A New Pathway of Lactose Degradation and Arginine Derivatization in Milk

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Abstract: Recently, N-δ-[5-(3-hydroxypropyl)-4-oxo-imidazolon-2-yl]-L-ornithine (PIO) was identified as a new arginine derivative, formed exclusively from the side-chain of peptide-bound arginine and degradation products of oligosaccharides with 1,4-glycosidic linkages, thus probably representing the major form of arginine derivatization in heated milk products. The formation mechanism of PIO was clarified via identification of a previously unknown C5 dicarbonyl precursor, namely 3,4-dideoxypentosulose (3,4-DDPs). 3,4-DDPs was isolated as the corresponding chinoxaline from reaction mixtures containing *N*-α-hippuryl arginine, lactose and *o*-phenylendiamine using semipreparative RP-HPLC. Identification was achieved using LC-MS as well as ^{1}H- and ^{13}C-NMR. The formation of 3,4-DDPs from lactose follows a new pathway of carbohydrate degradation in foods.

Keywords: arginine derivatization; lactose; 3,4-dideoxypentosulose; Maillard reaction

INTRODUCTION

During heating or storage of foods, proteins are modified by 1,2-dicarbonyls, formed as reactive degradation products from carbohydrates. Primary targets for 1,2-dicarbonyls are ε-amino group of lysine and the guanidino group of arginine. We recently isolated a new arginine derivative from a reaction mixture of lactose and *N*-α-hippuryl-L-arginine (Hip-Arg), namely 2-(2-benzoylamino-acetylamino)-5-[5-(3-hydroxypropyl)-4-oxo-imidazolon-2-yl]-L-ornithine (Hip-PIO). The free amino acid *N*-δ-[5-(3-hydroxypropyl)-4-oxo-imidazolon-2-yl]-L-ornithine (PIO) was prepared by heating *N*-α-BOC-arginine and lactose in the dry state, followed by mild hydrolysis with acetic acid [1]. PIO is exclusively formed from arginine and reducing carbohydrates with 1,4-glycosidic linkages, thus probably representing the major form of arginine derivatization in milk. The aim of the present study was to identify the possible precursor of PIO, a putative C5 carbonyl, which must be formed via a previously unknown degradation pathway from lactose.

EXPERIMENTAL

Chemicals. All chemicals were of highest purity available. PIO and Hip-PIO were synthesized according to [1]. Reference material of 3-deoxypentulose (3-DP) was synthesized according to [2]. 3-Deoxypentosulose (3-DPs) was synthesized according to [3].

Heating experiments. Lactose (17.1 mg) in 10 ml of 0.5M phosphate buffer, pH 7.0 was incubated in the absence and in the presence of 3.5 mg Hip-Arg for 2 h at 100°C. After cooling to room temperature, *o*-phenylendiamine was added and the derivatization was done in the darkness over night. The mixtures were membrane filtered and applied to analytical RP-HPLC according to Weigel *et al*. [4].

Liquid chromatography. Analytical and semipreparative RP-HPLC of 1,2-dicarbonyls as the corresponding chinoxalines was achieved after derivatization with *o*-phenylendiamine according to the methods described by Weigel *et al*. [4]. Hip-PIO was quantified via RP-HPLC with UV-detection according to [1].

NMR-spectroscopy. ^{1}H- and ^{13}C-NMR was performed in DMSO-d6 on a Bruker DRX500 instrument as described in [1] and [4].

Liquid chromatography/mass spectroscopy (LC-MS). LC-MS measurement was performed with a liquid chromatography system 1100 Series and a Mariner ESI-TOF mass spectrometer according to Mavric *et al*. [1] and Weigel *et al*. [4].

RESULTS AND DISCUSSION

The recently identified peptide-bound arginine derivative *N*-δ-[5-(3-hydroxypropyl)-4-oxo-imidazolon-2-yl]-L-ornithine (PIO) probably represents the main product of arginine derivatization in milk and milk products [1]. As can be seen in Figure 1, for the formation of PIO, the guanidino side chain of arginine reacted with a putative C5 1,2-dicarbonyl to give an imidazolinone ring structure. Up to now, such a C5-dicarbonyl with methylene moieties at C3 and C4 is not known in the literature as degradation product formed from lactose or maltose, although in general the formation of C5-carbonyls from disaccharides such as lactose or maltose well described. Troyano *et al*. [2] have reported on the formation of the small amounts of 3-deoxypentulose (3-DP) in heated and sterilized milk (Figure 1). Hollnagel and Kroh [3] were able to identify the quinoxaline of 3-deoxypentosulose (3-DPs) after heating starch in the presence of *o*-phenylendiamine (Figure 1). After isolation of 3-DP and 3-DPs according to methods described [2, 3] and incubation of the isolated dicarbonyls with *N*-α-hippuryl-arginine we were not able to detect Hip-PIO using RP-HPLC (data not shown). This indicates that the known carbonyls 3-DP and 3-DPs are not the precursors for PIO.

In order to detect 1,2-dicarbonyls formed from lactose during heating, a solution of the disaccharide was heated in the absence or in the presence of *N*-α-hippuryl-arginine and derivatized with *o*-phenylendiamine, respectively. Dicarbonyls trapped as the corresponding chinoxalines were analysed using RP-HPLC with UV-detection at 312 nm. As can be seen in Figure 2, heating of lactose resulted in one major compound (Peak X in Figure 2 A) formed together with 3-DPs. Significant smaller amounts of Peak X as well as of 3-DPs were formed when lactose was heated together with *N*-α-hippuryl-arginine (Figure 2 B), indicating reactions of the 1,2-dicarbonyls with the guanidino side chain of arginine. Similar results were obtained for maltose and lactulose, whereas after heating of monosaccharides like glucose or fructose, compound X was not detectable (data not shown). Using semi-preparative RP-HPLC, this unknown compound was isolated from a heated solution of lactose after derivatization with *o*-phenylendiamine. LC-MS showed a pseudomolecular ion [MH^+] at a *m*/*z* ratio 189, which enabled a calculation of an empirical formula of $C_{11}H_{12}N_2O$ with a theoretical mass of 188 Da. The structure could be unambiguously characterised by the means of one- and two-dimensional ^{1}H- and ^{13}C- NMR and by comparing of NMR data of our isolate with corresponding data of other chinoxaline published in the literature [4, 5]. Based on this, 2-(3′-hydroxypropyl)chinoxaline was identified as the main product formed during heating of lactose in absence of an amino compound. This chinoxaline results from the reaction of a previously unknown 1,2-dicarbonyl, namely 3,4-dideoxypentosulose (3,4-DDPs), with *o*-phenylendiamine. The formation of 3,4-DDPs as degradation product of

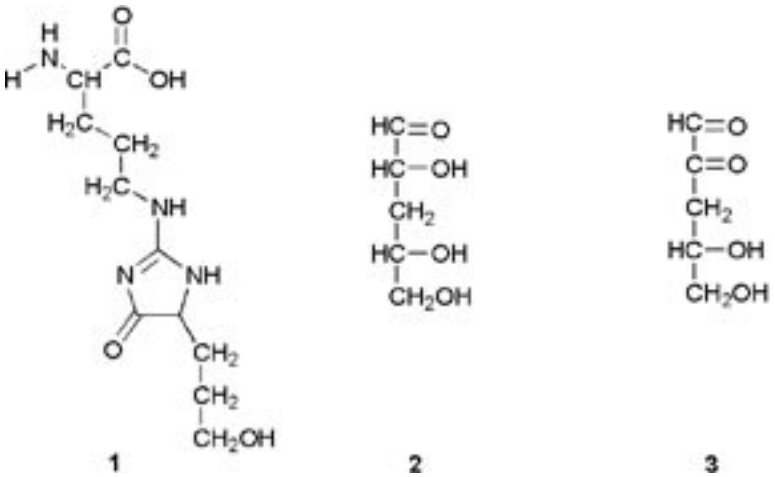

Figure 1. Compound **1**: *N*-δ-[5-(3-hydroxypropyl)-4-oxo-imidazolon-2-yl]-L-ornithine (PIO); compound **2**: 3-deoxypentulose (3-DP); compound **3**: 3-deoxypentosulose (3-DPs)

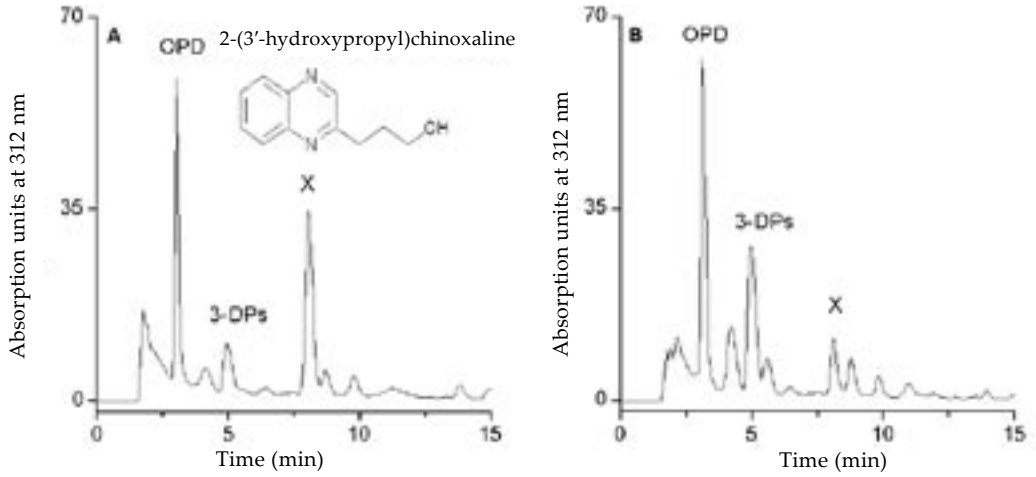

Figure 2. RP-HPLC with UV-detection of chinoxalines formed from 1,2-dicarbonyls with *o*-phenylendiamine. A: lactose (100°C, 2 h), B: mixture of lactose and *N*-α-hippuryl-arginine (100°C, 2 h)

lactose has not been described in the literature up to now. Independent synthesis of 3,4-DDPs and subsequent incubation with N-α-hippuryl-arginine unambiguously proved the formation of Hip-PIO, indicating that 3,4-DDPs represents the direct precursor for this new type of arginine derivatization [6]. We suggest that 3,4-DDPs is formed via 1,2-enolization of the reducing disaccharide resulting in transformation of the aldose to the corresponding ketose (i.e. lactose to lactulose), which then may form the 2,3-endiol, followed by elimination of the β-galactosyl moiety in the case of lactose from the C4 carbon of the sugar chain to a 4-deoxy-2,3-glucosone. This may explain the exclusive formation of 3,4-DDPs from 1,4-linked disaccharides, but further studies are necessary in order to explain how subsequent splitting of the monosaccharide backbone between C1 and C2 and final formation of the C5 dicarbonyl may proceed.

CONCLUSIONS

3,4-Dideoxypentosulose (3,4-DDPs) was identified as new degradation product formed exclusively from lactose and other disaccharides with 1,4-glycosidic linkages. 3,4-DDPs represents the direct precursor for a recently identified arginine derivative, namely *N*-δ-[5-(3-hydroxypropyl)-4-oxo-imidazolon-2-yl]-L-ornithine (PIO), which may represent the major form of arginine derivatization in milk. Studies concerning the mechanisms underlying the formation of 3,4-DDPs are underway in our laboratory.

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