NOVEL DETECTION METHOD OF NITRIC OXIDE USING HORSE Radish PEROXIDASE

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Nitric oxide (NO) readily makes corresponding complexes not only with ferrous iron but also with ferric iron. However, NO-ferric complexes of many heme proteins were unstable, while horseradish peroxidase formed the very stable NO-ferric porphyrin complex with a shift of the Soret band of the absorption wavelength from 396.5 nm to 420.0 nm. The concentration of NO in aqueous media could be monitored by measuring the absorption changes, and the detection limit was 10 nM. The simple procedure is convenient for concentration determination of NO solution.

KEY WORDS nitric oxide; horseradish peroxidase; Soret band; difference spectra

It is well established that nitric oxide (NO) plays an important role as a signal transmitter not only in the vascular endothelium but also in central and peripheral neurons and in phagocytic cells. To understand the mechanisms by which NO mediates various physiological and pathophysiological processes, it is necessary to monitor the change of NO formation quantitatively under physiological conditions. Thus, one often needs the standard curve of NO concentrations in aqueous media without complicated procedures.

Several NO detection systems have already been reported: the chemiluminescence assay based on the reaction with ozone in the gaseous phase, the aqueous chemiluminescence method using lumino/H$_2$O$_2$ system, the colorimetric assay of the nitric azo-coupling reaction (Griess reaction), the oxyhemoglobin (HbO$_2$) oxidation method, and the GC-MS detection assay. Direct determination of NO concentration in aqueous media is often done by the HbO$_2$ oxidation method. The method is based on the oxidation reaction of HbO$_2$ to methemoglobin (MetHb) by NO. However, there are two problems with this method. The first is that one should be extremely careful in providing HbO$_2$ solution without MetHb. In the case of using commercially available HbO$_2$ contaminated about 80% by MetHb, MetHb should be reduced to Hb by sodium thiocyanate, followed by formation of HbO$_2$, then it is necessary to purify HbO$_2$ by gel filtration. As a result, it should be difficult to reproduce the preparation of constant amounts of HbO$_2$. The second problem is that HbO$_2$ reacts with NO$_2^-$ to form MetHb. So it is intrinsically difficult to differentiate NO from its decomposed product.

Thus, we have examined the development of a novel NO detection method with a simple procedure. Ferric iron forms NO complexes with the same electron number as ferrous iron-CO complexes. The stability of the NO-ferric complexes of heme proteins depends on the steric structures of their apoproteins. That is, horseradish peroxidase (HRP), which is a commercially available heme protein with ferric iron, forms a very stable NO-ferric complex without any side-reactions, while other heme proteins, for example MetHb, which contain ferric iron, form...
unstable NO complexes.\textsuperscript{12}) This induces us to examine if HRP can be useful for detection of NO. In this communication, we report the simple and convenient method using HRP to detect NO in aqueous media.

Excess NO solution (2 mM) was added to the sodium phosphate buffer (pH 7.4) of HRP (1.5 µM; Sigma, USA). HRP formed the stable complex of NO, which induced the absorbance change of Soret band due to porphyrin (Fig. 1a). The Soret band shifted toward longer wavelength from 396.5 nm to 420.0 nm, and the two weak absorbance peaks of Q-band emerged at 527.0 nm and 558.5 nm. The difference spectra show the isosbestic point at 406.5 nm (Fig. 1b), and the $\varepsilon$ values\textsuperscript{13} were determined by adding several NO aliquots\textsuperscript{10} to HRP solution. The results show that

\begin{equation}
y = 0.0014773 + 0.13146x \quad R = 0.99973
\end{equation}

![Graph showing the standard curve of NO addition to HRP solution.](image)

Fig. 2. Standard Curve of NO Addition to HRP Solution
that the bigger spectral change was induced at 396.5 and 420.0 nm on the formation of NO-HRP complex. The standard curve was obtained by measurement at these two wavelengths (Figure 2). This method gave the same standard curve as HbO2 method. However, interestingly there was no difference between the use of deoxygenated buffer and the untreated buffer in this method. The result indicates that the procedure of this method is easier than that of HbO2 method. The detection limit was determined using the 10 cm path-length cell as 10 nM; that of the HbO2 method (50 nM) was lower than this HRP method. Also, MetHb can form the NO complex, but the spectral change was smaller than that between HRP and HRP-NO complex. When the commercially available hemeproteins Hb, MetHb and HRP were compared, HRP gave the most sensitive and simple assay system, so the HRP method is very useful in quantifying NO in standard solution.

Further studies on application to detection of NO in various biological samples (e.g. endothelial culture cells) are in progress.

REFERENCES AND NOTES

13) The ε values of HRP-NO complex were 4.9 x 10^5 and 7.7 x 10^5, and those of HRP were 6.6 x 10^6 and 3.3 x 10^6, at 396.5 nm and 420.0 nm, respectively. The ε value at the isosbestic point (406.5 nm) was 6.7 x 10^5.
14) Each one aliquot of NO (0.2 nmol) was 5 µl of sodium phosphate buffer solution (pH 7.4) and HRP solution was 0.1 µM in the same buffer. A cuvette with 10 cm path length was used, and the volume of the container was 38 ml.

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