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Hydrolysis of proteins performed at high temperatures and for short times with reduced racemization, in order to determine the enantiomers of D- and L-amino acids

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Abstract. Racemization of free amino acids is considerably lower than that of amino acids bound in peptide. In the same experimental conditions, the rate of racemization of free amino acids is only 20–80% of that of peptide bound amino acids. When using to traditional protein hydrolysis, racemization was 1.2–1.6 times as high as that obtained at high temperatures (160–180 °C), under conditions ensuring total hydrolysis of the protein. This lower degree of racemization may be explained by the fact that, at high temperatures, the protein hydrolyses more rapidly into

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free amino acids and the racemization of free amino acids is considerably slower that of amino acids bound in polypeptides. When hydrolysis is conducted at lower temperatures for longer times, the amino acids bound in the peptide chain are exposed for a longer time to the effects actually causing racemization. As a result, we may say that any factor which speeds up hydrolysis, will lower the degree of racemization.

Racemization was higher for proteins in milk powder than for pure proteins. This may be explained by catalysis of racemization by the heavy metals present. After 48 hours at 110 °C and in presence of 4 M barium hydroxide, all amino acids (whether free or bound in peptide) totally racemized. Therefore the racemization of tryptophan cannot be determined using barium hydroxide promoted protein hydrolysis. High temperature hydrolysis (at 160 °C for 45 to 60 minutes, at 170 °C for 30–45 minutes and 180 °C for 30 minutes) are recommended for those who would like to hydrolyse the protein for short times and to determine the degree of racemization occurring in the polypeptide chain, but do not wish to use enzyme hydrolysis.

1 Introduction

The role of optical activity in living organisms has long been known. The large group of biologically active molecules – such as the amino acids – are all optically active. Thus in order to know their roles in living organisms, we should be able to separate and determine their enantiomers. Recently, considerable effort has been devoted as to separation and quantitation of amino acid enantiomers. Among these is the archaeometric application whereby one can establish the age of archaeological relics based on racemization of amino acids, specifically the epimerization of isoleucine [15, 43, 52]. Another example of recent work is the study of the composition of extraterrestrial materials [12]. Recent research demonstrated that, due to technological intervention or the alteration of microbiological status, foods may contain sizeable amounts of D-amino acids [3, 8, 17, 23, 24, 25, 27, 30, 31, 32, 34, 36, 40, 42, 45]. Several articles dealt with the D-amino acid content of milk [4, 5, 6, 28, 16]. From these reports it became obvious that the appearance of D-amino acids is not solely due to technological interventions (heat treatment, heat preservation), but may also result from alteration of microbiological status.

When attempting to quantify amino acid enantiomers, it is not sufficient to separate these from each other. One also has to pay attention to the separation of these from the other amino acids and their derivatives. The amino acid derivative on which we decide to depend should be detectable

with good sensitivity. Lately, pre-column derivative formation has been used with fluorescent reagent, followed by Reversed Phase Chromatography (RPC) of the derivatives. Using these methods, the detection limits for the amino acids of interest are extremely low. On the other hand, the flexibility of this analytical method provides outstanding advantages [21, 38, 51]. Thus, automatic methods have been developed for the simultaneous determination of optically inactive *o*-phthalaldehyde (OPA)/mercapto-ethanol (ME) and α-amino acids [49], and of 9-fluorenyl-methyl chloroformate (FMOC-Cl) in the presence of α-amino and imino acids [2, 13]. The reaction of optically active (chiral) amino acids with chiral reagents yields dia-stereoisomer compounds. In theory, one should be able to separate these using a non-chiral column. If the chiral reagent is another amino acid, then the separation and determination of the diastereomer dipeptide may be achieved using ion-exchange column chromatography [12, 18, 19, 33].

Following derivative formation with chiral reagents, the enantiomers of protein building block amino acids may be separated in a single run using RPC. Since the chromatographic separation takes 50–70 minutes, it is of paramount importance that the analytical method be adaptable to full automation. Another prerequisite is that the derivative formation should be simple, proceeding in a short time at room temperature. The reaction between the optically active thiols, the OPA and the amino acids to be determined has been used to separate and quantify amino acid enantiomers [1, 7]. The use of chiral 1-(9-fluorenyl) ethyl chloroformate (FLEC) for the separation of enantiomers has the advantage of being able to form derivatives, not only with the α-amino acids, but also with the imino-acids [22].

It is very important to know whether or not racemization occurs during protein hydrolysis. If so, the results of the determination will be influenced adversely. Various studies reported that the degree of racemization during hydrolysis of peptide is dependent on protein type and amino acid background. It was found [26, 35, 41, 50] that amino acids bound in peptide racemize faster than free amino acids.

Several reports appeared in the literature [9, 29, 46, 54] dealing with the use of microwave technology in protein hydrolysis. Some authors reported excellent results [11, 14] using high temperatures and short times for the hydrolysis process. It appears that, during microwave promoted hydrolysis, significant racemization occurs, because microwaves have been purposely used [10] to trigger racemization of amino acids. Reports have been published describing the increase of D-enantiomers in foods [39] under the influence of microwave treatment. Racemization is no cause for concern if one does not wish to de-

termine the enantiomers of amino acids. However, if our aim is the separation and determination of amino acid enantiomers, the protein hydrolysis procedure selected should be such that the accompanying racemization is as small as possible. This is necessary since, in the case of significant racemization, we are unable to distinguish between the amino acid enantiomers initially present in the sample and those that appear during the hydrolysis process. Several methods have been developed [20, 47, 48] which restrict racemization occurring during hydrolysis. However, these proved to be lengthy and tedious. As a consequence, objective was to develop a protein hydrolysis method having the lowest possible degree of racemization, by using high temperatures for a short time duration.

2 Material and methods

Hydrolysis and processing of the hydrolysate. Pyrex reusable hydrolysis tubes having 8 mm I.D. (Pierce Chemical Company, Rockford, IL, USA) were used for hydrolysis of proteins or for treating free amino acids. Each tube can contain up to 8 ml of hydrolysing agent without making contact with the PTFE (polytetrafluoroethylene) sealing cup. 1 ml of 6 M hydrochloric acid (HCl) was added to each tube for preparation of protein and peptide hydrolysate. Each tube had two PTFE sealing caps to get complete leak-free operation during heating at 160, 170 or 180 °C.

Either 1 mg peptide, protein, or free amino acids or 20 mg fat-free milk powder was weighed into Pyrex tubes previously washed with hydrochloric acid and deionised water. One ml 6 M HCl was added to each sample (HCl was obtained from Pierce Chemical Company, Rockford, IL, USA) and nitrogen was bubbled for five minutes through the hydrolysing agent by glass capillary. After bubbling with nitrogen, the Pyrex tubes were immediately closed, and put into the heating oven at 160, 170 and 180 °C for 15, 30, 45 or 60 minutes. One sample of each examined material was hydrolysed at 110 °C for 24 h, according to the method of More & Stein [44], with 6 M HCL. Another sample was hydrolysed at 110 °C for 48 h using 4 M barium-hydroxide for determination of tryptophan from samples of food and feed. After hydrolysis, the tubes were cooled at room temperature and HCl was evaporated by lyophilization and the residue of the sample was dissolved in 0.01 M HCl. After the barium-hydroxide hydrolysis, pH of the hydrolysate was set to neutral with 1 M HCl, and the barium was removed from the hydrolysate in the form of barium-sulphate. During neutralisation, the temperature was held below 30 °C with the help of

a sodium chloride - ice mixture. Next all of the hydrolysates were filtered and stored at $-25\,^{\circ}\mathrm{C}$ until the analysis of D- and L-amino acid enantiomers by HPLC.

Materials tested. The following materials were used for testing the racemization during hydrolysis: Bovine ribonuclease, lysozyme, citochrom C, mare's milk powder, and individual free amino acids as follows: L-aspartic acid, L-glutamic acid, L-threonine, L-alanine, L-valine, L-phenylalanine, L-histidine and L-tryptophan. The protein content of milk powder (22.7%) was determined using a Kjel-Foss 16200 (Foss Electric, Denmark) rapid nitrogen analyser. The protein content was calculated from nitrogen % using a conversion factor of 6.38. Peptides and proteins were hydrolysed at varying temperature-time combinations. The free amino acids samples were subjected to the same temperature-time treatments. HPLC was used for determination of L-aspartic acid, L-glutamic acid, L-threonine, L-alanine, L-valine, L-phenylalanine, L-histidine and L-tryptophan content of the samples.

High performance liquid chromatography (HPLC) for separation and determination the D- and L-amino acids. *Instruments:* The chromatographic system was assembled from ISCO 100 DM syringe pumps (Isco Inc. Lincoln, Nebraska, USA) and a Rheodyne (Berkeley, California, USA) injector equipped with a $20\,\mu$l loop. The separation process was monitored and chromatograms stored on an ISCO Chem Research (Isco Inc. Lincoln, Nebraska, USA) system. The derivative formation and sample injection were performed manually. The excitation and observation wavelengths were 325 and 420 nm, respectively.

Reagents: Acetonitrile and methanol were purchased from Rathburn Ltd (Walkeburn, England). The AA standards, the *o*-phthalaldehyde and the TATG were obtained from Sigma Chemical Co., Inc. (St. Louis, MO). The buffers used for elution were prepared from mono- and disodium phosphate. The pH was adjusted with 4 M sodium hydroxide.

Synthesis of derivatives: The reaction was carried out in a $120\,\mu$l microvial which was placed in another vial (volume, 1.8 ml) that had TeflonR coating, internal cover plate, and a screw cap. The sample (free AA or protein hydrolysate evaporated by lyophilization), dissolved in $90\,\mu$l borate buffer (0.4 M; pH 9.5), was mixed with $15\,\mu$l of reagent (8 mg of *o*-phthalaldehyde (OPA) and 44 mg of 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (TATG) dissolved in 1 ml of methanol). The mixture was then homogenized by bubbling through

approximately 100 μl of nitrogen and left standing for 6 min. Then, 25 μl of the reaction mixture were injected into the analytical column. After injection, the system was rinsed three times with approximately 100 μl of a 70:30 acetone–water (v/v) solution. Synthesis of derivatives was performed manually and mixing of reagent solution was made with the aid of an IKA Vibro Fix instrument (Janke and Kunkel, IKA-WERK, Breisgau, Germany).

Separation and quantitation of the enantiomers: Separation of the enantiomers was made according to the method of Einarson et al. [6], using a reversed-phase analytical column packed with Kromasil octyl C-8 (250 × 5.6 mm internal diameter; 5 μm particle size, EKA Nobel AB, Bohus, Sweden). To increase the lifetime of the column, a safety column was fitted between the sample injector and the analytical column (RP-8, Newguard, 25 × 3.2 mm internal diameter, 7 μm particle size, EKA Nobel AB, Bohus, Sweden), and a cleaning column (C18, 36 × 4.5 mm internal diameter, 20 μm particle size, Rsil, EKA Nobel AB, Bohus, Sweden) was installed between the pump and the sample injector. In order to separate the enantiomers, the two component gradient system had the following composition: A = 40% methanol in phosphate buffer (9.5 mM, pH = 7.05) and B = acetonitrile. The flow rate was 1 ml/min, and the elution of the gradient as a function of time is shown below.

Table 1: Eluent composition applied for the separation of OPA-TATG derivatives of amino acid enantiomers

Time (min)	**A**[1]	**B**[2]
	%	
0	95	5
10	95	5
35	83	17
55	72	28
56	67	33
74	67	33
75	62	38

[1] 40% methanol in phosphate buffer (9.5 mM, pH = 7.05)
[2] Acetonitrile

3 Results

D-amino acid composition of bovine ribonuclease as related to time and temperature. Bovine ribonuclease was hydrolysed by 6 M HCl at 110 °C for 24 h and at 160, 170 and 180 °C for 15, 30, 45 and 60 minutes. The D-amino acid compositions of ribonuclease after hydrolysis at 110 °C for 24 h and at elevated temperatures for shorter times are in *Tables 2, 3* and *4.* The data in *Table 2* showed that both traditional hydrolysis (6 M HCl, 24 h, 110 °C) and high temperature–short duration hydrolysis, tryptophan almost completely decomposed.

Table 2: D-amino acid content of bovine ribonuclease hydrolysed by 6 M HCl at 160 °C for different times

Amino acid	6 M HCl 110 °C, 24 h	6 M HCl, 160 °C for			
		15 min	30 min	45 min	60 min
Asp	6.73	1.73	2.78	3.11	3.34
Glu	4.58	1.58	2.59	2.61	2.84
Thr	3.64	1.47	1.70	1.97	2.12
Ala	2.95	1.41	1.58	1.60	1.73
Val	2.34	1.22	1.29	1.51	1.54
Phe	3.28	2.13	2.47	2.93	3.21
His	1.96	0.92	1.41	1.52	1.64
Trp*					

The values refer to the percent of racemization expressed as the ratio (D/D+L)×100. Each values is the mean of triplicate determinations. Hydrolysis conditions: 6 M HCl 110 °C for 24 h and 160 °C for different times using Pyrex No. 9826 tubes.
*Almost totally decomposed during 6 M HCl hydrolysis at 160 °C for 15–90 min.

Table 3: D-amino acid content of bovine ribonuclease hydrolysed by 6 M HCl at 170 °C for different times

Amino acid	6 M HCl 110 °C, 24 h	6 M HCl, 170 °C for 15 min	30 min	45 min	60 min
Asp	6.73	2.23	3.02	3.62	3.89
Glu	4.58	1.97	2.74	2.93	3.28
Thr	3.64	1.99	2.16	2.54	2.84
Ala	2.95	1.69	1.99	2.22	2.54
Val	2.34	1.61	1.90	2.03	2.17
Phe	3.28	2.30	2.83	3.01	3.10
His	1.96	1.22	1.59	1.63	1.81

Data expressed as in *Table 2.*

Table 4: D-amino acid content of bovine ribonuclease hydrolysed by 6 M HCl at 180 °C for different times

Amino acid	6 M HCl 110 °C, 24 h	6 M HCl, 180 °C for 15 min	30 min	45 min
Asp	6.73	2.69	4.28	6.19
Glu	4.58	2.94	3.42	4.61
Thr	3.64	2.45	3.06	3.39
Ala	2.95	2.37	2.89	3.31
Val	2.34	1.99	2.43	2.70
Phe	3.28	2.97	3.12	3.78
His	1.96	1.77	2.09	2.53

Data expressed as in *Table 2.*

As a result, we shall not report this amino acid in the following tables. It is clear that, among the examined amino acids, the highest degree of racemization [D/(D+L)×100] is recorded for aspartic acid, in both traditional and short duration hydrolysis. This is followed in decreasing order by glutamic acid, threonine, phenyl-alanine, alanine, valine and histidine. At 160 °C racemization degree increases as hydrolysis time increases. In the case of every amino acid tested, the lowest racemization was recorded at 15 minutes hydrolysis times. Increasing the hydrolysis time from 15 to 60 minutes, racemization

increased from 1.73% to 3.34% in the case of aspartic acid, 1.58% to 2.84% for glutamic acid, 1.47% to 2.12% for threonine, 1.41% to 1.73% for alanine, 1.22% to 1.54% for valine, 2.13% to 3.21% for phenyl-alanine and 0.92% to 1.64% for histidine.

In earlier studies [14], it was reported that, protein hydrolyses performed at 160 °C for 15–45 minutes were insufficient for complete hydrolyses of proteins, and especially for breakage of the bond adjacent to Val, Ile and Leu. Therefore, for hydrolysis made at 160 °C, only the 60 minute times have practical importance. If we compare the racemization obtained after 60 minutes hydrolysis with results of the traditional method, it is found that the racemization degree for the traditional method, on the average, is 1.5 times as high as that of brief hydrolysis performed at 160 °C.

We reach similar conclusions if we analyse the data featured in *Tables 3* and *4*. When performing the hydrolysis at 170 °C, the hydrolysis reaction practically concludes after 45 minutes and after 60 minutes, even the very stubborn bonds adjacent to Val are broken. At 180 °C, 30–45 minutes are sufficient for complete hydrolysis. Therefore, when comparing results obtained during traditional hydrolysis, it is advisable to make comparisons with data obtained at 170 °C for 45 minutes and 180 °C for 30 minutes. Hydrolysis made at 160 °C for 60–90 minutes yields racemization similar to hydrolysis performed at 170 °C for 45 minutes. Hydrolysis performed at 180 °C for 45 minutes yields a racemization ca. 1.5 times as high as that of hydrolysis carried out at a lower temperature which results in total breakage of bonds. Obviously, both increasing temperatures (from 160 to 180 °C) and increasing time (from 15 to 60 minutes), produced a higher degree of racemization. However, at all three temperatures, continuation of hydrolysis until total hydrolysis of the peptide bond (e.g. at 180 °C for 30 minutes), produced a degree of racemization which represented only ca. 50–70% of that observed in the case of traditional hydrolysis.

Data in *Tables 2, 3* and *4* were subjected to analysis of variance with temperatures, times and amino acids representing main effects. All main effects plus interactions of temperature–time and temperature–amino acids were highly significant ($P<0.001$) sources of variance affecting degree of racemization. Increases of temperature and time of hydrolysis caused increases in racemization. The degree of racemization, when averaged over all time–temperature treatments, varied from 1.16% for His to 2.52% for Asp.

Degree of racemization values (average for all amino acids and for Asp) were fitted to a curvelinear and interactive function of time and temperature. The model explained 94 to 96% of the variation in the dependent variable. The function for Asp indicated that 3.6% racemization would occur at 60, 42 and

28 minutes, respectively, for 160, 170 and 180 °C temperatures. Corresponding times for average racemization were 70, 35 and 20 minutes to produce 2.33% racemization.

Influence of the hydrolysis method on the D-amino acid content of lysozyme, cytochrome C and milk powder. After the experiments with ribonuclease, we hydrolysed lysozyme, cytochrome C and milk powder using the traditional method. The results thus obtained were compared with the data obtained at 160 °C for 60 minutes, at 170 °C for 45 and 60 minutes and finally at 180 °C for 30 minutes. The degree of racemization was compared among the various hydrolysis conditions. The selection of the these time–temperature combinations was based on the time–temperature combinations required to produce total amino acid hydrolysis. The data in *Tables 5* and *6*, show that the degrees of racemization for lysozyme and cytochrome C were virtually identical to that obtained for ribonuclease at the same time and temperature.

Table 5: D-amino acid content of lysozyme (A), cytochrome (B) and milk powder (C) hydrolysed by 6 M HCl at different temperatures for different times

Amino acid	6 M HCl					
	110 °C for 24 h			160 °C for 60 min		
	A	B	C	A	B	C
Asp	6.62	7.01	7.89	3.27	3.42	4.15
Glu	4.58	4.61	5.93	2.79	2.84	3.61
Thr	3.62	3.74	4.38	2.29	2.31	3.14
Ala	2.99	3.21	4.02	1.69	1.65	2.13
Val	2.11	2.24	2.53	1.69	1.84	2.33
Phe	3.31	3.42	3.64	3.19	3.37	3.57
His	1.83	1.89	2.38	1.64	1.67	2.01

Data expressed as in *Table 2.*

In the case of milk powder, for each temperature–time combination, we obtained degree of racemization 15–25% higher than that of the three pure proteins. The higher racemization may be partially explained by the mineral matter content of milk powder. It is known that, with the exception of nickel, heavy metals catalyse the racemization of amino acids.

Table 6: D-amino acid content of lysozyme (A), cytochrome (B) and milk powder (C) hydrolysed by 6 M HCl at different temperatures for different times

Amino acid	6 M HCl					
	170 °C for 45 min			180 °C for 30 min		
	A	B	C	A	B	C
Asp	3.29	3.57	4.42	3.84	3.99	4.67
Glu	2.81	2.89	3.74	3.51	3.63	3.92
Thr	2.11	2.23	3.04	2.87	3.14	3.42
Ala	1.72	1.77	2.11	2.81	2.89	3.04
Val	1.71	1.82	2.27	2.54	2.57	2.82
Phe	2.89	3.11	3.60	2.97	2.83	3.20
His	1.52	1.60	1.99	1.79	1.93	2.11

Data expressed as in *Table 2.*

Racemization of free amino acids during hydrolysis. Racemization of free amino acids has been reported to be lower than that of amino acids bound in peptide. In order to test the hypothesis that there is difference between racemization of free amino acids and those bound in peptide, we have treated each free amino acid with 6 M HCl for various times, and at various temperatures. The results of this investigation are shown in *Table 7.*

When samples were treated for the same length of time, racemization increased with increased treatment temperature. Also, racemization increased with increased treatment times. The high temperature treatments yielded 20–55% less racemization than was observed for traditional treatment of 110 °C for 24 h. Even in the case of the sample treated at 180 °C for 30 minutes, the racemization, except of valine was only 70–90% of that seen for traditional treatment.

If we compare the racemization of free *(Table 7)* and peptide bound *(Tables 2, 3, 4, 5, 6)* amino acids, we find that the percentage racemization of peptide bound amino acids is 4 to 6 times as great as that of free amino acids at 110 °C for 24 h. When both free amino acids and peptide bound amino acids were subjected to the same high temperature, short time hydrolysis, the above ratio ranged from 5 to 7.

Table 7: Racemization of free amino acids treated by protein hydrolysis with different temperatures for different times

Amino acid	6 M HCl 100 °C for 24 h	6 M HCl					
		160 °C for		170 °C for		180 °C for	
		45 min	60 min	30 min	45 min	15 min	30 min
Asp	1.42	0.79	0.93	0.73	1.04	0.94	1.12
Glu	1.07	0.54	0.82	0.68	0.89	0.91	1.03
Thr	0.83	0.31	0.38	0.32	0.41	0.42	0.61
Ala	0.69	0.22	0.29	0.27	0.35	0.37	0.52
Val	0.54	0.19	0.25	0.17	0.24	0.37	0.57
Phe	0.72	0.21	0.27	0.20	0.31	0.32	0.47
His	0.47	0.11	0.18	0.10	0.19	0.21	0.33

Data expressed as in *Table 2.*

Racemization when using barium hydroxide promoted hydrolysis. It was observed earlier in this report that, during hydrolysis with 6 M HCl, tryptophan decomposed almost completely. If we wish to determine the degree of racemization for tryptophan, we must resort to a hydrolysis method which does not cause decomposition of Trp. Since we have been using the barium-hydroxide hydrolysis for the determination of Trp content of proteins, we decided to examine racemization associated with this process. We hydrolysed pure proteins (ribonuclease, lysozyme, cytochrome C), milk powder and free amino acids with 4 M barium hydroxide. We found that, in all materials examined (including free amino acids), all amino acids were fully racemized. In other words we were able to demonstrate the presence of 50% L- and 50% D-enantiomers. Because of this, barium hydroxide based hydrolysis may not be used to measure racemization of the tryptophan contained in proteins.

Conclusions and recommendations. Racemization of free amino acids was considerably lower than that of amino acids bound in peptide. In the same experimental conditions, the degree of racemization of free amino acids was only 20 to 80% that of peptide bound amino acids.

Traditional protein hydrolysis produced racemization is 1.5 times as high as that obtained at high temperatures (160–180 °C), under conditions ensuring total hydrolysis of the protein. This lower degree of racemization may be

explained by the fact that, at high temperatures, the protein hydrolyses more rapidly into free amino acids. Racemization of free amino acids is considerably less that of amino acids bound in polypeptides. Therefore, high temperature hydrolysis promotes conversion to the free state in which amino acids are less subject to racemization. When hydrolysis is conducted at lower temperatures for longer times, the amino acids bound in the peptide chain are exposed for a longer time to the effects actually causing racemization. As a result, we may say that factors which speed up hydrolysis, will lower the degree of racemization.

In the case of milk powder, racemization was higher than in the case of pure proteins. This may be explained by catalysis of racemization associated with the heavy metals present. After 48 hours at 110 °C and in presence of 4 M barium hydroxide, all amino acids (whether free or bound in peptide) totally racemized. Therefore the racemization of tryptophan cannot be determined using barium hydroxide promoted protein hydrolysis.

We recommend that protein samples be hydrolysed at high temperature for a short time (160 °C for 60 minutes and 170 °C for 45 minutes) for all those who would like to determine the degree of racemization occurring in the polypeptide chain, but do not wish to use enzyme hydrolysis.

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