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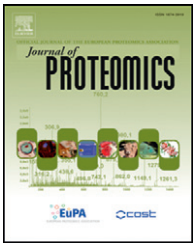

On-bead tryptic proteolysis: An attractive procedure for LC-MS/MS analysis of the Drosophila caspase 8 protein complex during immune response against bacteria☆

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ABSTRACT

This study aims to characterize the immune response against bacteria in Drosophila melanogaster. Obtaining a description of the *in vivo* state of protein complexes requires their isolation as a snapshot of physiological conditions before their identification. Affinity purification with streptavidin–biotin system is widely used to address this issue. However, because of the extraordinary stability of the interaction between streptavidin and biotin, the release of biotin-labeled bait remains a challenge.

We transfected Drosophila cells with a DNA construct encoding a biotin-tagged Dredd protein (ortholog of caspase 8). After affinity purification, different strategies were evaluated, and proteins analyzed by LC-MS/MS mass spectrometry. The on-bead digestion allowed the identification of more proteins associated to the Dredd complex than different protocols using competitive or acid elution.

A functional assay showed that a large part of the proteins specifically identified in the Dredd sample are functionally involved in the activation of the Imd pathway. These proteins are immune response proteins (BG4, Q9VP57), stress response proteins (HSP7C, Q9VXQ5), structural proteins (TBB1, CP190), a protein biosynthesis protein (Q9W1B9) and an antioxidant system protein (SODC). Our results clearly show that on-bead digestion of proteins is an attractive procedure for the study of protein complexes by mass spectrometry. This article is part of a Special Issue entitled: Translational Proteomics.

Abbreviations: cDNA, complementary deoxynucleotid acid; dsRNA, double strand ribonucleic acid; FA, formic acid; FLU, firefly luciferase activity arbitrary unit; NHS, N-hydroxysulfosuccinimide; PEG, polyethylene glycol; RT, room temperature.

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1. Introduction

In this work, we studied the antibacterial immune response against a microbial pathogen in Drosophila melanogaster. Studies carried out in this model, based on genetic analysis, have unraveled that two intracellular signaling cascades govern the reprogramming of gene expression following microbial infections: (1) the Toll pathway is activated upon fungal and Gram-positive bacterial infections; (2) the Imd pathway responds primarily to Gram-negative bacteria. Both pathways control the challenge-induced expression of hundreds of genes, which include the canonical antifungal and antibacterial peptides [see 1, for a review]. Genetic studies on the Imd pathway identified a dozen genes whose protein products are required for immune-induced transcription of antibacterial peptide genes [1]. Bacterial diaminopimelic acid-containing peptidoglycan recognized by the peptidoglycan recognition proteins LC (cell surface receptor) and LE (cytosolic receptor) activates the Imd pathway. Activation of the pathway leads to the initiation of a signal transduction cascade, mediated by Imd, Dredd (structurally related to vertebrate caspase-8), its adaptator Fas-associated death domain protein (FADD, also named BG4), TAK1-binding protein 2 (TAB2) and inhibitor of apoptosis 2 (dIAP2). Activation of this pathway results in the activation of the NF-κB precursor Relish and transcriptional induction of genes encoding antibacterial peptides [2–5]. RNAi based studies suggest that in this pathway, Dredd and FADD have two distinct roles. First, Dredd and FADD were shown to be required for immune-induced activation of Imd pathway, downstream of Imd protein but upstream of TAK1. Second, Dredd is thought to cleave and activate Relish [for a review, see 6]. However, for several of these proteins, the question of how they interact within the cascade has not been clearly addressed. In order to be fully effective, each of these proteins requires probably several unidentified molecular partners.

The stable, non-covalent interaction between avidin and biotin is one of the most common tools in chemistry and biology. Methods for derivatization of a variety of molecules (in particular, proteins) with biotin have been introduced, in order to allow their efficient recovery, immobilization and detection with avidin-based reagents [for a review, see 7]. Biotinylated proteins can be easily detected with avidine derivatives, and efficiently captured on avidin/streptavidin-coated solid supports such as resins or beads.

In order to identify the molecular partners of a protein of interest, widely used protocols consist of 1) biotin labeling of the protein of interest, 2) *in vitro* or *in vivo* incubation with candidate proteins, and 3) affinity purification of the complex. Then, proteins are eluted and, after enzymatic digestion, they are submitted to mass spectrometry analysis for identification.

The elution step is of a crucial importance in this workflow. Due to the extraordinary stability of the interaction between biotin and streptavidin ($K_D = 10^{-15}$ M) [8], the elution remains a challenge. An ideal elution should allow releasing all the proteins in a buffer whose composition and volume are suitable for shotgun proteomic analysis.

Many different approaches have been used to release the purified complexes [for a review, see 7]: (1) The use of cleavable spacer groups, such as N-hydroxysulfosuccinimide (NHS)-biotin, the linker NHS-photocleavable-LC-biotin and monodisperse PEG-based arms, is efficient in many cases. However, they may show insufficient stability in some biological fluids prior to purification, and may be less efficient when the spacer arm is buried in the protein complex. (2) Streptavidin molecules are also modified in order to allow elution under milder conditions. For an example, monomeric avidin, which displays a lower binding affinity for streptavidin, can be used. This is the basis of important methodologies in quantitative proteomics, such as ICAT methodology [9]. However, the lower affinity of avidin–biotin interaction prevents an efficient capture of biotinylated molecules in the presence of strong detergents. (3) Harsh elution conditions associated with competition with free biotin have also been used [10]. This method is compatible with protein analysis by SDS-PAGE and subsequent mass spectrometry analysis, and can be applied to the biotinylation of protein extracts, intact cells and organs. However, the use of SDS makes this protocol difficult to use in gel-free approaches.

Another possible strategy is to perform the enzymatic digestion directly on the beads, and to elute proteolytic peptides instead of whole intact proteins. This approach has been used for immunoprecipitated proteins [11–13]. In some reports [14–16] biotinylated proteins were directly digested with Lys-C and/or trypsin before elution. In the present work, we aim to assess the feasibility of on-bead proteolytic digestion for the identification of non-tagged molecular partners of a biotinylated protein.

To reach this goal, we stably overexpressed Dredd, a key player of Imd pathway activation [17], in *Drosophila* cells. This target protein had been coupled to a biotin-Tag, which allows affinity purification. Proteins co-purified with the Dredd protein were eluted or subjected to a direct on-bead tryptic proteolysis before mass spectrometry (MS) analysis. The biological significance of the identified proteins was assessed by knocking down the corresponding gene transcript through RNA interference, followed by a luciferase reporter assay which quantitatively measures the immune response against bacteria.

2. Material and methods

2.1. Cloning and plasmid construct

cDNA from the Dredd (FBgn0020381) gene was amplified by PCR and inserted into a pDONR221 vector (Invitrogen) using Gateway® cloning system according to the manufacturer's instructions.

Briefly, forward primer consisted of 31 nucleotide sequence containing attB1 sequence and gene specific sequence depleting Met codon in the beginning of coding sequence. Reverse primers contained gene specific primers, followed by triple Stop codons and attB2 sequence. By recombination reaction between PCR fragments and pDONR221, pENTRY-N-DREDD was obtained and sequenced. Then, we generated an insect expression vector pIZ/V5-His (Invitrogen)-backborn pDEST-BIO-N containing biotin-tag sequence (encoding the GLNDIFEAQKIEWHE peptide), a chloramphenicol resistance gene and the ccdB gene flanked by attR1 and attR2 sites, and *Drosophila* C virus inter ribosomal entry site (between gp1 and gp2) fused with