Abstract and keywords

The current thesis aims at the production of three enzymes, including beta-secretase 1 (BACE1; beta-site amyloid precursor protein cleaving enzyme 1), acetylcholinesterase (AChE), and butyrylcholinesterase (BChE). BACE1 is an integral membrane protein that plays a crucial role in the amyloid precursor protein's cleavage. The product is subsequently processed by y-secretase, producing amyloid-beta peptides and insoluble amyloid plaques in Alzheimer's patients (AD). According to the Annual Report of the Czech Alzheimer's Society, 158,000 patients were diagnosed in 2019. By 2050, this number is supposed to reach 300,000 patients. AChE inhibitors and N-methyl-D-aspartate receptor antagonists are currently the only alternatives for AD therapy. AChE is also a target enzyme in nerve agent (NA) poisoning (together with BChE). The Department of Toxicology and Military Pharmacy focuses on studying NA effects and medical protection against them. From this perspective, the production of AChE and BChE is essential for developing and evaluating newly synthesized cholinesterase (ChE) reactivators and inhibitors.

The thesis focuses on introducing the expression system Expi293 to produce human recombinant enzymes BACE1, AChE, and BChE. For each enzyme, the individual steps required to obtain a sufficient amount of active protein are described. Because BACE1 is a transmembrane protein, it was isolated from cells, while ChEs were secreted into the medium.

A simple purification protocol was established for each expressed enzyme, utilizing cobalt affinity chromatography, Sepharose4B/procainamide resins, and Hupressin resins for BACE1, AChE, and BChE, respectively. Purification of the enzymes was followed by protein identification, using gel electrophoresis, to determine the approximate molecular weight of the purified enzymes. Recombinant proteins were subsequently verified by western blotting with an anti-His antibody. Both methods showed a band of mature BACE1 at position ~75 kDa, a band of mature AChE at position ~70 kDa, and a band of mature BChE at position 100 kDa.

For each enzyme, kinetic properties were determined, including specific activity and Km and IC_{50} values for their selective inhibitors. The specific activity of human recombinant BACE1 was approximately 120,000 ± 4,000 $RFU \cdot min^{-1} \cdot \mu g^{-1}$. The K_m

value of 7-methoxycoumarin-4-acetyl-[Asn670, Leu671]-amyloid β/A4 precursor protein 770 fragment 667-676-(2,4-dinitrophenyl)Lys-Arg-Arg amide trifluoroacetate salt was not determined due to its poor solubility. Verubecestat IC_{50} was 1.765 ± 0.036 nM.

Human recombinant AChE specific activity was 20,000 $U \cdot min^{-1} \cdot \mu g^{-1}$. The K_m value of acetylthiocholine was 0.288 ± 0.020 mM. Donepezil IC_{50} was 7.834 ± 0.054 nM.

Human recombinant BChE specific activity was 50,000 $U \cdot min^{-1} \cdot \mu g^{-1}$. The K_m value for the butyrylthiocholine was 0.256 ± 0.003 mM. The IC50 value for the selective inhibitor ethopropazine was 0.477 ± 0.045 μM.

Both BACE1 and AChE were stabilized by lyophilization. Due to its known long-term stability, BChE was dissolved in 20 mM Tris buffer pH 7.5. All enzymes were stored at -80 °C, which ensured their long-term stability and activity for their further use.

Keywords: beta-secretase 1, acetylcholinesterase, butyrylcholinesterase, Alzheimer's disease, Expi293 expression system, protein production, purification, inhibitors