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A New Oxidase-Peroxidase Kit for Ethanol Assays in Alcoholic Beverages

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Summary

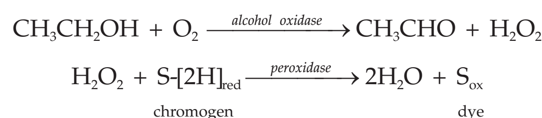
The paper presents analytical parameters of an enzymatic method for alcohol assay in alcoholic beverages by the use of a new kit based on alcohol oxidase and peroxidase catalyzed reactions (AOP). Sensitivity, linearity, accuracy and reliability of the proposed method were tested. It has been shown that results of AOP-assays of alcohol in beers, wines, and strong drinks are in good correlation with those obtained by alcohol dehydrogenase (ADH) method (relations of ADH to AOP values are 0.9197±0.012; correlation coefficient R=0.9983; p<0.0001).

Key words: ethanol, enzymatic analysis, alcoholic beverages, alcohol oxidase, peroxidase, methylotrophic yeast

Introduction

Ethanol assays are very important for control of fermentation processes and certification of alcoholic beverages as well as of alcohol-free sorts of beer. For this purpose, there are a lot of different physico-chemical and chemical approaches. They include gas chromatographic, densitometric and refractometric methods following a preceding distillation, as well as different variants of diffusion methods followed by colorimetric reaction with dichromate or some other oxidative agents. There exists also a number of modern enzymatic, chemo- and biosensor analytical approaches for alcohol determination *(1–12)*. All existing methods, both classical and modern, have some drawbacks. For example, a routine enzymatic method based on alcohol dehydrogenase (ADH) is sufficiently selective, sensitive, and simple in execution, but it is rather expensive due to high price of the enzyme and coenzyme NAD^+.

Another enzyme suitable for analysis of ethanol is alcohol oxidase (AOX) from methylotrophic yeasts *(13)*. This flavoprotein, in contrast to NAD^+-dependent ADH, does not depend on addition of any exogenous cofactor and the corresponding reaction is irreversible. During this reaction, hydrogen peroxide, which can be subsequently used as an oxidant in the coupled enzymatic reaction catalyzed by horseradish peroxidase, is formed. The principle of ethanol determination by alcohol oxidase-peroxidase (AOP) method is based on the measurement of the dye-product accumulation in peroxidative oxidation of chromogen by H_2O_2 generated from ethanol in AOX reaction:



AOX is not a very specific enzyme and besides ethanol can oxidize other primary alcohols, but most efficiently its natural substrate, methanol.

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A number of colorimetric variants of AOP-method are proposed, in which different chromogens are used: ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) *(14)*, a mixture of 4-aminoantipyrine (AAP) with 4-hydroxybenzosulfonate *(15)*, or mixture of AAP with phenol *(16)*.

Similar method for quantitative assay of alcohol in biological liquids was proposed by the authors of this paper *(17)* using a sensitive non-carcinogenic chromogen 3,3',5,5'-tetramethylbenzidine (TMB) *(18)* and a crude preparation of AOX. AOX is isolated from a catalase deficient mutant of the methylotrophic yeast *Hansenula polymorpha* capable to overproduce this enzyme during growth in glucose medium *(19,20)*. A corresponding analytical kit was developed by us for quantitative assay of alcohol content in human blood and serum *(17)*.

Here the results of the use of the elaborated enzymatic kit for alcohol assays in some alcoholic beverages (beer, wine and strong drinks) are described. Sensitivity, linearity, accuracy and reliability of the used approach have been studied.

Materials and Methods

Strain

Catalase-defective mutant *Hansenula polymorpha* C-105 *(19,20)* with impaired *g*lucose *c*atabolite repression of AOX synthesis (*gcr1, catX)* was used as a producer of alcohol oxidase for analytical purposes.

Cultivation and preparation of cell-free extracts

Cells of the mutant C-105 were cultivated in flasks on shaker (200 rpm) at 30 °C to the middle of the exponential growth phase (≈24 h) in the medium containing (in g/L): glucose, 10; $(NH_4)_2SO_4$, 3.5; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2$, 0.1; yeast extract, 3.0. The pH of the medium was adjusted to 5.5 with KOH.

After washing, freshly grown cells (about 15 g wet weight) were resuspended in two volumes of 0.05 M phosphate buffer, pH=7.5. Cells were disrupted in the presence of glass beads in a planetary disintegrator at 1000 rpm and 4 °C during 6 min. Whole cells and cell debris were removed by centrifugation at 13000 × *g* for 40 min. The supernatant was used as the cell-free extract for isolation of AOX. Activity of AOX was determined by the rate of hydrogen peroxide formation in reaction with methanol as recorded by the peroxidative oxidation of *o*-dianisidine in the presence of horseradish peroxidase *(21).*

Isolation of a crude preparation of alcohol oxidase (AOX)

AOX was isolated from a cell-free extract of the mutant C-105 by two step precipitation with ammonium sulfate (at 40 and 60 % of saturation) in the presence of 1 mM EDTA and 0.4 mM phenylmethylsulfonyl fluoride (PMSF) to inhibit proteases. At 40 % saturation a protein precipitate was discarded and the AOX-precipitate obtained at 60 % saturation was collected by centrifugation.

DEAE-cellulose column chromatography was performed as described earlier *(22).*

Analysis of AOX preparations by electrophoresis

Electrophoretic patterns of proteins from methylotrophic yeasts were obtained by native and sodium dodecyl sulfate (SDS) electrophoreses in 6 and 10 % PAG, respectively, in accordance with the methods of Davis *(23)* and Laemmli *(24)*. The gels were stained with Coommassie blue R-250.

Ethanol assays by the use of enzymatic kit »ALCOTEST«

The enzymatic kit consists of three components: *(i)* »Chromogen«, a mixture of chromogen and buffer components: *(ii)* »Enzymes«, a suspension of alcohol oxidase and peroxidase in ammonium sulfate solution and *(iii)* »Standard«, a calibrated ethanol solution, 10 g/L (stabilized). This kit is calculated for 100–400 assays (4–1 mL final volume of reaction mixture, respectively).

Preparation of reagent and standard solutions

The content of bottle 1 (»Chromogen«) was dissolved in 350 mL of distilled water at 100 °C and then cooled to room temperature. The final concentration of TMB was 0.067 mM in 0.05 M phosphate buffer, at pH=7.0. The solution was stable for two weeks at 20 °C, kept in the dark. The content of bottle 2 (»Enzymes« containing 27 units of AOX and 2 mg of horseradish peroxidase with RZ 3.0 in 0.5 mL of 60 % saturated ammonium sulfate) was dissolved in cooled solution of chromogen. Fresh solution was prepared each day. If the number of samples to be analyzed were less than 100 (4 mL reaction mixture) corresponding aliquots of the well stirred enzymes suspension and of the chromogen solution were mixed.

Ethanol solution from the bottle 3 (»Standard«) was diluted 100 fold to the concentration of 0.1 g/L and used the same day.

Sample preparation

Samples of analyzed alcoholic beverages were diluted with water to ethanol concentration of about 0.05–0.5 g/L. Usual dilution factor was 500–2000.

Analysis

To 0.1 mL aliquots of the sample, standards or water (for blank) in separate test tubes portions of 3.5 mL of reagent solution were added. The solutions were well mixed and left to stand at room temperature for 15 to 20 min. The reaction was stopped by addition of 0.5 mL of 0.8 M HCl and absorbance at 450 nm was read against the blank. As the assay was performed by the »fixed time« regime, timing of the beginning and termination of the assay is very important. Therefore, the reaction was started (and also ended) by adding respective reagent sequentially in 30 s intervals.

Calculations

$$\gamma = \frac{A_{sample}}{A_{standard}} \times 0.1 \times N$$

or

$$\varphi_{vol\%} = 0.13 \times \gamma \text{ (in respect to 96.5 \% ethanol)}$$

where mass concentration γ is expressed in g/L, N is dilution factor for initial sample and φ is volume fraction in %.

Alcohol assays were carried out *by alcohol dehydrogenase method* according to the instructions for enzymatic kit »TEST-Combination Blood Alcohol« produced by *Boehringer Mannheim* (Germany).

Samples of alcoholic beverages used for alcohol assays included 5 kinds of beer, 13 kinds of wine and 4 kinds of strong drinks, all the products of Lviv factories, Ukraine.

Results and Discussion

Selection of AOX producer and isolation of enzyme

Although the AOX content in wild type cells of methylotrophic yeasts is very high, there are some problems related to production of cheap preparations of the enzyme for AOP analytical methods: *i)* the requirement of the total absence of catalase activity which destroys hydrogen peroxide, the intermediate analytical product in ethanol assay; *ii)* avoiding autoproduction of hydrogen peroxide by purified AOX preparations by gradual oxidation of formaldehyde covalently bound to the protein during cell growth in the media supplemented with methanol and *iii)* enzyme stabilization.

Ignoring the first problem can result in lowered sensitivity of the analysis and the second factor causes false results in detection of ethanol. Stabilization of AOX preparations is also very important for construction of analytical kits for alcohol assays.

Different methods were proposed for removing catalase from AOX preparations needed for production of H_2O_2. In some of them the expensive chromatographic procedures are exploited *(25)* while others utilize some inhibitors, for example, 3-amino-1,2,4-triazole *(26)*, sodium azide *(27)* or anionic detergents *(13)*. It has to be mentioned that some of the inhibitors are non-specific, *e.g.* NaN_3 *(28)* and use of 3-amino-1,2,4-triazole raises other problems *(26)*. To overcome these drawbacks, it was decided to use the special mutant of methylotrophic yeast as an AOX producer that is totally devoid of catalase activity. As the catalase-defective mutants seemed to be unable to grow in methanol medium *(29,30)*, an additional regulatory mutation impairing catabolite repression of AOX synthesis by glucose and allowing constitutive AOX overproduction in glucose medium without methanol as an inducer *(19,20)* was introduced. AOX from glucose grown cells should also be free from covalently bound formaldehyde. Such adduct is always produced during cell cultivation in the media with methanol and leads to inhibition of enzyme activity and to the appearance of false analytical response. Therefore, the mutant C-105 (*gcr1 catX*) producing high level of AOX in glucose medium (up to 3.9–4.5 U/mg of protein in cell-free extract) and completely devoid of catalase activity, was used *(20)*.

The mutant cells grown in glucose medium were disrupted and cell-free extract was used for partial purification of AOX by two-step ammonium sulfate precipitation (Table 1). Using this simple procedure, enzyme preparation in a form of suspension in 60 % saturated $(NH_4)_2SO_4$, with specific activity of 7.5 U/mg, was obtained. This is close to activity of AOX purified by ion exchange chromatography – 10 U/mg *(22)* and of some commercial preparations of this enzyme. As shown by PAG electrophoresis, the isolated crude preparation of AOX is not homogenous (Figs. 1A and 1B), but still suitable for analytical application.

Isolated crude AOX preparation can be stored at 4 °C in 60 % saturated ammonium sulfate in the presence of 1 mM EDTA and 0.4 mM PMSF for at least 1 year without loss of activity.

Alcohol assays in alcoholic beverages by oxidase-peroxidase method

After optimization of analytical parameters for AOP method using TMB as a chromogen *(18)*, analytical kit »ALCOTEST« was elaborated for alcohol assays in blood *(17)*. Here, the possibility of using this kit for ethanol determination in alcoholic beverages, such as beers, wines and strong drinks, was tested.

Due to the high value of molar absorbance coefficient ($81.7 \cdot 10^3$ M^{-1} cm^{-1}) of the dye formed from TMB in peroxidative reaction of alcohol assay by AOP method *(17)* and high content of ethanol in alcoholic beverages

Table 1. Isolation and purification of alcohol oxidase (AOX) from *H. polymorpha* mutant strain C-105 (calculations of yields were carried out per 1 L of batch culture that corresponds to 15 g wet weight cells); 1 unit of AOX activity (U) is defined as 1 μmole of H_2O_2 produced per 1 min at 30 °C

Procedure	n (total protein)	Total AOX activity	Specific AOX activity	Yield
	mg	U	U/mg	%
Crude extract	450	2475	5.50	100
Precipitation by $(NH_4)_2SO_4$ (40-60 % of saturation)	233	1748	7.50	71
DEAE-cellulose chromatography	149	1520	10.2	61

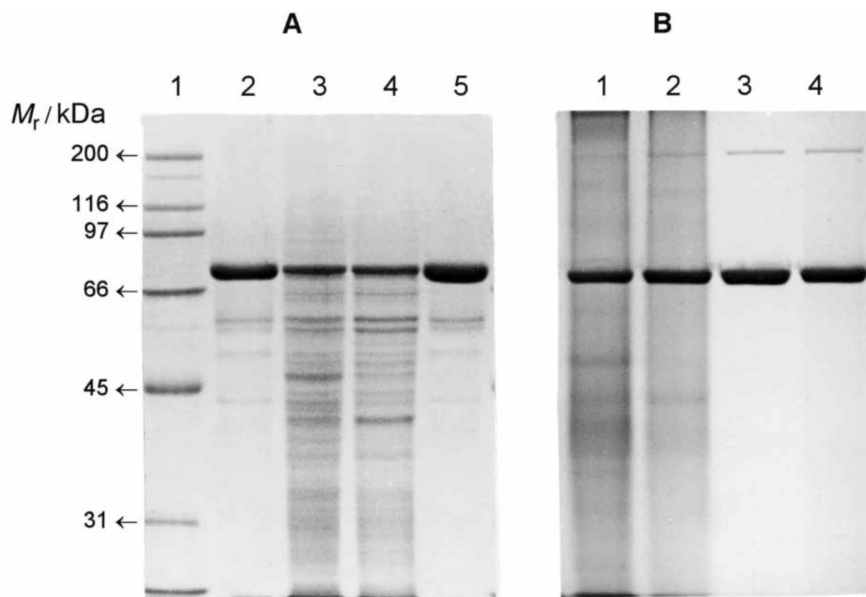

Fig. 1. Electrophoretic patterns of different preparations of alcohol oxidase (AOX) from methylotrophic yeast *H. polymorpha* C-105 (*gcr1 catX*) after SDS- (A) and native (B) electrophoreses in 10 % and 6 % PAG, respectively; **A)** lane 1 – protein standards; lane 2 – commercial preparation of AOX from *Pichia pastoris*, 5 μg of protein; lane 3 – cell-free extract, 30 μg of protein; lane 4 – crude preparation of AOX after second step of precipitation with ammonium sulfate (at 40–60 % of saturation), 20 μg of protein; lane 5 – AOX preparation after chromatography on DEAE-cellulose, 5 μg of protein. **B)** lane 1 – cell-free extract, 30 μg of protein; lane 2 – crude preparation of AOX after second step of precipitation with ammonium sulfate (at 40–60 % of saturation), 20 μg of protein; lane 3 – AOX preparation after chromatography on DEAE-cellulose, 5 μg of protein; lane 4 – commercial preparation of AOX from *Pichia pastoris*, 5 μg of protein

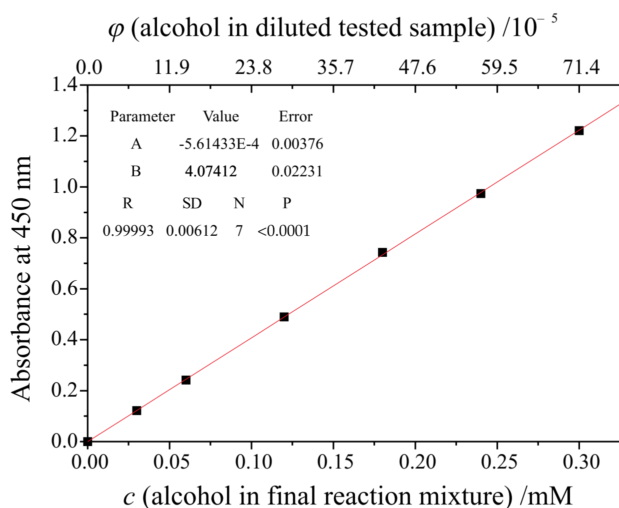

Fig. 2. Typical standard curve for ethanol assay by alcohol oxidase-peroxidase method; alcohol content is presented in different units: at the bottom axis – as concentration (in mmol/L) in a final assay mixture; at the top axis – as volume fraction in 10^{-5} in a diluted (usually 1000-fold) tested sample before its addition to an assay mixture (see Materials and Methods). Near the graph some statistical data are presented: parameters of linear regression (coefficients of equation Y=A + BX, where Y – optical density; X – alcohol content, mM; B – slope value), correlation coefficient (R) and standard deviation of the linear fit (SD) as well as number of data points (N) and P – probability (that R is zero)

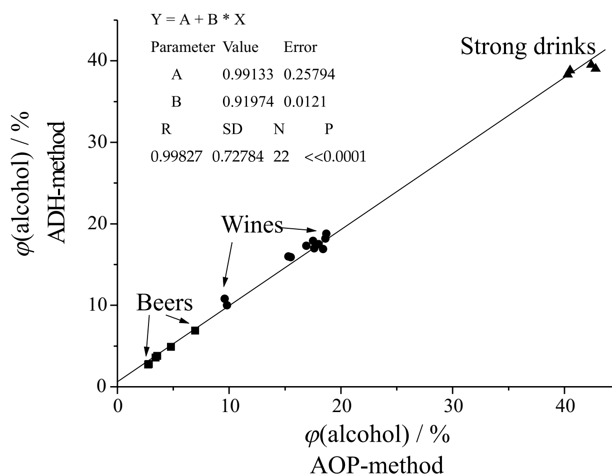

Fig. 3. Correlation between results of alcohol assays (volume fraction in %) in alcoholic beverages by alcohol oxidase-peroxidase (AOP) and alcohol dehydrogenase (ADH) methods; near the graph some statistical data are presented: parameters of linear regression (coefficients of equation Y=A + BX, where X and Y – alcohol content measured by AOP and ADH methods, respectively; B – slope value), correlation coefficient (R) and standard deviation of the linear fit (SD) as well as number of data points (N) and P – probability (that R is zero)

we usually use conditions of incomplete (5–10 %) conversion of analyte during analytical procedure (so-called »fixed reaction time« mode). Although the exploited sensitivity was 10–20 fold lower as compared with regime of complete termination (»end point«) of analytical reaction, it provides the possibility to test the content of alcohol as low as 10 nmole (0.46 μg) per 1 mL of reaction mixture. As shown in Fig. 2, the linear response of the absorbance of the resulting dye to the alcohol content is observed over the concentration range 0.01–0.3 mM (0.5–14 μg/mL) in the reaction mixture or 20–560 μg per 1 mL of analyzed solution that is equivalent to volume fraction of $2.6 \cdot 10^{-3}$–$72 \cdot 10^{-3}$ % ($R > 0.9999$; $p<0.0001$).

Reproducibility of parallel alcohol assays by AOP method is high enough; variation was determined to be 1–5.5 % in the middle of the linear part of the calibration curve.

Reliability of alcohol assays by AOP method was tested by parallel measurement of ethanol concentration in alcoholic beverages by ADH method (Fig. 3). It has been shown that results obtained by both methods are in good correlation (relations of ADH to AOP values are 0.920±0.012; correlation coefficient $R = 0.9983$; $p<0.0001$).

Thus, the experimental data confirm the possibility to use the analytical kit »ALCOTEST« based on AOP reactions for routine alcohol assays in alcoholic beverages. The proposed kit provides the accurate analytical characteristics and could be less expensive than ADH commercial kit due to the low cost needed for isolation of alcohol oxidase from AOX-overproducing catalase deficient mutant strain.

Acknowledgements

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Novi oksidazno-peroksidazni pripravak za određivanje etanola u alkoholnim pićima

Sažetak

U radu su navedeni parametri analitičkog postupka za određivanje etanola u alkoholnim pićima uporabom novog pripravka koji sadržava alkohol-oksidazu i peroksidazu (AOP postupak). Ispitana je osjetljivost, linearnost, točnost i pouzdanost predloženoga postupka. Pokazalo se da se dobiveni rezultati o količini alkohola u pivu, vinu i žestokim pićima podudaraju s onima dobivenim u postupku s alkohol-dehidrogenazom (ADH postupak). Odnos između vrijednosti dobivenih ADH i AOP postupkom iznosi 0,9197±0,012, a koeficijent korelacije R=0,9983; p<0,0001.