## Structural and functional studies of novel Ntn-amidohydrolases

## for potential antileukemic applications

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L-Asparaginases are enzymes that hydrolyze L-asparagine to L-aspartic acid and ammonia. Currently, type II L-asparaginases of bacterial origin are utilized in the therapy of acute lymphoblastic leukemia (ALL). Unfortunately, their administration is accompanied by severe toxicity, and those strong side effects emphasize an urgent need for new types of therapeutic L-asparaginases. Promising candidates for antileukemic agents may be found among type III L-asparaginases, represented by the *Escherichia coli* enzyme EcAIII, which belong to Ntn-amidohydrolases, i.e. are expressed as inactive precursors that develop their enzymatic activity upon autoproteolytic cleavage (into subunits α and β, Fig. 1).

###### **Obraz zawierający mapa, sztuka Zawartość wygenerowana przez sztuczną inteligencję może być niepoprawna.**

**Figure 1.**Immature EcAIII is a (A) homodimer consisting of two protomers (green and purple). Upon maturation, each protomer is divided (B) into two subunits, e.g., green (α) and blue (β). The solid spheres represent the catalytic Thr residue (or its substitution to Ala). (C) Active site of EcAIII.

New L-asparaginases belonging to the Ntn-hydrolase family were designed via mutagenesis and chimeragenesis, produced recombinantly, and characterized. Site-directed mutagenesis was carried out (i) in the EcAIII catalytic center, which possesses conserved residues important for substrate hydrolysis and for autoproteolysis, and (ii) in regulatory elements (such as a stabilization loop) important for maintaining proper geometry of the active site. In addition, random mutagenesis was performed in the interdomain linker region. Chimeric proteins, possessing one domain originating from EcAIII and another from its plant counterparts, were also designed and characterized.

Site-directed mutagenesis combined with bioinformatic analysis enabled the development of new EcAIII variants with enhanced catalytic efficiency (kcat), while the crystal structure of an EcAIII mutant revealed that the sodium-binding stabilization loop is a region that can be optimized to increase the catalytic activity of EcAIII. Crystallographic and enzymatic studies also demonstrated that site-directed mutagenesis in the active site can strongly affect EcAIII autoproteolytic maturation, and the same observation was made for the linker region. Biochemical studies confirmed that chimeric proteins possessing fragments of EcAIII combined with sequence motifs from plant proteins are stable and active enzymes, retaining the activity of their parental proteins.

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