**Novel methods using fluorophosphonate-biotin probe for quantification and profiling of serine hydrolases**

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Serine hydrolases are one of the largest and most diverse enzyme families, they mediate essential role in many biological processes. However, many of the serine hydrolases are still uncharacterized in regard to physiological substrates and functions. In addition, the expressions of serine hydrolases in several human tissues are not well characterized. Based on the conserved catalytic mechanism of serine hydrolases, an activity probe was developed, fluorophosphonate (FP)-biotin. FP-biotin reacts specifically with the active serine residue forming strong covalent bond. In the present study, we utilized FP-biotin in two approaches. The first approach is to develop a quantitative western blotting method to measure the expression level of recombinant serine hydrolases. The second approach is to characterize the expression of serine hydrolases in the synoviocyte.

The utility of FP-biotin in the analysis of serine hydrolases was assessed using porcine liver esterase (PLE). FP-biotin has reacted specifically with the catalytically active PLE, not with denaturated or inhibited PLE. This reaction resulted in complete inhibition of PLE by two fold higher concentration of FP-biotin, which is a racemic mixture of two stereoisomers and one stereoisomer is active. This confirms the 1:1 stoichiometric binding of the probe to PLE. Based on these properties, FP-biotin was utilized in the quantitative measurement of serine hydrolases using western blotting analysis and avidin-peroxidase. The linearity of detection was examined on three serine hydrolases; human carboxylesterase (CES) 1, butyrylcholinesterase and PLE. Similar response signals were obtained from the equimolar concentrations of these enzymes and excellent linearity was observed at the range of 0.4 – 3.4 pmol/lane ($r^2 > 0.99$). Accuracy and precision of the method were proved using PLE with recovery of 97.1 – 107.2% and relative standard deviation of 5.56%. PLE was selected as a calibration standard because of its high stability and commercial availability. The developed method was applied to measure the expression levels of four recombinant CES isozymes from human and cynomolgus macaque in S9 fraction of HEK293 cell homogenates. The expression levels of human CES1 and CES2, and cynomolgus macaque CES1 and CES2 were 2.51±0.1, 1.63±0.17, 0.79±0.09 and 1.37±0.13 pmol/5µg S9 protein, respectively. Based on these determinations, their hydrolytic activities were accurately assessed. Cynomolgus CESs showed lower hydrolysis activities for *p*-nitrophenyl esters than human CESs. The hydrolase

activities of CES2 isozymes were higher than CES1 in both species. Three to five folds faster hydrolysis for *p*-nitrophenyl butyrate than *p*-nitrophenyl acetate was observed in all CES isozymes except of cynomolgus CES1 that showed nearly same hydrolysis for both substrates. The developed method could be widely used for universal quantitative analysis of recombinant serine hydrolases.

Characterization of serine hydrolases expression in the synoviocytes would facilitate better understanding of the arthritis pathogenesis, discovery of new therapeutic targets and better design of intra-articular drug with predictable metabolism. The expression of serine hydrolases in the human fibroblast-like synoviocytes (HFLS) were analysed with FP-biotin probe, native-PAGE and LC/mass spectrometry. In addition, the rabbit synoviocytes (HIG-82) cells, were also analyzed and compared to the human synoviocyte (HFLS), since the rabbit arthritis models are commonly used in studying arthritis pathogenesis and evaluating arthritis therapies. In the native-PAGE analysis of HFLS cell homogenate, three protein bands (Band 1-3) that hydrolysed 4-methylumbelliferyl butyrate were detected and inhibited by FP-biotin. Protein band 1 and 2 showed limited substrate specificity to 4-MUB, while band 3 had broad substrate specificity. Distinct subcellular localization of the detected protein bands was observed. Two of these protein bands were conserved in HIG-82 and HFLS cell homogenates. FP-biotin was utilized to enrich and isolate the proteins from each detected bands in the HFLS cell homogenate by using streptavidin microbeads to be analysed by LC-mass spectrometry (MS). From the protein bands 1 and 2, several proteins were identified which have catalytic or regulatory functions. Two proteins that have hydrolytic activities were identified, neutral alpha glucosidase AB (GANAB) and transitional endoplasmic reticulum ATPase (TERA). Several annexins and chaperones were identified in the same band with these hydrolytic enzymes such as annexin A1, annexin A2, endoplasmin and 78 kDa glucose regulated protein. In the protein band 3, five proteins were detected that might be in association, including: protein disulfide isomerase (catalyse the formation of disulfide bonds), calreticulin (involved in protein folding), annexin A5 and 14-3-3 proteins epsilon and beta/alpha (regulatory proteins). However, no hydrolases were detected in this band, which might be attributed to its low expression level, in spite of its high hydrolytic activity. Further LC-MS analysis is required using affinity purification of FP-biotin-labelled peptides to increase the sensitivity of detection of low abundant serine hydrolases and to identify the new target proteins that covalently bound to FP-biotin. In addition, the hydrolytic activities of GANAB and ATPases require further investigation.