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Solid-Phase Reducing Agents as Alternative for Reducing Disulfide Bonds in Proteins

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Abstract

Disulfide reduction of *Kluyveromyces lactis* and *Aspergillus oryzae* β-galactosidases and β-lactoglobulin was assessed. Reduction was performed using one of two thiol-containing agents: dithiothreitol (DTT) or thiopropyl-agarose with a high degree of substitution (1000 μmol of SH groups/g of dried gel). Both reductants allowed an increase of three- (for *K. lactis* β-galactosidase) and fourfold (for *A. oryzae* β-galactosidase) in the initial content of SH groups in the lactases. Nearly sevenfold fewer micromoles of SH groups per milligram of protein were needed to perform the reduction of *K. lactis* β-galactosidase with thiopropyl-agarose than for the same reduction with DTT. However, for *A. oryzae* β-galactosidase, nearly twice as many micromoles of SH groups per milligram of protein were needed with thiopropyl-agarose than with DTT. Disulfide bonds in β-lactoglobulin were not accessible to thiopropyl-agarose, since this reduction was only possible in the presence of 6 *M* urea. These results proved that highly substituted thiopropyl-agarose is as good a reducing agent as DTT, for the reduction of disulfide bonds in proteins. Moreover, excess reducing agent was very simply separated from the reduced protein by filtration, making it easier to control the reaction and providing reduced protein solutions free of reductant. All these advantages substantially cut down the time required and therefore the cost of the overall process.

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Index Entries: Protein reduction; reducing agents; immobilized reductants; disulfide reductants; sulfhydryl residues; disulfide reduction; cystine bonds.

Introduction

Thiol groups are normally the most reactive groups found in proteins and can participate in a large number of reactions. Their reactivity is owing to the high nucleophilicity of the corresponding thiolate ions, which exist at reasonable concentrations at neutral to weakly alkaline pH values *(1)*. One of the most convenient ways of generating sulfhydryl groups is by reduction of native disulfides. Many proteins contain cystine disulfides that are not critical to structure or activity. In some cases, mild reducing conditions can free one or more SH groups for conjugation, immobilization onto thiol-reactive supports, or modification purposes *(2)*.

Disulfide reduction by means of thiol-disulfide exchange reactions can be done using thiol-containing compounds. The reaction is an easily reversible S-alkylation, performed in two stages. In the first stage, one molecule of the reducing agent undergoes disulfide exchange, cleaving the native disulfide and forming a new mixed disulfide. In the second stage, a second molecule of the thiol cleaves the mixed disulfide, releasing a free sulfhydryl and forming a molecule of oxidized reducing agent *(2,3)*. There are many reductants for cleaving disulfide bonds, but dithiothreitol (DTT) and dithioerythreitol (DTE) are usually selected. The mixed disulfides formed from the reaction of these compounds with an aliphatic disulfide are unstable and undergo an internal thiol-disulfide exchange leading to a stable six-membered ring, which drives the reaction toward completion *(4)*. Therefore, complete reduction is possible with much lower concentrations of DTT or DTE than when using monothiol systems. The reduction of β-galactosidase (also called lactase) from *Kluyveromyces lactis* has been extensively studied since this reduction dramatically improves the immobilization yield onto thiol-reactive adsorbents *(5)*. Although DTT produces a threefold increase in the initial content of SH groups of the enzyme, after the reduction step, excess reducing agent must be removed by gel filtration to avoid competition with the reduced enzyme during the immobilization process *(5)*. This gel filtration not only dilutes the reduced enzyme but also adds another step, making the scaling up of the immobilization process less feasible.

For these reasons, we hereby propose the use of a solid-phase reducing agent, thiopropyl-agarose (mercapto-hydroxypropyl ether-agarose) as an alternative reagent for the reduction of protein disulfides. The reduction process involves the formation of an insoluble mixed disulfide when a thiopropyl group from the support cleaves the disulfide from the protein. If the solid-phase reducing agent has an excess of thiopropyl groups, the SH group, a thiopropyl group close to the mixed disulfide, can launch a nucleophilic attack on the thiopropyl group involved in the mixed disulfide, forming a new disulfide bond and releasing the reduced protein (Fig. 1).

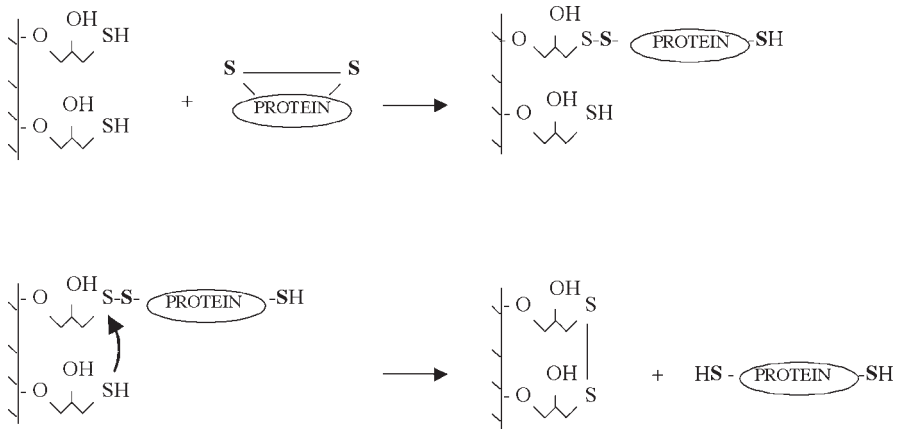

Fig. 1. Reduction of disulfide bonds in proteins with a solid-phase reducing agent.

The use of solid-phase reducing agents is not new and has many advantages over the use of soluble ones *(2,6)*: no liberation of contaminant products, easy separation from the reduced protein, easy regeneration, and the ability to reuse it many times. Here, we report our comparative studies on the reduction of disulfide groups in β-lactoglobulin and in *K. lactis* and *Aspergillus oryzae* β-galactosidases with soluble (DTT) and solid-phase (thiopropyl-agarose) reducing agents.

Materials and Methods

Chemicals

Sepharose 4B and PD-10 columns (Sephadex G-25) were from Pharmacia Biotech AB (Uppsala, Sweden). β-Galactosidase from *A. oryzae*, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), β-lactoglobulin from bovine milk, epichlorohydrine (1-chloro-2,3-epoxypropane), DTT, 2,2'-dipyridyl disulfide (2-PDS), and 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) were from Sigma (St. Louis, MO). β-Galactosidase from *K. lactis* (Maxilact LX-5000) was a generous gift from Gist Brocades (Cedex, France). A bicinchoninic acid (BCA) Protein Assay Kit was purchased from Pierce (Rockford, IL). All other products were of reagent or analytical grade.

Synthesis of Thiopropyl-Agarose

Thiol-agarose containing 1000 µmol of SH groups/g of dried gel was prepared essentially as described by Axén et al. *(7)*.

Titration of Thiol Groups

Thiol content of both thiopropyl-agarose and reduced proteins was determined spectrophotometrically by titration with 2-PDS (saturated solution, 1.5 m*M*) dissolved in 0.1 *M* sodium phosphate, pH 8.0 *(8)*. The quan-

titative determination of sulfhydryl groups in solution was also performed with DTNB *(9)*.

Protein Determination

Protein content was determined using the BCA assay *(10)*. Bovine serum albumin was used as the standard.

Activity Determination

β-Galactosidase activity was assayed using ONPG as a substrate in 20 m*M* potassium phosphate buffer, pH 7.0, containing 2 m*M* $MgCl_2$ and 0.1 *M* KCl for β-galactosidase from *K. lactis*, and 50 m*M* sodium acetate, pH 5.5, for β-galactosidase from *A. oryzae*. The rate of formation of free *o*-nitrophenol was determined at 405 nm. One unit of enzyme activity (EU) was defined as the amount of enzyme catalyzing the hydrolysis of 1 µmol of ONPG/min under the specified conditions.

Reduction of Proteins with DTT

β-Galactosidase from *K. lactis*

In experiment 1, aliquots of commercial extract (Maxilact LX-5000, 60 mg/mL, 1400 EU/mL) were incubated at room temperature with the same volume of reducing agent solution (with different concentrations of DTT) in 20 m*M* potassium phosphate, pH 8.0, in order to achieve ratios (micromoles of SH of reductant/milligram of protein, *R*) of 1.7 and 3.4. Periodically, samples were taken, diluted 10 times, and gel filtrated to remove excess DTT. The SH content was determined. In experiment 2, aliquots of enzyme were incubated with DTT for 30 min with a ratio of 3.4 (chosen according to the results of experiment 1). The mixtures were gently agitated at 24°C, and the influence of pH (7.0 or 8.0) and presence of salts (KCl or $MgCl_2$) was evaluated. Then, DTT was removed and the SH groups were quantified as described for experiment 1.

β-Galactosidase from *A. oryzae*

Aliquots of enzyme extract, partially purified by gel filtration on Sephadex G-100 (2.2 mg of protein/mL, 121 EU/mL), were incubated for different periods of time with aliquots of DTT in 20 m*M* sodium phosphate, pH 8.0, to give ratios of 3, 7, and 14. DTT was removed and SH groups were quantified as already described

β-Lactoglobulin

Aliquots of protein solution (4 mg/mL) with and without 6 *M* urea in 100 m*M* sodium phosphate, pH 8.0, were incubated with different amounts of DTT in order to achieve ratios from 1 to 25. At different time intervals the reducing agent was removed by gel filtration. The SH content was determined before and after the reduction process.

Reduction of Proteins with Thiopropyl-Agarose

Aliquots of the different proteins in buffer at pH 8.0 (the ionic strength and molarity of the potassium phosphate buffers were the same as the ones used for each protein during the reduction in solution, 20 m*M* for both β-galactosidases and 100 m*M* with and without urea for β-lactoglobulin) were incubated for different times (30 min to 20 h) with thiopropyl-agarose varying the ratio (*R*) from 0.3 to 25 (depending on the protein). In the case of β-galactosidase from *K. lactis*, the commercial extract was diluted five times (12 mg/mL) before the batchwise reaction with thiopropyl-agarose. In addition, β-lactoglobulin was also assayed in the presence of 6 *M* urea. After removal of the reducing agent by filtration on a sintered glass filter, thiol content was titrated as already described.

Stability of Thiol Groups Generated by Disulfide Reduction

After removal of the reducing agents (DTT or thiopropyl-agarose), aliquots of the reduced proteins were stored for different time periods in pH 8.0 buffer at room temperature and at 4°C. Then, remaining thiol content was quantified. In the case of β-lactoglobulin, the stability of the thiol groups was assayed in the presence and absence of 6 *M* urea.

Results and Discussion

Reduction of Protein with DTT and Thiopropyl-Agarose

A comparative study of the reduction process of disulfide moieties in model proteins (yeast and fungal β-galactosidases, β-lactoglobulin) with soluble (DTT) and solid-phase (highly substituted thiopropyl-agarose) reducing agents was accomplished. The degree of reduction achieved was mainly dependent on the ratio of micromole SH groups from the reducing agent/milligram of native protein (*R*). In the case of β-galactosidase from *K. lactis*, the reduction was also dependent on the phosphate buffer concentration, but independent of the presence of salts such as KCl and $MgCl_2$ (data not shown). The optimal conditions for enzyme reduction, which allowed a threefold increase in the SH group content, without affecting the specific activity, were 30 min of incubation with 100 m*M* DTT ($R = 3.4$) in 20 m*M* potassium phosphate at pH 7.0 or 8.0. The optimal conditions for the reduction of β-galactosidase from *K. lactis* with the solid-phase reducing agent, without affecting the specific activity, were not greatly different from conditions optimized for DTT: pH 8.0 and 2 h of incubation. Under these conditions, it was possible to increase the initial SH content threefold, but using a ratio of $R = 0.5$, nearly seven times fewer SH groups from thiopropyl-agarose were needed to achieve the same degree of reduction as was achieved by reduction in solution with DTT. In both reduction processes, it was confirmed by protein determination that there was no loss of protein, either during the gel filtration step or on the solid-phase reducing agent (Fig. 2).

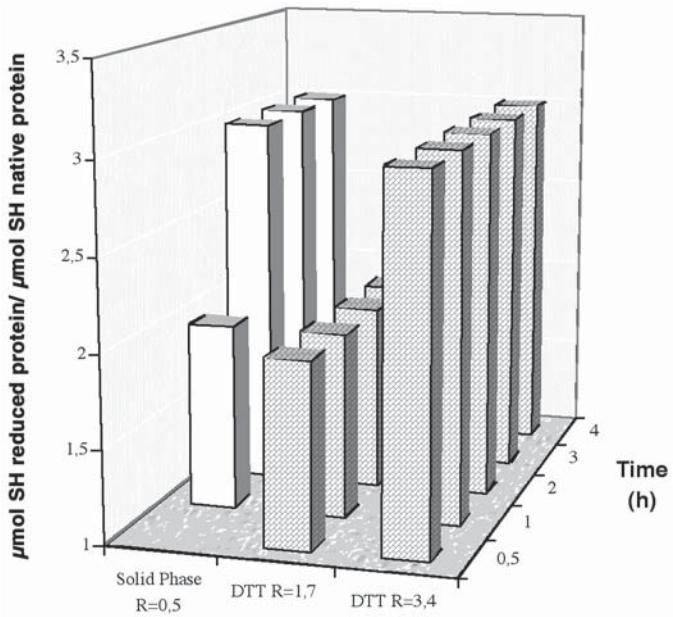

Fig. 2. Reduction kinetics of *K. lactis* β-galactosidase, with soluble (DTT) or solid-phase (thiopropyl-agarose) reducing agents. *R* = µmol of SH reductant/mg of protein. Data bars represent averages of at least three experiments.

There was a great difference between the reduction of acidic (*A. oryzae* β-galactosidase) and neutral (*K. lactis* β-galactosidase) lactases with thiopropyl-agarose. For *A. oryzae* β-galactosidase, it was necessary to use nearly twice as many SH groups per milligram of protein using thiopropyl-agarose than for the reduction in solution with DTT. In both cases, it was possible to increase the initial SH content fourfold without affecting specific activity (Fig. 3).

β-Lactoglobulin reduction was urea dependent. Reduction with DTT showed that the presence of urea modified the kinetics of the reduction process (e.g., the maximum reduction achieved with an *R* = 12.5 was six times faster than in the absence of denaturant agent). These results could not be explained by the instability of disulfide bonds in the presence of urea, since denatured protein (only treated with urea) was used as a blank. One possible explanation for these results could be that urea unfolds the protein tertiary structure, exposing disulfide groups formerly buried in the inner structure of the polypeptide chains, and making them more accessible to the reducing agent (Figs. 4 and 5).

The presence of urea dramatically improved the reduction of β-lactoglobulin with thiopropyl-agarose, since no reduction was achieved in the absence of the denaturing agent even when using ratios of micromoles of SH reductant/milligram of protein similar to those used with DTT (*R* = 16). However, in the presence of 6 *M* urea it was possible to achieve the same degree of reduction with thiopropyl-agarose as in solution with DTT

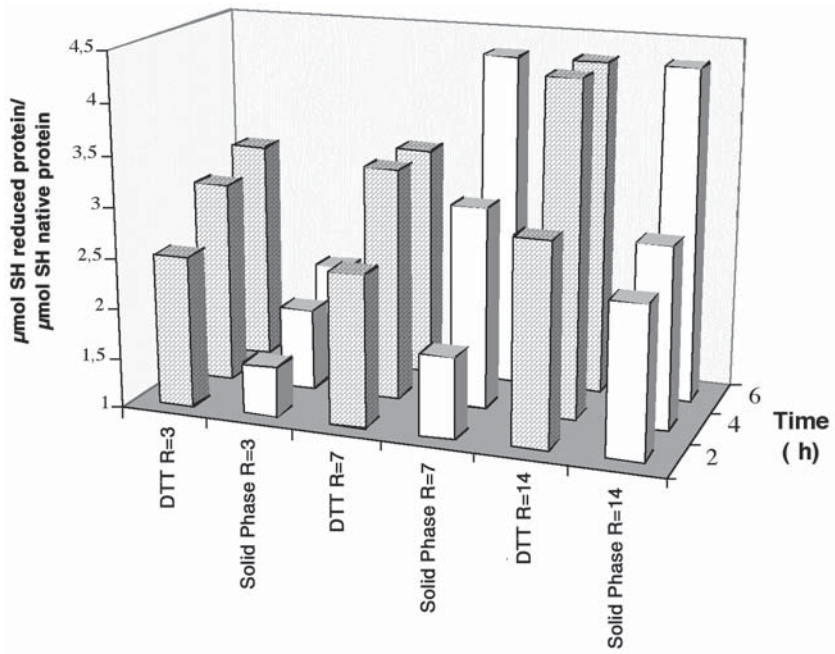

Fig. 3. Reduction kinetics of *A. oryzae* β-galactosidase, with soluble (DTT) or solid-phase (thiopropyl-agarose) reducing agents. *R* = µmol of SH reductant/mg of protein. Data bars represent averages of at least three experiments.

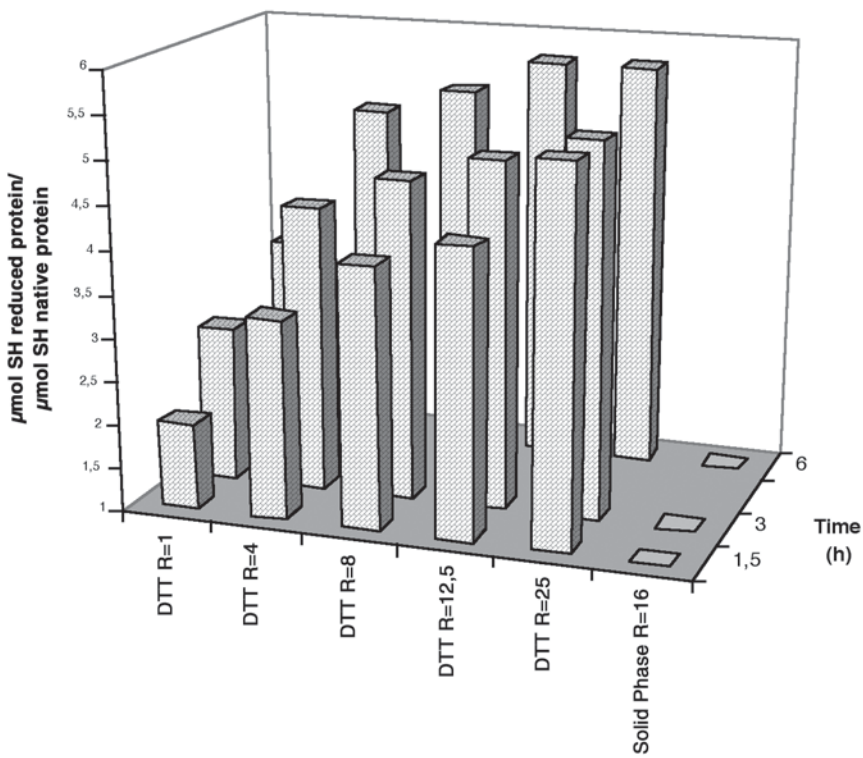

Fig. 4. Reduction kinetics for native β-lactoglobulin (in absence of urea). *R* = µmol of SH reductant/mg of protein. Data bars represent averages of at least three experiments.

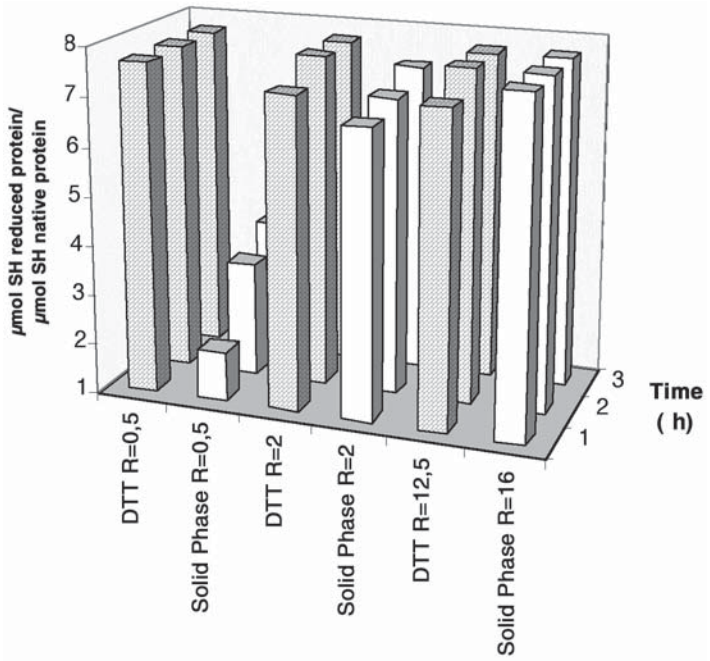

Fig. 5. Reduction kinetics for β-lactoglobulin in the presence of 6 *M* urea. *R* = µmol of SH reductant/mg of protein. Data bars represent averages of at least three experiments.

(Fig. 5). These results showed the critical importance of the steric effect when the reduction was performed with solid-phase reducing agents. This could be explained by the short distance between the SH group and the support surface (spacer arm) in thiopropyl-agarose. Therefore, only exposed disulfide groups could be reduced with this insoluble reducing agent under no denaturant conditions.

Stability of SH Groups Generated in Reduction Process

For *A. oryzae* β-galactosidase, the SH groups generated were very stable, since 100% remained unchanged after 18 h of incubation in the absence of reducing agents, at either 4°C or room temperature. In the case of *K. lactis* β-galactosidase and β-lactoglobulin, the stability of the generated SH groups was greater at lower temperature (Tables 1–3).

At 4°C, nearly 50% of the thiol groups generated in the neutral lactase had not been oxidized after 18 h of incubation in the absence of reducing agents (Table 1). Thermal stability of the SH groups is of great importance for the success of slow kinetic processes involving reduced enzymes, such as conjugation or covalent immobilization.

In the case of β-lactoglobulin, it was observed that the thiol groups generated were unstable in the presence of urea at both 4 and 25°C (Tables 2 and 3). However, the stability was much greater for the protein reduced with thiopropyl-agarose compared with that reduced with DTT, when the reduced protein was stored in the absence of urea. Under denaturing con-

Table 1
Stability at pH 8.0 of Thiol Groups Generated in *K. lactis* β-Galactosidase (After Reduction with Thiopropyl-Agarose or DTT)

Time (h)	Remaining thiol groups (%)			
	Thiopropyl-agarose		DTT	
	4°C	25°C	4°C	25°C
1	94	87	100	94
2	90	84	100	82
4	85	76	90	78
6	70	64	82	65
18	50	38	58	44

Table 2
Stability at pH 8.0 of Thiol Groups Generated in β-Lactoglobulin (After Reduction with Thiopropyl-Agarose)

Time (h)	Remaining thiol groups (%)			
	With urea		Without urea	
	4°C	25°C	4°C	25°C
1	88	68	95	77
2	84	48	88	73
4	60	25	82	60
6	52	10	84	50
20	10	—[a]	88	17

[a]No detectable SH levels.

Table 3
Stability at pH 8.0 of Thiol Groups Generated in β-Lactoglobulin (After Reduction with DTT)

Time (h)	Remaining thiol groups (%)			
	With urea		Without urea	
	4°C	25°C	4°C	25°C
1	82	50	90	75
2	70	30	84	63
4	44	15	75	47
6	35	10	50	36
20	7	—[a]	41	10

[a]No detectable SH levels.

ditions, the protein remained unfolded and this could explain the faster oxidation kinetics of the generated thiol groups, in spite of storage at low temperatures.

Conclusions

Thiopropyl-agarose performed excellently in the reduction of the model proteins, which it carried out as well as DTT. The conditions for the reduction process (micromoles of SH of reductant/milligram of protein, presence of denaturing agents, time) using DTT or thiopropyl-agarose were different depending on the location of the disulfide bridges within the protein molecule. The matrix of the solid-phase reducing agent generated a steric effect, allowing the reduction of only disulfide bonds exposed on the surface. This property will be very useful for the selective reduction of proteins containing both superficial and internal disulfide bridges, which is difficult to control with soluble reducing agents. In addition, the solid-phase reduction process proved to be an efficient and scalable method for reducing biocatalysts and has the potential for simplifying the scaling up of industrial applications, such as covalent immobilization and bioconjugation.

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