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ORIGINAL PAPER

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Microwave treatment of dietary gelatin does not generate *cis*-4-hydroxy-L-proline, an inhibitor of collagen biosynthesis

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Abstract Aqueous solutions (5 g/100 ml) of commercial preparations of (a) an enzymatic partial hydrolysate of gelatin and (b) type A gelatin were subjected to threefold heating to boiling in a domestic microwave oven at 750 W and to conventional heating. Then samples were totally hydrolyzed (6 M hydrochloric acid, 110 °C, 24 h) and investigated for the presence of eight possible stereoisomers of 3- and 4-hydroxyproline (Hyp) using capillary gas chromatography. Amino acids were analyzed as *N(O)*-trifluoroacetyl 2-propyl esters on Chirasil-L-Val and detected by selected ion monitoring mass spectrometry. Blanks of (a) and (b) were analyzed in parallel. Relative amounts of 5.0 ± 0.2% *cis*-4-D-Hyp were generated from native *trans*-4-L-Hyp as a result of total hydrolysis in all samples and independent of previous treatment. Notably, neither *cis*-3-L-Hyp nor *cis*-4-L-Hyp could be detected in either of the gelatin samples. Thus a report on the generation of antifibrotic and therefore potentially hazardous *cis*-3-L-Hyp and *cis*-4-L-Hyp from protein-bonded native *trans*-3-L-Hyp and *trans*-4-L-Hyp on microwave heating of infant formulae could not be confirmed.

Key words Amino acid epimerization · Infant formulae · Food proteins · Hydroxyproline stereoisomers · Gas chromatography-mass spectrometry

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Introduction

Microwave cooking, heating, tempering, and thawing of food is nowadays well established in the majority of private households, snack bars, and restaurants, as well as in the food processing industry [1, 2]. Taking this into account, a letter of Lubec et al. [3] attracted much attention, claiming that microwave heating of infant milk formulae generates potentially toxic D-proline (D-Pro) from L-proline (L-Pro), as well as *cis*-3-L-Hyp and *cis*-4-L-Hyp from native protein *trans*-3-L-Hyp and *trans*-4-L-Hyp (for structures of hydroxyproline stereoisomers, see Fig. 1).

The antifibrotic potential of *cis*-4-L-Hyp is well documented, as well as the fact that this amino acid can be incorporated into procollagen during collagen biosynthesis [4–6]. Further, *cis*-3-L-Hyp has been reported to exhibit antigenic and degradative effects on collagenous tissues [7, 8]. Therefore, hazardous effects (structural, functional, and immunological changes) on consumption of microwave treated food were postulated [3, 9]. However, neither the formation of D-Pro on microwave treatment of milk and milk-based infant formulae [10, 11] nor the toxicity of D-Pro on experimental animals could be confirmed [12].

The claim that *cis*-3-L-Hyp and *cis*-4-L-Hyp are formed in three microwave treated infant formulae investigated by Lubec et al. did not attract much attention. This is attributed to the fact that milk proteins are rich in Pro but do not contain Hyp. Thus, no Hyp was detected in milk or infant formulae based on milk proteins exclusively [10, 11]. However, abundant *trans*-4-L-Hyp and approx. 5% *cis*-4-D-Hyp were detected in one commercial infant formula declared as consisting of a mixture of soy protein, free amino acids (AAs), and hydrolyzed animal protein. Consequently, that animal protein was assumed to be gelatin [10]. Indeed, enzymatic partial hydrolysates of gelatin are used for the preparation of easily water-soluble and non-gelling diet formulations [13].

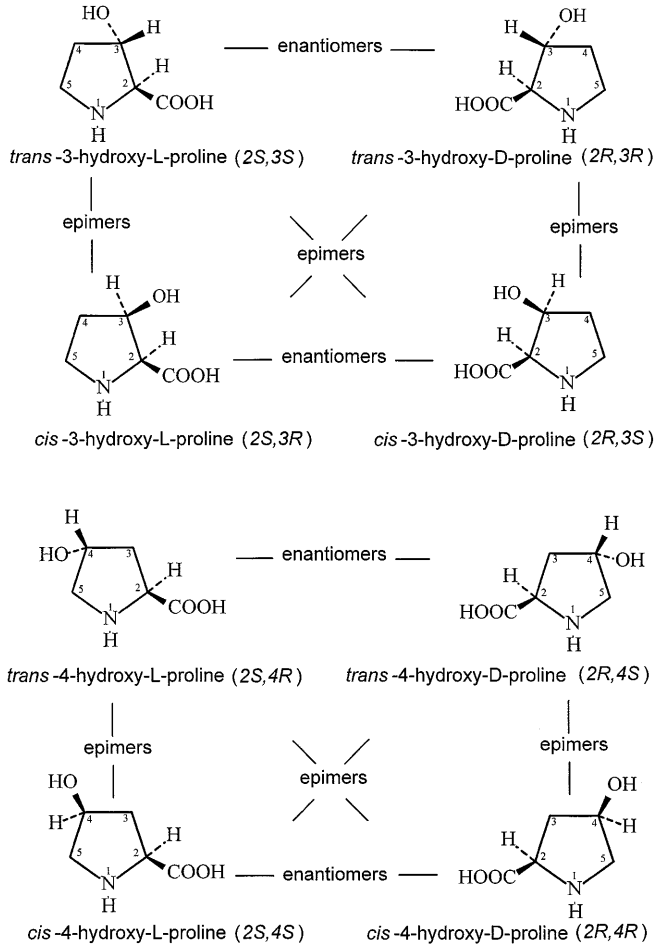

Fig. 1 Structures of the stereoisomers of 3- and 4-hydroxyproline and assignment of enantiomers and epimers

Gelatin is manufactured from collagenous raw materials such as bovine bones, pigskins, and cattle hides [13, 14]. Native collagen is synthesized as procollagen by ribosomal synthesis. Collagen's characteristic and abundant *trans*-4-L-Hyp is synthesized by enzymatic post-translational hydroxylation of specific proline residues [15]. Notably, in addition to *trans*-4-L-Hyp, low amounts of *trans*-3-L-Hyp (0.6–1.1%) were detected, for example in type IV collagen of basal membranes [16] and in type I collagen of renal cortex [17–19]. Therefore, increased urinary *trans*-3-D/L-Hyp ratios were suggested to be useful markers for a renal collagen disorder named Alport syndrome [20].

In the investigations carried out in order to verify the findings of Lubec et al., it was overlooked [10, 11] that the authors [3] actually did report that "...conventionally heated milk samples did not contain *cis*-3 or *cis* hydroxyproline. However, all three formulae contained *cis* stereoisomers of hydroxyproline after microwave treatment...". From the context of that report [3] and the references cited therein [21–23] it is obvious that "*cis*-3 or *cis*-4 hydroxyproline" refer to *cis*-3-L-Hyp and *cis*-4-L-Hyp, and not to *cis*-3-D-Hyp and *cis*-4-D-Hyp as assumed by Fay et al. and Marchelli et al. [10, 11]. Consequently, the question whether or not the *cis* isomers of 3- and 4-L-Hyp are generated from dietary gelatin by microwaves and/or are released on total hydrolysis from the natural *trans*-isomers was still open.

In order to clarify this question and in continuation of work on amino acid epimerization in the course of industrial gelatin manufacturing and processing [24], we decided to subject an enzymatic gelatin partial hydrolysate and pure gelatin to microwave treatment followed by acidic total hydrolysis. The possible isomerization of native hydroxyproline stereoisomers was investigated by chiral gas chromatography and highly sensitive and specific selected ion monitoring mass spectrometry.

Materials and methods

Chemicals, solvents and sample materials. All chemicals and solvents were of analytical grade: dichloromethane (DCM), 2-propanol (2-PrpOH), acetyl chloride (AcCl), hydrochloric acid (HCl), 32% (all from Merck, Darmstadt, Germany), sulfuric acid (H_2SO_4), 98% (Roth, Karlsruhe, Germany), trifluoroacetic acid anhydride (TFAA), 2,6-di-*tert*-butyl-*p*-cresol (BHT), and barium hydroxide octahydrate ($Ba(OH)_2 \cdot 8H_2O$) (Fluka Chemie, Buchs, Switzerland). Stereoisomers of Hyp (*cis*-3-DL-Hyp, *cis*-4-L-Hyp, *cis*-4-D-Hyp) were purchased from Sigma (St. Louis, Mo., USA), and *trans*-4-L-Hyp from Fluka.

The stereoisomers not commercially available (*trans*-3-DL-Hyp and *trans*-4-D-Hyp) were synthesized according to the literature [25]: to *cis*-3-DL-Hyp (5 mg) 0.2 M aqueous $Ba(OH)_2$ (0.5 ml) was added and the mixture was heated at 110 °C for 24 h. Suitable amounts of 2% H_2SO_4 were added in order to neutralize $Ba(OH)_2$. The barium sulfate precipitated was removed by centrifugation, the supernatant was dried in a nitrogen stream, and the residue, containing mainly *trans*-3-DL-Hyp, was used for derivatization.

A sample of *cis*-4-L-Hyp (5 mg) was heated in 8.77 M HCl in a sealed Reacti-vial (Wheaton, Millville, N.J., USA) at 130 °C for 72 h. Acid was removed in a stream of nitrogen and the remaining residue containing a mixture of *cis*-4-L-Hyp and *trans*-4-D-Hyp (ratio ca. 2:1) was used for derivatization.

Gelatin hydrolysate (Gelita-Sol, obtained by enzymatic partial hydrolysis, average molecular weight 3000 Da) was provided by the Deutsche Gelatine-Fabriken (DGF) Stoess, Eberbach, Germany. Gelatin (pure type A, made from pigskins) was from Ewald Werke, Sobernheim, Germany.

Sample preparation and derivatization. In separate experiments, Gelita-Sol (sample A) or gelatin (sample B) (5 g) in doubly distilled water (100 ml) were used for microwave treatment, for conventional heating and as control. A domestic microwave oven (Model HF 12520, Siemens AG, München; power output 750 W, according to IEC 705) was used. In experiment 1, samples were heated on a conventional electric hot-plate up to boiling temperature three times and were allowed to cool at room temperature in between. In experiment 2, samples were treated with 90 W power for 20 min (final temperature 50 °C) in order to test whether there was an intrinsic microwave effect besides the thermal effect. In experiment 3, samples were treated three times with 750 W power for 4 min up to boiling temperature. Samples were allowed to cool at room temperature in the interim periods. Samples were heated in open Erlenmeyer flasks in order to simulate common household conditions. Samples from experiment 4 were the untreated controls of gelatin partial hydrolysate and type A gelatin, respectively.

Table 1 Relative amounts (%) of *cis*-4-hydroxy-D-proline in total hydrolysates of aqueous solutions (5%) of Gelita Sol (A) and gelatin (B) subjected to microwaves (90 W and 750 W) and heated conventionally on a hot-plate in comparison to untreated controls. Two sample preparations, named A_1, A_2, and B_1, B_2, respectively, were analyzed in two parallel experiments ($n = 4$ injections); SD = standard deviation; (for sample treatment, see Materials and methods)

n	Experiment 1 (hot-plate)[a]		Experiment 2 (microwave 90 W)[a]		Experiment 3 (microwave 750 W)				Experiment 4 (control)			
	A_1	A_2	A_1	A_2	A_1	A_2	B_1	B_2	A_1	A_2	B_1	B_2
1	5.1	4.8	5.1	4.8	5.1	4.8	4.9	4.8	4.5	5.1	4.9	4.8
2	5.3	5.5	5.0	5.2	5.3	4.5	5.1	5.5	5.0	5.0	5.2	5.1
3	4.9	4.9	4.4	4.7	4.9	4.7	4.9	4.9	4.6	4.7	5.2	5.3
4	4.9	4.9	4.9	5.2	4.8	5.3	4.9	5.3	5.1	4.8	4.9	5.1
Average	**5.05**	**5.03**	**4.85**	**4.98**	**5.03**	**4.83**	**4.95**	**5.13**	**4.80**	**4.90**	**5.05**	**5.08**
SD	0.17	0.28	0.27	0.23	0.19	0.29	0.09	0.29	0.25	0.16	0.15	0.81

[a] B_1, B_2 not carried out (see Discussion)

For hydrolysis aliquots (1 ml) of samples of experiments 1–3 and of the controls (experiment 4) were transferred into 5 ml Reacti-vials (Wheaton). Then HCl (8.77 M, 2.17 ml) was added for carrying out total hydrolysis under standard conditions (6 M HCl at 110 °C for 24 h).

Besides the gelatin samples, a sample of enantiomeric pure *trans*-4-L-Hyp was treated under extremely acidic, heated conditions (10 mg of *trans*-4-L-Hyp in 1 ml 8.77 M HCl at 130 °C for 48 h) to test whether trace amounts of the *cis*-4-L-isomer were formed.

For conversion of AAs into *N(O)*-trifluoroacetyl 2-propyl esters, aliquots (200 µl) of the hydrolysates were used. HCl was removed in a stream of nitrogen, the antioxidant BHT (10 µl of a solution of 10 mg ml^{-1} in 2-PrpOH) and a mixture of 2-PrpOH/AcCl (4:1) (500 µl) were added. The tightly closed vials were heated at 100 °C for 1 h. Solvents were removed with N_2, DCM (200 µl) and TFAA (50 µl) were added, and the mixture was heated at 100 °C for 20 min. Solvents were removed in a stream of nitrogen. The residue was dissolved in DCM (200 µl) and portions of 0.6–1.0 µl were subjected to GC/MS at a split ratio of 1:50. MS was run in selected ion monitoring (SIM) mode.

Relative amounts (%) of *cis*- and *trans*-D-isomers were calculated according to Eq. 1 and 2:

$$cis\text{-D}\ (\%) = A_{cis\text{–D-isomer}} \times 100/(A_{cis\text{–D-isomer}} + A_{trans\text{–L-isomer}}) \quad (1)$$

$$trans\text{-D}\ (\%) = A_{trans\text{–D-isomer}} \times 100/(A_{trans\text{–D-isomer}} + A_{cis\text{–L-isomer}}) \quad (2)$$

where *A* is the peak area of the respective *cis*- or *trans*-D- or L-isomer.

For repeatability tests, each sample was subjected to GC/MS four times. The results shown are the average of two parallel preparation/derivatization procedures (see Table 1).

Capillary gas chromatography. A Shimadzu GC-17A gas chromatograph and a Shimadzu QP-5000 mass spectrometer were used. The column was a fused silica Chirasil-L-Val capillary column [26, 27] (25 m × 0.25 mm ID, Chrompack, Middelburg, The Netherlands). The carrier gas was helium at an inlet pressure of 80 kPa (flow rate 0.5 ml min^{-1}). The temperatures of injector and interface were 250 °C; samples were injected in split mode (split ratio 1:50). Temperature program: initial temp. 75 °C held for 8.5 min; heating rate A: 2.5 °C min^{-1} to 100 °C; rate B: 3.5 °C min^{-1} to 145 °C; rate C: 10.0 °C min^{-1} to 190 °C held for 4 min. Pressure program: initial pressure 5.0 kPa held for 8.5 min, increased at 0.2 kPa min^{-1} up to 7.0 kPa, increased at 0.3 kPa min^{-1} to 10.9 kPa, increased at 1.0 kPa min^{-1} to 15.0 kPa, held for 4.5 min.

GC/MS control and data processing were performed by a Shimadzu class 5000 workstation and class 5000 software.

Results

The complete separation of all stereoisomers of 3- and 4-hydroxyproline in a GC run is shown in Fig. 2.

No differences referring to the ratios of Hyp isomers were detected in total hydrolysates of aqueous solutions of either untreated (blank), microwave heated or conventionally heated Gelita-Sol (sample A) and gelatin (sample B). All hydrolysates contained *trans*-4-L-Hyp as the major component. Notably, no *cis*-4-L-Hyp was detected in total hydrolysates of either Gelita Sol (Fig. 3) or gelatin.

The average amount of *cis*-4-D-Hyp was about 5% relative to the sum of *trans*-4-L-Hyp and *cis*-4-D-Hyp in all cases (see Table 1). Relative to that sum, only traces of *trans*-3-L-Hyp (0.2–0.25%) were detected in acidic total hydrolysates of pure gelatin (sample B) (in untreated controls as well as in microwave treated samples), but not in the acidic total hydrolysates of sample A.

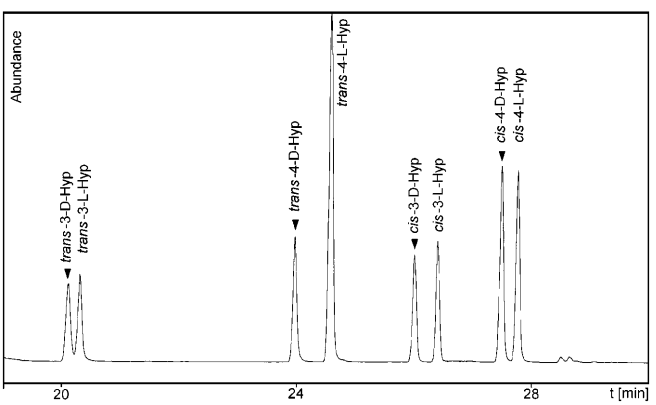

Fig. 2 GC/SIM-MS of a standard of all eight 3- and 4-hydroxyproline stereoisomers, separated on Chirasil-L-Val as *N(O)*-trifluoroacetyl-Hyp-2-propyl esters

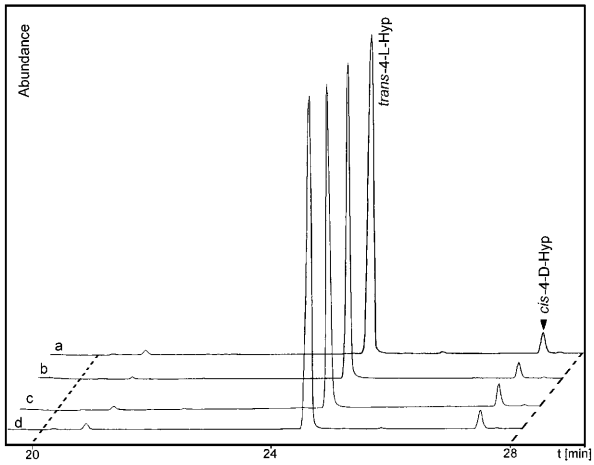

Fig. 3 GC/SIM-MS of total hydrolysates of aqueous solutions (5%) of Gelita-Sol focusing on Hyp stereoisomers, separated on Chirasil-L-Val as *N*(*O*)-trifluoroacetyl-Hyp-2-propyl esters: *a* conventionally heated on hot-plate, *b* microwave treated (90 W), *c* microwave treated (750 W), *d* untreated control (for details see Materials and methods)

Further, in a standard of *trans*-4-L-Hyp that for comparison had been treated under extreme conditions with 8.8 M HCl for 48 h at 130 °C, increased amounts of about 8% of *cis*-4-D-Hyp were found, but again no *cis*-4-L-Hyp was detected (detection limit ca. 0.1% of the peak area of *trans*-4-L-Hyp). These results, showing the partial epimerization of *trans*-4-L-Hyp providing *cis*-4-D-Hyp, corroborate data reported previously [10, 11, 24, 28].

Discussion

It was demonstrated that epimerization of *trans*-4-L-Hyp in dietary gelatin leads to the formation of *cis*-4-D-Hyp, and not to *cis*-4-L-Hyp. The formation of *cis*-4-L-Hyp *via* epimerization reactions would only be possible if significant amounts of *trans*-4-D-Hyp were present in the protein. However, that was not the case in the gelatin samples investigated and is very unlikely for other ribosomally synthesized food proteins. As far as we know, protein-bonded *cis*-4-L-Hyp has not yet been detected in food proteins.

The amounts of *cis*-4-D-Hyp formed in microwave treated, conventionally heated and in untreated control samples showed no significant differences ($P < 0.01$). Therefore, the gelatin sample (sample B) was exposed only to high microwave power (750 W) (see Table 1).

From the data it is concluded that the formation of about 5% of *cis*-4-D-Hyp from *trans*-4-L-Hyp is exclusively the result of acidic total hydrolysis that had to be carried out in order to release free AAs from gelatin. It might be that under the conditions described in the literature [3] the amounts of *cis*-4-D-Hyp formed in the course of the acid hydrolysis of the infant formulae were misinterpreted to be *cis*-4-L-Hyp. This is supported by the fact that the achiral TLC [22] and HPLC [23] methods reported to have been used for the detection and separation of *cis*-3-Hyp from *cis*-4-Hyp [3] are not suitable for the separation of the enantiomers *cis*-3-D-Hyp and *cis*-3-L-Hyp or the enantiomers *cis*-4-D-Hyp and *cis*-4-L-Hyp, respectively.

With regard to the reported formation of *cis*-3-L-Hyp, it should be pointed out that certain types of collagens and gelatin contain minor amounts of *trans*-3-L-Hyp (approx. 0.8% in type IV collagen). Acidic total hydrolysis under standard conditions, by analogy to *trans*-4-L-Hyp, should generate ca. 5% *cis*-3-D-Hyp from *trans*-3-L-Hyp, that is, about 0.01% with respect to *trans*-4-L-Hyp. This is beyond the detectability even under the highly sensitive instrumental conditions described here (detection limit ca. 0.1% of total Hyp). Further, analogous to the epimerization of *trans*-4-L-Hyp, no formation of *cis*-3-L-Hyp is to be expected on microwave treatment or heating of protein-bonded (or free) *trans*-3-L-Hyp. The presence of 5% of *cis*-4-D-Hyp in the total hydrolysates of all samples was shown to be an artifact due to acidic catalyzed epimerization of *trans*-4-L-Hyp. This removes all doubts of possible pathological effects of microwave treated foodstuffs containing collagen or its derivatives, referring to AA isomerization. Studies dealing with the incorporation of *cis*-Hyp into collagen only refer to the L-isomer [4, 6, 7, 29] and not to *cis*-D-Hyp.

In conclusion, a report on the isomerization of native, food protein-bonded hydroxyproline claiming the formation of potentially harmful, antifibrotic *cis*-4-L-Hyp (or *cis*-3-L-Hyp) on microwave treatment could not be confirmed. Since formation of D-Pro on microwave treatment of food and toxic effects of D-Pro on rats have also been disproved [10, 11, 12, 24, 30], there is no experimental evidence for the apprehensions concerning the microwave exposure of food proteins using common cooking practice. With regard to D-AAs in general, it should be stressed that they are common constituents in particular of fermented foods and beverages [31].

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