Purification and site-specific immobilization of genetically engineered glucose dehydrogenase on Thiopropyl-Sepharose

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The gene encoding glucose dehydrogenase (EC 1.1.1.47) from *Bacillus subtilis* was inserted in a plasmid 1.0 kb downstream from a *lac* promoter, resulting in a 70-fold higher production of the enzyme when expressed in *Escherichia coli*. A glucose dehydrogenase mutant containing a cysteine residue at position 44 could also be expressed at the same high level. This single cysteine residue was used as an 'affinity tag' to simplify the purification procedure as well as for site-specific immobilization of glucose dehydrogenase on Thiopropyl-Sepharose. This enzyme was purified to homogeneity with a final recovery of 65% and a specific activity of 240 U/mg. The oriented immobilization resulted in increased thermal stability.

Glucose dehydrogenase; Mutant; Purification; Covalent chromatography; Immobilization

1. INTRODUCTION

Glucose dehydrogenase (GlcDH) (EC 1.1.1.47) from *Bacillus subtilis* is a tetrameric enzyme catalyzing the oxidation of glucose to glucono-δ-lactone. Although this enzyme has been widely used for glucose determination both in free and in immobilized forms [1] as well as an NAD(H) regeneration enzyme [2], it has been difficult to obtain reasonable amounts of highly purified glucose dehydrogenase [3]. Recombinant DNA techniques have been utilized to facilitate the purification of several proteins using a variety of affinity tails combined with appropriate chromatographic procedures [4]. Recently, 4 cysteine residues were introduced into the C-terminus of galactokinase and the gene product could be purified on a column of Thiopropyl-Sepharose [5]. Similarly, subtilisin and IgG binding receptors have been genetically modified by introduction of cysteine residues [6,7]. A cysteine residue has also been introduced in glucose dehydrogenase at position 44 using site-directed mutagenesis. An activated NAD-analogue was covalently linked to this mutant (GlcDHcys^{44}) and the complex was used in a continuous regeneration system for NAD(H) [8].

The present paper describes the construction of expression vectors for native GlcDH and GlcDHcys^{44}, resulting in enhancement of their production by transformed *Escherichia coli* cells. Furthermore, the cysteine residue of GlcDHcys^{44} functioned as an 'affinity tag' and the purification was thereby improved and simplified since covalent chromatography on Thiopropyl-Sepharose could be utilized.

Covalent attachment of enzymes has been carried out by several methods, but all the methods have the disadvantage that heterogeneous orientation of the enzyme is obtained, which can interfere with the active site [9]. In this report, site-specific immobilization of GlcDH via the formation of a reversible disulphide bridge could be carried out simultaneous to the last purification step. The thermostability of immobilized and soluble GlcDH was also examined.

2. MATERIALS AND METHODS

2.1. *DNA constructions*

In construction of plasmid pGD1, a 2 kb *Xba*I fragment from plasmid pEF1 [10], carrying the *B. subtilis* glucose dehydrogenase gene, was inserted into the *Xba*I site downstream to the *lac* promoter of plasmid pUC19. The GlcDHcys^{44} gene obtained by site-directed mutagenesis as described previously [8] was inserted into plasmid pGD1 using *Hin*dIII and *Eco*RI, generating pGD1cys. *E. coli* JM 105 was used for standard transformation procedures.

2.2. *Expression, purification and enzyme assay*

Transformed cells were grown at 35°C in LB-medium supplemented with ampicillin. At OD_{550} ≈0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM and the culture was incubated overnight before harvesting by centrifugation. Cell pellets were resuspended in 50 mM imidazole buffer, pH 7.0, containing 20% glycerol and 1 mM DTT (buffer A), incubated with 0.5 mg lysozyme/ml for 30 min and sonicated on ice (6 × 30 s, output 6, Sonifer B-30, Branson Sonic Power). Cell debris was pelleted and ammonium sulfate was added to the supernatant to 40% saturation. Precipitated proteins were removed by centrifugation and the supernatant liquid applied to a column packed with Phenyl-Sepharose CL-4B (Pharmacia-LKB, Sweden) previously equilibrated with buffer A containing 1.7 M ammonium sulfate. The enzyme was eluted with a linear gradient from 1.7 M to 0 M

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Table I

Purification of GlcDHcys[44]

Sample	Volume (ml)	Protein (mg)	Activity (U)	Spect. act. (U/mg)	Recovery (%)
Sonication	21	690	17 400	25	100
Ammonium sulfate precipitation	25	470	16 500	35	95
Phenyl-Sepharose	65	79	14 200	180	82
Thiopropyl-Sepharose	20	48	11 400	240	65

7 g *E. coli* cells harboring plasmid pGD1cys, recovered from 1 liter fermentation broth, were extracted as described in Materials and Methods.

$(NH_4)_2SO_4$ in buffer A. Fractions with GlcDH activity were pooled, dialyzed against 50 mM imidazole buffer, 0.3 M NaCl, pH 7.5 (buffer B) and incubated with Thiopropyl-Sepharose 6B (Pharmacia-LKB) for 10 min. The gel was washed twice with buffer B containing 10 mM cysteine and the bound protein could then be eluted with 20 mM DTT in buffer B. The purification was followed on 12% SDS-PAGE and protein concentration measured using the Bio-Rad Protein Assay kit with BSA as standard. GlcDH activity was assayed according to Ramaley and Vasantha [11].

2.3. *Immunobilization and heat stability measurements*

Fifty milligrams of wet gel (Thiopropyl-Sepharose 6B) was mixed with 20 U GlcDHcys[44] in a total volume of 1.0 ml. The gel was sedimented by brief centrifugation, washed with 10 mM cysteine in buffer B; enzyme activity was measured in the supernatant (non-bound), in the washing solution, and on the gel slurry (activity in the immobilized system) with a Cary 2290 spectrophotometer, stirring when necessary. Coupling was also monitored by the release of 2-thiopyridone at 343 nm. To check the recovery, the enzyme was eluted from the gel by 20 mM DTT and measured for activity. Heat stability measurements were carried out at 50, 55 and 60°C in buffer B with immobilized and purified enzymes.

3. RESULTS AND DISCUSSION

3.1. *Expression and purification*

By combining the GlcDH gene with the strong *E. coli lac* promoter, the expression of glucose dehydrogenase from pGD1 and pGD1cys in *E. coli* resulted in approximately 10 mg enzyme per gram cells, which is almost 70 times higher than previously reported for other expression vectors [3,12]. Although the distance between the *lac* promoter and the start codon of GlcDH is nearly 1.0 kb, the overproduction was regulated by this promoter since cultivation without IPTG induction gave no enzyme at al.

GlcDHcys[44] was purified to apparent homogeneity using a simple 3-step purification scheme (Table I). Ammonium sulfate precipitation was used prior to separation on the hydrophobic column (Fig. 1). The last step, covalent chromatography on Thiopropyl-Sepharose (Fig. 2), could be utilized efficiently since this mutant has an 'affinity tag' consisting of an active cysteine on the surface of the enzyme. SDS-PAGE after this step showed a single polypeptide of molecular mass = 31 000 Da (Fig. 3). The absorbance at 280 nm was measured and the extinction coefficient calculated to 36 500 cm^{-1} M^{-1} per subunit. The overall yield was high: 48 mg pure enzyme per liter culture medium as opposed to a total of 30 mg from a 300-liter fermenter reported by Smith and Ramaley [3], and with a total recovery of 65%. The specific activity was found to be

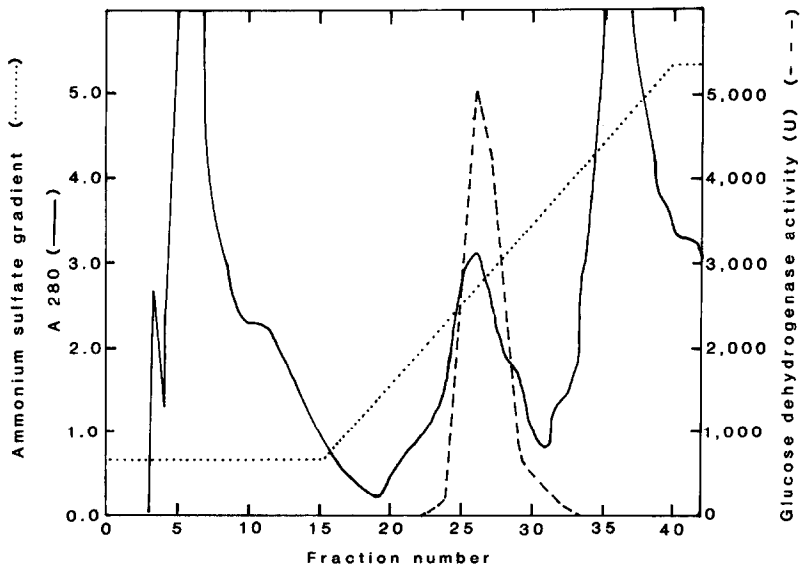

Fig. 1. Hydrophobic chromatography. Protein extract (23 ml) was applied to a 1.5 × 12 cm column of Phenyl-Sepharose CL-4B (flow rate: 20 ml/h, fraction volume: 10 ml).

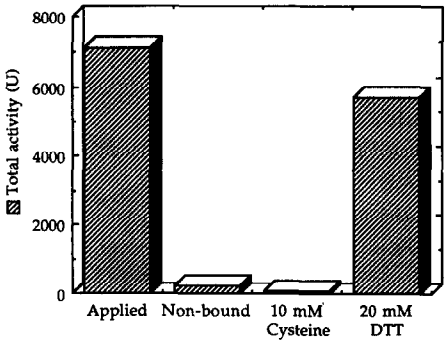

Fig. 2. Batch affinity purification of GlcDHcys[44] using 8 ml Thiopropyl-Sepharose 6B.

240 U/mg, which is between 50 and 70% of that found in other reports [11,12]. This lower value might be due to minor dissociation of the tetrameric form into dimers having much lower specific activity, as indicated by gel filtration experiments, since glucose dehydrogenase is sensitive and must be protected against this kind of inactivation. Glycerol and ATP have been used extensively for this purpose [11,12], but high ionic strength, such as 0.3 M NaCl, should also be sufficient [2].

The accessibility of solvent to the cysteine residues was also determined according to Ellman [13], which showed that all 4 cysteines (one per subunit) were fully exposed. Native glucose dehydrogenase could not be purified to homogeneity using this method since it lacks cysteine residues.

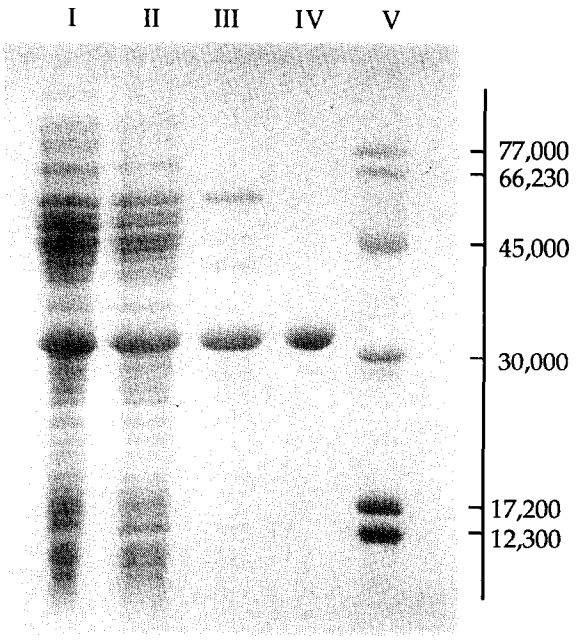

Fig. 3. SDS-PAGE after different purification steps. I, sonication; II, supernatant after 40% ammonium sulfate; III, hydrophobic chromatography; IV, Thiopropyl-Sepharose; V, molecular weight standards.

3.2. *Immobilization and heat stability of GlcDHcys[44]*

The oriented immobilization procedure for GlcDHcys[44] occurs via the formation of a reversible thiol bond between cysteine 44 and the matrix. This offers some important advantages such as: (i) reproducible immobilization, (ii) easy regeneration by reduction and oxidation, (iii) immobilization and purification can be achieved in a single step, (iv) covalent attachment occurs via an amino acid that is not essential for enzymatic activity; and (v) the oriented immobilization of a protein on e.g. a chip will facilitate future studying using optical procedures such as ellipsometry.

Reduced GlcDH was mixed and incubated with Thiopropyl-Sepharose for various periods of time at room temperature. The immobilization of GlcDH was monitored as a function of incubation time (Fig. 4). After approximately 15 min, complete immobilization was achieved as indicated by no further release of 2-thiopyridone and no detectable GlcDH activity in the supernatant. The coupling reaction is thus notably faster than for most naturally occurring thiol-containing proteins [14], indicating that the cysteines are very accessible. Optimal activity yield was obtained after 2 min when 40% of the GlcDH activity was retained on the gel. After this time, no further increase in the overall activity of the immobilized system was obtained despite the fact that more enzyme was bound to the matrix. Thiopropyl-Sepharose contains 20 μmol-2-pyridyl disulphide groups per ml gel. This high degree of substitution might explain the deactivation of immobilized GlcDH since multipoint attachment obviously reduces the activity. Since GlcDHcys[44] consists of 4 subunits, each with an 'active' cysteine, it is likely that more subunits are coupled to the gel, which might lead to less optimal interaction of the subunits with one another. When the immobilized enzyme was released by

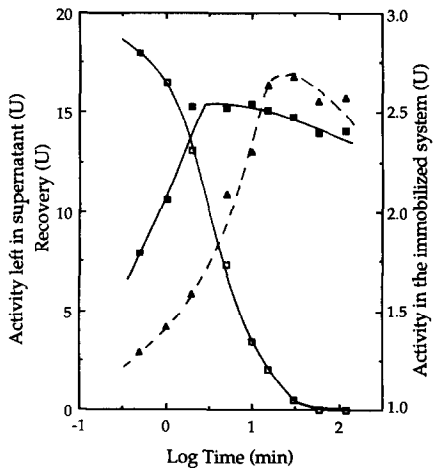

Fig. 4. Immobilization of GlcDHcys[44] on Thiopropyl-Sepharose 6B with time. Activity left in supernatant i.e., non-bound (□); activity in the immobilized system (■). Recovery of enzyme activity after subsequent addition of 20 mM DTT to the immobilized system (▲).

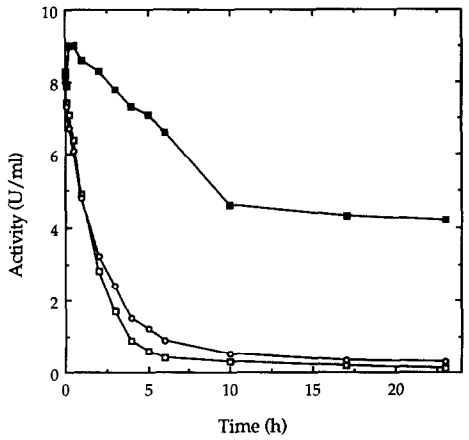

Fig. 5. Thermal stability against deactivation at 55°C of soluble native GlcDH (○); soluble GlcDHcys^{44} (□); and immobilized GlcDHcys^{44} (■).

reducing the disulphide bonds, only 80% of the activity could be recovered. Therefore, it is also conceivable that the weak subunit interactions are partly disrupted on the gel, causing a loss in specific activity.

In the thermal denaturation experiments (Fig. 5) about 90% of the initial activity of the soluble enzyme disappeared within the first 5 h, whereas only 20% of the activity was lost for the immobilized enzyme. After 10 h the deactivation process for the immobilized enzyme occurred slowly with nearly zero order kinetics. Similar results were obtained for different temperatures. Finally, the immobilized GldDHcys^{44} could be stored at room temperature for more than 5 months without any loss of activity, indicating its usefulness in practical applications. Overall, the results obtained demonstrate the usefulness of introducing an active cysteine residue in an enzyme, by site-directed mutagenesis, for site-specific immobilization and purification.

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