Changes of Amino Acid Composition and Lysinoalanine Formation in Alkali-Pickled Duck Eggs

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Duck eggs were pickled in alkali for 20 days to prepare Pidan. The extent of the degradation of compositional amino acids, the formation of lysinoalanine (LAL) in Pidan, and the relationship between the formation of LAL and the racemization values of D-serine and D-aspartic acid in Pidan albumen during the pickling period were investigated. Results showed that the remaining percentages of Cys, Arg, Lys, Ser, and Thr in albumen were much lower than that of the corresponding amino acid in yolk. The formation of LAL in albumen in the first stage was due to the speedy increase in the pH and the abundant formation of dehydroalanine (DHA) from cysteine. However, the formation of LAL in the later pickling period was related much more to the alkali-treating time than to the pH factor. Among the amino acids, cysteine was observed to be the most sensitive to alkaline and contributed mostly to the formation of LAL throughout the pickling period.

Keywords: *Alkaline treatment; duck egg; lysinoalanine; dehydroalanine; micellar capillary electrophoresis*

INTRODUCTION

Alkaline treatment is found effective in destroying toxins, such as aflatoxin, and functions of protein inhibitors and is usually used for plant protein extraction and preparations of textured protein foods, plant protein concentrates, and isolates (Ma, 1983). However, alkaline treatment causes the exceptional formations of lysinoalanine (LAL) and lanthionine, racemization of amino acids, degradation, and Maillard reactions especially when the protein-containing foods are thermally treated during the processing (Bohak, 1964; Masters and Friedman, 1979; Achor et al., 1981; Friedman and Masters, 1982; Liardon and Hurrel, 1983; Friedman and Liardon, 1985; Liardon and Ledermann, 1986). LAL and related cross-linked amino acids may be derived from the reaction of lysine with dehydroalanine (DHA) residues formed from substituted series, cystine, and cysteine residues in proteins through the nucleophilic reactions (Fletcher et al., 1963). However, the thus obtained crossing-linking product is indigestible by proteases, and the decline of digestibility of proteins and the loss of lysine are mainly the causes of nutritional losses of proteins (Friedman et al., 1984). In addition, racemization of compositional amino acids in alkali-treated duck eggs (Pidan), analyzed by micellar capillary electrophoresis, was found significant. The racemization values for serine, aspartic acid, glutamic acid, and phenylalanine in Pidan albumen alkali-pickled for 20 days were determined to be 41.84%, 35.74%, 19.92%, and 16.44%, respectively (Chang et al., 1998).

Friedman et al. (1981) and Liener (1994) have pointed out that the factors affecting LAL formation are pH, alkaline-treating time, and heating temperature as well as sources of protein samples. The formation of LAL in casein was initialized at pH 9.0, and therefore, 35% of LAL was determined when the pH was raised to 12.5. (Friedman et al., 1981). Cysteine was found to be the most unstable to alkaline and to be mostly destroyed when casein was alkali-treated at pH 9.0, 75 °C, for 3 h (Friedman and Masters, 1982). However, the decline of lysine was enhanced with the increasing pH (started at pH 9.0) and treating time accompanied with the formation of LAL (Ziegler, 1964; Corfield et al., 1967; Sternberg et al., 1975; Deng et al., 1990).

On the other hand, casein has been treated with alkaline, and the degree of racemization was observed to be increased with the increasing concentration of alkaline and reaction temperature. At the initial stage of alkaline treatment, a higher degree of racemization was determined as a result of the higher protein denaturation rate (Friedman and Liardon, 1985). The relationship between the formation of LAL and the racemization of amino acids in proteins is of particular interest in the food industry.

Micellar capillary electrophoresis, 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 a labile diastereomeric complex, respectively.

Pidan, a popular alkali-treated duck egg in Taiwan, is usually prepared by pickling the eggs in 4.2% NaOH/5.0% NaCl solution at ambient temperature for 20 days (Su and Lin, 1994). The racemization of amino acids and the formation of LAL in proteins such as casein and soy proteins have been intensively studied. However, formation of LAL in alkali-treated eggs has been rarely reported. Optimal separation systems for DL-amino acids have been established using β-cyclodextrin as the chiral selector (Tsai et al., 1998), and the separation conditions were found suitable for determining the racemization

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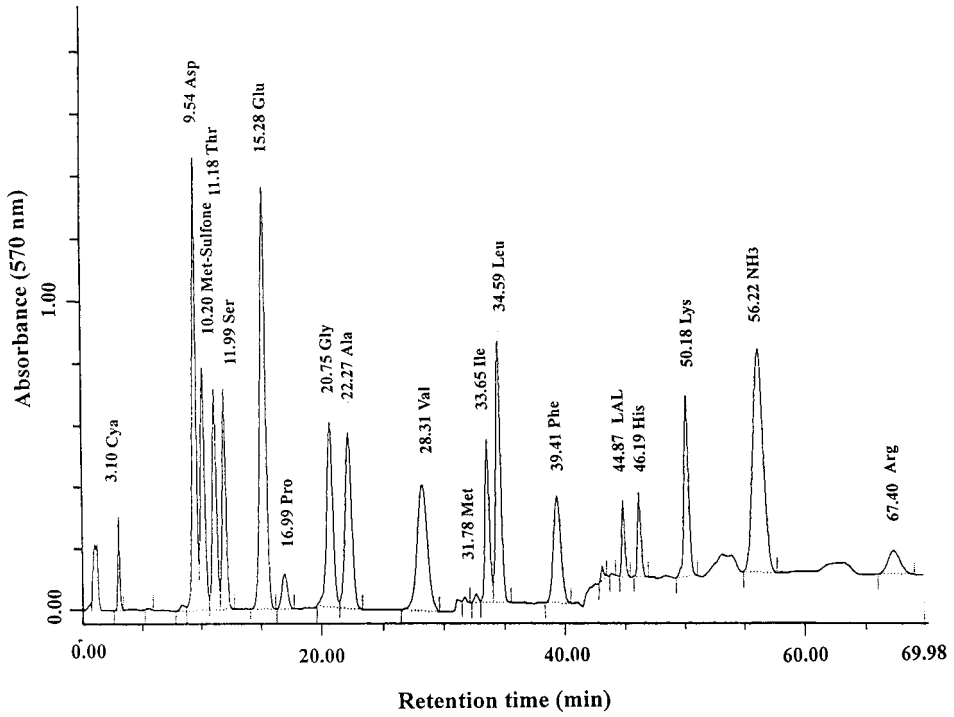

Figure 1. Chromatogram of compositional amino acids in Pidan albumen by an amino acid analyzer. Duck shell eggs were pickled in 4.2% NaOH/5% NaCl solution at ambient temperature to prepare Pidan.

values of compositional amino acids in Pidan (Chang et al., 1998).

The objective of this study is to investigate the changes of compositional amino acids in Pidan albumen (Figure 1) and yolk as well as the relationship between the formation of LAL and the degradation of cysteine, serine, and threonine at various alkali-treating stages. A study on the formation of LAL related to the contents of D-serine and D-aspartic acid in Pidan albumen is also going to be elucidated by micellar capillary electrophoresis.

MATERIALS AND METHODS

Materials. Fresh duck eggs purchased from a local supermarket were pickled in 4.2% NaOH/5.0% NaCl solution at ambient temperature for up to 20 days. Five eggs were collected each time at three-day intervals during this period. Egg albumen and yolk were separated carefully with a net, and their pH values were instantly reduced with 1 N acetic acid to their respective normals (8.95 and 5.84, respectively). The final pHs of albumen and yolk pickled for 20 days were 12.28 and 10.12, respectively (Chang et al., 1998). 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 determination of the pH of egg albumen and yolk was according to AOAC (1984).

Chemicals. Dansyl chloride (5-dimethylaminonaphthelene-1-sulfonyl chloride), boric acid, β-CD, 2-mercaptoethanol, lysinoalanine, D- and L-serine, and D- and L-aspartic acid were all purchased from Sigma (St. Louis, MO), whereas sodium dodecyl sulfate (SDS) was purchased from Bio-Rad (Richmond, CA). Sodium hydrogen carbonate, sodium hydroxide, and acetic acid were purchased from E. Merck (Darmstadt, Germany).

Protein Hydrolysis. Freeze-dried protein powders (control and alkali-treated albumen or yolk) were added with 88% formic acid and then were incubated overnight. Subsequently samples were added with performic acid (30% H_2O_2/88% formic acid = 1:9) and stayed for 1 h before being incubated at 0 °C in an ice bath for 4 h. The thus obtained samples were then vacuum-evaporated until dry, and the solids were hydrolyzed with 6 N HCl in the presence of 0.03% 2-mercaptoethanol under vacuum at 110 °C for 24 h. The removal of HCl from hydrolysates was conducted by vacuum evaporation. Then one part of the dried hydrolysates was dissolved in adequate volumes of distilled water and was subsequently applied to a capillary electrophoresis instrument to detect the racemization of amino acids posterior to the derivatization of dansyl chloride.

One part of the hydrolysates was dissolved in 0.2 M citrate buffer solution (pH 2.2) and was then applied to an amino acid analyzer to detect the changes of amino acid composition of egg proteins, both albumen and yolk, during the alkali-pickling period (20 days). Cysteine and cystine were all oxidized by performic acid to form cysteic acid which was quantitatively determined by an amino acid analyzer.

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 $\pm$ 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 composition was determined to be 200 mM SDS/75 mM β-CD/250 mM borate buffer solution (pH 9.5) for DL-aspartic acid and 200 mM SDS/75 mM β-CD/30% methanol/250 mM borate buffer solution (pH 9.5) for DL-serine (Chang et al., 1998). All of the BGEs were filtered through a 0.45 nm membrane prior to use. Deionized water was obtained from a Mili-Q system (Millipore, Japan).

Calibration curves for quantification of amino acids were prepared using samples of known concentration ($r^2 = 0.9997-0.9999$).

Racemization value = $100D/(D + L)$, where D and L are the amount of D- and the corresponding l-amino acid, respectively.

Table 1. Comparison of the Contents of Amino Acid in Fresh Albumen and Pidan Albumen[a]

amino acid	content (mmol/100 g of protein)		remained ratio[b] (%)
	fresh albumen	Pidan albumen	
Cys	21.25 ± 1.02	7.55 ± 0.74	35.5
Asp	68.56 ± 0.95	66.91± 1.08	97.6
Met	38.11 ± 1.02	37.92 ± 0.84	99.5
Thr	41.85 ± 0.69	36.86 ± 1.05	88.1
Ser	47.88 ± 1.11	35.04 ± 0.95	73.2
Glu	86.17 ± 0.86	84.53 ± 0.97	98.1
Pro	23.29 ± 0.86	23.57 ± 1.04	101.2
Gly	43.90 ± 1.21	43.02 ± 0.96	98.0
Ala	46.92 ± 0.54	46.08 ± 0.97	98.2
Val	53.60 ± 0.86	53.44 ± 0.92	99.7
Ile	29.08 ± 0.82	29.00 ± 0.93	99.7
Leu	52.41 ± 0.85	52.20 ± 0.98	99.6
Phe	29.16 ± 0.49	29.04 ± 0.85	99.6
His	11.31 ± 0.87	11.11 ± 1.12	98.2
Lys	39.73 ± 0.82	28.11 ± 0.84	70.8
Arg	20.67 ± 0.46	14.04 ± 0.86	67.9

[a] Duck eggs were pickled in 4.2% NaOH/5% NaCl solution for 20 days at ambient temperature. Data are the average of triplicate measurements. [b] Remained ratio = (content of amino acid in Pidan albumen/content of the corresponding amino acid in fresh albumen) × 100%.

Table 2. Amino Acid Composition of Pidan Yolk Alkali Pickled for 20 days[a]

amino acid	content (mmol/100 g of protein)		remained ratio[b] (%)
	fresh yolk	Pidan yolk	
Cys	16.83 ± 0.58	10.92 ± 0.89	64.9
Asp	90.50 ± 0.89	89.70 ± 1.01	99.1
Met	23.95 ± 0.54	23.93 ± 0.97	99.9
Thr	47.92 ± 0.87	47.82 ± 0.83	99.8
Ser	62.93 ± 0.68	52.29 ± 1.12	83.1
Glu	91.38 ± 1.24	91.56 ± 0.98	100.2
Pro	27.83 ± 0.58	27.72 ± 0.52	99.6
Gly	49.48 ± 0.68	50.12 ± 0.94	101.3
Ala	63.87 ± 0.57	64.12 ± 0.84	100.4
Val	55.78 ± 0.75	55.74 ± 0.67	99.9
Ile	39.81 ± 0.82	37.79 ± 0.67	99.9
Leu	62.66 ± 0.76	63.79 ± 0.98	101.8
Phe	21.02 ± 0.83	21.33 ± 0.98	101.5
His	17.26 ± 0.68	17.04 ± 0.85	98.7
Lys	53.81 ± 0.93	48.21 ± 0.82	89.6
Arg	40.54 ± 0.95	36.36 ± 0.84	89.7

[a] Duck eggs were pickled in 4.2% NaOH/5% NaCl solution for 20 days at ambient temperature. Data are the average of triplicate measurements. [b] Remained ratio = (content of amino acid in Pidan albumen/content of the corresponding amino acid in fresh albumen) × 100%.

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 always freshly prepared.

Quantification of Amino Acids and Lysinoalanine by High-Performance Liquid Chromatography. Protein hydrolysates, reacted with per-formic acid and thermally digested by 6 N HCl in the presence of 2-mercaptoethanol, were applied to an amino acid analyzer (Model 630, Beckman). The contents of amino acids and lysinoalanine were determined by a calibration curve constructed by known concentrations of corresponding amino acid and lysinoalanine.

Remained ratio (%) = (content of amino acid in Pidan albumen/content of corresponding amino acid in fresh albumen) × 100%.

RESULTS AND DISCUSSION

Change of Amino Acid Composition. Duck eggs were pickled in NaOH and NaCl solution, and the changes of the contents of compositional amino acids in egg albumen (Table 1) and yolk (Table 2) pickled for 20 days were determined. The contents (mmol/100 g of protein) of aspartic acid, glutamic acid, methionine, proline, glycine, alanine, valine, histidine, phenylalanine, isoleucine, and leucine in albumen were not apparently different before and after pickling treatment. However, the contents of cysteine, threonine, serine, lysine, and arginine were decreased remarkably to 35.5%, 88.1%, 73.2%, 70.8%, and 67.9%, respectively, after a 20-day alkaline treatment. The reduction of cysteine, serine, histidine, lysine, and arginine in yolk alkali-treated for 20 days, though less than that of the corresponding amino acid in the alkali-pickled albumen, was also remarkable, and the residual content percentages were determined to be 64.9%, 83.1%, 85.7%, 89.6%, and 89.7%, respectively (Table 2). The higher reduction percentage of amino acid in albumen than that of the corresponding amino acid in yolk, suggesting that some factors, such as, final pH, alkali-treating time, protein source, and other ingredients, coexisted with proteins (such as lipid is coexistent with proteins in the yolk), affected the reaction and degradation of amino acid in proteins during the alkaline treatment (Friedman and Masters, 1982; Friedman and Liardon, 1985). Cysteine, in both alkali-pickled egg albumen (Table 1) and yolk (Table 2), was found to be most sensitive to alkaline as a result of the formation of DHA and other products through the reaction of β-elimination (Fletcher et al., 1963). The other sources of DHA could be from serine and threonine, which are probably why those two amino acids have been degraded during the alkaline treatment (Asquith et al., 1969). Arginine is also sensitive to alkaline and degrades into urea and ornithine (Ziegier et al., 1967; Sanderson et al., 1978). Its remained ratio in Pidan albumen was 67.9% (Table 1) and in yolk was 89.7% (Table 2).

Achor et al. (1981) have pointed out that the contents of cysteine, lysine, serine, threonine, and arginine in the proteins of *Candida utiliz* treated with alkaline were also reduced remarkably. Similar results were also observed by Provansal et al. (1975) when sunflower proteins were treated with alkaline.

However, beside the above decomposition reaction of amino acids in proteins during alkaline treatment, racemization of compositional amino acid is also remarkable and results in the decrease of the nutritional values of proteins (Friedman et al., 1984). Racemization values of serine and aspartic acid in Pidan albumen were determined to be as high as 41% and 35%, respectively. Some essential amino acids such as phenylalanine, leucine, and valine also displayed 16.44%, 13.56%, and 4.09% of racemization values, respectively, in Pidan albumen (Chang et al., 1998). Thus, the decline of nutritional values of alkali-treated proteins is considered to be remarkable (Friedman et al., 1984).

Formation of LAL and Changes of Lysine, Cysteine, Serine, and Threonine. Table 3 presented the formation of LAL in egg albumen and yolk during the alkali-pickling period. LAL in albumen increased rapidly during the first 6-day pickling period and then gradually increased to about 7 mmol/100 g of protein at the end of the 20-day pickling period. The degree of the formation of LAL in proteins is closely related to the final pH of the proteins and the alkali-reacting time,

Table 3. Effect of Alkali-Pickling Time on the Contents of Lysinoalanine (LAL), Lysine, Cysteine, Serine, and Threonine in Duck Albumen and Lysinoalanine in Yolk[a]

pickling time (days)	content (mmol/100 g of protein)					
	albumen					yolk
	LAL	Lys	Cys	Ser	Thr	LAL
0	0.00 ± 0.00	39.73 ± 0.12	21.25 ± 0.26	47.88 ± 0.31	41.85 ± 0.14	0.00 ± 0.00
3	1.59 ± 0.10	37.89 ± 0.13	18.91 ± 0.12	46.20 ± 0.21	41.82 ± 0.14	0.31 ± 0.04
6	4.31 ± 0.11	32.03 ± 0.25	10.50 ± 0.14	45.01 ± 0.22	41.72 ± 0.16	0.59 ± 0.10
9	5.16 ± 0.11	31.96 ± 0.21	8.70 ± 0.24	43.32 ± 0.17	40.62 ± 0.16	0.91 ± 0.10
12	5.42 ± 0.14	31.28 ± 0.13	8.23 ± 0.21	40.58 ± 0.16	38.31 ± 0.17	1.42 ± 0.18
15	6.33 ± 0.18	28.13 ± 0.17	7.54 ± 0.20	37.45 ± 0.15	37.25 ± 0.16	2.03 ± 0.21
20	7.19 ± 0.14	28.11 ± 0.13	7.03 ± 0.15	35.04 ± 0.15	36.86 ± 0.10	2.21 ± 0.12

[a] Duck eggs were pickled in 4.2% NaOH/5% NaCl solution for 20 days at ambient temperature. Data are the average of triplicate measurements.

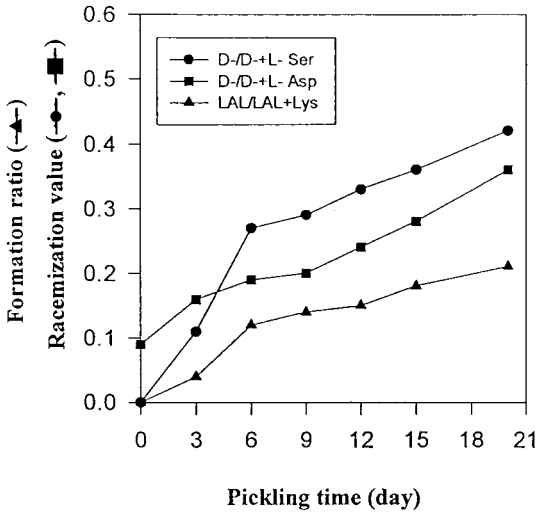

Figure 2. Comparison of the formation ratio of lysinoalanine and the racemization value of L-serine and L-aspartic acid in Pidan albumen as a function of pickling time. Formation ratio = content of LAL/contents of LAL and Lys; racemization value = $100 \times D/(D + L)$, where D and L are the contents of D- and the corresponding L-amino acid, respectively.

as well as the protein sources (Sternberg et al., 1975; Deng et al., 1990). The pH of egg albumen increased from the native (fresh egg) 8.95 to 11.73 after being pickled for 6 days and then was maintained approximately at 12 throughout the rest of pickling period (Chang et al., 1998). Thus, the formation of LAL at the initial pickling period (first 6 days) was mainly affected by the increase of the pH of albumen, while that of LAL at the later pickling period was apparently affected by the alkaline−protein reacting time factor. However, LAL in yolk exhibited a gradual increase throughout the pickling period and reached an amount of 2.1 mmol/100 g of protein at the end of the pickling period. A gradual increase in the pH of yolk was observed when eggs were alkali-pickled (Chang et al., 1998). The pH of yolk increased rapidly from the original 5.84 to 8.92 during the first 9-day pickling period and then increased gradually to 10.12 when alkali-pickled for 20 days. Therefore, the formation of LAL in yolk was considered to be mainly dependent on the degree of the increase in pH during the alkali-pickling period. Figure 2 presented the chromatogram of Pidan albumen alkali-pickled for 20 days by a amino acid analyzer. It was obvious that LAL was formed in albumen when duck shell eggs were treated with alkaline.

The relationship between the amount of the formation of LAL and the loss of lysine, cysteine, serine, and threonine was shown in Table 3. The amount of the LAL formed (1.59 mmol/100 g of protein) during the first 3-day pickling period was approximately 86% compared with that of lysine lost (1.84 mmol/100 g of protein), presenting that the main loss of lysine was due to the formation of LAL. Furthermore, the source of DHA, with which to form LAL, appeared mostly from the degradation of cysteine, compared with the loss of serine and threonine during the first 3-day pickling period. However, the quantity of lysine lost (7.70 mmol/100 g of protein) was much more than that of LAL formed (4.31 mmol/100 g of protein) during the first 6-day pickling period. Thus, the loss of lysine in this period was considered to be not only due to the formation of LAL but also apparently due to the alkaline-induced degradation reactions (Nashef et al., 1977; Friedman, 1994). Similarly, the loss of cysteine (10.75 mmol/100 g of protein) was far more than that of LAL formed (7.70 mmol/100 g of protein) during the first 6-day alkali-pickling period; thus the reactions other than DHA formation were considered to be the important pathways of cysteine degradation during the alkaline treatment. Therefore, it was obvious that most of the lost lysine and cysteine formed LAL in the early stage (first 6 days) of the alkali-pickling period of duck egg albumen. However, the proportions of lost lysine and cysteine in albumen forming LAL decreased with the increasing pickling period of time.

It has been reported (Fletcher et al., 1963) that DHA formed through the β-elimination reaction is unstable at high pHs and is liable to degradation to form pyruvic acid and ammonia as well as to reaction to form lanthionine and β-aminoalanine. The decrease in the loss of cysteine began to slow when egg albumen was pickled for more than 6 days; thus the degradation of cysteine appeared mainly to be affected by the pH factor (sharp increase in the pH of albumen) rather than by the pickling time factor. Similar results were also observed for the degradation of lysine during the pickling period in the present study. Serine and threonine were sensitive to alkaline, and they decreased progressively with the increasing pickling time (Table 3). Both of these two amino acids are responsible for the formation of DHA through the β-elimination and, thus, facilitate the formation of LAL (Fletcher et al., 1963). The quantity of lysine lost (11.62 mmol/100 g of protein) was far more than that of LAL formed (7.19 mmol/100 g of protein) during the alkali-pickling period, suggesting that about 60% of lost lysine formed LAL throughout the pickling period. Bohak (1964) has indicated that the degradation of cysteine, especially during the first 15 min, is faster than the formation of LAL when ribonuclease is alkali-treated in 0.2 N NaOH.

Formation of LAL and Racemization of L-Serine and L-Aspartic acid. L-Serine and L-aspartic acid in albumen racemized rapidly and remarkably when duck eggs were alkali-pickled (Chang et al., 1998). The formation of LAL and the racemization values of L-serine and L-aspartic acid were also increased with the prolonged alkali-treating time (Figure 2). D-Aspartic acid, formed at the 0-day pickling samples, could be due to the strong acid (6 N HCl) hydrolysis (Paquet and Ma, 1989). These three curves rose rapidly, indicating that the rapid formation of carbanions resulted from the rapid denaturation of albumen proteins through the removal of α-hydrogens from the asymmetric carbons of amino acids (Fletcher et al., 1963). Thus, it was obvious that the denaturation of proteins by alkaline caused simultaneously the formations of carbanions and LAL and the racemization of L-amino acids. Therefore, the rapid racemization of L-serine and L-aspartic acid could be treated as an indicator for the evaluation of protein deterioration during the alkaline treatment.

CONCLUSION

Duck eggs were alkali-pickled for 20 days to prepare the Chinese traditional egg products—Pidan—and the changes of the compositional amino acids and the relationship between the formation of LAL and the declines of cysteine, serine, and threonine were elucidated. The sharp increase in the pH of the albumen at the initial pickling period resulted in the rapid formation of LAL, suggesting that the formation of LAL in egg albumen was mainly affected by the increase of pH. In other words, the fact that incubation of albumen in shell eggs at high pH contributed less to the formation of LAL could be the result of the rapid decomposition of DHA and reaction of DHA with orinithine and ammonia to form lanthionine and β-aminoalanine, respectively, at high pH (Fletcher et al., 1963). In addition, the alkaline treatment of proteins loosens the protein compact structure by destroying the ionic bonds and hydrogen bonds and, thus, enhances the denaturation of proteins which facilitates the racemization of compositional amino acids (Liardon and Friedman, 1987). Woodard and Short (1973) fed rats alkali-treated soy proteins (1000 ppm LAL), and nephrocytomegalia in kidney was observed. The concentration of LAL formed in the Pidan albumen was as high as 28 mmol/100 mg of protein. Therefore, consumption of these alkali-pickled duck eggs should be reduced in daily life.

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