



## Analytical Methods

## Alcohol oxidase- and formaldehyde dehydrogenase-based enzymatic methods for formaldehyde assay in fish food products

Vladimir Sibirny<sup>a</sup>, Olha Demkiv<sup>b</sup>, Halyna Klepach<sup>b,c</sup>, Taras Honchar<sup>b</sup>, Mykhailo Gonchar<sup>b,d,\*</sup><sup>a</sup> Department of Biotechnology and Microbiology, Rzeszow University, Cwiklinskiej 2, 35-601 Rzeszow, Poland<sup>b</sup> Institute of Cell Biology, NAS of Ukraine, Drahomanov Street 14/16, Lviv 79005, Ukraine<sup>c</sup> Drohobych Ivan Franko State Pedagogical University, Ivan Franko Street 24, Drohobych 82100, Ukraine<sup>d</sup> Branch Campus of the Faculty of Biotechnology, Rzeszów University, ul. Sokołowska 26, 36-100 Kolbuszowa, Poland

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## ABSTRACT

For Gadoid fishes, formaldehyde can be generated in tissues in huge amounts during endogenous enzymatic degradation of natural osmoprotectant trimethylamine-*N*-oxide. This paper describes two enzymatic methods for assay of formaldehyde in fish food products using alcohol oxidase (AOX) and formaldehyde dehydrogenase (FdDH) isolated from the thermotolerant methylotrophic yeast *Hansenula polymorpha*. AOX-based method exploits an ability of the enzyme to oxidise a hydrated form of formaldehyde to formic acid and hydrogen peroxide monitored in peroxidase-catalysed colorimetric reaction. In FdDH-based method, a monitored coloured formazane is formed from nitrotriazolium salt during reduction by NADH, produced in formaldehyde-dependent reaction. It was demonstrated an applicability of both methods for assay of formaldehyde in fish products. The optimal protocols for analysis procedures have been elaborated and analytical parameters of both enzymatic methods have been established. The both methods were demonstrated that some fish products (hake and cod) contain high formaldehyde concentrations (up to 100 mg/kg wet weight).

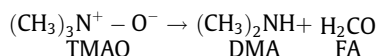
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## 1. Introduction

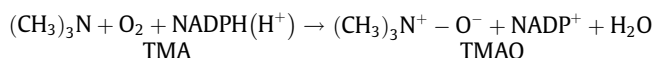
Formaldehyde (FA) is both a multi-tonnage industrial product and an essential metabolite of living systems (Gerberich, 1994). It is classified as a mutagen and a possible carcinogen (Feron et al., 1991). The teratogenic activity of FA is yet to be determined. High level of FA toxicity necessitates the control over the content of this substance in environment, industrial products, medical preparations, and even in some food products.

Recent research demonstrated the presence of FA in fruit, vegetables, meat and biological liquids of humans (Gerberich, 1994). The level of FA is especially high in some fish products (Rehbein, 1995). Frozen storage of fish is known to result in gradual decrease of edible qualities of products, in particular, in texture, smell, taste, and colour, especially if non-deep frozen fish is stored for a long time. These processes are especially active in Gadidae (Gadoid) fish: cod (*Gadus morhua*), pollack (*G. virens*), haddock (*G. aeglefinus*), whiting (*G. merlangus*), hake (*Merluccius* sp.) or mulsette (*Merluccius* sp.), in which FA content may reach as high as 210 or even 780 mg per 1 kg of wet weight which is of real danger to human health (Rehbein, 1995).

It was shown that both dimethylamine (DMA) and FA are products of the same enzymatic decomposition reaction of the natural component of many fish species – trimethylaminooxide (TMAO):



Both TMAO and betaine  $(\text{CH}_3)_3\text{N}^+ - \text{CH}_2 - \text{COO}^-$  play an important role in cell osmoregulation of salt-water fish (Tseng & Graves, 1998). The exact reactions of TMAO formation in fish tissues have not been determined, however, it is assumed to be formed from trimethylamine under the influence of specific  $\text{O}_2^-$  and NADPH-dependent monooxygenase:



It was revealed that the reactions of TMA accumulation are not unique for fish only; they may occur in other organisms as well. The evidence to this fact was found in recently discovered inherited fish-odour syndrome of humans, due to permanent secretion of trimethylamine with their sweat and urine (Zhang, Mitchell, & Smith, 1995).

It should be noted that the consumption of frozen fish products, in particular Gadidae fish food with high FA content leads not only to rapid aggravation of edible and organoleptic qualities of fish products but also to toxic influence of FA on human organism.

\* Corresponding author at: Institute of Cell Biology, NAS of Ukraine, Drahomanov Street 14/16, Lviv 79005, Ukraine. Tel.: +38 032 2612144; fax: +38 032 2611648.

E-mail address: [gonchar@cellbiol.lviv.ua](mailto:gonchar@cellbiol.lviv.ua) (M. Gonchar).

Moreover, the other product of TMAO-demethylation reaction, dimethylamine, is a potential carcinogen, as in the presence of food nitrates and bacterial enzymes in the gastrointestinal tract it is transformed into nitrosodimethylamine ( $(\text{CH}_3)_2\text{N}-\text{N}=\text{O}$ ), the carcinogenic activity of which due to interaction with DNA is indisputable. Besides, tissue metabolism of nitrosodimethylamine also results in the formation of FA, leading to hypermethylation of histones and genes' promoters which may be one of additional triggers for carcinogenesis via gene silencing (Belinsky, 2004; Esteller, 2007).

Different approaches have been proposed for FA assays (Bechmann, 1996; Demkiv et al., 2008), among them the most used are chemical and physico-chemical ones, although they are not void of serious drawbacks: low selectivity (chemical methods), necessity in analyte derivatization, costly equipment and trained personal (HPLC, mass-spectrometry, NMR). Recent achievements in the field of FA analysis include enzymatic and biosensor approaches based on the use of bacterial or yeast recombinant formaldehyde dehydrogenase (Ali et al., 2006; Ali et al., 2007; Demkiv, Paryzhak, Gayda, Sibirny, & Gonchar, 2007; Demkiv et al., 2008; Ho & Richards, 1990; Nikitina et al., 2007; Paryzhak, Demkiv, Schuhmann, & Gonchar, 2008) and yeast alcohol oxidase which is able to oxidise FA (Dmytruk et al., 2007; Gonchar, Strzelczyk, Maidan, Bieñ, & Sibirny, 1998; Sibirny et al., 2008).

This paper describes the development of two enzymatic methods to be used for FA assay in fish food products. Both methods use enzyme producers constructed by us earlier. One method is based on alcohol oxidase (AOX)–peroxidase coupled reaction while the second approach uses the recombinant yeast  $\text{NAD}^+$ - and glutathione-dependent formaldehyde dehydrogenase (FdDH). Both enzymes are isolated from the methylotrophic yeast *Hansenula polymorpha* cells: the mutant C-105 (*gcr1 catX*) overproducing AOX (Gonchar, Maidan, Pavlishko, & Sibirny, 2001) and the recombinant strain Tf-11-6 overexpressing *FLD1* gene coding for FdDH (Demkiv et al., 2007). It has been demonstrated that both approaches are applicable for assay of FA in fish products, although different methods for the sample preparation are required. The optimal protocols for analysis procedures have been elaborated, and analytic characteristics for both methods have been determined and compared with routine chemical approaches on the model solutions and real samples of fish food products.

## 2. Materials and methods

### 2.1. Materials

Nitrotetrazolium blue (NTB) were from Merck (Darmstadt, Germany); paraformaldehyde, Triton X-100, chromotropic acid, 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) were from Sigma–Aldrich; 3-(*N*-morpholino)propanesulfonic acid (MOPS), glutathione (reduced), phenazine methosulfate (PMS), tetramethylbenzidine hydrochloride (TMB), 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) were from Fluka (Buchs, Switzerland).  $\text{NAD}^+$  and NADH were obtained from Gerbu Biotechnik (Gailberg, Germany).

All other chemicals were of analytical reagent grade and all solutions were prepared using bi-distillated water.

### 2.2. Enzymes and enzymatic kit

Commercial horseradish peroxidase with specific activity 900 U/mg and RZ = 3.0 was from Aster (Lviv, Ukraine).

AOX with activity 7.5–10 U/mg was isolated and purified as described earlier (Gonchar et al., 2001) from the cells of the mutant

strain *Hansenula polymorpha* C-105 (*gcr1 catX*) impaired in glucose catabolite repression and void of catalase.

FdDH with activity 17 U/mg was isolated from the cells of the recombinant strain *H. polymorpha* Tf 11-6 constructed by us recently (Demkiv et al., 2007).

As a source of AOX, peroxidase and chromogen, the enzymatic kit ALCOTEST produced in small series by Institute of Cell Biology (Lviv, Ukraine) can be used.

### 2.3. Preparation of samples for analysis

For assay of FA in fish meat (muscle tissues of frozen hake and cod, as well as of freshly-killed carp), two different deproteinising procedures were used.

Procedure 1: 2.5 g of cut muscle tissue was pestled in a ceramic pot. After adding 10 ml water, the mixture was pestled again, mixed with 2 ml of Carrez reagent I (15%, m/V, aqueous solution  $\text{K}_3\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ ), 2 ml Carrez reagent II (30%, m/V, aqueous solution  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) and 34 ml of water (to obtain 50 ml volume) and stirred. Precipitated proteins were removed by filtering through folded filter Filtrak-88 (Germany). The filtrate was kept at +4 °C before the analysis.

Procedure 2: 5 g of cut muscle tissue was pestled in a ceramic pot, after adding 10 ml water the mixture was pestled again, treated with 2.5 ml 40% trichloroacetic acid (to 4% final concentration), mixed, and the total volume was adjusted by water to 25 ml. Precipitated proteins were removed by filtration, and filtrate was neutralised by concentrated solution KOH to pH 7.0 and kept at +4 °C before the analysis.

For chemical assay, TCA-extracts were diluted 4–8-folds.

### 2.4. Preparation of standard formaldehyde solution

FA solution for calibration was obtained by hydrolysis of the exact amount of paraformaldehyde in water aliquot (to 1 M concentration) in a sealed ampoule at 105 °C for 14 h and consequent dilution of the hydrolyzate to the required concentration.

### 2.5. Chemical assay of formaldehyde

#### 2.5.1. FA assay by Nash's method (Nash, 1953)

1 ml analysed extract (50–500 nmol  $\text{CH}_2\text{O}$ ) was treated with 1 ml 1 M  $\text{HClO}_4$  and 2 ml Nash's reagent (15% ammonia acetate in water containing 0.3% acetic acid and 0.2% acetyl acetone). The reaction mixture was heated at 58 °C for 5 min, cooled and photometrically measured at 400 nm against the blank sample, containing water instead of the analysed extract. The calculation of FA content (in mg per 1 kg of wet weight of the product) was performed using the calibration curve and taking into account the corresponding dilutions and initial batch of wet tissue.

#### 2.5.2. FA assay using chromotropic acid (Georghiou & Ho, 1989)

One millilitre of analysed extract (25–250 nmol  $\text{CH}_2\text{O}$ ) was treated with 2.5 ml 4% chromotropic acid in concentrated sulphuric acid, heated at 100 °C during 10 min, cooled and measured their optical density at 570 nm against blank containing water instead of the tested sample.

#### 2.5.3. FA assay using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) (Avigad, 1983)

One millilitre of analysed extract (5-fold diluted, containing 10–100 nmol FA) was treated with 3 ml 1% Purpald solution in 1 M NaOH. The samples were incubated at room temperature at continuous shaking for 15–20 min, and the optic density was measured at 548 nm against blank containing water instead of the tested extract.

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