reagents, loss in activity on reduction by mercaptoethanol, and electrophoretic studies confirm that the new inhibitor is proteinaceous in nature.

We could not unequivocally demonstrate the formation of an amylase–inhibitor complex during our studies. When inhibitor and human pancreatic amylase were chromatographed on Red-Sepharose, all the protein was eluted from the column whereas the native inhibitor was bound tightly to the immobilized ligand. While it should be expected that the inhibitor–enzyme complex would be eluted from Sephadex G-100 column earlier than the free enzyme and inhibitor, in our studies such a protein peak could not be identified. Instead, the inhibitor enzyme mixture was eluted in two protein peaks. The first peak with a V_e value corresponding to the native inhibitor had measurable amylase activity. The second peak corresponding to native human pancreatic amylase had neither amylase activity nor inhibitory activity. However, treatment of this fraction with mercaptoethanol resulted in the appearance of amylase activity. These data suggest that the two protein peaks obtained during gel chromatography of amylase–inhibitor mixture represent two types of complexes. The fraction with amylase activity eluted first during gel chromatography could represent a complex formed due to loose interaction of inhibitor and enzyme dissociating under assay conditions exhibiting amylase activity. The second protein fraction may represent a complex formed by strong noncovalent interaction between the inhibitor and amylase, which exhibited amylase activity on inactivation of the inhibitor by treatment with mercaptoethanol. Further studies are needed to substantiate this view.

Registry No. α-Amylase, 9000-90-2.

LITERATURE CITED

Abe, O.; Ohata, J.; Utsumi, Y.; Kuromizu, K. *J. Biochem.* **1978**, *83*, 1731.
Bernfeld, P. *Methods Enzymol.* **1955**, *1*, 149.
Blanco-Labra, A.; Iturbe-Chinas, F. A. *J. Food Biochem.* **1981**, *5*, 1.
Buonocore, V.; Petrucci, T.; Silano, V. *Phytochemistry*, **1977**, *16*, 611.
Chandrasekher, G.; Prasad Raju, D.; Pattabiraman, T. N. *J. Sci. Food Agric.* **1981**, *32*, 9.
Chandrasekher, G.; Pattabiraman, T. N. *Ind. J. Biochem. Biophys.* **1983**, *20*, 241.
Chaplin, M. F. *Biochem. J.* **1976**, *155*, 457.
Davis, A. B.; Hoseney, R. C. *Cereal Chem.* **1979**, *56*, 310.
Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.
Haynes, R.; Osuga, D. T.; Feeney, R. F. *Biochemistry* **1967**, *6*, 541.
Kneen, E.; Sandstedt, R. M. *Arch. Biochem.* **1946**, *9*, 235.
Lowry, O. H. Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1961**, *193*, 265.
Minamiura, N.; Kimura, Y.; Tsujino, K.; Yamamoto, T. *J. Biochem.* **1974**, *77*, 163.
Mundy, J.; Hejgaard, J.; Svendsen, I. *FEBS Lett.* **1984**, *167*, 210.
Petrucci, T.; Rab, A.; Tomasi, M.; Silano, V. *Biochem. Biophys. Acta* **1978**, *420*, 288.
Shainkin, R.; Birk, Y. *Biochim. Biophys. Acta* **1970**, *221*, 502.
Sharma, K. K.; Pattabiraman, T. N. *J. Sci. Food Agric.* **1982**, *33*, 255.
Shivaraj, B.; Pattabiraman, T. N. *Indian J. Biochem. Biophys.* **1980**, *17*, 181.
Strumeyer, D. H.; Malin, M. J. *Biochim. Biophys. Acta* **1969**, *184*, 643.
Takahashi, K.; Mizobe, F.; Chang, W. J. *J. Biochem.* **1972**, *71*, 161.
Takahashi, K.; Chang, W. J.; Ko, J. S. *J. Biochem.* **1974**, *76*, 897.
Warchalewski, J. R. *Nahrung* **1983**, *27*, 103.
Weselake, R. J.; MacGregor, A. W.; Hill, R. D. *Plant Physiol.* **1983**, *73*, 1008.

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Racemization Kinetics of Free and Protein-Bound Amino Acids under Moderate Alkaline Treatment

Rémy Liardon* and Simone Ledermann

Amino acid racemization was measured in α-lactalbumin, β-lactoglobulin, α-casein, lysozyme, and BSA and in a free amino acid mixture exposed to pH 9 at 83 °C for times ranging from 0.5 to 24 or 96 h. Inversion rate constants were determined for 14 to 15 amino acids in the six models. Conclusions derived from these data were as follows: (1) Free amino acids racemize about 10 times more slowly than bound residues. (2) The main driving force in free amino acid racemization is the electron-withdrawing ability of the side chain (σ^* constant), except for Asp, the inversion of which seems to involve an intramolecular assistance effect. (3) The racemization of bound residues is governed by both amino side chain effects and protein-related factors. (4) Bound amino acid racemization is also affected by alkali-induced denaturation of the proteins (e.g., partial hydrolysis).

INTRODUCTION

Structural and chemical changes occurring in food proteins on processing may produce undesirable nutritional effects. These include cross-linking (Provansal et al., 1975; Friedman, 1977; Finot, 1983), degradation (Asquith and Otterburn, 1977; Sen et al., 1977), and reactions with sugars (Finot, 1982) or with other food constituents such as polyphenols (Mauron, 1983; Nielsen et al., 1985). Recent investigations have focused on the racemization of amino acid residues, a process that may also be nutritionally detrimental (Hayase et al., 1973, 1975, 1979; Masters and Friedman, 1979; Friedman et al., 1981; Friedman and Masters, 1982; Liardon and Hurrell, 1983; Liardon and Ledermann, 1984; Jenkins et al., 1984). The conversion of L amino acids in food proteins into D isomers generates nonutilizable forms of amino acids, creates peptide bonds

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resistant to proteolytic enzymes, and forms unnatural amino acids that may be nutritionally antagonistic or even toxic (Freimuth et al., 1978; Masters and Friedman, 1980; Bunjapamai et al., 1982; Tovar and Schwass, 1983; Friedman and Gumbmann, 1984). An understanding of the factors influencing amino acid racemization in protein under food-processing conditions may contribute to the production of foods with optimal nutritional value and safety. Amino acid racemization occurs under various conditions of temperature, pH, and water activity (Hill and Leach, 1964; Ikawa, 1964; Manning, 1970; Hayase et al., 1975; Liardon and Hurrell, 1983) and might be activated by other food constituents like carbohydrates or lipids (Zumberge, 1979; Hayase et al., 1979). On the other hand, this reaction proceeds most readily at basic pH (Tannenbaum et al., 1970; Provansal et al., 1975; Liardon and Hurrell, 1983), and factors influencing the racemization rate of several amino acids under these conditions have been studied (Hill and Leach, 1964; Masters and Friedman, 1979; Friedman et al., 1981; Friedman and Masters, 1982).

However, the analytical means available frequently limited investigations to a few amino acids, often omitting the essential ones. Recently, more complete data have been reported. In an essentially qualitative study, Schwass and Finley (1984) described racemization in three proteins as a function of pH (3.3–12.8) and temperature (20–100 °C). Differences in susceptibility toward racemization were observed between the proteins: of the 11 amino acids investigated, serine appeared to be the most sensitive indicator of racemization. In a detailed kinetic study of alkali-induced racemization by Friedman and Liardon (1985), inversion rate constants were determined for 13 amino acids in soybean proteins exposed to 0.1 N NaOH at 75 °C; a survey of the influence of pH (8–14) and temperature (25–95 °C) on the extent of racemization in the same samples was also presented.

The extreme alkaline pH values of the latter study were not typical of actual food-processing conditions. Furthermore, the racemization kinetics were partly complicated by the simultaneous occurrence of alkaline protein hydrolysis and the degradation of some amino acids. The present work supplements our previous studies, investigating the racemization of free and protein-bound amino acids under milder alkaline conditions and evaluating the relative influence of protein-related and amino acid intrinsic factors on the inversion rates for the different species. Measurements were performed on five model proteins and on a mixture of free amino acids exposed to pH 9, at 83 °C, for various lengths of time.

EXPERIMENTAL SECTION

Materials. Five proteins were selected as models for studying bound amino acid racemization. α-Casein, α-lactalbumin, and β-lactoglobulin were purchased from Sigma Chemical Co. (St. Louis, MO), bovine serum albumin (BSA, fraction V) from Fluka (Buchs, Switzerland), and egg lysozyme from Serva (Heidelberg, FRG). The free amino acid model consisted of an equimolar mixture of 15 usual protein L amino acids.

Alkaline Treatment. Solutions of each protein (2 mg/mL) or of the free amino acid mixture (3.6 mg/mL) were prepared in 0.05 M borate buffer. The solutions were adjusted to pH 9.0 at room temperature. Aliquots (500 μL) of these solutions were transferred to small conical screw-capped reaction vials sealed with a septum. These were placed in an aluminum heating block (Pierce Chemical Co., Rockford, IL) and heated at 83 ± 1 °C, as measured with a thermocouple in a dummy vial. The effective pH at this temperature was 8.5. Heating times ranged from 0.5 to 24 h for the protein samples and from 2 to 96 h for the free amino acid model. Each assay was run in duplicate.

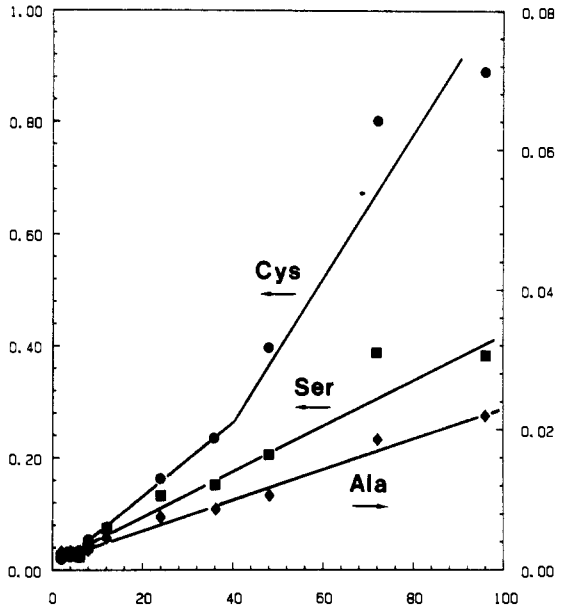


Figure 1. Racemization time course of free Ala, Ser, and Cys at pH 9, 83 °C. Arrows indicate ordinate axis. Note that for Cys the lines were drawn to indicate the slope change after approximately 40 h. The data do not permit a precise estimate of the time when the change actually occurs.

Sample Hydrolysis and Cleanup. After the alkaline treatment, samples were freeze-dried, redissolved in 2 mL of 6 N DCl, and transferred into hydrolysis ampules (Pierce Chemical Co.), and ethyl mercaptan (50 μL) was added as a reducing agent to convert cystine into cysteine. The samples were evacuated and heated for 24 h at 110 °C, after which the hydrolysate samples were vacuum dried on a rotary evaporator. The dry residues were redissolved in 1 mL of 0.2 M acetic acid and applied to small Dowex 50 (H^+) columns. After the buffer salts were washed from the resin with 10 mL of distilled water, the amino acids were displaced with 1 mL of 3 N NH_4OH followed by 1 mL of water into conical reaction vials and dried under nitrogen flow.

Racemization Measurement. The recovered amino acids were converted into *N*(*O*,*S*)-perfluoropropionyl isopropyl esters in a two-step reaction and analyzed by using the procedure described previously (Liardon et al., 1981). The amino acid enantiomers were separated on an OS 6411 fused silica capillary column (CGC Analytic, Moessingen, FRG) installed in a HP 5992 bench top GC/MS and detected by selected ion monitoring (SIM). The procedure allows the differentiation of D amino acid isomers formed during acid hydrolysis from those initially present in the samples and provides racemization values for these two contributions.

RESULTS

Amino acid inversion follows first-order kinetics obeying eq 1, where k is the rate constant, D/L the enantiomeric

$$k = 0.5 \ln [(1 + D/L)/(1 - KD/L)] = 0.5 f\{D/L\} \quad (1)$$

ratio at time t, and K the enantiomeric ratio at equilibrium. For all amino acid containing only one asymmetry center and for threonine, K is equal to 1. For isoleucine, K is equal to 0.79 (Nakaparksin et al., 1970). The D/L ratios determined for individual amino acids in the five proteins and the free amino acid mixture were used to determine values of $f\{D/L\}$ which were plotted against t according

Table I. Inversion Rate Constants of Free and Protein-Bound Amino Acids at pH 9, 83 °C

AA	free AA, 10^{-8} s^{-1}	lactalbumin, 10^{-7} s^{-1}	lysozyme, 10^{-7} s^{-1}	BSA, 10^{-7} s^{-1}	lactoglobulin, 10^{-7} s^{-1}	casein, 10^{-7} s^{-1}
Ala	3.0 (0.1)	2.3 (0.2)	5.6 (0.7)	7.5 (0.2)	2.8 (0.1)	3.6 (0.1)
Val	0.7 (0.1)	0.5 (0.1)	1.1 (0.1)	0.9 (0.3)	0.4 (0.05)	0.4 (0.01)
Leu	2.0 (0.2)	1.5 (0.1)	2.6 (0.3)	2.2 (0.1)	0.9 (0.1)	1.6 (0.1)
Ile	1.2 (0.1)	1.1 (0.1)	1.2 (0.3)	1.4 (0.3)	0.4 (0.05)	0.7 (0.1)
Met[a]	7.9 (0.4)	9.3 (0.5)	15.6 (0.9)	13.6 (0.5)	8.9 (0.5)	8.7 (0.3)
Met[b]		19 (2)				
Cys[a]	91 (2)	440 (50)	>1000	460 (10)	33 (2)	
Cys[b]	160 (30)	40 (4)	42 (8)	38 (8)		
Phe[a]	5.9 (0.3)	9.2 (0.5)	17.8 (0.7)	15.8 (0.4)	1.1 (0.2)	8.9 (0.4)
Phe[b]					5.0 (0.1)	
Tyr	5.2 (0.6)	8 (1)	13.0 (0.7)	11.0 (0.6)	3.5 (0.3)	4.7 (0.2)
Lys	4.0 (0.02)	4.2 (0.4)	10.7 (0.7)	11.4 (0.7)	4.1 (0.2)	9.9 (0.2)
Ser[a]	54 (2)	117 (3)	300 (30)	240 (10)	57 (1)	61 (2)
Ser[b]		75 (8)	59 (5)	67 (2)		
Thr[a]	21 (3)	18.3 (0.5)	62 (1)	41 (7)	18.7 (0.9)	18
Thr[b]			17 (2)			
Asp[a]	9.1 (0.3)	34 (2)	>195	90 (24)	29 (2)	51 (2)
Asp[b]		22 (2)	37 (1)	22 (2)	15.2 (0.7)	12 (2)
Glu	1.8 (0.7)	5.2 (0.3)	12.6 (0.5)	11.5 (0.2)	4.5 (0.5)	7.0 (0.4)
Trp	3.7 (0.9)		7 (2)			
Pro	2.7 (0.3)	5.7 (0.4)	<0.1	0.4 (0.1)	0.3 (0.1)	0.5

[a] Based on initial linear part of the racemization curve (or on complete curve if a straight line). [b] Based on second linear part of the curve.

Table II. Relative[a] Inversion Rate Constants of Free and Protein-Bound Amino Acids at pH 9, 83 °C

AA	free AA	lactalbumin	lysozyme	BSA	lactoglobulin	casein
Ala	1	1	1	1	1	1
Val	0.2	0.2	0.2	0.1	0.1	0.1
Leu	0.7	0.7	0.5	0.3	0.3	0.4
Ile	0.4	0.5	0.2	0.2	0.1	0.2
Met[b]	2.6	4.0	2.8	1.8	3.2	2.4
Met[c]		8.3				
Cys[b]	30	190	>180	60	12	
Cys[c]	53	17	8	5		
Phe[b]	2.0	4.0	3.2	2.1	0.4	2.5
Phe[c]					1.8	
Tyr	1.7	3.5	2.3	1.5	1.3	1.3
Lys	1.3	1.8	1.9	1.5	1.5	2.8
Ser[b]	18	50	54	32	20	17
Ser[c]		33	11	9		
Thr[b]	7	8	11	6	7	5
Thr[c]			3			
Asp[b]	3	15	>35	12	10	14
Asp[c]		10	7	2.9	5	3
Glu	0.6	2.3	2.3	1.5	1.6	1.9
Trp	1.2		1.3			
Pro	0.9	2.5	0	0.05	0.1	0.1

[a] Rates have been normalized with respect to k_{Ala}. [b] Based on initial linear part of the racemization curve (or on complete curve if a straight line). [c] Based on second linear part of the curve.

to eq 1, as shown in Figure 1.

The racemization time curves of all free amino acids were linear, as expected, with the exception of cystine, which appeared to double its racemization rate after 40 h of treatment (Figure 1); no satisfactory explanation was found for this. Compared to this simple behavior generally observed with the free amino acids, the racemization curves of protein-bound residues often exhibited more complex behaviors.

Inversion rate constants k were calculated from eq 1 by linear regression analysis. For curves exhibiting slope changes, different values of k were determined corresponding to the different linear sections. The results are reported in Table I. Relative rate constants normalized to that of Ala in each model are given in Table II.

The analytical procedure used in this study also provided values for the amounts of D amino acids formed during the acid hydrolysis of the proteins. Despite their apparent irrelevancy to the problem, these data were examined. For some amino acids, the extent of hydrolysis-induced racemization was found to depend on the duration of the alkaline treatment. This effect appeared to be related to some specific alkali-induced denaturation process of the proteins.

DISCUSSION

The mechanism of amino acid inversion under alkaline conditions proceeds via the abstraction of the α-hydrogen and the formation of a carbanion. The inversion rate is directly related to the formation probability and the stability of this intermediate state. This, in turn, is affected by the structural and electronic characteristics of the amino acid and its microenvironment. In this study, four factors were found to have an influence on racemization rates: (1) the free or bound state of the amino acid; (2) the amino acid side chain R; (3) the protein structure; (4) protein denaturation processes.

Free vs. Bound Amino Acids. Under neutral or alkaline conditions, the presence of the negatively charged α-carboxylate group would exert a destabilizing effect on the intermediate α-C carbanion. Therefore, free amino acids were expected to racemize at a slower rate than protein-bound residues (Smith and Evans, 1980). This assumption had been verified on several occasions with

Table III. Parameters of Free Energy Relationship between k and σ^*

model[a]		slope (ρ factor)	intercept	correln coeff
free amino acids	(1)	2.06 (0.15)	−0.09 (0.05)	0.969
casein	(1)	2.20 (0.35)	−0.10 (0.09)	0.900
	(2)	2.11 (0.44)	−0.15 (0.10)	0.850
lactoglobulin	(1)	2.20 (0.32)	−0.21 (0.10)	0.906
	(2)	2.10 (0.50)	−0.21 (0.11)	0.830
lactalbumin	(1)	2.75 (0.25)	0.08 (0.08)	0.960
	(2)	2.11 (0.26)	0.08 (0.06)	0.945
BSA	(1)	2.75 (0.23)	−0.16 (0.07)	0.966
	(2)	2.41 (0.34)	−0.17 (0.08)	0.927
lysozyme	(1)	2.93 (0.28)	−0.06 (0.09)	0.954
	(2)	2.29 (0.32)	−0.05 (0.07)	0.922

[a] Key: (1) all amino acids except Asp; (2) Asp, Ser, and Cys not included in the regression.

limited numbers of amino acids (Schroeder, 1974; Kriausakul and Mitterer, 1978; Sol, 1978). From the data in Table I, it appeared to apply to all amino acids. Under the chosen conditions, the racemization of bound residues was about 10 times faster than that of free amino acids, approximately the same as the ratio reported by Sol (1978) for the racemization of free and bound Ala, Leu, and Asp at pH 8 and 122.5 °C.

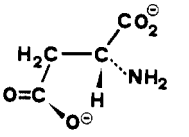
Influence of Amino Acid Side Chain. The electron-withdrawing ability (negative inductive strength) of the amino acid side chain is commonly evoked to explain differing racemization rates of amino acids. This relationship can be tested by plotting racemization rates against R group σ^* values, a measure of the inductive strength (Bada, 1971). Such plots have been reported by several authors for different conditions of racemization and on the basis of data of varying completeness. Rather poor correlations were obtained for racemization at low pH or in solid-heated protein (Smith et al., 1978; Liardon and Jost, 1981; Liardon and Hurrell, 1983), but alkali-induced racemization rates have generally shown much better correlations (Friedman and Masters, 1982; Friedman and Liardon, 1985).

In the present study, we were able to verify the validity of that relationship in both free and bound amino acid models. The logarithms of each amino acid rate constant relative to that of Ala were plotted against R group σ^* (Hansch and Leo, 1979) values for the different models (Figure 2). For the proteins, the position of both acid and amide forms of Asp and Glu was plotted. The omission of Pro from those plots was due to the unavailability of a σ^* value for the pyrrolidinyl group.

A distinct linear trend was observed in all six plots. The parameters of the least-squares regression lines are reported in Table III. The best correlation was obtained for the free amino acid model. In general, these results appeared to confirm the role of the R group inductive strength as a driving force in the racemization mechanism, but with one remarkable exception—in all six models, Asp appeared to racemize at a significantly higher rate than expected. (For this reason, Asp was not included in the regression calculation.) This had already been reported for alkali-induced racemization in proteins (Friedman and Masters, 1982; Friedman and Liardon, 1985), and it was suggested that the Asp-racemizing form in proteins may be the amide. However, since we show here that free Asp behaves in the same way, this explanation cannot hold.

As an alternative, we propose that the higher inversion rate for Asp is due to the participation of the β-carboxylate group in forming the incipient carbanion as illustrated in Chart I. This group could contribute to the abstraction of the α-proton via a five-membered ring transition state.

Chart I



Recently, Friedman and Masters (1982) reported that the activation energy for Asp racemization in alkali-treated casein (0.1 N NaOH, 65 °C) was considerably lower (20.8 kcal/mol) than for Phe, Glu, and Ala (28.8–32.4 kcal/mol). The same trend had been reported earlier by Sol (1978) for collagen-bound Asp, Ala, and Leu at pH 8. These results are consistent with the hypothesis of an intramolecular assistance effect. Moreover, this mechanism is expected only to be effective when the β-carboxylic group is completely dissociated, i.e. at pH 8 (Bada, 1971). In fact, under acid and neutral conditions, the Asp racemization rate correlated perfectly with the σ^* value of the undissociated β-carboxylic group (Liardon and Jost, 1981; Liardon and Hurrell, 1983).

In the case of Glu, the occurrence of a similar mechanism is less probable, since it would involve a six-membered ring transition state. Accordingly, Glu data points in the log k–σ^* plots were found to lie close to the regression lines, and the activation energy for Glu racemization, as reported by Friedman and Masters (1982), did not differ significantly from that of Ala or Phe.

Influence of Protein Structure. As discussed above, the data of Figure 2 and Table III tended to confirm the importance of R group inductive effect on the racemization rate of bound amino acids in all five model proteins. Yet the data also showed evidence of the influence of protein-related factors on the racemization process. In Table III, the correlation coefficients characterizing the regression lines for the various proteins were systematically lower than for the free amino acid model and the slopes of the lines (ρ factors) presented significant differences. Significant variations were also noticed for the five proteins in both the absolute magnitudes of the rate constants of the same amino acid and the relative order of the rate constants or the different amino acids (Tables I and II).

Of all amino acids, Cys and Ser appeared to be most sensitive to protein-related factors. The rate constants of Cys ranged from 3.3×10^{-6} to $>100 \times 10^{-6}$ s^{-1} and the relative rate calculated with respect to that of Ala from 12 to 190. Similar variations were observed with Ser, but to a lesser extent. The ρ factors in Table III obtained after excluding Ser and Cys from the regression presented less discrepancy, approaching the value for the free amino acid model. This seemed to confirm the dependence of Cys and Ser rate constants on protein-related factors.

In general, the available data did not explain the variations observed among the proteins. Nevertheless, a clue to the kind of structural factor capable of influencing the racemization rates was found for Pro. In α-lactalbumin, Pro was found to racemize more than 10 times faster than in any of the other proteins (Table I). To understand this, the environment of all Pro residues in the various proteins was examined. In particular, α-lactalbumin was found to contain only two Pro residues, in positions 24 and 67, with the following partial sequences (Dayhoff, 1972):

$$\overset{24}{\text{-Leu-Pro-Glu-}} \quad \overset{67}{\text{-Asp-Pro-His-}}$$

The four atoms involved in a peptide bond are known to form a relatively rigid structure, usually with trans configuration. X-Pro bonds have been shown to exist in both trans and cis configuration (Patel, 1973; Smith and Sol,

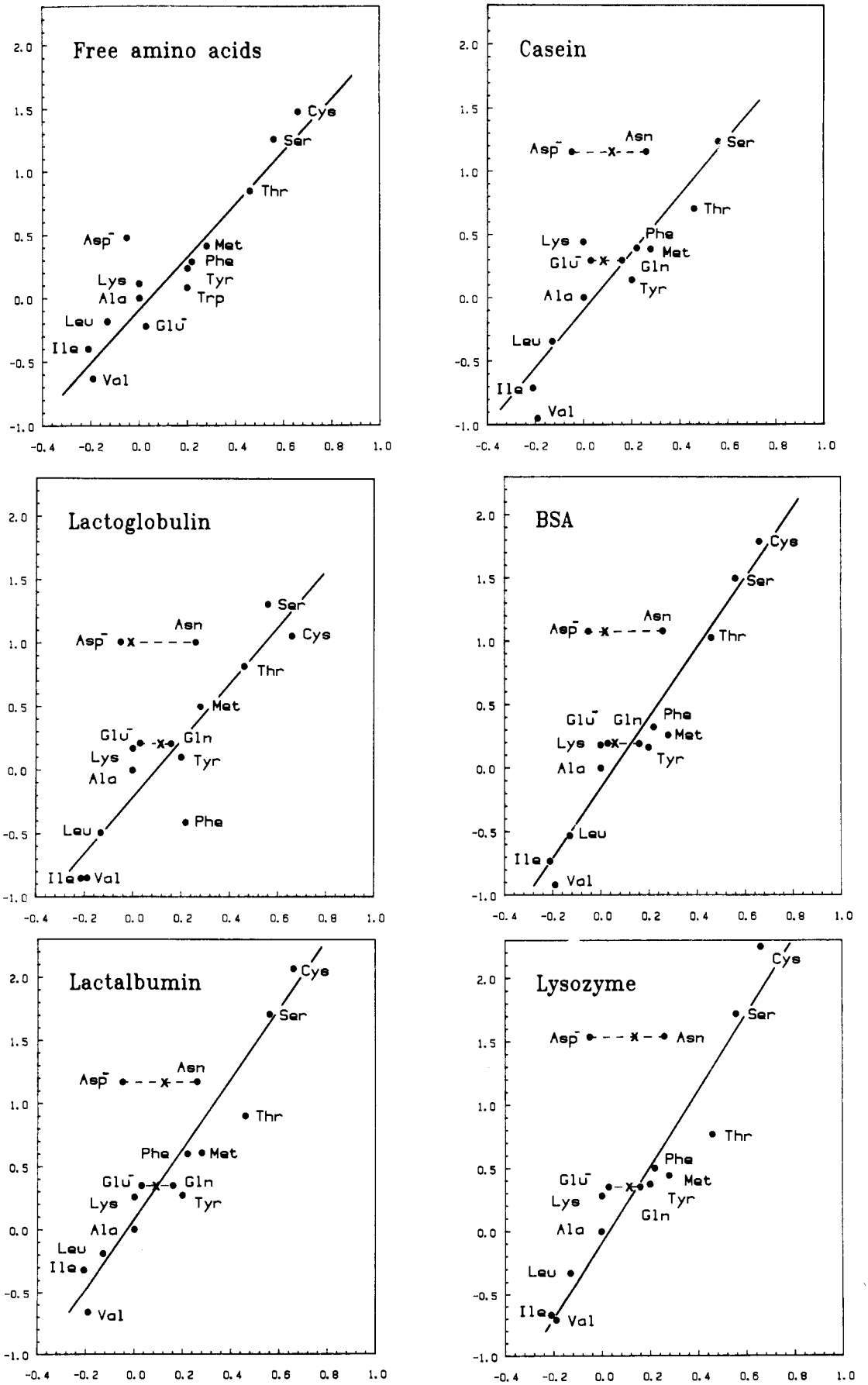


Figure 2. Relationship between the inductive constant (σ^*) of the amino acid side chain and the logarithm of the inversion rate constant (k) relative to that of alanine (k_{Ala}) for Val, Ile, Leu, Glu, Gln, Lys, Trp, Tyr, Phe, Met, Asp, Asn, Thr, Ser, and Cys in free amino acid and protein models heated at 83 °C in pH 9 buffer. The weighed average between Asp and Asn and between Glu and Gln based on the amino acid composition of the proteins are indicated by ×. The least-squares regression lines were calculated without including Asp/Asn data points. Inductive constants were taken from Hansch and Leo (1979).

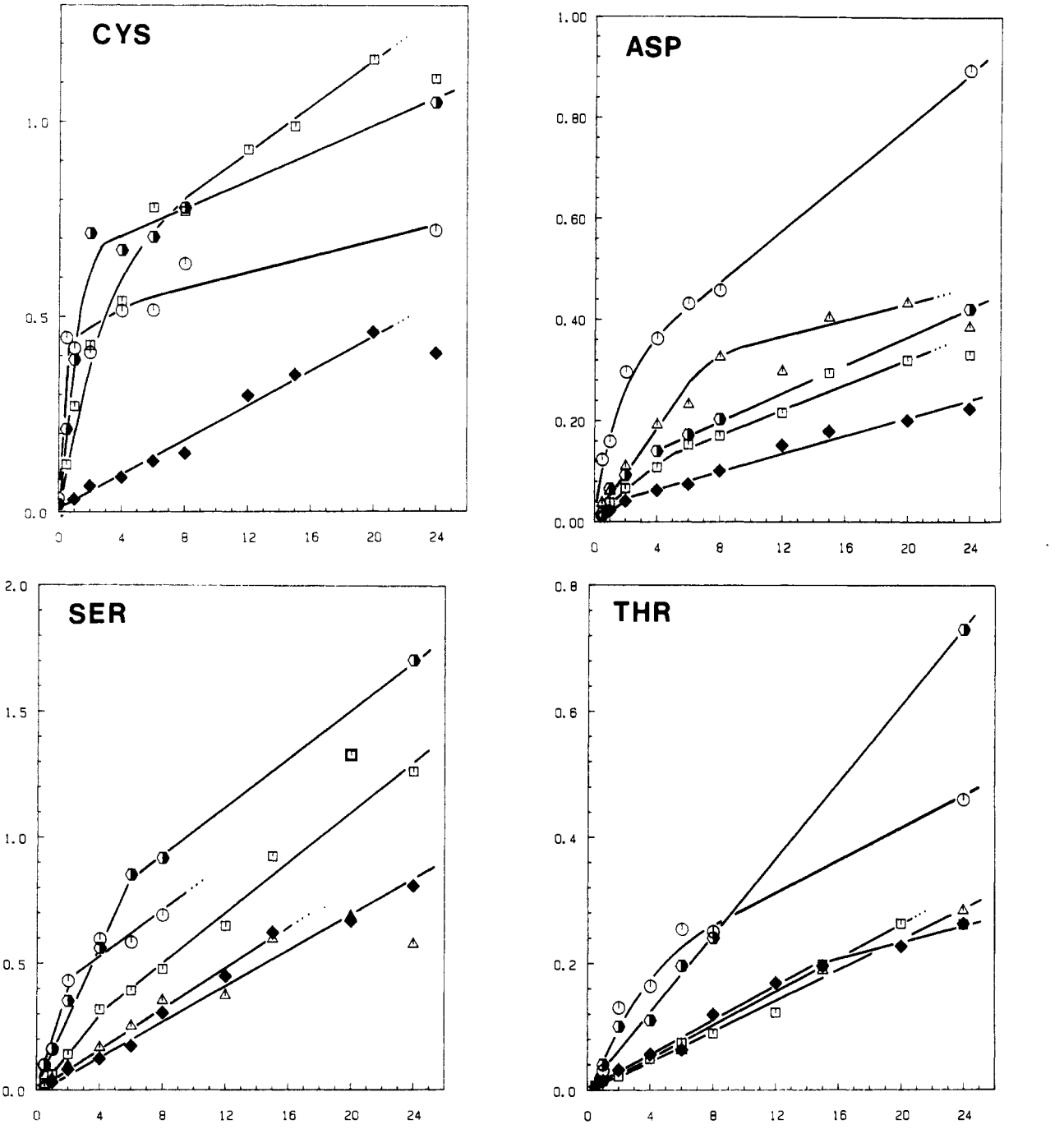


Figure 3. Racemization time course for Cys, Asp, Ser, and Thr in (□) α-lactalbumin, (◑) BSA, (○) lysozyme, (♦) β-lactoglobulin, and (△) casein exposed to pH 9 at 83 °C.

1980). In the case of Asp-Pro, the latter configuration places the Asp β-carboxylate group in the immediate vicinity of Pro α-hydrogen, as illustrated in Chart II. This arrangement would make it possible for the carboxylate group to participate in the formation of the Pro α-C carbanion. Among the five model proteins, the Asp-Pro combination was unique to α-lactalbumin. Therefore, we suggest that the higher inversion rate for Pro in this protein is due to the Asp assistance effect on Pro-67 residue.

Influence of Protein Denaturation on Amino Acid Racemization. For bound amino acid, the linear behavior expected from eq 1 often held only in the initial part of the racemization curves. Depending on the amino acid and the protein, this first period could extend from 30 min to several hours. At longer treatment times changes in kinetics would occur, presumably due to protein denaturation. Similar results have been reported in the literature, usually showing a decrease of the racemization rate for prolonged heat or alkaline treatment (Kriausakul and Mitterer, 1978; Sol, 1978; Masters and Friedman, 1979; Friedman and Masters, 1982; Friedman and Liardon, 1985). In the present study, a variety of different behaviors was observed, as illustrated in Figures 3–6. We will attempt to rationalize these observations.

The racemization curves of Asp/Asn, Cys, Ser, and Thr shown in Figure 3 present about the same pattern. After

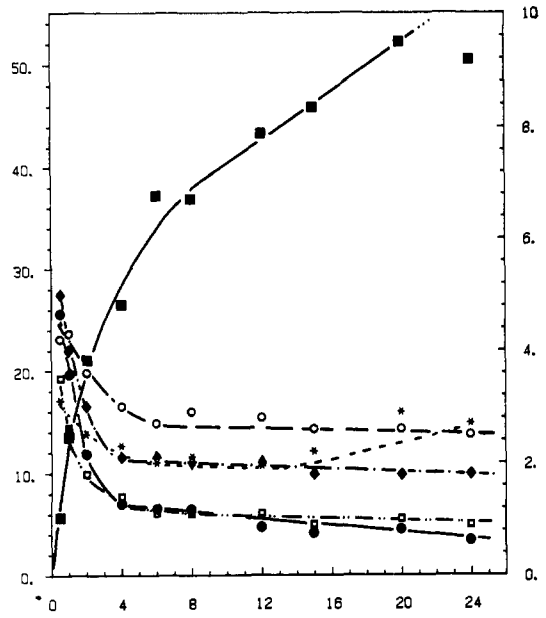


Figure 4. Influence of alkaline treatment duration on acid hydrolysis induced racemization of Cys N neighbors in α-lactalbumin. Left scale: (●) Met. Right scale: (○) Leu, (*) Lys, (♦) Ile, (□) Val. Also plotted for comparison: Cys alkali-induced racemization (■), left scale.

a period of time varying from one protein to another, a sharp decrease of the racemization rate appears to take place. These curves are quite similar to those obtained by Sol for collagen-bound Asp at pH 8 and 10 (Sol, 1978). This author explained the decline of the racemization rate by the progressive formation of slow-racemizing free Asp. For different reasons, however, this interpretation was not found entirely appropriate to explain the present results: (1) Under the treatment conditions chosen for this study, the complete liberation of amino acid residues was unlikely to occur. (2) The distinct bilinear shape of the racemization curves would indicate that beyond the inflection point no more change occurred in the protein, in contradiction to the concept of a progressive formation of free amino acids. (3) The rate constants calculated from the second linear part of the racemization curves were still much higher than those of the free amino acids (Table I).

On the other hand, the racemization rate of bond residues might conceivably be affected by protein partial hydrolysis. At pH 9, terminal residues carry either a free amino group or a dissociated carboxylic group, both of which would exert a negative influence on the stability of the incipient carbanion. Consequently, the inversion rate of residues moving from interior to terminal positions should decrease. This effect is expected to be particularly noticeable if peptide bond cleavage occurs at specific sites rather than randomly in the protein chain, and the racemization curves of the involved residues should resemble those in Figure 3. Supporting this hypothesis, Sine and Hass (1969) observed that muscle aldolase exposed to moderate alkaline conditions (pH 12.5 at 0 °C) hydrolyzed specifically at Ser, Thr, and possibly Pro N bonds. It was also established that N-terminal Ser racemized at a much slower rate than interior Ser (Noll et al., 1974).

No similar report was found for Asp/Asn or Cys in the literature, although protein hydrolysis in dilute acid is known to occur preferentially at Asp bonds (Schultz, 1967). In the case of Cys, the hypothesis of the specific cleavage of this residue was indirectly supported by acid hydrolysis induced racemization data (Figure 4). During acid hydrolysis, Cys has been found to promote the racemization

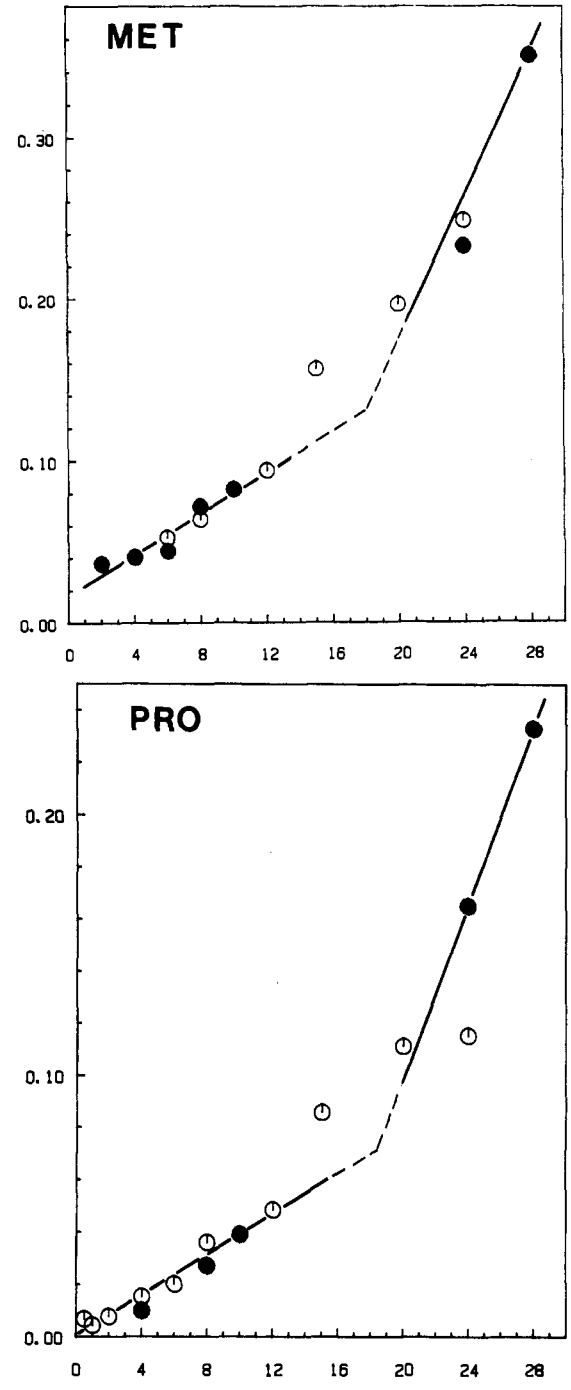


Figure 5. Racemization time course of Met and Pro in α-lactalbumin exposed to pH 9 at 83 °C: (○) first assay; (●) second assay.

of its N-bound neighbor by forming a thiazoline intermediate (Woivode et al., 1978; Liardon and Jost, 1981; Liardon and Ledermann, 1984). In Figure 4, the amount of hydrolysis-induced racemization of the various Cys N-bound neighbors in α-lactalbumin was plotted against the duration of the alkaline treatment. This plot shows that the racemization-inducing effect of Cys progressively vanished as a function of treatment time, as expected if the corresponding peptide bond had been cleaved. Furthermore, the complete disappearance of the Cys neighbor effect coincided with the beginning of the second linear part of Cys alkali-induced racemization curve. Similar observations were made on the other model proteins.

An additional aspect of the data illustrated in Figure 3 is the different behavior observed for the same residue in the various proteins. These discrepancies might reflect

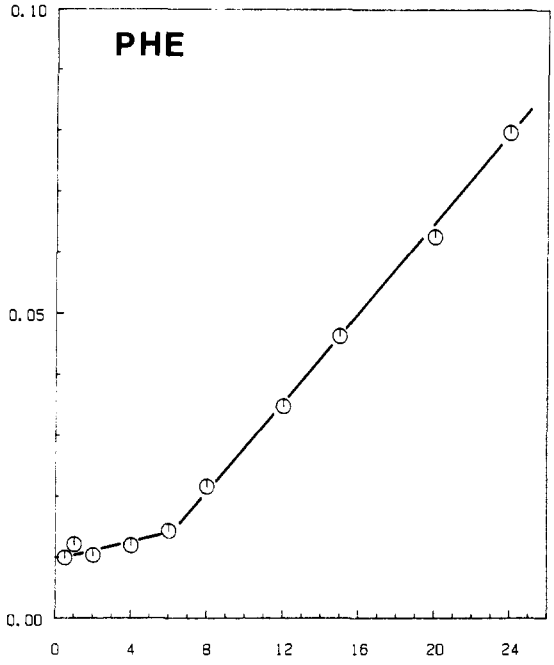


Figure 6. Racemization time course of Phe in β-lactoglobulin exposed to pH 9 at 83 °C.

differing susceptibilities of the proteins toward alkaline cleavage. The linear curves of Cys in β-lactoglobulin and of Ser and Thr in β-lactoglobulin and casein combined with low racemization rates would suggest the rapid hydrolysis of these two proteins at the sites of those residues. Conversely, BSA would appear to be particularly resistant to cleavage at Thr sites, and to a lesser extent at Ser sites.

In contrast to the cases discussed so far, Figures 5 and 6 show examples of amino acids whose racemization rate was found to increase at longer treatment times. Again it seemed that an explanation of this phenomenon was to be found in the selective hydrolysis of Asp/Asn, Cys, Ser, and Thr peptide bonds. For instance, α-lactalbumin contains a single Met residue in position 90:

$$\overset{90}{\text{-Asn-Asn-Ile-Met-Cys-Val-}}$$

In this sequence, the cleavage of both Asn–Ile and Met–Cys bonds would progressively liberate Ile-Met dipeptides in the alkaline medium. Racemization in dipeptides has been shown to be influenced predominantly by intramolecular assistance effects and to proceed at particularly high rates (Smith and Sol, 1980). This would explain the progressive increase of Met racemization rate with prolonged alkaline treatment.

As it turned out, a similar situation was found to exist for the two other examples illustrated in Figures 5 and 6, i.e. Phe in β-lactoglobulin

$$\overset{151}{\text{-Leu-Ser-Phe-Asn-}} \quad \text{(1 Phe out of 4)}$$

and Pro in α-lactalbumin.

$$\overset{67}{\text{-Asp-Pro-His-Ser-}} \quad \text{(1 Pro out of 2)}$$

The examination of the different protein sequences showed that other such dipeptides could be formed due to combinations of hydrolysis-prone residues. However, a significant upward bend in the racemization time course could only be observed in cases where this effect was not suppressed due to the presence in the protein of a large number of the residue in question.

CONCLUSION

Under alkaline conditions, the racemization of free amino acids is almost exclusively governed by the electronic characters of their different substituents and functional groups, with the exception of aspartic acid where the formation of the intermediate carbanion seems to be controlled by an intramolecular assistance effect. For bound residues, amino acid and protein structural factors appear to play an almost equivalent role. In spite of the complexity of the problem, it has been possible in some instances to propose a rationalization of the relationship observed between protein structure and racemization. However, many of the considerations developed in this report had to be based on indirect evidence. More specifically designed experiments will have to be carried out to confirm these conclusions.

Registry No. L-Ala, 56-41-7; L-Val, 72-18-4; L-Leu, 61-90-5; L-Ile, 73-32-5; L-Met, 63-68-3; L-Cys, 52-90-4; L-Phe, 63-91-2; L-Tyr, 60-18-4; L-Lys, 56-87-1; L-Ser, 56-45-1; L-Thr, 72-19-5; L-Asp, 56-84-8; L-Glu, 56-86-0; L-Trp, 73-22-3; L-Pro, 147-85-3; lysozyme, 9001-63-2.

LITERATURE CITED

Asquith, R. S.; Otterburn, M. S. *Adv. Exp. Med. Biol.* **1977**, *86A*, 93.
Bada, J. L. *Adv. Chem. Ser.* **1971**, *No. 105*, 309.
Bunjapamai, S.; Mahoney, R. R.; Fagerson, I. S. *J. Food Sci.* **1982**, *47*, 1229.
Dayhoff, M. D. *Atlas of Protein Sequences and Structures*; National Biomedical Research Foundation: Washington, DC, 1972.
Finot, P. A. *Diabetes* **1982**, *31*, 22.
Finot, P. A. *Nutr. Abstr. Rev.* **1983**, *53*, 67.
Freimuth, U.; Krause, W.; Doess, A. *Nahrung* **1978**, *22*, 557.
Friedman, M. In *Protein Crosslinking, Nutritional and Medical Consequences*; Friedman, M., Ed.; Plenum: New York, 1977; p 1.
Friedman, M.; Gumbmann, M. R. *J. Nutr.* **1984**, *114*, 2089.
Friedman, M.; Liardon, R. *J. Agric. Food Chem.* **1985**, *33*, 666.
Friedman, M.; Masters, P. M. *J. Food Sci.* **1982**, *47*, 760.
Friedman, M.; Zahnly, J. C.; Masters, P. M. *J. Food Sci.* **1981**, *46*, 127.
Hansch, C.; Leo, A. J. *Substituent Constants for Correlation Analysis in Chemistry and Biology*; Wiley Interscience: New York, 1979.
Hayase, F.; Kato, H.; Fujimaki, M. *Agric. Biol. Chem.* **1973**, *37*, 191.
Hayase, F.; Kato, H.; Fujimaki, M. *J. Agric. Food Chem.* **1975**, *23*, 491.
Hayase, F.; Kato, H.; Fujimaki, M. *Agric. Biol. Chem.* **1979**, *43*, 2459.
Hill, J.; Leach, S. J. *Biochemistry* **1964**, *3*, 1814.
Ikawa, M. *Biochemistry* **1964**, *3*, 594.
Jenkins, W. L.; Tovar, L. R.; Schwass, D. E.; Liardon, R.; Carpenter, K. J. *J. Agric. Food Chem.* **1984**, *32*, 1035.
Kriausakul, N.; Mitterer, R. M. *Science (Washington, D.C.)* **1978**, *201*, 1011.
Liardon, R.; Hurrell, R. F. *J. Agric. Food Chem.* **1983**, *31*, 432.
Liardon, R.; Jost, R. *Int. J. Peptide Protein Res.* **1981**, *18*, 500.
Liardon, R.; Ledermann, S. In *Chromatography and Mass Spectrometry in Nutrition Science and Food Safety*; Frigerio, A., Milon, H., Eds.; Elsevier: Amsterdam, 1984; p 7.
Liardon, R.; Ledermann, S.; Ott, U. *J. Chromatogr.* **1981**, *203*, 385.
Manning, J. M. *J. Am. Chem. Soc.* **1970**, *92*, 7449.
Masters, P. M.; Friedman, M. *J. Agric. Food Chem.* **1979**, *27*, 507.
Masters, P. M.; Friedman, M. *ACS Symp. Ser.* **1980**, *No. 123*, 165.
Mauron, J. In *Proceedings of the 6th International Congress of Food Science and Technology, Dublin*; McLoughlin, J. V., McKenna, B. M., Eds.; Boole Press Ltd.: Dublin, 1983; p 301.
Nakaparksin, S.; Gil-Av, E.; Oro, J. *Anal. Biochem.* **1970**, *33*, 374.
Nielsen, H. K.; De Weck, D.; Finot, P. A.; Liardon, R.; Hurrell, R. F. *Br. J. Nutr.* **1985**, *53*, 281.
Noll, B. W.; Jarboe, C. J.; Hass, L. F. *Biochemistry* **1974**, *13*, 5164.

Patel, D. J. *Biochemistry* **1973**, *12*, 667.
Provansal, M. M. P.; Cuq, J. L. A.; Cheftel, J. C. *J. Agric. Food Chem.* **1975**, *23*, 938.
Schroeder, R. A. Ph.D. Thesis, University of California, San Diego, 1974.
Schultz, J. In *Methods Enzymol.* **1967**, *11*, 255.
Schwass, D. E.; Finley, J. W. *J. Agric. Food Chem.* **1984**, *32*, 1377.
Sen, L. C.; Gonzales-Flores, E.; Feeney, R. E.; Whitaker, J. R. *J. Agric. Food Chem.* **1977**, *25*, 632.
Sine, H. E.; Hass, L. F. *J. Biol. Chem.* **1969**, *244*, 430.
Smith, G. G.; Evans, R. C. In *Biogeochemistry of Amino Acids*; Hare, P. E., Hoering, T. C., King, K., Eds.; Wiley: New York, 1980; p 257.
Smith, G. G.; Sol, B. S. *Science (Washington, D.C.)* **1980**, *207*, 765.
Smith, G. G.; Williams, K. M.; Wonnacot, D. M. *J. Org. Chem.* **1978**, *43*, 1.
Sol, B. S. M.S. Thesis, Utah State University, Logan, 1978.
Tannenbaum, S. R.; Ahern, M.; Bates, R. P. *Food Technol. (Chicago)* **1970**, *24*, 96.
Tovar, L. R.; Schwass, D. E. *ACS Symp. Ser.* **1983**, *No. 234*, 169.
Woivode, W.; Frank, H.; Nicholson, G. J.; Bayer, E. *Chem. Ber.* **1978**, *111*, 3711.
Zumberge, J. E. *Geochim. Cosmochim. Acta* **1979**, *43*, 1443.

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Comparison of the Amino Acid Composition of Two Commercial Porcine Skins (Rind)[1]

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The protein and amino acid contents of typical porcine skins (rind) produced in eastern and western Canada were compared to assess their protein quality and potential as a food or feed ingredient. Although wide ranges of values were found for moisture (43.1–76.3%) and each of the minerals analyzed, their total lipid content (12.8–47.4%) and the actual protein contents as determined by amino acid analysis did not differ significantly between eastern and western pig skins and ranged from 50.4 to 58.9% on a dry basis. The amino acid profiles from porcine skins from eastern and western Canada were similar, their calculated essential amino acid indices (32.9–35.9) were higher than previously reported, and all appeared limiting with respect to tryptophan, cyst(e)ine, tyrosine, and isoleucine. The mean residue weight for the amino acids in pig skin was 0.093777 μg/nmol, and correcting this mean residue weight for the absence of tryptophan and cyst(e)ine in protein hydrolysates resulted in a conversion factor, $F = 0.094021$ (μg). The chemical approach used in this study for evaluating protein quality of porcine skin was based on the direct chromatographic determination of its collagen and connective tissue contents. In this approach the content of collagen in pig skin (60–65.3%) was determined from the amounts of 5-hydroxylysine found and the content of total connective tissue proteins (70–82.5%) from the amounts of 4-hydroxyproline present.

INTRODUCTION

The definition of pork meat in the current Canadian Meat Inspection Regulations controlling meat products includes skin that normally accompanies the muscle after dressing, but excludes skin that has been detached primarily from the lard area of the back of dressed carcasses (Canada's Meat Inspection Act, 1979). Although such skins (rind) and skin trimmings have traditionally been utilized for the production of edible gelatin or for the manufacture of glue and luxury leather for shoes, garments, and upholstery (Heideman, 1979; Naghski, 1982), these uses consume only a minor portion of the pig skin supply. According to Asghar and Henrickson (1982), about 65% of the annual production of hide collagen as gelatin in the United States is consumed in edible products such as desserts, marshmallows, jellied meat, bakery foods, ice cream, and other products, while the remaining 35% is used by the photographic, metallurgical, cosmetic, and pharmaceutical industries (for reviews see: Chvapil, 1979; Rose, 1977; Wood, 1977; Naghski, 1982). The use of collagen as a feed supplement for animals and possibly as a food additive (Battista, 1975; Henrickson, 1980) in various meat products for human consumption holds promise, but its use will be related mainly to the nutritional quality of pig skin proteins and to the economics of the processes required.

Although numerous studies have described the distribution and occurrence of collagen types in skin tissues from several species, primarily human, bovine, and avian (for reviews see: Eastoe, 1967; Bornstein and Sage, 1980; Epstein, 1974; Miller and Gay, 1982; Eyre et al., 1984; Weiss, 1984; Light, 1985), and the use of skin collagen in the form of edible gelatin, there is a paucity of nutritional and compositional data on pig skin and its constituent proteins. Eastoe (1955) reported the amino acid composition of pig skin gelatin, prepared after alkaline pretreatment of the skin, gelatinization, alcohol coacervation of the gelatin and purification on ion-exchange resins. Chapman et al. (1959)

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