Comparative Study on Kinetics of Nonenzymatic Deamidation of Soy Protein and Egg White Lysozyme

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The kinetics of the deamidation reactions of soy protein and lysozyme followed an apparent first-order reaction. The deamidation rate for lysozyme at pHs above 5 was higher than that of soy protein. This may be because lysozyme has more Asn than Gln, while soy protein has more Gln than Asn. At pH 3, there was not much difference between the two proteins regarding their deamidation rates. This suggested that protein deamidation for both proteins may be more structurally sensitive at neutral or base conditions due to a succinimide formation mechanism. Under acidic conditions, it was believed that the deamidation of both proteins went via a direct hydrolysis pathway.

INTRODUCTION

The effect of chemical and enzymatic deamidation of food proteins on functional properties of proteins recently has been of great interest to the food industry (Shih, 1987, 1990, 1991). Vegetable proteins such as soy protein and wheat protein are usually rich in glutamine (Gln) and asparagine (Asn). Whitaker (1977) reported that up to one-third of the total amino acids of cereal proteins is glutamine. These glutamine and asparagine residues can be either enzymatically or chemically hydrolyzed to glutamic acid and aspartic acid. The deamidated protein has a lower isoelectric point and, therefore, its solubility increases in many mildly acidic food systems (Finley, 1975). It has been reported that levels of deamidation as low as 2–6% could enhance the functional properties of proteins (Matsudomi et al., 1985; Hamada and Marshall, 1989).

Nonenzymatic deamidation of proteins recently has been shown to occur during food processing such as twin-screw extrusion of wheat flour by Izzo et al. (1993a). They reported that increases in temperature and feed moisture enhanced deamidation and that deamidation was also favored at extremes of pH conditions.

The deamidation reaction during food processing also may have significant effects on the flavor and color development of foods. The ammonia molecule produced from the deamidation reaction can enter into a series of reactions resulting in its combination with various sugar degradation products and leading to the formation of amino carbonyls which produce flavor compounds such as pyrazines (Izzo et al., 1993b). These compounds have been widely studied and exhibited very important toasted, baked, or nutty notes in foods.

It is, therefore, important to understand the kinetics and mechanism of nonenzymatic deamidation during food processing. Several peptide model systems have been used to study the effects of pH, temperature, ionic strength, buffer solution, peptide composition, and sequence on the rate of nonenzymatic deamidation. However, data on protein deamidation are lacking (Robinson and Rudd, 1974). Knowledge of protien deamidation will be important for the development of a proper method to control the degree of deamidation for producing food products of desired properties.

It was the purpose of this paper to compare the kinetics of the deamidation reaction of soy protein with that of lysozyme at a wide range of pH conditions. Soy protein was chosen because of its high content of glutamine and asparagine. Lysozyme was chosen due to its well-established structure and sequence (Canfield, 1963; Blake et al., 1967; Hermann et al., 1970). Also, the lysozyme and soy protein have very different isoelectric points (Hamaguchi and Hayashi, 1972).

EXPERIMENTAL PROCEDURES

Material. Soy protein isolate (ARDEX DHV) was purchased from Archer Daniels Midland Co. (Decatur, IL). The protein content of the isolate was about 91.5% (oven dry basis) with 0.2% fiber content and 6.0% moisture content (wet basis). Ammonium chloride standard solution (0.1 M) and ionic strength adjustment solution for electrode ammonia determination were purchased from Orion Research, Inc. (Boston, MA). Deionized water was used for all reaction mixtures, and freshly distilled water was used in all of the ammonia determinations.

Lysozyme (L-6876) from chicken egg white (3× crystallized, dialyzed, and lyophilized powder) was purchased from Sigma Chemical Co. (St. Louis, MO). It was stored in a moisture barrier bottle under freezing temperatures prior to use.

Ammonia Determination. An electrode method was used for ammonia determination (Shih, 1990). A 4-mL portion of the deamidation reaction mixture was added to a centrifuge tube that contained an equal volume of 10% trichloroacetic acid (TCA). The mixture was well shaken to make sure all ammonia generated during deamidation dissolved into a TCA solution and all soluble protein precipitated. The mixture was then centrifuged. Five milliliters of clear solution from the centrifugation sample was diluted to 100 mL with distilled water before it was analyzed by an ammonia ion-selective electrode (Orion Research). A calibration curve was prepared using standard ammonium chloride solutions (10^{-6}–10^{-2} M).

A similar procedure was used for lysozyme deamidation; i.e., TCA was used to precipitate all soluble lysozyme. However, a special technique was used for sample preparation from some deamidated lysozyme samples at pH 9 and from all deamidated samples at pH 11. At pH 9.0 lysozyme started to precipitate and some deamidated samples formed a gel. The gel was chopped manually into small pieces, and 4 mL of gel (by measuring the total volume increase in TCA solution) was transferred to a centrifuge tube which contained a 4-mL TCA solution. The mixture was shaken for 5 min with a Vortex Genie 2 shaker (Scientific Industries, Bohemia, NY) to ensure that all ammonia in the gel was extracted into the TCA solution, and then the sample was centrifuged and the clear solution analyzed using the procedure described for soy protein samples. At pH 11, most lysozyme precipitated and formed a milk-like suspension because the isoelectric point for lysozyme is around this pH. The deamidated lysozyme at this pH became a foam-like solid which was squeezed manually with a stirring bar to release the clear

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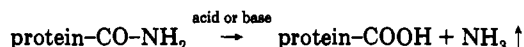
solution which contained the released ammonia during the deamidation reaction. A 4-mL clear solution was used for ammonia determination.

Complete Deamidation of Soy Protein and Lysozyme. To determine the total nonpeptide amide content of soy protein isolate, the isolate was subjected to total deamidation. Five grams of soy protein were suspended in 100 mL of 2 N HCl, and the mixture was refluxed for 3 h, after which time the total ammonia released was measured. Lysozyme had a much higher solubility, and 5 g of lysozyme was dissolved completely in 100 mL of 2 N HCl solution.

Conditions for Kinetic Studies. The following conditions were used for kinetic studies: pH for the reaction mixture, 3.0, 5.0, 7.0, 9.0, 11.0; temperature, 100, 115, 130 °C; time for sampling, 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 h. One gram of soy protein or lysozyme was suspended or dissolved in 17 mL of distilled water, and the pH was adjusted to the required value. The mixture was then transferred into an 18-mL Kimax brand glass tube with a black phenolic screw cap in which there is a PTFE-faced temperature- and pressure-resistant rubber liner (Fisher Scientific, Piscataway, NJ). To further prevent ammonia vapor loss, an additional 1 mm thick and temperature-resistant PTFE-faced silicone rubber flat disk septa (Fisher Scientific) was placed into the cap to secure the airtightness of the reaction tube. The reaction tubes were then placed in an oil bath at a required constant temperature. The tubes were regularly shaken to ensure uniform reaction of the heterogeneous protein suspension; samples were withdrawn at required intervals, and then the reaction was stopped using ice water. All kinetic studies were carried out in duplicate, and all samples were tightly capped and stored in a freezer before ammonia analysis was conducted. The data were analyzed using a linear regression.

Kinetic Calculation. *a. Determination of Reaction Order by Integration.* The following reaction can be representative for the protein deamidation reaction:



$$\text{protein-CO-NH}_2 \xrightarrow{\text{acid or base}} \text{protein-COOH} + \text{NH}_3\uparrow$$

Let R_A be the deamidation rate; R_A can be expressed as

$$R_A = d(C_A)/dt = kC_A^{\,n}$$

where C_A is the remaining amide concentration (mmol/g of protein) at time t (h), C_{A0} (mmol/g of protein) is the initial total amide concentration at time zero for the deamidation reaction and was determined according to the procedure described under Complete Deamidation of Soy Protein and Lysozyme, t is the total reaction time after the reaction started, and k is the reaction rate constant corresponding to each reaction order at the temperature of the reaction.

Using the integration method, the above equation can be converted to the following equations for the individual reaction order:

$$\text{zero order:} \quad C_{A0} - C_A = kt$$

$$\text{first order:} \quad \ln(C_A) - \ln(C_{A0}) = -kt$$

$$\text{second order:} \quad 1/C_A - 1/C_{A0} = kt$$

By plotting reaction time with C_A, $\ln(C_A)$, and $1/C_A$, respectively, the reaction order can be thus obtained from the plot that has the best linear relation.

b. Calculation of the Reaction Rate Constant (k) and Deamidation Half-Life ($t_{1/2}$). When the reaction rate law was obtained by the above-mentioned integration method, the reaction rate constant was calculated from the slope of the linearized rate law equation. The time required to have 50% of the total amide group deamidaated was named "half-life" for those deamidation kinetic studies, which was solved from the obtained law equation by substituting the reaction rate constant, k, with measured value and C_A with $0.5C_{A0}$, respectively.

RESULTS AND DISCUSSION

Figures 1–5 show that the overall deamidation reactions of both lysozyme and soy protein followed an apparent first-order reaction. The R^2s of first-order linear regression ranged from 0.985 to 0.995 for lysozyme and from 0.972 to 0.992 for soy protein. It was interesting to note that while the slope of each protein was different at different pHs, the reaction order did not change with pH. Also, the differences of slopes between two proteins increased as the pH increased. Since the slopes in Figures 1–5 related the reaction rate constants, the differences in slopes for two proteins over a wide range of pHs indicated that these two proteins showed very different deamidation rate constants.

Although the kinetics of protein deamidation have not been studied previously, many peptide model systems have

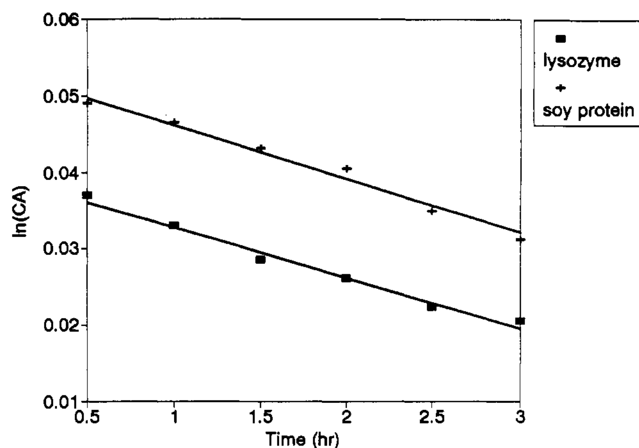


Figure 1. First-order rate plot for lysozyme and soy protein at a pH of 3 and a temperature of 100 °C.

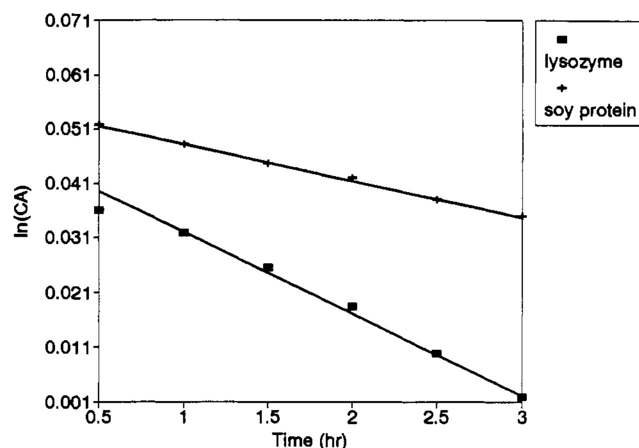


Figure 2. First-order rate plot for lysozyme and soy protein at a pH of 5 and a temperature of 100 °C.

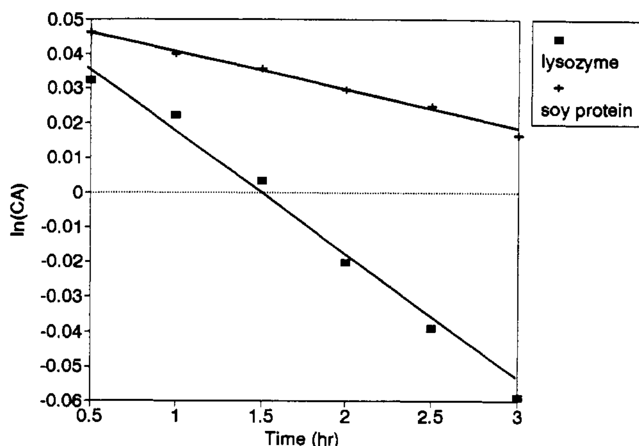


Figure 3. First-order rate plot for lysozyme and soy protein at a pH of 7 and a temperature of 100 °C.

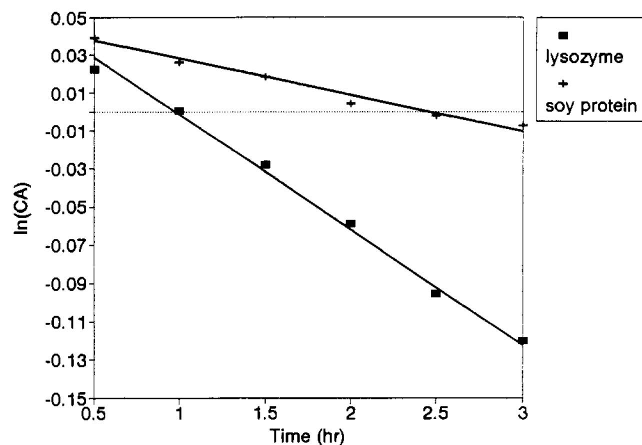


Figure 4. First-order rate plot for lysozyme and soy protein at a pH of 9 and a temperature of 100 °C.

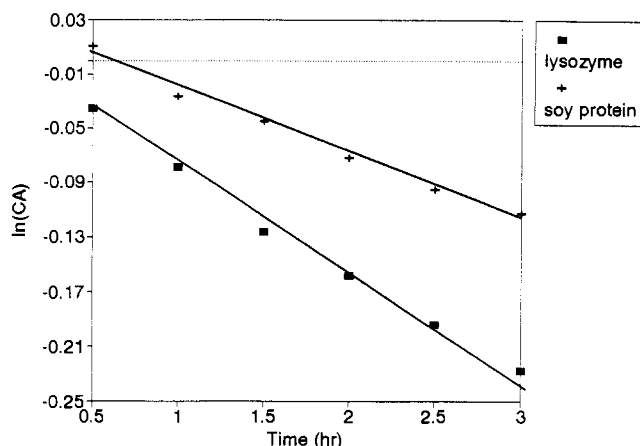


Figure 5. First-order rate plot for lysozyme and soy protein at a pH of 11 and a temperature of 100 °C.

been used for deamidation studies. Patel and Borchardt (1990a,b) observed a pseudo-first-order deamidation of several Asn peptides at a concentration of 0.0002 M, and Scotchler and Robinson (1972) reported a first-order deamidation of Gln pentapeptides at a concentration of 0.001 M. The 5% protein solution used in this study was calculated to be 0.0004 M in protein and 0.0064 M in terms of amide concentration, which was higher than the literature values.

Figure 6 shows that the deamidation rate constants for lysozyme at pHs above 5 were higher than those of soy protein. Several reasons, such as the difference in molecular weight, molecular shape, solubility, primary sequence, and three-dimensional structure between the two proteins, could contribute to the observed phenomena. For example, lysozyme has a much lower molecular weight than that of soy protein and is soluble under most pHs, while soy protein is only partially soluble under all pHs. The insolubility of soy protien may be an important factor contributing to its smaller deamidation rate constants. The sequence and conformation of soy protein is not known; therefore, it is not possible to understand exactly how the conformation of soy protein may affect its deamidation.

One factor that could explain the different deamidation rate constants between lysozyme and soy protein is their difference in Asn and Gln content. Lysozyme has more Asn than Gln (13% Asn; 3% Gln) (Hamaguchi and Hayashi, 1972), while soy protien has more Gln than Asn (18% Gln; 10% Asn) (Rackis et al., 1961). Robinson et al. (1974) observed that the deamidation of Asn was faster than that of Gln in model peptides of comparable or similar

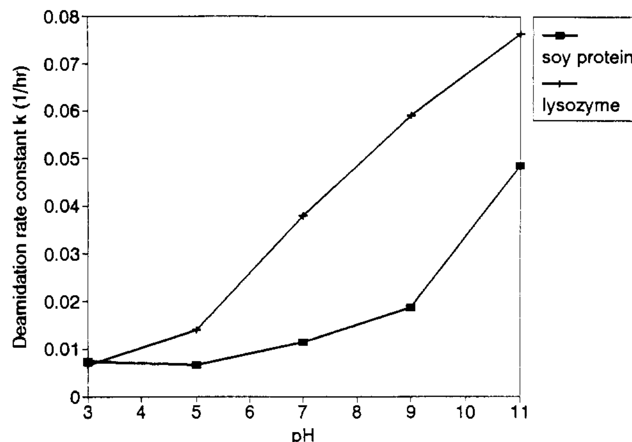


Figure 6. Comparison of deamidation rates of soy and lysozyme protein over a wide range of pHs.

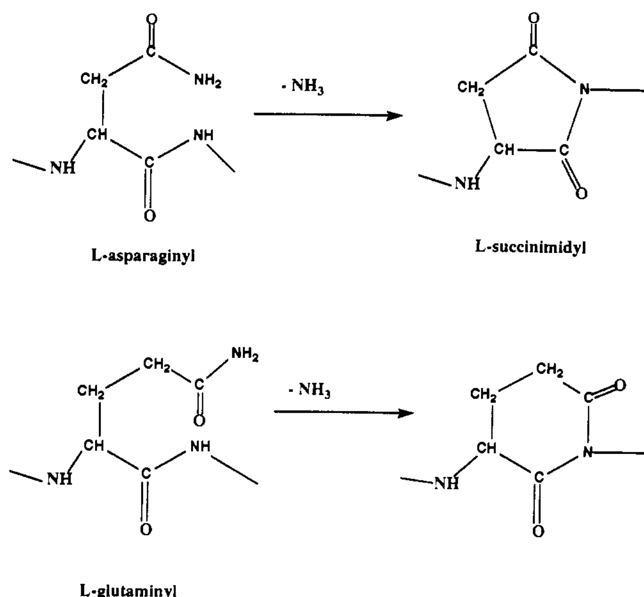


Figure 7. Deamidation of Asn and Gln via ring formation mechanisms.

sequence. On the bases of a model study, Wright and Robinson (1982) proposed that the difference between Gln and Asn deamidation was due to the greater distance from the adjacent peptide amide NH groups to the Gln side-chain amide group than that of Asn (Figure 7). As hydrogen donors, the NH groups could stabilize the tetrahedral oxyanion transition state of Asn during succinimide ring formation. On the other hand, the additional one methylene group of the Gln side chain makes the distance from these NH groups to the negatively charged oxygen of the oxyanion too long to form good hydrogen bonds.

Figure 6 shows that at pH 3 not much difference was observed between the two proteins regarding the deamidation rate. This indicated that protein deamidation for both proteins was more structurally sensitive at neutral or base conditions due to a succinimide formation mechanism. Bhatt et al. (1990) and Patel and Borchardt (1990a,b), using a hexapeptide model system, confirmed that deamidation of the Asn-Gly sequence in the peptide at neutral to alkaline pHs involved a cyclic imide intermediate which was hydrolyzed to yield the isoAsp and Asp peptides. They, however, reported that deamidation of the peptide at acidic conditions involved mainly the direct hydrolysis of the side chain rather than the formation of a cyclic imide intermediate. Furthermore, they found that the primary sequence did not change the overall

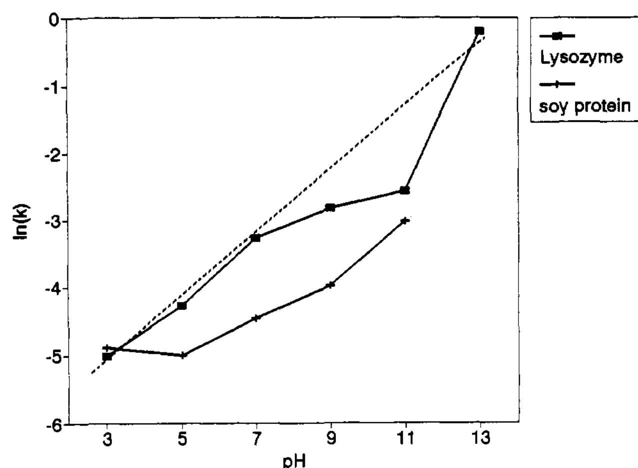


Figure 8. Retarding effects of protein insolubility at the isoelectric point on the rates of protein deamidation.

pathway of deamidation. However, they reported the primary sequence could influence the deamidation rates, the deamidation product ratio, and the tendency of peptides to undergo side reactions (e.g., attack of the Asn side chain on the peptide bond carbonyl resulting in peptide bond cleavage) as well as to form a cyclic imide intermediate at neutral to alkaline pHs. Under acidic conditions, the primary sequence did not appear to affect the deamidation rate or the exclusive formation of the Asp peptides as the deamidation product. The findings in their study were supportive of our observations; i.e., at pH 3 it was believed that the deamidation of both proteins went via a direct hydrolysis pathway. Therefore, the protein's primary sequence and its three-dimensional structure had limited effects on the deamidation rate as compared to the ring formation mechanism.

The logarithm of reaction rate constants of two proteins was plotted in Figure 8 with a pH profile. A 0.9 R^2 was obtained by regressing ln(k) and pH for reaction rates at pH 3, 5, 7, and 13 for lysozyme (dotted linear line). This indicated that the deamidation of lysozyme was first-order in relation to hydroxide ion concentration at these pHs, while no linear relationship was observed for soy protein over a wide range of pHs. The deviation from a linear relationship at pH 9 and 11 for lysozyme was a clear indication that the structural changes under these two pHs as observed during the investigation had retarding effects for the deamidation reaction, even though the reaction rates at these two pHs were still much higher than that at pH 7 or lower. The gel formation and aggregation of lysozyme at pH 9 and 11 caused the lysozyme to have a relatively fixed three-dimensional structure, which might be the cause of the retarding effects. Hamaguchi and Hayashi (1972) reported that the lysozyme in a solution at a pH of 5–9 was a self-associating system of the monomer–dimer, while at higher pHs polymers larger than the dimer appeared. Their finding is consistent with our observations.

Lysozyme is known to denature at pHs above 12 (Tanford and Wagner, 1954; Donovan et al., 1959; Ikeda and Hamaguchi, 1969). In the present study the lysozyme was a clear solution with a little yellow color at pH 13, both before and after deamidation reaction for up to 3 h. If the lysozyme was denatured at pH 13, it seemed that the denaturation did not affect the hydroxide ion concentration dependence of the deamidation rate.

Geiger and Clarke (1987) calculated the half-time of three Asn hexapeptides to range from 0.15 to 7.5 h at pH 7.7 at a temperature of 100 °C and reported that the neighboring group of Asn had a considerable influence on

Table I. Half-Life of the Deamidation Reactions of Soy Protein and Lysozyme at 100 °C As Influenced by pHs

pH	$t_{1/2}$ (h) soy protein	$t_{1/2}$ (h) lysozyme
3.0	92.4	103.8
5.0	103.4	49.9
7.0	60.3	18.3
9.0	36.9	11.7
11.0	14.3	9.1
13.0	a	0.8

[a] Experiment was not conducted at this pH for soy protein.

deamidation half-times. Lysozyme and soy protein were 18.3 and 60.3 h, respectively, under similar conditions (Table I). This indicated that the deamidation rates reported for peptides in the literature were higher than those for both the soy protein and the lysozyme. Patel and Borchardt (1990a) speculated that the rates of deamidation of some hexapeptides used in their studies probably represented the maximum rate of deamidation to be expected in proteins, assuming that side-chain participation does not significantly accelerate the reaction.

The results obtained by studies on small peptides under physiological circumstances cannot be straightforwardly applied to proteins, especially under food-processing conditions. The deamidation reaction at the specific site in a protein is expected to be dependent also on the local conformation and flexibility of the polypeptide (Capasso et al., 1989). During deamidation the protein structure would be expected to change due to heat effect under acidic and basic conditions, and the structural change would affect the deamidation rate. On the other hand, deamidation removes amides, generates acidic side chains, and changes the protein charge conditions, which would also cause the protein to alter its secondary and teritary structures. Matsudomi et al. (1982) reported that deamidation of gluten might induce conformational changes by increasing its electrostatic repulsion and decreasing the hydrogen bonding. Also, they found that the helix content and surface tension of gluten decreased as deamidation degree increased. Comparing the deamidation half-times of two proteins in the present study with those for peptides in the literature, the proteins studied were only $^1/_3$ to $^1/_{10}$ as fast as in the peptides and much faster than that of acetamide.

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