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Alcohol oxidase of the methylotrophic yeasts: new findings

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Abstract

The paper summarizes some of our recent findings in structure–function organization of the alcohol oxidase (AO) from two species of methylotrophic yeasts. As revealed by PAGE, in methanol-grown *Hansenula polymorpha* DL1 and *Pichia methanolica* MH4 along with AO, the non-flavin minor protein having AO activity and different type of subunits is expressed. HLPC analysis of the content of FAD and its modified analogue (mFAD) in the AO isoforms of *P. methanolica* MH4 and the occurrence of mFAD in AO-lacking mutants of *H. polymorpha* DL1 imply a biosynthetic origin of this cofactor. Restricted degradation of the flavin part of mFAD by sodium periodate has established the presence of xylitol instead of arabitol. Thus, mFAD having such a xylose fragment is a xylo-FAD, but not arabino-FAD as reported earlier.

Keywords: Methylotrophic yeasts; Alcohol oxidase multiplicity; Structure–function organization; Modified FAD

1. Introduction

Alcohol oxidase (AO, EC 1.1.3.13) catalyzes oxidation of methanol to formaldehyde and hydrogen peroxide in methylotrophic yeasts. This peroxisomal oligomeric flavoprotein contains noncovalently bound FAD and its modified analog, mFAD [1,2]. By using electrophoretic procedure, Lee and Komagata [3] first demonstrated that AO preparations from many methylotrophic yeasts consisted of two active bands: the minor and major proteins. Later, Bystrykh et al. [4] observed both major and minor proteins with AO activity in the AO preparations from *Hansenula polymorpha*. However, the authors could not separate these proteins and suggested that the minor protein is the AO octameric form which dissociates to the tetramers (the major protein) under alkaline pH values of the electrophoretic buffer. Recently, by routine chromatographic methods, we were able to separate the analogous proteins occurred in *H. polymorpha* DL1. The minor protein with AO activity, like to that of *H. polymorpha*, also presented in *Pichia methanolica* MH4 alongside the AO isozymes reported by us earlier [5]. In contrast to the minor protein with AO activity, these isozymes contained both FAD and mFAD. The latter was found only in methylotrophic yeasts [6–8]. Some authors suggested that mFAD appeared to be formed as a result of spontaneous

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modification of FAD in the active center [6,7,9]. Their NMR studies have shown that mFAD differs from FAD by stereoposition of the hydroxylic group in acyclic carbohydrate chain [6,9], thus suggesting that mFAD has a structure of arabino-FAD [9]. Herein, we report the new results on separation and partial characterization of the proteins with AO activity, including the AO isozymes, as well as the formation and structure of mFAD.

2. Experimental

2.1. Organisms and cultivation

The methylotrophic yeast *H. polymorpha* DL1 and its AO-lacking mutants *AOX14*, *AOX41*, and *AOX42* [10] from the culture collection of the Institute of Biochemistry and Physiology of Microorganisms (Russian Academy of Sciences) were used. We also used *P. methanolica* MH4 from the culture collection of the Institute of Biotechnology (Leipzig, Germany). *P. methanolica* MH4 was grown in an Ankum-2 fermentor (Scientific Manufacturing Association Biopribor, Pushchino) in the periodic regime at 30°C in minera1 medium [5] with 1% methanol, and samples were drawn at fixed intervals. *H. polymorpha* DL1 was grown at 37°C to mid-logarithmic growth phase. The yeast was also cultivated in the chemostatic regime at dilution rates 0.05, 0.1 and 0.16 h^{-1}, as described previously [5].

2.2. AO purification and characterization

AO was purified at 4°C. Wet biomass (10–20 g) was suspended in buffer A containing 50 mM Tris–HCl, 0.2 mM phenylmethylsulphonyl fluoride, pH 7.5 and disrupted in a French pressure cell. The crude extract was centrifuged at $10\,000 \times g$ for 30 min and the supernatant was fractionated with ammonium sulphate of 40–70% saturation. The precipitate was dissolved in a minimal volume of buffer A and dialysed against the same buffer. The dialysed solution was applied to a DEAE-Sepharose column (16 $\times$ 150 mm) equilibrated with buffer A and eluted for 4 h with a linear gradient of KCl (0–0.7 M) at flow rate of 120 ml/h. Fractions with AO activity were pooled and ammonium sulphate was added up to 40% of saturation. The solution obtained was applied to a Butyl-Toyopearl column (16×150 mm) equlibrated with buffer B (buffer A plus ammonium sulphate up to 40% saturation) and eluted for 3 h with a linear gradient of buffer A (0–100%) at flow rate of 120 ml/h. Pooled fractions having AO activity were concentrated on Amicon ultrafiltration membrane PM-30 (Pharmacia, Sweden). The concentrated preparation was applied to a Sepharose CL 6B column (16×1000 mm) eluted with buffer A at flow rate of 18 ml/h.

Visualization of proteins after PAGE was carried out with Coomassie blue or by activity with following reaction mixture: 70 ml of 50 mM Tris–HCl buffer (pH 7.0), 300 U of peroxidase, 0.5 ml of methanol, 5.5 mg of *o*-dianisidine. Gels were incubated at 30°C in the dark in this mixture and fixed by 7% acetic acid. The AO activity was measured spectrophotometrically at 460 nm in coupled assay with *o*-dianisidine and peroxidase as earlier described [4]. Protein concentration was determined according to Lowry et al. [11].

Molecular weight of the proteins was determined by either gradient (5–30%) PAGE under nondenaturating conditions or size exclusion chromatography (SEC) on a Superose 6 column. As electrode and separating buffers 50 mM Tris–HCl, pH 7.0, 7.5, 8.8 were used. A subunit weight of the proteins was determined by 15% SDS-PAGE according to Laemmli [12].

2.3. mFAD content determination

mFAD content in AO was determined by HPLC using a Gilson 121 fluorimeter (France) equipped with 450 nm excitation filter and 510–

650 nm emission filter. Each AO isoform (10–15 μg in 40 μl) was mixed with 20 μl of 15% trichloracetic acid and incubated at 4°C for 5 min in total darkness. Then the analyzed mixture was centrifuged at 15 000 × *g* for 10 min, and the supernatant was applied to a column (4 × 250 mm, 5 μm) of Lichrosorb RP18 (LKB, Sweden). Isocratic elution was carried out with 0.35 M potassium phosphate buffer supplemented with acetonitrile (90 ml/l) and methanol (50 ml/l), pH 2.5, at flow rate 0.5 ml/min [6].

2.4. Preparative isolation of modified flavins

To 2 ml of buffer solution of purified AO (10 mg/ml), 15% trichloracetic acid was added to a final concentration 5% and incubated 10 min in the absence of light. Denaturated protein was sedimented (30 min at 15 000 × *g*). A supernatant containing FAD and mFAD was applied to a Lichrosorb RP18 column (4 × 250 mm, 5 μm) and fractionated by isocratic HPLC in potassium phosphate buffer with addition of acetonitrile and methanol [6] at elution rate 0.4 ml/min under 280 nm. The mFAD-containing fractions were evaporated, then a dry residue was applied to the same column and washed with 20 ml H_2O and after elution with 50% CH_3OH evaporated. mFMN and m-flavin were obtained from purified mFAD according to Ref. [8].

2.5. Preparation of m-flavin for the MS-analysis

Chemical modification of flavins for MS-analysis was carried out as follows. 100 μg dry m-flavin or riboflavin dissolved in 3 ml absolute pyridine and 500 μl acetic anhydride was added and boiled on a water bath for 30 min in the dark. An acetylated product was purified from pyridine and minor admixtures on Lichrosorb RP18 column by using HPLC with a linear gradient of methanol (0–50%) for 30 min at 0.5 ml/min. Homogenous fractions of acetylated flavins were dried in a vacuum evaporator and analyzed on a Finnigan-MAT 8430 mass spectrometer (Bremen, Germany).

2.6. Restricted degradation of flavins and analysis of their fragments

Restricted degradation of riboflavin, FMN, m-flavin and mFMN was done by sodium periodate as described below. To 50 μl of water solution of flavin (3–8 nmol/ml according to spectrophotometric data), 50 μl glucose solution (0.4 g/ml) and 50 μl of sodium periodate solution (0.2 g/ml) were added. The reaction mixture was thoroughly mixed and after 10-min incubation at room temperature in the dark, 50 μl of sodium borohydride solution (0.15 g/ml) was added. After 5 min, 10 μl glacial CH_3-COOH was added and mixed. The presence of flavin fragments was analysed by isocratic HPLC as described in Section 2.3.

3. Results and discussion

3.1. An unkown alcohol oxidizing protein (x-AOP)

AO and x-AOP (the minor protein with AO activity) from *H. polymorpha* DL1 were first separated by us to electrophoretic homogeneity by using routine chromatographic methods. As seen in Fig. 1, the x-AOP did not dissociate into the AO during electrophoresis as reported ear-

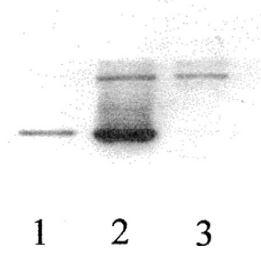

Fig. 1. 5% PAGE under native conditions. (1) The major protein, (2) cell-free extract of *H. polymorpha* DL1, (3) the minor protein stained on AO activity.

lier [4]. Our further studies on the purified AO preparation revealed some new features. SDS-PAGE showed that the x-AOP and AO consisted of different subunits Mr 77 and 75 kDa, respectively (Fig. 2), but not of one type subunits as reported earlier [4]. Moreover, our HPLC analysis revealed that the AO contained both FAD and mFAD, while no flavins were detected in the x-AOP. We also found that the value of molecular weight of the x-AOP was dependent on the method used: 680 kDa as determined by gradient PAGE (Fig. 3) and 480 kDa as determined by SEC. On the contrary, molecular weight of the AO was 450 kDa (Fig. 3) in both procedures. The results did not depend on the pH values of electrode and separating buffers. Perhaps, the x-AOP in opposite to the AO is able to aggregate under high local concentration during native PAGE. Previous communication about AO molecular weight equal to 758 kDa as determined by SEC appeared to be erroneous since the authors [4] have used inappropriate SEC column TSK G3000 SW applicable for fractionation of the proteins with molecular weights from 1 to 300 kDa. Hence, the difference in subunits type and the absence of FAD excludes the assignment of the x-AOP to the AO oligomeric form.

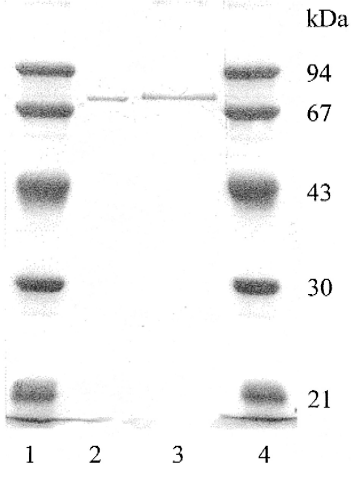

Fig. 2. 10% SDS-PAGE. (1 and 4) of marker proteins: phosphorylase b — 94 kDa, bovine serum albumin — 66 kDa, ovalbumin — 43 kDa, carbonic anhydrase — 30 kDa, trypsin inhibitor — 21 kDa; (2) the major protein, (3) the minor protein from *H. polymorpha* DL1.

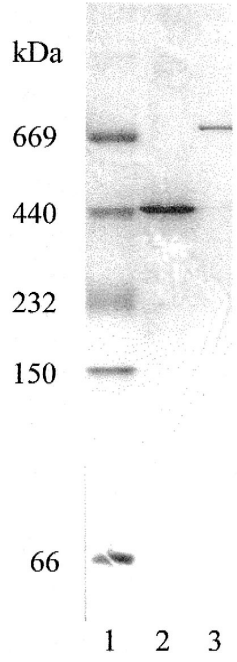

Fig. 3. 5–30% gradient PAGE under native conditions. (1) Marker proteins: thyroglobulin — 669 kDa, ferritin — 440 kDa, catalase — 232 kDa, alcohol dehydrogenase — 150 kDa, bovine serum albumin — 66 kDa; (2) AO; (3) the x-AOP.

3.2. AO multiplicity: possible reasons and consequences

Multiple AO isoforms have been found by us and other workers in several strains of *P. methanolica* [5,13]. These isoforms are designated from 1 to 9 according to their electrophoretic mobility (Fig. 4). All nine isoforms have identical molecular weights of the native protein (450 kDa) and subunits (75 kDa), but different charges (pI 4.18–5.2) and kinetic properties (Km 0.5–5.5, methanol as substrate) [5]. As suggested by the Japanese colleagues [13], the reason for such molecular heterogeneity is the occurrence of two *AOX* genes, dif-

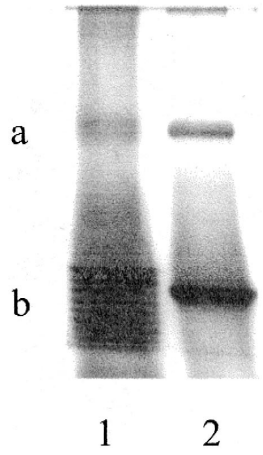

Fig. 4. 5% PAGE under native conditions. (1) Cell-free extract of *P. methanolica* MH4: (a) the x-AOP, (b) the AO isoforms. (2) Cell-free extract of *H. polymorpha* DL1: (a) the x-AOP, (b) AO. Gel was stained on AO activity.

fered in some amino acids substitutions in the proteins encoded. Evidently, the octameric AO isozymes are formed as a result of combination of two types of subunits being encoded by the appropriate *AOX* genes. Our studies on the separate isoforms isolated from AO of *P. methanolica* MH4 confirmed the occurrence of two types of subunits in the enzyme. According to HPLC analysis of the first and the ninth isoforms, they are homooligomers consisting of either α- or β-subunits, respectively. Alternatively, the intermediate isoform 5 is a heterooligomer comprising both types of the above subunits associated in the AO octameric structure in equimolar ratio [5].

Ellis et al. [14] have shown that *P. pastoris* possesses two highly homologous genes *AOX1* and *AOX2*, but having different promoters and transcription levels [15]. As followed from zymograms published by Lee and Komagata [3], the occurrence of two *AOX* genes in *P. pastoris* did not result in the AO isozymes formation, but the minor and major proteins with AO activity have been expressed by analogy to *H. polymorpha* DL1. It therefore can be suggested that the minor protein of *P. pastoris* is the x-AOP as a product of *AOX2* gene transcribed on a much lower level in comparison with the *AOX1* gene [15]. It is worth to note that in *H. polymorpha* DL1 and *Candida boidinii* having only one *AOX* gene [16, 17], two AO active proteins are expressed [3]. It cannot be excluded the existence of a specific gene encoding the x-AOP in the methylotrophic yeasts. The latter suggestion is supported by the presence the x-AOP in *P. methanolica* MH4 alongside with AO isoforms (Fig. 4), its α- and β-subunits, which are encoded by the specific genes [5,13]. Certainly, these data are not yet sufficient to answer the question: what is the function of the x-AOP? In this connection, there are interesting results of subcellular fractionation that indicate the peroxisomal localization of this protein (data not shown). Since the intervals between AO crystalloids are narrow [18,19], the x-AOP having the more molecular weight than AO is evidently presented directly in the AO crystalloids or located along the surface of peroxisomal membrane. Therefore, it has to be further explored whether the x-AOP is a structurally novel type AO or it is a new enzyme of methylotrophic yeasts.

3.3. Formation and distribution of mFAD between the AO isozymes

As mentioned above, the AO isoforms are flavoproteins. Analysis of three individual AO isozymes (1, 5, and 9) revealed that mFAD content varied unevenly during periodic cultivation of the yeast. During lag-phase, mFAD was present in the AO isoforms in trace amounts. In exponentially grown cells, mFAD content in isoform 1 increased faster (0–78%) than in isoform 5 (0–51%), and much faster than in isoform 9 (0–18%). At the stationary growth phase, isoform 1 contained the maximal amount of mFAD (78.7%). The change in the ratio mFAD/FAD in the AO isoforms has no effect on their electrophoretic mobility. The mFAD content in the AO isoforms also depended on dilution rate during chemostatic yeast cultiva-

tion. When the yeast was grown under dilution rate 0.05 h^{-1}, only isoform 1, containing 82% mFAD, was expressed. At 0.1 h^{-1}, the mFAD content was maximal in isoform 1 and minimal in isoform 9, as it was at stationary growth phase of the periodic cultivation. Alternatively, at 0.16 h^{-1}, mFAD was not detected in all nine AO isoforms [20].

Following a report about spontaneous FAD modification during incubation of purified AO of *H. polymorpha* DL1 in potassium phosphate buffer [6,7], we hypothesized that in *P. methanolica*, the AO isoforms have different FAD-modifying abilities. However, the initial mFAD content (about 7%) in the 1st, 5th, and 9th AO isoforms of this yeast was constant during 5 days of incubation. Moreover, on repeated incubation of the AO preparation from *H. polymorpha* DL1, we did not detect any spontaneous FAD modification. Furthermore, both FAD and mFAD were found in *H. polymorpha* DL1 mutants *AOX14*, *AOX41*, and *AOX42* (in average 7–12% mFAD) despite the fact that they lacked the AO activity [20]. Since there was no spontaneous FAD modification, we believe that in these mutants mFAD could appear only as a result of biosynthesis.

3.4. Postulated structure of mFAD

Previous NMR studies of the mFAD structure have demonstrated that this stereoisomer differs from classical FAD by configuration of the second or third carbon atoms in acyclic carbohydrate chain [6,8,9]. To clear up the mFAD structure, we have performed additional studies with the use of mass-spectrometry and chemical analysis.

According to the MS results, an acetylated m-flavin component of mFAD was identical to the spectrum of 2′,3′,4′,5′ tetraacetate riboflavin (Fig. 5) [21]. This also corresponded to the NMR evidence [6,8,9] of different stereoposition one of the hydroxylic group. Further determination of m-flavin structure was done by restricted degradation of C(OH)–C(OH) bonds with the use of sodium periodate. By using HPLC, the fragments obtained were compared with the fragments of FMN and riboflavin. The residual C atoms in carbohydrate moiety of the

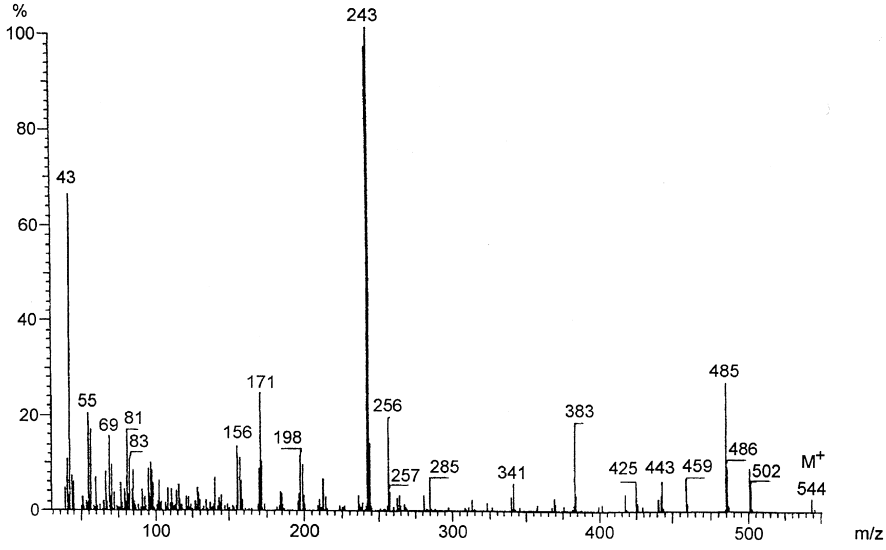

Fig. 5. Mass-spectrum of the acetylated m-flavin.

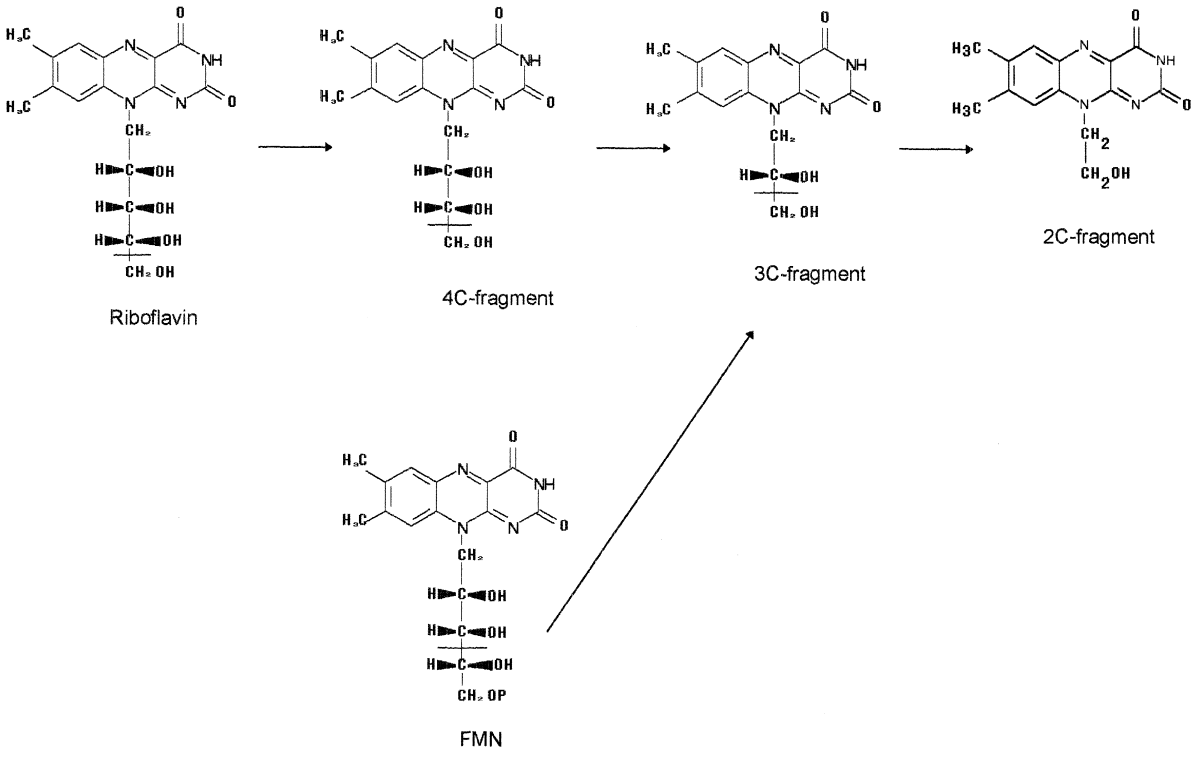

Fig. 6. Scheme of the restricted degradation of the flavin part by sodium periodate.

flavins were designated quantitavely as 4C, 3C and 2C (Fig. 6).

A restricted degradation of riboflavin or m-flavin yielded two even fragments and the different one (Fig. 7). The fragments 2 and 3 identical to those of FMN and mFMN were formed. During a complete degradation of all the above flavins the only equal fragment 3 with 2C-structure was formed [8] (Fig. 6). As known [22,23], a formation of the structural fragment 4C from FMN is not possible because of the presence of the phosphate residue in this flavin. Hence, the fragment 2 of FMN and other flavins (Fig. 7) has a 3C-structure (Fig. 6). Alternatively, the 4C-structure corresponds to the riboflavin fragment 1 (Fig. 7) which was absent upon FMN degradation. Since riboflavin and m-flavin are the isomers (Fig. 5), their fragments 1 and 1′ (Fig. 7) are also the isomers and its different chromatographic behaviour reflects the opposite configurations of C3 atom in carbohydrate chain (Fig. 8). Our analysis did not follow the configuration of C4 atom in the carbohydrate moiety. However, according to

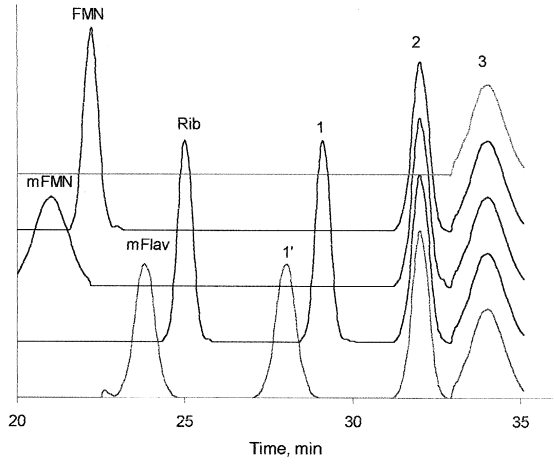

Fig. 7. Reverse-phase HPLC analysis of the carbohydrate fragments of degradation of riboflavin, FMN and their modified analogs. Rib — riboflavin, mFlav — m-flavin, (1′) 4C-fragment of m-flavin, (1) 4C-fragment of riboflavin, (2) 3C-fragment of flavins, (3) 2C-fragment of flavins.

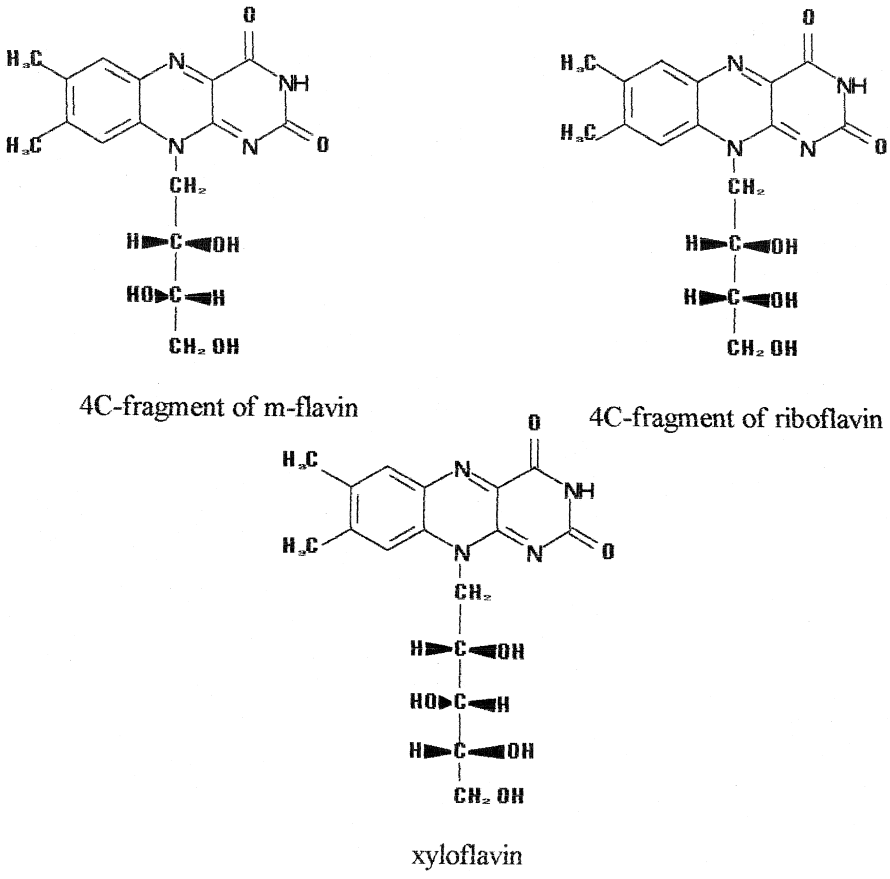

Fig. 8. The structure of 4C-fragment of m-flavin, 4C-fragment of riboflavin and xyloflavin.

Sherry and Abeles [8], the configuration of C4 atoms in m-flavin and riboflavin is the same. Therefore, m-flavin is a xyloflavin and mFAD having such a xyloflavin is a xylo-FAD (Fig. 8), but not an arabino-FAD as reported earlier [9].

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