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High-performance liquid chromatographic investigation of product formation in the horseradish peroxidase-enhanced chemiluminescence of luminol with different enhancers

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method for the investigation of the chemiluminescence-producing oxidation of luminol by the enzyme horseradish peroxidase is presented. Both the kinetics and the mechanism of product formation can be monitored. Special attention is paid to the mechanism of enhancement of the chemiluminescence by phenolic compounds, such as *p*-iodophenol, *p*-hydroxycinnamic acid and 6-hydroxybenzothiazole. The function of the enhancers was elucidated partially by the observation of a higher degradation rate of luminol. In addition, it was concluded that the mechanism of enhancement is probably different for the various enhancers, based on the product formation.

INTRODUCTION

Chemiluminescence immunoassays are very promising as a non-radioactive alternative to radioimmunoassays [1,2]. Recently, a large number of publications have reported various assays with a number of different chemiluminescent labels, such as isoluminol derivatives [3], acridinium esters [4] or peroxidase labels [5]. Chemiluminescence is inherently the most sensitive detection technique presently known. In addition a number of advantages, such as speed of detection, simple detection methods and the stability of reagents, make chemiluminescence very attractive as a detection technique in several biochemical disciplines. Chemiluminescence is based on a chemical oxidation reaction of luminol (LUM) by hydrogen peroxide. The oxidation reaction is catalysed by microperoxidase (a degradation product of cytochrome C), which yields a transient chemiluminescence signal with a fast decay in *ca.* 10 s or by the enzymes horseradish peroxidase [6] and, more recently, xanthin oxidase [7], which produce a long-term steady-state signal for several minutes or hours.

Horseradish peroxidase (HRP) (EC 1.11.1.7) is the most frequently used enzyme label in enzyme immunoassays. The endpoint of detection can be accomplished at neutral pH by colorimetric substrates, such as tetramethylbenzidine [8].

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or by chemiluminescent substrates at a more alkaline pH, such as LUM. In chemiluminescence detection, HRP has a number of advantages, in terms of sensitivity and reproducibility of the chemiluminescence measuring procedure, in comparison with the transient labels with very fast kinetics. In addition, the sensitivity can be increased dramatically by the use of enhancers, phenolic compounds such as 6-hydroxybenzothiazole [9], *p*-hydroxycinnamic acid or *p*-iodophenol (PIP) [10]. PIP in particular is a very effective enhancer (100-fold light increase) of the HRP–LUM–peroxide system. However, this so-called enzyme-enhanced chemiluminescence of HRP in the presence of LUM, hydrogen peroxide and PIP as enhancer is not fully understood [11].

When comparing the sensitivity of HRP labels with different detection methods, it appeared that chemiluminescence detection was no more sensitive than colorimetric detection [12], as might be expected from theoretical considerations. This observation initiated an investigation on the mechanism of enzyme-enhanced chemiluminescence in order to improve the detection limit of HRP labels used in chemiluminescent immunoassays. High-performance liquid chromatography (HPLC) appears to be a good method to investigate the enzymic reaction at the molecular level. This paper describes the first results of the HPLC study of the enzyme-enhanced chemiluminescence. Special attention is paid to the mechanistic role of the phenolic enhancers.

EXPERIMENTAL

Materials

HRP (Type VI), microperoxidase (MP-11) and 6-hydroxybenzothiazole were obtained from Sigma (Brunschwig, Amsterdam, The Netherlands). LUM and PIP were obtained from Aldrich (Brussels, Belgium). LUM was recrystallized from ethanol before use. *p*-Hydroxycinnamic acid and *m*-hydroxycinnamic acid were obtained from Janssen Chimica (Beerse, Belgium). All solvents were of analytical grade (Merck, Darmstadt, Germany).

HPLC equipment

The HPLC equipment consisted of the following components: an automatic injector (Model 231, Gilson, Meyvis, Bergen op Zoom, The Netherlands) with a 250-μl sample loop; two solvent-delivery systems (Model 2150, LKB, Pharmacia, Woerden, The Netherlands) controlled by a gradient controller (Model 2152, LKB); a diode-array detector (Model 1040A, Hewlett-Packard, Amsterdam, The Netherlands); a computing integration system (Model C1-10B, LDC/Milton Roy, Interscience, Breda, The Netherlands); and a personal computer (Model MPF 700, Multitech, Interscience) for data storage with integration software from Wico Electronics (Take Five program, version 3, Utrecht, The Netherlands). The column (150 mm × 4.6 mm I.D.) with 2-μm frits and Valco fittings (Chrompack, Middelburg, The Netherlands) was packed with Hypersil ODS (particle size 5

μm, Shandon, Zeist, The Netherlands) using a column-packing instrument (Shandon) according to the manufacturer's instructions.

HPLC conditions

The HPLC elution conditions used were as follows (where A + B = 100%): from 0 to 0.1 min a linear gradient from 0 to 35% B; from 0.1 to 4 min isocratic elution at 35% B; from 4 to 5 min a linear gradient from 35 to 65% B; from 5 to 10 min isocratic at 65% B; from 10 to 11 min a linear gradient from 65 to 100% B; and from 11 to 13 min isocratic at 100% B at a flow-rate of 1.0 ml/min. Mobile phase A consisted of 0.05 *M* sodium dihydrogen phosphate adjusted to pH 3.0 with phosphoric acid (0.1 *M*). Mobile phase B was methanol.

Enzyme assay

The enzyme reaction mixture (pH 8.5) was prepared as follows. To 4 ml of Amerlite signal reagent (Amersham) were added 2 ml of Amerlite signal reagent buffer (Amersham) containing 100 ng of HRP. The Amerlite signal reagent consisted of Amerlite signal reagent buffer in which Amerlite signal reagent tablets A (LUM and PIP) and B (perborate) were dissolved, according to the manufacturer's instructions (in the case of PIP as enhancer). In the case of other enhancers, such as 6-hydroxybenzothiazole, *p*-hydroxycinnamic acid and *m*-hydroxycinnamic acid, the signal reagent consisted of 16.6 μg of LUM, 5 μl of hydrogen peroxide and 20 μg of enhancer per ml of Tris buffer (0.05 *M*, pH 8.0). In the case of microperoxidase, 0.8 μg was added to the Amersham signal reagent. At $T = 0$, 4 ml of the signal reagent were added to 2 ml of buffer containing 2 μg of HRP. After 0, 15 and 30 min or 24 h, 550 μl were withdrawn from the reaction mixture and adjusted to pH 3 with 50 μl of phosphoric acid (0.1 *M*) to stop the enzymic reaction. From this solution, 200 μl were applied to the HPLC column.

RESULTS AND DISCUSSION

To monitor the enzymic degradation of the substrate LUM and the behaviour of the enhancer PIP, a reversed-phase HPLC gradient system has been developed in which both LUM and PIP can be observed. In order to determine a suitable wavelength for detection and integration, a three-dimensional plot (Fig. 1) was made of the reaction mixture at pH 8.5 before the addition of HRP (A) and after almost complete enzymic degradation (B). From this plot it can be concluded that single-wavelength detection at 230 nm is suitable for monitoring all the components involved and produced in the enzymic reaction.

Fig. 2 shows chromatograms of the reaction mixture containing LUM and PIP, with perborate as oxidant in Amersham buffer. Fig. 2A and B represent the situation before and 30 min after the addition of a relatively small amount of HRP (16 ng), respectively. Fig. 2B represents a typical chromatogram after enzymic degradation. Besides a decrease of the LUM peak to 72% of its original

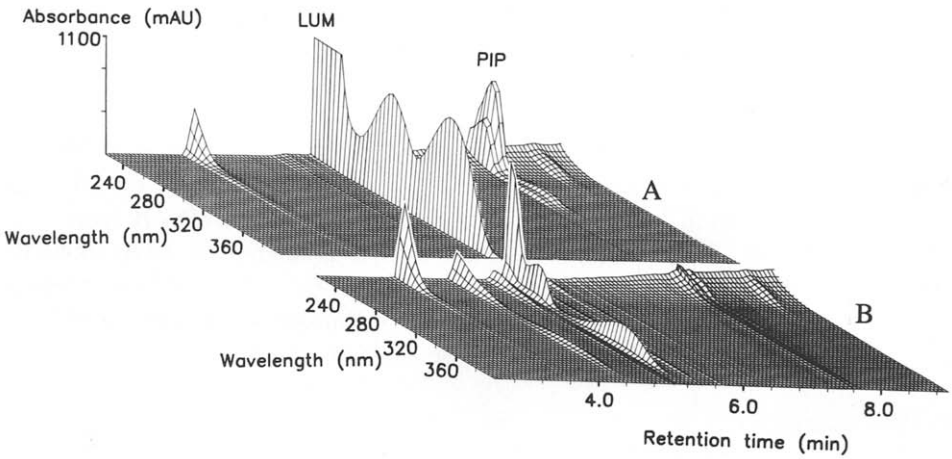

Fig. 1. Three-dimensional HPLC representations (retention time *versus* wavelength *versus* absorbance) of the reaction mixture of luminol (LUM), *p*-iodophenol (PIP) and peroxide before (A) and 30 min after (B) the addition of a relatively large amount (300 ng/ml) of horseradish peroxidase.

value, three newly formed peaks (indicated with A, B and C) are observed (at retention times of 3.85, 4.58 and 4.97 min), which are degradation products of LUM. This was confirmed by doing the same experiment without PIP, in which the same three peaks showed up (Fig. 2D). The PIP peak (at 11.15 min) has decreased to a constant level of *ca.* 70% of its original value, and an additional small peak is observed at a retention time of 10.85 min. When larger amounts of HRP were used in the assay total consumption of LUM was observed, accompanied by total consumption of PIP (Fig. 2C). At low amounts of HRP a constant signal was observed, whereas at a higher concentration the chemiluminescence signal decreased rapidly owing to the consumption of LUM and PIP. The ratio of the three degradation products of LUM appeared to be independent of the concentration of HRP. Also the absence of the enhancer PIP has no influence on the relative peak heights (Fig. 2D). In all cases the ratio is ca. 10:1.9:1.1 (A/B/C). The unexpected observation of three degradation products of LUM instead of only one, the 3-aminophthalate dianion, indicates that, besides the expected oxidation resulting in the production of chemiluminescence, other (dark) reactions may occur resulting in other products [13]. This was confirmed by a pH study, in which the ratio of the three peaks appeared to be strongly dependent on the pH. At pH 6.5, when no luminescence is produced, the ratio of the three peaks was 10:12:2 (data not shown). A similar product formation was observed in the transient chemiluminescence system with LUM, hydrogen peroxide and microperoxidase as catalyst. As shown in Fig. 3, the same ratio (10:2:11) was found again. This observation does not clarify the function of the enhancer PIP. The only conclusion that can be drawn about the enhancer is the increased degradation of LUM if the enhancer is present (*cf.* Fig. 2C and D).

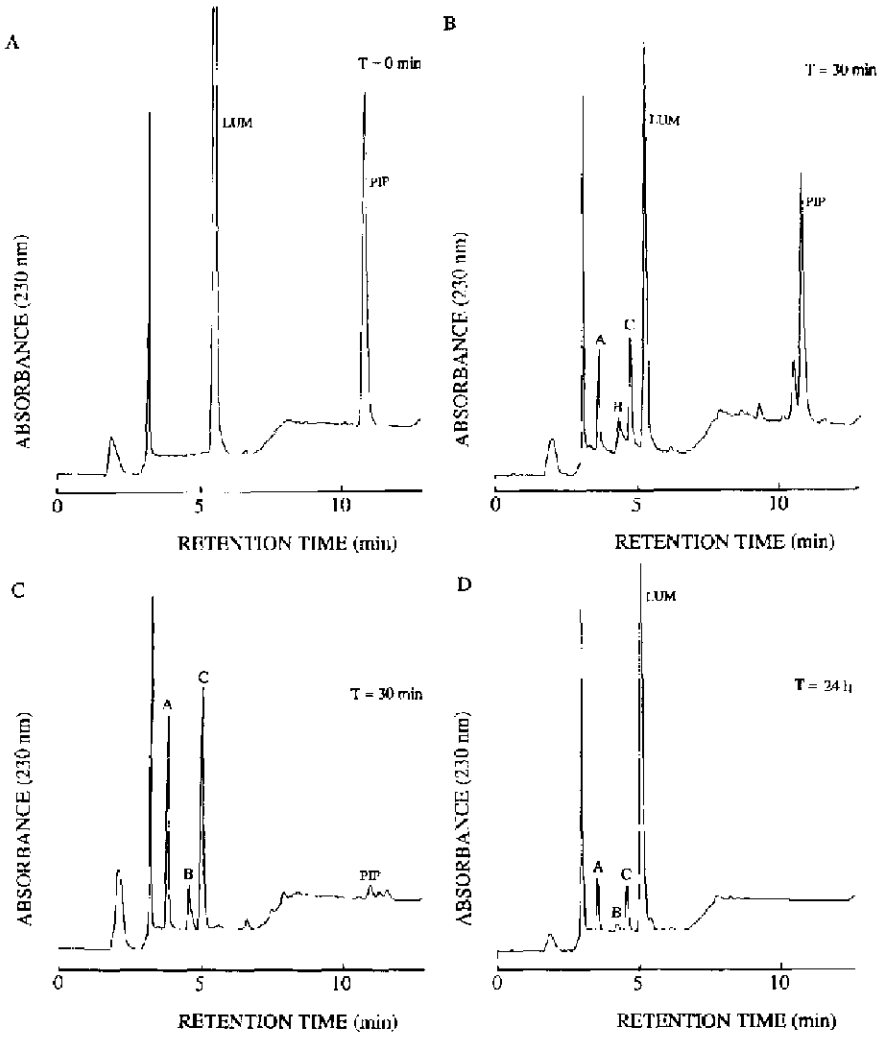

Fig. 2. Chromatograms of reaction mixtures of the enzyme-enhanced chemiluminescence [horseradish peroxidase, luminol (LUM), *p*-iodophenol (PIP) and peroxide]. (A) Before the addition of peroxidase; (B) 30 min after the addition of 16 ng/ml peroxidase; (C) 30 min after the addition of 300 ng/ml peroxidase; (D) 24 h after the addition of 300 ng/ml peroxidase in the absence of *p*-iodophenol.

To investigate further the mechanism of action of the enhancer, other enhancers were tested, including *p*-hydroxycinnamic acid and 6-hydroxybenzothiazole. These reactions were performed at pH 8.0. Fig. 4C and D show chromatograms of the reaction mixture containing the potent enhancer *p*-hydroxycinnamic acid before and after the addition of HRP, respectively. With this enhancer a degradation of LUM of 10% was observed, accompanied with almost total breakdown of the enhancer. Although the product formation was

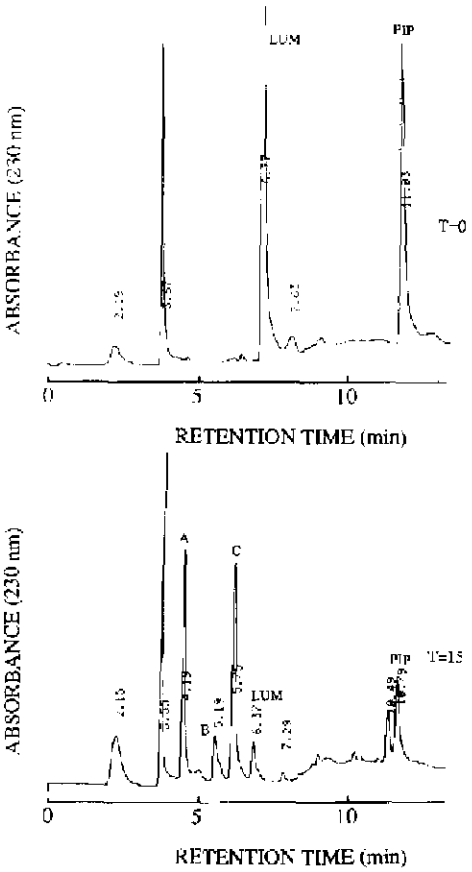

Fig. 3. Chromatograms of reaction mixtures of the transient chemiluminescence system: microperoxidase, lumino! (LUM), *p*-iodophenol (PIP) and peroxide. (Upper) Before the addition of microperoxidase; (lower) 15 min after the addition of 130 ng/ml microperoxidase.

rather low, only one degradation product of LUM was observed at 3.49 min. Based on the retention time this peak should be identical with peak A in Fig. 2. The effect of the inactive stereoisomer *m*-hydroxycinnamic acid is shown in Fig. 4A and B. In this case only minor degradation of LUM was observed, with no breakdown of the enhancer. Qualitatively similar behaviour was observed for the enhancer 6-hydroxybenzothiazole. As shown in Fig. 5, after addition of HRP the LUM peak decreased to 50% of its original value, and the enhancer peak disappeared completeley, which means that 6-hydroxybenzothiazole is probably a better enhancer than *p*-hydroxycinnamic acid. Again only one degradation product of LUM was detected, which is similar to that in Fig. 4 based on the retention time.

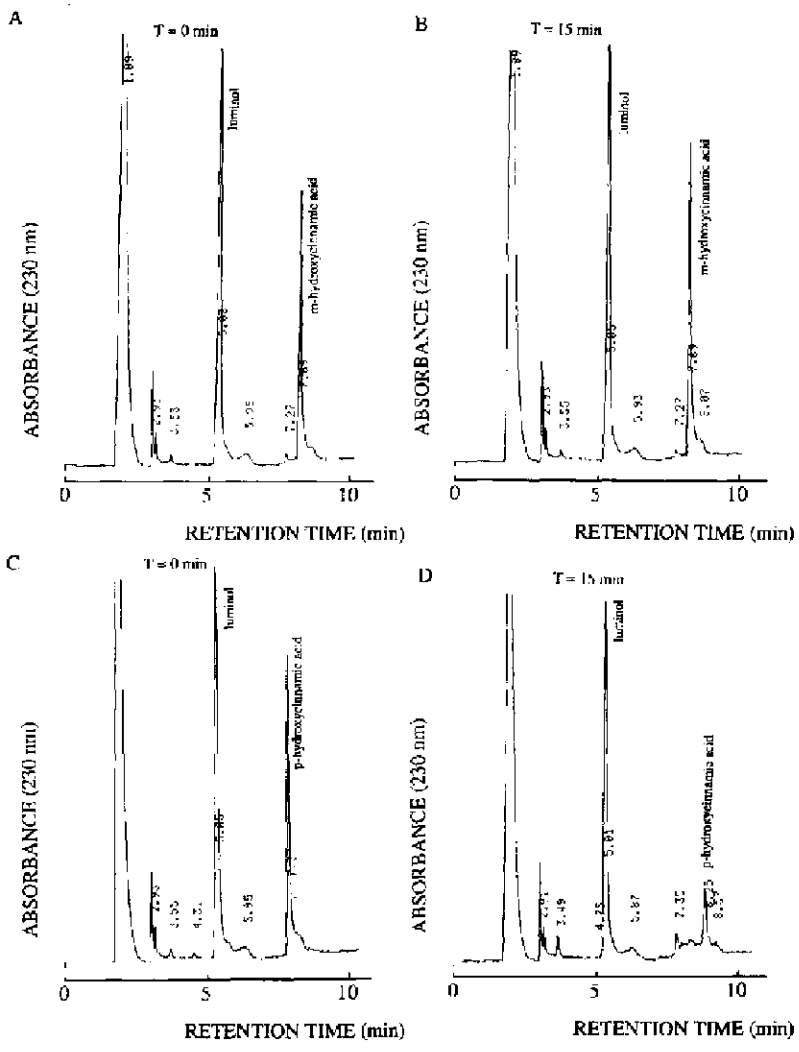

Fig. 4. Chromatograms of reaction mixtures of the enzyme-enhanced chemiluminescence (horseradish peroxidase, luminol, hydroxycinnamic acid and peroxide). (A) Before the addition of peroxidase in the presence of *m*-hydroxycinnamic acid; (B) 15 min after the addition of 300 ng/ml peroxidase in the presence of *m*-hydroxycinnamic acid; (C) before the addition of peroxidase in the presence of *p*-hydroxycinnamic acid; (D) 15 min after the addition of 300 ng/ml peroxidase in the presence of *p*-hydroxycinnamic acid.

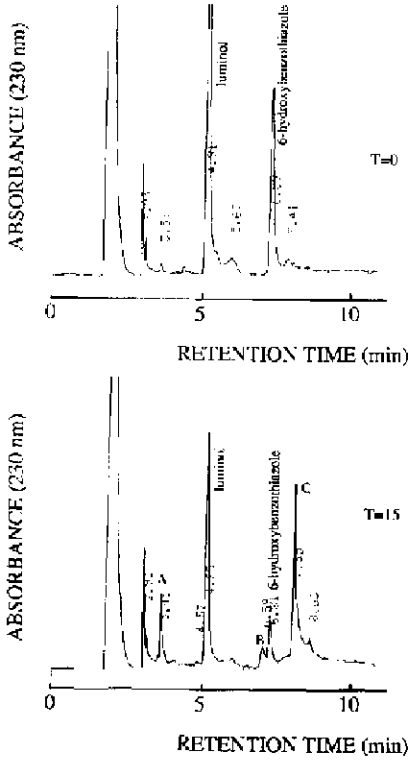

Fig. 5. Chromatogram of reaction mixtures of the enzyme-enhanced chemiluminescence (horseradish peroxidase, luminol, 6-hydroxybenzothiazole and peroxide). (Upper) Before the addition of peroxidase in the presence of 6-hydroxybenzothiazole; (lower) 15 min after the addition of 300 ng/ml peroxidase in the presence of 6-hydroxybenzothiazole.

CONCLUSIONS

From the results of the HPLC analysis of the enzymic reaction of HRP with LUM, PIP and peroxide, it is clear that the method presented here is suitable for investigating both the kinetics and the mechanism of action of enzyme-enhanced chemiluminescence. From the HPLC results it can be concluded that the mechanism of LUM peroxidation is strongly pH-dependent, at least between pH 6.5 and 8.5, which are appropriate pH values for colorimetric and chemiluminescence detection, respectively. In addition, it is observed that a number of oxidation products of LUM are formed. The role of the enhancers PIP, *p*-hydroxycinnamic acid or 6-hydroxybenzothiazole is not yet clear [14]. From the present study it is apparent that these compounds accelerate the turn-over of substrate molecules. Most probably they are not involved in the blocking of other oxidation pathways that may result in dark reactions, because the relative intensities of the products formed are independent of the presence of the enhancer in case of

PIP. The other enhancers, *p*-hydroxycinnamic acid and 6-hydroxybenzothiazole, probably act via another mechanism because only one product is formed.

From a pH study of the enzyme-enhanced chemiluminescence [15] it was concluded that the size of peak A in Fig. 2 is rather pH-independent and that product C shows the pH dependence expected for the LUM oxidation product, the aminophthalate dianion. However, a comparison between the relative retention time of peak A in Fig. 2 and 3B and that (3.5 min) of the LUM degradation product in Fig. 4D, indicates that peak A in Fig. 2 must be assigned to the aminophthalate anion. Several attempts to identify the degradation products of luminol by mass spectrometric techniques have failed.

To elucidate the entire mechanism, additional experiments will be necessary in which the PIP/LUM ratio is varied at different pH values, other enhancers are used, and the products formed are identified. To determine the kinetics more precisely more data points must be obtained in the time. This preliminary report will therefore be followed by more detailed HPLC studies to reveal the mechanism of the enzyme-enhanced chemiluminescence in order to increase the limit of detection of HRP labels in chemiluminescence immunoassays.

NOTE ADDED IN PROOF

Recently, we succeeded in the assignment of peak A to the aminophthalate dianion by mass spectrometry.

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