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Enzymatically oxidized lactose and derivatives thereof as potential protein cross-linkers

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Abstract—The enzyme galactose oxidase [EC 1.1.3.9] was applied to convert lactose, lactylamine and lactobionic acid into their corresponding 6′-aldehyde compounds. The potential protein cross-linking ability of these oxidized lactose and derivatives thereof was investigated using *n*-butylamine as the model compound. First, oxidized lactose gave double Maillard reaction products that were stable under mild alkaline conditions. Second, reductive amination of lactose followed by enzymatic oxidation gave cross-links that were stable under both neutral and alkaline conditions. Third, stable cross-links were obtained through enzymatic oxidation and amidation of lactobionic acid.

Keywords: Protein cross-linking; Lactose; Lactose dialdehyde; Galactose oxidase; Maillard reaction

1. Introduction

Cross-linking of proteins is an important method to modify their chemical, physical and functional properties.[1,2] Currently, protein cross-linking is generally achieved by: (i) chemical cross-linking with glutaraldehyde or formaldehyde and (ii) enzymatic cross-linking using transglutaminase.[3,4] The first method is rapid and inexpensive, but not allowed for food applications, whereas the second food-grade method is rather expensive and requires long reaction times or unfolding of the native proteins.[5] Therefore, a novel rapid and inexpensive enzyme mediated process for the preparation of a broad spectrum of cross-linked proteins for both food and non-food applications is highly desirable.

Carbohydrates are linked to proteins upon heating via the Maillard reaction, a common reaction in food processing.[6] The first step in the Maillard reaction is a condensation reaction between the aldehyde group of reducing sugars and the amino group of lysine residues of the protein, leading to glycosylation of the protein.[7] Oxidized carbohydrates containing two or more aldehyde groups can react with lysine residues of different proteins, leading to cross-linking. In recent studies, the proof of principle of galactose dialdehyde[8] and a range of other sugars[9] as a protein cross-linker has been described. Our attention is now focused on the use of lactose dialdehyde (LACTA; β-D-galacto-hexodialdo-1,5-pyranosyl-(1→4)-D-glucose) and other oxidized lactose derivatives as protein cross-linkers. 6′-Aldehydes of lactose derivatives can be conveniently generated by an oxidation with molecular oxygen catalyzed by the enzyme galactose oxidase.[10]

Cross-linking by LACTA and other lactose derivatives is especially interesting for the valorization of industrial whey protein mixtures, as they already contain lactose that can act as cross-linking agent upon oxidative enzymatic treatment in a cascade reaction.[11]

We investigated the potential of LACTA and other lactose derivatives as protein cross-linkers, using *n*-butylamine (H_2N–Bu) as the model compound, as it has the same structure as the lysine side chain. The structural and functional analysis of the model

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compounds gave more insight in the reaction mechanism, reaction conditions and product properties, which is essential for further investigation of protein cross-linking by lactose derivatives.

2. Results and discussion

2.1. Enzymatic oxidation and double Maillard reaction: lactose-based cross-linking

LACTA (**2**) was obtained by the selective oxidation of lactose (**1**) at the C-6′ position using the galactose oxidase/catalase [EC 1.11.1.6] enzyme system.[10,12] The reaction with *n*-butylamine at both aldehyde groups was followed by spontaneous dehydration at C-4′ and C-5′ to give di-imine **3** (Scheme 1). The straightforward enzymatic preparation of LACTA (**2**) and formation of its double Maillard product (**3**) with *n*-butylamine indicates its potential for protein cross-linking.

The ^{13}C NMR analysis of the reaction of LACTA with *n*-butylamine gave data that are characteristic for linkage to amines (Table 1). These characteristic NMR signals can be used as references to monitor protein coupling, especially when ^{13}C-enriched compounds are used.[15] The ^{13}C NMR spectra of lactose (**1**), LACTA (**2**) and di-imine **3** were assigned based on literature data of similar structures.[13]

The signal of C-6′ shifts from 61.2 to 88.6 ppm when lactose (**1**) is oxidized to LACTA (**2**, the aldehyde at C-6′ is in the hydrate form in water). The double reaction of LACTA (**2**) with butylamine leads to a shift of C-1 (β) from 96.2 to 91.7 ppm. The signal of the imine at C-6′ of **3** was found at 158.2 ppm.[8,14] In addition, due to the dehydration at C-4′/C-5′ signals appear in the vinylic region of **3** (115 and 148 ppm).

As shown by in situ ^{13}C NMR, the double linkage of *n*-butylamine to LACTA (**2**) proceeds in 2 h at 60 °C when excess *n*-butylamine is used. After evaporation of excess *n*-butylamine, pure di-imine **3** is obtained. The use of an organic solvent (methanol or ethylene glycol) and only 2 mol of *n*-butylamine gave similar results.

Table 1. Key ^{13}C NMR data of compounds **1**, **2**, **3**, **6**, **7**, **10** and **13**[a]

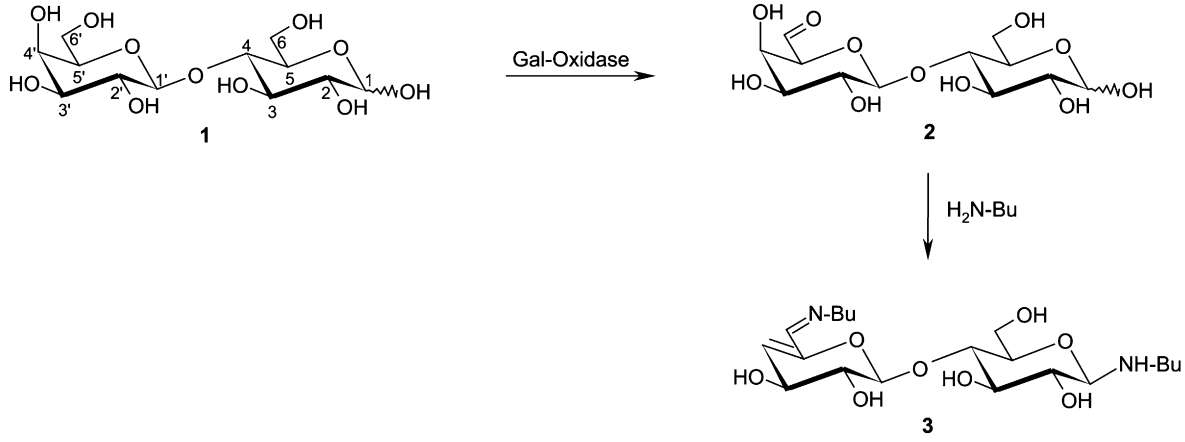
Compound	C-1	C-4′	C-5′	C-6′
1[b]	α: 92.0 (~40%) β: 95.9 (~60%)	68.7	75.5	61.2
2[b]	α: 92.3 (~40%) β: 96.2 (~60%)	68.5	77.5	88.6
3	α: not observed β: 91.7	114.8	148.1	158.2
6	50.5	114.8	147.8	159.7
7	50.1	106.5	146.0	56.6
10	175.0	114.1	147.7	158.8
13	175.1	114.2	147.6	159.0

[a] Data may vary slightly at different concentrations. Typical values were 298 K, 100 mg compound/mL CD_3OD.
[b] Dissolved in 20% D_2O/H_2O. Methanol was used as an internal reference ($\delta = 49.0$ ppm).

^{13}C NMR analysis also showed that the reaction at C-6′ is faster than at C-1 and that the dehydration at C-4′/C-5′ occurs readily after the formation of the imine at C-6′, similar to the dehydration of galactose dialdehyde with L-proline as described by Schoevaart and Kieboom.[15,16] The rate-limiting step in the formation of di-imine **3** is the amination reaction at C-1.

As mixtures of whey protein and lactose are obtained as aqueous solutions, the applicability of LACTA as cross-linker in water is of great interest. When 2.5 mol *n*-butylamine was added to an aqueous solution of LACTA, the formation of the imine at C-6′ was complete in 1 h at rt. The dehydration at C-4′/C-5′ and imine-formation at C-1 were found to proceed at a slower rate in water than in organic solvents, but were both completed after 24 h. These results show that the double Maillard reaction of LACTA can be performed in aqueous solutions, which enhances its practical applicability.

As glycosylamines are sensitive to hydrolysis in water,[17] the stability of di-imine **3** was determined.



Scheme 1. Formation of di-imine **3** by the double Maillard reaction of lactose dialdehyde with *n*-butylamine.