

NEW AFFINITY SORBENTS FOR PURIFICATION OF RECOMBINANT PROTEINS WITH THE USE OF CHITIN-BINDING DOMAIN AS AN AFFINITY TAG

**Denis V. Kurek, Sergey A. Lopatin,
*Vladimir E. Tikhonov, Valery P. Varlamov**

Centre "Bioengineering" RAS,

*A. N. Nesmeyanov Institute of Organoelement Compounds RAS,
Moscow, Russia
E-mail: Svoboda_rus@mail.ru

Abstract

With the increasing amounts of recombinant proteins production the problem of effective purification of produced proteins became one of major topics for research nowadays. Affinity chromatography proved to be the most dominant method for this aim and application of chitin based affinity sorbents in combination with use of chitin-binding domain as an affinity tag for purification of recombinant proteins showed its efficiency even in comparison with commercially available systems.

Key words: *chitin based sorbents, affinity chromatography, chitin-binding domain.*

1. Introduction

A variety of recombinant proteins have found now the numerous applications in medicine and biological and biomedical researches. These applications require the highly purified proteins only. Therefore, a purification process represents an important part of recombinant proteins production, and affinity chromatography has become the most powerful tool for this purpose. Affinity tagging of proteins helps to overcome some limits and disadvantages of chromatography process. Affinity tags can be defined as exogenous amino acid sequences with a high affinity to specific biological or chemical ligand. Several affinity tags are now available, and the most common ones are oligohistidine tags used in association with immobilized metal-ion affinity chromatography (IMAC) method. Even though His-tags have proved to be efficient, the alternative tags are constantly developed since the affinity tagging technique has become a very useful method for purification of recombinant proteins bearing N-terminal affinity tags of different origin. Fusion of an affinity tag to recombinant protein significantly improves and simplifies its purification process. Unfortunately, the major disadvantage of His-tags and IMAC method of purification is a lack of high selectivity to recombinant proteins bearing chitin binding domain (ChBD) [1, 2].

Chitin, [(1→4)-2-acetamido-2-deoxy-β-D-glucan], is the most abundant naturally occurring poly(aminosaccharide), while chitinases are enzymes widely distributed among different mammalian bacterial and fungal species. Chitinases contain chitin-binding and catalytic domains so that they can bind to and hydrolyze chitin.

The studies described here were initiated to develop a number of affinity chitin based sorbents capable of separating D-amino acid oxidase from *Trigonopsis variabilis* (DAAO, EC 1.4.3.3) fused with N-terminal ChBD of chitinase A1 (EC 3.2.1.14) from *Bacillus circulans*.

2. Materials and methods.

2.1. Sorbent synthesis

Crab shell chitin (Mv 500 kDa) of practical grade, chitosan of average viscosity molecular weight of 75 kDa (degree of deacetylation 75%) were purchased from ZAO "Bio-progress" (Moscow region, Russia). Crab shell chitin or chitosan and a calculated amount of epichlorohydrin were mixed in 0.2 M Na₂CO₃ and stirred at room temperature for 12 hours. A product was filtered off and washed with an excess of distilled water.

2.2. Enzyme preparation

Recombinant DAAO fused with mutant ChBD from chitinase A1 was prepared in accordance with the previously disclosed method [3]. Specific D-amino acid oxidase activity was determined towards D-alanine in all fractions. The activity expressed as an amount (mg) of D-alanine converted to pyruvic acid by one mg of protein in 50 mM Na-P buffer (pH 8.0) at 37 °C per minute.

2.3. Chromatography protocol

A sorbent was packed into 20x40 mm glass column. The column was equilibrated with 50 mM sodium phosphate buffer (Na-P) pH 8.0. A crude cells extract was saturated

with 65% ammonium sulfate. The precipitated proteins were dissolved in Na-P buffer and loaded onto a column. The column was washed with Na-P buffer containing different NaCl concentrations, and fraction of 5 ml were collected, dialyzed, lyophilized, and analyzed by 12% SDS-PAGE electrophoresis.

3. Results and discussion

The wild type ChBD of chitinase A1 consists of 45 amino acid residues (Ala⁶⁵⁵ – Gln⁶⁹⁹) and has a compact globular structure shown in **Figure 1**. This domain was used as the affinity tag for recombinant proteins purification by means of affinity chromatography method and the use of the commercially available sorbent Chitin Beads (New England Biolabs, USA). Since it was shown in literature that the wild type ChBD domain bound to a native crystalline chitin practically irreversibly under nondenaturing conditions, the separation of wild type ChBD from Chitin Beads required a special technique of enzymatic hydrolysis of a link between the target protein and ChBD. In current research DAAO fused with N-terminal ChBD (Mw 45 kDa), subjected to the point mutation (Try⁶⁸⁷ to Phe⁶⁸⁷), expressed by *E. coli*, was used as a model recombinant protein. This point mutation leads to decrease of binding strength of ChBD and it made possible application of this domain as an elutable affinity tag. Since *E. coli* expressed also the multiple extra cellular proteins, the crude protein mixture was concentrated and partly purified by means of precipitation with saturated ammonium sulfate solution. A crude enzyme mixture was placed onto a chitinous sorbent and the purified model protein was eluted and analyzed as it is described in the experimental part.

As a starting point for affinity purification of fusion protein sorbent X-1 was synthesized. Sorbent X-1 represented a semi crystalline chitin prepared by means of swelling of

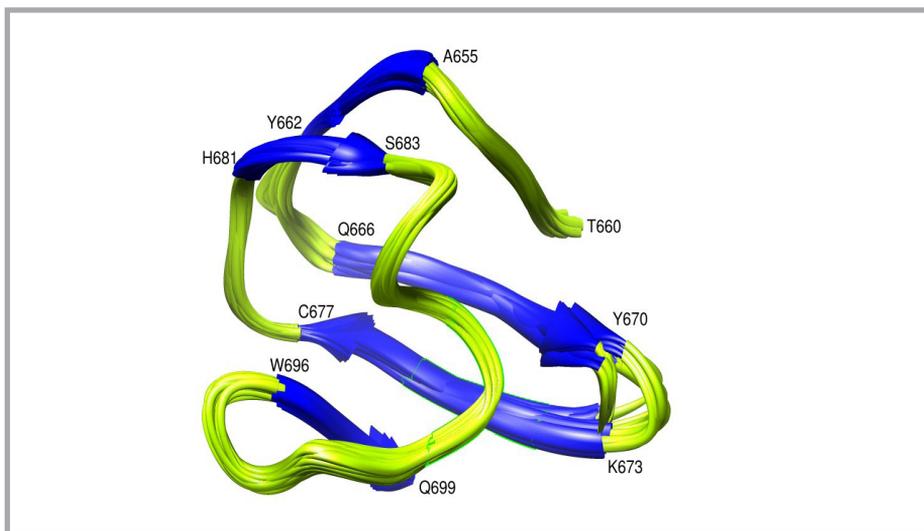


Figure 1. Schematic structure of chitin-binding domain ChBD_{ChiA1}.

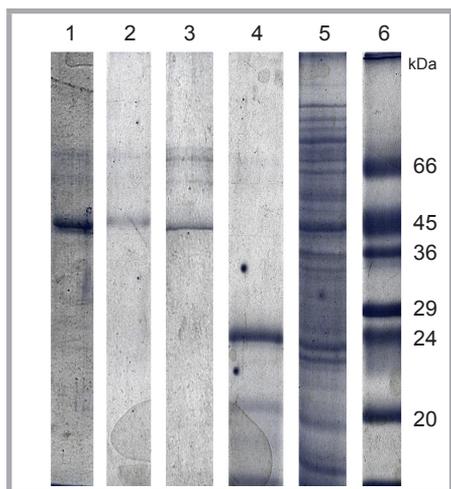


Figure 2. SDS-PAGE electrophoresis of eluted fractions. Lanes 1 - 4: sorbents (1 - X-1; 2 - X-1.20Epy; 3 - X-1.40Epy; 4 - X-1.60Epy); 5 - crude cell extract; 6 - molecular weight markers.

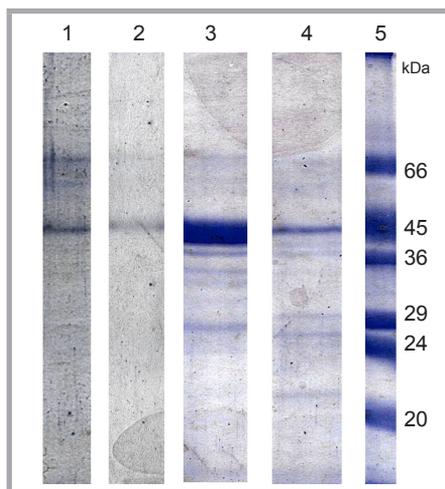


Figure 3. Specificity of the sorbents: SLC.20Epi (lane 1), X-1.20Epy (lane 2) and Chitin Beads (lanes 3 and 4: two different fractions were used); lane 5 - molecular weight markers.

the crystalline crab shell chitin in 85% phosphoric acid followed by the complete washing with an excess of distilled water. Recent bacterial ChBD studies have shown major contribution of the hydrophobic interactions to an interaction between ChBD from chitinase A1 and chitin. So that, as the first step of the current research an influence of NaCl concentration (0 - 2 M) on a nonspecific hydrophobic binding of waste proteins and other contaminants to sorbent X-1 was investigated to find out if the waste proteins and contaminants could be completely separated from the target enzyme. The results obtained allowed to optimize the mobile phase composition. If 2 M NaCl solution in 50 mM phosphate buffer (pH 8.0) was used as the effluent, the target recombinant enzyme bound to sorbent X-1 only. The application of 1mM phosphate buffer allowed the complete elution of the target enzyme. SDS-PAGE electrophoresis confirmed the high specificity of sorbent X-1 towards protein containing ChBD affinity tag (**Figure 2**, lane 1). Unfortunately, the main disadvantage of sorbent X-1 was its mechanical softness which did not allow a multiple usage of this sorbent.

In order to prepare a sorbent having an increased mechanical strength, semi crystalline chitin was subjected to cross-linking by epichlorohydrin (ECH). As a result, three cross-linked chitinous products have been prepared: sorbent X-1.20Epy, sorbent X-1.40Epy, and sorbent X-1.60Epy. These products differed in an amount of ECH used in the cross-linking reaction: 20, 40 and 60 mol.% of ECH per acetylglucosamine unit were used, respectively.

The applications of the above mentioned ECH-modified sorbents for purification of the recombinant DAAO showed a significant decreasing in the sorption capacity as the

Table 1. Results of chromatography experiments.

Affinity sorbent	Specific DAAO activity of crude protein extracts, U/mg	Specific DAAO activity of eluted fractions, U/mg	Purification fold	Capacity, µg/ml
X-1	0.78	10.1	13.0	150
X-1.20Epy	0.62	8.0	12.9	150
X-1.40Epy	0.71	6.32	8.9	150
X-1.60Epy	0.75	0.36	0.48	-
SLC.20Epy	0.71	8.1	11.5	1000
Chitin beads (New England Biolabs)	0.74	9.0	12.1	2000

increased amounts of ECH were used (**Figure 2**, lanes 2 - 4; **Table 1**). As a possible explanation, it was supposed that changes in polysaccharide chains caused by epichlorohydrin modification could result in the enhanced hydrophobicity of the sorbents. The experiments showed that the usage of 20 mol.% ECH did not influence a binding ability of the tag so that sorbent-X-1.20Epy could be used in further researches.

In addition to the above mentioned method, an alternative approach to preparation of an affinity sorbent was elaborated. In the approach water soluble chitosan samples of different molecular weights were completely acetylated following to Hirano's method (acetic acid anhydride in water/acetic acid solution). The obtained regenerated amorphous chitins (SLC) were cross-linked with 20 mol.% ECH at the same conditions described in the experimental part. It was found that only the usage of a chitosan with a molecular weight not less than 75 kDa, a cross-linked SLC of the appropriate mechanical properties could be obtained. It should be mentioned that chitosan itself could not be used for separation of a model enzyme since ChBD could not bind to chitosan but could to chitin. Thus, Sorbent SLC.20Epy was prepared. The sorbent was compared with the commercial Chitin Beads sorbent in the processes of D-amino acid oxidase bearing mutant ChBD purification (**Table 1**). The results obtained revealed that SLC.20Epy proved to be the promising material for the purpose claimed although it possessed a relatively reduced enzyme binding capacity, while Chitin Beads displayed an insufficient enzyme specificity (**Table 1**, **Figure 3**).

4. Conclusions

Thereby, the application of the mutation type chitin-binding domain from chitinase A1 has been demonstrated. A series of affinity sorbents possessing high specificity to the affinity tag have been synthesized, and it has been determined that chitosan is a perspective and convenient material for affinity sorbent preparations. The results obtained may represent a base for creation of a more efficient affinity chromatography technique for purification of the recombinant proteins bearing N-terminal chitin-binding domain as an affinity tag.

5. Acknowledgments

- *We gratefully acknowledge Dr. M.A. Eldarov (Centre “Bioengineering” RAS) for valuable advice and providing model recombinant proteins.*
- *This work was supported by the RFBR grant no. 07-04-12204*

6. References

1. **Graslund S., Nordlund P., Weigelt J., et al.;** (2008), *Nature Methods*. V. 5. № 2. P. pp. 135-145.
2. **Kurek D.V., Lopatin S. A., Varlamov V. P.;** (2009), *Appl. Biochem. Microbiol.* V. 45, №1, P. pp. 5-13.
3. **Redo V.A., Eldarov M.A., Zhgoun A.A., Khatuntseva S.A., Zeinalov O.A., Skryabin K.G.;** RF patent № 2310688, 20.11.2007.
4. **Hardt M., Laine R.;** *Arch. (2004), Biochem. Biophys.* V. 426. P. pp. 286-297.