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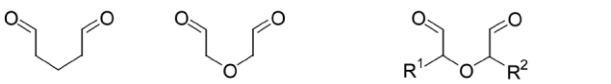
Glutaraldehyde Cross-link Analogues from Carbohydrates

The effectiveness of glutaraldehyde as protein cross-linker is unbeaten by any cross-linker known today. Surprisingly, no efforts have been made to improve the properties of glutaraldehyde via derivatisation. From the group of carbohydrates, periodate oxidised derivatives with cross-linking power have been reported. We demonstrate that these are based on the 1,5-dialdehyde structure, analogous with glutaraldehyde. Diglycolaldehyde and periodate oxidised carbohydrates containing this basic 1,5-dialdehyde moiety show excellent cross-linking properties. Although these cross-linkers are less reactive than glutaraldehyde, quantitative cross-linking is effected without undesired colouration and enzyme deactivation: effects often encountered with glutaraldehyde. These are the first results from an investigation in the use of carbohydrates for the alternative and inexpensive cross-linking of proteins.

Keywords: 2,2'-Oxy-bisacetaldehyde, Sodium metaperiodate; Protein cross-linker

1 Introduction

The production of starch from both corn and potatoes and the preparation of cheese from milk generates large amounts of proteins for which no high value application is possible yet. Improvement of the properties of these proteins is of major importance from an economical and environmental point of view. Cross-linking of proteins is an important method to increase both viscosity and gelation [1] in aqueous food and non-food applications. Additionally, unwanted sensitising side-effects of proteins are reduced in this way. Up to now the most investigated method is the linkage of lysine and glutamine residues by transglutaminases [2], though this method is quite costly. The aim of this investigation is the development of glutaraldehyde cross-link analogues for new and inexpensive processes for the production of viscosity increasing and gelating products from bulk proteins, by using the carbohydrates already present therein (Scheme 1).



Scheme 1. Glutaraldehyde analogues.

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Previously [3] we investigated the use of the enzyme galactose oxidase in the presence of galactose, where galactose dialdehyde as bifunctional cross-linking agent was formed. With the aid of ^{13}C labelling and NMR it was proven that spontaneous Amadori (and Maillard) reactions lead to protein cross-linking. Such procedures are still far from application in the large scale treatment of proteins due to the sluggish rate of this reaction.

A closer comparison of properties and molecular structures of proteins treated with the non-foodgrade glutaraldehyde gave insight in how this successful cross-linker worked [4]. In many cases the 5-carbon chain length of glutaraldehyde proved to be essential for the highly favourable six-membered piperidine ring formation through the two aldehyde groups and the amino group of protein lysine residues. Additionally, aldol reactions take place between individual glutaraldehyde molecules with formation of di-, tri-, and oligo-glutaraldehydes, leading to a perfect fit between two cross-linkable protein lysine residues. This inspired us to look for ways of transforming carbohydrates into 1,5-dialdehyde-containing structures.

Enzymatic oxidation of pentoses, which would yield C-5 dialdehydes, is not possible with galactose oxidase, because these pentoses are no substrates for this enzyme. Other enzymatic oxidations, to generate dialdehydes from carbohydrates, are not available. For that reason we chose sodium periodate, which is well-known for its dialdehyde producing power in carbohydrates [5] and which was also applied on an industrial scale.

Research Paper

Reports on the use of oxidised carbohydrates for cross-linking established practical procedures, but never disclosed the molecular basis for the cross-linking of proteins. Mostly formation of a strong imine linkage [6] was suspected to be the first step of the cross-linking by subsequent reductive amination [7]. In the case of dextran polyaldehyde, one-step cross-linking was first reported by *Kobayashi* et al. [8]. In a recent report for the first time, the spontaneous cross-linking capabilities of periodate oxidised lactose were recognised [9]. We will rationalise how oxidised carbohydrates can become effective cross-linkers, by a comparison with glutaraldehyde and show that it is even possible to cross-link a protein with its own glycosidic residues.

The choice of carbohydrates was determined by their availability and cost. Regarding the goal of cross-linking major protein waste streams, we opted for two disaccharides. Protein waste from cheese making (whey) contains large amounts of lactose which is therefore an obvious choice and also very cheap. Sucrose is another inexpensive and widely available sugar. In addition, periodate oxidised polysaccharides such as starch and cellulose are attractive as cross-linkers, because they are abundant and cheap. We have limited our choice to prove their cross-linking properties to the highly water soluble dextran (1,6-glucan) for practical reasons.

In order to study cross-linking experimentally we used a model system with enzymes rather than the above mentioned bulk proteins. Enzyme activities can be determined quickly in large numbers with standard spectrophotometric methods. Aggregation of these proteins with a precipitant followed by cross-linking produces so called cross-linked enzyme aggregates (CLEA®) [10]. The exact cross-link percentage and therewith the successfulness of the carbohydrate-based cross-linker can be calculated from the amount of free enzyme left in solution.

2 Experimental

To determine the reactivity of the cross-linkers, tests were performed at both room temperature and 4°C at concentrations varying from 10 mM to 1 M and incubation times of 1 h up to 24 h. Typical precipitants were ammonium sulphate, polyethylene glycol (PEG) and 1,2-dimethoxyethane (DME). Optimal conditions were compared with optimal conditions known for glutaraldehyde.

*Diglycolaldehyde (**2**) via ozonolysis*

2,5-dihydrofuran (7 g) was dissolved in 100 mL water (1 M) and subsequently chilled in an ice bath. Ozone was bubbled through (4 h) until ^{13}C NMR showed no more starting material. After flushing with nitrogen, the solution was stored at 4°C, ready for use.

*Periodate oxidised carbohydrates and alcohols (**2, 4, 5, 6**)*

To a 20 mL solution of 500 mM diglycerol, lactose, sucrose or *cis,trans*-1,2-hexanediol 2.13 g of $NaIO_4$ per equivalent was added. After stirring for 1 h, 80 mL acetone was added and the mixture cooled on ice. Filtration and evaporation of the acetone yielded a 20 mL solution of the 1,5-dialdehyde cross-linkers. Solutions could be further concentrated up to 1 M by evaporation of water *in vacuo* at room temperature.

*Dextran cross-linker (**7**)*

1.65 g of dextran (*M* 100–200 kD) was dissolved in 50 mL water, then 3.85 g $NaIO_4$ (360 mM) was added. After stirring for 1.5 h at room temperature the dextran polyaldehyde was dialysed against 1 L of demineralised water giving an end volume of 56 mL (~ 29 mg/mL).

Cross-linking analysis

To 10 μL *Candida antartica* lipase B (CaLB, Novozyme, Bagsvaerd, Denmark), 5 μL water and 10 μL cross-linker (1 M) were added. Then 75 μL DME was used to precipitate the protein giving a final concentration of 100 mM of cross-linker.

Assay: 900 μL water was added to 100 μL of precipitated enzyme mixture to dissolve remaining free enzyme. Of this mixture of cross-linked and free enzyme 25 μL was added to a cuvet giving the sum of activities. Centrifugation for 5 min removed all cross-linked enzyme. An assay of 25 μL of the supernatant yields the uncross-linked enzyme activity. Substracted from the mixed activity it provides the percentage of cross-linking.

Self-cross-linking of proteins

To an enzyme solution an equal volume of 100 mM sodium periodate was added. After 1 h the pH was adjusted to pH 7. This solution was then precipitated with a tenfold excess of DME and incubated. The determination of cross-linking grade is as described above.

Lipase assay

Candida antarctica lipase A [EC 3.1.1.3] activity was assayed with *p*-nitrophenyl propionate as substrate (7.8 mg in 1 mL ethanol; 10 μL per mL assay buffer). Assay buffer (100 mM potassium phosphate, pH 7.4) contained 0.4 mM 4-nitrophenyl propionate at 25°C. The reaction was monitored at 400 nm. Blank reaction rate: ΔA 0.00318 min^{-1}. Additionally (slow) hydrolysis of

triacetine (2%, v/v) in 50 mM Tris buffer pH 7.4 and 40°C was performed monitored by 0.1 M NaOH titration.

Phytase assay

Hydrolysis of *para*-nitrophenyl phosphate (0.4 mM) by *Aspergillus niger* phytase [EC 3.1.3.8] in 0.1 M acetate pH 4.5 monitored at 400 nm gave the activities of phytase. The blank reaction at pH 4.5 is negligible.

Galactose oxidase assay

A solution containing galactose (200 mM, prepared 1 day in advance to allow for mutarotation), *o*-tolidine (1.75 mM), peroxidase (60 units/mL) and 100 mM potassium phosphate at pH 7.3 was used to detect *Dactylium dendroides* galactose oxidase [EC 1.1.3.9] activity. The oxidation of *o*-tolidine was monitored at 425 nm.

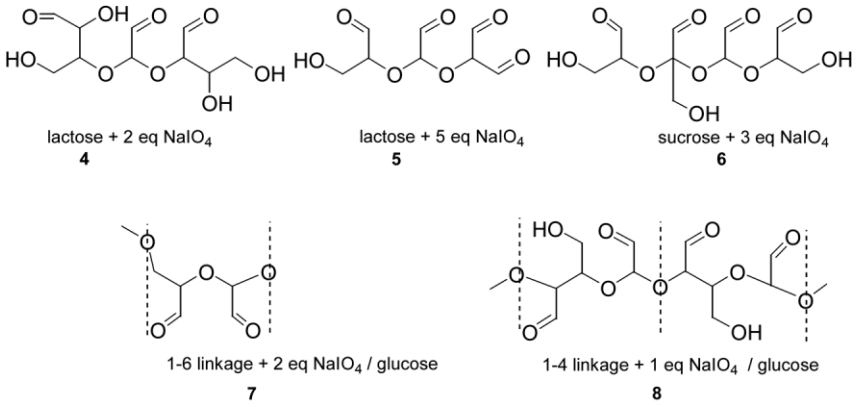
3 Results and Discussion

3.1 Glutaraldehyde derivatives

Treatment of monosaccharides with an excess of periodate leads to formaldehyde and formic acid. Polysaccharides, disaccharides and alkylglucosides, in contrast, possess a glycosidic linkage, which is not broken upon periodate treatment. Periodate oxidation produces one or more 2,2'-oxy-bisacetaldehyde or diglycolaldehyde units **3** (which have the required 1,5-dialdehyde resemblance with glutaraldehyde (**1**). These chiral C5-dialdehyde structures can form the basis for the cross-linking capabilities of periodate treated carbohydrates. The most simple carbohydrate-derived glutaraldehyde-like derivative is diglycolaldehyde itself (**2**) which is easily synthesised by ozonolysis of 2,5-dihydrofuran or periodate treatment of diglycerol. Although compound **2** is known for almost 75 years [11] and applied in synthesis, its cross-linking powers were never discovered. When comparing NMR spectra of aqueous glutaraldehyde and diglycolaldehyde solutions, the same complex acetal formation is observed.

With two equivalents of periodate, lactose is oxidised to several dialdehydes of which compound **4** has the desired 1,5-dialdehyde unit (Scheme 2). Compound **4** represents the average oxidation state, because a mixture of various grades of oxidation will be formed, up to complete oxidation. With 5 equivalents of periodate, lactose is converted to the tetraaldehyde **5** containing three overlapping 1,5-dialdehyde moieties. It is yet unclear to what extent more than one dialdehyde unit contributes to the cross-link. Probably, multiple cross-linker molecules are necessary in the cross-linking cascade, as is known for glutaraldehyde. Sucrose, in comparison with lactose, requires only three equivalents of periodate for complete transformation into the tetraaldehyde **6** containing three overlapping or two independent 1,5-dialdehyde units. Treatment of dextran (1→6-linked α-D-glucose moieties) with two equivalents of periodate yields **7** containing one 1,5-dialdehyde moiety per glucose unit. For polysaccharides such as starch (amylose part) and cellulose with 1→4-linked β-D-glucoses, only one equivalent of periodate is necessary per glucose whereby each 1,5-dialdehyde unit originates from two adjacent glucose units (**8**).

Interestingly, sugar residues on proteins can also act as cross-linker upon treatment with periodate. Oxidised glycosidic residues are well known [12] and in a separate investigation their reduction in the presence of diamines in order to effect cross-linking is described. In accordance with the present results, it was noticed that spontaneous cross-linking of the proteins took place upon storage after periodate treatment through "self-cross-linking" of the formed 1,5-dialdehydes. In some cases, this happened in a quantitative yield, possibly depending on the degree of glycosylation of the enzyme.



Scheme 2. Formation of 1,5-dialdehyde moieties by periodate oxidation of lactose (**4** and **5**), sucrose (**6**), (1→6)-glucans (**7**), and (1→4)-glucans (**8**). The areas between the dotted lines in **7** and **8** correspond with the former glucan glucose units, i.e. each glucose unit of (1→6)-glucans leads to a 1,5-dialdehyde moiety (**7**) whereas two adjacent glucose units of (1→4)-glucans are required for one such 1,5-dialdehyde moiety (**8**).

3.2 Cross-linking with glutaraldehyde analogues

As a model protein for the cross-linking measurements, *Candida antarctica* lipase A (CaLB) was chosen. This enzyme has a high industrial interest and many applications in organic synthesis. The formaldehyde, that is released in some of the polyalcohol oxidations, as is shown by ^{13}C NMR, did not lead to cross-linking in blank reactions. For all the glutaraldehyde analogues **1**, **2**, **5**, **6** and **7** up to quantitative cross-linking was observed (Fig. 1). Only typical conditions (100 mM, room temperature) are displayed, although good cross-linking was found at concentrations down to 10 mM and temperatures of 4°C. The carbohydrate-based 1,5-dialdehyde cross-linkers performed well, with diglycolaldehyde **2** and dextran derived poly-1,5-dialdehyde **7** performing best. Maximum cross-linking was reached within 1 h with compound **2**, making it the fastest O-containing glutaraldehyde derivative. Its cross-linking rate is, at the same concentration, about ten times slower than that of glutaraldehyde. Compound **7** reached complete cross-linking of enzyme in a few hours, which is in accordance with previous findings [8]. In contrast to glutaraldehyde, **2** did not reach 100% cross-linking with CaLB. However, with diglycolaldehyde **2** and the dextran-derived polyaldehyde **7** hyperactivation was noticed: although not all of the activity was captured upon cross-linking, up to 200% absolute activity was detected. This phenomenon is well known for cross-linked enzyme aggregates (CLEAs) [13].

Higher concentrations of cross-linker lead to faster cross-linking. However, high concentrations of glutaraldehyde are normally detrimental to enzyme activity. This effect was not noticed with carbohydrate-derived cross-linkers: up to 1 molar concentrations gave normal enzyme activities. Furthermore, glutaraldehyde produces

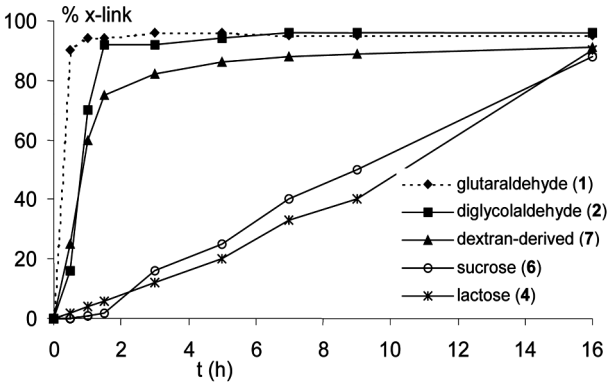


Fig. 1. Cross-linking of the enzyme CaLB in DME with glutaraldehyde versus carbohydrate based cross-linkers (100 mM at room temperature).

dark coloured products, especially at high concentrations. This is not observed for the carbohydrate analogues demonstrating that in the cross-linking cascade less conjugated bonds are formed. Glutaraldehyde normally forms pyridine-like structures. When exchanging an oxygen atom for a methylene group, double bond formation – and thereby colourisation – is partially prevented. Some known pathways in the glutaraldehyde cross-linking cascade are thus excluded for the carbohydrate analogues. Between pH 4.5 and pH 10 good cross-linking results are obtained. Obviously, cross-linking based solely on Schiff base formation cannot explain these findings. Glutaraldehyde shows the same pH pattern, demonstrating the similarities in structure and reaction pathways. An experiment with hexanedial showed that not seven- but six-membered ring formation is essential for cross-link reactions, because in the case of hexanedial only very slow, partial cross-linking was observed. The stability of the carbohydrate cross-link was good. For the dextran-derived analogue some release of free enzyme was observed, however. There have been reports of the slow release of dextran polyaldehyde cross-linked proteins [6]. Most likely, the glycosidic link in the dextran polyaldehyde is susceptible to hydrolysis. In a hydrolysis test, an acylase CLEA was completely dissolved in two days at pH 8.5. At neutral pH the bond is stable. With diglycolaldehyde (**1**) and the disaccharide based analogues (**5** and **6**) hydrolysis was not observed.

In analogy with the results found for several carbohydrates, we tested if periodate treated glycosylated proteins could be cross-linked with their own oxidised glycosidic residues. Three enzymes were investigated: phytase, galactose oxidase (GalOx) and *Candida antarctica* lipase (CaLB). After oxidation with periodate the enzymes were precipitated and incubated at room temperature. Within hours (Tab. 1) substantial cross-linking was achieved for phytase and galactose oxidase. Both enzymes are known to be significantly glycosylated. The lipase, with much fewer glycosidic residues, required longer cross-linking times as expected.

Tab. 1. Self cross-linking of periodate treated enzymes.

Enzyme	Time [h]	Temperature	Absolute activity	% Cross-link
Phytase[a]	6	RT	33	98
GalOx[a]	3	RT	65	90
CaLB[a]	16	RT	77	91

a) Glycosidic residues on protein surface.

4 Conclusions

Periodate treated lactose, sucrose and dextran gave glutaraldehyde analogues with interesting and useful protein cross-linking properties. The most straightforward glutaraldehyde analogue diglycolaldehyde (**2**), was found to be the most reactive one, being about ten times less reactive than glutaraldehyde. This moderation of the reactivity is constructive, however, because the glutaraldehyde's high reactivity is often detrimental to enzyme activity and protein integrity. Typical glutaraldehyde colouration is absent in the analogues, reflecting the deviation from the known cross-linking cascade. Carbohydrate-based cross-linkers are expected – like glutaraldehyde – to cross-link non-specifically, which makes their applicability broad. In addition it was proven that glycosidic residues on proteins could be used to cross-link the proteins themselves, with only sodium periodate as reactant. Future structural investigation with ^{13}C labelled sugars will provide further insight into the interaction between cross-linker and protein. Utilisation of other carbohydrates will give additional insight and possibly even more effective cross-linkers. The ease with which carbohydrate-based glutaraldehyde analogues are prepared, will make them an attractive source for protein cross-linking.

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