A novel chemoenzymatic glycosylation strategy: application to lysozyme modification

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Abstract Hen egg lysozyme has been non-specifically glycosylated using a novel two-step strategy. First, a number of sucrose molecules have been chemically bound to the protein surface lysines, then the glycosidic chains have been enzymically lengthened, using a glycosyltransferase. For this task, a fructosyltransferase and a levansucrase have been tested, the latter appearing as the most effective one. In all cases, reactions have been optimised and several degrees of modification have been obtained. Finally, the effects of the modifications on lysozyme hydrophobicity, hydrolytic activity, hydrolysis substrate affinity and thermostability have been assessed.

Key words: Glycosylation; Lysozyme; Glycosyltransferase; Hydrophobicity; Stability

1. Introduction

Nowadays, the major role of protein surface properties (i.e. hydrophobicity and charge) on biocatalysts behaviour seems to be clear. Many techniques have been proposed to modify enzyme surface or microenvironment and, therefore, enzyme functional properties. The most frequent is the covalent binding of appropriate molecules to the enzyme's surface aminoacids. Among the many compounds which can be used as modifiers, glycosides are outstanding as regards both the number of reports and the improvements in enzyme functionality (i.e. thermostability) achieved [1–3].

In the present work, we have undertaken the derivatization of a model enzyme, lysozyme, with a hydrophilic glycoside, whose length has been progressively increased by a combination of chemical and enzymic techniques. First, sucrose molecules have been chemically bound to the enzyme and then glycosyltransferase enzymes have been used to progressively lengthen the glycosidic chain. Two glycosyltransferases were chosen according to availability and low cost criteria and their ability to catalyze the lengthening reaction was studied. This novel modification approach is especially interesting, since it allows the production of enzymes linked to variable length glycosides, using mild reaction conditions. Furthermore, it opens up an interesting field for future research on glycosyltransferases, discovering new applications and illustrating that this kind of enzyme may be active on non-natural donors and/or acceptors.

The modification reactions have been thoroughly studied and optimized, in order to get a range of glycosylation degrees. Then, hydrophobicity, hydrolytic activity and thermostability of the produced lysozyme species have been investigated, in an attempt to enlighten the effects of enzyme surface glycosylation on these characteristics.

2. Materials and methods

2.1. Materials

Hen egg lysozyme (EC 3.2.1.17), cyanogen bromide and *Micrococcus lysodeikticus* were obtained from Sigma. Radioactive-labelled sucrose was from NEN Research Products. Fructosyltransferase from *Aspergillus niger* and levansucrase from *Bacillus subtilis* (industrial partially purified enzymes) were kindly provided by Eridania Beghin-Say. All other reagents were analytical grade.

2.2. Assay of lysozyme hydrolytic activity

Controls and samples were previously diluted in osmosed water to 40 μg of protein/ml. Then, 80 μl of diluted controls and samples were added to 2 ml of a *M. lysodeikticus* suspension prepared in a 0.1 M potassium phosphate buffer (pH 6.24). The decrease in the optical density at 450 nm and 30°C was recorded for 2 min in a temperature-controlled diode array spectrophotometer Hewlett-Packard 8452A. One lysozyme activity unit is defined as the amount of enzyme producing an optical density decrease of 0.001/min, at 450 nm, pH 6.24 and 30°C.

2.3. Glycosylation procedure

2.3.1. First step: chemical binding of sucrose. Sucrose was activated by treatment with cyanogen bromide [4], after which the suitable amounts of lysozyme, in order to achieve the desired sucrose/lysozyme molar ratios in the reaction mixture, were added (pH 9.0). The coupling reaction was allowed to occur for 16 h at 4°C and stopped by addition of glycine. The modified enzyme was purified and freeze-dried. Control solutions were prepared without addition of CNBr.

2.3.2. Second step: enzymic lengthening of glycosidic chain. Fructosyltransferase from A. niger: A 0.04 mM solution of the previously sucrose-bound lysozyme (6 mol of sucrose/mol of lysozyme) was prepared in 66 mM sodium phosphate buffer (pH 5.5). The suitable amounts of sucrose, in order to achieve the desired final sucrose concentrations, and 3.2 μg/ml (18 U/ml) of fructosyltransferase were added. The reaction mixture was kept at 40°C for 16 h and the enzyme was eventually purified and freeze-dried. Control solutions were prepared without addition of fructosyltransferase.

Some attempts on optimizing the lengthening reaction were carried out using higher amounts of fructosyltransferase (36 U/ml) and shorter reaction times (1 h) at a higher temperature (55°C).

Levansucrase from B. subtilis: A 0.14 mM solution of sucrose-bound lysozyme (6 mol of sucrose/mol of lysozyme) was prepared in 50 mM potassium phosphate buffer (pH 6.0). Suitable amounts of this solution were treated with 0.13 mg of levansucrase/ml (5 U/ml) at 37°C in the presence of several sucrose concentrations and during different reaction times. Modified enzyme was purified and freeze-dried. Control solutions were prepared without addition of levansucrase.

2.3.3. Purification of modified lysozyme. Lysozyme in control solutions and samples was separated from residual reagents by cation exchange chromatography, using a Mono-S column connected to an FPLC (Fast Protein Liquid Chromatography) system, both from Pharmacia LKB. The column was eluted at 1 ml/min with a gradient of 30 mM sodium acetate buffer (pH 5.0) as eluent A and the same buffer containing 1 M NaCl as eluent B. Protein fractions were collected, dialysed and freeze-dried.

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2.3.4. Determination of glycosylation degrees. Sugar fixed onto the lysozyme surface was detected by addition of a certain amount of suitable radioactive sucrose to the reaction mixture and measurement of the radioactivity left onto the enzyme after purification, using a Liquid Scintillation System Beckman LS 1801. Uniformly ^{14}C-labelled sucrose was used for the chemical binding step and sucrose ^{14}C-labelled on fructose for the enzymic lengthening step.

2.3.5. Properties of modified enzymes. Hydrophobic/hydrophilic balance of the enzymes: Native and modified lysozyme hydrophobicity was evaluated from the reversed phase chromatography profiles obtained in an HPLC Hewlett-Packard 1090 equipped with a Nucleosil C18 5-μm column (220 × 2.1, SFCC). The column was eluted at 1 ml/min with a gradient of H_2O/0.1% TFA as eluent A and acetonitrile/0.1% TFA as eluent B. After a 5-min step of 100% A, a linear gradient from 0 to 100% B in 15 min was applied. Eluted proteins were evaluated from the on-line measurement of the optical density at 280 nm.

Thermal stability: Samples and controls were diluted in osmosed water to 0.1 g/l. Tight-closed tubes containing 0.5-ml aliquots were placed in a water bath at 80°C, taken out at regular time intervals and cooled down in ice. The residual activities were measured and referred to the initial values.

3. Results and discussion

3.1. Modification of lysozyme: procedure set-up and optimization

In a first step, sucrose molecules were chemically fixed onto the lysozyme surface. This disaccharide is the natural acceptor in the transfer reaction catalysed by the glycosyltransferases involved in the next step of the proposed glycosylation strategy. Sucrose was activated by addition of cyanogen bromide, producing imidocarbonates which reacted at alkaline pH with the enzyme amino groups (ε-amino from lysine and terminal α-amino) [5]. Glycoside activation by cyanogen bromide has often been applied in enzyme immobilization [5] and enzyme stabilization attempts by polysaccharide binding [6–13].

Several molar sucrose/enzyme ratios were used in the reaction mixture leading to a range of modification degrees being obtained (Fig. 1). Saturation of all lysozyme lysines was reached when 500 mol of sucrose/mol of enzyme were added to the reaction mixture, though relatively high modification

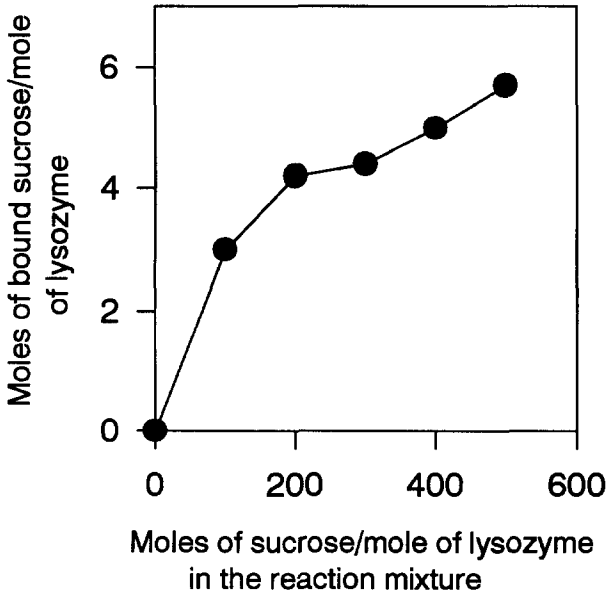

Fig. 1. Degrees of chemical binding of sucrose to lysozyme.

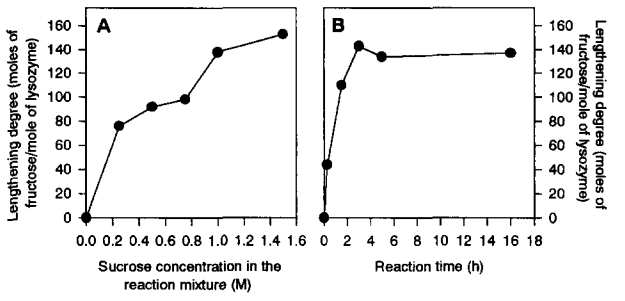

Fig. 2. Lengthening of the lysozyme-bound glycosidic chains by levansucrase from *B. subtilis*. (A) Lengthening degree as a function of sucrose concentration in the reaction mixture (37°C, pH 6.0, 16 h reaction time). (B) Lengthening degree as a function of reaction time (37°C, pH 6.0, 1 M sucrose).

degrees were also obtained in the presence of lower sucrose concentrations. The relatively low number of lysozyme lysines (six), their spacement and their superficial location [14] in the folded protein structure explain the good sucrose-binding results obtained.

Although in the present work this method has been preferentially used with sucrose, it could work on other glycosides containing free hydroxyl groups. Maltose has also been successfully tested as a modifier: up to 3 mol of maltose/mol of lysozyme were grafted, at maltose/lysozyme molar ratios in the reaction mixture from 50 to 250.

In a second step, enzymic lengthening of the previously lysozyme-bound sucrose molecules was carried out. A number of fructose molecules were transferred from a donor, the sucrose, to the chemically grafted glycosidic chains. The first enzyme utilized to catalyze this reaction was the fructosyltransferase from *A. niger* (EC 2.4.1.9), which transfers fructose residues from sucrose to other sucrose molecules or to its analogues. This enzyme cleaves the β-1–2 bond between fructose and glucose and transfers the former to the acceptors, synthesizing a new β-1–2 bond. Sucrose is the fructosyltransferase natural acceptor, but this biocatalyst has also been reported to work on other sugars and primary alcohols [15].

Fructosyltransferase was able to catalyze the transfer of fructose onto the chemically glycosylated lysozyme; transfer rates at 40°C (Table 1) seemed to increase with the donor (sucrose) concentration in the reaction mixture, up to levels (2 M) at which no lengthening was observed, possibly due to fructosyltransferase inhibition by the substrate. Nevertheless, quite low chain extension (<5 mol of fructose/mol of lysozyme) were obtained in all cases with this enzyme. Neither the use of a higher transferase concentration (36 U/ml) nor the combina-

Table 1
Enzymic lengthening of the lysozyme-bound glycosidic chains by fructosyltransferase from *A. niger*

Sucrose concentration (M)	Fructosyltransferase concentration (u/ml)	Reaction time (h)	Temperature (°C)	Lengthening degree (mol of fructose/mol of lysozyme)
0.25	18	16	40	2
0.75	18	16	40	5
2.0	18	16	40	0
0.75	36	16	40	5
0.25	18	1	55	2

Table 2
Hydrophilic/hydrophobic balance of glycosylated lysozyme: elution times from a C18 HPLC column (eluents: water-0.1% TFA/acetonitrile-0.1% TFA)

Molecules bound per molecule of lysozyme	Elution time (min)
0	9.86
3 sucrose	9.71
4 sucrose	9.68
5 sucrose	9.67
6 sucrose	9.74
6 sucrose + 110 fructose	9.20
6 sucrose + 140 fructose	9.05

tion of high temperatures (55°C) and short reaction times (1 h) improved the results. The low extent of chain extension obtained may be explained by the occurrence of steric hindrances: the high molecular weight of the modified lysozyme (~16,400 Da) compared with that of the natural acceptor (342 Da) could prevent the proper interaction between the fructosyltransferase and the substrate.

Another glycosyltransferase, the levansucrase from *B. subtilis*, was tested to lengthen the glycosidic chain fixed onto the lysozyme molecules. This enzyme cleaves the β-1–2 bond between glucose (G) and fructose (F) in sucrose and synthesizes a new β-2–6 bond between the fructose residue and acceptors containing a terminal fructose, forming levan-like polymeric structures ($G(F)_n$).

The efficiency of levansucrase was assessed in the presence of a number of sucrose (fructose donor) concentrations in the reaction mixture (Fig. 2A). Lengthening degrees were quite high (up to 150 mol of fructose/mol of lysozyme) and increased with the initial sucrose concentration, tending to a stationary level from 1 M sucrose. The development of the reaction with time (Fig. 2B) was studied at a sucrose concentration of 1 M; this concentration was high enough to allow good reaction rates, but not so high as to increase the viscosity of the reaction mixture to an extent at which operating and purification difficulties could arise. A maximum polymerization degree was achieved after 2 h reaction time, after which it remained quantitatively steady, although the possibility of internal rearrangements of the glycosidic chains should not be neglected.

Levansucrase appeared then as a much more adequate tool for the intended reaction than the previously tested fructosyltransferase, although both enzymes were less efficient in the present case than in their natural reaction conditions. The smaller size of levansucrase (~50,000 Da) compared with fructosyltransferase (180–210,000 Da) would minimize the aforementioned steric hindrances related to the bulky substrate considered. Besides, fructosyltransferase does not build up glycosidic chains containing more than four fructose units [16], even when it operates on its natural acceptor, while levansucrase naturally produces a polymeric compound [17].

3.2. Properties of modified lysozyme

3.2.1. Surface hydrophobicity. The surface characteristics of enzymes (i.e. hydrophilic/hydrophobic balance) play a very important role in determining biocatalyst physico–chemical and catalytic properties, such as thermostability, resistance to proteases, solubility or activity.

Changes in enzyme surface hydrophilic/hydrophobic balance after binding of glycosides have been assessed from the chromatographic profiles obtained after passage of the produced enzymes through a reversed phase C18 HPLC column. Variations in the elution times have been observed in all cases (Table 2). Binding of hydrophilic molecules (glycosides) brought about a faster elution, which meant an increase in the global hydrophilic character of the protein. The more important the modification, the stronger were the effects on enzyme surface hydrophobic/hydrophilic balance.

3.2.2. Hydrolytic activity and kinetic parameters. The hydrolysis of the copolymer of *N*-acetyl-D-glucosamine (NAG) and *N*-acetylmuramic acid (NAM), major cell wall component, was utilized to assess the catalytic activity of glycosylated lysozyme (Table 3). In all cases, a decrease in hydrolytic activity was detected, very noticeable (up to 90%) for the lysozyme linked to the longest glycosidic chains.

The loss of enzymic activity after chemical modification is a phenomenon frequently encountered in the literature dealing with this topic [1,2,4,7,8,10,12,18,19]. It can be justified by enzyme conformational changes or steric hindrances to the enzyme-substrate contact provoked by modifiers, especially when large molecules are involved. Since lysozyme is a low molecular weight protein, it is not surprising that chemical modification of its surface provokes significant modification of enzyme activity.

The enzyme–substrate affinity of glycosylated lysozyme in the hydrolysis reaction has been evaluated from the values of the kinetic parameter K_M, obtained from Lineweaver–Burk plots. A very slight tendency to an increase in affinity can be detected for the enzymes chemically bound to a certain amount of sucrose molecules. This tendency, not very pronounced, could be attributed to the appearance of non-covalent interactions (hydrogen bonds or van der Waals interactions) between the hydrophilic compound linked to lysozyme surface and the substrate, since both sucrose and NAG-NAM polymer include electronegative atoms. This kind of interaction could contribute to stabilize reaction intermediates.

Table 3
Hydrolytic activity, Michaelis constants and thermal stability of modified lysozyme

Molecules bound per molecule of lysozyme	Activity (units/mg protein)	Residual activity (%)	K_M (mg/ml)	Half-life time (min)	Variation in half-life time (%)
0	42 000	100	0.21	7.4	–
3 sucrose	33 800	80.4	0.19	7.0	− 5.4
4 sucrose	28 000	66.7	0.17	6.0	−18.9
6 sucrose	21 200	50.5	0.20	5.4	−27.0
6 sucrose + 110 fructose	3 900	9	0.45	3.0	−59.5
6 sucrose + 140 fructose	5 200	12	0.40	2.3	−68.9

When longer glycosidic chains were bound to the enzyme, the effect of steric hindrance prevailed over the hypothetical stabilizing non-covalent modifier–substrate interactions, bringing about a decrease in enzyme–substrate affinity. This phenomenon has already been reported after chemical modification of enzymes with polysaccharides [3,11].

3.2.3. Thermal stability in aqueous solution. The results (Table 3) showed a decrease in thermal stability when increasing the global hydrophilic character of the enzyme by glycosylation of its surface. A generally accepted enzyme thermostability mechanism in aqueous solution proposes that the contact between the enzyme hydrophobic surface clusters and the surrounding water provokes the biocatalyst deactivation at high temperatures [20]. Protection of these hydrophobic clusters from water contact would contribute to increase enzyme thermostability, and a number of reports deal with the development of chemical modification techniques using this strategy [19,21–23]. In the present work, neither the kind nor the location of the attached compounds seemed appropriate to avoid the destabilizing water–protein surface interactions; moreover, the high water-binding ability of the grafted glycosides could contribute to increase them, explaining the tendency observed in the half-life times of glycosylated lysozyme.

The presented work opens a wide scope for future research on chemoenzymatic protein modification strategies and new applications of glycosyltransferases. It would be interesting to apply the ideas developed here to other biocatalysts, for which more positive effects on catalytic properties might be expected after modification. The small size of lysozyme favours the occurrence of steric hindrances and conformational deformations and these destabilizing effects would be less important in larger proteins. Furthermore, the ability of lysozyme to recognize certain carbohydrates may lead to non-specific interactions between the modifiers and the enzyme substrate, and complicate the interpretation of the data.

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