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Saccharide induced racemization of amino acids in the course of the Maillard reaction

Rapid Communication

H. Brückner, J. Justus, and **J. Kirschbaum**

Interdisciplinary Research Center, Institute of Nutritional Science, Department of Food Sciences, University of Giessen, Federal Republic of Germany

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Summary. The formation of D-amino acids on heating aqueous solutions of protein L-amino acids at pH 2.5 and pH 7.0 together with glucose, fructose or saccharose was investigated by enantioselective gas chromatography. The saccharide induced partial racemization (epimerisation) of L-amino acids is attributed to the Maillard reaction.

Keywords: D-amino acids – Maillard reaction – Nonenzymic browning – Racemization mechanism – Saccharides (glucose, fructose, saccharose) – Gas chromatography – Chirasil-L-Val

Introduction

The conversion of free or protein- or peptide-bound physiological L-amino acids into their mirror images (enantiomers) named D-amino acids is of great interest from nutritional and physiological points of view (Friedman, 1999). This process of the change of the chirality ("handiness") of amino acids is commonly referred to as racemization (or epimerisation if several chiral centers are involved), although in the strict sense racemic amino acids consist of equal amounts of D- and L-amino acids. From an experiment on heating L-Pro together with a mixture of fructose and glucose in aqueous acetic acid (Erbe and Brückner, 2000) it was assumed that D-amino acids were generated from L-amino acids in the course of the so-called Maillard reaction. That reaction is defined as interaction of reducing sugars with amino components leading to flavor compounds and melanoidins.

Intermediates of the Maillard reaction formed *in vivo* have also attracted attention as they lead to so-called advanced glycated end products in the body (Finot et al., 1990; Ledl and Schleicher, 1990).

Here we report about our study on heating aqueous solutions of common protein L-amino acids together with the saccharides glucose, fructose, or saccharose, present data on quantities of the D-amino acids formed and propose mechanisms for their generation.

Materials and methods

Instrumental

For gas chromatography (GC) a Model 14 A instrument (Shimadzu, Kyoto, Japan) with flame ionization detector (FID) was employed. For the separation of amino acid enantiomers a chiral fused silica capillary column Chirasil-L-Val® (propionyl-L-valine tert. butylamide polysiloxane), 25 m × 0.25 mm ID, film thickness 0.12 μm (from Chrompack, Middelburg, The Netherlands) and helium as carrier gas were used. Full details of analytical procedures have been reported previously (Brückner and Hausch, 1989). Relative quantities of D-amino acids (% D) were calculated from peak areas of L- and D-amino acid derivates according to the equation % D = 100 D/(D + L).

Chemicals

For racemization experiments an amino acid standard solution (no. AA-S-18, from Sigma Chemicals, St. Louis, MO, USA) was used comprising 2.5 μmol/ml L-Ala, L-Arg, L-Asp, L-Glu, Gly, L-His, L-Ile, L-Leu, L-Lys, L-Met, L-Phe, L-Pro, L-Ser, L-Thr, L-Tyr, L-Val, and 1.25 μmol/ml $(L\text{-Cys})_2$ (cystine). D(+)glucose (Glc) and D(−)fructose (Frc) were from Sigma, and D(+)saccharose (Sac) from Fluka, Buchs, Switzerland. Dowex 50 X 8–400 cation exchanger (Sigma) was washed thoroughly with 1 M NaOH, 1 M HCl, and water before used.

Experiment A (pH 2.5)

Aliquots (3 × 2.5 ml) of the amino acid standard solution were transferred into three conical flasks and evaporated to dryness at 40°C *in vacuo* using a rotatory evaporator. To the residues either 278 mM glucose, or 278 mM fructose, or 146 mM saccharose (2.5 ml) in 5% acetic acid (pH 2.5) were added and aliquots (4 × 0.5 ml) were transferred from each flask into 1-ml vials. Closed vials were heated at 100°C for time periods of 24–96 h, cooled at ambient, and centrifuged at 3,500 × *g* for 15 min. Supernatants were directly subjected to cation exchange treatment as described below. The control experiments (heating at 100°C for 96 h) were carried out analogously with the exception that no saccharides had been added to 5% acetic acid.

Experiment B (pH 7.0)

Aliquots (3 × 2.5 ml) of the amino acid standard solution were transferred into three conical flasks, 4 M aqueous ammonia (500 μl) was added to each flask and the solutions were evaporated to dryness as described above. To the residues either 278 mM glucose, or 278 mM fructose, or 146 mM saccharose (2.5 ml) in pH 7.0 buffer (prepared from 1 M sodium acetate titrated to pH 7.0 with 1 M HCl) were added, and pH 7.0 was maintained by addition of 4 M NaOH (20 μl). Aliquots representing one fivth of the total volume (4 × 504 μl) were transferred from each flask into 1-ml vials. Samples were heated and centrifuged as described in experiment A. Supernatants were diluted with water (3 ml),

pH 2.3 was adjusted with 1 M HCl and solutions were subjected to cation exchange treatment. Control experiments were carried out with no saccharides added to the buffer.

Treatment of analytes

Analytes resulting from experiments A and B were passed through glass columns, filled with cation exchanger (0.5 cm × 5 cm). The resins were washed with water (10 ml), amino acids adsorbed were eluted with 4 M aqueous ammonia (3 ml), and the effluents were evaporated to dryness at 40°C *in vacuo*. Amino acids were converted into volatile *N*(*O*)-trifluoroacetyl-1-propyl esters using acetyl chloride in 1-propanol and trifluoroacetic anhydride in dichloromethane and analysed by GC-FID as described (Brückner and Hausch, 1989; Erbe and Brückner, 2000).

Results

Data on the relative quantities of D-amino acids formed in experiment A are presented in section (A) of Table 1. As can be seen D-amino acids were

Table 1. Relative quantities of D-amino acids (%D) formed on heating (100°C) of a mixture of L-amino acids (2.5 mM) in 278 mM glucose (Glc), 278 mM fructose (Frc), or 146 mM saccharose (Sac) in (A) experiment A (pH 2.5), and (B) experiment B (pH 7.0) (n = 2–3). In the controls (heated for 96 h at 100°C) the saccharides were omitted

Amino acid		24 h			48 h			72 h			96 h			
		Glc	Frc	Sac	Glc	Frc	Sac	Glc	Frc	Sac	Glc	Frc	Sac	control
(A)	D-Ala	2.2	4.1	3.6	6.5	8.7	7.9	7.4	16.7	12.9	11.9	22.0	16.9	9.4
	D-Val	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4	0.0	1.1	1.9	0.0	0.2
	D-*allo*-Ile	0.0	0.0	0.0	0.9	0.0	0.0	1.1	1.8	1.3	2.0	3.4	1.9	0.4
	D-Pro	1.8	4.2	3.6	6.6	10.2	8.8	9.1	20.5	16.2	14.6	26.4	22.1	1.9
	D-Ser	9.2	10.7	10.6	21.2	21.9	22.3	27.1	33.3	31.2	35.1	42.9	36.1	25.2
	D-Leu	1.2	1.5	1.2	4.0	4.5	4.4	5.1	10.0	8.1	9.1	14.7	10.6	2.5
	D-Asp	15.3	17.1	16.4	29.3	30.3	31.2	37.5	42.2	40.5	43.5	46.1	44.1	39.7
	D-Met	2.9	5.5	4.2	8.6	11.7	9.4	10.8	20.1	17.5	17.6	28.0	20.9	6.8
	D-Phe	3.0	3.6	3.7	8.1	8.9	14.2	11.0	19.5	16.4	16.5	25.7	20.4	6.1
	D-Glu	7.5	15.6	10.8	13.8	19.3	16.0	13.1	18.9	19.6	15.6	26.8	18.3	10.2
	D-Tyr	3.2	3.9	4.4	8.8	8.7	9.9	9.8	16.1	14.7	14.2	22.6	17.1	6.1
	D-Lys	1.3	2.0	2.1	3.9	5.8	4.4	6.1	9.3	8.4	8.4	15.6	9.1	4.1
(B)	D-Ala	0.0	1.3	0.0	1.5	1.9	0.0	1.6	2.8	0.0	1.7	4.0	3.2	0.0
	D-Val	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	D-*allo*-Ile	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	D-Pro	1.1	0.0	0.0	1.2	1.0	0.0	1.1	1.4	1.8	1.8	1.9	2.5	1.5
	D-Ser	4.4	3.8	5.0	8.4	5.6	8.1	9.9	9.0	10.8	12.5	12.2	13.4	14.0
	D-Leu	0.0	0.0	2.2	0.0	0.0	0.0	0.6	0.0	0.0	0.9	1.0	0.9	0.0
	D-Asp	3.9	5.5	3.6	12.1	12.8	9.7	16.6	21.2	15.7	21.2	29.1	23.3	3.0
	D-Met	0.0	2.0	0.0	2.3	3.9	0.0	3.3	4.3	2.9	3.4	5.7	3.8	3.5
	D-Phe	0.8	0.0	1.3	1.7	1.2	5.4	2.0	3.0	2.0	2.6	2.5	4.2	2.1
	D-Glu	4.2	4.9	9.1	13.8	10.0	10.5	14.1	14.5	15.3	n.d.	18.4	22.3	2.0
	D-Tyr	3.0	1.7	2.7	1.4	1.8	3.5	2.5	2.7	2.6	2.8	3.1	3.2	4.2
	D-Lys	0.0	0.0	4.0	1.2	0.0	1.5	2.1	1.8	9.6	2.3	2.2	2.4	2.0

Note that enantiomers of Arg, His, $(Cys)_2$ and D-Thr (if formed) could not be analysed; *n.d.* not determinable.

already formed in the control heated for 96 h, and amounted to 25.2% D-Ser, 39.7% D-Asp, 10.2% D-Glu, and 9.4% D-Ala. Lower amounts of other D-amino acids were also detected. On addition of saccharides quantities of D-amino acids increased. For example, on heating with fructose for 96 h, quantities of 42.9% D-Ser, 46.1% D-Asp, 26.8% D-Glu, and 22.0% D-Ala were detected. Amounts of the other D-amino acids increased also drastically, e.g. D-Pro from 1.9% (control) to 26.4%.

The data of experiment B on heating L-amino acids at pH 7.0 are presented in section (B) of the Table. Amounts of D-amino acids detected in the control heated for 96 h at pH 7.0 are much lower in comparison to the control heated at pH 2.5. Again addition of fructose and heating for 96 h lead to an increase of the D-amino acids formed, furnishing 29.1% D-Asp and 18.4% D-Glu, whereas in the control 3.0% D-Asp and 2.0% D-Glu were determined. The relative high amounts of 14.0% D-Ser in the control remained almost unchanged on addition of saccharides. Moderate quantities of the D-enantiomers of Ala, Pro, Leu, Met, Phe, and Lys were also generated.

Discussion

Although certain amounts of D-amino acids were already formed in the control experiments their quantities increased on addition of the saccharides. Albeit largest amounts of D-amino acids were generated by saccharides under acidic conditions, high or moderate amounts were also produced under neutral conditions.

The reaction of reducing sugars, such as glucose and fructose, with amino componentes, such as amino acids, is known as Maillard reaction or nonenzymic browning reaction. Since an intensive browning at pH 2.5 as well as pH 7.0 was observed at the heating experiments with saccharides it is assumed that, indeed, the Maillard reaction proceeded.

As to the racemization mechanism it is postulated that the reaction of amino acids with glucose or fructose starts with the reversible formation of labile Schiff bases. Proton abstraction takes place from the C^{α}-atom of the L-amino acid and a more or less planar carbanion is formed, possibly stabilized by conjugative delocalisation of the electron pair. Reattachment of the proton can take place at both sides of the carbanion resulting in its partial racemization (epimerisation). The degree of racemization depends in particular on steric and electronic properties of the amino acid side chains. The reaction proceeds with intramolecular rearrangements of glycosyl amino acids yielding relatively stable 1-amino-1-deoxy-2-ketoses (Amadori compounds) and 2-amino-2-deoxyaldoses (Heyns compounds), respectively. The amino acids therein might racemize again and can be eliminated e.g. from Amadori compounds with formation of 1- and 3-desoxyosons which are precursors of heterocyclic flavor compounds and melanoidins. Amino acids released at various stages of the Maillard reaction can participate again or undergo further reactions such as Strecker degradation.

Although Schiff bases are formed from amino acids and reducing sugars, heating with nonreducing saccharose notably yielded also D-amino acids (cf. Table). This is explained by the cleavage of saccharose, furnishing equal amounts of glucose and fructose.

The data prove that D-amino acids are formed on heating aqueous solutions of L-amino acids and saccharides. The range of about pH 2.5–7.0 is representative for many foods of plant and animal origin. Presence of amino acids together with saccharides is common. Thus the study provides also a feasible explanation for the generation of free D-amino acids in foods or biological systems which is not depending on microorganisms or racemases (Brückner and Schieber, 2000; Erbe and Brückner, 2000). The proposed racemization mechanisms might be extended on the reaction of amino acids with reactive carbonyl compounds in general.

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Authors' address: Prof. Dr. Hans Brückner, Interdisciplinary Research Center, Institute of Nutritional Science, Department of Food Sciences, Justus-Liebig-University of Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Federal Republic of Germany, Fax: +49-641-9939149, E-mail: hans.brueckner@ernaehrung.uni-giessen.de

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