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The oxidation of oxytocin and vasopressin by peroxidase/H_2O_2 system

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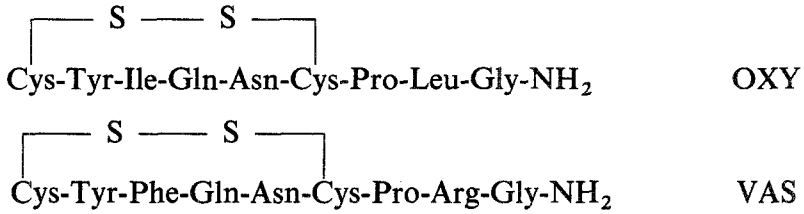
Summary. Oxytocin and vasopressin are oxidized by horseradish peroxidase and by lactoperoxidase, in the presence of hydrogen peroxide. Spectrophotometric measurements are indicative of the formation of dityrosine. Kinetic parameters indicate that the affinity of horseradish peroxidase is slightly higher for oxytocin with respect to vasopressin and that the two hormones are better substrates for both peroxidases than free tyrosine.

Keywords: Amino acids – Oxytocin – Vasopressin – Peroxidase – Dityrosine

Introduction

Recently we have undertaken some investigations on enkephalins and esorphins as substrates of mammalian and horseradish peroxidases (Rosei et al., 1991a). Opioid peptides, all sharing the characteristic of a tyrosine residue at the amino terminus, are easily oxidized by the above mentioned enzymes, giving rise to dimers that are linked through a molecule of dityrosine (Rosei et al., 1991a, Foppoli et al., 1991). It seemed to us interesting to explore the effect of plant and mammalian peroxidases on tyrosine when the amino acid was localized internally to the peptide sequence. For this reason we have focused our attention on two hormones, oxytocin (OXY) and vasopressin (VAS), whose functional role is well established (Bisset, 1976). The two nonapeptides present the following amino acid sequence:



In this work we provide evidence that the nonapeptides OXY and VAS are substrates for plant and mammalian peroxidases.

Materials and methods

OXY, VAS, horseradish peroxidase (HRP; 1100 U/mg) and lactoperoxidase (LPO; 79 U/mg) were purchased from SIGMA. Peroxidases concentrations were determined spectrophotometrically using the following millimolar extinction coefficients: 91 at 403 nm for HRP (Bayse et al., 1972) and 114 at 412 nm for LPO (Pruitt et al., 1980).

Hydrogen peroxide solutions were freshly prepared diluting a 30% stock solution (Merck); the concentration was measured using a molar extinction coefficient of 72.4 at 230 nm (Nelson et al., 1972).

All the other chemicals were analytical grade.

Spectrophotometric measurements were performed with a Varian DMS 200 spectrophotometer using thermostated cuvettes at 25°C; spectra were recorded automatically every two minutes.

The rate of dityrosine formation was estimated measuring the absorbance at 315 nm as a function of time (Sawahata et al., 1982).

Except where indicated, the assay mixture for kinetic measurements contained: 0.2 mM substrate, 0.1 mM H_2O_2, 7.37 U of HRP or 2.6 U of LPO, 0.1 M potassium phosphate buffer (pH 8) in a volume of 1 ml.

The reaction was started by the addition of the enzyme whereas an identical mixture without peroxidase was used as reference. Suitable blanks of OXY or VAS in presence of H_2O_2 ruled out a non-enzymatic oxidation of the substrates. For the chromatographic analysis, the experiments were carried out in 0.2 M sodium borate buffer (pH 8.5) in thermostated bath at 37°C (Amadò et al., 1984). The mixture contained 0.5 mM substrate, 1 mM H_2O_2 and 0.3 μM (16.5 U) HRP in a final volume of 1 ml. After enzyme addition, the mixture was incubated with shaking at 37°C; the reaction was stopped at various times by adding sodium metabisulfite to destroy H_2O_2. For amino acid analysis, samples (1 ml) were treated with an equal volume of 12 M HCl and hydrolyzed for 20 h at 120°C. Amino acids were quantitatively determined by ion-exchange chromatography with a Carlo Erba 3A29 Amino acid Analyzer, according to the procedure already described (Rosei et al., 1989).

Results

The spectra at various times of an incubation mixture of OXY with HRP/H_2O_2 system are reported in Fig. 1. The hormone was easily oxidized, giving rise to the formation of an absorption spectrum with maxima at about 290 nm and 315 nm that can be attributed to dityrosine production (Amadò et al., 1984, Andersen, 1964, Rosei et al., 1991a). Such spectral characteristic is identical to that exhibited by an authentic sample of dityrosine (Andersen, 1964). The reaction showed to be rather fast, reaching the completeness in about 10 minutes. The spectra obtained utilizing VAS were substantially similar; the reaction proceeded however more slowly (see inset Fig. 1).

Kinetic measurements indicated that the initial rate, measured as increase of absorbance at 315 nm in presence of a fixed concentration of H_2O_2, was linearly related to enzyme concentration (Fig. 2).

In Table 1 the kinetic parameters and the apparent Km values for the oxidation of OXY and VAS by HRP/H_2O_2 are reported. The affinity of the enzyme for OXY was found to be slightly higher, the apparent Km values being 0.37 mM for OXY and 0.91 mM for VAS. The kinetic parameters for oxidation

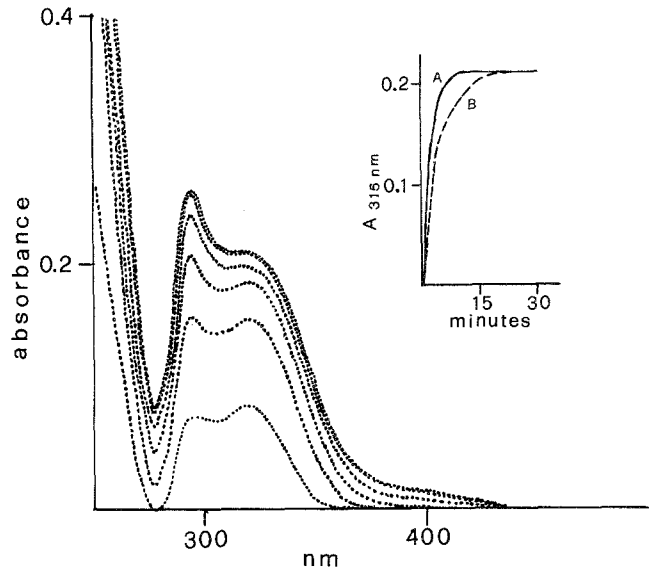


Fig. 1. Spectral modification of oxytocin oxidized by HRP/H_2O_2 system. Spectra were recorded every two minutes, from 0′ to 10′. Inset: absorbance at 315 nm as function of the incubation time. **A** oxytocin, **B** vasopressin

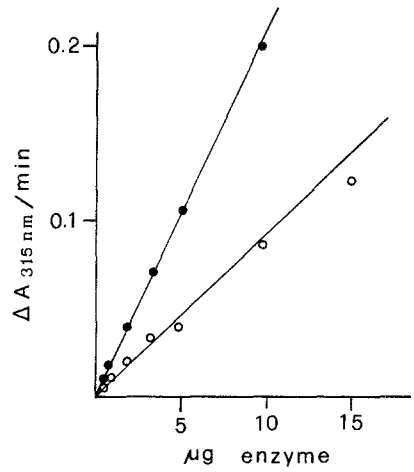


Fig. 2. Reaction rate as function of HRP concentration. The initial rate was calculated as Δ of absorbance at 315 nm/min. Full circles = oxytocin, empty circles = vasopressin

of the two nonapeptides in function of various concentration of H_2O_2 were also determined; the data obtained are reported in Table 2. For these determinations, H_2O_2 concentrations ranging from 5 to 50 μM were used; the Km values are quite similar for both substrates.

In order to ascertain whether peroxidase action was able to perform the linkage between two molecules of OXY or VAS, a large-scale incubation of the hormone in presence of HRP/H_2O_2 was carried out. The hydrolysis of the incubation mixture and the subsequent analysis by amino acid analyzer allowed to detect the presence of dityrosine into the specimens. In fact, a peak with

retention time of 57 minutes completely overlapped with the peak of an authentic sample of dityrosine.

The dityrosine formed was quantitatively determined; the amount and the time course formation of the dimer are reported in Fig. 3. A parallel decrease of tyrosine was observed, but a residual amount of this amino acid was yet present after 2 hours incubation.

Table 1. Kinetic parameters of OXY and VAS oxidation by horseradish peroxidase

Substrate	Km (mM)	Kcat (Vmax/EU)	Kcat/Km
Oxytocin	0.37	30.27	81.81
Vasopressin	0.91	17.54	19.27
Tyrosine	3.30	11.82	3.58

Analysis was performed at saturating H_2O_2 concentration. Vmax is expressed as Δ of absorbance at 315 nm/min.

Table 2. Kinetic parameters of OXY and VAS oxidation by horseradish peroxidase in function of H_2O_2 concentration

Substrate	Km (μM)	Kcat (Vmax/EU)	Kcat/Km
Oxytocin	4.6	13.77	2.99
Vasopressin	6.0	9.23	1.54

The tests were performed at saturating substrate concentration. Vmax is expressed as Δ of absorbance at 315 nm/min.

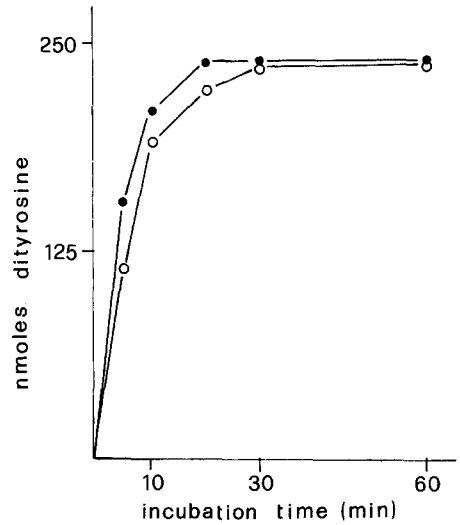


Fig. 3. Time course formation of dityrosine. Dityrosine amount is expressed as nmoles recovered in hydrolysates of incubation mixtures of oxytocin (full circles) or vasopressin (empty circles), performed as described under Methods

Table 3. Kinetic parameters of OXY and VAS oxidation by lactoperoxidase

Substrate	Km (mM)	Kcat (Vmax/EU)	Kcat/Km
Oxytocin	0.71	9.26	13.04
Vasopressin	0.40	4.82	12.05

Analysis was performed at saturating H_2O_2 concentration. Vmax is expressed as Δ of absorbance at 315 nm /min.

Since previous experiments demonstrated that peptides with a tyrosine residue at the N-terminus were also substrates for lactoperoxidase (Rosei et al., 1991a), we performed a series of experiments to test whether OXY and VAS could be substrates also for the mammalian peroxidase.

The results indicated that both peptides function as hydrogen donors for this enzyme, showing spectral changes identical to those exhibited with HRP and reported in Fig. 1. Also in this case, the reaction rate linearly increased with the raise of enzyme concentration. Kinetic parameters calculated for the oxidation of the two hormones by LPO are reported in Table 3. The apparent Km values are very similar to those reported for HRP.

Discussion

Kinetic measurements indicate that oxytocin and vasopressin are easily oxidized by the HRP/H_2O_2 system and that tyrosine, when bound to a peptide chain even if internal to the sequence as in this instance, functions better as hydrogen donor with respect to free tyrosine. All kinetic data indicate that the two hormones are preferred as substrates with respect to tyrosine. The results parallel previous finding obtained with tyrosinase/O_2 (Rosei et al., 1989, 1991b) and HRP/H_2O_2 system (Foppoli et al., 1991, Rosei et al., 1991a) demonstrating that opioid peptides were oxidized by the mentioned enzymes more easily than tyrosine itself.

Since the action of tyrosinase and peroxidase is brought about through the generation of a radical on tyrosine ring (Gross et al., 1959, Takahashi et al., 1989), any factor stabilizing the presence of the radical can actually favour the oxidation of the amino acid (Rosei et al., 1991a, Foppoli et al., 1991). The presence of the peptidic bond, favouring the electronic flow toward the carboamide bond (Dixon, 1984) and thus facilitating the formation of the radical, can be considered a propitius factor for the oxidation of the phenolic ring.

The results reported in the present paper confirm the achievement that the catalysis of these oxidative enzymes toward tyrosine is improved if the amino acid is linked through a peptidic bond. The sorrounding molecule of the peptide chain however exerts a hampering effect, in fact when tyrosine is at the N-terminus of the amino acidic sequence, as in opioid peptides, the affinity of peroxidases for the amino acid is higher (Rosei et al., 1991a). The affinity of HRP is higher for OXY rather than for VAS. This may be explained by the fact that

in the latter hormone an arginine residue is located in proximity of the phenolic ring of tyrosine. The presence of a strong basic group, as the guanidic one, was previously demonstrated as an unfavourable factor for peroxidase action (Foppoli et al., 1991). Lactoperoxidase, on the contrary, exerted a similar effect on the two peptides (Km: 0.71 mM for OXY and 0.40 mM for VAS).

Our effort to isolate the dimers of both peptides has been unfruitful up to now. We are investigating whether these compounds may be labile products because of their steric hindrance or could copolymerize to form high molecular weight aggregates during the oxidation. Bailey et al. (1991) demonstrated indeed that in peroxidase-catalyzed reactions of tyrosine molecules, the formation of polymers could actually occur.

In the present work, a low H_2O_2 concentration has been employed in order to cut down the possible oxidation of the disulphide bridge (Matheis et al., 1984). Other authors (Chany et al., 1982) have also found that in these conditions the S-S bridge is only slightly oxidized.

The fact that mammalian peroxidases can react with and transform peptide molecules could be, in principle, a matter of real interest. Indeed the dimers of enkephalins, whose formation by the action of myeloperoxidase we previously demonstrated in vitro (Rosei et al., 1991a), have been found to suppress the respiratory burst of polymorphonuclear leukocytes (Rabgaoui et al., 1993).

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