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Chorion peroxidase-mediated NADH/O₂ oxidoreduction cooperated by chorion malate dehydrogenase-catalyzed NADH production: a feasible pathway leading to H₂O₂ formation during chorion hardening in *Aedes aegypti* mosquitoes

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Abstract

A specific chorion peroxidase is present in *Aedes aegypti* and this enzyme is responsible for catalyzing chorion protein cross-linking through dityrosine formation during chorion hardening. Peroxidase-mediated dityrosine cross-linking requires H₂O₂, and this study discusses the possible involvement of the chorion peroxidase in H₂O₂ formation by mediating NADH/O₂ oxidoreduction during chorion hardening in *A. aegypti* eggs. Our data show that mosquito chorion peroxidase is able to catalyze pH-dependent NADH oxidation, which is enhanced in the presence of Mn²⁺. Molecular oxygen is the electron acceptor during peroxidase-catalyzed NADH oxidation, and reduction of O₂ leads to the production of H₂O₂, demonstrated by the formation of dityrosine in a NADH/peroxidase reaction mixture following addition of tyrosine. An oxidoreductase capable of catalyzing malate/NAD⁺ oxidoreduction is also present in the egg chorion of *A. aegypti*. The cooperative roles of chorion malate/NAD⁺ oxidoreductase and chorion peroxidase on generating H₂O₂ with NAD⁺ and malate as initial substrates were demonstrated by the production of dityrosine after addition of tyrosine to a reaction mixture containing NAD⁺ and malate in the presence of both malate dehydrogenase fractions and purified chorion peroxidase. Data suggest that chorion peroxidase-mediated NADH/O₂ oxidoreduction may contribute to the formation of the H₂O₂ required for chorion protein cross-linking mediated by the same peroxidase, and that the chorion associated malate dehydrogenase may be responsible for the supply of NADH for the H₂O₂ production.

Keywords

Chorion peroxidase; Malate dehydrogenase; H₂O₂; NAD⁺; NADH; Nicotinamide adenine dinucleotide; Dityrosine

1. Introduction

Peroxidase plays multiple physiological roles in living organisms. These enzymes occupy a special position in biochemistry and enzymology and are among the most extensively studied enzymes. In addition to their primary peroxidative reaction cycle with H₂O₂ as oxidizing agent, some peroxidases show an oxidase activity mediating the reduction of O₂ to superoxide (O₂^{•−}) and H₂O₂ by substrates such as epinephrine, dihydroxyfumarate, reduced

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nicotinamide adenine dinucleotide (NADH) and indole-3-acetic acid [1–7]. There are also reports that discuss the catalase activity of the peroxidases [8,9], especially from fungi [10–12]. Data from our recent study demonstrate the presence of a specific peroxidase in mosquito eggs and the involvement of this enzyme in chorion protein cross-linking through dityrosine formation, which contributes to the formation of a hardened chorion in mosquitoes [13,14]. Floodwater mosquitoes, such as the yellow-fever mosquitoes of *Aedes aegypti*, oviposit on substrates at the edge of water, and the eggs hatch only after being flooded into water following adequate rainfall. The time period for these eggs in the environment varies enormously depending upon weather conditions. Consequently, formation of a hardened and protective chorion that is resistant to desiccation and other environment adversities is critical for the survival of these eggs before hatching.

The involvement of peroxidase in chorion protein cross-linking during chorion hardening is based on the progressive increase in peroxidase activity in the developing eggs, the localization of the enzyme within the chorion in mature eggs, and the detection of dityrosine in the hydrolysate of the hardened chorion [13,14]. The catalytic cycle of peroxidases is initiated with the oxidation of the resting enzyme by H_2O_2 to an active form termed compound I that is two oxidizing equivalents above its resting state, and two subsequent one-electron oxidations of reducing agents, involving the formation of compound II intermediate, return the compound I to its resting state [15]. During peroxidase-mediated chorion hardening, the enzyme, after being oxidized by H_2O_2 , oxidizes tyrosine residues on protein to tyrosine radicals that interact to form dityrosine, thereby resulting in chorion protein cross-linking that contributes to the formation of a rigid and insoluble chorion in mosquitoes. To be able to oxidize tyrosine, peroxidase must first be oxidized by H_2O_2 ; consequently, there must be mechanism(s) leading to the formation of H_2O_2 during chorion peroxidase-catalyzed protein cross-linking. In this communication, we provide data that suggest a feasible biochemical pathway for the formation of H_2O_2 involving both chorion peroxidase and a malate/NAD⁺ oxidoreductase (MAD) during mosquito chorion hardening.

2. Materials and methods

2.1. Chemicals and enzymes

Catalase (from bovine liver, 48 000 units/mg protein), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), disodium DL-malate, H_2O_2 (30%), horseradish peroxidase (HRP), manganese chloride ($MnCl_2$), β -NAD⁺, β -NADH, phenazine methosulfate (PMS), phenylmethylsulfonyl fluoride (PMSF), superoxide dismutase (from bovine kidney, 4,950 units/mg protein) and L-tyrosine were from Sigma (St. Louis, MO). Dityrosine was synthesized enzymatically by mixing HRP with tyrosine and H_2O_2 [13], and purified by reverse-phase HPLC using a reverse-phase (C_{18}) column (1.0×25 cm).

2.2. Chorion peroxidase purification

Aedes aegypti black-eyed Liverpool strain mosquitoes used in this study were reared according to a described method [13]. Mosquito ovaries with mature eggs were dissected from *A. aegypti* at 72 h following a bloodmeal and placed in 10 mM phosphate buffer (pH 6.5). The procedures for the purification of chorion peroxidase were the same as those described in our recent study [14]. Protein was determined by a colorimetric method [16].

2.3. Chorion peroxidase-mediated NADH oxidation and effects of pH and Mn^{2+} on NADH oxidation

A reaction mixture (0.3 ml) consisting of 0.1 mM NADH and varying amounts of purified chorion peroxidase (0, 2 and 5 μ g) was prepared in 0.1 M phosphate buffer (pH 7.5) and incubated at 25°C. Oxidation of NADH in the reaction mixture was monitored

spectrophotometrically at 340 nm. The effect of pH on peroxidase-mediated NADH oxidation was based on the rate of NADH oxidation in the NADH/chorion peroxidase reaction mixture (0.3 ml) prepared in 0.1 M citrate buffer (pH, 4.5–6.5), phosphate buffer (pH, 7.0–7.5) or Tris buffer (pH 8.0–8.5), respectively. The effect of Mn^{2+} on chorion peroxidase-catalyzed NADH oxidation was also based on the rate of NADH oxidation in the NADH/chorion peroxidase reaction mixtures (0.3 ml) in 0.1 M phosphate buffer (pH 7.5) containing 0, 40, 80, 160 and 240 μM MnCl_2 , respectively.

2.4. Formation of $\text{O}_2^\bullet$ and H_2O_2 during chorion peroxidase-mediated NADH oxidation

Formation of $\text{O}_2^\bullet$ and H_2O_2 during NADH oxidation by peroxidase was based on production of dityrosine in a NADH/peroxidase reaction mixture after addition of tyrosine. A reaction mixture (0.3 ml) consisting of 0.1 mM NADH, 80 μM Mn^{2+} and 5 μg chorion peroxidase was prepared in 0.1 M phosphate buffer (pH 7.5), and incubated at 25°C. At 10 min after incubation, 0.1 ml of 1.5 mM tyrosine was added into the reaction mixture, and the reaction mixture was incubated for an additional 10 min. The reaction was stopped by mixing 0.4 ml of 0.8 M formic acid into the reaction mixture. The sample was centrifuged at 20 000×g for 15 min, and the supernatant was analyzed by HPLC with electrochemical detection (HPLC-ED) to determine the formation of dityrosine [13]. $\text{O}_2^\bullet$ and H_2O_2 formed in the reaction mixture may oxidize NADH directly or through peroxidative pathway, and their role in enhancing NADH oxidation was assessed by changes of NADH oxidation rate in the presence of either 40 units of catalase or superoxide dismutase in the reaction mixture.

2.5. Detection of chorion MAD

Chorion sediments from 3000 ovary pairs were treated with 1% Triton X-100 plus sonication, and the solubilized chorion proteins were extensively dialyzed against 10 mM phosphate buffer (pH 7.5) containing 1 mM PMSF. The sample was chromatographed on a Q-cellulose column (2.5×12 cm), and proteins were eluted with a linear potassium phosphate (0–250 mM, pH 7.5). The active MAD fractions were pooled, washed and concentrated using a stirred cell with a membrane at molecular mass cut-off of 30 000 (Millipore). The concentrated enzyme fractions were chromatographed on an UNO-Q column (7×35 mm, Bio-Rad), and the active fractions that were devoid of peroxidase activity were concentrated and used for MAD activity assays. The presence of MAD in the concentrated fractions was further verified by native polyacrylamide gel electrophoresis of the sample with subsequent substrate staining in a solution containing MTT, PMS, NAD^+ and malate [17].

The MAD activity was assayed spectrophotometrically at 340 nm. A reaction mixture (0.3 ml) consisting of 0.3 mM NAD^+ , 2 mM malate and 6 μg of MAD fraction was prepared in 0.1 M phosphate buffer (pH 7.5) and incubated at 25°C. Increase in absorbance at 340 nm was continuously monitored for 10 min. A reaction mixture with heat-inactivated MAD and a reaction mixture without malate served as controls. NADH is easily oxidized at the working electrode during HPLC-ED analysis. Therefore, accumulation of NADH in the above reaction mixtures was also verified by HPLC-ED at an oxidative potential (850 mV) of the working electrode.

2.6. MAD/chorion peroxidase-mediated H_2O_2 formation

The sequential actions of MAD and chorion peroxidase in mediating H_2O_2 formation with NAD^+ and malate as initial precursors were determined by incubation of malate and NAD^+ in the presence of chorion MAD fraction with subsequent addition of purified chorion peroxidase and tyrosine. The accumulation of H_2O_2 in the reaction mixture was based on detection of dityrosine. The ability to generate H_2O_2 by crude chorion proteins was also

evaluated by mixing urea-solubilized chorion proteins with NAD⁺, malate and tyrosine at the same time and formation of H₂O₂ was based on the detection of dityrosine in the reaction mixture after a 40 min incubation.

3. Results

3.1. Oxidation of NADH by chorion peroxidase under different conditions

There was no apparent decrease in absorbance at 340 nm in a NADH solution at weak basic conditions during a short incubation period, but a much rapid absorbance decrease at 340 nm was observed when chorion peroxidase was incorporated into the NADH solution (Fig. 1). The rate of absorbance decrease was approximately proportional to the amounts of chorion peroxidase in the reaction mixture (Fig. 1), indicating that the absorbance decrease of the reaction mixture at 340 nm results from chorion peroxidase-mediated NADH oxidation.

Based on the rate of NADH oxidation, two optimum pHs for chorion peroxidase-mediated NADH oxidation, one at weak acidic (pH 5.0) and the other at slightly basic conditions (pH 7.5), were determined (Fig. 2). At pH above 8.5, decrease in NADH was due primarily to the nonenzymatic NADH oxidation (not shown). Because pH 7.5 is close to physiological pH conditions, the subsequent NADH oxidation reactions were conducted in 0.1 M phosphate buffer at pH 7.5.

Mn²⁺ had no noticeable effect on enhancing NADH oxidation in the absence of chorion peroxidase (Fig. 3, line 1), but it significantly increased chorion peroxidase-mediated NADH oxidation at its applied concentrations of 40, 80 and 160 μ M, respectively, with a maximum enhancing effect at 80 μ M (Fig. 3, lines 4–6). However, a higher concentration of Mn²⁺ inhibited peroxidase-mediated NADH oxidation (Fig. 3, line 2).

3.2. Formation of H₂O₂ during chorion peroxidase-mediated NADH oxidation

At the applied reaction conditions, oxidation of NADH (0.1 mM final concentration in 0.3 ml) was completed at 10 min after incubation in the presence of 5 μ g chorion peroxidase and 80 μ M Mn²⁺ (Fig. 4A). Disappearance of NADH in the reaction mixture was also verified by HPLC-ED (not shown). When tyrosine was added to the above reaction mixture at 10 min after NADH oxidation and then incubated for an additional 10 min, production of dityrosine was observed in the reaction mixture (Fig. 4B). Oxidation of tyrosine by peroxidase requires H₂O₂; consequently, production of dityrosine demonstrates the accumulation of H₂O₂ in the reaction mixture. In contrast, no dityrosine was produced during a 20 min incubation in a reaction mixture containing tyrosine, chorion peroxidase and Mn²⁺ in the absence of NADH (Fig. 4C), indicating that H₂O₂ is derived from the NADH/O₂ oxidoreduction process.

When the NADH/chorion peroxidase reaction mixture in the presence of 80 μ M of Mn²⁺ was incubated in the presence of superoxide dismutase, the rate of NADH oxidation was decreased (Fig. 5). A similar effect was observed when catalase was incorporated into the NADH/chorion peroxidase/Mn²⁺ reaction mixture (Fig. 5).

3.3. Detection of chorion MAD

Progressive increase in absorbance at 340 nm was observed when the concentrated MAD fractions devoid of chorion peroxidase activity after UNO-Q column separation were mixed with NAD⁺ and malate (Fig. 6A, line 1). Increase in absorbance at 340 nm was not observed when heat-inactivated MAD fraction was incorporated into the reaction mixture (Fig. 6A, line 2) or when malate was omitted in the reaction mixture (Fig. 6A, line 3). Analysis of the above reaction mixtures using HPLC-ED revealed the accumulation of NADH in the

reaction mixture with active MAD fraction (Fig. 6B) and the absence of NADH in the reaction mixture with heat-treated MAD fraction (Fig. 6C). Native electrophoresis of the concentrated protein sample with subsequent MAD activity staining in a solution containing MTT, PMS, NAD⁺ and malate resulted in the detection of a positive MAD band (Fig. 6D, lane 1). The MAD band was not observed when the native gel with separated protein sample were incubated in the staining solution but without either malate or NAD⁺ (Fig. 6C, lanes 2 and 3). These data demonstrate the presence of a MAD in the mosquito chorion.

3.4. Formation of H₂O₂ by MAD/peroxidase-mediated reactions

The sequential actions of MAD and chorion peroxidase in H₂O₂ formation with malate and NAD⁺ as starting substrates were demonstrated by the accumulation of NADH in a NAD⁺/malate reaction mixture in the presence of MAD fraction (Fig. 7A), the disappearance of NADH in the reaction mixture at 20 min after addition of chorion peroxidase and Mn²⁺ (Fig. 7B), and the production of dityrosine following addition of tyrosine into the same reaction mixture (Fig. 7C). Triton-solubilized chorion proteins had fairly high MAD activity, but this nonionic detergent was not efficient on solubilizing chorion peroxidase because of a low chorion peroxidase activity in the Triton-solubilized chorion proteins. In contrast, saturated urea was effective in solubilizing both MAD and chorion peroxidase, and both enzymes were active in the presence of the high concentration of urea. When malate, NAD⁺ and tyrosine were mixed with the urea-solubilized chorion proteins at the same time and incubated at 25°C, formation of dityrosine was also observed in the reaction mixture after 40 min incubation (Fig. 7D).

4. Discussion

Peroxidase-catalyzed chorion protein cross-linking through dityrosine formation contributes to the formation of a hardened chorion in *A. aegypti* eggs [13]. Involvement of peroxidase in eggshell protein cross-linking has also been suggested in *Drosophila* [18–20]. Although there have been no critical analysis of chorion hardening in other insects, peroxidase-mediated eggshell or chorion protein cross-linking is likely to be a critical physiological event in oviparous insects whose embryos require protection from environmental factors during embryonic development in the environment. H₂O₂ is necessary for peroxidase-catalyzed chorion protein cross-linking through dityrosine production; therefore, there must be pathway(s) leading to the production of H₂O₂ during chorion hardening in insects. Our data suggest that during chorion hardening H₂O₂ may be derived from NADH/O₂ oxidoreduction mediated by chorion peroxidase in *A. aegypti* eggs or that this pathway may be one of the H₂O₂ generating mechanisms, and a MAD that also is present in the chorion of *A. aegypti* eggs may be responsible for the supply of NADH.

In plants, several peroxidase isozymes are present in the cell wall. These enzymes catalyze cell wall protein cross-linking and polymerization of cinnamic alcohols, the precursors of lignin [21–26]. Plant cell wall peroxidases were able to oxidize NADH to produce H₂O₂; therefore, these enzymes were suggested to be responsible for the production of H₂O₂ by mediating NADH/O₂ oxidoreduction during lignification [26]. There were also reports suggesting the presence of a cell wall-bound MAD and the role of the enzyme in H₂O₂ generation through the supply of NADH during lignification [27,28]. The ability of chorion peroxidase to enhance NADH oxidation and H₂O₂ production and the presence of a MAD in the solubilized chorion proteins provide a reasonable basis for suggesting a feasible H₂O₂ generating pathway involving both chorion peroxidase and MAD during chorion protein cross-linking in *A. aegypti* eggs.

The mechanism of the peroxidase-mediated NADH oxidation and H₂O₂ formation is likely to be similar to those described for HRP [29–31]. It is considered that H₂O₂, in catalytic

amounts that may be derived from autoxidation of NADH if no exogenous H_2O_2 is added, is required to initiate NADH oxidation by peroxidase [29,30]. Once a small amount of H_2O_2 is present, it oxidizes peroxidase to compound I that in turn oxidizes NADH to $\text{NAD}^\bullet$. $\text{NAD}^\bullet$, once formed, can react with O_2 , leading to the formation of $\text{O}_2^\bullet$ that can either dismutate to form H_2O_2 , or react with NADH to produce $\text{NAD}^\bullet$ and H_2O_2 . In addition, $\text{NAD}^\bullet$ could be oxidized by $\text{O}_2^\bullet$ to produce relatively stable NAD^+ and H_2O_2 , respectively. The following describes the proposed overall reaction mechanisms involved in the chorion peroxidase-mediated NADH/ O_2 oxidoreduction (Eqs. 1–7).

Although NADH can be oxidized by O_2 , it proceeds slowly at physiological pH. However, the small amount of H_2O_2 derived from NADH autoxidation seems adequate enough to initiate a chain reaction of peroxidase-mediated NADH oxidation. In this process, peroxidase promotes H_2O_2 formation indirectly through its one-electron oxidation product of $\text{NAD}^\bullet$ [29,30]. To be able to oxidize tyrosine, chorion peroxidase must be activated by H_2O_2 to compound I. Therefore, detection of dityrosine in the NADH/chorion peroxidase reaction mixture in the presence of tyrosine indicates not only the accumulation of H_2O_2 , but also the formation of chorion peroxidase compound I in the reaction mixture.

In *A. aegypti* eggs, the overall chorion hardening process takes about 2–3 h after oviposition; therefore, a rapid production of H_2O_2 is necessary during chorion hardening. The ability of chorion peroxidase to mediate H_2O_2 formation by accelerating the NADH/ O_2 oxidoreduction process, and the formation of dityrosine in the NADH/chorion peroxidase reaction mixture in the presence of tyrosine provides a reasonable basis to suggest the involvement of chorion peroxidase in promoting H_2O_2 formation through NADH oxidation and O_2 reduction during chorion hardening. In the mosquito chorion, the proximity between chorion peroxidase and its substrates and the possibility of H_2O_2 to defuse (from its site of

production) within the chorion layer might allow H₂O₂ to be used more efficiently for protein cross-linking during chorion hardening. Mn²⁺ enhances NADH oxidation in the presence of chorion peroxidase, but it seems apparent that Mn²⁺ alone has no effect on facilitating NADH oxidation because it does not accelerate NADH oxidation in the absence of chorion peroxidase. The effect of Mn²⁺ in stimulating NADH oxidation may be due to its interaction with O₂, in which Mn²⁺ reduces O₂[•] to H₂O₂ with itself being oxidized to Mn³⁺ and Mn³⁺ may then oxidize NADH to NAD[•] (Eqs. 8 and 9); thereby accelerating NADH oxidation and H₂O₂ formation [32].

However, this mechanism cannot explain why a relatively high concentration of Mn²⁺ inhibits NADH oxidation. It is possible that Mn²⁺ at high concentration may react more easily with H₂O₂ leading to the formation of hydroxyl radical (Eq. 10), which may inactivate the chorion peroxidase.

In summary, results from this study suggest that (i) chorion peroxidase in *A. aegypti* is capable of mediating NADH oxidation leading to the formation of H₂O₂, (ii) the NADH/O₂ oxidoreduction mediated by chorion peroxidase represents a feasible pathway for H₂O₂ production required during chorion hardening, and (iii) a chorion MAD is present in the egg chorion, and the enzyme might be responsible for the supply of NADH for H₂O₂ generation during chorion hardening.

Acknowledgments

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Fig. 1. Oxidation of NADH by chorion peroxidase. A reaction mixture of 0.3 ml consisting of 0.1 mM NADH and 0, 2 or 5 μ g of purified chorion peroxidase was prepared in 0.1 M phosphate buffer (pH 7.5) and incubated at 25°C. The absorbance decrease at 340 nm was continuously monitored for 12 min. Lines 1, 2 and 3 illustrate the relative rate of NADH oxidation in the reaction mixtures in the presence of 0, 2 and 5 μ g of chorion peroxidase, respectively.

Fig. 2.

Effect of pH on chorion peroxidase-catalyzed NADH oxidation. The reaction mixtures containing 0.1 mM NADH and 5 μ g chorion peroxidase were prepared in either 0.1 M citrate (pH 4.5–6.5), phosphate (pH 7.0–7.5) or Tris buffer (pH 8.0–8.5), and absorbance decrease at 340 nm was continuously monitored. Bar graph shows the decrease in absorbance at 340 nm in the reaction mixtures during 12 min incubation at indicated pH conditions. The absorbance at the beginning of the reaction (zero time) was 0.26–0.27.

Fig. 3.

Effect of MnCl_2 on peroxidase-mediated NADH oxidation. A reaction mixture of 0.3 ml consisting of 0.1 mM NADH, 5 μg chorion peroxidase, 40–240 μM MnCl_2 was prepared in 0.1 M phosphate buffer and incubated at 25°C. Absorbance decrease at 340 nm due to NADH oxidation was continuously monitored for 12 min. Line 1 shows the absorbance change of the NADH solution in the presence of 80 μM Mn^{2+} without chorion peroxidase and line 3 illustrates level of absorbance decrease in the NADH/chorion peroxidase reaction mixture without Mn^{2+} as controls. Lines 2, 4, 5 and 6 are the absorbance decreases in the NADH/chorion peroxidase reaction mixtures in the presence of 240, 160, 40 and 80 μM of Mn^{2+} , respectively. Mn^{2+} at 80 μM showed its maximum enhancing effect on peroxidase-mediated NADH oxidation.

Fig. 4. Formation of H_2O_2 during peroxidase-mediated NADH oxidation based on dityrosine formation. Line graph shows the decrease in absorbance at 340 nm in a reaction mixture containing 0.1 mM NADH, 5 mg chorion peroxidase and $80 \mu\text{M Mn}^{2+}$, and the arrow indicates the time when tyrosine was added to the reaction mixture (A). Chromatograms illustrate the formation of dityrosine in a peroxidase/NADH/ Mn^{2+} mixture after an additional 10 min incubation following incorporation of tyrosine (B), and the inability to produce dityrosine in a tyrosine/chorion peroxidase/ Mn^{2+} reaction mixture in the absence of NADH at 20 min after incubation (C). A C_{18} reverse-phase column ($4.6 \times 150 \text{ mm}$) and a mobile phase consisting of 96% 0.1 M citrate buffer (pH 3.2) and 4% acetonitrile with a flow rate of 0.6 ml min^{-1} were used for the separation. The working electrode was maintained at 875 mV with Ag/AgCl/KCl as a reference electrode.

Fig. 5.

Effects of superoxide dismutase and catalase on chorion peroxidase-catalyzed NADH oxidation. A reaction mixture containing 0.1 mM NADH, 80 μ M Mn^{2+} and 5 μ g purified chorion peroxidase in the presence of 40 units of either superoxide dismutase or catalase was incubated at 25°C, and decrease in absorbance at 340 nm was monitored for 5 min. Lines 1 and 2 show absorbance decrease in the reaction mixture in the presence of 40 units of superoxide dismutase and catalase, respectively. Line 3 illustrates the absorbance decrease in the same reaction mixture without superoxide dismutase and catalase as a control.

Fig. 6.

Detection of chorion malate dehydrogenase (MAD). Line graph (A) shows the absorbance changes during incubation (10 min at 25°C) in a 0.3 ml reaction mixture containing 2 mM malate and 0.3 mM NAD^+ prepared with 0.1 M phosphate buffer (pH 7.5) in the presence of 6 μg active MAD fraction (line 1), in the presence of heat-inactivated enzyme fraction (line 2) or in the presence of active enzyme, but in the absence of malate (line 3). Chromatograms illustrate the accumulation of NADH in the above reaction mixtures with active MAD fraction (B) and the absence of NADH in the reaction mixture with heat-inactivated enzyme fraction (C), respectively. Conditions of HPLC-ED analysis were identical to those described in Fig. 4. Native gel electrophoresis (6% polyacrylamide) analysis of the MAD fraction with substrate staining (D) shows the detection of the MAD band after incubation of the gel in a MTT and PMS solution in the presence of malate and NAD^+ (lane 1), and inability to detect the MAD band after incubation of the gel in the same solution without either NAD^+ (lane 2) or malate (lane 3).

Fig. 7. MAD/chorion peroxidase-mediated H_2O_2 and dityrosine formation. Chromatograms show the production of NADH in a reaction mixture (0.3 ml) containing 0.3 mM NAD^+ , 0.5 mM malate and 6 μg of MAD fraction at 10 min after incubation at 25°C (A), the disappearance of NADH in the reaction mixture at 20 min after addition of 5 μg of chorion peroxidase and 80 μM Mn^{2+} (B), and the production of dityrosine in the same reaction mixture at 15 min after addition of tyrosine (C). Chromatogram D shows the formation of dityrosine in a reaction mixture containing 0.3 mM NAD^+ , 0.5 mM malate and 0.5 mM tyrosine in the presence of 0.3 mg urea-solubilized chorion proteins at 40 min after incubation. Conditions of HPLC-ED analysis were identical to those described in Fig. 4.