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APPLICATIONS OF NANODIAMONDS

Applications of Nanodiamonds for Separation and Purification of Proteins

V. S. Bondar', I. O. Pozdnyakova, and A. P. Puzyr'

Institute of Biophysics, Siberian Division, Russian Academy of Sciences, Akademgorodok, Krasnoyarsk, 660036 Russia

e-mail: bondvs@mail.ru

Abstract—Recombinant apoobelin and recombinant luciferase are separated from bacterial cells of *Escherichia coli* with the use of detonation nanodiamonds. The application of nanodiamonds has a number of points in its favor, namely, (i) simplifies the procedures for purifying the proteins, (ii) decreases the time of their separation to 30–40 min, (iii) eliminates the necessity of using special chromatographic equipment, and (iv) makes it possible to prepare high-purity apoobelin and luciferase materials with protein yields of 35–45 and 45–60%, respectively. The possible mechanisms of interaction of protein molecules and nanodiamond particles are analyzed. © *2004 MAIK "Nauka/Interperiodica".*

1. INTRODUCTION

Detonation nanodiamonds [1] are of particular interest to biochemists, because these materials exhibit unique physicochemical properties, such as a highly developed surface of particles (270–280 m^2/g) with a large number of surface ionogenic (carboxyl, carbonyl, hydroxyl, ether) groups, hydrocarbon fragments, and metal microimpurities [2, 3]. Taken together, these factors allow one to treat nanodiamonds as new sorbents suitable for the separation and purification of proteins.

In this work, we used nanodiamonds to separate recombinant Ca^{2+}-activated photoprotein apoobelin and recombinant luciferase from bacterial cells of *Escherichia coli*.

2. SAMPLE PREPARATION AND EXPERIMENTAL TECHNIQUE

The nanodiamonds used in our experiments were synthesized at the Department of Physics of Finely Dispersed Materials, Krasnoyarsk Research Center [3].

The choice of the proteins under investigation was motivated by the fact that they belong to luminescent systems of different types, substantially differ in functional molecular structure, and can be separated by different methods. The Ca^{2+}-activated photoproteins are light-emitting EF proteins that occur in marine Coelenterata organisms and generate photons in the visible spectral region upon interaction with calcium ions [4]. These proteins are stable enzyme–substrate complexes that consist of a small single-subunit apoprotein molecule (nearly 20 kDa), a substrate (coelenterazine) molecule, and oxygen. Luciferases are light-emitting proteins contained in marine luminous bacteria. These proteins are heterodimers (consist of α and β subunits), have a molecular mass of approximately 80 kDa, and involve flavin as a cofactor in the structure [5]. The apoobelin and luciferase genes were cloned and expressed in *Escherichia coli* cells, which permitted us to separate the producer strains of these proteins [6, 7]. Photoproteins and luciferases have been widely used as light-emitting indicators in bioluminescent analysis [5, 8].

Upon expression of particular genes in *Escherichia coli* cells, recombinant proteins synthesized by the cells are accumulated in the form of insoluble aggregates, so-called inclusion bodies [9]. This property underlies the method of separating these proteins, including apophotoproteins [10, 11]. The universally accepted technique of purification involves separation of the inclusion body fraction after cell disruption (for example, by ultrasound or a French press), extraction of the recombinant protein from this fraction by a highly concentrated denaturing chaotropic agent (urea, guanidine–HCl), chromatographic purification, and protein refolding after removal of the chaotropic agent. The purification of recombinant apoobelin according to such a procedure takes at least two days [6, 11]. On the other hand, many recombinant proteins, such as luciferases, can be accumulated in a cytosol fraction of synthesizing cells without the formation of inclusion bodies [7]. Compared to proteins, luciferases have a more complex structure and are more sensitive to disrupting factors, for example, to chaotropic agents and heavy metal ions. Therefore, these features should be taken into account in the purification of luciferases. These proteins have been purified without denaturing agents by using a procedure that includes several chromatographic stages and takes at least two or three days [5].

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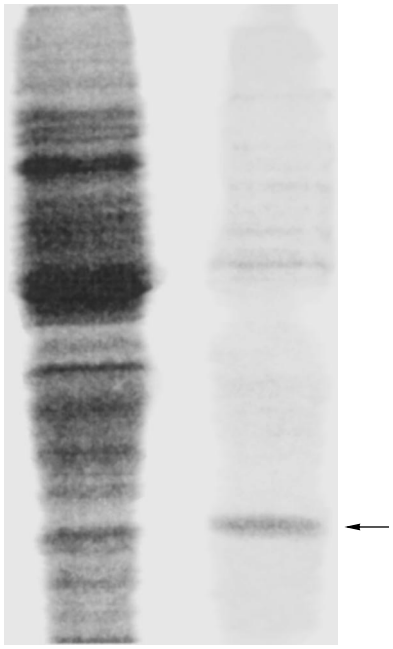

Fig. 1. Electrophoresis images of the recombination apoobelin samples at the stages of separation from bacterial cells of *Escherichia coli* with the use of nanodiamond particles: (*1*) the initial extract and (*2*) the final product. The arrow indicates the position of the apoobelin.

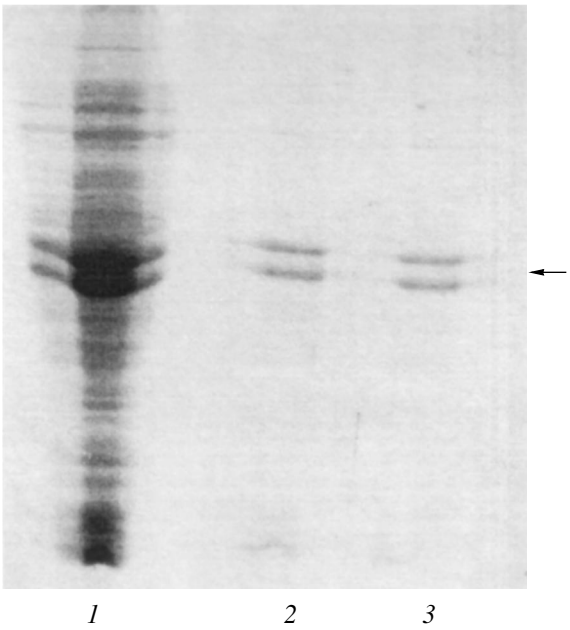

Fig. 2. Electrophoresis images of the recombination luciferase samples at the stages of purification from a biomass of bacterial cells of *Escherichia coli* with the use of nanodiamond particles: (*1*) the initial extract and (*2*, *3*) the final luciferase products prepared upon sequential desorption of the enzyme from the nanoparticle surface with an eluating buffer. The arrow indicates the positions of the α and β luciferase subunits.

3. RESULTS AND DISCUSSION

The apoobelin protein was separated with the use of nanodiamonds according to the following procedure. Cell proteins were extracted from a biomass with a chaotropic agent (6 M urea) for 1 h. The cell debris was removed by centrifugation. The nanodiamond particles were resuspended in a supernatant and then sedimented through centrifugation. The sediment of particles with adsorbed proteins, including apoobelin, was washed twice with a buffer in order to remove unadsorbed inert proteins. The apoobelin protein was desorbed from the particle surface with a buffer containing SH reagent (dithiothreitol) at a concentration of 10 mM. The apoobelin product prepared according to the above procedure has a high degree of purity (Fig. 1), and the apoobelin yield is no less than 35–45%.

The technique of purifying the luciferase protein involved the following stages. The biomass was disrupted in water by ultrasound, and the cell debris was removed through centrifugation. The nanodiamond particles were added to the supernatant, stirred, and sedimented through centrifugation. The sediment was washed twice in order to remove unadsorbed inert proteins. The luciferase protein was desorbed from the particle surface with a 20-mM desorbing buffer. According to the electrophoretic data (Fig. 2), the final product has a high degree of purity and the luciferase yield is 45–60%.

It should be noted that the technique of protein purification with the use of nanodiamonds is characterized by high rapidity. After preparing the initial extracts, the separation procedure takes no more than 30–40 min. It seems likely that this technique can be more correctly regarded as adsorption–desorption chromatography in the bulk. The technique enables one to simplify the separation of proteins substantially, to decrease the time consumption, and to eliminate the necessity of using special chromatographic equipment. The advantage of nanodiamond application is that, in addition to efficient protein separation, the protein can be concentrated, because it can be desorbed using very small amounts of an eluent. For example, from cells with a very low apoobelin concentration (30–300 μg per 1 g of biomass), it is possible to extract a concentrated, virtually homogeneous apoobelin material with a yield of 40–50% for 30–40 min. This cannot be achieved in the case of column chromatography, because compounds are washed out with an eluent whose volume is determined by the retention parameters of the column and the mass transfer. It should also be noted that the use of nanodiamonds ensures the complete separation of luciferase from endogennic NADH : FMN oxidoreductase. This fact is very important, because contamination with oxidoreductase, which contributes to the operation of a luminescence bienzyme system [5], reduces the quality of luciferase in analytical applications.

The data obtained in purifying the apoobelin and luciferase proteins with the use of nanodiamonds allow us to propose possible mechanisms of the interaction of protein molecules with the particle surface. An analysis of the data on the elution of apoobelin with the SH reagent and investigations into the treatment of the nanoparticle surface with a selective blocker of SH groups, namely, 5,5'-dithiobis(2-nitrobenzoic acid), and a chelator (EDTA) of bivalent ions demonstrate that protein molecules can interact with nanodiamonds through the formation of S–S bridges (approximately 10% of molecules) and coordination bonds (approximately 40% of molecules). The remaining protein molecules (50%) most likely interact with nanodiamond particles through other mechanisms. For example, multipoint interaction of protein molecules with different functional groups on the particle surface is a very possible mechanism. Apparently, the interaction can occur through the formation of ionic, hydrophobic, and covalent bonds (except for S–S bonds) or their possible combinations. This is confirmed by the fact that repeated treatment or an increase in the SH reagent concentration do not lead to an increase in the apoobelin yield at the elution stage, and the luciferase desorption efficiently proceeds with an increase in the buffer molarity. The occurrence of the interaction between proteins and particles through different mechanisms should be recognized more as an advantage, as it allows us to treat nanodiamonds as universal sorbents capable of providing different chromatographic operations.

In the above cases, protein purification with nanodiamonds was performed in the bulk. The use of nanodiamonds as a new material for column chromatography also seems to hold considerable promise. Actually, we prepared a nanodiamond-based sorbent that makes it possible to carry out the column chromatographic analysis at normal pressures. The applicability of this sorbent to the adsorption–desorption of protein molecules was demonstrated using the cytochrome C protein as an example.

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