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Hydrazide as a ligand moiety in immobilized metal ion affinity chromatography
Separation of BO–IMI and BODIPY–hydrazide

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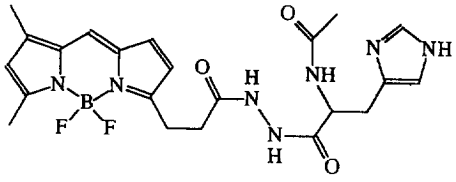
Abstract

BODIPY hydrazide (BO–HZ, a commercially available fluorescent dye) and BO–IMI (obtained by coupling the hydrazide moiety of BODIPY to the carboxyl group of *N*-acetylhistidine) were separated on three forms of a Sepharose–iminodiacetic acid column: Cu(II), Ni(II) and Zn(II). Whereas BO–IMI eluted first on the Cu(II) and Ni(II) columns (a pH gradient from 7.0 to 2.0 was applied), it eluted last on the Zn(II) column. BO–HZ eluted from the Zn(II) column without displacing this metal. The explanation suggested for these results is that BODIPY hydrazide undergoes strong, bidendate binding only to the Cu(II) and Ni(II) columns. © 1997 Elsevier Science B.V.

Keywords: Detection, electrophoresis; Hydrazides; BO–IMI; BODIPY–hydrazide

1. Introduction

A fluorescent reagent called 'BO–IMI' (structure is shown below) is available for covalent labeling of phosphate monoesters [1,2].



BO-IMI

In this labeling reaction, the phosphate moiety of the target substance is activated with a water soluble carbodiimide for attack by BO–IMI. This yields a phosphoroimidazolide product in which the imidazole moiety of BO–IMI has replaced an oxygen atom of the phosphate group. The reaction takes place under aqueous conditions at room temperature and is specific for phosphomonoesters. While carboxylic acids are also activated by the carbodiimide, they do not form a hydrolytically-stable conjugate with BO–IMI. To date the reaction has been applied to nucleotides [1], some phosphate metabolites [1], and the phosphoprotein pepsin [2]. In all cases, the BO–IMI-labeled analyte was detected by capillary electrophoresis with laser-induced fluorescence detection (CE–LIF).

Here we report the separation of BO-IMI and BODIPY-hydrazide (BO-HZ), a synthetic precursor of BO–IMI, by immobilized metal ion affinity

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chromatography (IMAC) on a Sepharose–iminodiacetic acid (IDA) column. We were motivated to conduct this study for two reasons. First, it was hoped that IMAC might provide an efficient way to remove any traces of BO–HZ and some other impurities from BO–IMI at the end of the synthetic procedure for this latter compound. This could minimize the background of interfering peaks when a trace analyte is detected via labeling with BO–IMI. Second, IMAC potentially could serve as well to remove residual BO–IMI from BO–IMI-labeled analyte at the conclusion of the labeling reaction.

IMAC was introduced by Porath [3], has been reviewed [4–6], and has been studied in detail more recently (e.g., [7–10]). Most applications have involved peptides or proteins, where the side chain of histidine and the *N*-terminal NH_2-CHR-CONH group can be the two predominant ligands. Nevertheless, many factors can play a role such as type of metal, degree of metal loading, pH, and the overall structural features of the peptide or protein, potentially including a contribution from its other nucleophilic groups [10]. For proteins engineered to possess a hexa-histidine *N*-terminus, a Ni(II)-nitrilotriacetic acid form of IMAC, introduced by Hochuli et al. [11,12], has become important, e.g. [13]. The behavior of a hydrazide in IMAC apparently has not been reported before.

2. Experimental

2.1. Materials and chemicals

Chelating Sepharose Fast Flow (Sepharose-IDA) was purchased from Pharmacia (Uppsala, Sweden). Zinc sulfate and copper sulfate were obtained from Mallinckrodt (St. Louis, MO, USA). Nickel sulfate and sodium acetate were from Aldrich (Milwaukee, WI, USA). Ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA) was obtained from Fisher Scientific (Pittsburgh, PA, USA). 4,4-Difluoro-5,7-dimethyl-4-bora -3a,4a-diaza -3-indacenepropionyl hydrazide (BODIPY hydrazide, BO–HZ) was obtained from Molecular Probes (Eugene, OR, USA). BO–IMI was synthesized as described [1]. The acetate buffer was 0.1 *M* sodium acetate, pH 4.0, and the phosphate buffer was 0.1 *M* sodium phosphate, pH 7.0.

2.2. Separation of BO–IMI and BO–HZ by IMAC

Sepharose–IDA in ethanol (1.5 ml) was packed into a 10 ml disposable pipet plugged with glass wool. After the column was washed with 20 ml of distilled water, 10 ml of 0.2 M metal sulfate (copper, nickel or zinc) were applied. The unbound metal ions were washed off with 20 ml each of acetate and distilled water, and the column was equilibrated with 20 ml of phosphate. A 100 μl sample which contained 10 μg of BO–IMI and 10 μg of BO–HZ in phosphate was loaded onto the column and eluted with a stepwise pH gradient, starting from phosphate and followed by acetate–HCl buffers: pH 6, 5, 4, 3 and 2. The fractions were tested by CE–LIF.

2.3. Capillary electrophoresis

The capillary electrophoresis system, which is fitted with a laser-induced fluorescence detector (argon ion laser; 488 nm excitation, 520 nm detection) has been described [1]. The separation was achieved in a 65 cm (40 cm to detector)×75 μm I.D. capillary at 25 kV with anodic siphoning injection (column elevated 5 cm for 20 s). The running buffer is 0.01 *M* 2-(N-morpholino)ethanesulfonic acid, 0.0035 *M* tris(hydroxymethyl)aminomethane, 10% acetonitrile, pH 6.2.

2.4. Preparation of zinc-free solvents and tubes

Water and buffers for the following experiment involving zinc measurements were made metal-free by extraction with dithizone in chloroform as described [14]. Residual chloroform was removed by N_2 bubbling. Nitric acid (15%) soaking followed by washing with metal-free water was used to make the test tubes metal-free.

2.5. BO-HZ on Zn(II)-IMAC followed by Zn measurement

Sepharose-IDA gel (1.5 ml) was packed into a 5 ml disposable pipet and washed with 20 ml water. After adding 2 ml of 0.2 *M* $ZnSO_4$, 20 ml of acetate

and 50 ml of phosphate were applied to wash out unbound zinc. BO–HZ (15 μg) in 0.5 ml of phosphate was loaded onto the column and eluted with 25 ml of phosphate. Fractions were collected and analyzed for zinc content by atomic absorption spectroscopy.

3. Results and discussion

Target substances bound on an IMAC column typically are eluted by a gradient to a lower pH, or by the addition of imidazole, ammonium acetate, or a chelating agent like EDTA. For our purposes, involving preparative or analytical purification, a pH gradient was most attractive. Based on the extensive literature concerning IMAC of histidine-containing substances, we started the separation at pH 7.0 in 0.1 *M* sodium phosphate buffer, and selected to first test a Ni(II) form of IMAC.

Neither BO–IMI nor BO–HZ migrated on the Sepharose–IDA at pH 7.0 (visual observation of the yellow–green band of sample on the top of the column bed, which can be enhanced by exposure to a UV lamp, providing 365 nm), so we began a stepwise pH gradient employing 0.1 *M* sodium acetate buffer (HCl treated). The separation was monitored more quantitatively by collecting fractions and testing them off-line by CE–LIF (data not shown). These conditions nicely resolve the two compounds by Ni(II)–IMAC, with BO–IMI eluting much earlier than BO–HZ, as shown in Fig. 1A.

Similar testing of Cu(II) and Zn(II) forms of Sepharose–IDA gave the chromatograms displayed in Fig. 1B and 1C, respectively. As seen, the two compounds are separated under both of these conditions as well, but with a change in the order of retention on the Zn(II) column. We did not optimize any of the separations, since they were all adequate for our purposes. These chromatograms show that the decreasing order of retention is Cu(II)>Ni(II)> Zn(II) for BO–IMI, and for BO–HZ it is Cu(II)~ Ni(II)>Zn(II). When Belew et al. [15] tested several proteins on a TSK polymer IDA column, eluting with a linear gradient of imidazole, they similarly observed the retention order Cu(II)>Ni(II)>Zn(II). However, Porath reported the order Cu(II)>Zn(II)> Ni(II) for serum proteins on a Sepharose–IDA

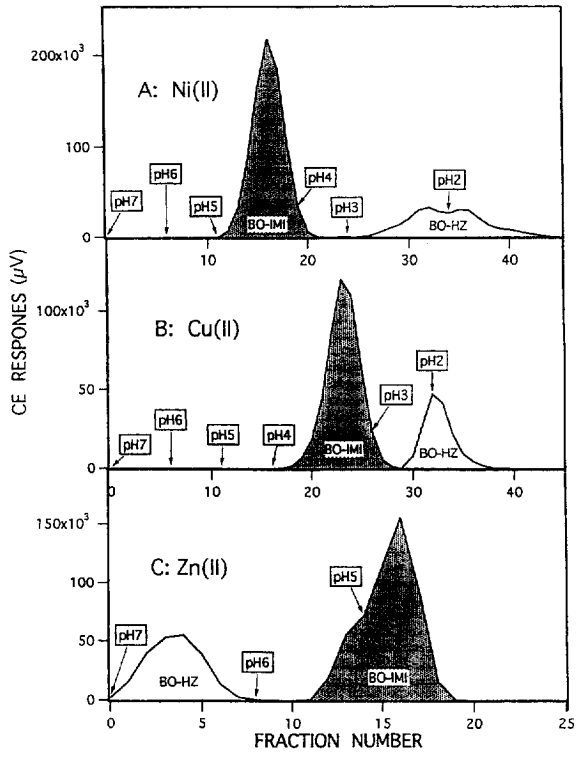

Fig. 1. IMAC chromatograms obtained by loading a Sepharose-IDA column in the Ni, Cu, or Zu form, as indicated, with a solution of BO-IMI and BO-HZ at pH 7.0, and eluting with stepwise changes to lower pH while monitoring the colored bands visually, and also collecting 2 ml fractions for measurement of BO-IMI and BO-HZ by CE-LIF.

column [3], as did Andersson and Sulkowski [16]. Anyway, the complexity of proteins and the variation in analytes and conditions among these studies makes it difficult to make meaningful comparisons.

We wish to speculate, in part, on the mechanisms producing the separations shown in Fig. 1. All three metal ions are medium–soft in their polarizability [5], so this concept does not seem to be helpful. It is known that hydrazides tend to bind as bidentates to metal ions [17–19], whereas imidazole acts as a monodentate ligand, so this could explain the stronger retention of BO–HZ than BO–IMI on the Ni(II) and Cu(II) packings. This speculation is represented in Fig. 2. Similarly, Hansen et al. [20] have pointed out that the *N*-terminal end of a peptide, in principle, may bind as a bidentate (α-

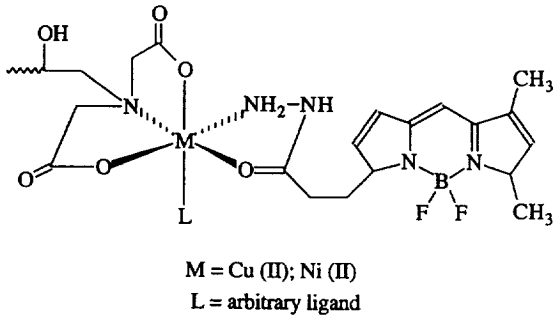

Fig. 2. Postulated bidendate binding of BO-HZ to the Ni(II) and Cu(II) forms of Sepharose-IDA. L=arbitrary ligand.

amino and nearby carbonyl) to Sepharose-IDA–Cu(II).

The weak retention of the BO–HZ on the Zn(II) column could be due either to a very weak (e.g., from monodentate binding) or strong binding of the Zn(II) by BO–HZ. In the latter case, BO–HZ would have eluted rapidly by stripping the Zn(II) from the IDA chelate, a general mechanism that has been called 'metal ion transfer' for IMAC [8]. We ruled out the latter mechanism by observing no displacement of zinc by BO–HZ from the Zn(II) column. Others have observed that peptides lacking histidine fail to retain on a Sepharose–IDA–Zn(II) column, even when they possess an *N*–terminal α-amino group [16,20].

The strong binding of a hydrazide on a Sepharose–IDA–Ni(II) or –Cu(II) column might be exploited. For example, many substances contain one or more carboxylic acid groups, and this group can be converted easily to a hydrazide, potentially enabling the substance then to bind reversibly on such a packing. If successful, this would expand the variety of extrinsic groups useful as 'affinity handles' in IMAC, which presently comprise multi-histidine groups [11–13] and certain di- or tripeptide sequences containing a terminal histidine [21,22]. A second, general suggestion is that a simple hydrazide like acetylhydrazide might be employed as an alternative eluent for an IMAC column. A final suggestion for future experiments is to test the behavior of hydrazides on other versions of IMAC, such as *tris*(carboxymethyl)ethylenediamine (TED; [5]) and nitrilotriacetic acid (NTA; [11]) columns.

4. Conclusion

BO–IMI and BO–HZ, which are imidazole and hydrazide species, respectively, can be separated readily on a Sepharose-IDA column. When the column is in the Ni(II) or Cu(II) form, BO–IMI elutes first, whereas BO–HZ elutes first on the Zn(II) form of the column. Potentially the usefulness of IMAC columns can be broadened by taking advantage of hydrazide as a ligand.

Acknowledgments

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