REACTION BEHAVIORS OF GLYCINE UNDER SUPER- AND SUBCRITICAL WATER CONDITIONS

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Abstract. The influence of temperature and pressure on the dimerization and decomposition of glycine under simulated hydrothermal system conditions was studied by injecting a glycine solution into water in the sub- and supercritical state. The experiments at five different temperatures of supplied water – 250, 300, 350, 374, and 400 °C – were performed at 22.2 and 40.0 MPa. At 350 °C, experiments under 15.0–40.0 MPa were conducted. Diglycine, triglycine (trace), diketopiperazine, and an unidentified product with a high molecular mass (433 Da) were the main products of oligomerization. The results show that temperature and pressure influence the extent of dimerization and decomposition of glycine. The maximum of dimers formation was observed at 350 and 375 °C at 22.2 and 40.0 MPa, respectively, and coincided with a high rate of glycine decomposition. Glycine, alanine, aspartic acid, as well as other amino acids, were obtained by injecting a mixture of formaldehyde and ammonia. The results support the oligomerization and synthesis of amino acids in a submarine hydrothermal system.

Keywords: glycine, high pressure, high temperature, hydrothermal system, oligomerization, origin of life, supercritical water

1. Introduction

A carbon isotopic study reported that photosynthesis must have existed as a biochemical process about 4.0 billion years ago (Mojzsis *et al.*, 1996). Significant numbers of large (up to 500 km in diameter) impactors (Sleep *et al.*, 1989; Lyons and Vasavada, 1999) and micrometeorites (Maurette, 1998) continued to hit the Earth-moon system, during the period of 4.5 to 3.8 billion years ago (Ga), producing enough shock energy to boil the ocean. It has been suggested that the surface temperature of the ocean declined from 90–100 °C at 4.1 Ga to 30–50 °C at 3.5 Ga (Bengtson, 1994). Hydrothermal vents were widespread and active on the ocean floor at that time.

A hypothesis concerning the origin of life in a hydrothermal system was proposed in 1981 (Corliss *et al.*, 1981; Baross and Hoffman, 1985). Fluids circulate through pores and fractures of oceanic crust, penetrate to the front of magma and then ejected back into the ocean through hydrotermal vents where temperatures

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reach up to 350–400 °*C* (Gamo *et al*., 1996; Holm, 1992). Therefore, hydrothermal vents are considered to be flow reactors for abiotic synthesis of organic compounds and oligomerization of monomers. Under these conditions decomposition of organic monomers and polymers also occur. By heating mixtures of various amino acids to 240 °C at 26.5 MPa, it was found that alkylamines are the main products among nitrogen compounds (Bada *et al*., 1995; Miller and Bada, 1988). Repeated circulation of glycine through the hot (200–250 °C) and cold (0 °C) regions in a flow reactor at 24.0 MPa showed protection of peptide bond and elongation of oligopeptides (Imai *et al*., 1999). Synthesis of diketopiperazine, di- and triglycine was reported by circulating glycine solution without catalyst and tetra- and hexaglycine in the presence of divalent ion (copper). When a mixture of glycine and alanine was used, an exponential growth of the oligopeptides was observed (Ogata *et al*., 2000). For reviews concerning the chemical evolution of the origin of life see also Oro *et al*. (1990) and Rode (1999).

Therefore, it is necessary to examine whether the formation of biopolymers from monomers can occur under super- and subcritical water conditions. In our preliminary work we observed oligomerization of glycine in supercritical water (Alargov *et al*., 2001). By injecting a glycine solution into water in a supercritical state (pressure 39.5 MPa and temperature ∼400 °C), diglycine, triglycine (trace amount), diketopiperazine, and an unidentified product with a high molecular mass (433 Da) were obtained in a flow reactor. Here we report a more detailed investigation on the influence of temperature and pressure on the processes of condensation and decomposition of glycine in simulated hydrothermal conditions.

2. Materials and Methods

The reactor apparatus was made of Hastelloy C, a nickel-based alloy, and it consisted of two parts; the supercritical water (SCW) supply system and the sample injection system (Figure 1). In the SCW supply system, the water is pressurized by the pump P-1 (controlled by a pressure-regulating valve, V-1), and heated by an electric heater. In the sample injection system, a solution of glycine (at room temperature) is compressed to a pressure slightly higher than that in the SCW supplying system to allow injection. Injection is controlled by the valve V-2. The flow rate of fluid in the SCW supply system is controlled by adjusting the capacity of pump P-1 and it was kept constant at 10 mL min^{-1}. The experiments were conducted at various temperatures at this flow rate, measured at the injection point prior to injection. After mixing for reaction, the fluid is cooled down externally and leaves the system. Two sample injection systems were used. One was a hand-operated system, which allowed injection of 12 mL of solution, and the other was a HPLC pump providing a constant flow rate. After injection, the initial 15 mL was discarded and then 100 mL was taken as a sample in the former case. Glycine concentration at the outlet was determined to be 0.065 M in the control experi-

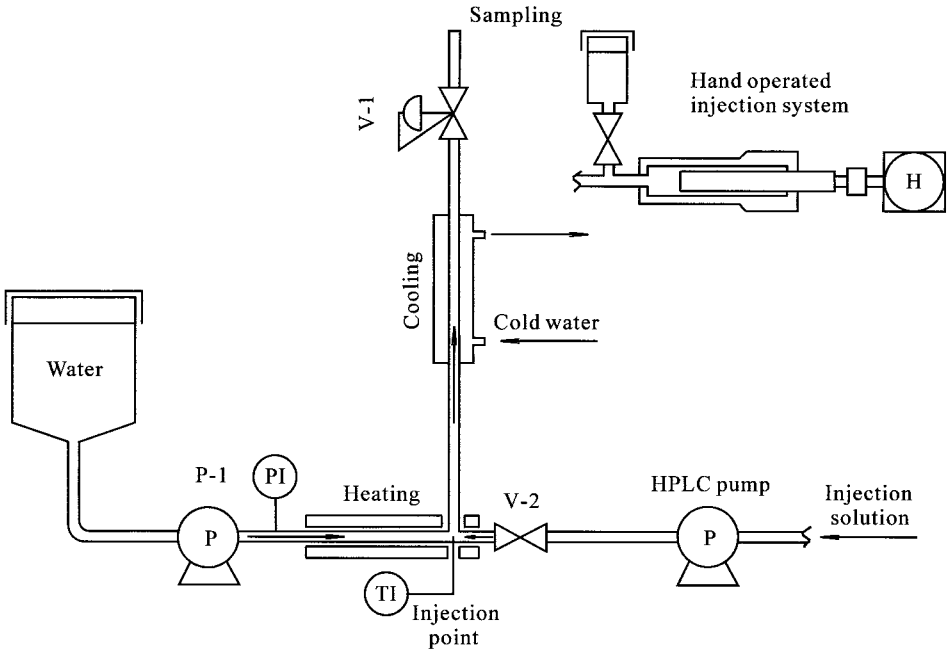


Figure 1. Scheme of the flow reactor simulating a submarine hydrothermal system: P-1 – pump; PI – pressure indicator; TI – temperature indicator; V-1 – pressure regulating valve; V-2 – valve.

ment performed at 27 °C. The flow rate of the latter injection system was kept at 5.0 mL min^{-1}. The concentration of glycine at the outlet in the control experiment performed at 28 °C was 0.22 M. Concentrations of triglycine and diketopiperazine at the outlet in the control experiment performed at room temperature were 0.024 and 0.05 M, respectively. After mixing, the temperature of the fluid dropped to 15–25 °C. The residence time of the injection solution for reaction at high temperature, i.e., the time required to pass from the injection point to the cooler, was calculated to be about 10 sec.

The experiments at five different initial temperatures of supplied water – 250, 300, 350, 374, and 400 °C – were performed at 22.2 MPa with the hand-operated injection system and at 40.0 MPa with the HPLC-pump. At 350 °C of supplied water experiments at 15.0, 20.0, 25.0, 30.0, 35.0, and 40.0 MPa were conducted with the latter sample injection system. The experiments with triglycine and diketopiperazine were also carried out by the latter sample injection system. A mixture (1:1(v)) of formaldehyde (37%) and ammonia (28%) was injected into water at various high temperatures (300, 350, 374, and 400 °C) in our experiments. In the control experiment with the mixture of formaldehyde/ammonia, performed at 24.7 °C, no amino acids were detected. Glycine was purchased from Nakalai tesque, Inc., Kyoto, Japan; triglycine was obtained from Sigma Chemical, Co., and diketopiperazine was obtained from Wako Pure Chemical Industries, Ltd. Pure

water (Milli-Q) was used for sample preparation. Analysis of the reaction mixture was performed using a Shimadzu HPLC apparatus with an auto-injector system. A Shodex Asahipak column ODP-50 6E (6.0 mm ID, 150 mm L) was employed. The mobile phase consisted of 5 mM KH_2PO_4 and 2.5 mM $C_6H_{13}SO_3Na$, and the pH was maintained at 2.7 with H_3PO_4 (Kalonia *et al.*, 1989; Tucker *et al.*, 1989). The flow rate of the mobile phase was 0.5 mL min^{-1}. The compounds were detected by monitoring the absorbance at 200 nm. As standards, glycine and its oligomers up to hexaglycine, were purchased from Sigma. The reaction mixtures were also analyzed by thin layer chromatography (TLC) in two solvent systems: phenol–methanol-buffer solution (a water solution containing 6.3% (wt) sodium citrate and 3.7% (wt) monobasic sodium phosphate) – acetic acid (2000:1500:400:1) and n-butanol–methanol–water–28% ammonia (10:10:5:2 (v)). The compounds were visualized by spraying with a ninhydrin and chlorine - *o*-tolidine. For hydrolysis 0.4 mL of 37% HCl was added to 0.4 mL of sample and the mixture was heated at 110 °C for 24 hr in a sealed tube.

Amino acid analysis was entrusted to Toray Research Center, Inc. Sample solution (50 μL) was placed in a test tube, and was dried *in vacuo*. Hydrochloric acid (6 N, 100 μL) was added, the tube was flame sealed, and the sample was hydrolyzed at 135 °C for 3 hr. The tube was opened, the solution was dried *in vacuo*, and the residue was redissolved in water (250 μL). An aliquot of the solution (50 μL) was subjected to amino acid analysis. The analysis was performed on a Hitachi L-8500 amino acid analyzer (Hitachi, Ltd., Tokyo, Japan). Amino acids were detected by on-line post column reaction with ninhydrin.

Identification and quantification of glycine (**1**), diketopiperazine (**2**), diglycine (**3**) and triglycine (**4**) were made by HPLC (Figure 2). The presence of compounds identified by HPLC was confirmed by thin layer chromatography (TLC). Unidentified compound **5** was ninhydrin positive and had high molecular mass (MS: m/z = 433, 366, 351, 337, 327, 284, 269, 255, 245, 202, 187, 173, 163, 120, 105 (100%). After hydrolysis, the reaction mixtures were studied again by HPLC and TLC. It was established that the peaks of oligoglycines disappeared after hydrolysis. On the TLC plate, only a single spot of glycine was detected.

3. Results and Discussion

3.1. Effect of temperature on oligomerization and decomposition of glycine

Figure 3 shows the temperature dependence of the products (diglycine and diketopiperazine) and the raw material (glycine) concentrations at constant pressure. It can be seen from the figure that oligomerization of glycine started at a relatively low temperature with the formation of diglycine. This temperature was determined to be about 250 °C at both 22.2 and 40.0 MPa, at which pressures a trace amount of diglycine was detected.

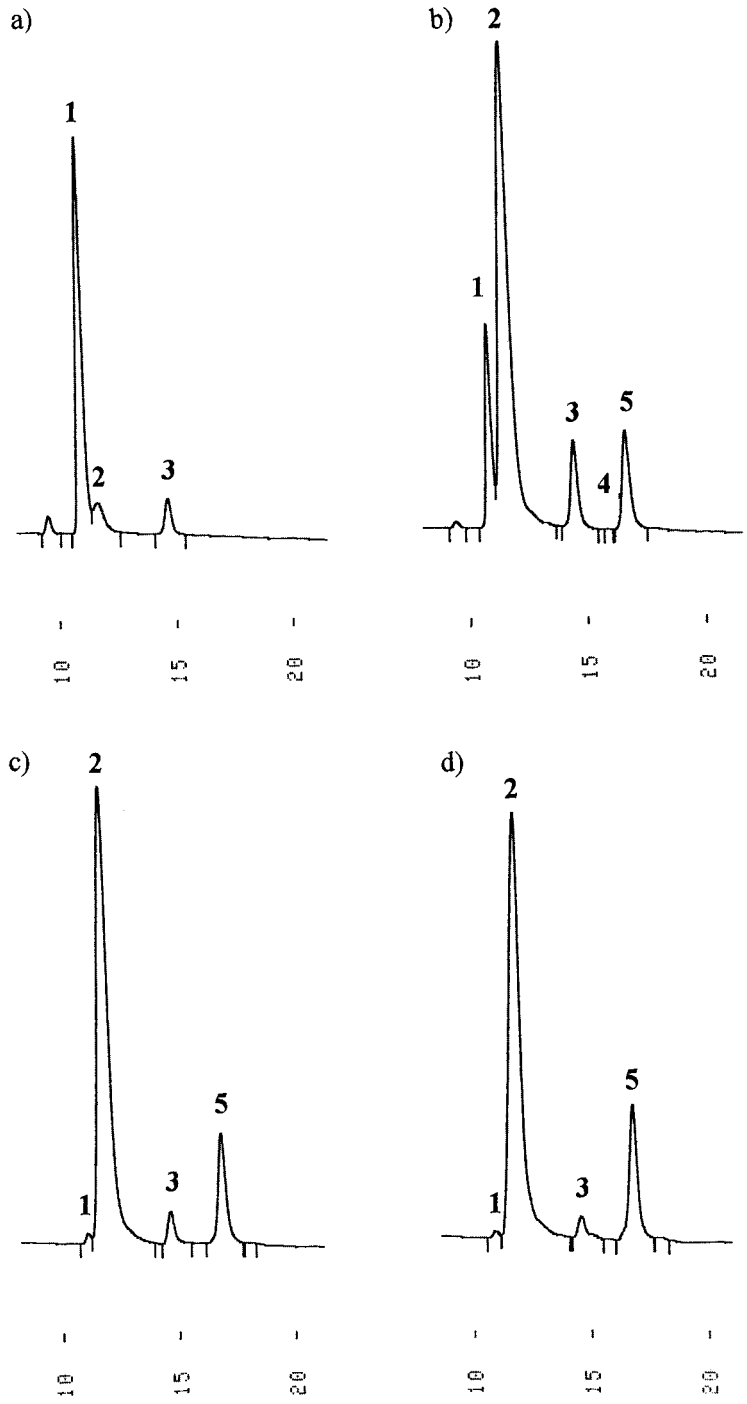
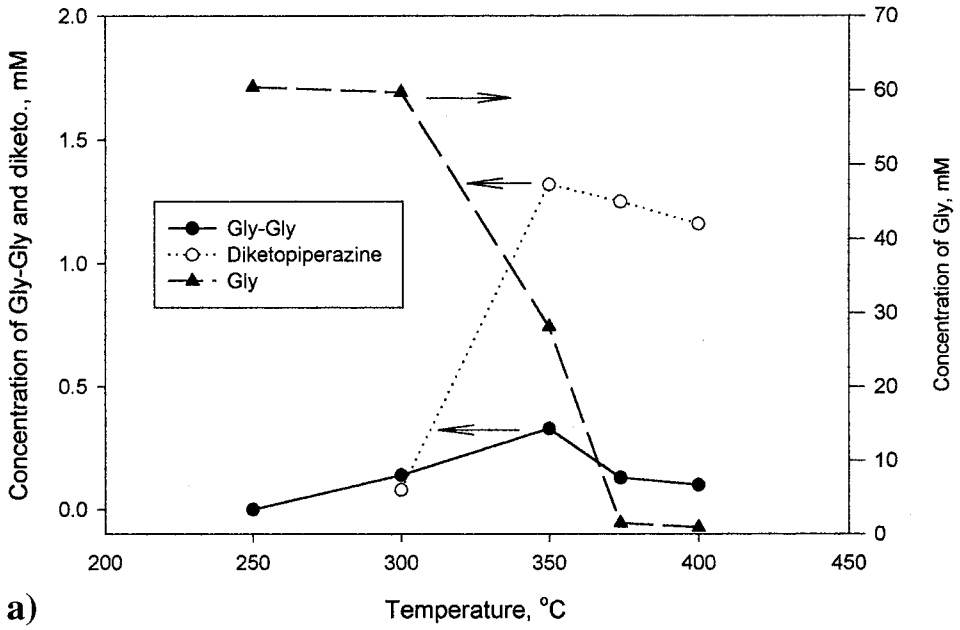


Figure 2. HPLC chromatograms of reaction mixtures obtained in experiments of oligomerization of glycine at different temperatures at 22.2 MPa using a hand-operated sample injection system: a) 300 °C; b) 350 °C; c) 374 °C; d) 400 °C. **1** – Glycine; **2** – diketopiperazine; **3** – diglycine; **4** – triglycine; **5** - unknown.



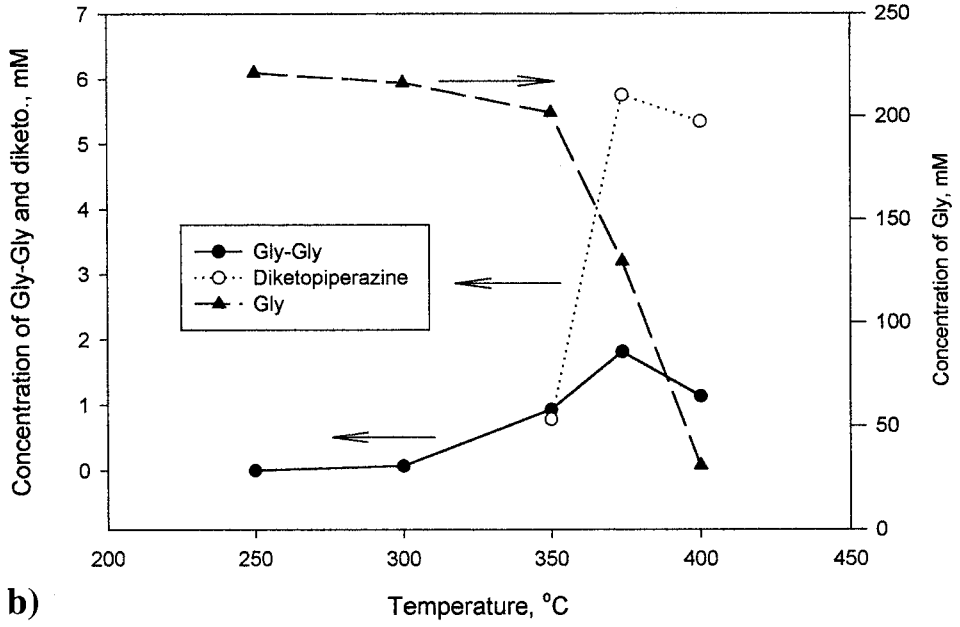


Figure 3. Plots of the concentrations of diglycine, diketopiperazine and glycine in reaction mixtures after experiments of oligomerization of glycine at different temperatures and constant pressure: at 22.2 MPa using a hand-operated sample injection system, at 40.0 MPa using an HPLC pump as the sample injection system. The arrows show the right or left ordinate.

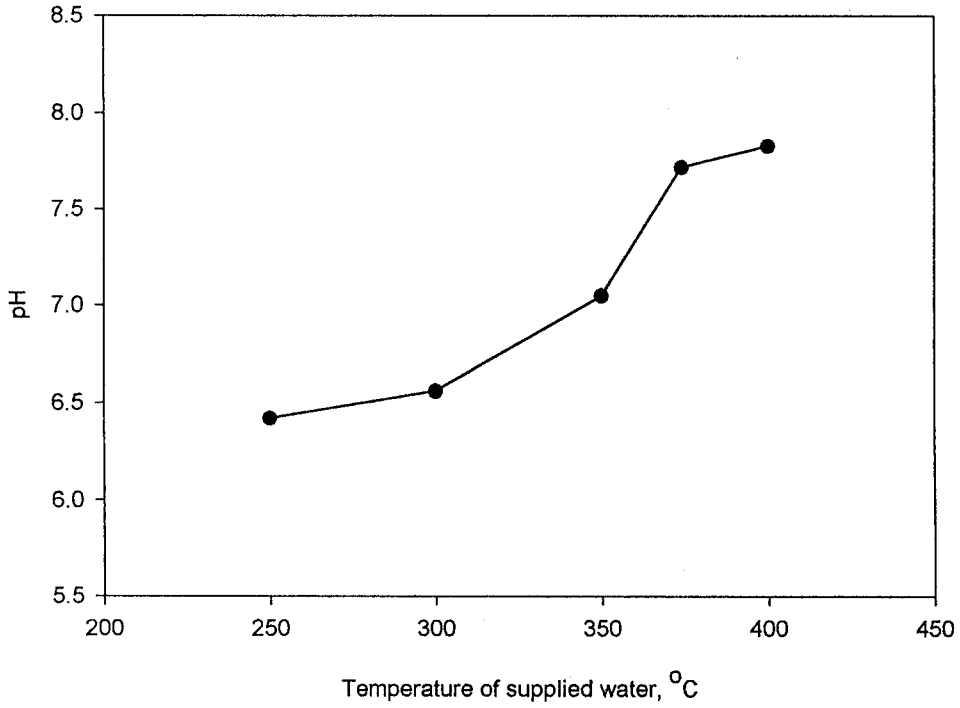


Figure 4. Plot of pH as a function of temperature after glycine oligomerization reactions at 40.0 MPa using an HPLC pump as the sample injection system.

With an increase of temperature of roughly 50 °C, the formation of diketopiperazine was observed, probably due to dehydration of diglycine. The maximum concentrations of these products were obtained at 325–374 °C at 22.2 MPa and 350–400 °C at 40.0 MPa. At these temperatures, triglycine was also revealed from the HPLC spectra, but at very low concentrations; 0.02–0.04 mM. Possible mechanisms of formation of triglycine are chain elongation or opening of the diketopiperazine ring with addition of one glycine molecule (Nagayama *et al.*, 1990).

The optimum temperature for the formation of glycine oligomers was found to coincide with that for rapid decomposition of glycine. The pH of reaction mixtures also increased with temperature, due to the formation of methylamine and other amines (Figure 4). The reaction mixture was very complex and further detailed studies are needed to eluccidate its composition. It is possible, that some amino acids be obtained due to the interaction of glycine with the products of decomposition (Ivanov, 1983). Decomposition of methylamine is also possible, probably via the formation of ammonia and formaldehyde (Bada *et al.*, 1995).

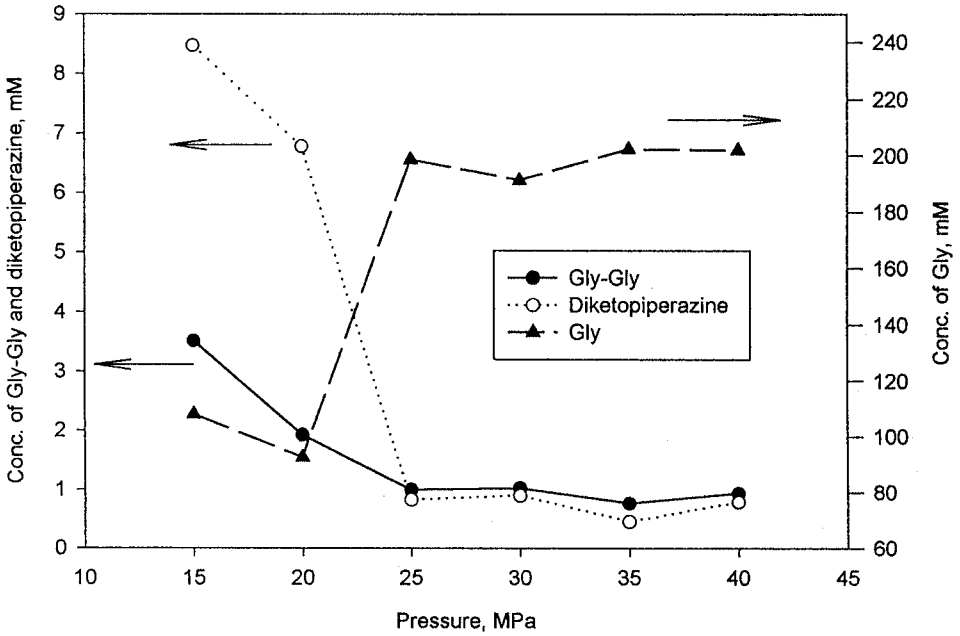


Figure 5. Plot of the concentrations of diglycine, diketopiperazine and glycine obtained after injection of glycine at different pressures at 350 °C. The arrows show the right or left ordinate.

3.2. Effect of pressure on oligomerization and decomposition of glycine

From the plot of the concentrations of diglycine and diketopiperazine in the reaction mixtures obtained at different pressures at a constant temperature of 350 °C, it can be concluded that pressure also affected the ratios of products (Figure 5). At relatively low pressures (15.0 and 20.0 MPa), the extent of decomposition of glycine was higher. Decreasing pressure also led to the formation of diglycine and diketopiperazine in high concentrations (3.51 and 8.47 mM, respectively). At pressures of 15.0 and 20.0 MPa, the formation of triglycine was also observed, reaching a concentration of 0.02 mM. Increasing the pressure to 25 MPa resulted in reduction of the extent of glycine decomposition. Additional studies are necessary for an explanation of this abrupt transition of the behavior of glycine and its derivatives. It is also interesting to note that under these conditions (over 25 MPa) the concentration of diglycine was higher than that of diketopiperazine.

3.3. Effect of temperature on decomposition of triglycine and diketopiperazine

The behavior of triglycine and diketopiperazine as reaction components involved in oligomerization of glycine was also studied.

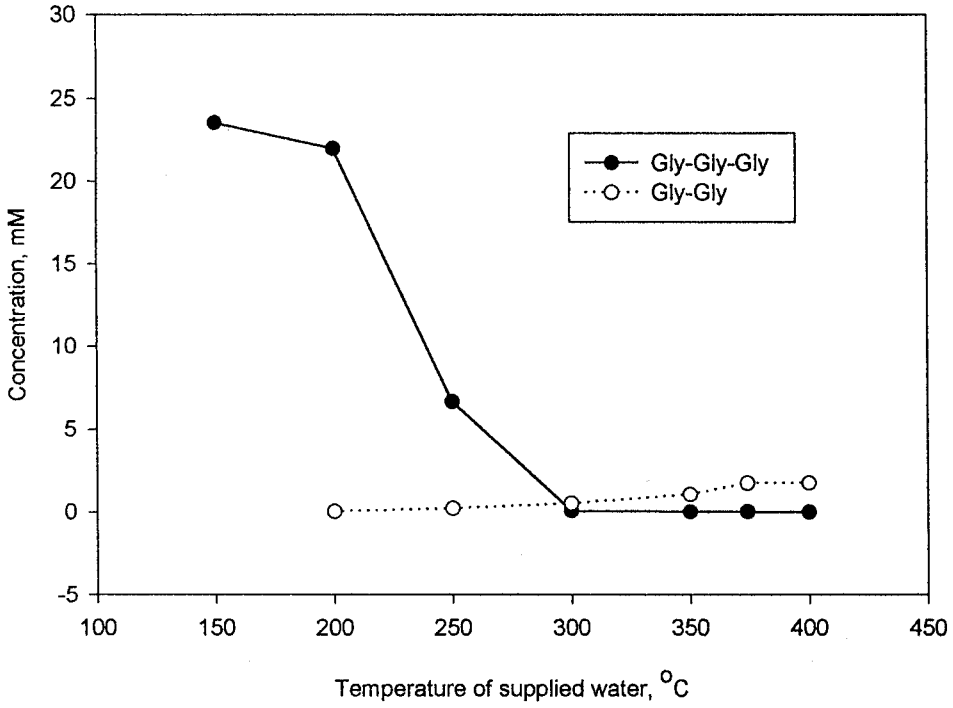


Figure 6. Plots of the concentrations of triglycine and diglycine obtained after injection of triglycine at different temperatures at 40.0 MPa, using an HPLC pump as the sample injection system.

It was established that decomposition of triglycine started at comparatively low temperatures (Figure 6). The main products were diketopiperazine and glycine. Decomposition occurs through diketopiperazine formation from the N-terminus of triglycine (Steinberg and Bada, 1983). It was found that there was no direct formation of hexaglycine due to the fast rate of diketopiperazine formation. Diglycine was also revealed. It is formed from glycine condensation and maybe small extent from the ring-opening of diketopiperazine. At 374 and 400 °C, the formation of an unknown product **5** was observed.

Diketopiperazine was very stable under these experimental conditions (Figure 7). Even at 400 °C, only a small portion of the total amount of it was hydrolyzed to yield diglycine. At 350, 374 and 400 °C, a trace amount of glycine was detected by TLC. At 400 °C the formation of the unknown compound **5** was detected by HPLC.

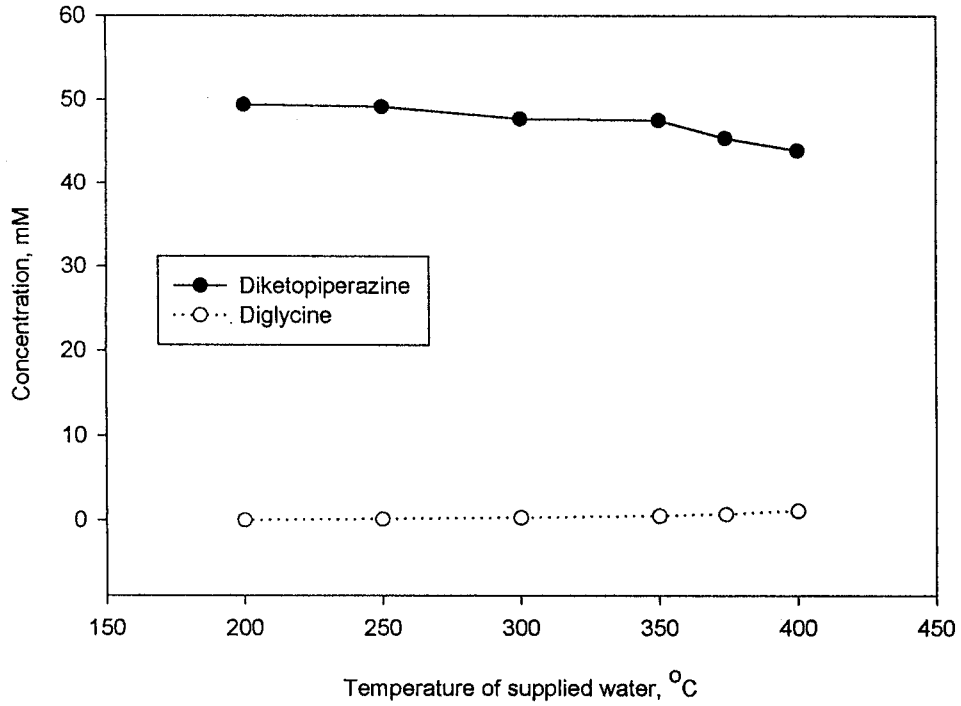


Figure 7. Plots of the concentrations of diketopiperazine and diglycine obtained after injection of diketopiperazine at different temperatures at 40.0 MPa using an HPLC pump as the sample injection system.

TABLE I

Concentrations of some amino acids obtained after injecting a mixture of formaldehyde and ammonia into water at different temperatures

Amino acid	Concentration (μmol mL^{-1}/sample)			
	300 °C	350 °C	374 °C	400 °C
Aspartic acid	–	0.0176	0.0073	0.0071
Threonine	–	0.0038	0.0145	0.0259
Serine	–	0.0431	0.0956	0.0607
Glutamic acid	0.0746	0.2125	0.0041	0.0041
Glycine	0.3230	0.4540	0.8105	1.1335
Alanine	–	0.0630	0.1894	0.3469
Isoleucine	–	0.0346	0.1421	0.3346
Leucine	–	0.0177	0.0063	–

3.4. SYNTHESIS OF AMINO ACIDS AFTER INJECTION OF A MIXTURE OF FORMALDEHYDE AND AMMONIA

Amino acid formation may also take place in or near the hydrothermal vents. Seven amino acids were obtained upon heating formaldehyde and ammonia at 185 °C for 8 hr (Fox and Windsor, 1970).

The concentrations of some amino acids in the reaction mixtures, as determined by amino acid analysis, are shown in Table I. Glycine, alanine, isoleucine, leucine, threonine, serine, aspartic acid and glutamic acid were the main amino acids obtained. At temperatures 374 and 400 °C, sarcosine, β-alanine, β-aminobutyric acid, γ-aminobutyric acid and ornithine were also revealed.

Amino acids synthesis and oligomerization take place under the same temperature and pressure conditions, and so some peptides with a random composition may be formed.

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