Racemization Kinetics of Free and Protein-Bound Lysinoalanine (LAL) in Strong Acid Media. Isomeric Composition of Bound LAL in Processed Proteins

Rémy Liardon,*,† Mendel Friedman,‡ and Georges Philippossian†

Nestlé Research Center, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland, and Western Regional Research Station, U.S. Department of Agriculture, 800 Buchanan Street, Albany, California 94710

The racemization kinetics of free and protein-bound lysinoalanine (LAL) in deuterated hydrochloric acid was determined by measuring the deuterium incorporation in LL- and LD-LAL diastereomers by GC/MS, and a method was developed to determine LAL isomeric composition in processed proteins. The method includes a limited acid hydrolysis of the samples in 6 N DCl, thus avoiding the complete racemization of LAL residues, and the measurement of unlabeled and labeled LAL isomer distributions. A range of heated or alkali-treated proteins of plant or animal origin were analyzed. All samples contained LL- and LD-LAL in similar amounts with, however, a significant stereoselectivity in favor of LD-LAL in samples subjected to moderate processing conditions (low temperature, short time, or low alkaline pH). A minimum relative concentration of ca. 40% was found for LL-LAL in the least-treated proteins. Computer modeling indicated a possible difference in the heats of formation between LL- and LD-LAL isomers which could explain the observed asymmetric distributions. The presence of both LAL isomers in similar proportions in all proteins indicates that the postulated two-step mechanism for LAL formation applies for both cyst(e)ine and serine–phosphate as precursors.

INTRODUCTION

Lysinoalanine (LAL), an unnatural cross-linking amino acid, is formed in food proteins under heat or by using alkaline conditions (Bohak, 1964; Sternberg et al., 1975). Due to its property of inducing nephrocytomegaly in the rat, this compound has been the subject of numerous investigations which have been extensively reviewed (Finot, 1983; Friedman et al., 1984a; Maga, 1984). More recently, the chelating ability of LAL has been observed in vitro on metal ion dependent enzymes (Hayashi, 1982; Pearce and Friedman, 1988; Friedman and Pearce, 1989) and confirmed in vivo in rats (Furniss et al., 1985; Furniss, 1988).

Since LAL contains two asymmetric centers, four stereoisomers can exist (Tas and Kleipool, 1976). However, with the exception of situations where a significant racemization of lysine is expected, only LL- and LD-LAL diastereomers should be present in food proteins in proportions depending on LAL formation pathway.

The most commonly accepted mechanism for the alkali-induced formation of LAL is a two-step reaction: first, formation of dehydroalanine by α,β-elimination from cyst(e)ine or serine; second, addition of the α-amino group of a lysine residue on the newly formed double bond (Masters and Friedman, 1980). In the addition step, LL and LD isomers should be formed in equal proportion. Alternatively, a one-step substitution mechanism has been proposed for the reaction of serine–phosphate with lysine in heated milk casein (Friedman, 1977). This reaction would result in the specific formation of LL-LAL.

Feron et al. (1978) observed that the capacity of LD-LAL to induce nephrocytomegaly was about 10 times higher than that of the LL isomer, and Friedman and Pearce (1989) reported that the relative toxicity of the isomers parallels to some extent their affinities for copper ions in vitro. This raised the question of the distribution of the two diastereomers in food proteins. Until now, however, the direct determination of LAL isomeric composition was not possible due to the complete racemization of the alanine moiety of LAL during acid hydrolysis (Tas and Kleipool, 1976). Using an indirect approach, De Weck-Gaudard et al. (1988) recently determined the composition of urinary-excreted LAL in rats fed heat- or alkali-treated proteins. In all cases, LL-LAL was found to be excreted in much higher proportion (80–93%) than LD-LAL. However, this result could be interpreted as reflecting the resistance of LD-LAL residues to proteolytic cleavage in the intestinal tract rather than the distribution of LAL stereoisomers in the proteins.

In view of these results, a new approach was developed for the direct determination of LAL isomers in processed proteins. The method is based on a limited acid hydrolysis of the samples, avoiding the complete racemization of LAL, and on the use of deuterium labeling to discriminate racemized LAL molecules from those that have retained the initial configuration (Liardon et al., 1981). The method was validated by measuring the racemization kinetics of free and bound LAL in acid media. Subsequently, it was used for the determination of LAL isomeric composition in a series of proteins subjected to varying conditions of pH and temperature.

EXPERIMENTAL PROCEDURES

Synthesis of Lysinoalanine (LAL). LAL dihydrochloride salt was prepared as described by Pintauro et al. (1985). This sample had a molecular rotation $[M]^{20}_D$ of 34.7° (c 2, 2 N HCl) corresponding to a mixture of about 40% LL-LAL and 60% LD-LAL (Tas and Kleipool, 1976). This was confirmed by GC/MS (39.9% LL-LAL). The discrepancy with respect to the expected theoretical composition (50% LL-LAL) was probably due to the final crystallization step, as the two isomers differ in their solubility in water (Tas and Kleipool, 1976).

Test Proteins. The following proteins were analyzed for their composition in LAL isomers.

Milk Proteins. Alkali-treated whey protein (AL-WP) was obtained by heating 5% whey protein (ultrafiltrated lactoserum,

† Nestlé Research Center.
‡ U.S. Department of Agriculture.

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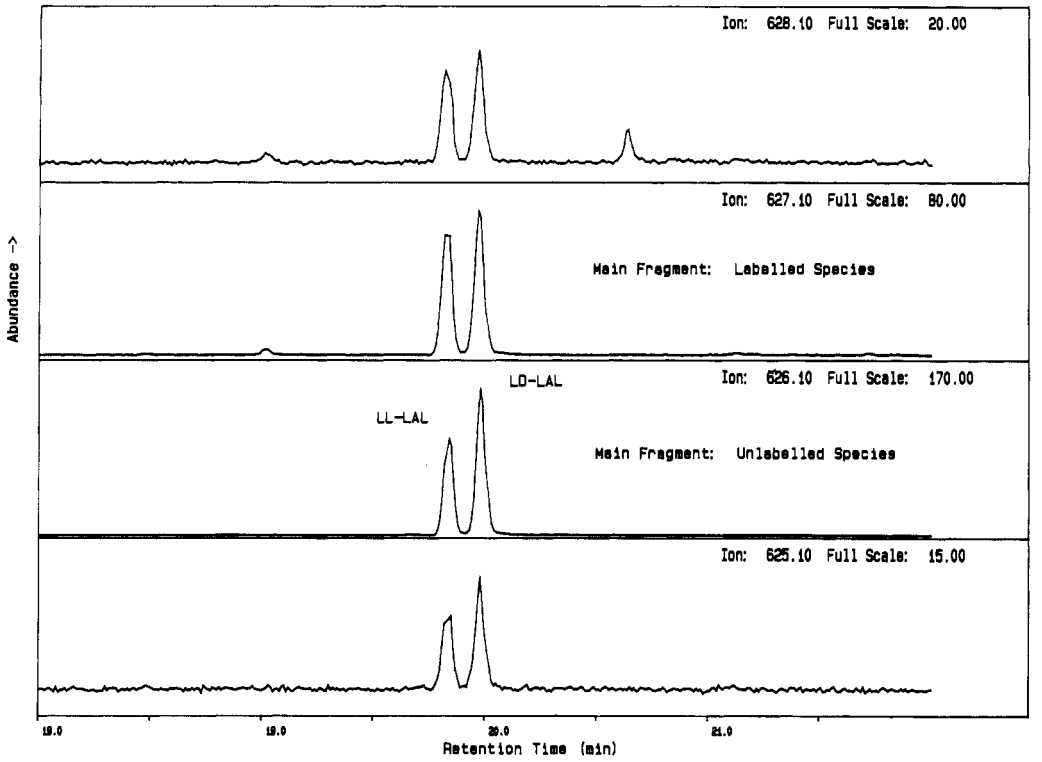


Figure 1. Mass chromatogram of alkali-treated whey protein (AL-WP) after partial hydrolysis in 6 N DCl (4 h, 110 °C). The nonracemic distribution of protein-bound LAL is clearly visible from the profile of the unlabeled main fragment ion at m/z 626, even before the raw abundances are corrected for the contribution of natural heavy isotope and parallel fragmentation.

Guigoz SA, Vuadens, Switzerland) in 0.1 N NaOH at 60 °C for 5 min, followed by precipitation at pH 3.9, washing the precipitate with water, and freeze-drying; LAL content was 10 540 mg/kg. Heated casein (H-CAS) was obtained by precipitation at pH 4.6 of skim milk sterilized in can at 115 °C for 55 min, followed by washing with water and freeze-drying; LAL content was 1544 mg/kg. Moderately alkali treated casein (AL-M-CAS) was obtained by heating 5% casein (Kliba, Kaiseraugst, Switzerland) in 0.05 N NaOH (adjusted to pH 9.75) at 60 °C for 4 h, followed by precipitation at pH 4.6, washing with water, and freeze-drying; LAL content was 779 mg/kg. Severely alkali treated casein (AL-S-CA) was obtained by heating 5% casein in 0.1 N NaOH at 80 °C for 2 h, followed by neutralization and freeze-drying; LAL content was 14 800 mg/kg. An experimental infant formula (H-IMF) with the same composition as Alprem (Nestlé SA, Vevey, Switzerland) was heated for 5 min at 148 °C by UHT treatment, aseptically filled into cans and then further heated for 10 min at 121 °C; LAL content was 550 mg/kg of crude protein (N × 6.25).

Plant Proteins. Samples of soy protein isolate (Promine D, U.S. Biochemical Co., Cleveland, OH) treated under various conditions of pH (pH 8–14), temperature (25–95 °C), and time (10–480 min) were obtained as described by Friedman (1982) and Friedman et al. (1984b, 1985). The corresponding LAL contents, which covered a wide range of concentrations (0–40 000 mg/kg), have been reported in the cited papers. Similar conditions of treatment were also applied to samples of lima bean trypsin inhibitor (LBI; Bowman–Birk type).

Miscellaneous. In addition to these proteins, various samples of plant and animal proteins (see list in Table III) were submitted to the same alkaline treatment, namely 3 h at 75 °C in 0.1 N NaOH, followed by neutralization and freeze-drying (Liardon and Friedman, 1987).

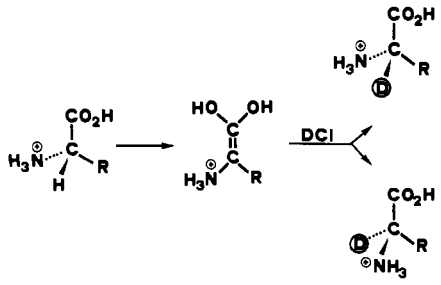
Kinetic Assay of LAL Racemization. *Free LAL.* Small volumes (ca. 1 mL) of 1 or 6 N DCl were equilibrated for 15 min at 100 or 110 °C (±1 °C) in a thermostated bath. At the end of this period, 50 μL of an LAL solution (200 mg/10 mL of D_2O) was added. The mixture was further heated for several hours while samples (50 μL) were taken at regular intervals for analysis. When the assay was repeated for DCl concentrations varying between 0.01 and 2 N, the analyses were restricted to samples heated for 6 h. Samples for analysis were placed in 1-mL conical vials and dried under a stream of nitrogen.

Bound LAL in Milk Proteins. Heated milk casein (H-CAS), alkali-treated casein (AL-S-CAS), or whey protein (AL-WP) was dissolved in 6 N DCl in D_2O at 2 mg/mL. Aliquots (2 mL) were transferred into sealed conical reaction vials and heated on a heating block at 100 °C. At varying time intervals, two vials were removed from the block for analysis. After cooling, the samples were dried under vacuum on a rotary evaporator. The dry residues were redissolved in 0.1 N HCl and transferred into small Dowex 50 (H^+) cartridges. After washing with 10–15 mL of distilled water, the amino acids were eluted with 4 N NH_4OH into 1-mL conical vials and dried under a stream of nitrogen.

Analysis of LAL Isomers. *Sample Derivatization.* Free LAL samples or amino acids recovered from Dowex cartridges were converted into *N*(O,S)-perfluoropropionylisopropyl esters in a two-step reaction: (1) esterification in 2-propanol/acetyl chloride followed by (2) acylation with perfluoropropionic anhydride (PFPA). The details of the procedure have been described elsewhere (Liardon et al., 1981). After the excess reagent was evaporated, the amino acid derivatives were redissolved in 25–50 μL of ethyl acetate followed by 0.5–1 mL of hexane.

Sample Analysis. The analysis of the derivatized samples was performed on a HP 5995 bench-top GC/MS system with a 30 m × 0.3 mm (i.d.) DB-5 fused silica capillary column. Sample introduction was made by on-column injection. Column temperature was programmed from 60 to 250 °C at 6 °C/min. Ion source temperature was set at 250 °C, and the ionizing voltage was 70 eV. The mass spectrometer was run in the selected ion monitoring mode (SIM). The monitored ions were m/z 625, 626, 627, and 628, and their abundances were measured at the retention times of the two LAL isomers (Figure 1). The abundances determined for m/z 626, 627, and 628 were corrected for naturally occurring heavy isotopes and for the contribution of the parallel fragmentation leading to m/z 625 in the mass spectrum of unlabeled LAL. The distribution of LAL isomers was directly derived from the corrected abundances of the main fragment ion m/z 626 for unlabeled species and m/z 627 for labeled species.

Chart I



RESULTS AND DISCUSSION

Principle of the Method. As shown in Chart I, the inversion of an amino acid molecule in acid solution is a first-order reaction involving the exchange of the α-hydrogen. Due to this feature, performing the reaction in a deuterated (or tritiated) medium leads to a direct labeling of the inverted molecules (Manning, 1970; Woiwode et al., 1978; Liardon et al., 1981).

The kinetics of deuterium incorporation is related to the inversion kinetics through the equation

$$-\ln (1 - X) = kt$$

where X is the incorporation level of the heavy isotope at time t and k the rate constant of the inversion reaction. The mathematical development leading to this equation is presented in the Appendix.

On this basis, a method was developed whereby free or protein-bound LAL was heated in deuterated hydrochloric solutions and analyzed by GC/MS in selected ion monitoring mode. The use of a capillary GC column allowed the separation of the two diastereomers, while detection by mass spectrometry permitted the discrimination of unlabeled from labeled species.

Through this approach it was possible to determine simultaneously (i) the deuterium incorporation level in the two diastereomers and (ii) the isomeric composition of unlabeled and labeled LAL. By use of the above equation, the racemization kinetics of LAL was established from the deuterium incorporation levels. The isomeric composition of unlabeled LAL by contrast was a direct measurement of the initial composition in the sample, prior to acid treatment.

A condition for this approach to be applicable for the determination of LAL isomer distribution in proteins was to interrupt the acid hydrolysis before the racemization of LAL was complete. Although such limited hydrolysis might have resulted in an incomplete liberation of LAL residues, it was assumed that the composition of the free LAL would still reflect the overall isomer distribution in the protein.

Racemization Kinetics of Free LAL. To validate our method, we investigated the racemization kinetics of free LAL in acid solution. Synthetic LAL consisting of a 4:6 mixture of the LL and LD isomers was heated at 100 °C for various lengths of time in 1 or 6 N DCl and analyzed by GC/MS. Figure 2 shows a plot of the resulting values of ln $(1 - X)$ against reaction time. As can be seen, the data points are perfectly aligned on two straight lines corresponding to the two acid concentrations. On the basis of the above equation the inversion rate constants were calculated by regression analysis. The resulting values are reported in Table I.

Determinations were also made at other DCl concentrations, on the basis of single time treatment (average of two measurements). The influence of DCl concentration on LAL inversion rate is illustrated in Figure 3. The

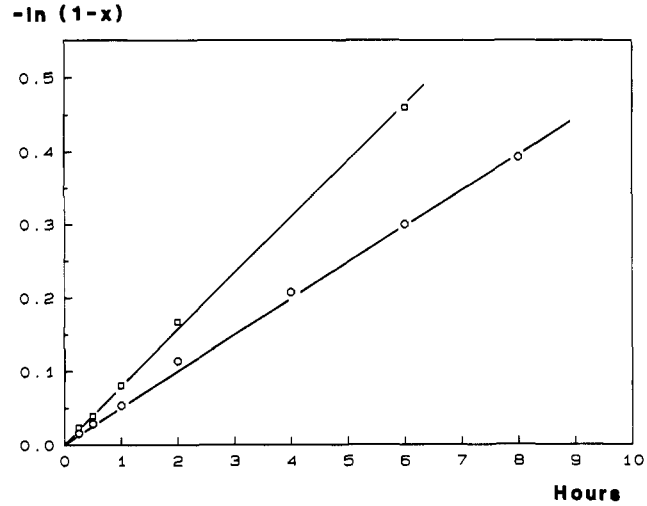


Figure 2. Kinetics of free LAL inversion in 1 N (□) and 6 N (○) hydrochloric acid at 100 °C.

Table I. Inversion Rate Constant[a] of Free and Protein-Bound LAL in Acid Solutions

sample	H^+ concn, N	temp, °C	k [10^{-5}/s]
free LAL	1.0	100	2.31 (0.06)
free LAL	6.0	100	1.35 (0.02)
free LAL	6.0	110	4.23 (0.04)
heated casein (H-CAS)	6.0	110	2.10 (0.18)
severely alkali treated casein (AL-S-CAS)	6.0	110	2.03 (0.12)
alkali-treated whey protein (AL-WP)	6.0	110	2.08 (0.11)

[a] Rate constants were calculated by regression analysis of plots shown in Figures 2 and 4. Values in parentheses are standard deviations.

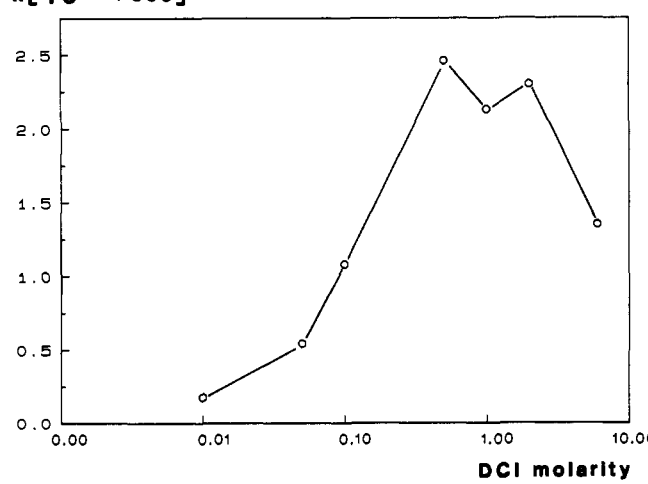


Figure 3. Influence of deuterated hydrochloric acid concentration on LAL inversion rate constant at 100 °C.

increase taking place between pH 2 and pH 0 was expected on the basis of theoretical considerations (Bada, 1972) and is in good agreement with the data reported by Smith and Sivakua (1983) for alanine. On the other hand, the leveling off and decrease of the rate constant at higher acid concentration is less readily explained. As this range of acid conditions has seldom been explored, comparative data for other amino acids are not available. The isomerization constant in 6 N DCl was also determined at 110 °C, the usual temperature for protein hydrolysis. The resulting value (Table I) indicated a 3-fold increase with respect to that at 100 °C.

The data in Table I show that, in acid solutions, LAL

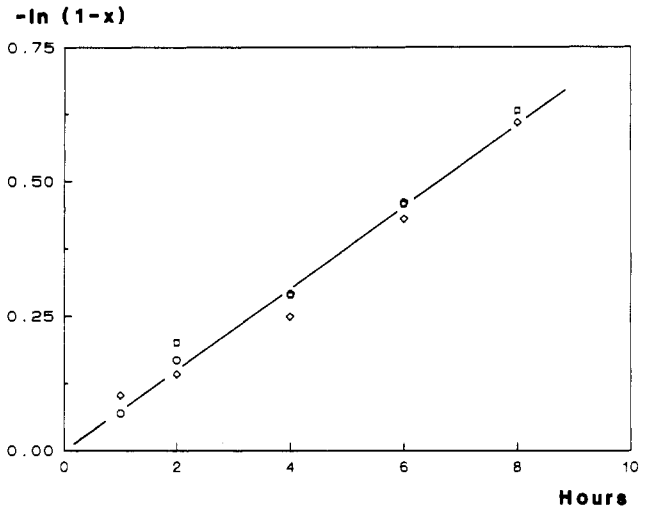


Figure 4. Kinetics of protein-bound LAL inversion during acid hydrolysis (6 N HCl, 110 °C) of heated milk casein (□), alkali-treated casein (◇), and alkali-treated whey protein (○).

racemizes 100–1000 times faster than natural amino acids (Frank et al., 1981; Liardon and Jost, 1981). The probable explanation of this behavior is a stabilization of the intermediate carbanion due to the electron-withdrawing capacity of the protonated secondary β-amino group, as reported for 2,3-diaminopropionic acid (Jacobson et al., 1974). This stabilizing effect will affect only the asymmetric carbon of the alanine moiety. Therefore, it can be assumed that the inversion rate of the lysine moiety is similar to that of free lysine (ca. 10E-7/s) and does not play any significant role in LAL racemization under acid conditions.

Racemization Kinetics of Protein-Bound LAL. By use of the same approach, the inversion kinetics of protein-bound LAL was determined during the acid hydrolysis (6 N DCl, 110 °C) of three protein samples with various LAL content induced by different pretreatments: alkali-treated whey protein (AL-WP, 10 540 ppm), alkali-treated casein (AL-S-CAS, 14 800 ppm), and heated milk casein (H-CAS, 1544 ppm). The resulting time curves are shown in Figure 4, and the corresponding rate constants calculated by regression analysis are reported in Table I. No significant difference can be seen between the values obtained for the three proteins, indicating the absence of any influence of the nature of the protein, the pretreatment, or LAL content. Surprisingly, the inversion rate of protein-bound LAL was lower by a factor of 2 than that of free LAL under the same conditions. In general, protein-bound amino acids have been found to racemize in strong mineral acids at the same rate or slightly faster than free amino acids (Liardon and Jost, 1981).

LAL Isomer Composition. As already mentioned, the distribution of unlabeled LAL isomers after limited hydrolysis in DCl theoretically should correspond to the initial composition in the sample. This was verified with the free LAL samples from the racemization kinetics assays. The results are reported in Table II. As can be seen, the ratio of unlabeled LAL isomers reflected very accurately the composition of the synthetic LAL used for these assays. On the other hand, a 1:1 ratio was found for the labeled isomers, as could be expected from the inversion mechanism illustrated in Chart I.

By use of the same approach, the distribution of LAL isomers was determined in the various model proteins, as reported in Table II. In a first examination, the two isomers appeared to form in comparable proportions in all samples, supporting the postulated two-step mechanism of LAL formation. Conversely, these results rule out the substitution mechanism suggested by Friedman (1977) for LAL formation in heated milk protein, involving serine–phosphate, since it would lead to the specific formation of LL-LAL.

Table II. Isomeric Composition of Free and Protein-Bound LAL

sample		isomeric ratio[a]
free LAL[b]	assay 1	0.41(0.01)
	assay 2	0.40(0.01)
	assay 3	0.40(0.01)
	assay 4	0.41(0.01)
bound LAL		
alkali-treated whey protein	(AL-WP)	0.39(0.02)
moderately alkali treated casein	(AL-M-CAS)	0.43(0.03)
severely alkali treated casein	(AL-S-CAS)	0.50(0.01)
sterilized infant milk formula	(H-IMF)	0.45(0.01)
heated casein	(H-CAS)	0.47(0.02)

[a] LAL isomeric ratio defined as [LL-LAL]/{[LL-LAL] + [LD-LAL]}. These values are based on the distribution of nondeuterated LAL isomers after hydrolysis in DCl. Values in parentheses are standard deviations. [b] Sample prepared by synthesis. Isomeric ratio: 0.40 (see Experimental Procedures).

Table III. Isomeric Composition of Protein-Bound LAL after Severe Alkali Treatment

protein[a]	LAL content,[b] g/16 g of N	isomeric ratio[c]
casein	4.40	0.51 (0.05)
lactalbumin	5.38	0.51 (0.002)
wheat gluten	0.95	0.50 (0.002)
bovine hemoglobin	3.36	0.50 (0.003)
bovine serum albumin	8.52	0.50 (0.002)
fish protein concentrate	2.75	0.50 (0.004)
soybean protein	3.18	0.49 (0.004)

[a] Alkali treatment: 3 h at 75 °C in 0.1 N NaOH. [b] Determined by ion-exchange chromatography on an amino acid analyzer. [c] LAL isomeric ratio defined as [LL-LAL]/{[LL-LAL] + [LD-LAL]}. Values in parentheses are standard deviations.

These results also confirm the conclusion of our recent study of the isomeric composition of urinary LAL in the rat (De Weck-Gaudard et al., 1988). As it is now established that the proteins fed to the rats contained the two LAL isomers in about equal amounts, the asymmetric distribution of urinary-excreted LAL could only result from a discrimination at the absorption level.

However, on closer examination of the data in Table II, it could be seen that, except for severely alkali treated casein, LAL isomer distribution exhibited small but significant discrepancies with respect to the expected 1:1 racemic ratio. This observation indicated that the formation mechanism of LAL may include some stereoselective feature, possibly depending on (a) the nature of the protein leaving groups [OH, OPO_3H_3 from serine; SH, SR from cyst(e)ine]; (b) treatment conditions; or (c) the microenvironment of LAL amino acid precursors (Liardon and Friedman, 1987).

In an attempt to clarify this point, we analyzed a series of proteins of different nature treated under various conditions of temperature, pH, and time. Table III shows isomer ratio values measured for several proteins submitted to the same severe alkali treatment. These data show no difference between the various proteins, all of them containing a racemic mixture of LL- and LD-LAL.

On the other hand, results obtained for samples of soy protein and lima bean trypsin inhibitor (LBI) submitted to various conditions of treatment show a distinct influence of the reaction conditions on LAL isomer ratio (Figures 5–7). In samples subjected to milder treatment (low pH

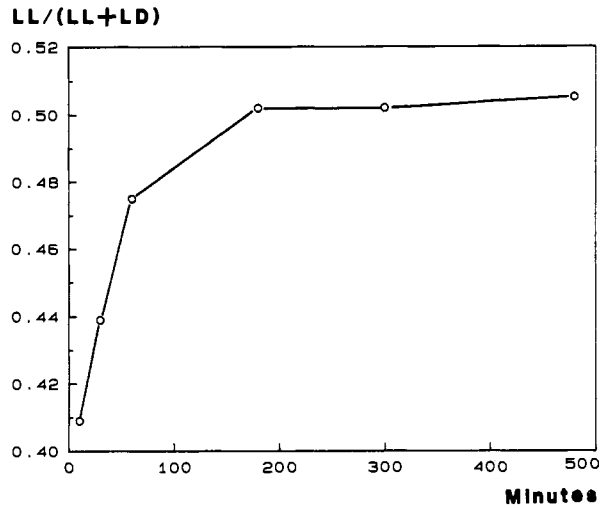


Figure 5. Influence of treatment time on LAL isomer ratio in soybean protein treated at 75 °C in 0.1 N NaOH.

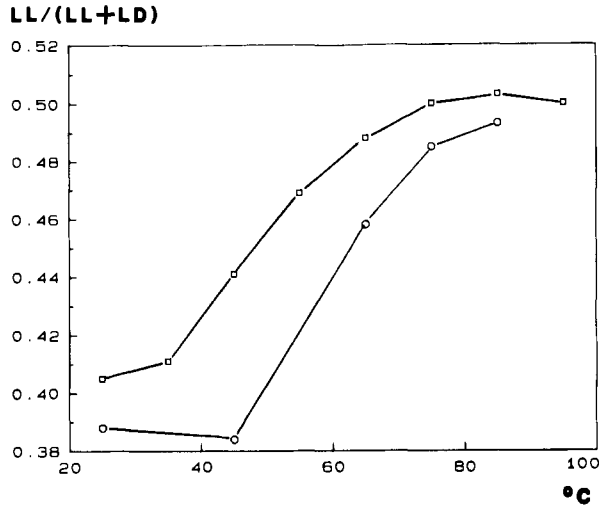


Figure 6. Influence of temperature on LAL isomer ratio in soybean protein (□) and lima bean trypsin inhibitor (LBI) (○) treated for 3 h in 0.1 N NaOH.

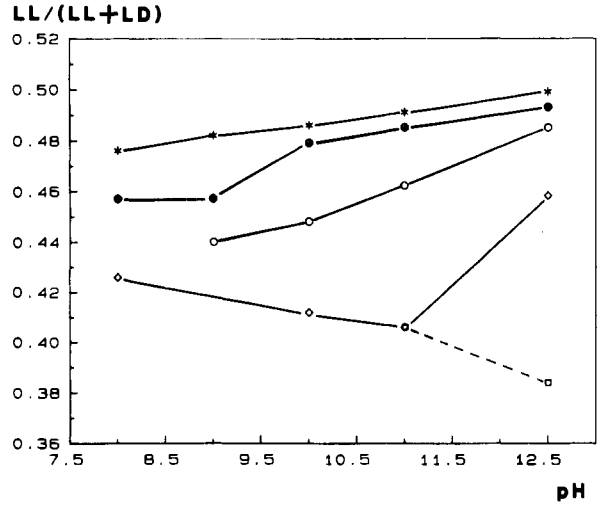


Figure 7. Influence of pH on LAL isomer ratio in soybean protein treated for 3 h at 75 °C (*) and lima bean trypsin inhibitor (LBI) treated for the same length of time at 45 (□), 65 (◇), 75 (○), and 85 °C (⊕).

or temperature or short reaction time), we find evidence for a preferred formation of LD-LAL, while the proportions of the two isomers tend to become equal for more severe reaction conditions. Interestingly, the isomer ratio measured in the least-treated samples is approximately the same for LBI and soy protein (0.38–0.40).

An attempt was made to understand the stereoselectivity of LAL formation by computer-based calculation of the difference in stability of the protein-bound LAL diastereomers. On the basis of the results of Hasegawa et al. (1981) and due to computing limitations LAL models were restricted to situations where the reacting residues (cystine or serine and lysine) were separated by 0, 1, or 2 intercalary amino acid residues. The conformational analysis was performed by using the SYBIL molecular modeling program. Calculations of the lowest energy conformers for the various LAL models were based on the Tripos force field (White et al., 1977; Vinter et al., 1987) and refined with the quantum mechanical model AM1 (Austin Model 1; Dewar et al., 1985).

Although the different methods of calculation did not converge, they predicted small but significant differences in the heat of formation between LAL diastereomers for most configurations of the reacting residues. On the basis of these results, we could conclude with reasonable confidence that the formation of LAL diastereomers is governed by some stereoselectivity. However, when considering the calculated geometries of the LAL models, it appeared impossible to assign these energy differences to defined structural factors, e.g., steric factors or hydrogen bonding.

As already mentioned, computation models were limited to reacting partners separated by up to 2 residues. Intuitively, however, increasing the distance between these partners is expected to result in a reduced difference in stability of the protein-bound diastereomers, hence in a vanishing stereoselectivity. This assumption is supported by the lower stereoselectivity found for heated casein (Table III). In this protein, not only are most lysine and serine–phosphate residues quite distant (Whitney et al., 1976) but LAL can also be formed by intermolecular reactions (Hasegawa et al., 1981). By contrast, in the other model proteins (lactalbumin, LBI), for which higher stereoselectivities have been observed, cysteine and lysine residues are in close vicinity (Whitney et al., 1976; Tan and Stevens, 1971).

Concerning the dependence of LAL isomer composition on reaction conditions, the most probable explanation is the alkali-induced racemization of the alanine moiety after LAL formation. As discussed earlier in this paper, the presence of an amino substituent in the β-position makes this species more prone to racemization than normal amino acids. Another possible explanation would be the racemization of lysine residues prior to reaction with dehydroalanine, leading to the formation of the DL-LAL isomer, analytically indistinguishable from LD-LAL. By contrast, the racemization of serine or cyst(e)ine residues prior to the formation of LAL could not contribute to the observed evolution since their chirality is lost in the formation of the intermediary dehydroalanine. Alternatively, the factor(s) responsible for the asymmetric formation of LAL might also be eliminated as a result of protein denaturation (Liardon and Friedman, 1987).

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APPENDIX

The unimolecular inversion process of LAL can be represented by the chemical equation

$$A \rightleftharpoons \text{transition state} \rightleftharpoons B \quad \text{or} \quad A \underset{k}{\overset{k}{\rightleftharpoons}} B$$

Considering the equality of the forward and backward rate constant, the distribution of A and B at time t is given by

$$a(t) = a(0) + X[b(0) - a(0)]/2 \tag{1}$$

where $a(0)$, $b(0)$ are the molar fractions of A and B at time 0 respectively [$a(0) + b(0) = 1$]; $a(t)$, $b(t)$ are the molar fractions of A and B at time t, respectively; and X is the proportion of A or B molecules having reached the transition state between time $t = 0$ and t (X comprised between 0 and 1).

In the deuterated medium, molecules leaving the transition state are automatically labeled. Therefore, the molar fraction of labeled A at time t [$a^*(t)$] is given by

$$a^*(t) = X[a(0) + b(0)]/2 = X/2 \tag{2}$$

However, since a molecule leaving the transition state has the same probability of taking the A or B configuration, we can write

$$a^*(t) = b^*(t) \tag{3}$$

$$a^*(t) + b^*(t) = X \tag{4}$$

Furthermore, label incorporation levels (I) in A and B at time t can be defined as

$$I(A) = a^*(t)/a(t) \tag{5a}$$

$$I(B) = b^*(t)/b(t) \tag{5b}$$

Combining eqs 5a and 5b with eqs 2 and 3 gives

$$I(A)a(t) = I(B)b(t) = X/2 \tag{6}$$

Kinetically, the incorporation of the label in A can be expressed by the differential equation

$$\begin{aligned} da^*/dt &= k[a(0) - a^*(t) + b(0) - b^*(t)]/2 \\ &= k[1 - 2a^*(t)]/2 \end{aligned} \tag{7}$$

Integrating eq 7 between $t = 0$ and t gives

$$kt = -\ln [1 - 2a^*(t)] \tag{8}$$

or, in combination with eq 4, 5a, or 5b

$$\begin{aligned} kt &= -\ln [1 - 2I(A)a(t)] = -\ln [1 - 2I(B)b(t)] \\ &= -\ln [1 - X] \end{aligned} \tag{9}$$

Remarks: 1. Equation 4 leads to another definition of X, namely, the overall incorporation level of the label in A and B considered as one species. 2. Considering that no conditions have been imposed for the isomer distribution at $t = 0$, it appears that the determination of the inversion rate constant on the basis of label incorporation does not require the availability of pure isomers. Indeed, this determination could even be made with a completely racemized mixture.

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