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# Experimental and bioinformatic approach to identifying antigenic epitopes in human α- and β-enolases

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ABSTRACT

Human α- and β-enolases are highly homologous enzymes, difficult to differentiate immunologically. In this work, we describe production, purification and properties of anti-α- and anti-β-enolase polyclonal antibodies. To raise antibodies, rabbits were injected with enolase isoenzymes that were purified from human kidney (α-enolase) and skeletal muscle (β-enolase). Selective anti-α- and anti-β-enolase antibodies were obtained by affinity chromatography on either α- or β-enolase-Sepharose columns. On Western blots, antibodies directed against human β-enolase, did not react with human α-isoenzyme, but recognized pig and rat β-enolase. To determine what makes these antibodies selective bioinformatic tools were used to predict conformational epitopes for both enolase isoenzymes. Three predicted epitopes were mapped to the same regions in both α- and β-enolase. Peptides corresponding to predicted epitopes were synthesized and tested against purified antibodies. One of the pin-attached peptides representing α-enolase epitope (the C-terminal portion of the epitope 3 - $S^{262}$PDDPSRYISPDQ$^{273}$) reacted with anti-α-enolase, while the other also derived from the α-enolase sequence (epitope 2 - $N^{193}$VIKEKYGKDATN$^{205}$) was recognized by anti-β-enolase antibodies. Interestingly, neither anti-α- nor anti-β-antibody reacted with a peptide corresponding to the epitope 2 in β-enolase ($G^{194}$VIKAKYGKDATN$^{206}$). Further analysis showed that substitution of $E^{197}$ with A in α-enolase epitope 2 peptide lead to 70% loss of immunological activity, while replacement of $A^{198}$ with E in peptide representing β-enolase epitope 2, caused 67% increase in immunological activity. Our results suggest that $E^{197}$ is essential for preserving immunologically active conformation in epitope 2 peptidic homolog, while it is not crucial for this epitope's antigenic activity in native β-enolase.

## 1. Introduction

Enolase (E.C. 4.2.1.11) is a dual function enzyme essential for cellular processes. It acts as 2-phospho-D- glycerate hydro-lyase in glycolysis pathway, and as phosphoenolpyruvate hydratase in gluconeogenesis pathway [1]. This highly conserved protein retains similar catalytic function in prokaryotes and eukaryotes [2]. In many organisms, including primates and lower mammals, enolase is responsible for both catabolic and anabolic processes. Enolase is enzymatically active as a dimer. Three types of subunits, α, β and γ, each encoded by a separate gene can form a dimer. Both homo- and heterodimers are formed. Expression of α, β and γ subunits is regulated developmentally and in a tissue-specific manner [1–3]. The α homodimer is found in human fetus and adult tissues such as lung, liver, adipose tissue, pancreas, spleen and kidney. The β isoenzyme is present in tissues with high energy requirements such as heart and skeletal muscle (αβ and ββ isoenzymes), while γ-enolase is found in neuronal and neuroendocrine cells (αγ and γγ isoforms). The ββ enolase accounts for 3% of soluble proteins in human skeletal muscle and more than 90% of overall enolase activity [3,4].

Some highly conserved proteins sometimes perform multiple functions, often very different from their well-known “classical” activities.

*Abbreviations:* AP, alkaline phosphatase; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray injection; HRP, horse radish peroxidase; IgG, immunoglobulin G; LC, liquid chromatography; MeOH, methanol; MS, mass spectrometry; OPD, ortho-phenylenediamine; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; pNPP, para-nitrophenyl phosphate; SDS, sodium dodecylsulfate; TBST, 20 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween-20; UPLC-Q-TOF-MS, ultrapressure liquid chromatography, quadrupole-time-of-flight mass spectrometer; WB, western blotting

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Such proteins were recently named “moonlighting” proteins [5]. A considerable number of glycolytic pathway enzymes, including enolase, exhibit non-glycolytic functions [6]. In eukaryotic cells enolase is primarily located in the cytosol, where besides its catalytic function it participates in a regulation of the cell morphology and is interacting with the cytoskeleton [7]. The enolase has also been detected in mammalian cell nuclei where it is participating in the transcriptional regulation of genes involved in cells' morphological transformation and proliferation [8,9]. The α-enolase has been implicated in numerous diseases [2,10] including metastatic cancer [11,12], neurodegenerative diseases [13], autoimmune disorders [14–16] and in bacterial infections [17]. Additionally, there are reports describing α-enolase as being expressed at the cell surface of nonpathogenic and pathogenic microorganisms [18–20]. The disease-related role of α-enolase is mostly attributed to its immunogenic capacity, DNA-binding ability, and plasmin/plasminogen receptor function [10]. It is important to note that enolase primary and tertiary structures are highly conserved. Alignment of human enolase isoenzymes shows approximately 83–84% of sequence identity and 91–93% of similarity. However, some structural differences between enolases of human tissue-specific variants and lower-mammals exist [2,3,21].

For all enolase isoforms, the active site and subunit interface is most conserved and composed of the same amino acids [2]. However, short variable region localized at the surface of the molecule is probably responsible for enolase interaction with other macromolecules [22]. Moreover, this variable region may also act as a site of the enolase distinct antigenic epitopes. This notion has been supported by the identification of non-cross-reacting antibodies recognizing different enolase isoenzymes in patients with auto-immunological diseases. The sera of patients with lupus erythematosus and hypophysitis do not react with rabbit muscle enolase but recognize human α-enolase [23,24]. It is noteworthy to add that α-enolase is a common autoantigen in several autoimmune diseases [25–27], a feature related to noncatalytic properties of this protein. The α-enolase also functions as a plasminogen receptor, a modulator of the cell growth and differentiation by downregulating c-myc protooncogene expression, a structural protein in the lens of some species, and possibly as a suppressive lymphokine [10,15,28,29]. The mass spectrometry offers the best accuracy in detecting α-enolase in biological samples and in discriminating it from other proteins [30,31]. However, the abundance of α-enolase in autoimmune diseases renders it less attractive as a biomarker.

On the other hand, the human muscle-specific enolase may become useful marker in diagnosis of the muscule-related diseases. During cardiac and skeletal muscle development the β-enolase progressively replaces α-isoform and becomes an early marker of the myogenic differentiation [7,32]. For this reason, β-enolase was described as a sensitive histological marker in rhabdomyosarcoma [4]. Muscle-specific enolase deficiency was observed in metabolic myopathy [33]. An increase of the β isoenzyme occurs in the serum of the patients with both acute myocardial infarction and exercise-induced muscle damage [34,35]. Furthermore, detection of the β-enolase in bloodstains after traumatic skeletal muscle injury has a potential to be used in a forensic practice [36]. Only a few reports refer to immunochemical detection of β-enolase in biological material [32,35–38]

In this work bioinformatic and empirical approaches were used to identify antigenic epitopes in human α- and β-enolases. The gained knowledge was used to raise specific antibodies against these two enolases. Polyclonal antibodies recognized some of the predicted epitopes.

The gained knowledge will aid us in obtaining specific α-and β-enolase monoclonal antibodies and help in developing diagnostic test enabling differentiation of these two isoenzymes.

## 2. Materials and methods

### 2.1. General

All chemicals were of the analytical grade. 2-Phospho-D-glycerate was purchased from Fluka (FlukaAnalytical, St. Gallen, Switzerland). The molecular mass protein markers for SDS-PAGE were from Bio-Rad (Bio-Rad Polska, Warsaw, Poland). Other reagents were purchased from Sigma-Aldrich (Sigma-Aldrich Poland, Poznan, Poland). LC/MS-grade acetonitrile, water, and formic acid were from Baker Company (J.T. Baker, Griesheim, Germany). Secondary antibodies for ELISA and Western blotting were purchased from Promega (Promega Corp., Madison, USA). The Horse Radish Peroxidase (HRP) substrate, o-phenylenediamine, was acquired from Promega, while the alkaline phosphatase (AP) substrate, p-nitrophenyl phosphate, came from Sigma-Aldrich. The EZ-Link™ NHS-PEG Solid Phase Biotynylation Kit: *spin columns* and fluorescein-labeled streptavidin were obtained from Thermo Scientific (Thermo Scientific, Rockford, USA).

The human tissues for enolase purification were obtained as histologically normal tissues from Department of General, Vascular and Transplantation Surgery of Wroclaw Medical University. Kidney as a blood-group mismatched and not immunogenic organ from a healthy individual (positive opinion No. KB-516/2005 issued by the Bioethics Committee of Wroclaw Medical University) and samples of human tibialis anterior muscle as postoperative material, in accordance with Polish legal requirements under the license issued by the Commission of Bioethics of Wroclaw Medical University (positive opinion No. KB-516/2005). The fresh rat skeletal muscle samples were obtained from the Animal Laboratory of the Wroclaw Medical University, and pig skeletal muscle samples were purchased at the local slaughterhouse. New Zealand rabbits were purchased from local rabbit breeding farm. All experiments involving rabbits were performed at the animal facility at the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wroclaw. Animals were cared for in accordance with criteria approved by the Institutional Animal Ethical Committee of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wroclaw (LKE 53/2009).

### 2.2. Purification of α- and β-enolases

At all stages of the enzyme purification, the magnesium sulfate and temperature set at 4 °C were used to prevent the loss of the enolase activity. Magnesium ions are essential for both catalysis and stability of the enolase dimer [2,39]. Purification of β-enolase from human muscle tissue was performed according to the procedure described earlier [40]. Briefly, 110 g of frozen striated muscle was homogenized in deionized water containing 3 mM $MgSO_4$ and protease inhibitors PMSF (phenylmethylsulfonyl fluoride) and aprotinin (2 μg/ml). Homogenization was performed in PT3100D homogenizer (DanLab, Poland) at 4 °C for 10 min. The crude protein extract was incubated at 53–54 °C for 3 min, then cooled to 4 °C and denatured proteins removed by centrifugation at 9000 g for 45 min. The supernatant was fractionated with ammonium sulfate at 4 °C, by adding solid salt in small portions and by stirring gently. The mixture was left overnight at 4 °C after reaching 60% saturation of $(NH_4)_2SO_4$ and precipitated proteins were centrifuged at 9000 g for 45 min and discarded. Protein precipitation was continued by adding solid $(NH_4)_2SO_4$ up to 80% saturation, and after overnight storage at 4 C the precipitated proteins were centrifuged. The pellet was dissolved in 20 mM Tris-HCl buffer pH 9.0, containing 3 mM $MgSO_4$ and 1 mM β − mercaptoethanol and dialyzed against this buffer. In the following step, the sample was loaded on a DEAE-Sephadex A-50 column (30 × 3 cm) equilibrated with the same buffer. Under these conditions, β-enolase did not adhere to this ion-exchanger, and fractions containing enolase activity were eluted (0.08 ml/min flow-rate) with equilibrating buffer and precipitated with ammonium sulfate. The pellet was dissolved in 10 mM phosphate (Na+) buffer pH 6.4,