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Review

Laccase-catalyzed oxidative polymerization of aniline dimer (N-phenyl-1,4-phenylenediamine) in aqueous micellar solution of sodium dodecylbenzenesulfonate

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ABSTRACT

We have studied oxidative polymerization of aniline dimer (N-phenyl-1,4-phenylenediamine) catalyzed by high-redox potential laccase isolated from the fungi *Trametes hirsuta* (*Wulfen*) *Pilát* CF-28. Enzymatic aniline dimer polymerization was performed in aqueous micellar solution of sodium dodecylbenzenesulfonate, the atmospheric oxygen serving as an oxidizer. The resultant dispersion was stable for at least 6 months. The products synthesized were characterized using Fourier transform infrared and UV–vis spectroscopies. MALDI TOF analysis has shown that aniline dimers polymerize to mainly form aniline oligomers with the *m/z* ratio up to 2180, which corresponds to a polymerization degree of 24 (in terms of aniline subunits). Enzymatically formed aniline oligomers consist for the most part of para-directed units in the form of emeraldine salt. The end product structure depends on the reaction medium pH. Transmission electron microscopy has revealed granular nanoparticles of the reaction product.

Contents

1. Introduction

Laccase (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2), a multicopper enzyme belonging to the family of blue oxidases, catalyzes the one-electron oxidation of various organic substances to produce radicals [1,2]. Moreover, the atmospheric oxygen serves as an oxidizing agent. Laccases find potential application in organic synthesis including biotransformations of organic substrates into

Abbreviations: PANI, polyaniline; PPDA, N-phenyl-1,4-phenylenediamine; SDBS, sodium dodecylbenzenesulfonate; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; FTIR, Fourier transform infrared; TEM, transmission electron microscopy; MALDI TOF, matrix assisted laser desorption ionization coupled to time-of-flight; CMC, critical micelle concentration; HPR, horseradish peroxidase; NHE, normal hydrogen electrode.

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polymers [3–6]. The using of laccase in synthetic organic chemistry has increased due to its broad substrate specificity. In organic chemistry laccase can be employed for the oxidation of functional groups; the coupling of phenols and amines; the construction of carbon–nitrogen bonds and so on.

The use of laccase enables the environmentally friendly polymerization of aniline with better characteristics as compared to the polymer produced by the traditional chemical synthesis.

Polyaniline is one of the most important conducting polymers due to its stability, rather simple production, a low cost of the monomer and the possibility to change its optical and electrical properties by varying the degree of protonation and oxidation. Because of its physicochemical properties, PANI can be used in lightweight power sources [7], light-emitting diodes [8], for corrosion and static discharge protection [9], in sensors [10] and many other fields.

The commercial application of the PANI prepared by chemical oxidation of aniline with ammonium persulfate as oxidant is mainly hampered by environmentally unfriendly harsh conditions of the synthesis (highly acidic pH values and great amount of the oxidizing agent used) and poor solubility of the polymer in common organic solvents. Both problems can be eliminated by means of biotechnology, namely, by synthesizing PANI with enzymes as catalyst in the presence of certain templates. The use of templates improves the processability of the PANI obtained. As templates, negatively charged polyelectrolytes and anionic surfactant micelles are most commonly employed [11,12]. Plant peroxidases isolated from various sources like horseradish [13], soybeans [14] and palm trees [15], as well as fungal laccases [16] are mainly used in aniline polymerization. Peroxidases catalyze the reaction of aniline oxidation with hydrogen peroxide. However, they lose their initial activity at hydrogen peroxide concentration above 1 mM and at pH values below 4.0. At the same time, in laccase-catalyzed aniline polymerization, the atmospheric oxygen serves as oxidant. Besides, unlike many peroxidases, fungal laccases show acid stability which enables the synthesis of conducting PANI at low pH values. Aniline oxidation in acidic solution occurs by way of radical cations, of which aniline dimers then form [17,18]. The radical formed during the initial stage of the oxidative polymerization reaction can be localized either on the nitrogen atom or on the ortho- or para-carbons, which resulted in structural heterogeneity of the reaction products. The further oxidation of aniline dimers results in the formation of radicals that couple to produce aniline oligomers, and thus the polymeric chain is growing. Among all the forms of PANI only the emeraldine salt is conductive. Chemical polymerization is an autocatalytic process, the reaction of aniline dimer formation being rate-limited [19]. Unlike chemical polymerization, laccase-catalyzed PANI synthesis occurs without an induction period and by another mechanism [20]. It is essential that the mechanism of aniline dimer oxidative polymerization should be elucidated to illuminate the molecular mechanism of laccase-catalyzed aniline polymerization.

The purpose of the given work was to study the oxidation reaction of aniline dimer (PPDA) catalyzed by high-redox potential laccase in aqueous micelles formed from sodium dodecylbenzenesulfonate (SDBS) and investigate the physico-chemical properties of the products obtained.

2. Materials and methods

2.1. Materials

PPDA, N-phenyl-1,4-phenylenediamine (Aldrich, USA); SDBS, dodecylbenzenesulsonic acid sodium salt, sodium hydroxide (Fluka, Italy); Na_2HPO_4, citric acid anhydrous (Riedel-de Haen, Germany), tetrahydrofurane (VWR, Austria); ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid (Sigma, Germany) were used without further purification. The other chemicals and solvents were of analytical grade or better, and also used as received. Aniline was purchased from ChemMed (Russia) and purified by double distillation in vacuum.

All solutions were prepared with the water deionized with the help of a "Simplicity" system (Millipore, USA). Laccase was isolated from the culture liquid of the fungi *Trametes hirsuta* (*Wulfen*) *Pilát* CF-28 as describe in [21]. The enzyme was purified to homogeneity according to SDS-electrophoresis. The activity of laccase was measured spectrophotometrically, using 10 mM catechol as a chromogenic substrate ($\lambda = 410$ nm, $\varepsilon = 740\ M^{-1}\ cm^{-1}$), at 24 °C in 0.1 M Na–citrate–phosphate buffer, pH 4.5. One unit of activity is defined as the amount of laccase oxidizing 1 μm of substrate per min. The specific activity of the enzyme preparation was about 200 U/mg of protein.

2.2. Methods

2.2.1. Enzymatic polymerization

As PPDA is poorly soluble in water solutions and its concentration depends on pH of the medium, the enzymatic reactions were performed in experimentally saturated solutions of aniline dimer in 10 mM Na–citrate–phosphate buffer (maintained at pH 3.8) at room temperature with constant stirring. Under these conditions, PPDA concentration was about 1–1.5 mM. SDBS concentration was 10 mM, unless otherwise stated. The oxidation reaction was initiated by adding laccase. The syntheses of PANI/SDBS complexes were carried out under the air saturated condition for 1.5 h to complete the reaction polymerization. There was no significant change in pH during the reaction. The enzyme activity in the medium was 0.4 U/ml. The reaction of PPDA oxidation was monitored using a Shimadzu UV-mini 1240 spectrophotometer (Japan).

The enzyme long-term stability was determined in 50 mM Na–citrate–phosphate buffer (pH 3.8) and at the same enzyme concentration. The kinetic measurements were performed at 25 °C. The enzyme operating stability was recorded spectrophotometrically by the rate of ABTS oxidation ($\lambda = 420$nm, $\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$), equal aliquots of the solutions tested being taken at certain time intervals.

Depending on the purpose of experiment, either the initial aqueous aniline oligomers/SDBS dispersion obtained by enzyme-catalyzed synthesis or the surfactant-free product was used. To do this, the product of the enzymatic synthesis was first treated with 3% aqueous ammonia for 3 h, and then diluted with the equal volume of ethanol. The precipitate was separated by centrifugation, washed with deionized water, and dried for 48 h at 70 °C.

2.2.2. Characterization

FTIR-spectra of dedoped products of the enzymatic PPDA polymerization in KBr pellets were recoded with a Nicolet Magna-750 spectrophotometer. The morphology of the product was studied by transmission electron microscopy using JEM-100CX/SEG (Jeol, Japan). The sample of the aqueous dispersion was preliminary dialyzed against deionized water to separate excess salts, whose crystals could cause image distortion, and then spotted onto templates. Electrochemical experiments were carried out at room temperature in a glass cell using a three-electrode system connected to a potentiostat (BAS CV-50W, Electrochemical analyzer, Bioanalytical System, West Lafayette, IN). A glass carbon electrode (BAS) was used as the working electrode. The reference and counter electrodes were Ag/AgCl (BAS) and 1 mm platinum wires, respectively.

MALDI-TOF spectra of the dedoped products were acquired on a Brucker Daltonics Microflex MALDI mass spectrometer. A 1 μl

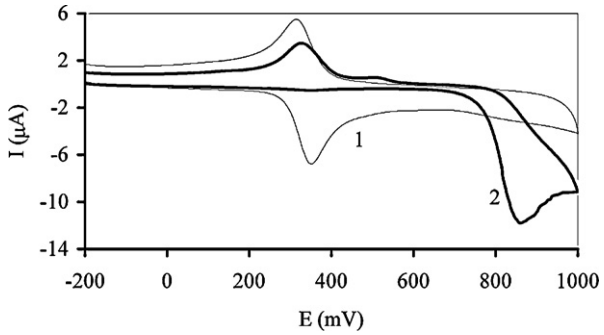

Fig. 1. Cyclic voltammograms of aniline dimer (1) and aniline (2) recorded in buffer solutions. The experimental conditions: scan rate – 100 mV/s; 10 mM Na–citrate–phosphate buffer (pH 3.8); experimentally saturated solution of aniline dimer diluted with buffer (1:200); [aniline] = 2×10^{-5} M.

sample of aniline dimer oxidation products in tetrahydrofurane (1.2 mg/ml) was applied to the target in two experiments. The spectra were recorded in the reflection mode. The instrument was calibrated against peptides with known molecular mass from 700 to 3500 Da.

3. Results and discussion

We earlier reported that laccase from the fungi *Trametes hirsuta* catalyzes the polymerization of aniline with a good yield of conducting polymer [16,20,22,23]. The products of aniline oxidation are water-insoluble, which hamper studying the polymerization reaction. Therefore, we have used the template-assisted method of enzyme-catalyzed PANI synthesis, which enables the formation of stable aqueous dispersion of PANI/template complexes and investigation of the polymerization reaction. As a soft template, aqueous micelles formed from SDBS were used. The anionic micellar template secures the appropriate alignment of protonated aniline molecules to obtain linear polymers and provide charge-compensating anions for the main chain of the conducting polymer. The same approach was also used in the given work to study oxidative polymerization of aniline dimer.

3.1. Enzymatic oxidation of PPDA in aqueous micellar solution

The redox potential of aniline dimer is much lower than the potential of aniline oxidation (Fig. 1). Since the redox potential of T1 site of *T. hirsuta* laccase is very high (780 mV vs NHE), laccase-catalyzed reaction of PPDA oxidation is thermodynamically preferential as compared to aniline oxidation. We have found that in aqueous SDBS micellar solution laccase-catalyzed PPDA oxidation resulted in formation of a stable dark green dispersion. Time-dependent changes in UV–vis absorption spectra of PPDA/SDBS complex and the spectra of aniline dimer are shown in Fig. 2a and b, respectively. It is noteworthy that the rate of enzymatic oxidation of PPDA in aqueous SDBS solutions is significantly higher than the rate of aniline oxidation under the same conditions [20]. Characteristic electron absorption bands are well seen at 800–1100 nm and 410–420 nm wavelengths, which indicate the formation of a polaron in the polyaniline chain. The enzymatic oxidation of aniline dimer was performed at SDBS concentration of 10 mM, which is higher than the critical micelle concentration (CMC) for this surfactant (1.6 mM). The variation of SDBS concentration in the reaction medium at a fixed pH value and saturating concentration of aniline dimer showed that after 20 min the absorbance of the solution at λ = 950 nm is significantly lower at surfactant concentrations of 2 mM and 20 mM than at 6 mM and 10 mM concentrations (Fig. 3). There was no enzymatic polymerization at SDBS concentrations below CMC. The laccase-catalyzed oxidation of aniline dimer under the same experimental conditions but without SDBS resulted in the formation of brown precipitate of an irregular structure, which indicates an important role of templates and their influence on the end product nature.

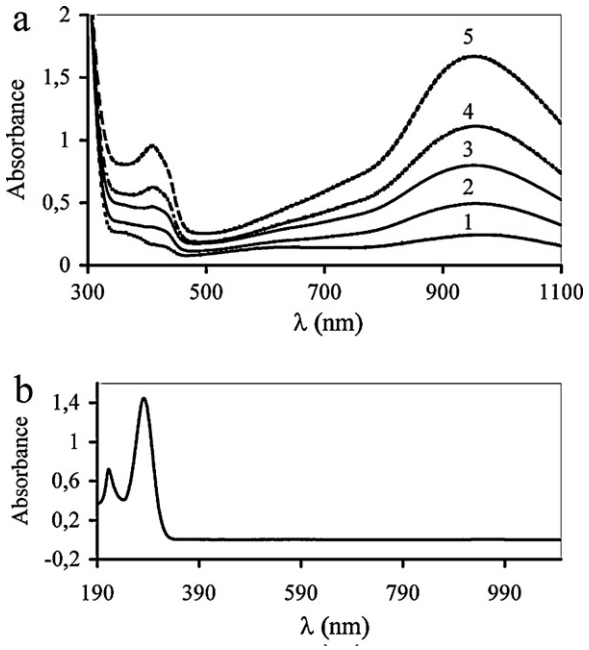

Fig. 2. (a) Evolution of UV–vis absorption spectra of products of laccase-catalyzed PPDA/SDBS complex oxidation with reaction time: (1) 1 min; (2) 5 min; (3) 10 min; (4) 20 min; (5) 30 min. The samples were diluted with 10 mM solution of SDBS in buffer (1:20). Experimental conditions: experimentally saturated solutions of aniline dimer in 10 mM Na–citrate–phosphate buffer (maintained at pH 3.8); [SDBS] = 10 mM; [laccase] = 0.4 U/ml. (b) Spectrum of aniline dimer solution in 10 mM Na–citrate–phosphate buffer. Experimentally saturated solution of aniline dimer was diluted with buffer (1:100).

The titration of PANI/SDBS complex with sodium hydroxide resulted in dedoped polyaniline, the color of the solution changing from dark green to blue. UV–vis spectra of the complex at various pH values are shown in Fig. 4. At pH values higher than 3.6, the intensity of absorption bands at 1100 nm and 420 nm, which are typical of emeraldine salt, gradually decreased and completely disappeared at pH > 9.0. At the same time appeared a peak with a maximum at 560 nm, which is indicative for the formation of

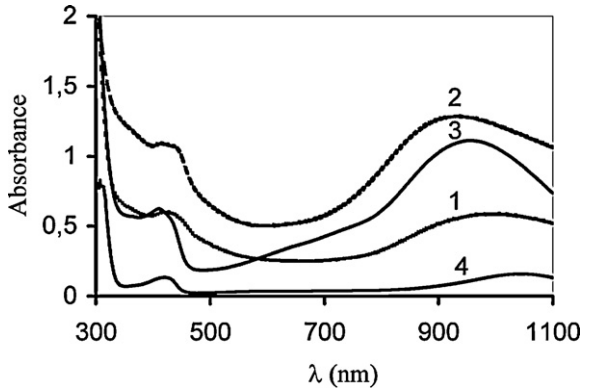

Fig. 3. UV–vis absorption spectra of products of laccase-catalyzed aniline dimer polymerization at different SDBS concentrations: (1) 2 mM; (2) 6 mM; (3) 10 mM; (4) 20 mM. The samples were diluted with corresponding solution of SDBS in buffer (1:20). Experimental conditions: 10 mM Na–citrate–phosphate buffer (maintained at pH 3.8); experimentally saturated solution of aniline dimer; [laccase] = 0.4 U/ml; reaction time – 20 min.

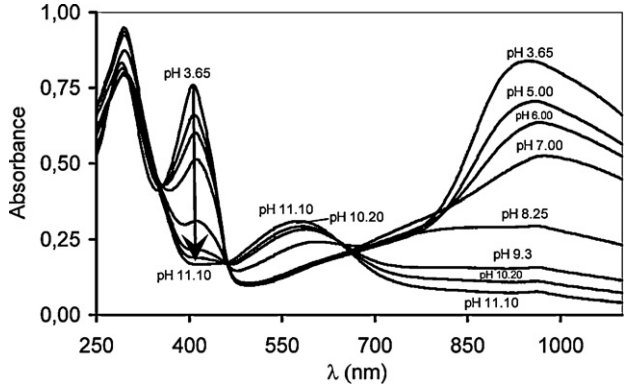

Fig. 4. Effect of pH on UV–vis spectra of enzymatically synthesized PANI/SDBS complexes. The samples were diluted with 10 mM solution of SDBS in buffer (1:60). The pH of PANI/SDBS solution was varied by titration with sodium hydroxide or phosphoric acid. Experimental conditions: 10 mM Na–citrate–phosphate buffer (pH 3.8); experimentally saturated solution of aniline dimer; [SDBS] = 10 mM; [laccase] = 0.4 U/ml; reaction time – 1.5 h.

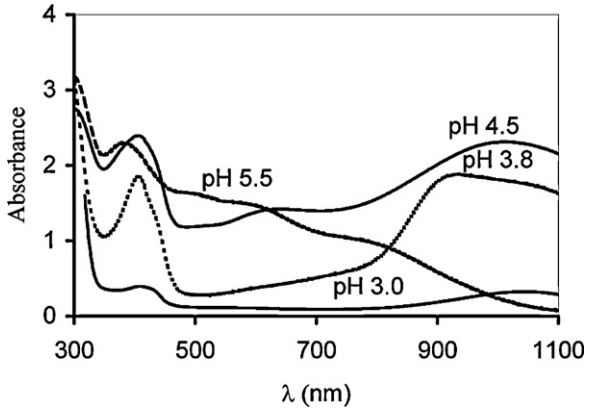

Fig. 6. UV–vis spectra of the products of laccase-catalyzed aniline dimer polymerization carried out at different pH. The samples were diluted with 10 mM solution of SDBS in buffer (1:20). Experimental conditions: 10 mM Na–citrate–phosphate buffer; experimentally saturated solution of aniline dimer; [SDBS] = 10 mM; [laccase] = 0.4 U/ml; reaction time – 1.5 h.

emeraldine base. Titration process is completely reversible up to pH 7.

3.2. Laccase stability

T. hirsuta laccase is a rather stable and active enzyme in the pH range from 3.0 to 5.0. At higher pH values, the enzyme solution is catalytically inactive. For the enzymatic synthesis, it is essential to know the enzyme stability in the course of the reaction. In this work, we have studied the laccase stability and compared the enzyme stability at the same pH values in the absence of substrate and in the absence of both substrate and surfactant (Fig. 5). Laccase inactivation in the synthesis (curve 1) proceeds markedly faster than in the buffer solution containing neither SDBS nor PPDA (curve 2). The enzyme completely lost its activity after 1 h of PPDA oxidation in micellar solution, whereas in the buffer solution its activity was higher than 60% of the initial during the whole experiment. Besides, the enzyme denatured quickly and completely in 10 mM SDBS containing no aniline dimer (curve 3). However, laccase preserves its activity, when enzymatic polymerization of aniline is performed in SDBS micellar solutions [20], which can be related to the reaction of the protonated substrate with negatively charged surfactant molecules. As a result, the denaturing effect of the latter decreases and the enzyme remains active.

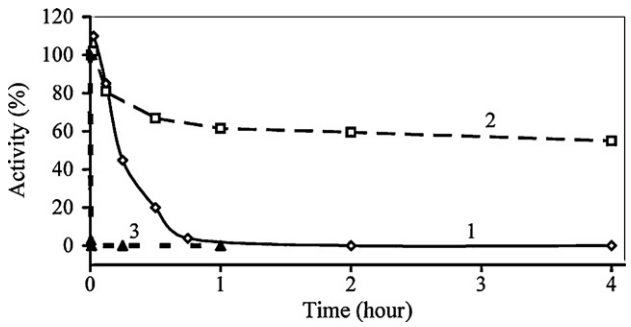

Fig. 5. Stability of laccase under different condition: (1) operating stability (standard synthesis conditions – 10 mM SDBS and PPDA saturated solution; (2) stability in 10 mM Na–citrate–phosphate buffer (pH 3.8); (3) stability in 10 mM SDBS solution in 0.01 M Na–citrate–phosphate buffer (pH 3.8).

3.3. Effect of pH on the PPDA enzymatic oxidation in micellar media

The effect of pH on laccase-catalyzed polymerization of aniline dimers was carried out in the pH range 3.5–5.5. The spectra of the products synthesized for the equal time at different pH values are shown in Fig. 6. As can be seen, a product corresponding to conducting emeraldine salt is synthesized at a high rate on PPDA enzymatic polymerization only at pH 3.8. At pH 3.0 the rate of the product synthesis decreases to a great extent, whereas at pH values higher than 4.5, the reaction rate increases but the spectra of the reaction products vary. No absorption band related to conducting PANI is observed in the spectrum of the product synthesized at pH 5.5. This spectrum resembles that of the branched PANI synthesized using horseradish peroxidase (HPR) [24]. The color of the reaction mixture on enzymatic PPDA polymerization in SDBS micellar solutions differ visibly depending on pH values: it was dark green at pH 3.8 and dark brown at 5.5. Thus the differences in the electronic spectra of the products of laccase-catalyzed PPDA oxidation enable us to assume that emeraldine salt of PANI is only formed at pH 3.0–4.0.

3.4. FTIR analysis and TEM investigations

To characterize the product of enzymatic PPDA oxidation in detail FTIR spectra were recorded (Fig. 7). The spectrum of the dedoped product has bands at 1596 cm^{-1} and 1500 cm^{-1} characteristic of C–C stretching vibration in quinoid diimine and benzenoid

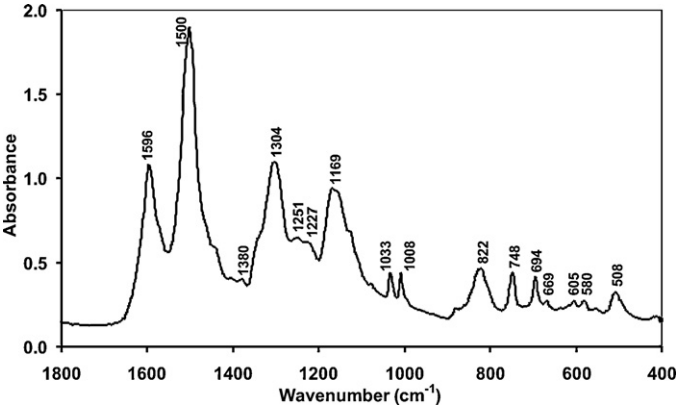

Fig. 7. FTIR spectra of dedoped PANI/SDBS complex prepared at pH 3.8.

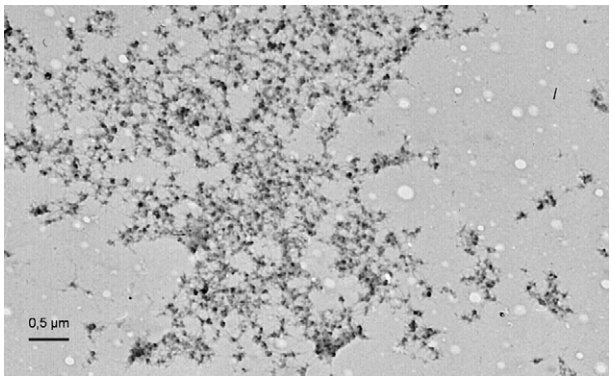

Fig. 8. TEM micrographs of laccase-catalyzed synthesized PANI/SDBS complexes. Experimental conditions: 10 mM Na–citrate–phosphate buffer; experimentally saturated solution of aniline dimer; [SDBS] = 10 mM; [laccase] = 0.4 U/ml. The sample was preliminary dialyzed against deionized water.

diamine units of the polymer, respectively [25]. The peak at 1304 cm^{-1} is due to C–$N_{aromatic}$ stretching vibration. The peaks at 822 cm^{-1} and 1171 cm^{-1} indicate the head-to-tail linking of PPDA molecule on enzymatic polymerization [26–29]. The bands at 1033 cm^{-1} and 1008 cm^{-1} (asymmetric and symmetric stretching of the sulfonate group of SDBS) are due to SDBS which could not be completely removed [30,31].

The morphology of PANI/SDBS complexes was studied by transmission electron microscopy (Fig. 8). PANI granules were 50–100 nm in size and were mostly aggregated.

3.5. Determination of molecular mass of aniline oligomers by laser desorption ionization

It is well-known that PANI is insoluble in most polar and nonpolar common solvents. However, the product obtained in the given work by laccase-catalyzed PPDA polymerization completely dissolved visibly in tetrahydrofurane. This could be explained by a low degree of aniline dimer polymerization. The analysis of the synthesized products was performed by the method of matrix assisted laser desorption ionization (MALDI) coupled to time-of-flight (TOF). The most important aspect of this experiment is the sample preparation process developed to optimize the mass spectral quality. Since SDBS molecules obstruct accurate interpretation of mass-spectra, the polymer produced by enzymatic PPDA oxidation was first precipitated with ethanol in order to remove the bulk of surfactant. The precipitate was then treated with aqueous ammonia solution, washed extensively with deionized water, collected by centrifugation (12,000 × *g*, 10 min), vacuum dried and dissolved in tetrahydrofurane.

The mass-spectrum of the products of PPDA polymerization is shown in Fig. 9. In the course of the enzymatic reaction form aniline oligomers with a mass range of 365–2174 *m/z* characteristic of 4–20-mers with different variations of the end groups. The peak with *m/z* = 184 is an indication of unreacted aniline dimer. The peaks of oligomers are a multiple of the mass of aniline dimer whose *m/z* value is 182–184. Each of the oligomers is present in the form of three series of ions in the mass spectrum. These products may be the result of either the enzymatic-catalyzed synthetic procedure or the limited hydrolysis of quinonoid diimine groups. They may have —NH_2; —NO; =NH; —NO_2; =O; —C_6H_5 terminal groups. The first product in aniline dimer oxidative coupling is tetramer – the smallest repetitive unit of emeraldine. The peak with *m/z* = 365 may be attributed to a tetramer of B—N=Q—N—B—NH—B—NH_2 or B—NH—B—NH—B—N=Q=NH structure, where B and Q stand for a benzenoid and quinonoid unit, respectively. However, the mass spectra also contain other peaks, which could be related to aniline tetramer with —NO and —NO_2 terminal groups and their combination with =N=H; —NH_2; —C_6H_5 end groups.

The main peaks with *m/z* = 365, 546, 727, 907, 1087, 1267, 1450, 1632, 1813, 1993 and 2174 could belong to aniline oligomers with a polymerization degree of 4–22 in terms of aniline subunits with *m/z* = 90–92. The mass spectra contain some peaks with different *m/z* values around each oligomer which correspond to its various redox states and different end groups.

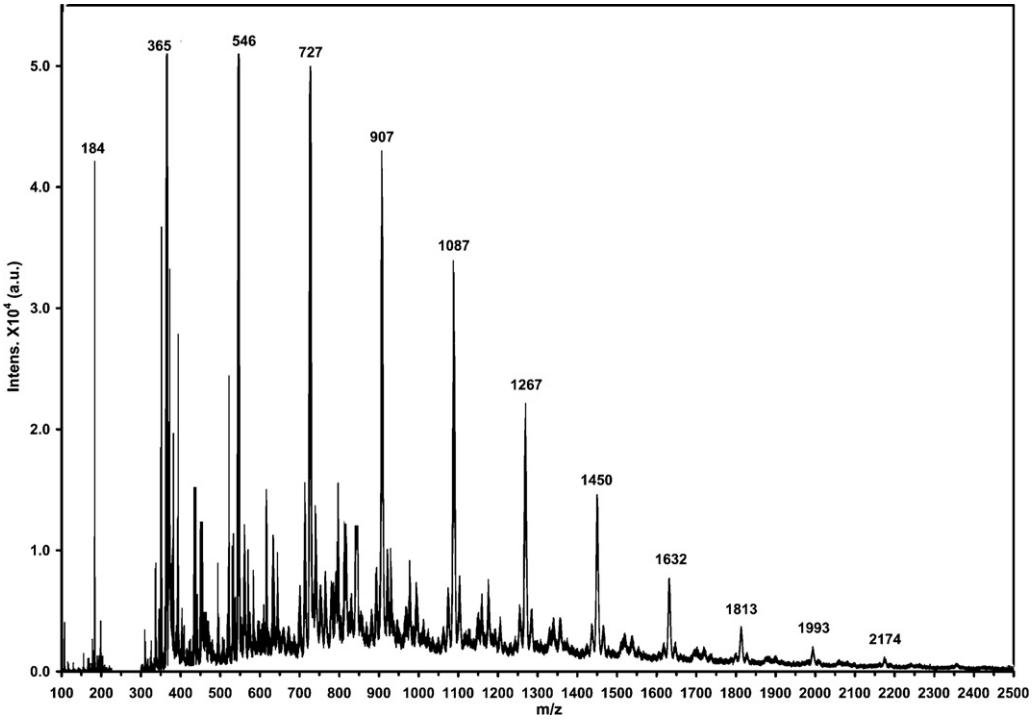

Fig. 9. Laser-desorption ionization mass spectrum of dedoped polyaniline in tetrahydrofurane.

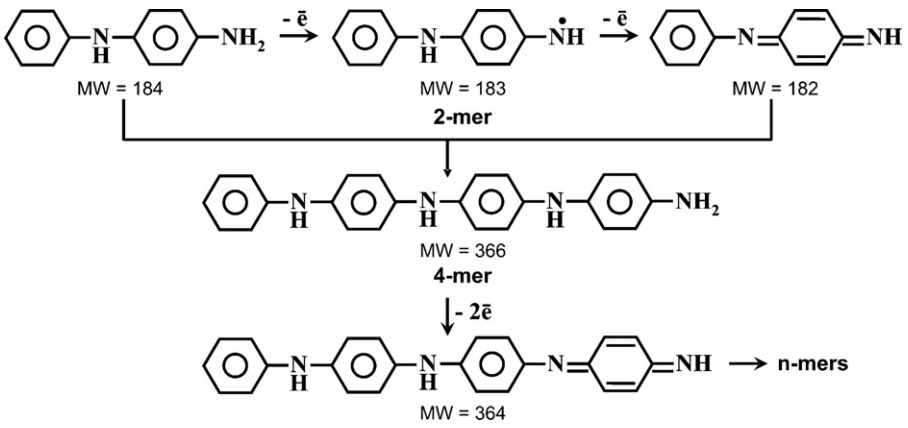

Fig. 10. Scheme of laccase-catalyzed oxidative coupling of aniline dimer.

The low degree of aniline dimer polymerization might be associated with its low concentration in solution. As aniline dimer is the initial substrate in laccase-catalyzed oxidative coupling, the main stages of the reaction can be conceived as follows (Fig. 10).

4. Conclusion

High-redox potential laccase from the fungi *T. hirsuta* has been used as a catalyst in the reactions of aniline dimer oxidative coupling in aqueous SDBS micellar solutions. UV–vis and FTIR measurements have shown head-to-tail coupling of aniline dimers to form emeraldine salt as the reaction product. The rate of laccase-catalyzed aniline dimer oxidation in SDBS micellar solutions is markedly higher than the rate of aniline oxidation under the same conditions. Thus aniline dimer formation may be the rate-limiting stage of the enzyme-catalyzed aniline polymerization. MALDI-TOF spectra of tetrahydrofurane-dissolved products of aniline dimer enzymatic oxidation are evidence for aniline oligomers with the polymerization degree 4–22 in terms of aniline subunits. The aqueous dispersion of aniline oligomers is stable for at least 6 month. TEM measurements have shown that polyaniline nanoparticles have a granular shape.

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