Quantification of Racemization of Amino Acids in Alkaline-Treated Duck Eggs by Micellar Capillary Electrophoresis

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Duck eggs were pickled in 4.2% NaOH/5% NaCl solution for 20 days to prepare the traditional Chinese Pidan. The extent of racemization of compositional amino acid in egg albumen and yolk over the alkaline pickling period was investigated with micellar capillary electrophoresis (MCE) using β-cyclodextrin as chiral selector. The racemization value of amino acids in egg albumen was in the order serine > aspartic acid > glutamic acid > phenylalanine > leucine > valine > threonine = isoleucine, whereas the order in egg yolk was aspartic acid > glutamic acid > phenylalanine > leucine > valine. Therefore, the tendency of amino acid racemization appeared to be closely related to the properties of its residual side chain, as well as the pH and alkaline treating period. Moreover, racemization of most of the amino acids was remarkably induced by the alkaline treatment during the initial pickling period.

Keywords: *Alkaline treatment; duck egg; racemization value;* DL*-amino acid; micellar capillary electrophoresis (MCE)*

INTRODUCTION

Alkaline treatment is a common process for protein extraction, processing of textured protein foods, and preparation of plant protein concentrates (Ma, 1983). However, it generally causes exceptional formation of lysinoalanyl and lanthionyl residues, racemization and degradation of amino acid residues, and Maillard reactions, especially when the protein-containing foods are thermally treated during the processing (Masters and Friedman, 1979; Achor et al., 1981; Friedman and Masters, 1982; Liardon and Hurrel, 1983). Moreover, most of these reactions are the main causes of nutritional losses (Friedman et al., 1984).

Friedman and Liardon (1985) have pointed out that the factors affecting racemization are pH, alkaline treating time, and heating temperature, as well as the sources of protein samples. Casein has been treated with alkali, and the degree of racemization was observed to increase with both increasing alkaline concentration and increasing reaction temperature. At the initial stages of alkaline treatment, a higher degree of racemization resulted from higher protein denaturation rates (Friedman and Masters, 1982). Furthermore, racemization of free amino acids has been observed to be more less significant than that of amino acid residues in the proteins (Liardon and Ledermann, 1986). The racemization of compositional amino acid residues in proteins not only lowered the nutritional value of proteins but decreased the protein digestibility as well (Friendman et al., 1984). Thus, chiral analysis of amino acids is of particular interest to the food industry.

Micellar capillary electrophoresis (MCE), based on the differential partitioning of analytes between the micelle and the surrounding aqueous phase (Terabe et al., 1989, 1993), has been reported to be effective in the separation of enantiomers (Ozaki et al., 1995; Tsai et al., 1998). Enantiomeric separation can be achieved by using a chiral environment that interacts with the enantiomers, either before or during the separation process, forming stable diastereoisomers or labile diastereomeric complexes, respectively.

Pidan, alkali-treated duck eggs, are quite popular in Taiwan, and are usually prepared by pickling the eggs in a 4.2% NaOH/5.0% NaCl solution at ambient temperature for 20 days (Su and Lin, 1994). Although the racemization of amino acids in proteins, such as casein and soy proteins, has been intensively studied, DL-amino acid residue formation in alkali-treated eggs has rarely been reported. In the previous research (Tsai et al., 1998; Chang et al., 1998), optimal separation systems for free DL-amino acids have been established using β- or γ-cyclodextrin as the chiral selector.

The objective of this study is to investigate and compare the racemization value and the racemization rate constant of compositional amino acid in egg albumen and yolk during the alkaline pickling period. The first phase of the study determined the rates of racemization of amino acid residues in egg albumen and yolk. Subsequent studies were conducted to evaluate the racemization values of amino acid residues in both egg albumen and yolk during the alkaline pickling period.

MATERIALS AND METHODS

Materials. Fresh duck eggs purchased from a local supermarket were pickled in a 4.2% NaOH/5.0% NaCl solution at ambient temperature for up to 20 days. Five eggs were collected each time at 3-day intervals during this period. Egg albumen and yolk were separated carefully with a net, and the pH values of these substances were instantly reduced with 1 N acetic acid to their respective normal pH level (8.95 and 5.84, respectively). The samples thus obtained were dialyzed against 100 volumes of deionized water for 3 days in a cold room (4 °C) and were then freeze-dried (Eyela FD-5N, Rikakikai Co., Japan). The pH of egg albumen and yolk were determined according to an AOAC (1984) method.

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Chemicals. Dansyl chloride [5-(dimethylamino)naphthelene-1-sulfonyl chloride), boric acid, β- and γ-cyclodextrin (CD), 2-mercaptoethanol, and all D- and L-amino acids were purchased from Sigma (St. Louis, MO), whereas sodium dodecyl sulfate (SDS) was purchased from Bio-Rad (Richmond, CA). Sodium hydrogen carbonate, sodium hydroxide, methanol, acetone, and acetic acid were purchased from E. Merck (Darmstadt, Germany).

Protein Hydrolysis. Freeze-dried protein powders were hydrolyzed with 6 N HCl under vacuum at 110 °C for 24 h. The removal of HCl from hydrolysates was conducted by vacuum evaporation. The dried hydrolysates were then dissolved in adequate volumes of distilled water and were subsequently applied to a capillary electrophoresis instrument to detect the racemization of amino acids after the derivatization of dansyl chloride.

Apparatus and Electrophoretic Conditions. All of the experiments were carried out on a capillary electrophoresis instrument P/ACE system 5500 (Beckman, Palo Alto, CA), equipped with a diode array detector monitoring a wavelength of 254 nm. An uncoated fused silica capillary (Beckman, total length 47 cm, effective length 40 cm, i.d. = 75 μm) was pretreated successively with 0.1 M hydrochloric acid and 0.1 M sodium hydroxide for 10 min each and then rinsed with deionized water and background electrolyte (BGE) solution prior to use. The separation column was kept at a constant temperature of 25.0 ± 0.1 °C by means of a fluorocarbon liquid continuously circulated through the cartridge, and the applied voltage was 15 kV. Sample introduction was performed using the pressure option for 5 s. Data collection was carried out with the Gold Chromatography data system version 8.1.

The BGE compositions were determined to be 200 mM SDS/75 mM β-CD/250 mM borate buffer solution (pH 9.5) for DL-valine, -glutamic acid, -aspartic acid, -isoleucine, -leucine, and -phenylalanine (Tsai et al., 1998); 200 mM SDS/75 mM β-CD/30% methanol/250 mM borate buffer solution (pH 9.5) for DL-serine and -threonine (Chang et al., 1998); 100 mM SDS/75 mM β-CD/250 mM borate buffer solution (pH 9.5) for DL-arginine; and 100 mM SDS/75 mM β-CD/200 mM acetate buffer solution (pH 5.0) for DL-histidine. All of the BGE products were filtered through a 0.45 nm membrane prior to use. Deionized water was obtained from a Milli-Q system (Millipore, Japan).

Calibration curves for quantification of amino acids were prepared using samples of known concentration ($r^2 = 0.9995-0.9999$).

Derivatization of Amino Acids. Dansyl chloride was used to derivatize the DL-amino acids according to the method described by Nergo et al. (1987). Usually, 100 mL of 500 mM $NaHCO_3$ in deionized water and 100 mL of 20 mM dansyl chloride in acetone were added to 10−40 mg of free amino acid dissolved in 100 mL of deionized water in a screw-capped Pyrex tube. The samples were reacted in darkness for 40 min at 65 °C. Dansyl chloride solution was freshly prepared for each batch.

Determination of Racemization Rate. Amino acid racemization rate was determined according to the following equation (Liardon et al., 1981):

$$2tk = \ln(1 + D/L)/(1 - D/L)$$

where t is alkali-treating time, k is the racemization rate constant, and D/L is the ratio of D-amino acid to the corresponding L-amino acid.

RESULTS AND DISCUSSION

Change of pH during Pickling Period. Duck eggs were pickled in NaOH and salt solution, and the progressive changes of pH in the egg albumen and yolk were determined. As shown in Figure 1, pH values increased sharply during the initial pickling stage; the pH of the egg albumen and yolk increased from the original 8.95 and 5.84 to the final 12.28 and 10.12, respectively, after the total 20-day pickling period.

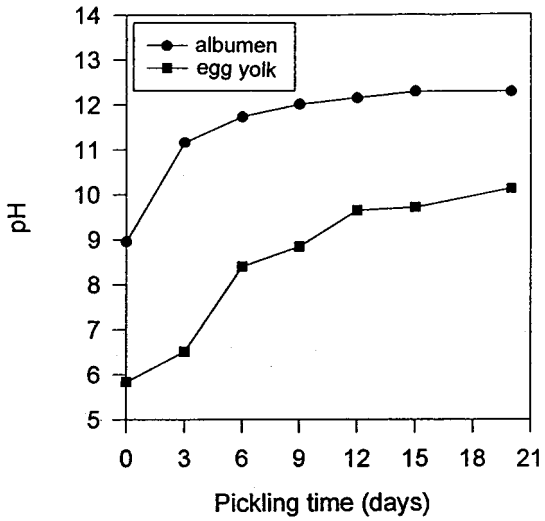

Figure 1. Change of pH values in duck egg albumen and yolk during alkaline pickling period. Pickling solution: 4.2% NaOH/5.0% NaCl solution.

Racemization of Amino Acids. Protons are removed from the asymmetric carbons of the amino acids and negatively charged carbanions are formed when proteins are treated with alkaline. However, when carbanions are bound with protons, D- and L-enantiomers are equally formed and the theoretical racemization value (amount of D-amino acid/amounts of D- and L-amino acids) is 50% (Master and Friedman, 1979; Friedman and Master, 1982; Liardon and Hurrel, 1983; Friedman and Liardon, 1985; Jenkins et al., 1984). Amino acids such as aspartic acid, phenylalanine, glutamic acid, leucine, serine, and valine are liable to cause racemization during alkali treatment (Masters and Friedman, 1979). Enantiomeric separations of Dns-DL-amino acids obtained from hydrolyzed Pidan egg albumen (Figure 2) and yolk (Figure 3) were conducted using 200 mM SDS/75 mM β-CD/250 mM borate as BGE. Enantiomers of Dns-DL-valine, -glutamic acid, -aspartic acid, -isoleucine, -leucine, and -phenylalanine from both egg albumen and yolk were also separated as well, and the resolution values (Rs) were all larger than 1.0, as reported previously (Tsai et al., 1998). Enantiomers of Dns-serine and -threonine from Pidan egg albumen (Figure 4A) and yolk (Figure 4B) were separated with 200 mM SDS/75 mM β-CD/30% methanol/250 mM borate (pH 9.5) as BGE. The formation of D-serine in egg albumen alkali-pickled for 20 days was extremely high, although it was not observed in the yolk. The separated DL-amino acids from duck egg albumens and yolk hydrolysates sampled at various pickling periods were then quantified and the racemization values were calculated. As shown in Table 1, racemization values of aspartic acid and serine in egg albumen were detected to be 16.33 and 11.48%, respectively, after the first 3-day pickling period. It progressed successively and remarkably with the increased pickling time to 35.74% for albumen and 41.81% for yolk after the full 20-day pickling (Table 1A). However, D-enantiomers of glutamic acid, aspartic acid, and leucine were all detected in the fresh egg albumen, which could have been induced by the acid (6 N HCl) hydrolysis during thermolysis process (Paquet and Ma, 1989). The compositional amino acids which formed D-enantiomers in

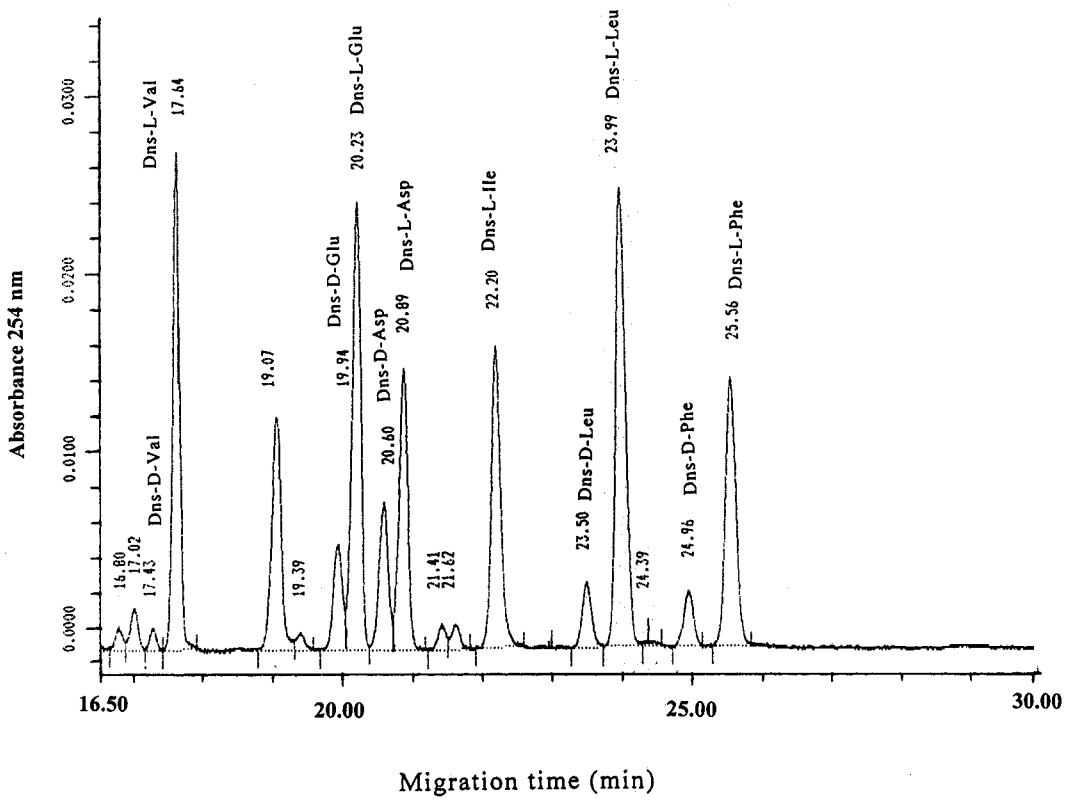

Figure 2. Enantiomeric separations of amino acids in egg albumen alkali-pickled for 20 days. Amino acids were dansyl derivatized before application to separation. Separation solution: 200 mM SDS/75 mM β-CD/250 mM borate buffer (pH 9.5).

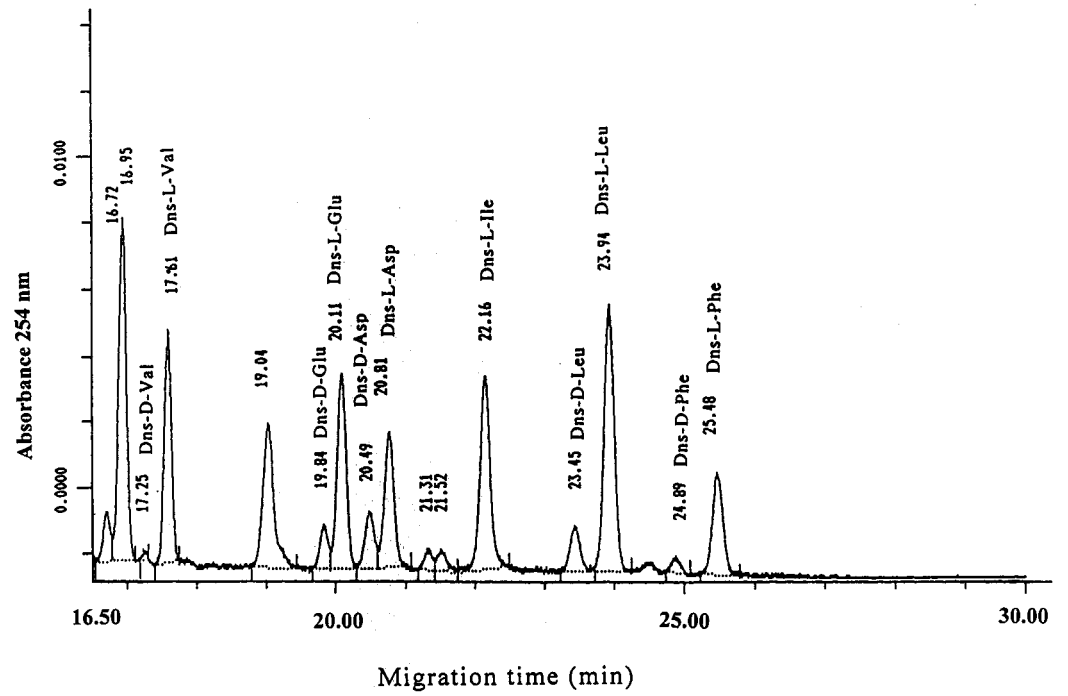

Figure 3. Enantiomeric separation of amino acids in yolk alkali-pickled for 20 days. Amino acids were dansyl derivatized before application to separation. Separation solution: 200 mM SDS/75 mM β-CD/250 mM borate buffer (pH 9.5).

duck egg albumen after the 20-day pickling period were serine, aspartic acid, glutamic acid, phenylalanine, leucine, and valine (Table 1A). Formation of racemization during the alkaline treatment has previously been shown to be closely related to the residual side chain of amino acid and source of protein during the alkaline treatment (Paquet and Ma, 1989; Liardon and Friedman, 1987). Racemization values of four alkali-treated proteins (casein, soy globulin, wheat gluten, and lactoglobulin) were previously determined, with those of phenylalanine, alanine, aspartic acid, and glutamic acid reported to be significant, whereas those of leucine, valine, proline, serine, and threonine were insignificant or of trace levels (Masters and Friedman, 1979). Bunjapamai et al. (1982) and Schwass and Finley (1984) have also indicated that serine showed the highest

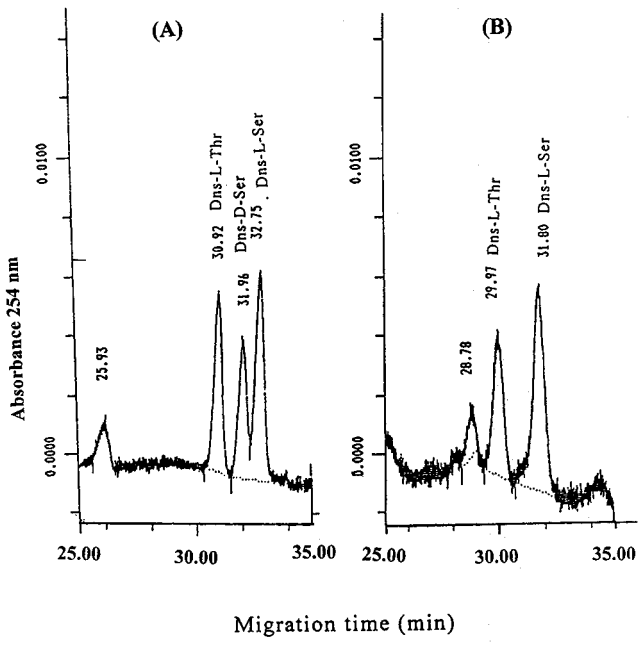

Figure 4. Electropherograms of racemization of serine and threonine in egg albumen (A) and yolk (B) alkali-pickled for 20 days. Amino acids were dansyl derivatized before application to separation. Separation solution: 200 mM SDS/75 mM β-CD/30% methanol/250 mM borate buffer (pH 9.5).

Table 1. Effect of Alkali-Treating Time on the Racemization Values[a] of Amino Acids in Egg Albumen (A) and Yolk (B) during the Alkaline Pickling Period[b]

amino acid	racemization values[a,c] (%) after alkali treatment of						
	0 days	3 days	6 days	9 days	12 days	15 days	20 days
				(A) Egg Albumen			
Val	0.00	0.00	1.94	2.02	2.99	3.41	4.09
Glu	2.93	7.04	8.36	12.16	12.97	17.57	19.92
Asp	9.01	16.33	18.76	20.37	24.32	28.42	35.74
Ile	–[d]	–	–	–	–	–	–
Leu	1.81	5.90	6.90	8.82	9.81	11.05	13.56
Phe	0.00	5.52	5.65	7.41	10.02	11.24	16.44
Ser	0.00	11.48	26.81	28.82	33.27	35.70	41.81
Thr	–	–	–	–	–	–	–
				(B) Egg Yolk			
Val	0.00	0.00	0.00	0.00	0.00	3.02	3.92
Glu	2.01	6.81	7.02	9.54	10.09	13.10	17.92
Asp	5.87	15.92	15.99	16.65	17.79	20.20	29.78
Ile	–	–	–	–	–	–	–
Leu	1.76	4.99	6.01	7.49	8.20	10.38	12.18
Phe	0.00	0.00	0.00	0.00	0.00	9.86	14.13
Ser	–	–	–	–	–	–	–
Thr	–	–	–	–	–	–	–

[a] Racemization value = $100D/(D + L)$, where D and L are the amounts of D- and L-amino acids, respectively. [b] Separation conditions: 200 mM SDS/75 mM β-CD/250 mM borate (pH 9.5) for Dns-DL-valine, -glutamic acid, -aspartic acid, -isoleucine, -leucine, -phenylalanine; 200 mM SDS/75 mM β-CD/30% methanol/250 mM borate (pH 9.5) for Dns-DL-serine and -threonine. [c] Each value is an average of triplicate samples. [d] –, not detectable.

racemization value in alkali-treated leaf protein concentrate, soy protein isolate, and lactalbumin. The proposed explanation for this was due to the speedy removal of α-hydrogen to form carbanion (Smith and Silva de Sol, 1980). Therefore, serine was considered to be a useful indicator for detecting racemization in the alkali-treated proteins. The higher racemization level of aspartic acid is due to the action of the β-carboxyl group, which forms a penta-ring structure and thus enhances the removal of the α-hydrogen, leading to the speedy formation of carbanions (Liardon and Ledermann, 1986). A similar result with casein was reported by Friedman and Master (1982).

Consistent with the results in the present study, remarkable racemization of aspartic acid and serine in the heat- and alkali-treated bovine plasma albumin and chicken meat was found by Liardon and Hurrell (1983). The racemization values of compositional amino acids in duck egg yolk (Table 1B) after the 20-day pickling period were compared. These values were all remarkably lower than those of amino acids in egg albumen. The cause of the racemization of valine and phenylalanine in the yolk was observed during the later stage of alkaline treatment (after a 15-day pickling period), probably owing to their low pH sensitivity (Table 1B). The D-enantiomer of serine, which was formed in large quantity in alkali-treated egg albumen (Table 1A), was not detected in the alkali-treated yolk proteins (Table 1B). A reasonable explanation for this would be due to the approximately one-third content of fat in yolk and to the hydrolysis of most of the serine residues which belong to phosvitin to form dehydroalanine through β-elimination during the alkaline pickling period (Friedman et al., 1984). The final pH of yolk was only 10.12, lower than that of egg albumen (pH 11.15) pickled for only 3 days. However, aspartic acid displayed a high racemization value (29.78%) in such a low pH (Table 1B). Thus, the racemization value of each amino acid in protein is considered to be dependent on the protein source, coexistent substances (such as lipids that coexist with proteins in yolk), and sensitivity to pH (the removal speed of α-hydrogen to form carbanion).

Racemization Rate. Glutamic acid, phenylalanine and leucine (Figure 5A) and serine and aspartic acid (Figure 5B) in duck albumen all showed higher racemization rates over the alkali-pickling period. The higher racemization rate of these amino acids was considered to be a result of their higher pH sensitivity, increasing carbanion formation, as well as the high degree of protein hydrolysis induced by the alkaline treatment (Liardon and Friedman, 1987). However, valine exhibited a much lower racemization rate (Figure 5A), and threonine and isoleucine did not show any racemization during the 20-day pickling period (Table 1A).

The racemization rate of amino acids in yolk showed almost the same trend as that of amino acids in egg albumen, except that of serine. Alkali-treating time and alkaline concentration in pickling solution were considered to be the major factors affecting the final pH, the conformation, and the degree of protein hydrolysis of alkali-treated products. Friedman and Liardon (1985) have reported that the racemization rate of each amino acid in proteins was remarkably affected by the property of amino acid residue, electron-withdrawing property (σ^*), and location of the amino acid in proteins. Table 2 indicates that the racemization rate constant of amino acid varies with the pickling period. For instance, glutamic acid, leucine, and phenylalanine showed median inversion rate constants [$(22-25) \times 10^{-8}$ S^{-1}) at the first 3-day pickling; however, they decreased to $(9-13) \times 10^{-8}$ S^{-1} by the end of the 20-day pickling period, whereas serine and aspartic acid retained high racemization rates throughout the pickling period. Such variations are consistent with those reported by Friedman and Liardon (1985).

Enantiomeric separation of DL-arginine was conducted, although only L-arginine was detected, revealing

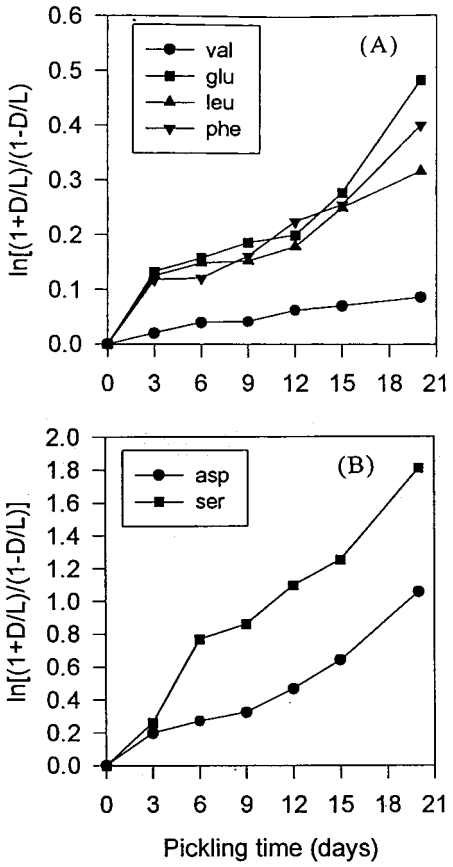

Figure 5. Racemization rate of valine, glutamic acid, leucine, and phenylalanine (A) and serine and aspartic acid (B) in egg albumen during alkaline pickling period.

Table 2. Racemization Rate of Constants[a] of Amino Acids in Egg Albumen during Alkali-Pickling Period

	rate constant ($\times 10^{-8}$ S^{-1})	
amino acid	first 3 days[b]	15−20 days[c]
Val	3.9	12.5
Glu	25.4	13.9
Asp	38.0	30.6
Leu	22.1	9.1
Phe	22.6	11.5
Ser	50.3	52.3

[a] $k = \ln(1 + D/L)(1 - D/L)/2t$, where t is alkaline treating time and D/L is the ratio of D-amino acid to the corresponding L-amino acid. [b] Values based on the racemization induced in the first 3 days. [c] Values corresponded to the slopes of the racemization curves at the longer treatment for 15−20 days.

that the D-enantiomer was not formed during the 20-day pickling period (Figure 7A). The D-enantiomer of histidine was also not detected using 100 mM SDS/75 mM β-CD/250 mM acetate buffer (pH 5.0) as BGE (Figure 7B).

Conclusion. Racemization of amino acids in Pidan egg albumen and yolk were very remarkable during the alkaline pickling period. Higher pH in egg albumen in the final products resulted in the more apparent racemization of the compositional amino acids. About 41% of serine was racemized to D-enantiomer during the 20-day pickling period. D-Enantiomers of essential amino acids, such as leucine, phenylalanine, and valine, were all determined, and the nutritional value of Pidan was supposed to be reduced. In the present study, the racemization rate was found to vary with the amino acid species and the alkaline-treating time, suggesting that

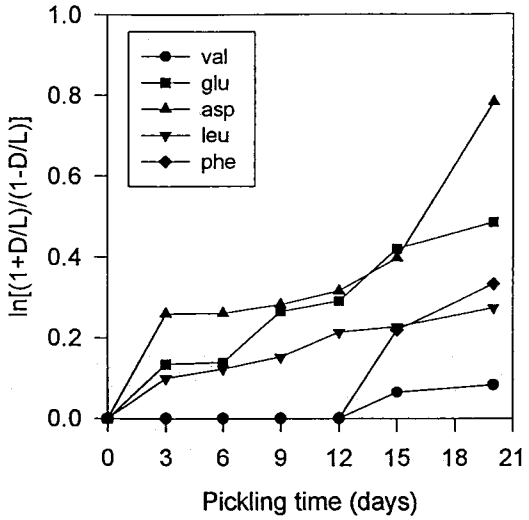

Figure 6. Racemization rate of valine, glutamic acid, leucine, phenylalanine, and aspartic acid in yolk during alkaline pickling period.

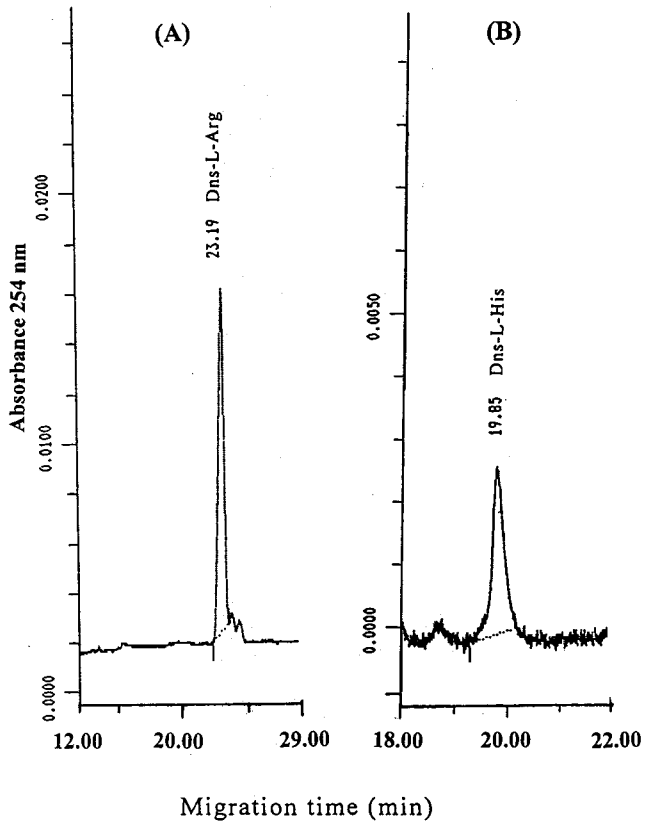

Figure 7. Electropherograms of racemization of arginine (A) and histidine (B) in egg albumen alkali-pickled for 20 days. Amino acids were dansyl derivatized before application to separation. Separation solution: 100 mM SDS/75 mM β-CD/250 mM borate buffer (pH 9.5) for arginine; 100 mM SDS/75 mM β-CD/200 mM acetate buffer (pH 5.0) for histidine.

the formation of D-enantiomers was closely related to the alkaline concentration, temperature, treating time, and position of amino acid in the proteins. The degree of protein hydrolysis could also be an important factor (Liardon and Friedman, 1987). An interesting result that was observed is that serine, which was quickly racemized in the egg albumen, did not show any D-enantiomer formation in the yolk proteins. Further investigations of the compositional changes of amino

acids and the formation of lysinoalanine during Pidan preparation are needed.

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