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Identification of specific interaction of juvenile hormone binding protein with isocitrate dehydrogenase

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Juvenile hormone (JH) is essential for multiple physiological processes: it controls larval development, metamorphosis and adult reproduction. In insect hemolymph more than 99% of JH is bound to juvenile hormone binding protein (JHBP), which protects JH from degradation by nonspecific hydrolases and serves as a carrier to supply the hormone to the target tissues. In *Galleria mellonella* hemolymph, JHBP is found in a complex with lipid-binding high molecular weight proteins (HMWP) and this interaction is enhanced in the presence of JH. In this report, we present studies on the interaction of JHBP with low molecular weight proteins (LMWP) in the hemolymph. Using ligand blotting we found that JHBP interacts with a protein of about 44 kDa. To identify the protein that preferentially binds JHBP, a LMWP fraction was applied to a Sepharose-bound JHBP and, after washing, the column was eluted with free JHBP acting as a specific competitor or with carbonic anhydrase as a negative control. The eluted proteins were separated by SDS/PAGE and analyzed by mass spectrometry. Isocitrate dehydrogenase was identified as a component of the supramolecular complex of JHBP with hemolymph proteins.

Keywords: JHBP, protein–protein interaction

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INTRODUCTION

Juvenile hormone binding protein (JHBP) transports juvenile hormone (JH) from *corpora allata* to target cells and serves as a pool of JH in the hemolymph. JH regulates growth, development, metamorphosis and also stimulates reproduction of insects. The presence of JH maintains insects in the larval stage and its absence allows metamorphosis to occur (Gilbert *et al*., 2000). In hemolymph, three separate groups of proteins may bind JH, lipophorins, hexameric proteins, and low molecular weight proteins of approximately 30 kDa. The most abundant high molecular weight lipophorins with up to 50% lipid content belong to the family of large lipid transfer (LLT) proteins and contain two or three apolipoproteins: apolipophorin I (apoLp-I), apolipophorin II (apoLp-II) and apolipophorin III (apoLp-III) with molecular weights of about 220–250, 80, and 17–20 kDa, respectively. The second group of high molecular weight proteins with about 15% lipid content belongs to the superfamily of hexameric larval hemolymph proteins (LHP) (Gilbert *et al*., 2000). The third group of JH binding proteins, low molecular weight JHBP (25–32 kDa), is of special interest as these proteins bind 99% of JH in the hemolymph despite constituting below 1% of the total protein content of hemolymph (Hidayat & Goodman, 1994).

Lepidopteran JHBPs are monomeric with the molecular mass in the range 25–35 kDa (Kort & Granger, 1996). JHBP from *Galleria mellonella* is a glycoprotein (Duk *et al*., 1996). Structurally it resembles the folding pattern of some mammalian lipid-binding proteins, namely bactericidal permeability-increasing protein (BPI) and cholesteryl ester transfer protein (CETP), with a similar organization of one cavity and a disulfide bond between a long helix and a β-sheet. JHBP reveals, therefore, a fold used by nature for hydrophobic-ligand binding proteins (Kolodziejczyk *et al*., 2008). The JHBP molecule undergoes a profound conformational transition upon binding JH as judged from its changing electrophoretic mobility, UV spectra, sedimentation coefficient and its resistance against proteolysis (Wieczorek & Kochman, 1991; Krzyżanowska *et al*., 1998). It has been suggested that such a structural change might have a physiological significance for hormone signal transmission (Wieczorek & Kochman, 1991).

The concentration of hemolymph proteins often exceeds 100 mg/ml (Ożyhar & Kochman, 1987). This creates favorable conditions for protein–protein interactions, some of which might be functional. In *G. mellonella* hemolymph, JHBP is found in a complex with lipid binding proteins of high molecular weight, apolipophorin, arylphorin and hexamerin (Zalewska *et al*., 2009). These proteins bind JHBP, both free and in a complex with JH. However, this binding is stronger in the presence of JH. It has also been shown that JHBP binds to fat body membrane proteins. ATP synthase has been identified as a JHBP binding protein and this interaction is specific and occurs with high affinity (K_d = 0.86 nM) (Zalewska *et al*., 2009).

In this report, we focused our attention on low molecular weight proteins (LMWP) which might be bound to JHBP. Two types of experiments were designed: ligand blotting and JHBP affinity chromatography. By the use of ligand blotting, we found that JHBP interacts with a protein of about 44 kDa. Using Sepharose-coupled JHBP column chromatography and mass spectrometry analysis, we have identified NADP dependent isocitrate dehydrogenase (NADP-IDH) as a JHBP binding protein. Interaction of JHBP with NADP-IDH may be involved

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Abbreviations: HMWP, high molecular weight proteins; IDH, isocitrate dehydrogenase; JH, juvenile hormone; JHBP, juvenile hormone binding protein; LMWP, low molecular weight proteins

in the formation of a complex with other hemolymph proteins but further studies are needed to determine the role of this interaction.

MATERIALS AND METHODS

Chemicals. Juvenile hormone III (10R,S-JH III) was purchased from Sigma. 10-[^{3}H]-labeled JH III was purchased from Polatom (Poland).

Insects. *Galleria mellonella* (Lepidoptera, Pyralidae) larvae were reared in constant darkness at 30°C on a semi-artificial diet prepared as described by Sehnal and Slama (1966).

Purification of juvenile hormone binding protein and juvenile hormone binding activity assay. The hemolymph from 4th day, VIIth instar larvae was collected into a plastic tube containing a few crystals of 1-phenyl-2-thiourea and stored at –20°C.

JHBP was purified from hemolymph by immunoaffinity chromatography (Wieczorek *et al.*, 1996). The JH-binding activity was determined with a charcoal assay in the presence of 0.1% gelatin, as described previously (Ożyhar & Kochman, 1987).

Separation of hemolymph low molecular weight proteins (LMWP) from high molecular weight proteins (HMWP). Seven milliliters of hemolymph was applied onto a Sephadex G-200 column (K 16/100, Pharmacia) equilibrated with 10 mM Tris buffer, 100 mM NaCl, 0.25 mM 1-phenyl-2-thiourea, pH 7.3. The flow rate was 17 ml/h and 7-ml fractions were collected. Fractions from the ascending part of the second absorption peak (A_{280}), containing LMWP (Fig. 1A), were combined and used for ligand blotting analysis.

SDS polyacrylamide gel electrophoresis. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) (Laemmli, 1970) under reducing or non-reducing conditions. To obtain non-reducing conditions, we did not boil the sample and reducing agents were not added to minimize irreversible protein denaturation. Gels consisted of a 4% stacking gel and a 12% resolving gel. Electrophoresis was carried out until the dye front reached the bottom of the gels. Gels were stained with Coomassie Brilliant Blue R or with silver for visual observation (Shevchenko *et al.*, 1996).

Western blotting. Proteins were separated by SDS/PAGE under reducing conditions and were transferred to nitrocellulose transfer membrane (Millipore) for 1 h at 150 V. The membranes containing transferred proteins were first incubated in a buffer (10 mM Tris, 150 mM NaCl, pH 7.5, supplemented with 3% non-fat dry milk) for 1 h at 25°C. After blocking, the nitrocellulose membranes were treated with a 1:25 000 dilution of polyclonal anti-JHBP. Polyclonal antibodies against JHBP were obtained as previously described (Rodriguez Parkitna *et al.*, 2002). Then, the membranes were washed three times with the buffer and incubated with 1:5000 dilution of goat anti-rabbit polyclonal antibodies conjugated with horseradish peroxidase (HRP) (Sigma). The Western blotting assay was developed using a peroxidase detection kit (ECL plus Western Blotting Detection System, Amersham).

Ligand blotting. Proteins were separated by SDS/PAGE under non-reducing conditions and were transferred to nitrocellulose membrane (Towbin *et al.*, 1979) for 1 h at 150 V. The membranes containing transferred proteins were first incubated in a blocking buffer as described above. After blocking, the membranes were incubated for 1 h in a 10 mM Mops, 100 mM NaCl pH 7.2 buffer containing either JHBP (1 mg/ml) or JHBP (1 mg/ml) plus 20 μM JH (preincubated for 0.5 h at 4°C) or with BSA (1 mg/ml) as a negative control. Next, the membranes were washed three times with the buffer, and afterwards treated with a 1:25 000 dilution of polyclonal anti-JHBP and washed again three times with the buffer. Then, the membranes were incubated with 1:5000 dilution of goat anti-rabbit polyclonal antibodies conjugated with horseradish peroxidase (HRP) (Sigma). The ligand blotting assay (Daniel *et al.,* 1983) was developed using a peroxidase detection kit (ECL plus Western Blotting Detection System, Amersham).

Coupling of CNBr-activated Sepharose with JHBP. JHBP was coupled to Sepharose beads accord-

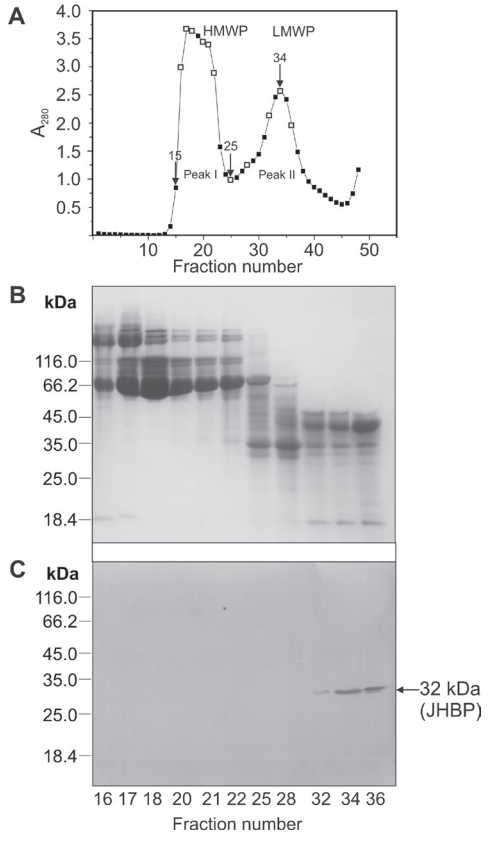

Figure 1. Gel filtration chromatography of hemolymph proteins on Sephadex G-200 and analysis of the presence of juvenile hormone binding protein (JHBP) in the obtained fractions
(A) Sephadex G-200 chromatography. Seven milliliters of hemolymph from *G. mellonella* was subjected to gel filtration on Sephadex G-200 column (K26/100, Pharmacia) equilibrated and eluted with stock buffer (10 mM Tris, 100 mM NaCl, pH 7.3) containing 0.25 mM 1-phenyl-2-thiourea. Fractions of 7 ml were collected. Selected fractions (open squares) were applied to SDS electrophoresis. Fractions (15 to 24) from the first absorption peak contained HMWP. Fractions (25 to 34) from the ascending part of the second absorption peak indicated by arrows were combined (representing a portion of LMWP) and used in further experiments (see Fig. 2). **(B)** SDS/PAGE analysis. Fractions obtained by gel filtration (40 μg protein/lane) were subjected to SDS/PAGE and stained with Comassie Brilliant Blue. (**C**) Western blotting analysis. Fractions eluted from Sephadex G-200 column were separated by SDS/PAGE (40 μg protein/lane) and then transferred to nitrocellulose membrane. The membrane was treated with polyclonal antibodies against JHBP (1:25 000). Binding was visualized with secondary goat anti-rabbit HRP-conjugated antibody (1:5 000) and a chemiluminescence system. Arrow indicates position of JHBP (32 kDa).

ing to the manufacturer's instructions (Sigma). Briefly, 1 mg (0.75 ml) of JHBP in 0.1 M $NaHCO_3$, pH 8.3, was mixed with 0.25 ml of CNBr-activated Sepharose beads in suspension and left overnight at 4°C with gentle shaking. After washing with the buffer and blocking with ethanolamine, pH 8.0, the beads were stored in 0.04% NaN_3 at 4°C before use.

Affinity chromatography on JHBP-Sepharose. Hemolymph proteins were separated on a Sephadex G-200 column equilibrated with a 10 mM Mops, 100 mM NaCl buffer, pH 7.2, and fractions designated as LMWP were combined from the ascending part of the second 280 nm absorption peak (Fig. 1A). Ten milliliters of LMWP (1.8 mg/ml) was incubated with 0.2 ml of a suspension of Sepharose-bound JHBP. After incubation for 1 h at 4°C, the beads were washed with 6 vol. of the buffer and the bound protein complexes were eluted by competitive elution with JHBP (0.3 mg, 0.4 ml) or with carbonic anhydrase (0.33 mg, 0.4 ml) as a negative control. Eluted proteins were applied to SDS/PAGE and detected by Coomassie Blue staining and silver staining. Protein bands were excised and identified using tandem mass spectrometry (MS).

Mass spectrometry and database searching. The peptide mixture obtained by a standard in-gel tryptic digestion procedure (including reduction and alkylation) was applied to an RP-18 precolumn (Waters, NanoACQUITY, 20 mm × 180 μm) using water containing 0.1% trifluoroacetic acid as a mobile phase and then transferred to a UPLC RP-18 column (Waters, NanoACQUITY, 250 mm × 75 μm) using an acetonitrile gradient (0–60% in 120 min) in the presence of 0.05% formic acid at a flow rate of 150 nl/min. The column outlet was directly coupled to the ion source of an Ion Cyclotron Resonance spectrometer (LTQ-FTICR, Thermo Electron) working in the regime of data-dependent MS to MS/MS switch. The resulting mass spectra were used to search the nonredundant protein database of the National Center of Biotechnology Information (NCBI version 20080422) using the MASCOT (matrixscience.com) search engine (eight-processor on-site license). Protein mass was unrestricted. The search parameters for peptide mass tolerance were ± 40 ppm and for fragment mass tolerance ± 0.8 Da, with an allowance made for one missed semiTrypsin, fixed modifications of cysteine through carbamidomethylation and variable modification through methionine oxidation.

The above MS analysis and database search was performed by the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa, Poland.

RESULTS

Gel filtration chromatography of hemolymph proteins and JHBP profile

Hemolymph proteins from *G. mellonella* were fractionated on a Sephadex G-200 column and separated into two main A_{280} absorption peaks, the first containing HMWP and the second LMWP, as shown in Fig. 1A. Then, the protein fractions were analyzed by SDS/PAGE (Fig. 1B) and Western blotting (Fig. 1C) to detect JHBP. JHBP was found primarily in fractions 34–36. A very weak band was also present in fraction 32. In the conditions of this experiment, JHBP does not comigrate with proteins of higher molecular weight. For analysis of the interaction of LMWP with JHBP fractions 25–34 were combined and used for ligand blotting experiments.

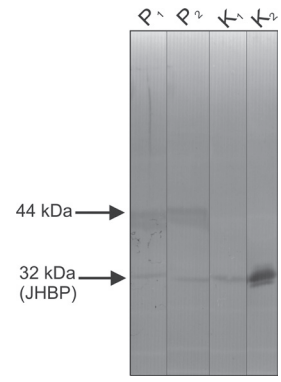

Figure 2. Ligand blotting analysis of JHBP or JHBP–JH complex binding to hemolymph LMWP
LMWP (40 μg) (lanes: P_1, P_2 and K_1) and 0.2 μg of JHBP (lane: K_2) were separated by SDS/PAGE under non-reducing conditions designed to minimize irreversible protein denaturation and were transferred to nitrocellulose membrane. The binding experiment was performed in the absence (lane K_1) or presence of JHBP (lane P_1) or JHBP–JH complex (lane P_2) as ligands. The membrane was treated with polyclonal antibodies against JHBP (1:25 000). Binding was visualized with HRP-goat anti-rabbit (1:5 000) as a secondary antibody and a chemiluminescence system. Arrows indicate positions of protein interacting with JHBP and JHBP–JH complex (44 kDa) and of free JHBP (32 kDa).

Ligand blotting analysis reveals a protein interacting with JHBP

In order to determine whether JHBP may interact with a protein(s) present in the LMWP fraction, a ligand blotting analysis was performed (Daniel *et al.*, 1983). As shown in Fig. 2, among several proteins present in this fraction (Fig. 1B), only a protein band of about 44 kDa interacts with JHBP and its position is clearly distinct from that of JHBP detected in LMWP or in control sample (Fig. 2, compare lanes P_1 and K_1). An analogous experiment performed in the presence of JH revealed almost the same intensity of the 44 kDa band in the

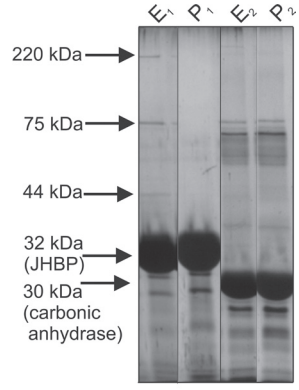

Figure 3. Interaction between LMWP and JHBP analyzed by affinity chromatography
LMWP (18 mg in 10 ml) was incubated with JHBP immobilized on Sepharose beads (0.2 ml). After incubation the beads were exhaustively washed. Proteins eluted by JHBP (0.3 mg, 0.4 ml) or carbonic anhydrase (0.33 mg, 0.4 ml) as specific and nonspecific competitors are shown in lane E_1 and E_2, respectively. Eluates (28 μl) were analyzed by SDS/PAGE and stained with silver reagent. JHBP (lane P_1) and carbonic anhydrase (lane P_2) used for elution were electrophoresed to show their mobility. Arrows indicate positions of JHBP-binding proteins (220, 75 and 44 kDa) and competitors, JHBP (32 kDa) and carbonic anhydrase (30 kDa).

Table 1. JHBP-binding hemolymph protein isolated by affinity chromatography and identified using tandem mass spectrometry

M_r SDS/PAGE [kDa]	Protein (organism)	NCBI Accession number	Calculated M_r [kDa]	No. of peptides identified	Sequence coverage [%]
44	Isocitrate dehydrogenase (*Bombyx mori*)	Q2F681	46.2	4	12.25

LMWP fraction interacting with JHBP (Fig. 2, compare lane P_1 with P_2). Small differences in the electrophoretic mobilities between samples P_1 and P_2 are due to imperfections in electrophoretic conditions. In a control experiment, where bovine serum albumin (BSA) was used instead of JHBP, no binding of the antibody against JHBP to the 44 kDa protein was observed (Fig. 2, lane K_1). For comparison, the electrophoretic mobility of purified JHBP is shown in lane K_2. Its microheterogeneity is due to different extents of glycosylation of hemolymph JHBP (Duk *et al.*, 1996).

Identification of JHBP-binding protein with affinity chromatography and mass spectrometry analysis

Affinity chromatography was used to confirm that the hemolymph LMWP fraction contains a protein which binds JHBP. Combined LMWP fractions (from the ascending part of the second absorption peak, see Fig. 1A) were applied on a JHBP-Sepharose column and after excessive washing with a buffer, the column was eluted with JHBP acting as a specific competitor or with carbonic anhydrase as a control (nonspecific) ligand. The eluted proteins were separated by SDS/PAGE and analyzed by mass spectrometry. For control elution we chose carbonic anhydrase because in SDS/PAGE this protein is clearly separated from JHBP and from the protein of about 40 kDa present in LMWP. When the gel was stained with Coomassie Brilliant Blue, only traces of bands corresponding to molecular masses of 220 kDa and 75 kDa were detected as eluted by JHBP (not shown). Because of that, silver staining, a more sensitive method of protein detection, was used. Analysis of the SDS/PAGE results after silver staining revealed three distinct protein bands eluted by JHBP (Fig. 3, lane E_1). Their positions correspond to molecular masses: 220 kDa, 75 kDa and 44 kDa. The protein bands corresponding to 220 kDa and 75 kDa have already been identified as HMWP interacting with JHBP (Zalewska *et al.*, 2009). In the LMWP fraction a new band of 44 kDa was identified. This protein band was excised from the gel and subjected to ESI-FTICR-MS analysis and identified as NADP-dependent isocitrate dehydrogenase (Table 1, Fig. 4). The mass spectrometric analysis detected 50 of the 408 amino acids of isocitrate dehydrogenase (12.25%), basing on the amino-acid sequence from *Bombyx mori* (Lepidoptera). The genomic sequence or the amino-acid sequence of the enzyme for *G. mellonella* are not known yet.

DISCUSSION

As shown above the affinity chromatography of LMWP on JHBP-Sepharose column revealed three protein bands specifically eluted by JHBP and several other

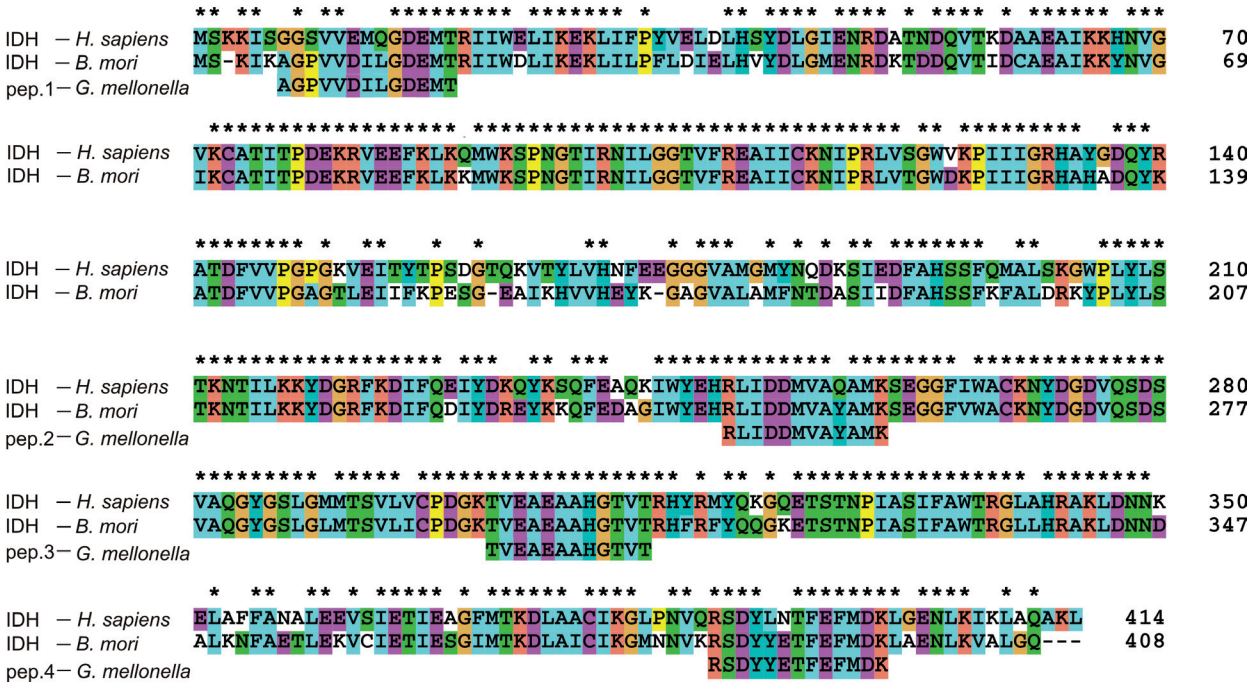

Figure 4. Amino-acid sequence comparison of human and silk moth cytosolic NADP-IDH and of the peptides obtained from MS analysis

Amino-acid sequences of cytosolic NADP-IDH from *Homo sapiens* (O75874) and *B. mori* (Q2F681) and four peptides derived from 44 kDa protein band (pep. 1, 2, 3, 4 — *G. mellonella*) were aligned using the ClustalW program (Thompson *et al.*, 1994). "*" denotes identical residues.

nonspecifically eluted bands that were present in the JHBP and carbonic anhydrase eluates. Positions of the specifically eluted protein bands correspond to molecular masses: 220 kDa, 75 kDa and 44 kDa. The proteins corresponding to 220 kDa and 75 kDa have already been identified as interacting with JHBP (Zalewska *et al*., 2009). The protein band of 220 kDa corresponds to an apolipophorin subunit, the major component of lipophorin, which mediates the transport of various types of lipids in the hemolymph. The protein band of 75 kDa is an arylphorin subunit belonging to the superfamily of hexameric larval hemolymph proteins (LHP).

As judged from tandem mass spectrometry and database searches, four peptides identified from the protein of 44 kDa have sequences identical with NADP dependent isocitrate dehydrogenase (IDH) from *B. mori* (Fig. 4). These peptides cover 12.25% of the enzyme sequence composed of 408 amino-acid residues. Although *G. mellonella* IDH primary structure is not known, it is noteworthy that IDH sequence is highly conserved during evolution (Fig. 4). In eukaryotic organisms, the NADP-IDHs represent a highly conserved group of proteins. Substantial degree of identity of the amino-acid sequence is observed between the cytosolic and mitochondrial forms of the enzyme as well as between organisms with different phylogenetic origins. *B. mori* cytosolic NADP-IDH compared with the human cytosolic form of NADP-IDH is 76% identical (Fig. 4). Therefore, based on the MS data we believe that NADP dependent isocitrate dehydrogenase is the protein found in a complex with JHBP.

Eukaryotic cells express different isoforms of isocitrate dehydrogenase (IDH), an enzyme which catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate utilizing either NAD or NADP as cofactors. NAD-dependent IDH (EC 1.1.1.41) is a mitochondrial enzyme and its role is well known, as it catalyzes a key step in the tricarboxylic acid cycle. It is composed of α, β, and γ subunits, and the enzyme is allosterically regulated by ADP (Ceccarelli *et al*., 2002). The physiological roles of the NADP-dependent IDHs (NADP-IDHs) (E.C.1.1.1.42), which function as homodimers, are not clearly understood. NADP-IDHs are present in mitochondria, cytoplasm and peroxisomes. Mitochondrial NADP-IDH is an additional source of α-ketoglutarate and NADPH (Jo *et al*., 2001; Contreras-Shannon *et al*., 2005). Non-mitochondrial NADP-IDHs apparently function to provide NADPH for biosynthetic reactions and for thiol-based antioxidant systems (Lu *et al*., 2008). Cytosolic NADP-IDH is one of the major producers of NADPH required for fatty acid and cholesterol biosynthesis in vertebrates (Koh *et al*., 2004). In *Saccharomyces cerevisiae*, there are three homologous but genetically distinct and differentially compartmentalized NADP-IDH isoenzymes: mitochondrial, cytosolic and peroxisomal (Lu & McAlister-Henn, 2010). In contrast to yeast, mammalian cells contain a single non-mitochondrial isoenzyme of NADP-IDH that localizes to both the cytosol and peroxisomes (Minard *et al*., 1998; Geisbrecht & Gould, 1999). This non-mitochondrial mammalian NADP-IDH contains a type I peroxisomal targeting sequence, an Ala-Lys-Leu tripeptide at the carboxyl terminus (Jennings *et al*., 1994; Geisbrecht & Gould, 1999). However, the mechanisms and conditions influencing the distribution of this enzyme in the two different cellular compartments are still unknown.

IDH is also present in extracellular spaces, e.g. in bronchoalveolar lavage fluid, as was demonstrated by Guo *et al*. (2005). IDH was also found in the plasma (Ellis & Goldberg, 1971; Chung *et al*., 2001). These findings suggest extracellular metabolic functions of IDH (Mitchell *et al*., 2008). A protein map of *Drosophila melanogaster* larvae hemolymph was obtained by Guedes *et al*. (2003) and IDH was identified there.

It has been demonstrated that IDH may interact with other proteins. An interaction between NAD-IDH and mitochondrial α-ketoglutarate dehydrogenase was shown by Porpaczy *et al*. (1987). Hatakeyama *et al*. (2004) using S100A12-affinity chromatography identified cytosolic NADP-IDH as an S100A12-binding protein. This protein is a member of the S100 family of proteins containing two EF-hand calcium-binding motifs. S100 proteins have been found to interact in a Ca^{2+}-dependent manner with proteins involved in cell proliferation and differentiation, cellular architecture, signal transduction, and intracellular metabolism (Hatakeyama *et al*., 2004).

It is worthy of note that NADP-IDH is expressed mainly in lipogenic tissues such as liver and adipocytes (Koh *et al*., 2004), whereas JHBP is expressed in insect fat body, a tissue functionally resembling liver. Earlier, it has been shown that the cell membrane ATP synthase interacts with JHBP and presumably participates in JHBP export from the site of its synthesis to the hemolymph (Zalewska *et al*., 2009). This process was found to be JH-dependent. Similarly, the binding of JHBP to apolipophorin, arylphorin and hexamerin was different in the presence and absence of JH, which suggested that these proteins participate in transport of the JHBP-JH complex to the target tissues. This allowed us to postulate a model of JHBP transport from fat body cells to the target tissues in a JH-dependent manner (Zalewska *et al*., 2009). However, the interaction between IDH and JHBP does not seem to be dependent on the JH presence in the experimental conditions applied.

The JHBP molecule has a unique fold resembling the folding motif found in mammalian lipid binding proteins, bactericidal permeability-increasing protein (BPI) and cholesteryl ester transfer protein (CETP) (Beamer *et al*., 1997; Qiu *et al*., 2007), which also have an affinity to phospholipids. The molecule of JHBP contains two cavities (E, W) of almost the same size (Kolodziejczyk *et al*., 2008). There are some arguments, including ligand docking and chemical modification studies, that the W cavity forms the binding site for the hydrophobic JH molecule, with the N-terminal peptide of nine amino acids forming a lid which covers the cavity after hormone binding (Kolodziejczyk *et al.,* 2008). Since the stoichiometry of JH binding to JHBP is 1:1, the role of the second cavity is a subject of discussion. However, it is known that this cavity is more hydrophilic at its edges than the W cavity (Kolodziejczyk *et al*., 2008). It seems that it can bind a phospolipid molecule. In this context it is interesting to note that IDH can interact with proteins which exhibit an affinity to phospolipids. Namely, analysis of *D. melanogaster* protein–protein interactions with a yeast two hybrid system has revealed that NADP-IDH interacts with six proteins, including a 14-3-3 protein (Giot *et al*, 2003). 14-3-3 protein isoforms have been found to associate to membranes (Martin *et al*., 1994; Roth *et al*., 1994) and phospholipid vesicles (Roth *et al*., 1994). Thus it is conceivable that JHBP may compete for a phospholipid bound to hemolymph lipid binding proteins forming a transient complex, and that this complex may contain IDH.

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