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Carbonyl-reducing enzymes as targets of a drug-immobilised affinity carrier

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ABSTRACT

Proteins, peptides and nucleic acids are commonly isolated and purified in almost all bioscience laboratories. Methods based on molecular recognition are currently the most powerful tool in separation processes due to their selectivity and recovery. The aim of this study was to prove the versatility and the ability of an affinity carrier containing the immobilised ligand oracin (previously developed by our workgroup) to selectively bind carbonyl-reducing enzymes. These enzymes play an important role in metabolic pathways of various endogenic compounds and xenobiotics. Many important drugs, such as doxorubicin, daunorubicin, haloperidol and the model anticancer drug oracin, are metabolised by carbonyl-reducing enzymes. The functionality of the presented carrier was demonstrated with pure recombinant enzymes (AKR1A1, AKR1B1, AKR1B10, AKR1C1, AKR1C2, AKR1C3, AKR1C4, CBR1 and CBR3) as well as with two model biological samples (cell extract from genetically modified *Escherichia coli* and pre-purified human liver cytosol). Enzymes that show an affinity toward oracin were efficiently captured, gently eluted using 150 mM ammonium hydroxide and subsequently identified by MS. The method is highly selective and robust and may be applied to the purification and identification of various carbonyl-reducing enzymes from any biological sample.

1. Introduction

Although genome-sequencing projects have provided a substantial amount of crucial information, they offer rather limited insights into the biochemical function of encoded proteins. Conversely, conventional biochemical methods that can clarify important aspects of the role of proteins in biochemical pathways also suffer from some shortcomings. Specifically, some of these methods cannot cope with the extremely high complexity of biological samples, which results in minor proteins being masked by highly abundant proteins. Therefore, sensitive separation techniques for the direct and selective recognition of target biomolecules in biological samples are increasingly needed [1,2].

Chemical proteomics represents an intersection of organic chemistry, structural biology, biochemistry, cell biology and mass-spectrometry [2]. Two major types of experiments combine various recognition techniques for protein separation with mass spectrometry to enable detailed structural analysis and characterisation: (I) activity-based probe profiling (ABPP) using active site-directed probes that covalently bind the target protein [3] and (II) compound-centric chemical proteomics (CCCP) utilising small molecules (e.g., substrates, inhibitors or cofactors) immobilised on an inert biocompatible matrix to form affinity carriers. In essence, this method combines classical drug affinity chromatography [4] with mass spectrometry [5].

The CCCP technique enables the identification of molecular targets that interact with a small molecule covalently bound to the solid phase of the carrier. This method can imitate conditions that resemble the *in vivo* environment to identify the biomolecules that interact with the immobilised drug molecules. This bioanalytical approach was successfully applied to determine the target or off-target (may be responsible for side-effects) proteins of clinically used drugs (e.g., flavopiridol [6], roscovitine [7], gefitinib [8]). The CCCP approach has also been widely used to identify kinase inhibitors and study enzymes from the group of kinases (important in signalling pathways) [9–11]. In addition, other types of drugs, such as tacrolimus [12], methotrexate [13] and CB30865 [14]), have been similarly utilised. Similarly, endogenous substances, such as oxalate [15,16], certain types of prostaglandin J_2 [17] and

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L-arginine [18], have been efficiently utilised. The procedure was also used to determine the molecular targets of biologically active compounds, such as petrosapongiolide M [19], ampicillin [20], jacalin [21] and resveratrol [22,23].

The ability of a biofunctionalised carrier with covalently bound oracin to capture bioactive molecules with carbonyl-reducing activity was clearly demonstrated in a previous study [24]. Some of the enzymes from this protein group play an important role in the metabolism of various endogenous (e.g., steroids, prostaglandins) and xenobiotic (e.g., oracin, anthracyclines) compounds and are thus involved in various pathological processes, such as hormone-dependent cancers and metabolic syndrome [25,26]. However, many are poorly characterised, and thus, their study may provide new significant insights regarding the aforementioned roles.

In this study, we optimised a previously published separation protocol for the isolation of carbonyl-reducing enzymes [24] and demonstrated the versatility and selectivity of an oracin-immobilised affinity carrier to isolate desired bioactive molecules, even from complex biological samples. This approach can be used to obtain these enzymes at the required purity and amount to perform detailed MS analysis without limitations.

2. Experimental procedures

2.1. Materials

Oracin was obtained from the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic). The following chemicals were purchased from Sigma–Aldrich (Prague, Czech Republic): 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), *N*-hydroxysulfosuccinimide sodium salt (S-NHS), bis-(3-aminopropyl)amine (BAPA), D-glucose-6-phosphate sodium salt, $NADP^+$, glycerol, acrylamide, N,N′-methylene-bis-acrylamide, *N*,*N*,*N*,*N*-tetramethylenediamine (TEMED), acetonitrile (HPLC grade), trifluoroacetic acid (TFA) and bovine serum albumin (BSA). 6-Bromohexanoic acid and Coomassie Brilliant Blue G-250 were obtained from Fluka (Buchs, Switzerland), and glucose-6-phosphate dehydrogenase (from baker's yeast [*Saccharomyces cerevisiae*]) was obtained from Roche (Penzberg, Germany). The Precision Plus protein standard was purchased from Bio-Rad (Hercules, CA, USA). Sequencing-grade trypsin was obtained from Promega (Madison, WI, USA). All of the other chemicals were of the highest available purity grade.

2.2. Carrier with covalently immobilised oracin for the isolation of carbonyl-reducing enzymes

An affinity carrier was prepared according to Škarydová et al. [24]. The surface of the SiMAG-COOH microparticles (Chemicell, Berlin, Germany) was modified with BAPA molecules using the carbodiimide coupling method. Subsequently, the ligand, namely oracin, was immobilised via ester aminolysis.

2.3. Preparation of recombinant forms of proteins

The carbonyl-reducing enzymes AKR1A1, AKR1B1, AKR1B10, AKR1C1, AKR1C2, AKR1C3, AKR1C4, CBR1 and CBR3 were prepared in an *Escherichia coli* expression system according to previously published methods [27].

2.4. Preparation of human liver cytosol

Human liver samples were obtained from the Cadaver Donor Programme of the Transplant Centre of the Faculty of Medicine (Hradec Králove, Czech Republic) in accordance with Czech legislation. The preparation of the human liver subcellular fraction from the obtained tissues was previously described by Škarydová et al. [28].

2.5. Sample preparation

2.5.1. Sample A

Solution of 17.6 μg of pure recombinant carbonyl-reducing enzymes (AKR1A1, AKR1B10, AKR1C1, AKR1C2, AKR1C3, AKR1C4, CBR1 or CBR3) in 200 μl of buffer A (60 mM Na-phosphate buffer, 1.1 mM EDTA, 150 mM NaCl, and 10% (v/v) glycerol, pH 7.4).

2.5.2. Sample B

Mixture of 5 μg of AKR1C3 and 5 μg of CBR1 in a total volume of 200 μl of buffer A.

2.5.3. Sample C

E. coli cells overexpressing AKR1C3 were disrupted with the BugBuster® Protein Extraction Reagent (Novagen, Madison, WI, USA), and the obtained supernatant was mixed with buffer B (60 mM Na-phosphate buffer, 1.1 mM EDTA, 150 mM NaCl, 10% (v/v) glycerol, and 0.01% Triton X-100, pH 7.4) at a ratio of 1:1 (v/v). The total volume of the samples used for separation on the affinity carrier was 200 μl.

2.5.4. Sample D

One millilitre of human liver cytosol separated by size-exclusion chromatography (Section 2.6).

2.6. Size-exclusion chromatography

One millilitre of human liver cytosol (10 mg/ml) was concentrated by centrifugation at 7000*g* and 4 °C in an Amicon test tube to a volume of 100 μl and separated on a Superdex G 75/200 GL column (Amersham Biosciences, Uppsala, Sweden) that was previously equilibrated with buffer B. The flow rate of the mobile phase was 0.5 ml/min, and the volume per fraction was 1 ml. The fraction with a major portion of proteins larger than 37 kDa removed was subjected to affinity separation.

2.7. Affinity chromatography

A previously described protocol for carbonyl-reducing enzymes [24] was modified in terms of the buffer composition and elution conditions (all changes were the results of an optimisation process and are stressed in Table 1). The chelating agent EDTA was added to all of the buffers at a concentration of 1.1 mM. The concentration of NH_4OH in the elution buffer was increased from 45 to 150 mM, and each elution step was prolonged from 1 to 10 min by mixing on a rotator. The separation protocol for complex biological samples was further modified by the addition of a blocking step with 1% bovine serum albumin (BSA) and the addition of 0.01% Triton-X100 into binding buffer.

2.7.1. Separation protocol using oracin-affinity carrier

One milligram of the affinity carrier was washed with buffer A. Subsequently, sample A or B was loaded on the equilibrated affinity carrier. After incubating the sample with the carrier for 1 h under gentle mixing on a rotator, the unbound enzyme was removed, and the carrier was washed several times with buffer A. The captured enzyme molecules were eluted with 200 μl of elution buffer (150 mM NH_4OH, 1.1 mM EDTA, and 10% (v/v) glycerol, pH 11) by mixing for 10 min on a rotator. The affinity carrier was then washed with buffer A and prepared for subsequent use. The separation process was performed in a cold room at 8 °C. All of the fractions acquired during the isolation were analysed by