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# Differential Proteins of the Optic Ganglion in *Octopus vulgaris* Under Methanol Stress Revealed Using Proteomics

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**Abstract** An analytical approach using the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) technique separated the proteome from the optic ganglia of *Octopus vulgaris* (OVOG). Approximately 600 protein spots were detected from the extraction when applying 150 μg protein to a 2D-PAGE gel in the pH range 5.0–8.0. Compared to the control, significant changes of 18 protein spots were observed in OVOG under the stress of native seawater containing 2% methanol for 72 h. Among these spots, we found that eight were down-regulated and ten were up-regulated in the gels, which were further identified using both peptide mass fingerprinting and database searches. Significant proteins such as glyceraldehyde-3-phosphate dehydrogenase, alpha subunit of succinyl-CoA synthetase, alcohol dehydrogenase, and long-chain specific acyl-CoA dehydrogenase were up-regulated proteins, whereas putative ABC transporter was a down -regulated protein. These differential proteins at the level of subcellular localization were further classified using LOCtree software with a hierarchical system of support vector machines. We found that most of the differential proteins in the gel could be identified as mitochondrial proteins, suggesting that these protective or marker proteins might help to prevent methanol poisoning via the mitochondria in the optical ganglia. The results indicated that both beta-tubulin and beta-actin were potential biomarkers as up-regulated proteins for monitoring methanol toxicosis associated with fish foods such as octopus and shark.

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## Introduction

Methanol is normally used as an industrial solvent and has also been introduced as an alternative automotive fuel, called biofuel [1]. With the increase of methanol use, extensive good comprehension of its toxic mechanism is very necessary and significant [2], since methanol may be inhaled in the toxic vapor evaporating from the product surface. Every day, a large number of methanol poisoning incidents related to the consumption of methanol-contaminated drink occur in the world. Methanol is known to affect both the visual and the central nervous systems (CNS). In certain mammals, especially humans, the well-known toxicity of methanol is thought to be due to its metabolites such as formic acid and formate, which act as metabolic poisons and cause histotoxic hypoxia and subsequently vasodilation [3]. Formate has been demonstrated to inhibit oxidative phosphorylation by decreasing both the intracellular synthesis of ATP and the ratio of $NAD/NADH^{+}$. Primates, such as humans, are particularly sensitive to methanol toxicity compared to other animals, whereas lower mammals have the ability to rapidly oxidize the formic acid to carbon dioxide. Bradycardia, shock, widespread vasodilation, and anuria are serious prognostic signs of methanol toxicity [4].

*Octopus vulgaris* is a mollusk belonging to the class Cephalopoda in China. It is widely used as food. Another mollusk, *Aplysia*, which is not edible because of its high toxicity in the bowels, is often used as a typical biological model [5, 6], since various optical ganglia (OG) are easily separated to carry out complex work in neural science. Though *O. vulgaris* is not a typical biological model for neural science, its optical ganglion is much larger than that of other mollusk and separated easily for scientific research. In addition, an important question whether the methanol utilizes food pathway to impact on eyesight in human is still unclear. It is very significant work to try to find some differential proteins for functional research in the optical ganglia from the various animal models under stress of methanol. In this study, we used a proteomic approach to search for differential proteins from the OVOG under methanol stress. Little proteomic work has been done on the CNS in mollusks (especially cephalopods) under methanol stress. Following the identification and analysis of those differential proteins, we might be able to further understand methanol toxicology in the CNS of various mollusks and also possibly in higher animals such as humans.

## Materials and Methods

### Chemicals

Ampholines with a pH range of 5.0–8.0 were obtained from the Amersham Biosciences Co. (Sweden), methanol from Merck Co. (Germany), and matrix α-cyano-4-hydroxy-cinnamic acid (CHCA) and most of the electrophoresis reagents from Sigma Co. (Cleanse, USA), and other inorganic chemicals were analytical grade reagents and came from various commercial sources in China.

### Animals and Methanol Exposure

*O. vulgaris* were purchased from the Xiamen Fish Company, Xiamen, China. After acclimatization for 2 days, five healthy *O. vulgaris* of approximately the same size (about

100 g) were exposed to seawater containing methanol. Meanwhile, five animals were maintained in seawater without methanol to serve as the control group. Following Aziz et al. [7], we used methanol concentrations ranging from 2% to 5% in native seawater. After several pre-experiment tests with 5% methanol, we found that all the *O. vulgaris* died within 30 min, but they remained alive under a 2% methanol stress up to 72 h. For this reason, the exceptional appearances of *O. vulgaris* such as unresponsive, undynamic, and costive behavior were observed at the 2% methanol level in the experiments. The *O. vulgaris* were sampled for gas chromatography (GC) and proteomic analysis when exposed to 2% methanol for 72 h.

## Methanol Determination Using Gas Chromatograph (GC)

Optic ganglion (OG) size is much larger than that of other neural ganglions in *O. vulgaris*. Likely *Aplysia*, experimental OGs are easily separated by normal anatomic method in *O. vulgaris*. The OGs were popularly obtained using normal separation techniques [8, 9] under a dissecting microscope and then saved at 4 °C for 30 min. The 1:1 proportion (*w*/*w*) of OG tissue to water was mixed to be mashed into serosity equably for protein extraction, giving the best ratio (*w*/*w*) for obtaining maximum content of the protein in OG. The mixture was transferred into a 1.5-mL polypropylene tube for 15-min sonication, and then, the suspension sample was centrifuged at 12,000×*g* for 10 min. Finally, the supernatant samples were collected for methanol determination using GC (Varian Co., Modell CP-3800) equipped with a flame ionization detector (FID; 280 °C) according to a procedure described previously. The capillary column of the GC analyzer was equipped with a CP-WAX52CB column (30 m× 0.32 mm DF=0.25 μm, Varian). One microliter of the sample or the standard methanol solution was injected (injector 250 °C) into the GC machine with its FID (280 °C). After 2 min, the temperature was increased from 50 to 100 °C at 5 °C/min. Other parameters maintained were: column flux, 0.5 mL/min; air, 350 mL/min; $H_2$, 35 mL/min; and $N_2$, 30 mL/min. The quantitative analysis of methanol in the tissue was carried out using the normal standard curve method. We also observed that the methanol with analytical purity, as standard sample, contains still micro-impurity compounds that can be determined by GC.

## Protein Sample Preparation

The lysis solution consisted of 7 M urea, 4% CHAPS, 2 M thiourea, 60 mM dithiothreitol (DTT), 10 mM Tris, 1 mM EDTA, 0.5% carrier ampholyte pH 3.0–10.0 [9–11], and 0.0002% bromophenol blue. At a 1:5 (*w*/*v*) proportion, a proper volume of lysis solution was added to a group of OG and then the mixture was homogenized using an agitator at 4 °C and maintained for 15 h at 4 °C for complete protein release before centrifugation at 13,000×g for 35 min. In order to obtain a high resolution of protein spots in the gel, the lipid layer on the surface of the liquid suspension sample was carefully removed and the protein sample in the middle layer was collected with a pipette for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [9]. In order to improve protein solubility, 0.5% carrier ampholyte pH 3.0–10.0 must be added into the lysis solution for protein sample preparation, which can bind to a little free salt from the cytoplasm and increase protein solubility for obtaining high-quality map of 2D-PAGE [9–11].

## 2D-PAGE

2D-PAGE was performed according to the procedure described by Chen et al. [10]. In brief, 150 μg of protein sample was loaded on to 13-cm strips, and isoelectric focusing

was carried out with carrier ampholyte (pH 5.0–8.0) for 10,000 Vh. Before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the gel strips were equilibrated for 15 min in an equilibration buffer (6 M urea, 30% glycerol, 50 mM Tris–HCl, 2% SDS, and 1% DTT [pH 8.8]). The strips were then placed on top of the polyacrylamide gel ($T$=12%), and electrophoresis was performed at a constant power of 25 mA/gel until the bromophenol blue reached the bottom of the gel. For each protein sample (control or methanol-treatment), three experimental replicates were subjected to 2D-PAGE.

## Image Capture and Analysis

The proteins on the gels were visualized using silver staining as described previously [11]. The stained gels were scanned with an Image Scanner II apparatus (GE Healthcare). Digitized images of the gels were analyzed using the ImageMaster 2D Platinum software (version 5.0). Protein spots were detected and matched between different samples, and volume values of corresponding spots were obtained according to the program instructions. To eliminate gel-to-gel variation, the individual spot volume of each gel was normalized relative to the total valid spot volume. The differentially expressed spots between the control and methanol-treatment groups were selected with a criterion of $P<0.05$ obtained by Student's $t$ test.

## In-Gel Digestion of Protein Spots

Differentially expressed protein spots were picked manually and enzymatically digested in-gel according to the procedure followed the method of Huang et al. [12] with some modifications. The gel was dehydrated with acetonitrile and dried in a vacuum centrifuge prior to the addition of sequencing-grade trypsin. Digestion was carried out at 37 °C for 8–12 h, and peptides were recovered by sequencing extractions with 25 mM ammonium bicarbonate, 50% acetonitrile/0.1% trifluoroacetic acid (TFA), and 100% acetonitrile, and all steps were repeated one more time.

## Protein Identification

The peptide extracts were mixed with an equal volume of matrix CHCA saturated with 50% acetonitrile/0.05% TFA, spotted onto a MALDI target plate, and analyzed using a Reflex III MALDI-TOF mass spectrometer (Burker Daltonik, Germany). The peptide spectra were recorded in the reflector mode, calibrated using acid peptide [residues 26, M ($H^+$)=2,961.5], synthesized using an automated peptide synthesizer (Model PS3), and then purified using HPLC in order to remove other proteins/peptides. The accuracy of molecular ion mass determination was better than ±0.1 Da, up to a mass of 6,000 Da.

Spectra were calibrated using trypsin autodigested ion peaks ($m/z$=842.510 and 2,211.1046) as internal standards. The peptide mass fingerprinting (PMF) data were used to search for candidate proteins using MASCOT (http://www.matrixscience.com) software. And the search parameters were set up as follows: the databases were NCBInr; the minimum number of matched peptides was four; fixed modification of carbamidomethylation and variable modification of methionine oxidation; the monoisotope masses were used; the number of missed cleavage sites allowed was up to 1; and the maximal mass tolerance was 100 ppm.

### Network Analysis

To model the signaling networks being affected by methanol treatment in *O. vulgaris*, the gene object corresponding to each modulated protein was subjected to network analysis using the ingenuity pathways analysis (IPA) software (Ingenuity Systems, http://www.ingenuity.com). The genes mapped to the biological networks were ranked by scores indicating the probability that a collection of genes equal to or greater than the number in the network could be achieved by chance alone, and a score of 3 was used as the cutoff for identifying gene networks significantly affected by methanol.

## Results and Discussion

### Observation of *O. vulgaris* Behavior Under Methanol Stress

Compared to the control group, it was found that the experimental *O. vulgaris* group exhibited erratic swimming pathways and a few individuals also spurted their ink under the methanol stress, indicating that their exceptional behavior was a hyperirritable response to the methanol toxicity. They then gradually died over 72 h under the 2% methanol stress, while they remained alive in the control group. After dissection, we found that both organs and tissues in the experimental group showed evident pathological changes, such as lessening of OG, nigrescence of kidney, and lax eye coordination, compared to the control groups. This indicated that the methanol had strong effects on the regular working organs of the *O. vulgaris*. It was possible that the OG also expressed many acutely significant differential proteins under the methanol stress. We also believed that one or more of these differential proteins could be used as an important tool/marker in monitoring the pollution level of methanol in seawater.

### Analysis of Methanol Residue in OG

A standard curve formula [$Y=-19.967+49604X$ ($R^2=0.9988$)] for the analysis of methanol was established using GC. The chromatographic peaks among 1 μL double-distilled water, 2% methanol, and methanol with chromatographic purity were analyzed using GC. With reference to the distilled water control (Fig. 1a), we found that the experimental methanol showed three chromatographic peaks in seawater, indicating that two of these peaks came from the solvent and the remaining one from the methanol (Fig. 1b). Figure 1c is a chromatographic peak of methanol with chromatographic purity in the absence of the solvent peaks. Comparing Fig. 1b and Fig. 1c, the retention time of the methanol was calculated to be 3.25 min.

In addition, compared to the control (Fig. 1d), we found that the retention time of the chromatographic peaks of OVOG determined using GC did not correspond to that of methanol (Fig. 1c), indicating that the control *O. vulgaris* contained a small amount of methanol in its OG. Further analysis revealed that there were four chromatographic peaks in the experimental OG (Fig. 1e) and, compared to the control (Fig. 1d), the retention time of a differential peak shown in Fig. 1e was calculated to be 3.25 min, indicating that the methanol including solvent could be transferred into the OVOG under the methanol stress in seawater. Based on the area of the methanol peak (648.25 mV s) in Fig. 1d and a standard curve formula, the methanol content from the experimental OG was calculated to be 1.35% (*w*/*w*). However, the real methanol content should be somewhat higher than the

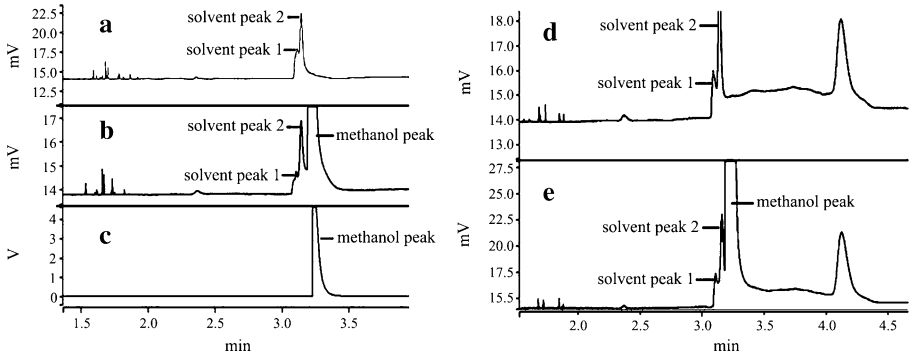


**Fig. 1** Methanol residues determined with gas chromatography in OGOV. **a** Solvent (distilled water), **b** methanol solvent, **c** methanol with high purity, **d** control, **e** methanol-treatment

data obtained because its metabolism was ignored. The results indicated that the methanol was easily moved into the OVOG and might quickly damage eyesight. It is possible that human eyesight might also be impaired greatly and quickly by the consumption of even small amounts of methanol.

## 2D-PAGE Analysis of OG

Using 2D-PAGE, we obtained the OG proteome maps of control (Fig. 2a) and experimental group (Fig. 2b). Each map had approximately 600 protein spots, which mainly distributed in the pH range of 5–8 and a molecule weight range of 14.4–116 kDa. Quantitative image analysis revealed 18 protein spots showing statistically significant changes (decrease or increase, $P<0.05$) in the methanol-treated versus the control. Figure 3 summarizes the differential spots in Fig. 2: eight spots [spot1 (s1), s2, s3, s5, s6, s7, s8, s9] were down-regulated while 10 spots (s4, s10, s11, s12, s13, s14, s15, s16, s17, s18) were up-regulated. In addition, the relative abundance of these protein spots calculated by software was shown in Fig. 4.

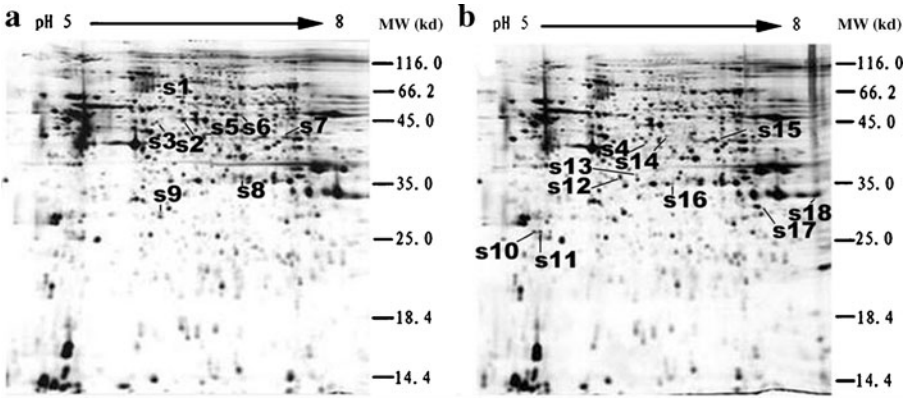


**Fig. 2** 2D-PAGE patterns of OGOV protein extraction. **a** Control, **b** methanol-treatment. Both gels were silver-stained. The *number-marked spots* refer to the proteins with a modified expression level after 2% methanol exposure for 72 h

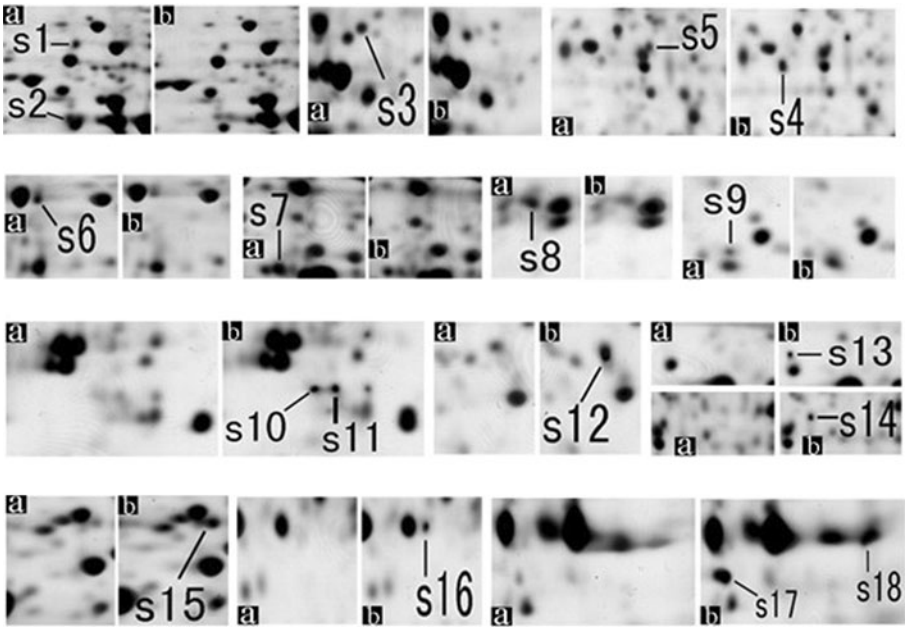


**Fig. 3** Magnified images of protein spots that showed significantly different changes between the **a** control and **b** methanol-treatment groups

## Identification of Differential Proteins

The differential spots shown in Fig. 3 were further identified using MALDI-TOF MS and database search, and the results are summarized in Table 1. Both s4 (beta-tubulin) and s12 (beta-actin) showed the most significant matching grade with scores of 109 and 88, and other spots showed somewhat lower scores than these. We note that information within the database concerning the world halobios is lacking, and thus, it is difficult to obtain high scores for the spots identified in Fig. 3, especially for mollusks such as *O. vulgaris*.

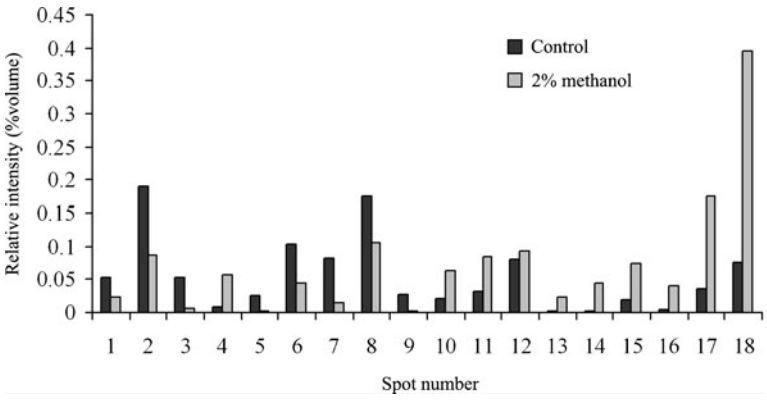


**Fig. 4** Spot intensity of differential proteins analyzed with the ImageMaster 2D Platinum software against the spot numbers of differential proteins shown in Fig. 2

**Table 1** Identification of proteins differentially expressed in response to methanol treatment in OVOG

| Spot | Accession number | Score | Taxonomy | Molecular weight | p*I* | Description | Subcellular localization |
|---|---|---|---|---|---|---|---|
| s1 | gi\|116183745 | 50 | *Vibrio* sp. Ex25 | 47,230 | 5.64 | Hypothetical protein VEx2w_02003713 | Organellar |
| s2 | gi\|11596258 | 48 | *Pneumocystis carinii* f. sp. carinii | 57,417 | 6.09 | Yer049wp-like protein | Organellar |
| s3 | gi\|149116505 | 46 | *Shewanella baltica* OS223 | 51,867 | 5.70 | Succinic semialdehyde dehydrogenase | Mitochondria |
| s4 | gi\|37651156 | 109 | *Octopus vulgaris* | 44,466 | 5.54 | Beta-tubulin | Cytoplasmic |
| s5 | gi\|26987644 | 45 | *Pseudomonas putida* KT2440 | 40,204 | 6.27 | Hypothetical protein PP_0908 | Extra-cellular |
| s6 | gi\|150003021 | 48 | *Bacteroides vulgatus* ATCC 8482 | 51,890 | 6.70 | Inosine-5′-monophosphate dehydrogenase | DNA binding |
| s7 | gi\|148257395 | 76 | *Bradyrhizobium* sp. BTAi1 | 33,479 | 6.67 | Putative ABC transporter (ATP binding protein) | Mitochondria |
| s8 | gi\|57640612 | 50 | *Thermococcus kodakaraensis* KO | 32,061 | 8.01 | Heat shock protein HtpX | Extra-cellular |
| s9 | gi\|90424260 | 48 | *Rhodopseudomonas palustris* BisB18 | 26,457 | 6.04 | Putative outer-membrane protein precursor | Extra-cellular |
| s10 | gi\|3126825 | 62 | *Felis catus* | 13,246 | 6.93 | Glyceraldehyde-3-phosphate dehydrogenase | Cytoplasmic |
| s11 | gi\|13473039 | 54 | *Mesorhizobium loti* MAFF303099 | 22,254 | 5.29 | Hypothetical protein mll3521 | Cytoplasmic |
| s12 | gi\|125995175 | 88 | *Balaenoptera acutorostrata* | 20,570 | 4.87 | Beta-actin | Cytoplasmic |
| s13 | gi\|86748273 | 56 | *Rhodopseudomonas palustris* HaA2 | 36,467 | 6.25 | Alcohol dehydrogenase superfamily, zinc-containing | Mitochondria |
| s14 | gi\|116694650 | 48 | *Ralstonia eutropha* H16 | 42,479 | 5.59 | Acyl-CoA dehydrogenase, long-chain specific | Mitochondria |
| s15 | gi\|15896329 | 46 | *Clostridium acetobutylicum* ATCC 824 | 43,912 | 8.80 | Uncharacterized secreted protein | Mitochondria |
| s16 | gi\|85858307 | 53 | *Syntrophus aciditrophicus* SB | 24,741 | 6.33 | Ferridoxin | Nuclear |
| s17 | gi\|32034771 | 69 | *Actinobacillus pleuropneumoniae* serovar 1 str. 4074 | 30,591 | 6.66 | COG0074: Succinyl-CoA synthetase alpha subunit | Mitochondria |
| s18 | gi\|108763856 | 47 | *Myxococcus xanthus* DK 1622 | 25,499 | 7.77 | Oxidoreductase, short chain dehydrogenase/reductase family | Mitochondria |

Microtubules are one of the cytoskeletal structures which exist for cell division and cell movement [13]. The microtubule is a lateral aggregation of protofilaments consisting of a chain of both stable α- and β-tubulin dimers. Each dimer chain has specific combination sites to bind to various drugs which treat neural diseases. Actin is a microfilament and an important component in the cytoskeleton [14]. It is found that four out of six actins are α-actins, and the remaining two are β- and γ-actins. Actins play many important roles in muscle shrinking, amoeboid movement, and the cell signal pathway and are very significant proteins in methanol toxicology. We suggest, therefore, that these differential proteins have potential as biomarkers for understanding methanol toxicity in OG. Unlikely *Aplysia*, it is well known that *O. vulgaris* is not a typical biological model in neural science which makes it difficult to further identify these differential proteins including its function and get quite satisfying both experimental results and relative datum, especially in halobios.

## Subcellular Localization of Differential Proteins

In order to understand biological functions, LOCtree software [15, 16] assists in predicting the subcellular localization of differential proteins. The results (summarized in Table 2) indicate that most of the differential proteins were mitochondrial proteins (adding up to 38.89%) and that five of the seven mitochondrial proteins were related to energy metabolism for decreasing methanol toxicity. We suggested that an important function for reducing toxicity came from the mitochondria in the OG, which might be an effective target for reducing methanol toxicity in the OG and improving failing eyesight levels in humans [17]. The biological functions of the remaining differential proteins [18, 19] need to be further proofed based on a great deal of experimental researches, such as enzyme activity determination and immunoreaction, assisted by analytical software.

## Protein Networks Involved in Methanol Treatment

To investigate whether the differentially expressed proteins interacted biologically, ingenuity pathways analysis (IPA) software was used to generate network diagrams to elucidate signaling pathways in the *O. vulgaris* impacted by methanol based on the proteomics results. Eighteen differential proteins (Table 1) were all searched with their corresponding accession numbers for their exact gene counterparts in IPA. These gene counterparts were then imported to the IPA module. IPA mapped these proteins for their corresponding gene objects in the ingenuity pathways knowledge base, and six genes were eligible for the network generation. These genes are corresponding to four up-regulated proteins such as alcohol dehydrogenase 1B (class I) beta polypeptide, acyl-CoA dehydrogenase long chain, glyceraldehyde-3-phosphate dehydrogenase, and actin beta

**Table 2** Subcellular localization of the differential proteins

| Subcellular localization | Spots | Sum | Percentage (%) |
|---|---|---|---|
| Mitochondria | s3, s7, s13, s14, s15, s17, s18 | 7 | 38.89 |
| Cytoplasmic | s4, s10, s11, s12 | 4 | 22.22 |
| Extra-cellular | s5, s8, s9 | 3 | 16.67 |
| Nuclear | s16 | 1 | 5.56 |
| DNA binding | s6 | 1 | 5.56 |
| Organellar | s1, s2 | 2 | 11.11 |

and two down-regulated proteins such as aldehyde dehydrogenase 5 family, member A1 and inosine 5′-monophosphate dehydrogenase 2. As a result, IPA generated only 1 network of 35 genes (maximum number of genes per network), six of which were focus genes (Fig. 5). Among these six proteins, four were up-regulated while two were found down-regulated in the proteomics experiments.

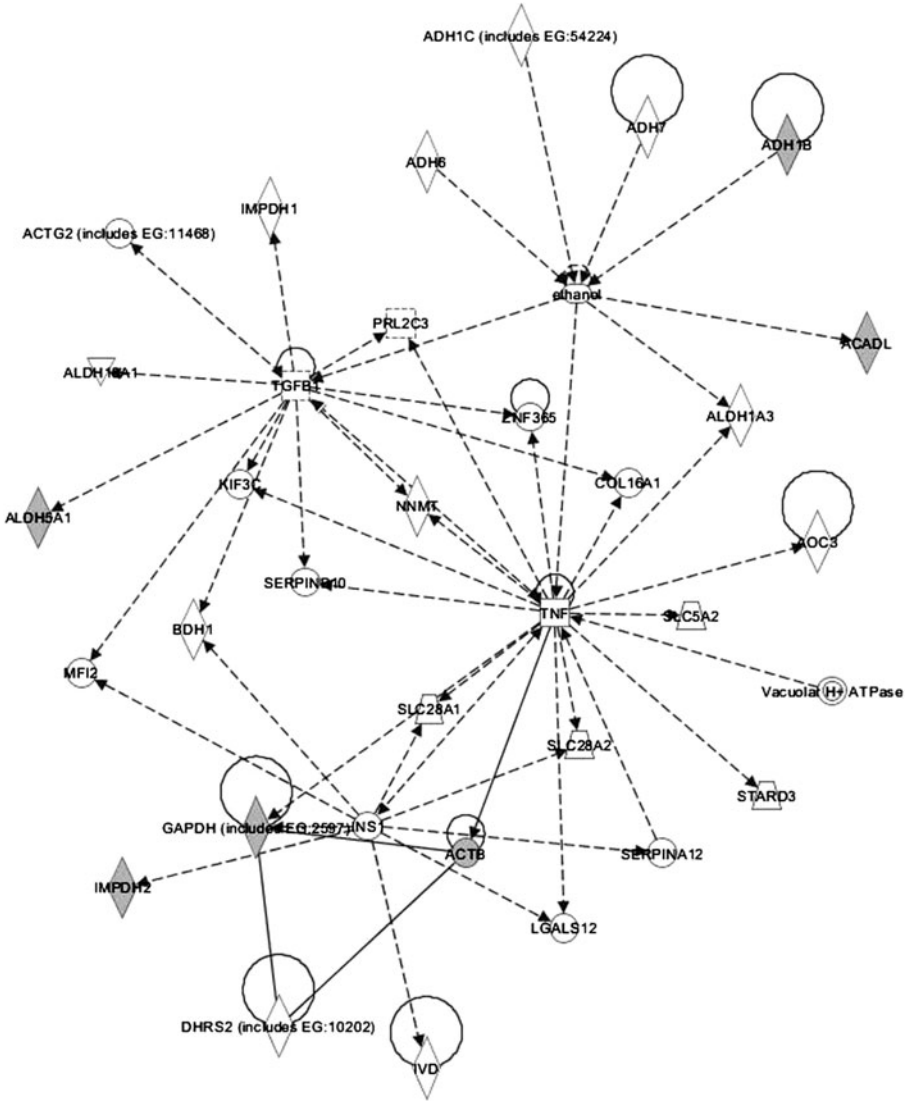


**Fig. 5** Network analysis of differentially expressed proteins performed using the IPA software. Proteins are represented as *nodes*. *Nodes in gray* represent the modulated proteins detected by proteomics. Proteins represented by *white nodes* were not observed. *Solid lines* indicate direct interactions or regulation, while *dashed lines* indicate indirect effects mediated by additional proteins

## Conclusion

An analytical approach, 2D-PAGE, is effectively developed to separate proteome from the OVOG under the stress of methanol, giving approximately 600 protein spots. Compared to the control, 18 proteins spots with significant changes are further selected and identified by proteomic methods such as PMF, showing that eight were down-regulated and ten were up-regulated in the gels.

These differential proteins at the level of subcellular localization were further classified using LOCtree software with a hierarchical system of support vector machines. In addition, IPA software was also used to generate network diagrams to elucidate signaling pathways in the *O. vulgaris* impacted by methanol based on the proteomics results.

Here, we indicated that both beta-tubulin and beta-actin were potential biomarkers as up-regulated proteins for monitoring methanol toxicosis associated with fish foods such as octopus and shark as well as humans. It is noticed that one of the important factors for the failing eyesight level in humans came from the food such as fish contaminated with methanol.

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