fibroin as a model compound. Results show that serine (Ser), aspartic acid (Asp) and other complex amino acids, obtained initially in significant amount, gradually decreased as reaction time and temperature were increased. At temperature higher than 523 K, it is likely that Gly and Ala underwent further decomposition. Regarding the effect of various additives, no significant effect on the yield of amino acids was 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. The effect of alkalinity and acidity, the production of amino acids was enhanced in either acidic or basic conditions.

Key words: Near-Critical Water, Proteins, Amino Acids, Hydrolysis, Waste Resource Recovery

INTRODUCTION

Application of high-temperature and high-pressure (HTHP) water to recover useful materials from various organic wastes, with the general purpose of mitigating environmental pollution, has recently been attracting attention of many researchers and environmentalists. As a recognized environmentally benign solvent, the use of water offers ecological benefits for clean industrial processes. Chemical reactions in sub- and supercritical water media have attracted considerable interest [Shaw et al., 1991; Savage et al., 1995]. To date, numerous articles have been written on the application to chemical synthesis and decomposition [Holliday et al., 1998; Ahn et al., 1998], and have been summarized by Savage [1999]. Moreover, the use of sub- and supercritical water is an emerging technique that can provide complete decomposition of organic [Goto et al., 1999] and hazardous wastes such as PCB and dioxins [Staszak et al., 1987; Yamasaki et al., 1980; Anitescu et al., 2000]. This has also been applied as a medium for recovery of useful materials from various organic wastes such as plastics [Arai et al., 1999], cellulose [Sasaki et al., 1998; Park et al., 2002], and proteins [Yoshida et al., 1999; Kang et al., 2001; Daimon et al., 2001].

Most of our recent works deal with the application of near-critical water, utilizing its potential for hydrolysis reaction, to recover amino acids from proteinaceous fish-derived wastes [Kang et al., 2001; Daimon et al., 2001]. Amino acids have wide uses and applications in pharmaceuticals, food products, animal nutrition, and cosmetic industries. As medicine, they can be used for the treatment of various diseases such as renal, gastrointestinal, endocrinal, and dermal among others. In the food industry, amino acids are utilized as taste enhancers and animal feeds. If each amino acid could be separated individually, these can also be reagents for the synthesis of new materials including electronic-related chemicals (*e.g.*, for liquid crystals, exposure liquids for color copiers). Regarded as fish-derived wastes, impurities are present in great amounts. The presence of

such compounds might affect hydrolysis reaction, thereby retarding amino acid formation from the hydrolysis of fish-derived wastes. For these it is difficult to examine the accurate reaction mechanism of hydrolysis. For this reason, a model compound is necessary to investigate the general behavior of formation of amino acids from proteinaceous materials. Silk fibroin, a protein that can be obtained in nearly pure state, is a good candidate material for this purpose. This has been used as a model compound in early protein studies [Becker et al., 1997].

This paper elucidates the behavior and mechanism of amino acid formation from the hydrothermal treatment of proteins, taking silk fibroin as a model. Specifically, the behavior of formation of glycine (Gly), alanine (Ala), serine (Ser), and aspartic acid (Asp), abundantly present in silk fibroin was investigated. Initial reaction stages were analyzed by using a semi-batch reactor with rapid sample injection system at stable reaction conditions. The effects of reaction parameters were then analyzed in detail with a batch reactor. Investigation of the effect of additives that may be present in various proteinaceous wastes such as fish-derived wastes was also carried out.

EXPERIMENTAL SECTION

1. Properties of Silk Fibroin

Silk fibroin powder was provided by Gifu Bio-Industrial Institute (Gifu, Japan). No pretreatment procedures were performed on the sample. Elemental analysis (using an EA-1108 instrument, Fisons Instruments) performed on silk fibroin showed the presence of the following elements: 44% carbon, 6.6% hydrogen, 17% nitrogen and 32.4% others. Results of acid hydrolysis using 6-N HCl solution showed silk fibroin to contain mostly Gly and Ala, at mole fraction of 27 and 26%, respectively as shown in Fig. 1. Lucas et al. [1962] reported silk fibroin to consist of the following repetitive sequence of amino acids:



2. Experimental Apparatus and Methodology

The batch experiments were carried out in a reactor made of SUS-

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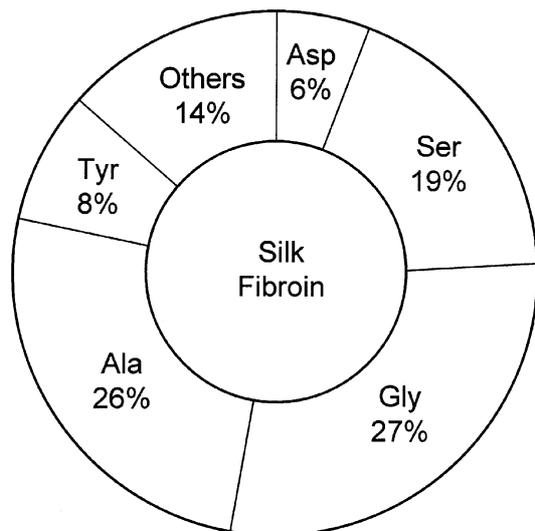


Fig. 1. Amino acid composition of silk fibroin determined by 6N-HCl acid hydrolysis.

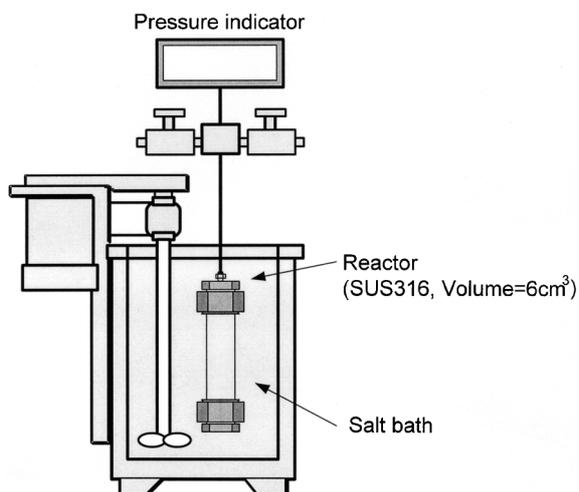


Fig. 2. Schematic diagram of batch reactor apparatus.

316 having an inside volume of about 6 cm^3 ; the schematic diagram is shown in Fig. 2. The allowable pressure is restricted by the operating temperature. At 373 K, it can tolerate a pressure of up to 45 MPa. At a higher temperature of 723 K, the operating pressure should not exceed 35 MPa. Experiments were conducted over a temperature range of 473 to 573 K, at corresponding saturated vapor pressures of 1.4 to 9 MPa, respectively. The reaction time was from 2 to 62 min. In each experimental run, about 0.05 g sample and 5 g of deionized water (weight ratio=1 : 100) were charged into the reactor. The reactor was sealed with Swagelok, and then the air inside was purged by using Argon gas. The reactor was immersed into the preheated molten salt bath containing a mixture of potassium nitrate and sodium nitrate set at desired temperature. It takes about 2 min to reach the desired temperature and pressure after soaking into the salt bath. After the desired reaction time had elapsed, the reactor was plunged into a water bath to bring them quickly to room temperature, effectively ceasing any occurring reactions.

For the study of initial stage of decomposition of silk fibroin, ex-

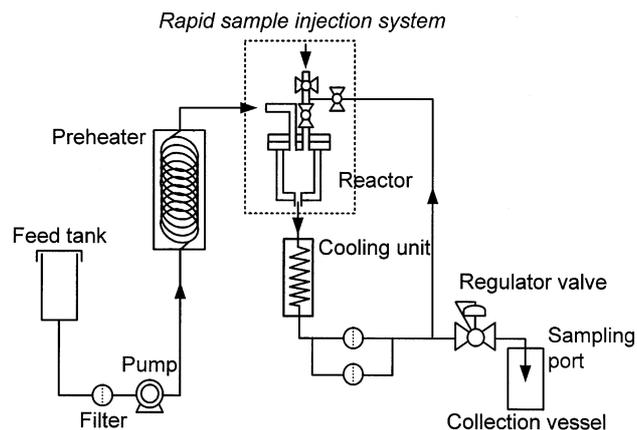


Fig. 3. Schematic diagram of semibatch reactor apparatus equipped with rapid sample injection system.

periments were conducted in a semi-batch reactor with rapid sample injection system. The schematic diagram of the apparatus is shown in Fig. 3, and is described in detail elsewhere [Kang et al., 2001]. The 100-cm^3 reactor and other connected parts are made of Inconel 625 (Ni, Cr, Mo and Fe alloy). This can be operated at the maximum temperature of 450 °C and a maximum pressure of 40 MPa. A 60- μm filter is placed before the inlet pump and pressure regulator. The same procedure as have been previously described was adopted. In brief, deionized water is being delivered by a high-pressure pump (NP-CX-40, NSK Corp.) at a constant flow rate of 40 cm^3/min . The water passes through the preheater before entering the reactor vessel. In each run, about 0.04 g of sample was placed inside a mesh capsule before introducing to the injection port. After the sample was injected into the reactor, the first 96 cm^3 water was removed. This corresponds to the volume of the pipe in the reactor downstream of the sampling port. In all experiments, the TOC of the first 96 cm^3 water collected was negligible, indicating the absence of any organic compounds. Then, sample was continuously collected at the desired sampling period of 1 min (in this study). Sampling was done continuously for a period of 1 min; after the time had elapsed, the sampling container was switched with a new one for the next sampling period.

3. Analytical Methods

The amino and organic acid content of the reaction products was determined by using an amino acid analyzer (LC-10AD, Shimadzu Corp.) and an organic acid analyzer (LC-10A, Shimadzu Corp.), respectively. Analytical conditions of each amino and organic acids used in this experiments are summarized in Table 1. The amino acid analyzer is a combination of an ion-exclusion column (Shimadzu Corp.) and post-column labeling methods with spectrofluorophotometer (Shimadzu Corp.). In sample preparation for amino acid analysis, filtration was performed with an ultra-filtration membrane (30,000 fractional molecular weight, Millipore Ultra Free C3) to maintain good performance of the chromatographic system. The quantities of 17 kinds of amino acids (presented here according to elution order) - namely, aspartic acid (Asp), threonine (Thr), serine (Ser), glutamine (Glu), proline (Pro), glycine (Gly), alanine (Ala), cystine (Cys), valine (Val), methionine (Met), isoleucine (Ileu), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), histidine (His), lysine (Lys) and arginine (Arg) - were determined in each analyti-

Table 1. Analytical conditions of amino and organic acids used in this experiment

	Amino acids	Organic acids
Column	Shim-pack amino-Na	Shim-pack SCR-102H
Mobile phase	Amino acid mobile phase kits (Na type)	5 mM p-toluene sulfonic acid (C ₇ H ₈ O ₃ S)
Flow rate	0.6 mL/min	0.8 mL/min
Column temperature	312 K	313 K
Detector	RF-10A	CDD-6A

cal run.

The organic acid analysis system consists of an ion-exclusion column and electroconductivity detector. The objects to be analyzed are aliphatic carboxylic acids, hydroxycarboxylic acids, keto-carboxylic acids, and other organic acids having dissociation constants (pK_a) of 2-5 and carbon numbers of 5 or less. TOC of the products was measured with a TOC-5000A (Shimadzu Corp.).

RESULTS AND DISCUSSION

1. Initial Stages of Silk Fibroin Decomposition

The behavior of amino acid production from silk fibroin during initial stages of reaction was investigated initially by using the semi-batch reactor apparatus equipped with rapid sample injector. The chromatogram of standard solution containing 17 kinds of amino acids is shown, as an example, in Fig. 4. The trend on the production of each amino acid at 523 K (25 MPa) from silk fibroin as shown in Fig. 5 illustrates that high-molecular-weight amino acids were easily removed from the sequence of amino acids in proteins. It shows that Asp, Ser and other complex amino acids, obtained initially in significant amount, gradually decreased as time progressed. On the contrary, production of relatively simple, low-molecular-weight amino acids such as Ala and Gly became more significant. The behavior of protein decomposition under hydrothermal conditions agrees with our previous results on the study of hydrothermal treatment of fish-derived wastes using the same apparatus [Kang et al., 2001]. At high temperature, as demonstrated by the results at 573 K, the amounts of Gly and Ala decreased. It is also likely that Gly and Ala under-

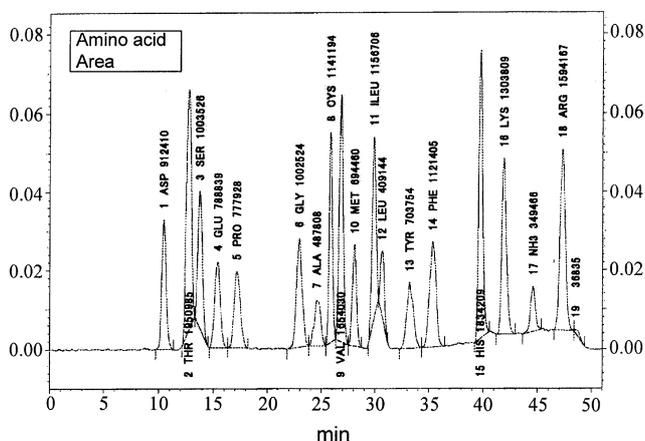


Fig. 4. Chromatogram of standard solution containing amino acids.

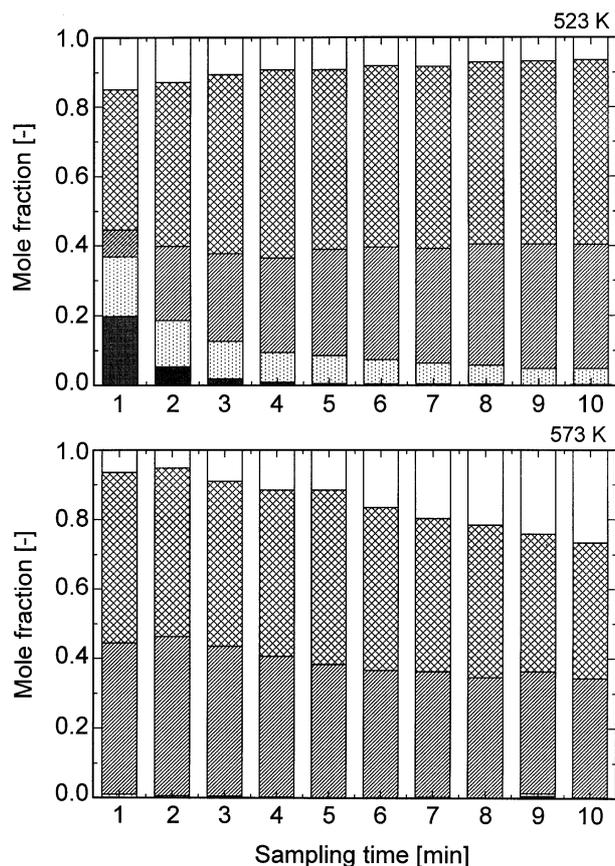


Fig. 5. Behavior of amino acid composition during initial stages of hydrothermal decomposition of silk fibroin at 523 and 573 K [■ Asp ▨ Ser ▩ Ala ▤ Gly □ Others].

went further decomposition, as evident from its decreasing amount as time progressed.

2. Detailed Study of the Behavior of Silk Fibroin Decomposition

Based on the result obtained using the semi-batch reactor apparatus, a detailed study of the behavior of formation of Gly, Ala, Ser and Asp from the decomposition of silk fibroin in near-critical water was performed. A summary of the time and temperature-dependent behavior of silk fibroin decomposition is shown in Fig. 6. Low temperature and short reaction time favor formation of Ser and Asp. At 473 K, while Ser increases, Asp decreases with time, reaching zero in 62 min. As the temperature increases to 523 K, Ser and Asp decrease, while Gly and Ala increase. The increasing amount of Gly and Ala at 523 K is presumably due to the higher ion product of water, favoring hydrolysis reaction. In the case of Ser and Asp, thermal decomposition occurs, thus, lowering the yield. It is likely that simple, low-molecular amino acids such as Gly and Ala are formed by decomposition of Ser and Asp. This interesting phenomenon merits further investigation.

At 573 K, the yields of Gly and Ala decrease, apparently due to their further decomposition to other organic compounds such as organic acids. This is near the optimum conditions ($T=543$ K, 5.51 MPa) obtained by Yoshida et al. [1999] in the study of hydrolysis of fish meat. As stated in their work, the reason for high yield of amino acids by hydrolysis at this condition is the high ion product

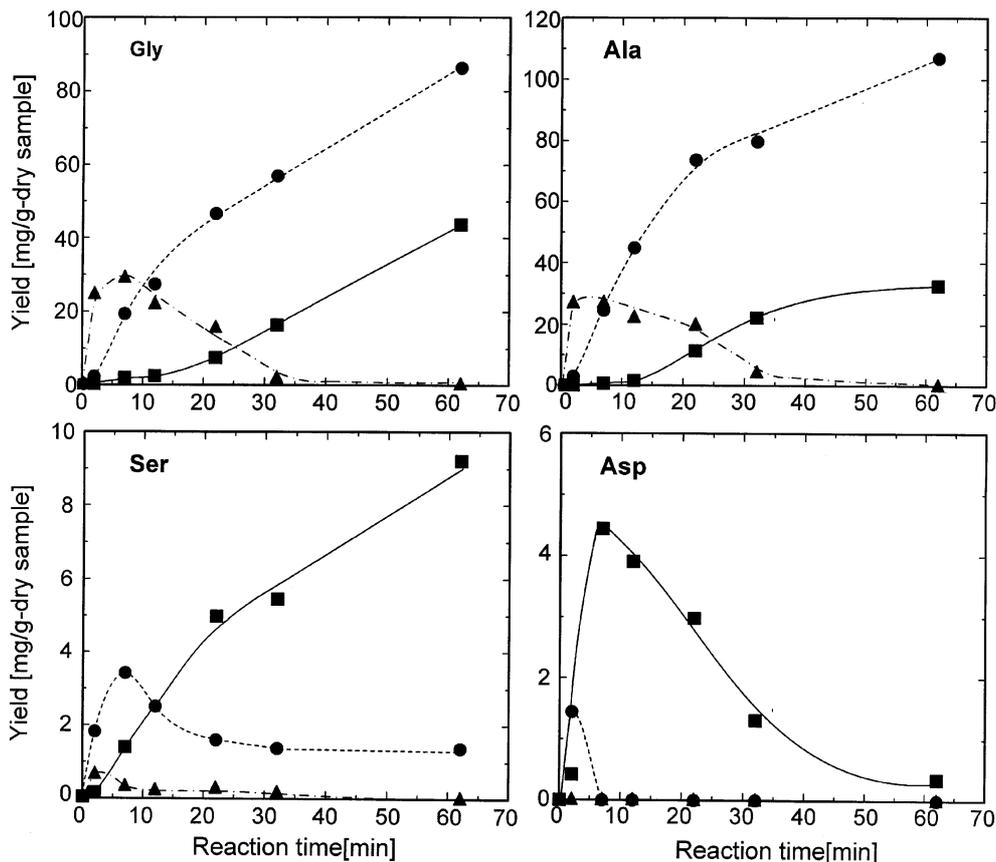


Fig. 6. Behavior of Gly, Ala, Ser and Asp formation from hydrolysis of silk fibroin under hydrothermal conditions [■-473 K (1.4 MPa), ●-523 K (4 MPa), ▲-573 K (9 MPa)].

of water. In general, high ion product of water favors hydrolysis reaction. The temperature that gives the maximum yield of amino acids agrees with that with the maximum K_w . At temperatures below 523 K and at high pressure and temperature ($T > 523$ K), the values of K_w are relatively low. This is expected to be the reason for the

low yield of amino acids at these temperature ranges. At high temperature, the yield of amino acids is low because decomposition is favored over production of amino acids. Among the decomposition products are organic acids including formic acid, lactic acid, acetic acid and propionic acid, which are produced by removal of amino group from various amino acids. Regarding this, experiments to investigate decomposition of amino acids were carried out. The first-order plots of decomposition of each amino acid, shown in Fig. 7, illustrate that high-molecular-weight amino acids such as Ser and Asp decompose faster than Gly and Ala.

3. Effect of Various Additives

Also investigated was the effect of additives that may be present in various proteinaceous wastes such as fish-derived wastes. These additives include low-molecular-weight carboxylic acid (formic acid), salt (NaCl), fatty acid (oleic acid), alkali (represented by NaOH). All experiments were conducted at 523 K (4 MPa) and reaction time of 32 min. The fraction of additives in solution was fixed at 5 mol%. A summary of the results for the yield of amino acids is shown in Fig. 8. Oleic acid, a fatty acid, has no significant effect on the yield. A slight increase in the yield was observed with the addition of NaCl. It was reported that NaCl further increases the ionic activity of H_2O under near-critical conditions [Penninger et al., 2000]. Addition of 5 mol% formic acid (FA) and NaOH significantly increased the yield of amino acids. A three-fold increase was observed with FA, while the yield in the presence of NaOH increased four times.

4. Influence of Alkalinity and Acidity of Solution

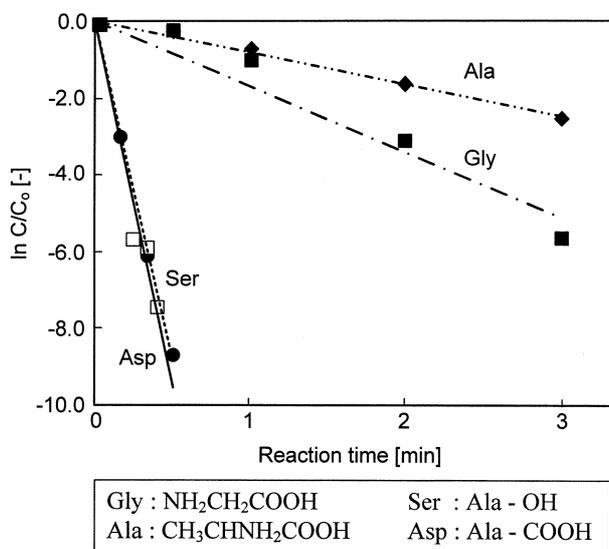


Fig. 7. First-order plot of decomposition rate of Gly, Ala, Ser and Asp in subcritical water (573 K, 20 MPa).

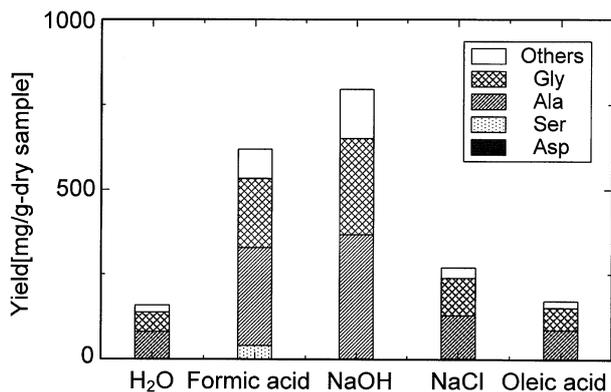


Fig. 8. Effect of various additives (in 5 mol% aqueous solution) on the yield of amino acids from silk fibroin (T=523 K, P=4 MPa, reaction time=32 min).

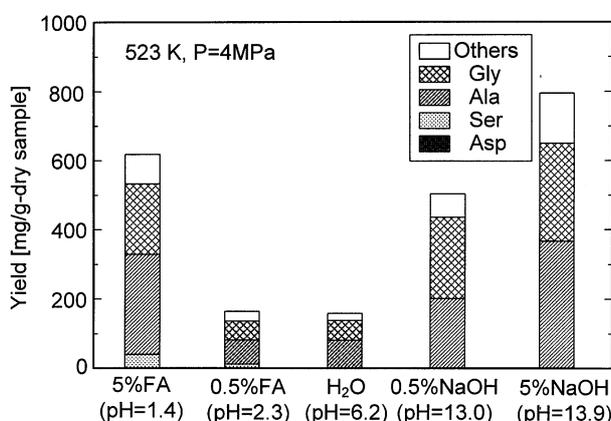


Fig. 9. Effect of pH on the yield of each amino acid (Note: Formic acid (FA) and NaOH were used to adjust the pH).

From the results of the effect of FA and NaOH, it is likely that the yield could be related to the acidity and alkalinity of the solution. The effect of alkalinity and acidity of solution is investigated by adjusting pH using FA and NaOH, and the results are shown in Fig. 9 compared with that of pure H₂O. The production of amino acids was enhanced in both acidic and basic conditions. The more basic or acidic the solution, the better is the yield of amino acids, with strong basic conditions favoring hydrolysis compared to acidic ones.

CONCLUSIONS

The behavior of amino acid (i.e., Gly, Ala, Ser and Asp) production from silk fibroin was elucidated in this study. Initial reaction stages were analyzed by using a novel reactor for quick injection of solid materials into a reactor at pre-existing hot, compressed water. The effects of reaction parameters were then analyzed in detail with a batch reactor. The behavior of fibroin decomposition in the presence of various compounds (i.e., short-chain carboxylic acids, fatty acids, NaCl, etc.) which may be present in proteinaceous wastes such as fish-derived wastes were also investigated.

Results show that Ser, Asp and other complex amino acids, obtained initially in significant amount, gradually decreased as reac-

tion temperature and time were increased. On the contrary, production of relatively simple, low-molecular-weight amino acids such as Gly and Ala became more significant. At high temperature, as demonstrated by the results at 573 K, the amounts of Gly and Ala decreased. It is also likely that Gly and Ala underwent further decomposition; thus, decomposition of each amino acid at 573 K was also studied.

Regarding the effect of various additives, no significant effect on the yield of amino acids was observed with the addition of oleic acid. Addition of 5 mol% NaCl slightly increased the yield. FA and NaOH, in great amount, have significant effect on the yield. The result could be related to the alkalinity and acidity of the solution; thus, further experiments on the effect of pH were carried out by adjusting the amount of FA and NaOH in the solution. The production of amino acids was enhanced by either acidic or basic conditions.

This research lays the groundwork for further investigation of the decomposition behavior of proteins under near-critical conditions, a promising research topic on environmental and industrial viewpoint.

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REFERENCES

- Ahn, S. H., Joung, S. N., Yoo, K. P., Noh, M. J., Han, J. H. and Han, S. O., "Oxidation Characteristics of Phthalic and Adipic Acids by Supercritical Water," *Korean J. Chem. Eng.*, **15**, 390 (1998).
- Anitescu, G. and Tavlarides, L. L., "Oxidation of Aroclor 1248 in Supercritical Water: A Global Kinetic Study," *Ind. Eng. Chem. Res.*, **39**, 533 (2000).
- Arai, K. and Adschiri, T., "Importance of Phase Equilibria for Understanding Supercritical Fluid Environments," *Fluid Phase Equilibria*, **158**, 673 (1999).
- Becker, M. A., Magoshi, Y., Sakai, T. and Tuross, N. C., "Chemical and Physical Properties of Old Silk," *Studies in Conservation*, **42**, 27 (1997).
- Daimon, H., Kang, K., Sato, N. and Fujie, K., "Development of Marine Waste Recycling Technologies Using Sub- and Supercritical Water," *J. Chem. Eng. Japan*, **34**, 1091 (2001).
- Goto, M., Nada, T., Kodama, A. and Hirose, T., "Kinetic Analysis for Destruction of Municipal Sewage Sludge and Alcohol Distillery Wastewater by Supercritical Water Oxidation," *Ind. Eng. Chem. Res.*, **38**, 1863 (1999).
- Holliday, R. L., Jong, B. Y. M. and Kolis, J. W., "Organic Synthesis in Subcritical Water Oxidation of Alkyl Aromatics," *Journal of Supercritical Fluids*, **12**, 255 (1998).
- Kang, K., Quitain, A. T., Daimon, H., Noda, R., Goto, N., Hu, H. and Fujie, K., "Optimization of Amino Acids Production from Waste Fish Entrails by Hydrolysis in Sub- and Supercritical Water," *Can. J. Chem. Eng.*, **79**, 70 (2001).
- Kang, K., Quitain, A. T., Urano, S., Daimon, H. and Fujie, K., "Rapid Sample Injection in Semi-batch Hydrothermal Treatment of Solid Wastes," *Ind. Eng. Chem. Res.*, **40**, 3717 (2001).

- Lucas, F., Shaw, J. T. B. and Smith, S. G., "Some Amino Acid Sequences in the Amorphous Fraction of the Fibroin of *Bombyx mori*," *The Biochemical Journal*, **83**, 164 (1962).
- Park, J. H. and Park, S. D., "Kinetics of Cellobiose Decomposition Under Subcritical and Supercritical Water in Continuous Flow System," *Korean J. Chem. Eng.*, **19**, 960 (2002).
- Penninger, J. M. L., Kersten, R. J. A. and Baur, H. C. L., "Hydrolysis of Diphenylether in Supercritical Water: Effects of Dissolved NaCl," *J. Supercrit. Fluids*, **17**, 215 (2000).
- Sasaki, M., Kabyemela, B., Malaluan, R., Hiroshi, S., Takeda, N., Adschiri, T. and Arai, K., "Cellulose Hydrolysis in Subcritical and Supercritical Water," *J. Supercrit. Fluids*, **13**, 261 (1998).
- Savage, P. E., Gopalan, S., Mizan, T. I., Martino, C. J. and Brock, E. E., "Reactions at Supercritical Conditions: Applications and Fundamentals," *AIChE J.*, **41**, 1723 (1995).
- Savage, P. E., "Organic Chemical Reactions in Supercritical Water," *Chem. Rev.*, **99**, 603 (1999).
- Shaw, R. W., Brill, T. B., Clifford, A. A., Eckert, C. A. and Franck, E. U., "Supercritical Water: A Medium for Chemistry," *Chem. Eng. News*, **12**, 26 (1991).
- Staszak, C. N., Malinkowski, K. C. and Killilea, W. R., "The Pilot-scale Demonstration of the MODAR Oxidation Process for the Destruction of Hazardous Organic Waste Materials," *Environ. Prog.*, **6**, 39 (1987).
- Yamasaki, N., Yasul, T. and Matsuoka, K., "Hydrothermal Decomposition of Polychlorinated Biphenyls," *Environ. Sci. Technol.*, **14**, 550 (1980).
- Yoshida, H., Terashima, M. and Takahashi, Y., "Production of Organic Acids and Amino Acids From Fish Meat by Subcritical Water Hydrolysis," *Biotechnol. Prog.*, **15**, 1090 (1999).