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Determination of protein-bound 2-amino-6-(2-formyl-1-pyrrolyl)-hexanoic acid ("pyrraline") by ion exchange chromatography and photodiode array detection

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Bestimmung von proteingebundener 2-Amino-6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)-hexansäure („Pyrralin") mittels Ionenaustauschchromatographie und Photodiodenarray-Detektion

Zusammenfassung. Die Bestimmung des proteingebundenen Maillard-Produktes Pyrralin, eines Lysinderivats mit Pyrrolstruktur, gelang in enzymatischen Hydrolysaten von Proteinen mittels Ionenaustauschchromatographie und kombinierter Photodiodenarraymessung und Ninhydrinanfärbung. Das Verfahren erlaubt die gleichzeitige Bestimmung von Pyrralin neben den übrigen Hydrolysataminosäuren sowie säurelabilen Verbindungen wie den Amadori-Produkten und Tryptophan bis zu Gehalten von 500 µg pro kg Protein. Pyrralingehalte in kontrolliert erhitzten Proben stiegen zunächst linear mit der Erhitzungszeit und nahmen nach starkem Erhitzen deutlich ab. Unter drastischen Erhitzungsbedingungen (110 °C/5 h) reagierten 5,6% der Lysinreste zum Pyrrolaldehyd.

Summary. Following enzymic hydrolysis, sensitive and unambiguous determination of the protein-bound Maillard compound pyrraline, a lysine derivative with a pyrrol structure, was achieved by ion-exchange chromatography with photodiode array measurement and subsequent ninhydrin detection. The method allows the simultaneous quantification of pyrraline in addition to the common amino acids, as well as acid-labile components such as the Amadori products and tryptophan, at levels lower than 500 µg/kg protein. Values of pyrraline in controlled heated samples first increased linearly with heating time, followed by a significant decomposition after long-term heating. In severely heat-treated samples (110° C for 5 h), up to 5.6% of the initial lysine residues had reacted to the pyrrolaldehyde.

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Introduction

Since its first description in 1912 [1] the so-called Maillard reaction between amines and reducing carbohydrates has been intensively studied. For decades, attention was focused mainly on the reactive behaviour of the carbonyl compound. To date, a large number of sugar degradation products have been identified [2, 3]. In comparison, only little information is available concerning the formation of protein-bound Maillard reaction products. In the early 1970s the so-called Amadori products lactuloselysine and fructoselysine (**1**, Fig. 1) were isolated and identified as the first known derivatives resulting from the reaction of the ε-amino group of protein-bound lysine and lactose [4]. Oxidative degradation of these aminoketoses leads to the formation of carboxymethyllysine (**2**, Fig. 1) which could be detected in acid hydrolysates of milk proteins [5]. Recently, Sell and Monnier [6] isolated pentosidine (**3**, Fig. 1) from collagen and thus proved the direct participation of the Maillard reaction in protein crosslinking during ageing of proteins in vivo.

The compound "pyrraline" (**4**, Fig. 1) was first identified by Nakayama et al. [7] and a few years later by Miller et al. [8] in heated reaction mixtures of lysine and glucose. Sengl et al. [9] as well as Schüßler and Ledl [10] succeeded in the determination of the acid-labile lysine derivative **4** in alkaline hydrolysates of proteins that had been reacted with glucose. Free pyrraline proved to be a mutagen [11] and was found to be a strong competitive inhibitor for

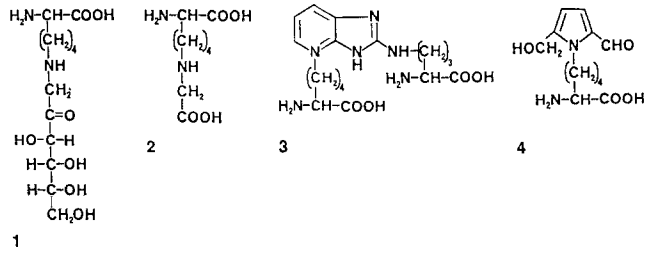

Fig. 1. Compounds **1–4** referred to in the text

gastrointestinal proteases [12]. The most recent investigations [13, 14] indicate that the hydroxy group in the α-position of the pyrrolic ring is easily substituted by alcohols and mercaptanes. Thus, crosslinking of proteins might proceed by bonding of protein-bound cysteine and the pyrrol aldehyde **4**. In this paper an alternative procedure is described for the determination of **4**, based on the combination of photodiode array measurement and subsequent ninhydrin detection after ion exchange chromatography. The method allows the simultaneous quantification of **4**, with the Amadori products and common amino acids, including tryptophan, in enzymic hydrolysates.

Materials and methods

Chemicals. Pepsin and pronase were from Merck (Darmstadt, FRG). The suspension of aminopeptidase was from Boehringer (Heidelberg, FRG). Prolidase and an amino acid standard solution were from Sigma (Deisenhofen, FRG). All other chemicals were from Merck (Darmstadt, FRG). A pure standard sample of pyrraline was provided by Dr. M. Sengl, University of Munich.

Preparation of heated samples. Powdered casein/lactose samples (CLP) were prepared by freeze-drying a mixture of 3000 mg Hammersten-casein and 5260 mg lactose monohydrate in 100 ml of synthetic milk ultrafiltrate [15] after adjusting the pH to 6.80 (final water content 2.5% by mass). Skim milk was obtained from the Bavarian State Dairy (Weihenstephan, FRG). Skim milk powder (SMP) was prepared by freeze-drying (final water content 3.4% by mass). Samples of CLP and SMP (1 g each) were heated in stoppered test tubes (10 × 100 mm, Pyrex, France). Heating was performed in a sandbath placed in a drying oven to avoid variations in temperature. Heating time was 1–7 at 90° and 100° C.

Enzymic hydrolysis. Heated samples were hydrolysed enzymically as described in [16] with some modifications [17].

Alkaline hydrolysis. Heated samples (20 mg each) were hydrolysed with 4.5 ml 5 mol/l sodium hydroxide for 16 h at 120° C [9].

Acid hydrolysis. Heated samples were hydrolysed in the presence of 6 mol/l hydrochloric acid as described in [17].

Amino acid analysis. Amino acid analysis was performed with an Alpha Plus amino acid analyser (LKB Biochrom, Cambridge, UK), using a stainless steel column (150 × 4 mm) filled with ion exchange resin DC4A-spec (Laborbedarf KG, Olching, FRG). Composition of buffers and reagent and running conditions, are described in [18]. However, contrary to the reported separation program the buffer flow rate was set at 20 ml/h. The running time of buffer 1 was set at 5 min at 37° C; buffer 2 was set at 22 min at 65° C. All other program steps remained unchanged. The separated amino acids were first detected using a photodiode array detector PDA 991 (Waters Millipore, Milford, Massachusetts, USA) connected to the outlet of the column, simultaneously measuring a wavelength range from 240 to 330 nm to detect pyrraline, tyrosine and other aromatic amino acids. After flowing through the PDA detector, common amino acids were detected after reaction with ninhydrin at 570 nm; coil temperature was 135° C. Data acquisition and peak integration were evaluated using the PDA software and Maxima chromatography software (Waters Dynamic Solutions, Ventura, CA, USA), respectively, on two NEC 386 AT.

Results and discussion

The high performance liquid chromatography methods published to date have only been suitable for the quantification of pyrraline along with some aromatic amino acids in alkaline hydrolysates. The purpose of this study was to establish conditions for chromatography, detection as well as hydrolysis, which would enable the simultaneous determination of **4** along with the common amino acids as well as other acid- or alkaline-labile compounds. Direct determination of **4** by ion exchange chromatography and ninhydrin detection under the conditions described recently [18] was not possible: **4** and phenylalanine eluted with identical retention times (Fig. 2 a). The elution position of **4** was determined after injection of a pure standard preparation of pyrraline. Varying chromatographic parameters such as running time, pH and ionic strength of the buffers or the column temperature had no effect on the separation of **4** and phenylalanine.

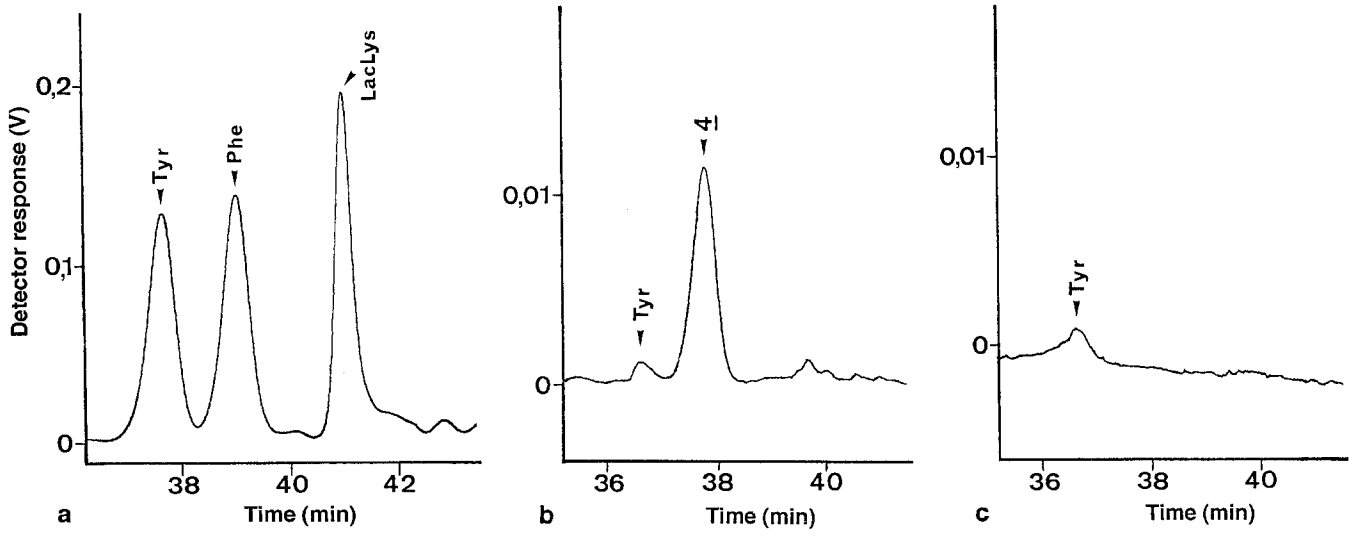

Fig. 2 a–c. Chromatograms (detail) of enzymic hydrolysates (**4**, pyrraline; LacLys, lactuloselysine). **a** SMP sample (100° C for 2 h), detection at 570 nm after ninhydrin reaction; **b** SMP sample (100° C for 2 h), PDA detection at 297 nm; **c** SMP sample (unheated), PDA detection at 297 nm

Table 1. UV Absorption characteristics of various amino acids

Amino acid	λ_{max} (nm) [solvent]	log ε	Reference
Tyrosine	274.5 [water, pH 6]	3.15	[21]
Phenylalanine	242 [water]	1.86	[21]
	257	2.25	
	267	1.91	
Tryptophan	280 [water]	3.8	[21]
Pyrraline **4**	265 [water]	3.78	[7]
	297	4.15	
	265 [CH_3OH/water, 4+1]	3.78	[19]
	295	4.15	

Consequently, the direct ultraviolet (UV) detection of **4** was attempted. Comparison of the UV spectras and absorption maximas of phenylalanine and pyrraline (Table 1) showed that most sensitive detection of **4** would be achieved at 297 nm. A photodiode array detector (PDA) was therefore used for monitoring the eluent after it left the ion exchange column. Measurement of absorption was carried out simultaneously within a wavelength range of 240–330 nm. After data acquisition, single chromatograms were extracted from the complete data file and peak areas integrated using the PDA software. In this way it was possible to quantify phenylalanine (254 nm), tyrosine (274.5 nm), tryptophan (280 nm) and **4** (297 nm) at the corresponding absorption maximas. By connecting the outlet of the PDA detector to the inlet of the reaction coil, subsequent ninhydrin detection was achieved. The use of this combined detection method enabled the sensitive and unambiguous determination of pyrraline along with all common amino acids including tryptophan, as well as the Amadori products of lysine, in enzymic hydrolysates of heated samples of CLP and SMP (Fig. 2 a, b). In the chromatogram obtained after detection at 297 nm, **4** eluted as a well-resolved peak immediately after tyrosine (Fig. 2 b). At this wavelength phenylalanine exhibited no detectable absorption (Fig. 2 c). Pyrraline was identified by comparing the retention time and UV spectrum with a standard sample [19]. The absorption maximum of **4** determined in the standard sample as well as in hydrolysates was 297 nm, which was identical to the value for λ_{max} reported by Nakayama et al. [7].

With reference to the different molar absorptivities ($\varepsilon_{PyrL,\,297}$; $\varepsilon_{Tyr,\,274,5}$; see Table 1), the molar pyrraline content of the heated samples (c_{PyrL}) could be calculated according to Beer's law by comparing the peak area of **4** at 297 nm ($A_{PyrL,\,297}$) with the peak area of an external standard of tyrosine (standard concentration $c_{Tyr\text{-}STD}$), integrated from a 274.5 nm chromatogram ($A_{Tyr\text{-}STD,\,274,5}$):

$$C_{PyrL} = \frac{A_{PyrL,\,297}}{A_{Tyr\text{-}STD\,274,5}} \times \frac{\varepsilon_{Tyr,\,274,5}}{\varepsilon_{PyrL,\,297}} \times C_{Tyr\text{-}STD} \cdot \qquad (1)$$

Using this relation, quantification of **4** can be achieved without the use of a stable standard of the lysine derivative. Validity of the fomula was proved by injection of various standard samples of tyrosine and **4**, containing defined amounts of the amino acids. Identical values of pyrraline obtained after calculation via the external standard as well as with Eq. (1) showed that influences of buffer composition (pH 6.40; 1.5 mol/L Na^+) on the absorption properties of **4** and tyrosine were negligible. The detection limit for pyrraline was between 1 and 2 pmol. Simultaneous injection of pyrraline and samples containing up to 500 µg protein hydrolysate enabled detection of **4** at levels as low as 0.5–1 mg/kg. Concentrations of **4** and corresponding peak areas showed good linear correlation ($r = 0.997$) within the range of 5 pmol to 25 nmol per injection.

For the measurement of protein-bound **4**, enzymic hydrolysis of the heated samples was performed. Thus, complete and careful protein digestion was achieved. With the exception of proline, the recovery of amino acids after enzymic hydrolysis was comparable with the results obtained after acid hydrolysis, even for severely heat-treated samples. Deviations from the mean values of **4** determined after multiple hydrolysis of individual samples and subsequent chromatography were between 9.3% (5 mg/kg protein) and 3.1% (2650 mg/kg protein). In contrast to this, the conditions used for alkaline hydrolysis seemed not to be suitable for a direct determination of **4**. After neutralization of the alkaline hydrolysate with concentrated hydrochloric acid and subsequent lyophilization, large amounts of sodium chloride were formed. To obtain a complete solution, the dry hydrolysates had to be diluted with large volumes of buffer (0.2 mol/L sodium citrate, pH 2.2 [18]) before injection. Thus, sensitive determination of **4** after alkaline hydroly-

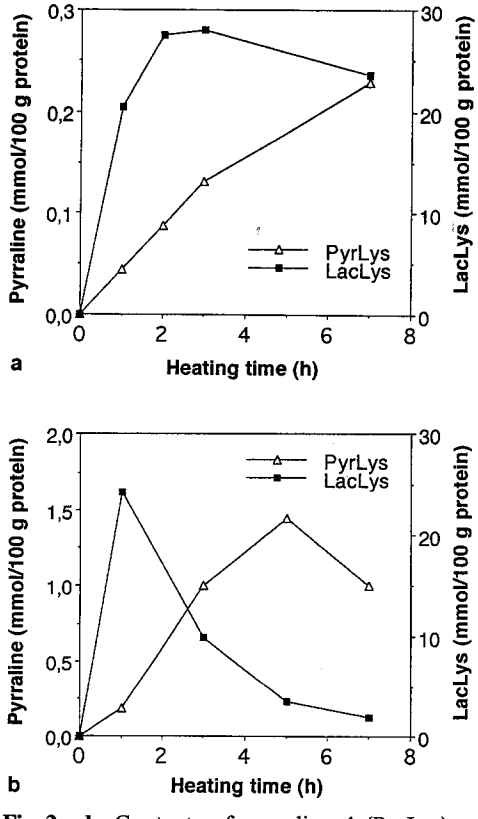

Fig. 3 a, b. Contents of pyrraline **4** (PyrLys) and lactuloselysine (LacLys) in enzymic hydrolysates of heated CLP samples. **a** 100° C; **b** 110° C

sis was impossible. Quantitative determinations of **4** and the Amadori product lactuloselysine in enzymic hydrolysates of heated CLP-samples are shown in Fig. 3 a, b.

In the samples heated to 100° C (Fig. 3 a), the lactuloselysine contents increased during a heating time of 1 to 3 h. Prolonged heating times up to 7 h led to a decrease in the Amadori product, indicating degradation of the aminoketose as the Maillard reaction progressed. Parallel to the formation of the Amadori product, linearly increasing amounts of **4** were found, but no increase in the formation of **4** was observed that correlated with the start of the decrease in lactuloselysine. Even under severe heating conditions (110° C; Fig. 3 b), leading to the continuous decomposition of lactuloselysine, no enhanced formation of **4** was measured. Again, the content of **4** increased linearly up to values of 1.45 mmol/100 g during heating for 1 to 5 h, followed by a remarkable decrease after heating for 7 h. Similar tendencies were observed for hydrolysates of heated samples of SMP. Probably as a result of higher water content, 2.96 mmol of **4** per 100 g was measured in severely heat-treated SMP samples before degradation. Compared with an initial lysine value of 53 mmol per 100 g protein, up to 5.6% of the lysine residues had reacted to form the protein-bound pyrrolaldehyde **4.** Significantly lower values reported in the literature (only 0.1% of initial lysine reacting to form **4** [10]) might be due to earlier degradation of **4** during preparation of the sample under investigation (β-casein/glucose-solution, 120° C, 2.5 h).

The observation that the formation of **4** is independent of the degradation of the Amadori product indicates that **4** might originate from direct reactions between unmodified lysine residues and sugar degradation products, mainly 3-deoxyglucosone [20]. Parallel to the main glycosylation pathway leading to stable aminoketoses, small amounts of highly reactive α-dicarbonyl compounds must be formed which could react with free ε-amino groups to form heterocyclic compouns such as the pyrrolaldehyde **4.**

No information is available yet concerning the mechanisms that lead to the observed degradation of **4** during prolonged heat treatment. Whether and to what extent crosslinking reactions between protein-bound **4** and thiols such as cysteine are responsible for a decrease in the content of **4** and a possible protein oligomerization is now under investigation. Further studies are also in progress to obtain data for a kinetic interpretation of the formation and decomposition of **4.** The level of **4** might serve as a sensitive indicator of the extent of the Maillard reaction in foods subjected to severe heat treatment or long-term storage.

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References

1. Maillard LC (1912) CR Acad Sci Ser 154:66–68
2. Ledl F, Schleicher E (1990) Angew Chem Int Ed Engl 29:565–594
3. Ledl F (1990) In: Finot PA, Aeschbacher HU, Hurrell RF, Liardon R (eds) The Maillard reaction in food processing, human nutrition and physiology. Birkhäuser, Basel, pp 19–42
4. Möller AB, Andrews AT, Cheeseman GC (1977) J Dairy Res 44:259–266
5. Büser W, Erbersdobler HF (1986) Milchwissenschaft 41:780–785
6. Sell DR, Monnier VM (1989) J Biol Chem 264:21597–21602
7. Nakayama T, Hayase F, Kato H (1980) Agric Biol Chem 44:1201–1202
8. Miller R, Olsson K, Pernemalm PA (1984) Acta Chem Scand 38B:689–694
9. Sengl M, Ledl F, Severin T (1989) J Chromatogr 463:119–125
10. Schüßler U, Ledl F (1989) Z Lebensm Unters Forsch 189:32–35
11. Omura H, Jahan N, Shinohara K, Murakami H (1983) In: Waller GR, Feather MS (eds) The Maillard reaction in foods and nutrition. ACS Symp Ser 5:537–563
12. Öste RE, Miller R, Sjöström H, Noren O (1987) J Agric Food Chem 35:938–942
13. Ledl F, Klein E (1991) In: Baltes W, Eklund T, Fenwick R, Pfannhauser W, Ruiter A, Thier HP (eds) Strategies for food quality control and analytical methods in europe. Behr's Verlag, Hamburg, pp 309–314
14. Klein E, Ledl F, Bergmüller W, Severin T (1992) Z Lebensm Unters Forsch 194:556–560
15. Jenness R, Koops J (1962) Neth Milk Dairy J 16:153–164
16. Schmitz J, Zahn H, Klostermeyer H, Rabbel K, Watanabe K (1976) Z Lebensm Unters Forsch 160:377–381
17. Henle T, Walter H, Klostermeyer H (1991) Z Lebensm Unters Forsch 193:119–122
18. Henle T, Walter H, Krause I, Klostermeyer H (1991) Int Dairy J 1:125–135
19. Schüßler U, Ledl F (1989) Z Lebensm Unters Forsch 189:138–140
20. Kato H (1962) Agric Biol Chem 26:187–192
21. Weast RC (ed) (1971) Handbook of chemistry and physics, 52nd edn. CRC, Cleveland