Effect of Peptide Bond Cleavage on the Racemization of Amino Acid Residues in Proteins

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The extent of alkali-induced racemization of L-amino acid residues to D-amino acids was measured by a gas chromatographic–mass spectrometric technique in eight food proteins: casein, bovine hemoglobin, bovine serum albumin, fish protein concentrate, lactalbumin, soybean proteins, wheat gluten, and zein. To obtain supportive evidence on the factors influencing the racemization process, a parallel study was also carried out on the extent of racemization of amino acid residues in a series of polyamino acids. The results indicate that the amino acids in the proteins and polyamino acids differed significantly in their susceptibility to racemization. The extent of racemization of a number of amino acid residues in the proteins appears to have been influenced by concurrent peptide bond cleavage, as measured by a ninhydrin assay. Mechanistic explanations are offered to account for the observed influence of the structural environment of an amino acid residue on its succeptibility to racemization. The studies described should help in devising food-processing conditions that minimize undesirable nutritional consequences of racemization.

Alkali-induced racemization of food proteins often impairs nutritional value and safety by generating D-amino acids that are nutritionally antagonistic and toxic and D–L, L–D, and D–D peptide bonds that are poorly digested (Friedman et al., 1984a–c).

In a previous study (Friedman and Liardon, 1985), we reported that exposing soybean proteins to alkaline conditions for various time periods and temperatures increased racemization of L-amino acid residues to D isomers. Relative susceptibilities of most amino acids were correlated with a linear free energy relationship. This relationship was based on plots of the ratio of the logarithm of the rate constant for the racemization of any amino acid to the same logarithm for alanine, the simplest amino acid with an asymmetric carbon atom, against σ^*, a parameter that measures electron-donating or inductive effects of the ammino acid side chains. Mechanistic explanations were offered to account for the observed influence of these variables on racemization kinetics.

In a related study Liardon and Ledermann (1986) showed that the nature of the proteins and the denaturation processes occurring during the alkaline treatment influenced significantly the racemization kinetics. To further clarify the factors that may influene the racemization of peptide-bound amino acid residues, it was of interest to find out whether peptide bond cleavage concurrent with alkali-induced racemization would influence the extent of inversion of amino acid residues. Such studies were carried out with a series of food proteins and polyamino acids to compare the extent of racemization of the same amino acid residue in different microenvironments. The results demonstrate the paramount importance of structural features in influencing the extent of racemization of peptide-bound amino acids.

MATERIALS AND METHODS

Materials. The following polyamino acids were obtained from Sigma Chemical Co., St. Louis, MO: poly-L-asparagine (MW 9000, DP 80), poly-L-aspartic acid sodium salt (MW 20000, DP 130), poly-L-histidine (MW 9000, DP 60), poly-L-lysine hydrobromide (MW 55000 DP 260), poly-L-serine (MW 5000–10000, DP 55–110), and poly-L-tyrosine (MW 100000, DP 600). Soybean proteins were obtained from Ralston Purina, St. Louis, MO, and the other proteins from ICN Nutritional Biochemicals Corp., Cleveland, OH.

Alkali Treatment of Proteins. A 0.1 N NaOH solution (500 mL) in a 1-L Erlenmeyer flask was brought to the desired temperature (25–95 °C) in a water bath. To this solution was added 10 g of protein, and the flask was shaken until a uniform suspension was formed. The flask was then stoppered with a Nalgene cap, and the top was covered with aluminum foil to keep the cap from popping. Next, the flask was placed into the preheated water bath for a specified period timed from the addition of protein. At the end of the reaction period, the flask was removed and cooled under running water to room temperature. The final pH was checked, and the solution was divided into two equal parts. Half was dialyzed (dialysis tubing molecular weight cutoff, 12500), first against 0.01 N acetic acid and then against water for about 3 days with five changes of water per day. The dialyzate was then lyophilized. The other half was neutralized with HCl to pH 7 and then lyophilized.

Variations in Procedure. *Time Study.* To 3 L of preheated 0.1 N NaOH was added 60 g of soybean proteins. Periodically, 500-mL samples were removed and treated as described above.

Soy Protein Study. Because the dialyzed samples were prepared for an earlier study (Friedman and Liardon, 1985) and the neutralized ones for this one, they came from different batches rather than from a single reaction mixture.

Alkali Treatment of Polyamino Acids. For each polyamino acid, each of four 20-mg samples was dissolved or suspended in 10 mL of preheated (75 °C) 0.1 N NaOH. The samples were then heated in a water bath at 75 °C while being shaken for 30, 60, 120, or 180 min. Each reaction mixture was then dialyzed against deionized distilled water for 2 days using dialysis tubing with a molecular weight cutoff of 3500. The material was then freeze-dried.

Ninhydrin Assay. The amino group content of untreated and alkali-treated proteins was measured by an improved ninhydrin assay described previously (Friedman et al., 1984b). Leucine was always run as a standard because it produces stoichiometric amounts of the ninhydrin

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0021-8561/87/1435-0661$01.50/0

Table I. Effect of Alkali Treatment (0.1 N NaOH, 75 °C, 3 h) on the Ninhydrin Color Yield of Eight Food Proteins

protein	ninhydrin color yield (Leu equiv)			hydrolytic susceptibility: C-A/A	size distribn: B-A/C-A
	untreated controls (A)	alkali-treated, dialyzed (B)	alkali-treated, neutralized (C)		
bovine serum albumin	0.632 (0.022)[a]	0.570 (0.024)	0.634 (0.022)	ca. 0	
lactalbumin	0.512 (0.020)	0.557 (0.017)	0.626 (0.021)	0.2	0.39
bovine hemoglobin	0.502 (0.023)	0.570 (0.022)	0.717 (0.025)	0.4	0.32
fish protein	0.519 (0.026)	0.551 (0.033)	0.858 (0.042)	0.7	0.09
casein	0.402 (0.016)	0.533 (0.015)	0.868 (0.001)	1.2	0.28
soybean protein	0.244 (0.011)	0.475 (0.015)	0.878 (0.019)	2.6	0.36
wheat gluten	0.226 (0.018)	0.227 (0.009)	0.835 (0.009)	2.7	0.00
zein	0.076 (0.003)	0.148 (0.003)	0.635 (0.016)	7.4	0.13

[a] Average from triplicate determinations. Values in parentheses are standard deviations from the mean for three separate (triplicate) determinations.

chromophore (Ruhenmann's purple) in the ninhydrin reaction (Friedman and Williams, 1974). The reported values are in terms of leucin equivalents defined as

$$\text{Leu equiv protein} = \frac{A_{570\text{nm}}/\text{mg protein N}}{A_{570\text{nm}}/\text{mg Leu}}$$

where $A_{570\text{nm}}$ is the absorbance of the ninhydrin chromophore at 570 nm. Protein nitrogen is Kjeldahl nitrogen determined by a standard method (AOAC, 1980).

Isomeric Analyses. Analysis of the D and L composition of proteins or poly(amino acids) was carried out in duplicate by a gas chromatographic–mass spectrophotometric (GC–MS) technique (Liardon et al., 1981). Samples (2–3 mg) of untreated and alkali-treated proteins or poly(amino acids) in 6 N DCl were hydrolyzed in evacuated ampules for 24 h at 110 °C. The solutions were then evaporated to dryness with the aid of an aspirator. The dry residues were redissolved in 1 mL of 0.2 M acetic acid and applied to small Dowex 50 (H^+) columns. The buffer salts then were washed from the resin with 10 mL of distilled water. The amino acids were displaced into conical reaction vials with 1 mL of 3 N NH_4OH followed by 1 mL of water and dried under a stream of nitrogen. The dry residues were first treated with 250 μL of 2-propanol–acetyl chloride (100/20, v/v) and then heated for 30 min at 110 °C. The excess reagent was removed under nitrogen and 150 μL of perfluoropropionic acid anhydride (PFPA) added to the residue. Next, the samples were heated for 15 min at 110 °C and the excess PFPA was removed under a stream of nitrogen. For the complete derivatization of histidine, the sample was then treated with 50 μL of isobutyl chloroformate (IBCF) and maintained for 10 min at 110 °C. The residues were finally dissolved in 100 μL of ethyl acetate and the resultant mixtures analyzed by the GS–MS technique described elsewhere (Liardon et al., 1981; Liardon and Hurrell, 1983). This technique provided racemization values corrected for acid hydrolysis induced contributions.

RESULTS AND DISCUSSION

Influence of Protein Hydrolysis on Amino Acid Racemization. The generally accepted mechanism for the racemization of an amino acid under alkaline conditions involves a hydroxide ion initiated removal of a proton from the α-carbon of an amino acid to form a carbanion intermediate (Masters and Friedman, 1979; Friedman and Masters, 1982; Liardon and Hurrell, 1983; Liardon and Ledermann, 1984; Friedman and Liardon, 1985). The trigonal carbon of the carbanion, which has lost the original asymmetry of the α-carbon, can recombine with a proton from the environment to regenerate a tetrahedral carbon. If recombination takes place at an equal rate on either side of the carbanion, the product is an equimolar mixture of the amino acid D and L isomers.

The results in the cited references indicate that the extent of racemization of a protein's various amino acid residues is strongly influenced by the inductive property of the amino acid side chain that is attached to the asymmetric carbon atom. In fact, plots of a parameter that gives a quantitative measure of the inductive or electron-donating power of the R group (Hansch and Leo, 1979) against racemization rate constants are generally linear. In addition, the results cited show that protein-related factors influence the racemization of amino acid residues. Protein conformation, steric and electronic effects, and alkali-induced competitive reactions with certain amino acid residues have been postulated to affect the racemization kinetics (Friedman and Liardon, 1985).

More recently, it was found that partial hydrolysis of the proteins during relatively mild alkaline treatment influenced the racemization kinetics of certain residues (Liardon and Ledermann, 1986). It was therefore of interest to determine how hydrolysis and racemization might be related. This was realized by measuring in parallel the extent of peptide bond cleavage (ninhydrin color yield) and of racemization in a series of protein samples submitted to various alkaline conditions. In addition, the extent of racemization was determined both in dialyzed samples containing only intact proteins and large peptides and in neutralized samples including all possible protein fragments resulting from the hydrolytic process. The results obtained for a set of eight different proteins exposed to the same alkaline conditions are reported in Tables I and II. Table I shows the ninhydrin color yield of the control proteins (A) and the alkali-treated dialyzed (B) and neutralized (C) samples. The differences in color yield between treated and control samples clearly indicate that proteins were partially hydrolyzed during treatment. The extent of hydrolysis, however, appeared to depend strongly on the protein, as shown by the color yield ratio of neutralized to control samples (C-A/A), which ranged from 0.0 for bovine serum albumin (BSA) to 7.4 for zein.

Further differences could be observed in the size distribution of the hydrolysis products. Depending on the protein, the relative abundance of the high molecular weight fragments, as given by the B-A/C-A ratio, varied from zero to more than 50% of the total hydrolysis products. This parameter did not seem to be related to the protein's susceptibility to hydrolysis.

Table II shows the racemization values measured for each amino acid in the eight treated food proteins. In general, differences in racemization in the neutralized and dialyzed samples were expected to depend on the extent of protein fragmentation. In fact, the observed differences were generally small and with no apparent relationship to the hydrolysis data in Table I. In particular, the differences between neutralized and dialyzed samples were equally small for BSA and zein, the two extreme proteins

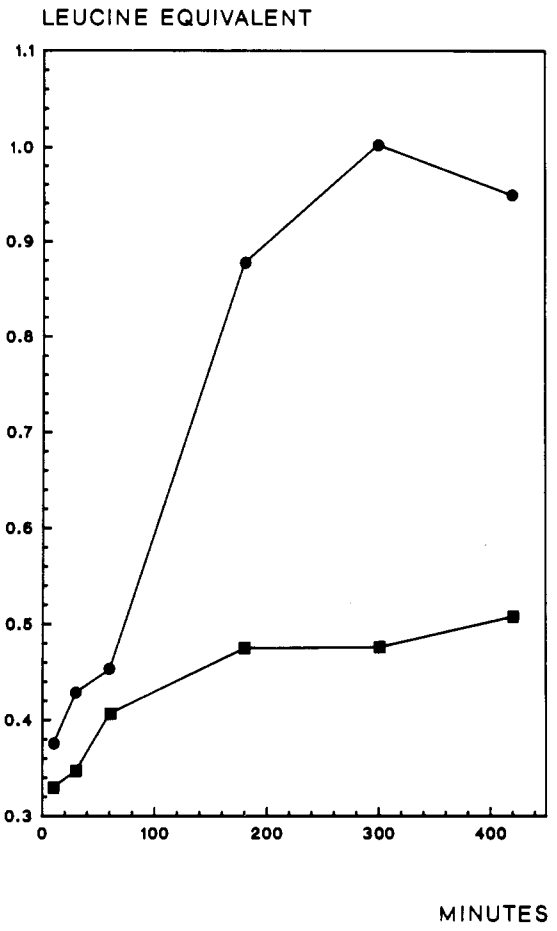

Figure 1. Comparison of ninhydrin color yields (leucine equivalents) of dialyzed (■) and neutralized (●) soybean proteins treated with alkali (0.1 N NaOH, 75 °C) for the indicated time periods.

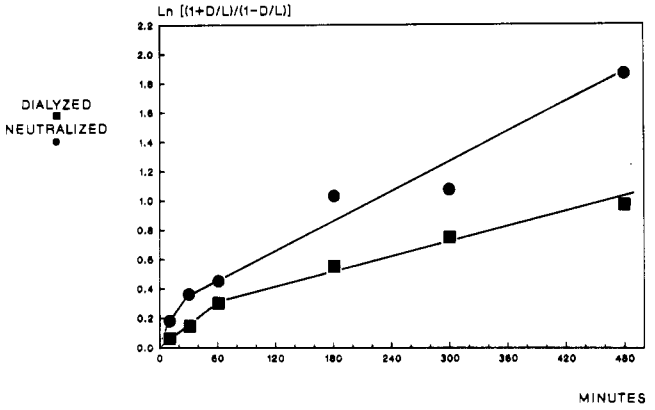

Figure 2. Comparison of methionine racemization of dialyzed (■) and neutralized (●) soybean proteins exposed to alkali (0.1 N NaOH, 75 °C) for the indicated time periods.

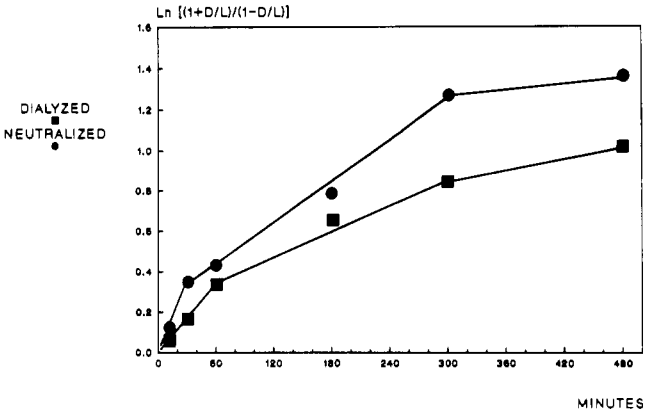

Figure 3. Comparison of phenylalanine racemization of dialyzed (■) and neutralized (●) soybean proteins exposed to alkali (0.1 N NaOH, 75 °C) for the indicated time periods.

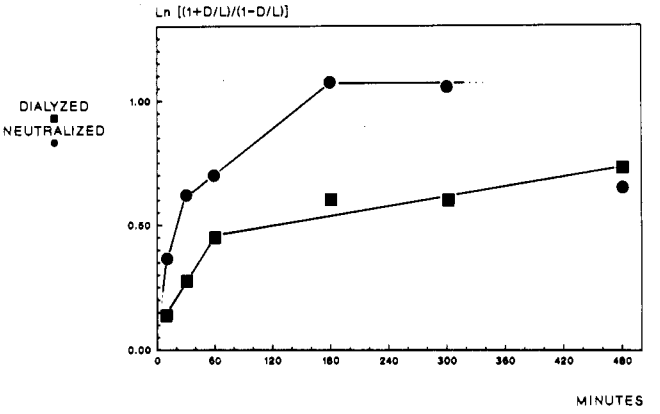

Figure 4. Comparison of threonine racemization of dialyzed (■) and neutralized (●) soybean proteins exposed to alkali (0.1 N NaOH, 75 °C) for the indicated time periods.

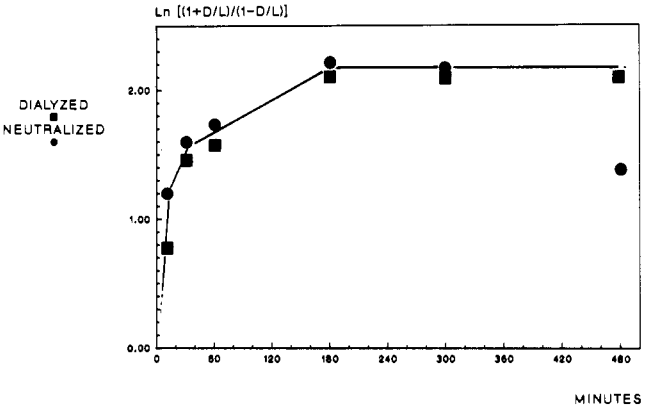

Figure 5. Comparison of serine racemization in dialyzed (■) and neutralized (●) soybean proteins exposed to alkali (0.1 N NaOH, 75 °C) for the indicated time periods.

on the hydrolysis susceptibility scale.

In the soybean proteins, however, racemization differed significantly in the neutralized and dialyzed samples. This led us to study in more detail the hydrolysis and racemization kinetics in these proteins, following the approach described previously (Friedman and Liardon, 1985).

Soybean Protein Time Study. Table III and Figure 1 show the influence of time of the ninhydrin color yield for soybean proteins exposed to 0.1 N NaOH at 75 °C. The hydrolysis of the proteins proceeded almost linearly over the first 3 h and then leveled off. A probable explanation for this behavior is that under the chosen conditions only some peptide bonds are sufficiently labile to be cleaved. Supporting this hypothesis, Sine and Hass (1969) observed that muscle aldolase exposed to 0.1 N NaOH at 0 °C hydrolyzed specifically at Ser, Thr, and Pro peptide bonds.

In our experiments, the proportion of high molecular weight peptides tended to decrease with time, indicating that limited further hydrolysis of the primary fragments occurred.

The influence of time on the extent of racemization in the two sets of soybean proteins exposed to 0.1 N NaOH is shown in Table IV. Table V shows inversion rate constants calculated as described previously (Friedman and Liardon, 1985). The rate constants in the neutralized samples were systematically higher than in the dialyzed samples. The most significant differences were observed for Met, Phe, Thr, and Pro, while Leu, Lys, Ser, and Tyr

Table II. Alkali-Induced Racemization in Eight Proteins Exposed to 0.1 N NaOH for 3 h at 75 °C [Racemization: 100[D/(D + L)]]

amino acid		casein	lactalbumin	gluten	zein	fish protein	soy protein	bovine hemoglobin	bovine serum albumin	rel racemizn range[c]
Ala	1[a]	15.6	13.9	19.2	22.0	19.2	18.5	20.7	17.5	1.00
	2[b]	14.8	14.9	18.0	22.5	19.5	13.1[e]	23.6	16.7	
Val	1	2.5	2.6	3.8	4.5	3.1	3.0	3.2	3.8	0.15–0.22
	2	2.6	2.7	4.3	5.2	3.1	1.9[e]	3.8	4.1	
Leu	1	7.8	5.5	7.2	7.5	6.9	7.7	7.1	6.5	0.34–0.50
	2	6.9	4.6	7.1	8.2	6.7	5.0[e]	9.3	6.8	
Ile	1	3.5	3.2	4.1	5.4	3.5	4.7	*d*	5.4	0.18–0.31
	2	3.2	3.0	3.9	5.5	3.6	3.0	5.7	4.5	
Cys	1	*d*	28.9	28.4	42.5	21.6	*d*	*d*	30.3	1.13–2.08
	2	*d*	35.2	35.5	44.9	24.1	21.0	23.9	29.8	
Met	1	26.2	34.4	33.4	26.6	32.1	32.2	23.7	19.9	1.14–2.47
	2	22.9[e]	30.3[e]	32.8	27.2	30.4	21.3[e]	27.1[e]	21.4	
Phe	1	24.6	24.0	24.5	32.3	28.0	27.2	25.8	30.9	1.25–1.77
	2	24.3	24.6	24.4	32.5	28.0	23.8	30.4	29.2	
Lys	1	6.8	7.2	10.1	7.7	11.6	13.5	12.6	10.2	0.44–0.65
	2	9.9	7.1	8.7	8.2	11.5	9.1[e]	14.0	9.6	
Asp	1	28.8	21.6	27.7	43.7	24.1	31.9	29.7	18.5	1.06–1.85
	2	29.7	23.6	23.6	39.6	26.0	29.6	24.3	19.3	
Glu	1	17.7	20.7	33.0	35.8	16.8	22.5	15.6	19.7	0.75–1.72
	2	21.6	18.2	31.5	34.1	20.9	19.6	21.2	20.0	
Ser	1	40.4	48.2	46.7	48.1	41.8	44.5	46.1	48.1	2.19–3.47
	2	41.6	46.0	40.7	39.9[e]	42.4	44.0	39.8	40.8[e]	
Thr	1	29.3	30.7	30.6	35.1	33.7	32.9	23.1	30.6	1.12–2.21
	2	29.4	27.4	29.5	37.6	31.9	22.6[e]	33.5[e]	31.8	
Pro	1	*d*	*d*	*d*	*d*	0.4	*d*	*d*		
	2	*d*	*d*	*d*	*d*	0.03	*d*	*d*		
Tyr	1	15.8	19.4	18.9	34.8	18.2	12.9	13.9	22.7	0.59–1.17
	2	14.3	18.3	20.1	36.2	14.4	14.5	16.7	22.5	

[a] Neutralized samples. [b] Dialyzed samples. [c] Relative racemization range for eight food proteins = $(R_{AA}/R_{Ala})_{min} - (R_{AA}/R_{Ala})_{max}$, where R_{AA} = extent of racemization (D/L ratio) of any amino acid and R_{Ala} = extent of racemization of alanine. [d] Below detection limit. [e] Statistically different ($P < 0.05$) from neutralized sample.

Table III. Effect of Time of Alkali Treatment (0.1 N NaOH, 75 °C, 3 h) on the Ninhydrin Color Yield of Soybean Proteins

time, min	ninhydrin color yield (Leu equiv) dialyzed (B)	ninhydrin color yield (Leu equiv) neutralized (C)	hydrolytic susceptibility: C-A/A	size distribn: B-A/C-A
10	0.330 (0.006)[a]	0.376 (0.033)	0.54	0.65
30	0.347 (0.015)	0.429 (0.018)	0.75	0.56
60	0.407 (0.006)	0.453 (0.002)	0.86	0.78
180	0.475 (0.015)	0.878 (0.019)	2.60	0.36
300	0.476 (0.006)	1.003 (0.006)	3.23	0.31
420	0.508 (0.000)	0.949 (0.030)	2.89	0.37
unheated control (A)	0.244 (0.011)			

[a] Average of triplicate experiments. Values in parentheses are standard deviations.

showed almost no difference. This is further illustrated by the racemization time curves in Figures 2–6.

It is worth noting that the extent of racemization of Ser and Thr in soybean proteins as a function of time (Table IV) appears to level off and then decrease. The appearent decrease, if not an artifact, would imply that preferential hydrolytic destruction of one of the isomers may be taking place after a long exposure to alkaline conditions. (See related discussion below on the racemization of polyamino acids.)

In a study based on protein models, Liardon and Ledermann (1986) related features observed in the racemization kinetics of some amino acids to the hydrolytic cleavage of specific peptide bonds in the protein sequence. In the present situation, the soybean proteins consisted of a mixture of proteins of unknown structure, making it more difficult to rationalize the results illustrated in Figures 2–6. Generally, greater discrepancies were observed between racemization rates of neutralized and dialyzed samples in the early state of the treatment than after 3 h or more. This confirmed earlier observations indicating that protein fragmentation under alkaline conditions eventually induces a decrease of the racemization rates (Kriausakul and Mitterer, 1978; Sol, 1978; Masters and Friedman, 1979; Friedman and Masters, 1982; Friedman and Liardon, 1985; Liardon and Ledermann, 1986).

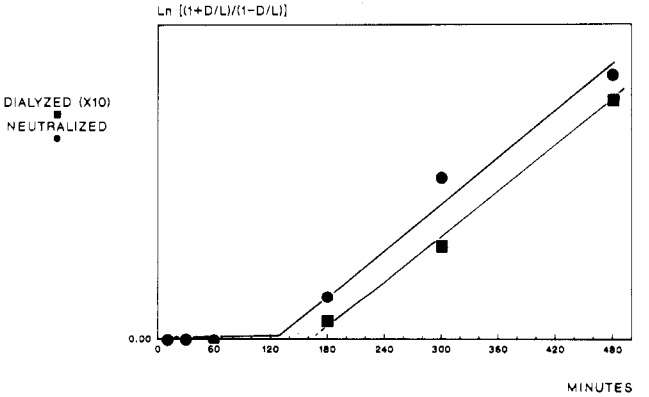

Figure 6. Comparison of proline racemization in dialyzed (■) and neutralized (●) soybean proteins exposed to alkali (0.1 N NaOH, 75 °C) for the indicated time periods.

However, the racemization curves of Pro in Figure 6 illustrate the opposite behavior. In both dialyzed and neutralized samples, Pro racemization started only after ca. 3 h of treatment. This suggests that the racemization

Table IV. Effect of Time on Racemization in Alkali-Treated (0.1 N NaOH, 75 °C) Soybean Proteins (Racemization: 100[D/(D + L)])

amino acid		time, min 10	30	60	180	300	380
Ala	1[a]	2.9	6.3	9.4	18.5	24.3	28.8
	2[b]	1.3	3.0[d]	6.6[d]	13.1[d]	17.9[d]	21.9[d]
Val	1	0.4	0.8	1.5	3.0	4.5	6.9
	2	0.2	0.3	1.0	1.9[d]	2.7[d]	4.2[d]
Leu	1	1.2	2.2	3.4	7.7	10.9	13.5
	2	0.7	1.0	2.4	5.0[d]	7.2[d]	9.4[d]
Ile	1	[c]	1.3	1.8	4.7	7.0	9.9
	2	0.2	0.6	1.2	3.0	3.9[d]	5.6[d]
Met	1	[c]	8.9	18.1	32.2	32.9	42.2
	2	2.9	7.0	13.1[d]	21.3[d]	26.6[d]	31.2[d]
Phe	1	5.5	14.6	17.4	27.2	36.0	37.1
	2	3.1[d]	7.6[d]	14.5[d]	23.8	28.5[d]	31.9[d]
Lys	1	1.8	3.6	5.1	13.5	15.7	20.5
	2	0.8	2.0	4.3	9.1[d]	12.3[d]	16.3[d]
Asp	1	19.6	25.9	26.8	31.9	35.5	35.6
	2	13.5	20.9	25.4	29.6	32.0	36.1
Glu	1	3.5	11.6	12.8	22.5	27.0	31.0
	2	2.6[d]	6.1[d]	11.9	19.6	24.3	28.0
Ser	1	35.0	40.0	41.2	44.5	44.3	37.5
	2	27.1[d]	38.4	40.0	44.0	43.9	43.8
Thr	1	15.5	23.2	25.2	32.9	32.6	24.0
	2	6.5[d]	12.3[d]	18.3[d]	22.6[d]	22.6[d]	26.0
Pro	1	[c]	[c]	[c]	0.4	2.5	4.0
	2	[c]	[c]	[c]	0.03[d]	0.4[d]	0.4[d]
Tyr	1	3.4	5.2	10.0	14.8	25.0	42.0
	2	3.8	4.8	8.4	14.5	19.0[d]	23.1[d]

[a] Neutralized samples. [b] Dialyzed samples (Friedman and Liardon, 1985). [c] Below detection limit. [d] Statistically different ($P < 0.05$) from neutralized sample value.

Table V. Amino Acid First-Order Racemization Rate Constants (10^{-6} s^{-1}) in Alkali-Treated Soybean Proteins (0.1 N NaOH, 75 °C, Time Study)

amino acid		neutralized (total protein)	dialyzed (high-MW fraction)
Ala		24.3 (2.6)[b]	19.4 (1.1)[c]
Val		3.8 (0.1)	2.2 (0.5)
Leu		7.8 (0.6)	6.2 (1.3)
Ile		5.4 (0.7)	3.9 (0.4)
Met	1[a]	>150	37.3 (0.9)
	2	28	16
Phe	1	96 (1)	47 (1)
	2	28	17
Lys		13.5 (0.5)	12.4 (1.2)
Asp	1	>420	>260
	2	16.0 (0.5)	11.2 (0.5)
Glu	1	75 (5)	37 (1)
	2	13 (1)	14
Ser		>1000	>650
Thr	1	310	52 (3e
	2	25.3 (0.8)	5.5
Tyr		23 (3)	18 (4)
Pro		1.9 (0.3)	0.2

[a] Whenever applicable, rate constants have been derived from the first (1) and second (2) linear part of the racemization time course. [b] Values in parentheses are standard deviations of the slope of the regression lines. [c] From Friedman and Liardon (1985).

of Pro only occurs after some hydrolysis has taken place. In addition, the apparent racemization rate in the dialyzed samples was about 10 times lower than in the neutralized sample, indicating that racemizing Pro was mainly to be found in the smaller protein fragments. These conclusions are in good agreemet with those derived from the racemization kinetics of Pro in α-lactalbumin exposed to moderate alkaline conditions (Liardon and Ledermann, 1986). They suggest that racemization in Pro would only occur at a significant rate when it is in terminal position.

Soybean Protein Temperature Study. The data showing the influence of temperature on the hydrolysis of soybean proteins exposed to 0.1 N NaOH are reported in Table VI. Hydrolysis remained quite limited at lower temperatures but increased exponentially above 65 °C. The size distributions seemed to indicate that the fragmentation into smaller peptides proceeded more slowly than the total hydrolytic process.

Table VI. Effect of Temperature of Alkali Treatment (0.1 N NaOH, 3 h) on the Ninhydrin Color Yield of Soybean Proteins

temp, °C	ninhydrin color yield (Leu equiv) dialyzed (B)[a]	neutralized (C)[a]	hydrolytic susceptibility: C-A/A	size distribn: B-A/C-A
25	0.318 (0.006)	0.314 (0.034)	0.29	ca. 1
35	0.309 (0.004)	0.357 (0.012)	0.46	0.90
45	0.346 (0.005)	0.394 (0.020)	0.61	0.68
55	0.379 (0.008)	0.480 (0.033)	0.96	0.57
65	0.432 (0.013)	0.587 (0.029)	1.41	0.55
75	0.475 (0.015)	0.878 (0.019)	2.6	0.36
85	0.691 (0.033)	1.026 (0.032)	3.20	0.57
95	0.815 (0.031)	1.498 (0.024)	5.14	0.46
unheated control (A)	0.244 (0.011)			

[a] Average of triplicate determinations. Values in parentheses are standard deviations.

The corresponding racemization data are reported in Table VII. The differences between the dialyzed and he neutralized samples tended to be greater in the higher temperature range. This trend, particularly marked for Thr, Met, and Pro, appears to be related to the extent of protein hydrolysis.

Conclusion of the Soybean Protein Study. Our data support a relationship between the hydrolysis and racemization processes. In general, racemization appears to proceed more quickly in peptides liberated in the early stage of the protein hydrolysis than in the intact protein. However, further fragmentation into smaller peptides and, eventually, free amino acids appears to lead to markedly reduced racemization rates. Exceptionally, the racemiza-

Table VII. Effect of Temperature of Alkali Treatment (0.1 N NaOH, 3 h) on Racemization of Soy Proteins (Racemization: 100[D/(D + L)])

amino acid		temp, °C							
		25	35	45	55	65	75	85	95
Ala	1[a]	c	c	2.8	4.0	9.8	18.6	28.0	36.7
	2[b]	0.3	0.5	2.0	3.9	9.9	17.0[d]	24.5[d]	32.7[d]
Val	1	c	c	c	c	1.5	3.2	6.1	12.6
	2	0	0.1	0.3	0.5	1.0	2.3[d]	4.2[d]	8.6[d]
Leu	1	0.7	1.0	1.1	1.4	3.8	7.4	12.0	22.0
	2	0.2	0.3	0.9	1.6	3.1	6.5	10.4	16.8[d]
Ile	1	c	c	c	c	2.0	3.7	8.1	15.8[d]
	2	0.03	0.2	0.6	0.9	1.8	3.4	6.5[d]	11.4[d]
Met	1	c	c	c	5.5	17.6	30.6	41.2	47.5
	2	1.0	1.6	5.1	9.8	18.2	27.1[d]	32.3[d]	39.4[d]
Phe	1	1.3	3.2	4.2	9.8	18.6	27.6	39.7	40.0
	2	0.8	1.8	5.2	10.8	19.7	27.9	33.3[d]	39.3
Lys	1	c	1.2	2.0	3.3	6.5	12.2	22.5	31.7
	2	0.3	1.5	1.4	3.0	6.7	11.9	17.8[d]	27.0
Asp	1	6.6	12.5	17.0	22.9	27.6	29.0	37.2	43.9
	2	5.9	9.3	17.1	20.1	27.7	31.6	34.8	41.2
Glu	1	1.1	1.7	3.6	6.7	10.9	24.1	27.7	37.1
	2	0.4	1.4	4.8	9.1[d]	16.2[d]	22.5	28.8	36.9
Ser	1	14.8	23.0	34.2	39.9	40.9	42.8	43.8	43.6
	2	13.8	20.4	33.9	38.9	42.2	40.5	42.8	41.0
Thr	1	3.5	8.4	13.5	26.2	32.8	35.2	30.4	33.3
	2	2.8	6.5	11.3	15.8[d]	21.9[d]	23.3[d]	21.0[d]	31
Pro	1	c	c	c	c	c	c	0	3.5
	2	c	c	c	c	cc	c	0.5	1.2[d]
Tyr	1	c	c	c	c	12.1	15.1	31.7	68.6
	2	1.7	2.6	3.8	5.8	10.8	16.8	24.1[d]	33.6[d]

[a] Neutralized samples. [b] Dialyzed samples (Friedman and Liardon, 1985). [c] Below detection limit. [d] Statistically different ($P < 0.05$) from neutralized sample value.

tion of Pro occurred mainly in the lower molecular weight fragments. The situation is even more complex. The alkali-induced racemization and peptide bond hydrolysis are accompanied by the formation of inter- and intramolecular lysinoalanine cross-links (Friedman et al., 1984a,c). Since such cross-links are derived from cystine, serine, and lysine residues, they alter both the conformation and size of the peptide chains. They could, therefore, also influence the extent of racemization. This aspect requires further study.

Influence of Protein Structure on Amino Acid Racemization. The influence of protein type on the racemization rate of bound amino acids was also investigated in this study. Table II shows variations among the different food proteins in absolute extent of racemization of each amino acid, as well as in the relative racemization susceptibilities normalized to alanine. (See the last column in Table II).

These observations were complemented by measuring racemization in several polyamino acids exposed to 0.1 N NaOH at 75 °C. This corresponds to effective pH 11.3 (Friedman and Liardon, 1985). With the exception of polyhistidine, which was only partially soluble, the listed polyamino acids completely dissolved in the alkaline medium. Table VIII shows the D/L ratios observed for four different treatment times. For each of these periods, racemization increased significantly with time in Lys, Asp, Asn, Glu, Ser, Tyr, and His.

In related experiments, Steinberg et al. (1984) report the following rate constants ($\times 10^{-6}$ s^{-1}) for the racemization of Asp in polyaspartic acid and Ser in polyserine at 100 °C: pH 6.65, Asp 5.0, Ser 5.8; pH 7.65, Asp 4.4, Ser 9.9. In our results (Table IX), the racemization rate constant for Asp in polyaspartic acid at effective pH 11.3 was 4.4, identical with the value reported by Steinberg et al. at pH 7.65. Our corresponding rate constant for Ser in polyserine was about 40 times greater, however. The difference in the two findings may be explained by (a) the observed influence of pH on the reaction medium on the ionization of the β-COOH group of Asp and the OH group of Ser and (b) the effects of this ionization on the stabilities of the

Table VIII. Racemization of Alkali-Treated Polyamino Acids[a,b]

amino acid	enantiomeric compn, % (D/L ratios × 100)				
	untreated controls	treatment time, min			
		30	60	120	180
Asp	9.4	8.7	11.8	11.3	14.1
Asn	21.0	31.1	34.7	34.5	34.8
Glu	0.1	0.7	1.4	3.6	5.2
Lys	0.2	0.7	0.9	2.1	2.0
Ser	0.7	29.5	29.3	30.9	37.5
Tyr	1.8	2.5	3.9	5.6	5.7
His	0.5	2.3	3.8	c	5.6

[a] Corrected for hydrolysis-induced racemization, except for Tyr. [b] Conditions of treatment: 20 mg of polyamino acid in 10 mL of 0.1 N NaOH, 75 °C for the indicated time periods. [c] Not determined.

Table IX. Comparison of Calculated Racemization Rate Constants for the Inversion of the Same Amino Acid Residue in Soybean Proteins and in Polyamino Acids[a,b]

amino acid	rate const ($k \times 10^{-6}$), s^{-1}		remarks for polyamino acids
	soybean protein[c]	polyamino acid	
Lys	12.4 (1.2)	2.6 (0.3)	
Asp	>260[d]	4.4 (1.3)	8% D-Asp in control poly-Asp
Asn[e]		>61	based on first 2 data points; 17% D-Asn in control poly-Asn
Glu	37.2 (1.3)[f]	4.9 (0.3)	
Ser	>650	>165	based on first 2 data points
Tyr	18 (4)	5.5 (0.4)	
His[e]		9.1 (0.5)	based on first 3 data points

[a] Conditions: 0.1 N NaOH, 75 °C. [b] Values in parentheses are standard deviations of the slope of the regression line. [c] Data from Friedman and Liardon (1985). [d] Average value for asparagine and aspartic acid residues. [e] Racemization of asparagine and histidine residues in soy proteins could not be determined experimentally. [f] Average value for glutamine and glutamic acid residues.

carbanions during the racemization process (Boehm and Bada, 1984; Steinberg et al., 1984).

Results for Ser racemization are also complicated by the possibility of concurrent side reactions during exposure to alkali. Attack of OH^- ions on L-Ser generates a carbanion and a dehydroprotein. Reprotonation of the carbanion or hydration of the dehydroprotein produces D,L-Ser. The double bond of the dehydroprotein can also participate in nucleohilic addition reactions with active hydrogen bearing protein functional groups to produce cross-linked proteins, as discussed in detail elsewhere (Friedman, 1977; Masri and Friedman, 1982). In addition, the dehydroprotein can undergo hydrolysis via an imino protein intermediate to pyruvic acid, ammonia, and a protein fragment. Analogous transformations can also occur with Thr side chains.

The observed racemization of Asp and Asn residues also deserves special comment. Under the reaction conditions used, β-carboxyl groups of Asp were all ionized. The negative charge on the β-COO^- residues would be expected to electronically repel incoming, negatively charged OH^- ions and to destabilize the negative charge on the carbanion intermediate. In contrast, the β-carboxamide group ($CONH_2$) of Asn residues should racemize faster than corresponding Asp residues in alkaline solution (Robinson et all., 1973; Scotchler and Robinson, 1974; Masters and Friedman, 1979; Steinberg et al., 1984). Results in Tables VIII and IX show that this is indeed the case.

Table IX compares rate constants for the observed racemization of selected amino acid residues in soybean proteins and in polyamino acids, calculated as previously described (Friedman and Masters, 1982; Liardon and Hurrell, 1983). The results show that (a) Ser in soybean protein racemizes about 4 times faster than in polyserine. (b) Ser in both soybean protein and in polyserine is the fastest racemizing amino acid. (c) Asp in soybean protein racemizes about 60 times faster than in polyaspartic acid. (d) Asn in polyasparagine racemizes about 14 times faster than Asp in polyaspartic acid. (e) Tyr in soybean protein racemizes 2–3 times faster than in polytyrosine.

The limited data from this study show different extents of racemization of the same amino acid residue in a polyamino acid and in a food protein. although the reasons for this effect are not immediately apparent, the data suggest that the predominant factors influencing racemization in polyamino acids may not be the same as those influencing proteins. In any case, our findings strikingly illustrate the need for caution in drawing mechanistic conclusions about the racemization of amino acids in structurally different microenvironments (such as free amino acids, small peptides, polyamino acids, proteins) unless the extent of racemization was measured under identical conditions.

In summary, this and related studies demonstrate the paramount importance of "structural" features in the racemization of amino acids, peptides, and proteins. These features include the nature of the amino acid side chains; the pK values of ionizable functional groups; the amino acid sequence; the conformation of polyamino acids and proteins; hydrogen-bonding, hydrophilic, or hydrophobic interactions; the ionic strength of the solution; and solvent effects near the asymmetric centers. The apparent importance of structural features suggests that the primary amino acid sequence of a protein largely dictates the amino acid's susceptibility to racemization. Future studies should be directed to defining the role of these variables in the racemization process.

ACKNOWLEDGMENT

We thank Feli Bautista, Simone Ledermann, and Carol Levin for excellent technical assistance.

Registry No. Poly-L-asparagine, 28088-48-4; poly-L-asparagine, SRU, 26894-34-8; poly-L-aspartic acid, 25608-40-6; poly-L-aspartic acid, SRU, 26063-13-8; poly-L-histidine, 26062-48-6; poly-L-histidine, SRU, 26854-81-9; poly-L-lysine, 25104-18-1; poly-L-lysine, SRU, 38000-06-5; poly-L-serine, 25821-52-7; poly-L-serine, SRU, 25821-94-7; poly-L-tyrosine, 25619-78-7; poly-L-tyrosine, SRU, 25667-16-7.

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Received for review October 1, 1986. Revised manuscript received May 11, 1987. Accepted June 22, 1987.