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MINI REVIEW

Activity assays for flavoprotein oxidases: an overview

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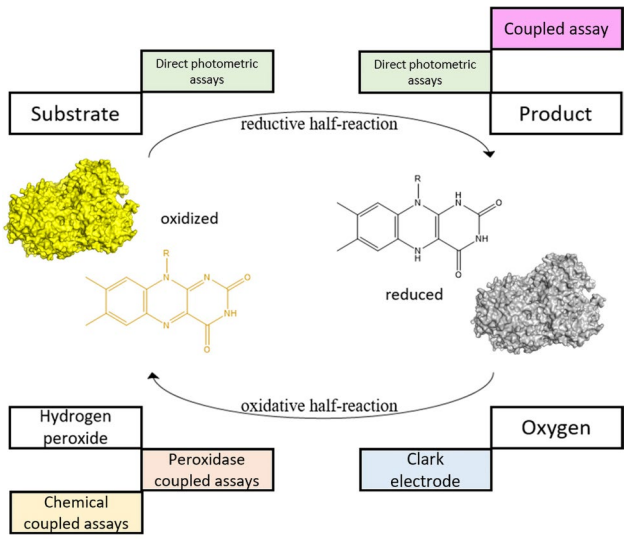
Abstract

Flavoprotein oxidases have found many biotechnological applications. For identifying and improving their characteristics, it is essential to have reliable and robust assay methodology available. The methodologies used to monitor their activity seem to be scattered in the literature and seem often selected based on convenience. Due to the diversity of reactions catalyzed by flavoprotein oxidases, it is virtually impossible to recommend a single activity assay. A literature analysis of 60 recent papers describing flavoprotein oxidases revealed that continuous spectrophotometric assays, in particular colorimetric assays, are the preferred choice, as they are facile, scalable and allow for better interpretation of data than discontinuous assays. Colorimetric assays typically rely on the extinction coefficient of a monitored chromogenic product, which can be highly variable depending on the experimental conditions. Therefore, it is important to determine the extinction coefficient under the specific experimental conditions used, rather than taking it directly from the literature. To provide a guideline and assist in standardization, this review describes the most commonly utilized activity assays for flavoprotein oxidases, along with their respective merits and limitations.

Graphical Abstract



Activity assays for flavoprotein oxidases

Key points

- *Researchers should be more aware of limitations of activity assays.*
- *Extinction coefficients should be determined for the appropriate experimental setup.*
- *New robust activity assays are desired.*

Extended author information available on the last page of the article

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Keywords Oxidase activity assays · Horseradish peroxidase · Chromogenic assays · Electrochemical assays · Photometric assays

Introduction

Over the past century, the utilization of enzymes as catalytic tools in biotechnology has significantly increased, substituting or enhancing traditional chemical processes across various industries (Chapman et al. 2018; Robinson 2015). Additionally, enzymes have found applications in other areas, such as in food processing and (bio)sensing (Kurbanoglu et al. 2020; Sindhu et al. 2020). The remarkable selectivity of enzymes, coupled with their capability to operate under mild conditions, has driven their widespread adoption (Chen & Arnold 2020). The interest in developing enzyme-based processes has especially increased due to recent efforts to achieve climate neutrality and replace unsustainable chemical process (Aslam et al. 2020; Chen et al. 2023; Cipolatti et al. 2019).

Redox enzymes form the class of enzymes that are capable of performing oxidations and reductions, often displaying exquisite regio- and/or enantioselectivity. A major group of redox enzymes can be classified as flavoenzymes: enzymes that contain a flavin cofactor to facilitate redox catalysis.

Flavoenzymes are one of the most utilized type of enzymes, with their application spanning various industries since the 1970 s (Pimviriyakul & Chaiyen 2020). Flavoprotein oxidases, which merely rely on molecular oxygen as electron acceptor, are perhaps the most cost-effective redox enzymes. In contrast with many other redox enzymes, these biocatalysts do not depend on (expensive) coenzymes and solely produce hydrogen peroxide as a by-product (Martin et al. 2020). This by-product can be easily eliminated by the use of a catalase thereby regenerating some molecular oxygen. In other applications, the produced hydrogen peroxide itself is the desired product or it can also be utilized for completing a cascade reaction that involves a peroxide-dependent enzyme (Habib et al. 2017). Examples of flavoprotein oxidase-based applications can be found in glucose biosensors (Mandpe et al. 2020), valorization of waste products (Binoy et al. 2022), and discovery of therapeutic inhibitors (Youdim et al. 2006). A famous example of an industrially widely applied flavoprotein oxidase is glucose oxidase. This oxidase was one of the first oxidases adopted in biotechnological processes several decades ago and can nowadays be found in numerous applications (Wong et al. 2008).

All-in-all, the above illustrates the importance of and interest in flavoprotein oxidases. For developing or improving oxidase-based applications, experimental research on flavoprotein oxidases is an active field and reliable assays to monitor oxidase activity are indispensable. Whereas the interpretation and documentation of flavoprotein oxidase activity is well addressed in literature (Bisswanger 2014; Lauterbach et al. 2023; Pleiss 2021; Schomburg et al. 2002) and an ongoing endeavor (Apweiler et al. 2010; Range et al. 2022), the various different methodologies for obtaining said data can only be found scattered throughout literature. Despite the existence of some excellent books on the topic (Bisswanger 2019; Egbuna et al. 2022; Eisenthal & Danson 2002) and reviews about specific oxidases (Reis & Binda 2023; Rosini et al. 2018), the choice for a certain method often appears to depend on the availability and convenience for the researcher. A thorough comparison of described methods seems to be missing while a well-informed choice for a certain activity assay could benefit research. With this review, we attempt to give an overview of the commonly used flavoprotein oxidase activity assays. With a critical analysis of the pros and cons of the various approaches, it may serve as a guide for choosing a particular assay for research.

In the last decades, a variety of oxidase assays has been reported in literature. While some of these assays may also be applicable for other types of redox enzymes, such as copper-dependent oxidases, we focus our review on assays that were demonstrated with one or more flavoprotein oxidases. An analysis of 60 recent publications (since 2020) detailing flavoprotein oxidases reveals a wide array of different activity assays employed (SI1). Most of the described assays are based on one of the following three specific catalytic properties of the oxidases: (1) the production of hydrogen peroxide (Keston 1956), (2) the consumption of molecular oxygen (Smith & Camerino 1963), or (3) product formation (De Jong et al. 1992) (Fig. 1). The analysis revealed that the majority of assays utilized are continuous (51 out of 60), allowing for real-time monitoring of the reaction, rather than discontinuous, which involve quenching the reaction at specific intervals (Harris & Keshwani 2009). This observation aligns with existing literature recommending continuous assays, as assays can exhibit lag or burst phases due to hysteretic effects (Eisenthal & Danson 2002; Frieden 1979). These artifacts can be accounted for and corrected in continuous assays, but they often remain undetected in discontinuous assays. Additionally, spectrophotometric-based assays are the most frequently used (55 of 60), most likely due to their user-friendly nature. Recommending a specific assay is nearly impossible as a choice needs to be based on

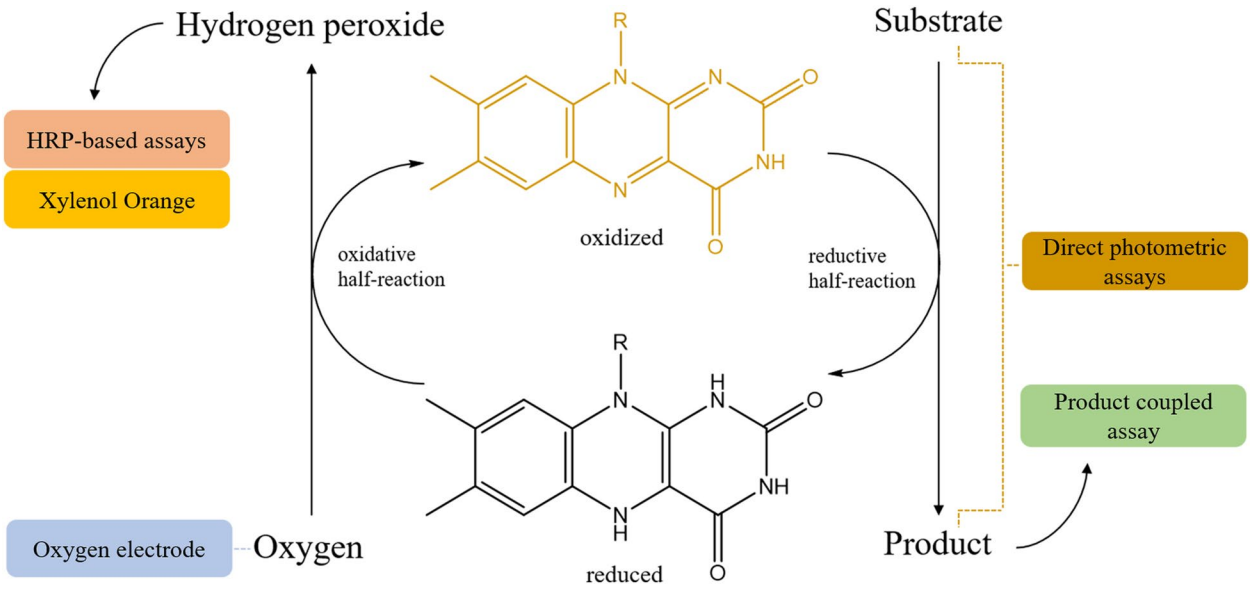

Fig. 1 Simplified representation of a flavoprotein oxidase-catalyzed reaction and the respective activity assays described in this review. The chemistry behind each type of assay is indicated

sensitivity, cost, convenience, specifics of the reaction and many more aspects. Nonetheless, a good understanding of the pros and cons of the available assays is important to efficiently perform research, such as efforts in directed evolution and high-throughput screening of flavoprotein oxidases (Atkin et al. 2008; Ebrahimi Fana et al. 2023; Rembeza et al. 2022).

The accuracy of these high-throughput screening campaigns is often dependent on the precision of the activity assay used, highlighting the importance of a well-thought-out decision for the utilized activity assay (Wang et al. 2021). To provide a guideline in choosing an appropriate flavoprotein oxidase assay, this review aims to describe the most used spectrophotometric and electrochemical assays. The information on these oxidase activity assays may also be used for other oxidases, such as copper-dependent oxidases. Other types of activity assays such as calorimetry, radiometric, and liquid chromatographic assays fall out of the scope of this review and are well-documented elsewhere (Eisenthal & Danson 2002; Falconer et al. 2021; Welling et al. 1994).

Spectrophotometric assays

Spectrophotometric assays are known for their user-friendly nature, requiring minimal amounts of reagents while also being applicable in high-throughput screening (Prodanović et al. 2020; Sunoqrot et al. 2021; Viña-Gonzalez et al. 2019). The assays utilize widely available laboratory equipment, such as spectrophotometers or microplate readers, which offer significant potential for scalability. This likely explains why spectrophotometric assays, encompassing colorimetric and fluorometric assays, are the most utilized assays in recent literature (SI 1). The assays rely on the detection of an increasing or diminishing chromophore or fluorophore (Eisenthal & Danson 2002). These easily detectable compounds are formed or depleted either directly due to the flavoprotein oxidase reaction or via a coupled reaction. To clarify, a chromophore is a chemical with a distinctive light absorbance feature, which, in this context, can extend outside of the visible light spectrum. A chromogen is the, often, non-detectable precursor or product of the chromophore. Similarly, fluorophores and fluorogens follow the same principle, with the distinction being that they produce a change in fluorescence signal rather than in light absorbance.

Colorimetric assays

The sensitivity of colorimetric assays relies on the extinction coefficient of the detected chromophore, the wavelength at which the chromophore can be monitored and, in the case of a coupled reaction, its stoichiometric relationship with the reactant. Due to advances in instruments, cuvettes, and microplates, influences of the measured wavelength is less influential for the sensitivity (Capelle et al. 2007; Perkampus 2013). Further, it is important to note that, in most cases, the extinction coefficient of the monitored chromophore needs to be experimentally determined. This is because it can vary depending on the reaction conditions, or the experiment requires the detection of a different absorbance optima than reported in literature, to avoid overlapping with absorbance of one of the other components in the reaction.

The extinction coefficient can be determined using the Beer-Lambert law (1).

$$\text{Absorbance} = \varepsilon * \text{path length} * \text{concentration} \quad (1)$$

Fluorometric assays

Fluorometric assays follow the same principles as the colorimetric assay but monitor the depletion or emerging of fluorophores instead of chromophores. They are often more sensitive than colorimetric assays as the fluorescent measurements suffer from less background signal than absorbance measurements (Gul & Gribbon 2010). Its sensitivity relies on the fluorescent signal of the fluorophore and, in case of a coupled reaction, on its stoichiometric relationship with the initial reaction and its monitored wavelength. Similar to the need for determining the exact extinction coefficients of chromophores, it is essential to prepare calibration curves for the assayed fluorophore.

In short, the sensitivity of spectrophotometric assays can be mainly judged on the extinction coefficient or fluorescent yield of the followed chromophore or fluorophore and in case of a coupled reaction, its stoichiometric relationship with the flavoprotein oxidase reaction.

Direct spectrophotometric assay: substrates and products

Ideally, either the substrate or product of the flavoprotein oxidase-catalyzed reaction is a chromophore or fluorophore. In this way the reaction can be followed under any suitable condition for the oxidase, requiring no additional reagents, avoiding pipetting steps, and keeping the cost down. It appears to be the preferred type of activity assay as it is consistently used when available. The extinction coefficient of the monitored chromophore needs to be experimentally determined as it can vary depending on the reaction conditions. For example, vanillin is the product of several oxidase-catalyzed reactions and its formation can be monitored at 340 nm. Yet, the extinction coefficient of vanillin at this wavelength is highly pH-dependent, changing by an order of magnitude depending on the pH of the solution (García-Bofill et al. 2019; Jin et al. 2007; van den Heuvel et al. 2001). Thus, for each buffer, it is advisable to determine the exact extinction coefficient of vanillin.

For aromatic substrates or products, such as vanillin, the absorption spectrum can also vary depending on the pH (Fraaije et al. 1995). Therefore, accurate determination of the absorption maximum also needs careful consideration. This highlights the importance of determining the extinction coefficient and the absorption maximum, rather than taking it from literature. Examples of well-known flavoprotein oxidases catalyzing reactions that result in a change in absorbance due to the substrate or product being a chromophore are NAD(P)H oxidases (Koh et al. 2009), vanillyl alcohol oxidases (De Jong et al. 1992), eugenol oxidases (Jin et al. 2007), and 5-hydroxymethylfurfural oxidase (Dijkman & Fraaije 2014). In principle, also hydrogen peroxide that is typically formed by action of a flavoprotein oxidase can be monitored as chromophore as it absorbs around 240 nm (ε_{240}: 40 M^{-1} cm^{-1}) (Bergmeyer 1974; Bickar et al. 1982). Unfortunately, this is a relatively low value for an extinction coefficient. Two hundred forty nanometers is in a region where also other components (e.g., substrate, product, and protein) absorb light, which prohibits the use of this characteristic of hydrogen peroxide. It would also rely on a highly pure oxidase sample, devoid of catalase activity.

Coupled reactions

Unfortunately, the majority of flavoprotein oxidase-catalyzed reactions do not inherently involve reactants that are or form chromophores or fluorophores. This prevents direct spectrophotometric activity measurements as described above. In order to monitor these reactions, an auxiliary reaction, either enzymatic or non-enzymatic, can be utilized to monitor and determine the rate of the reaction (Duggleby 1983). This can be in the form of a spontaneous reaction of a formed product with an added reactant (Gauillard et al. 1993) or via an additional (bio)catalyst and non-interfering second substrate (Bergmeyer 1965). The utilized coupled indicator reaction is preferably irreversible, sensitive and rapid to ensure precision (Easterby 1973; McClure 1969; Yang & Schulz 1987). In practical applications, this is typically achieved by incorporating an excess of indicator reactants (Cleland 1979; García-Carmona et al. 1981; Rudolph et al. 1979). Even so, a lag phase often occurs until the auxiliary reaction reaches its maximal catalytic rate (Eilertsen & Schnell 2018). Often this artifact is negligible, as the auxiliary reaction is relatively fast or, in the case of a continuous assay, one can correct for the lag phase. In a discontinuous assay this can, however, introduce an underestimation of the oxidase activity (Eisenthal & Danson 2002).

Spontaneous coupled reactions, in which a chemical reagent is used, are dependent on the product of the flavoprotein oxidase and are typically discontinuous. In literature, such assays have mainly been used for amino acid oxidases, in which the formed α-keto acid reacts with 2,4-dinitrophenylhydrazine, producing a chromophore which absorbs light at around 445 nm (Katane et al. 2020, 2007; Nagata et al. 1988). For most flavoprotein oxidases this approach is not an option because they produce different types of products. Therefore, more generic methods have been developed. These methods commonly rely on an auxiliary enzyme that reacts with the flavoprotein oxidase reaction product

to either produce or deplete a chromophore or fluorophore. Among these approaches are assays utilizing an NAD(P)H-dependent enzyme, often a dehydrogenase (Gitomer & Tipton 1983). However, these are again very specific for the products of respective flavoprotein oxidase reactions. A more generic oxidase assay is the approach of using a peroxidase in combination with a flavoprotein oxidase. The peroxidase will utilize the hydrogen peroxide produced by most flavoprotein oxidases (Mattevi 2006). There is only one flavoprotein oxidase class known that does not form hydrogen peroxide as reduced dioxygen species: some NADH oxidases reduce molecular oxygen to water (Higuchi et al. 1993; Lopez de Felipe & Hugenholtz 2001; Stanton & Jensen 1993; Tanabe 1979).

Peroxidase-based assays

Spectrophotometric assays based on an auxiliary peroxidase rely on the peroxidase-catalyzed oxidation reaction that typically results in formation of a radical as product (Veitch 2004). A number of peroxidase substrates have been identified that are suitable for spectrophotometric monitoring of such coupled reactions. The peroxidase typically generates a chromophore or fluorophore. Since these assays do not consume the product generated by the oxidase, they can even provide insight into product inhibition, although they cannot detect the rarer inhibition by hydrogen peroxide (de la Mata et al. 2000; Kleppe 1966). As mentioned above, peroxidase-based assays can be used for almost all flavoprotein oxidases relying solely on the hydrogen peroxide produced by almost all flavoprotein oxidases (Mattevi 2006) (Fig. 2). The generic character of this type of assay can simplify workflows as the same assay can be utilized for screening different flavoprotein oxidases and/or different substrates.

In literature, the peroxidase utilized in peroxidase-based assays is consistently horseradish peroxidase (HRP). This is most likely due to it having the lowest reported K_M (5 µM) (Gilabert et al. 2004) for hydrogen peroxide among the reported peroxidases in the BRENDA database, combined with high k_{cat} values for its substrates, greatly surpassing rates of flavoprotein oxidases. Flavoprotein oxidases typically exhibit k_{cat} values in the range of 1 to 100 s^{-1} (Mattevi 2006). The difference in turnover number between the flavoprotein oxidase and HRP, as mentioned before, is vital to follow the reaction precisely. This is also the reason why HRP and its reactants are often added in excess during the enzyme activity assays. The use of HRP as peroxidase for peroxidase-based assay may also be due to its longstanding commercial availability at a relatively

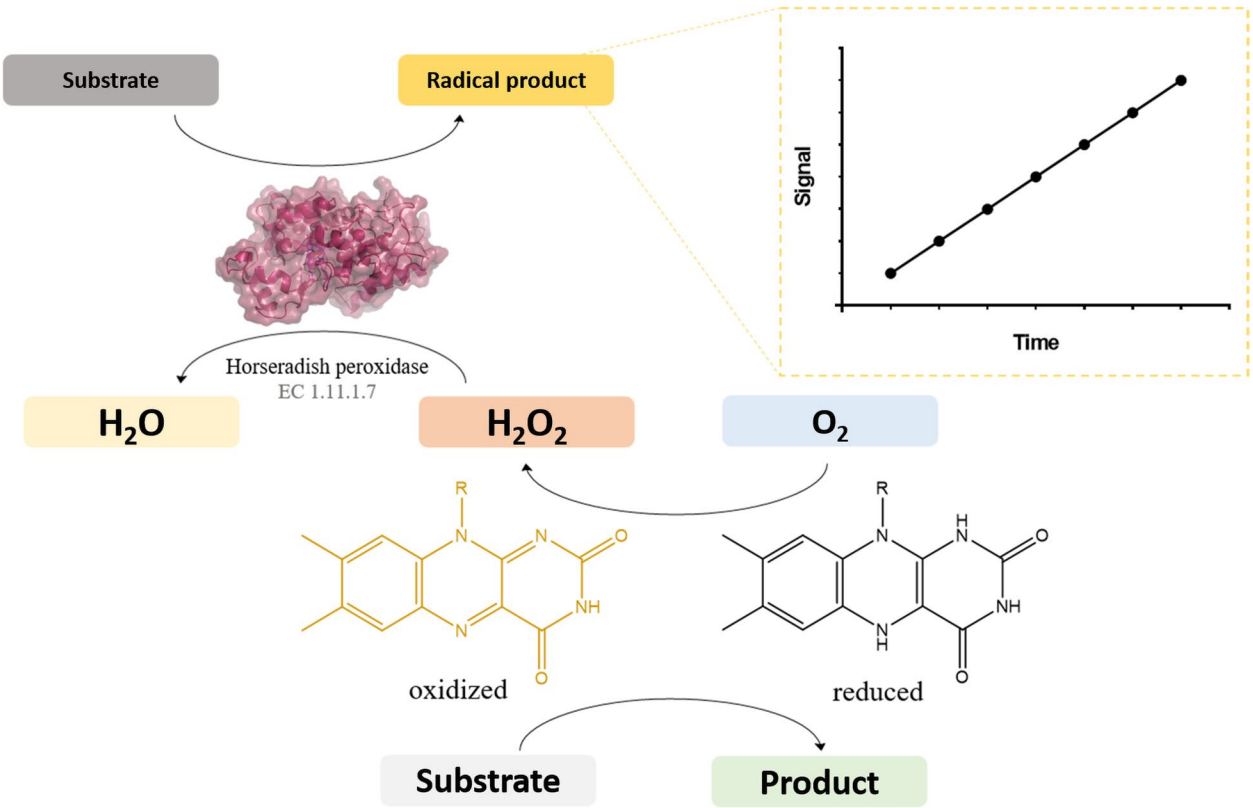

Fig. 2 General reaction scheme of a flavoprotein oxidase, together with a coupled horseradish peroxidase reaction

low price, which goes hand-in-hand with the development of various assay methods based on HRP.

As mentioned before, this assay can be utilized for most flavoprotein oxidases, but the use of an auxiliary enzyme brings its limitations, as the working range of the assay is limited to the working range of HRP. In practical terms this implies that the assay should be performed at temperatures below 45 °C and ideally around 28 °C, in accordance with HRP's temperature working range (Lavery et al. 2010). The pH working range of the assay is harder to define as the pH optimum for activity of HRP is slightly different depending on the used chromogen/fluorogen (Karasyova et al. 2003; Lavery et al. 2010; Munoz-Munoz et al. 2007; Nagaraja et al. 2009). HRP has been used for assays at pH 4–9, while its pH optimum for activity is around pH 6 (Schomburg et al. 2012). The broad pH range employed in assays is explained by the fact that typically an excess of HRP is used with respect to the amount of oxidase activity. This is also easily achieved due to the relatively high turnover number of HRP (200 s^{-1} at pH 6) (Violante-Mota et al. 2010).

There are variants of HRP described in literature with broader working ranges or higher turnover numbers (Cherry et al. 1999; Morawski et al. 2001) but these are thus far not utilized in activity assays due to the difficulty of expression of the HRP gene, despite recent developments in alternative expression systems (Chauhan & Kang 2018; Zhao et al. 2023). In fact, HRP is mainly offered as an enzyme purified from the roots of horseradish due to the poor expression as recombinant protein. In literature there are also alternative peroxidases, from other sources, described with broader working ranges or higher turnover number (Brissos et al. 2017; Pećanac et al. 2025; van Bloois et al. 2010), but these are thus far also not utilized in activity assays, most likely due to their obscurity or commercial unavailability.

Reactants that inhibit HRP, including thiols and heavy metals (Sariri et al. 2006; Zatón & Ochoa de Aspuru 1995; Zollner 1999), or flavoprotein oxidase substrates or products that are also accepted by HRP, such as specific phenolic compounds (Patel et al. 1997), are unsuitable for use in HRP-coupled oxidase assays. HRP is also reported to be unstable at high hydrogen peroxide concentrations (Baynton et al. 1994; Hernández-Ruiz et al. 2001; Morales-Urrea et al. 2023); these concentrations are however rarely met in activity assays as it typically only involves measurements of initial rates. Finally, the use of antioxidants, like L-ascorbic acid (Baker 1998), is not recommended with HRP-based assays as they react with the hydrogen peroxide. This results in an underestimation of oxidase activity. We also observed that commercially available HRP is not entirely pure and frequently contains trace amounts of other enzymes, among which are likely plant-derived oxidases. This underscores the importance of conducting a control experiment with the tested substrate as these contaminations can lead to false positives.

The true potential and limitations of HRP-based assays, however, are determined by the choice of chromogenic or fluorogenic substrate employed in the assay. The choice of these cosubstrates heavily influences the usability and sensitivity of the assay. An analysis of literature reporting on flavoprotein oxidases suggests that the selection of these peroxidase substrates is primarily driven by convenience, as no discernible trend could be identified (Fig. 3B, SI 2). There are a few well-established peroxidase substrates that are used in most cases. Recent literature (post 2020) shows the utilization of four popular HRP-based assays in which *o*-dianisidine, 2,2-azino-bis(2-ethylbenzthiazoline- 6-sulfonic acid) (ABTS), 4-aminoantipyrine (4-AAP) coupled with a phenolic compound or Amplex Red is utilized as chromogen or fluorogen. HRP displays high peroxidase activity (Feng et al. 2008; Glettenberg & Niemeyer 2009; Kamal & Behere 2003; Ugarova et al. 1981) for all these compounds while exhibiting a relatively low K_M for hydrogen peroxide. All of these assays are designed to include an excess of the chromogen/fluorogen, with their sensitivity determined by the extinction coefficient (or fluorescent yield) and the stoichiometric relationship between the resulting chromophore or fluorophore and hydrogen peroxide. Recommending an appropriate set of chromogens/fluorogens to use for an HRP-based assay is challenging as each has its own merits and a decision needs to be made based on sensitivity, cost, safety, stability of chromophore or fluorophore. To give a guideline, below, a summary is provided of the most utilized HRP-based assays. An overview of the different systems is shown in Fig. 3 and a list of chromogens/fluorogens, and their properties are given in Table 1.

O-Dianisidine as peroxidase substrate

The development of HRP-based oxidase assays began in the mid- 1950 s as a method to allow measurements of blood glucose levels using glucose oxidase (Keston 1956). Herein, *o*-dianisidine was used as chromogen, and quickly after this publication, the assay was utilized for the characterization of oxidases (Crowne & Mansford 1962; Farmer et al. 1960; McComb et al. 1957). The assay relies on the stoichiometric 1:1 oxidation of *o*-dianisidine with hydrogen peroxide by HRP, which results in a brownish colored product with an absorbance maximum at 460 nm (Blaedel & Uhl 1975; Claiborne & Fridovich 1979a). The extinction coefficients in older literature are reported to be 30 mM^{-1} cm^{-1} at 460 nm (Lebedeva et al. 1977; Moller & Ottolenghi 1966; Rogozhin et al. 2000; Ugarova et al. 1981) and 15 mM^{-1} cm^{-1} at 450 nm (Savitsky et al. 1994). These values, however, are questionable as newer literature utilizing this assay follows formation of the product at 436 nm with a reported extinction

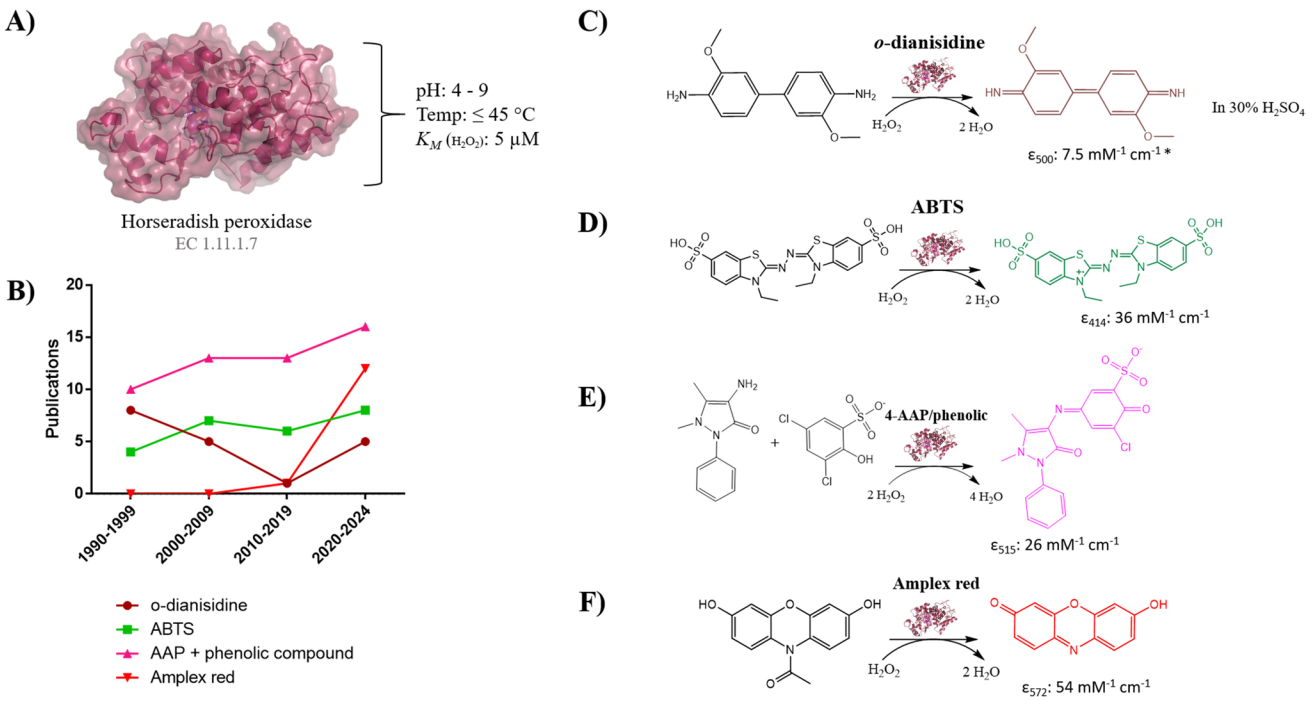

Fig. 3 A Structure and properties of horseradish peroxidase (PDB: 1HCH). **B** Observed trend in the last 34 years in the use of different HRP-based assays for measuring flavoprotein oxidase activity (data is shown in SI 2). Amplex Red also includes Amplex UltraRed. **C** *O*-dianisidine oxidation catalyzed by HRP with hydrogen peroxide. **D** ABTS oxidization catalyzed by HRP with hydrogen peroxide. **E** 4-AAP and DCHBS oxidization catalyzed by HRP with hydrogen peroxide. **F** Amplex Red oxidization catalyzed by HRP with hydrogen peroxide

coefficient of 8.31 mM^{-1} cm^{-1} (Adachi et al. 2010; Geueke et al. 2007; Jimbo et al. 2003; Yang et al. 2005). Still, due to inconsistency in reported extinction coefficients without proper explanations, the instability of the formed dye (Dohnal & Zyka 1974) and reported pH sensitivity (Claiborne & Fridovich 1979a), it is highly recommended to make a calibration curve for the assay under the required conditions instead of taking an extinction coefficient from literature. The working pH range of the assay is reported to be between pH 4 and pH 7 (Blecher & Glassman 1962). *O*-dianisidine is poorly soluble in water and is typically solubilized in methanol. To avoid using an organic cosolvent, the water-soluble salt, *o*-dianisidine dihydrochloride, can better be utilized. Such *o*-dianisidine solution displays an absorbance maximum at 440 nm with an extinction coefficient of 13 mM^{-1} cm^{-1} (Khan et al. 2024). The instable formed dye can be stabilized by converting the assay to a discontinues format where 30–50% H_2SO_4 is added after the reaction (McComb & Yushok 1958). This stabilizes the formed dye, reaching half-life values of up to 161 h (Gabler et al. 2000). The addition of an acid shifts the maximal absorbance for oxidized *o*-dianisidine to 500 nm with an extinction coefficient of 7.5 mM^{-1} cm^{-1} (Dave et al. 2023; McComb & Yushok 1958) and for *o*-dianisidine dihydrochloride to 540 nm with an extinction coefficient of 9.6 mM^{-1} cm^{-1} (Lehmann et al. 1974; Schosinsky et al. 1974). These extinction coefficients appear to be consistent in newer literature (Alapati & Handanahal 2020; Dave et al. 2023; Rayapati et al. 2024). Different acids can be used to stop the reaction and stabilize the formed chromophore, but these will slightly alter the absorbance maxima and the extinction coefficients (Porstmann et al. 1981). A more pressing limitation is that both *o*-dianisidine and its soluble analogue, although debated (Golka et al. 2004), are classified as carcinogenic (Martin & Kennelly 1981). This might explain its decline in recent years despite its low price (Table 1). The assay still finds use in screening campaigns where oxidase activity is screened for in cell extracts. In such experiments, one takes advantage of *o*-dianisidine acting as an inhibitor for catalases, native in most laboratory strains, minimizing interference caused by hydrogen peroxide consumption by these catalases (Claiborne & Fridovich 1979b; Gabler et al. 2000).

ABTS as peroxidase substrate

The desire to find alternative non-carcinogenic chromogens for oxidase assays led to the discovery of 2,2-azino-bis(2-ethylbenzthiazoline- 6-sulfonic acid) (ABTS) in the early 1970 s. It was described as being four times more sensitive than *o*-dianisidine, water soluble, not carcinogenic and a

Table 1 Commonly used chromogens and fluorogens in HRP-based assays

Chromogen(s)	λ_{max} (nm)	ε (mM^{-1} cm^{-1})	Price per 100 µL reaction (€)	Recommended use	References
o-Dianisidine (+ H_2SO_4)	436 500	8.31[a] 7.5	0.05×10^{-4} (0.2 mM) 0.2×10^{-4} (1 mM)	Cheap and good for disrupted cell screenings Discontinuous version recommended	(Adachi et al. 2010; Geueke et al. 2007; Jimbo et al. 2003; Yang et al. 2005) (Dave et al. 2023; McComb & Yushok 1958)
o-Dianisidine dihydrochloride (+ H_2SO_4)	440 540	13[a] 9.6	0.3×10^{-4} (1 mM) 0.5×10^{-4} (1.5 mM)	Cheap, water soluble and good for disrupted cell screenings Discontinuous version recommended	(Khan et al. 2024) (Lehmann et al. 1974; Schosinsky et al. 1974)
o-Tolidine (+ acid)	630[b]	*n.d*	1×10^{-4} (1 mM)	Not recommended	(Đurić & Deletić, 2020)
p-Anisidine	458	*n.d*	0.7×10^{-4} (1 mM)	Not recommended	(Avila & de La Guardia 1997)
p-Cresol	277[b] (Decrease)	1.7[a]	0.8×10^{-4} (1 mM)[c]	Not recommended	(Hewson & Dunford 1976a, 1976b)
o-Phenylenediamine	418[b]	16.7[a]	0.5×10^{-4} (3.7 mM)	Not recommended	(Fornera & Walde 2010)
3,3′-Diaminobenzidine	352[b]	*n.d.*[a]	9×10^{-4} (0.36 mM)	Not recommended	(Cohen 1973; Sannia et al. 1991)
ABTS	414	36	5.5×10^{-4} (100 µM)	Water soluble, user-friendly and multiple absorbance optima	(Childs & Bardsley 1975; Wang & Reckhow 2016)
4-Chloro- 1-naphthol	550[b]	*n.d*	10×10^{-4} (4 mM)	Membrane staining/membrane activity screenings	(Conyers & Kidwell 1991; Ulyashova et al. 2011)
2′,7′-Dichlorodihydrofluorescein diacetate	502 (Can also be used as fluorogen)[b]	91 (Stoichiometric relationship of 5 with hydrogen peroxide)	10×10^{-4} (0.25 mM)	Not recommended	(Chen et al. 2010; Köchli & Von Wartburg 1978; Setini et al. 2005)
AAP/phenol	505	6.58[a]	1.7×10^{-4} (1.5 + 2 mM)	Not recommended	(Doukyu et al. 2008; Mortarino et al. 1996; Motoyama et al. 2022; Sugiura et al. 2021; Yamashita et al. 2000)
AAP/4-hydroxybenzoic acid	500	5.3	1.5×10^{-4} (1.2 + 2 mM)	Not recommended	(Iwamoto et al. 1996; Meiattini et al. 1978)
AAP/phenol- 4-sulfonic	490	5.56	4×10^{-4} (0.4 + 25 mM)	Not recommended	(Vojinović et al. 2004, 2007)
AAP/2,4,6-tribromo- 3-hydroxybenzoicacid	510	29.4	0.9×10^{-4} (0.75 + 2 mM)[c]	Appears to be in use for *L*-amino acid oxidases	(Braun et al. 1992; Trinder & Webster 1984)
AAP/DCHBS	515	26	0.3×10^{-4} (0.1 + 1 mM)	Water soluble, good cost, sensitive balance and produces a stable signal	(Boverio et al. 2023; Fossati et al. 1980; Tjallinks et al. 2023a)

Table 1 (continued)

Chromogen(s)	λ_{max} (nm)	ε (mM^{-1} cm^{-1})	Price per 100 µL reaction (€)	Recommended use	References
Phenol-Red (+ NaOH)	610	*n.d*	0.4×10^{-4} (1 mM)	Not recommended	(Johnston et al. 2008; Pick & Keisari 1980)
Fluorogen(s)	$\lambda_{ex}/\lambda_{em}$ (nm)				
Scopoletin	360/465 (decrease)	*n.d*	3200×10^{-4} (2 mM)[c]	Not recommended	(Andreae 1955; Brotea & Thibert 1988; Corbett 1989)
Homovanillic acid	320/420	*n.d*[a]	20×10^{-4} (1 mM)	Cheap fluorogen option	(Faccio et al. 2010; Guilbault et al. 1967)
Luminol	351/415	Depended on solution[a]	30×10^{-4} (100 µM)	Not recommended	(Díaz et al. 1996; Dure & Cormier 1964)
Amplex Red	563/587	54 (572 nm)	700×10^{-4} (50 µM)	Extreme high sensitivity	(Mohanty et al. 1997; Zhou et al. 1997)
Amplex UltraRed	568/581	> 54[a] (570 nm)[b]	2500×10^{-4} (100 µM)	Extreme high sensitivity and stability at lower pH values	(Invitrogen, 2009)

Price per reaction is based on only the price of the chromogen found at www.merck.com (08/2024). The amount of chromogen or fluorogen is based on the protocol paper added in the table together with a total reaction volume of 100 µL. Prices are taken from solid versions of chromogen or fluorogen, kit prices are not checked, and prices are calculated based on the lowest quantity that is available for purchase. [a]Check the extinction coefficient with a calibration curve as reports are inconsistent, it is highly sensitive to the used conditions, or use in literature always is accompanied by a calibration curve. [b]Max absorbance is either highly dependent on conditions or could not be confirmed [c]Concentration could not be verified

highly stable chromophore ($t_{1/2}$: 48 h) (Erel 2004; Gawehn et al. 1970; Werner et al. 1970). However, it is important to note that subsequent studies revealed that ABTS is mutagenic, which asks for cautious handling of this compound (Hosoda et al. 1986). The assay relies on the one-electron oxidation of ABTS by HRP with a 2:1 stoichiometric relation with hydrogen peroxide (Cai et al. 2018; Rodríguez-López et al. 2001) to form the corresponding radical, $ABTS^+$. ABTS itself can be detected at 340 nm with an extinction coefficient of 36 mM^{-1} cm^{-1} and its blue-green radical has a broad absorbance spectrum and can be followed at 414 nm with an extinction coefficient of 36 mM^{-1} cm^{-1} (Childs & Bardsley 1975), or at one of its other peaks (ε_{650}: 10.2 mM^{-1} cm^{-1}; ε_{732}: 13.7 mM^{-1} cm^{-1}; and ε_{820}: 10.8 mM^{-1} cm^{-1}) (Wang & Reckhow 2016). The radical is also frequently monitored at 405 nm (ε_{405}: 31.6 mM^{-1} cm^{-1}), enabling more precise measurements (Pinkernell et al. 1997, 2000). Having such a broad absorbance spectrum to follow the chromophore enhances the utility of the assay. For each experimental set-up, an optimal wavelength, minimizing interferences with other reaction components, can be chosen. ABTS is a more expensive chemical when compared with *o*-dianisidine (Table 1) but the price per reaction remains low. It suffers, however, from a few notable artifacts. The stability of the produced radical is highly pH-dependent; at higher pH values, the radical can react with hydrogen peroxide, regenerating ABTS (Barr & Aust 1993). Also, maintaining an excess of ABTS is crucial, as ABTS to hydrogen peroxide ratios of 0.5 can lead to overoxidation, producing $ABTS^{2+}$, which precipitates from solution as a pale yellow color (Ilyasov et al. 2020; Kadnikova & Kostić, 2002; Majcherczyk et al. 1999). The stability of the $ABTS^+$ chromophore is also influenced, although limited, by light, resulting in errors after relatively long exposure times (Liberato et al. 2018). Furthermore, commercial ABTS-HRP kits have been found to contain impurities of ABTS precursors and analogues, leading to artifacts at pH values above 8 (Zhang & Hess 2020), underscoring the importance of maintaining a proper pH for the assay. Despite these pH-dependent artifacts, the literature does not clearly define an optimal pH working range for the assay. While some sources suggest a pH range of 3–6.5 (Cano et al. 1998), other studies report successful use of the assay at higher pH values (up to pH 8) (Kamathewatta et al. 2020; Kopacz et al. 2011; Mendes et al. 2016; van Hellemond et al. 2008). The aforementioned artifacts, although important to take into account, are often relatively slow and cause minimal error in measuring initial activities. A more important limitation of the HRP-based ABTS assay is the tendency of $ABTS^+$ to react with antioxidants (Arnao et al. 1996; Ilyasov et al. 2020; Re et al. 1999), phenolic compounds with electron-donating substituents (Osman et al. 2006), β,γ-unsaturated aryl ketones (Koo et al. 2025), and L-amino acids (Zheng

et al. 2016). This reactivity renders the assay unusable if one of the reactants of the original reactions interacts with the ABTS radical. Clearly, negative and positive controls are vital to be able to use this assay type. The HRP-based ABTS assay continues to be widely employed (Fig. 3B) for both the characterization (Abrera et al. 2020; Kamathewatta et al. 2020; Punthong et al. 2022) and screening (Prodanović et al. 2020) of flavoprotein oxidases, likely due to its commercial availability, well-established protocols, and simplicity.

4-Aminoantipyrine in combination with a phenolic compound as peroxidase substrates

In 1969, Trinder proposed the use of 4-aminophenazone, later replaced by 4-aminoantipyrine (4-AAP), together with phenol as a non-carcinogenic alternative to *o*-dianisidine (Trinder 1969). Its subsequent use and popularity in glucose oxidase-coupled assays to measure blood glucose levels gave rise to the name "Trinder reagents" in medical papers (Bauminger 1974; Emmerson et al. 1973; Lott & Turner 1975). The assay gives rise to a pink quinoneimine dye which is stable, exhibits no signal loss within the first 30 min (Artiss et al. 1981), and has a stoichiometric relationship of 1:2 with hydrogen peroxide (Vojinović et al. 2004). The formed chromophore has a maximal absorbance at 505 nm and reported extinction coefficients in comparable conditions ranging from 6.58 to 13.8 mM^{-1} cm^{-1} (Doukyu et al. 2008; Mortarino et al. 1996; Motoyama et al. 2022; Sugiura et al. 2021; Yamashita et al. 2000), underscoring the necessity of determining an extinction coefficient tailored for the specific experimental conditions. In older literature, it was preferred over ABTS as it is reported to be less pH sensitive (Drozd et al. 2016) and more stable, and the formed chromophore is less reactive than the ABTS radical (Lott & Turner 1975; Pennock et al. 1973; Sharp 1972).

Due to the stability of the formed chromophore, the pH range from the assay is reported to be dependent on the pH working range of HRP, giving it a larger pH range than the aforementioned ABTS-based assay. Despite that the use of 4-AAP and phenol was initially proposed as alternative to avoid the use of carcinogenic chemicals, it was found that phenol is also toxic (Gami et al. 2014). To circumvent the use of phenol, alternatives were quickly reported that offer reduced toxicity, enhanced sensitivity, and improved water solubility (Barham & Trinder 1972; Meiattini et al. 1978; Trivedi et al. 1978; Wong et al. 1981). Notable examples still being used today are as follows: the less toxic and more water soluble phenol- 4-sulfonic acid (ε_{490}: 5.56 mM^{-1} cm^{-1}) (Vojinović et al. 2004, 2007), the more sensitive and less toxic 2,4,6-tribromo- 3-hydroxybenzoicacid (ε_{510}: 29.4 mM^{-1} cm^{-1}) (Trinder & Webster 1984) and the more sensitive, water soluble, and less toxic 3,5-dichloro-2-hydroxybenzenesulfonic acid (DCHBS) (ε_{515}: 26 mM^{-1} cm^{-1}) (Fossati et al. 1980). The produced dye of the 4-AAP/DCHBS HRP-based assay (Fig. 3E) appears to be resilient as the reported extinction coefficients in literature remains consistent across various experimental conditions (Boverio et al. 2023; Callejón et al. 2015; Lim et al. 2006; Tjallinks et al. 2023a). The assay is affected by several notable artifacts, including the vulnerability of the phenolic compound to oxidative degradation during storage (Ngo & Lenhoff 1980). The majority of artifacts, however, arise from the secondary non-enzymatic reaction between the 4-AAP radical and the phenolic chromogen. Strong reducing agents, like *p*-diphenols, can react with the 4-AAP radical to produce *p*-quinones, preventing color formation (Tarasek et al. 2020) and giving rise to false negatives with the assay. Multiple different chemicals have been reported to react with the radical intermediate with no clear explanation (Genzen et al. 2016; Karon et al. 1998; Witte et al. 1978), and some chemicals are known to give rise to false positives with the assay. These potential artifacts again stress the importance of performing negative and positive controls while using the HRP/4-AAP/phenol assay. The 4-AAP-based assays remain popular (Fig. 3B) due to their low cost (Table 1), good sensitivity, stability of the produced chromophore and safety.

Amplex Red as peroxidase substrate

When compared with chromogenic assays, fluorescence-based measurements generally show less background interference (Gul & Gribbon 2010). Accordingly, during the early stages of HRP-based assay development, several fluorophores and fluorogens were proposed (Andreae 1955; Black & Brandt 1974; Guilbault et al. 1967; Keston & Brandt 1965) and used to measure enzyme activity (Flohé & Brand 1969; Wellner & Lichtenberg 1971). One of the first developed assays was an assay involving scopoletin (6-methyl- 7-hydroxy- 1,2-benzopyrone). This aromatic compound exhibits a loss of fluorescence upon oxidation by HRP (Andreae 1955), setting it apart from the previously mentioned HRP-coupled assays, as it results in a decline in signal rather than in a gain. The stoichiometry of the reaction between scopoletin and hydrogen peroxide is 1:1, and the fluorophore can be followed by excitation at 360 nm with an emission at 465 nm (Andreae 1955; Brotea & Thibert 1988; Corbett 1989). Scopoletin is described to be stable for more than 30 min at 25 °C (Lichtenberg & Wellner 1968) but it can quickly become unstable and breaks down due to temperature and pH changes, resulting in high background signals and noise (De la Harpe & Nathan 1985; Ramasarma 1982). A solution to stabilize the remaining scopoletin signal was to quench the reaction by increasing the pH to ~ 10 (Corbett 1989), making the assay discontinuous.

These complications may explain its disappearance in recent literature in favor for HRP-based assays using other fluorophores such as luminol (Díaz et al. 1996; Dure & Cormier 1964), homovanillic acid (Guilbault et al. 1967) and Amplex Red. Described first in the late 90's (Mohanty et al. 1997; Zhou et al. 1997), to our knowledge, Amplex Red and its derivative Amplex UltraRed are the most sensitive fluorogens for HRP-based oxidase assays. HRP oxidizes Amplex Red to form resorufin, with a stoichiometric relation of 1:1 with hydrogen peroxide. Resorufin can be excited at 563 nm and emits at 587 nm or can be followed with its absorbance maxima at 572 nm with an extinction coefficient of 54 mM^{-1} cm^{-1} (Zhou et al. 1997). The stability of resorufin, which retains more than 95% of its signal after 4 h at 25 °C (Mohanty et al. 1997; Zhou et al. 1997), coupled with the high sensitivity of the assay, has established the Amplex Red assay as the gold standard for hydrogen peroxide detection. Consequently, the assay has become the benchmark for HRP-based methods in the study of flavoprotein oxidases, contributing to its increased prevalence in recent literature. Amplex Red is relatively costly compared to the assays mentioned previously (Table 1) raising the question whether the extra sensitivity compensates for the cost, as most flavoprotein oxidases can also be assayed with the less costly and less sensitive assays described above. A few notable artifacts plaguing the Amplex Red-based assay should be named. Since resorufin is a substrate of HRP (Brotea et al. 1989), albeit with turnover rates 30 times lower than those of Amplex Red, the ratio of Amplex Red to hydrogen peroxide needs to be at least five to prevent the overoxidation of resorufin (Mohanty et al. 1997; Zhou et al. 1997). Commercial Amplex Red is known to contain trace amounts of resorufin, which, in a self-catalyzing reaction with oxygen under light exposure, can create superoxide and hydrogen peroxide, which subsequently react with Amplex Red to produce more resorufin (Summers et al. 2013; Zhao et al. 2012). Since this is known to happen even by exposure to room light, the stock of Amplex Red needs to be stored under dark conditions. The signal caused by this artifact is relatively low and often overshadowed by any oxidase activity. However, when extreme sensitivity is required, the assay can be done in a discontinuous way in the dark, by measuring only an endpoint and avoiding light exposure. Further, the assay is incompatible with NAD(P)H (Zhao et al. 2011), as it reacts with resorufin. Also, the use of peroxynitrite in the assay should be avoided as HRP can use it to oxidize Amplex Red (Dębski et al. 2016). A more recent analogue of Amplex Red, known only as Amplex UltraRed, has been developed to avoid most of the previously mentioned artifacts. It exhibits reduced photosensitivity, enhanced sensitivity, and a broader pH operating range. Resorufin, the fluorescent product of the Amplex Red assay, maintains stable emission between pH 6 and pH 10 (with detectable signals as low as pH 5) and has an optimal sensitivity between pH 7.5 and 8 (Towne et al. 2004). Amplex UltroxRed, the fluorescent product of the Amplex UltraRed assay, demonstrates stable fluorescent emission from pH 5 to pH 10, with detectable signals starting at pH 4 (Invitrogen, 2009; Zhu et al. 2010). Amplex UltraRed also has a stoichiometric relation of 1:1 with hydrogen peroxide and its product Amplex UltroxRed has excitation/emission maxima of ~ 568/581 nm (Bulina et al. 2006). The extinction coefficient of Amplex UltroxRed is reported to be higher than that of resorufin and studies employing this assay typically generate calibration curves to establish the appropriate extinction coefficient or fluorescent yield under specific experimental conditions (Muellers et al. 2023). Both Amplex Red and Amplex UltraRed suffer from artifacts at high hydrogen peroxide concentrations (> 100 µM), which translates in practical terms that incubation times with the assay should be short or oxidase concentrations should be low (Towne et al. 2004). Amplex Red appears to be more used than Amplex UltraRed in papers describing flavoprotein oxidases (10:1, SI 2); this is most likely due to Amplex UltraRed being more costly than Amplex Red (Table 1) and due to its shorter time on the market. However, if a lower pH is required and the sensitivity of the Amplex Red is needed, Amplex UltraRed is the recommended fluorogen.

Xylenol orange

The hydrogen peroxide generated by flavoprotein oxidase-catalyzed reactions can also be monitored through non-enzymatic methods. By utilizing one or more redox-active compounds, similar chromogen/chromophore reactions can be catalyzed as described before. A commonly used non-enzymatic coupled assay is the xylenol orange assay (Fig. 4), in which hydrogen peroxide oxidizes the ferrous ion, producing a ferric radical which reacts with the xylenol orange dye (3,3′-bis(N,N-bis(carboxymethyl)aminomethyl)-*o*-cresolsulfonephthalein) in a ~ 1:4 stoichiometric relationship to form a chromophore complex (Gupta 1973). The reaction proceeds under highly acidic conditions, making the assay discontinuous. The resulting chromophore, which develops after 30 min, is reported to be highly stable, persisting for over 25 h and can be monitored at 560 nm (Gay et al. 1999b). However, its stability can vary depending on specific experimental conditions (Gay et al. 1999a, 1999b). The assay was initially reported in the 1970 s (Gupta 1973), although an earlier Japanese paper mentions its potential (Otomo 1965). The assay was further developed in the early 1990 s (Jiang et al. 1990) and has seen extensive use across various fields (Eymard & Genot 2003; Nourooz-Zadeh et al. 1995). Its use for measuring flavoprotein oxidase activity increased significantly following its use in the substrate specificity screening of *para*-phenol oxidases (Ewing et al.

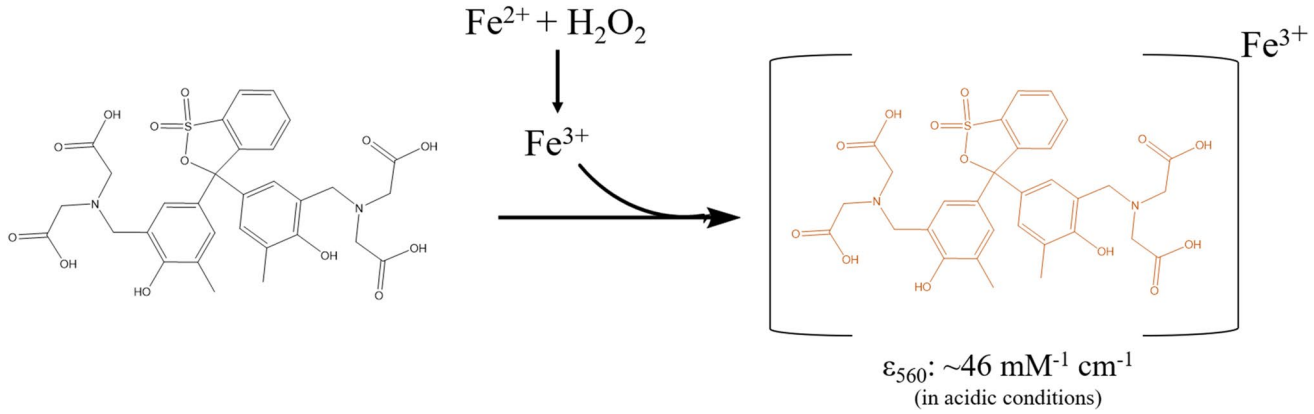

Fig. 4 Oxidation of ferrous to ferric iron by hydrogen peroxide under acidic conditions followed by complex formation of xylenol orange with ferric iron to form a chromophore complex. The reported extinction coefficient is an estimation and is highly variable based on used experimental conditions

2018). Unlike HRP-based assays, this method is suitable for use with phenolic compounds (Henriksen et al. 1999), which explains why it was chosen for studying oxidases acting on aromatic compounds. Another advantage of the discontinuous xylenol orange-based assay is that it allows for the determination of pH optima (Eggerichs et al. 2023a, 2023b). The extinction coefficient of the assay is highly dependent on the conditions, such as the acid used (Gay & Gebicki 2002), and needs to be experimentally determined for each experimental setup. It is reported to be around 43 mM^{-1} cm^{-1} (Gay et al. 1999a) and can be influenced by adding organic cosolvents (DMSO, ethanol, and methanol) (Rhee et al. 2010) or certain carbohydrates (glucose, sorbitol) (Gay & Gebicki 2000). As a result, the assay is not suited for carbohydrate oxidases since the organic substrate and/or product may affect the chromophore properties. The assay is also known to be inhibited by various substrates, and its use is reviewed in detail elsewhere (Bou et al. 2008). In brief, the assay suffers from reproducibility problems (Gay et al. 1999a) as even the source of xylenol orange can affect its extinction coefficient. The use of the assay in literature appears to be mainly for the use with *para*-phenol oxidases, such as vanillyl alcohol oxidase (Eggerichs et al. 2023a, 2023b; Ewing et al. 2018) and related flavoprotein oxidases (Frezzini et al. 2023; Viña-Gonzalez et al. 2016, 2015).

Electrochemical assays: the oxygen electrode

Numerous electrochemical enzyme activity methods have been reported in literature (Eisenthal & Danson 2002). One particular electrochemical assay appears to have been used for decades to monitor flavoprotein oxidase activity. Described in 1956 (Clark Jr, 1956), the Clark electrode significantly advanced the measurement of molecular oxygen in solution by simplifying the process and broadening its accessibility for widespread application. It can follow the oxygen consumption in solution of the flavoprotein oxidase-catalyzed reaction by utilizing a negatively charged platinum cathode and an Ag/AgCl reference electrode, with a thin layer of saturated KCl and a separating Teflon membrane (Charlton et al. 1963). Due to the use of a separating Teflon membrane (5–15 µm) (Friese 1980; Ultman et al. 1981), the reaction solution rarely interferes (Fork 1972) with the measurements and is reliable at different pH values and temperatures reaching 95 °C (Pouvreau et al. 2008). Coupled with the inherent oxygen consumption of all flavoprotein oxidases (Mattevi 2006), this assay stands as a true generalist in comparison to the previously described assays. The method also utilizes a stirring bar, which assures proper mixing and is beneficial for reactions using viscous solutions or reagents. The sensitivity of the electrode is influenced by various factors, such as the diameter of the cathode, the thickness of the Teflon membrane, the precision of the setup, and the age of the cathode (Eisenthal & Danson 2002). An improper setup of the electrode can result in an uneven membrane, which decreases sensitivity and accelerates electrode degradation due to its susceptibility to poisoning (Fork 1972). Clearly, it requires some experience of the user to set up the assay correctly. An improper setup or an ageing electrode can be seen by its relatively high background noise. A normal background noise of the electrode, caused by the slow consumption of oxygen by the electrode, is reported to be around 5–10 nM O_2 (Pouvreau et al. 2008). The acceptable amount of background noise is dependent on the activity of the flavoprotein oxidase, and the method is reported to be unreliable for oxidases with K_M values around or below 100 nM (Lundsgaard et al. 1978; Pouvreau et al. 2008). Its response times varies from commercially available electrodes but is reported to be as low as 1 ms (Hertz & Barenholz 1973), being sufficient for flavoprotein oxidase assaying. The reason

why the assay is being less utilized than spectrophotometric assays can be contributed to several factors. First, the assay requires relatively large amounts of reactants. Nowadays, reaction chambers of commercially available instruments are in the 1-mL range. The assay is not scalable to a microtiter format, rendering the assay unusable for high throughput screening. Also, a dedicated instrument is needed for such measurements. Thus, the assay is less user-friendly when compared with spectrophotometric assays. The method is mainly utilized for determining pH optima and oxygen affinity, as seen in recent work from our research group (Tjallinks et al. 2023a, 2023b). Although various alternative methods for monitoring oxygen consumption in microtiter plates have been reported (John et al. 2003; Ladner et al. 2015; Wesolowski et al. 2008), they are, to our knowledge, not frequently used for measuring flavoprotein oxidase activity.

Concluding remarks

This review aims to provide guidelines in selecting an appropriate activity assay and to motivate the reader to critically asses the continued use of assays based solely on convenience or tradition within laboratories. Due to the versatility of flavoprotein oxidases it is nearly impossible to recommend a specific activity assay, as it needs to be decided case by case. However, a few general remarks can be made. If it is possible to directly follow the reaction, then this is generally preferred over coupled reactions as it avoids many of the artifacts mentioned in this review. It is also recommended to prefer continuous assays over discontinuous assays as it gives more insight in kinetic behavior and avoids possible hysteric effects. The choice between spectrophotometric and electrochemical assays can be made based on viscosity of the mixture, availability of the reagents/instruments and possible interferences of the assay. If reagents are not limiting, conditions require stirring, extreme pH values, high temperatures, or one of the reagents is known to interfere with either HRP or hydrogen peroxide, then the oxygen electrode is a good option. Otherwise, spectrophotometric assays should be generally preferred due to their scalability and easier workflow. When using phenolic compounds, the xylenol orange assay is a more reliable option than HRP-coupled assays since HRP is known to react with a wide range of phenolic compounds. The choice between the chromogen or fluorogen used in HRP-based assays should be decided based on cost, required sensitivity, work safety, and stability of the produced signal. *O*-Dianisidine is the cheapest option, but it is carcinogenic, suffers from many artifacts, and should preferably be used in its discontinuous form. ABTS is a well-defined and user-friendly option but should be used at slightly acidic pH values. AAP/DCHBS is cheap and continuous and produces a stable chromophore but due to its AAP radical intermediate it is known to give rise to false positives and negatives. Amplex Red is the most sensitive chromogen but comes at a high cost. When Amplex Red is preferred but the conditions of the reactions are slightly acidic, then the even more expensive Amplex UltraRed should be considered. For most continuous HRP-based assays, it is important to note that the extinction coefficient or fluorescent yield of the produced chromophore/fluorophore is often dependent on the conditions in which it forms, making it preferable to determine it experimentally for the specific required conditions. Albeit for the more stable chromogens/fluorogens, this is less vital as small deviations in the extinction coefficient often only translate in small deviations in the found k_{obs} values.

With the increasing implementation of high-throughput screening approaches, there is a growing demand for activity assays that can be performed directly in crude cell extracts or with permeabilized cells, thereby omitting enzyme purification steps. Most of the assays described in this review should be compatible with such approaches. However, such extracts often contain native catalases, peroxidases, and other compounds, which may interfere with assay outcomes. The inclusion of specific inhibitors, such as hydroxylamine (Eggerichs et al. 2024), may therefore be necessary to suppress the native consumption of hydrogen peroxide. Also, the high affinity of HRP for hydrogen peroxide may lower the risk of interfering activities. Clearly, appropriate controls are essential when setting up such high-throughput oxidase activity measurements.

With the exception of Amplex UltraRed, the assays reviewed here are relatively dated, and over the years, numerous artifacts and limitations have been identified in these methods. With the identification of novel flavoprotein oxidases, bringing new distinct reaction conditions and reagents, it is likely that additional challenges with these assays will be identified. These considerations require continuous refinement and optimization for the current assays or development of new robust assays. Promising assays may also lay hidden in literature. For example, a novel activity assay with potential for high-throughput scalability involves the use of a redox-sensitive green fluorescent protein tethered to a peroxidase which can be used to probe oxidase activity in whole cells. This methodology has been demonstrated for the identification of pyranose oxidase mutants (Herzog et al. 2020). In another study, it was shown that the H_2O_2-sensitive transcriptional regulator, OxyR, can be used in detecting cells that harbor oxidase activity (Kardashliev et al. 2021). Such cell-based methods allow screening of a large number of cells for oxidase activity but are not yet suitable for detailed analyses of enzyme performance.

Overall, we hope that this review encourages researchers to critically evaluate their choice of activity assays for flavoprotein oxidases or other redox enzymes, to develop new assay methodologies, and to explore underutilized or overlooked assays described in the literature.

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