

Control of the Amount of Free Molecular Iodine in Iodine Germicides

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Abstract

Horseradish peroxidase has been used to generate iodine compositions that comprised principally free molecular iodine.

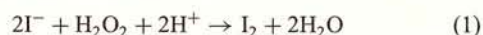
The concentration of free molecular iodine in these enzyme-based compositions ranged from 44 to 63% of the thiosulphate titratable iodine; this is substantially higher than the corresponding value for the povidone-iodine preparation betadine. The biocidal efficacy of these compositions was proportional to the concentration of free molecular iodine. Iodine compositions with relatively low total iodine concentrations but high levels of free molecular iodine (20-175 ppm) killed *Staphylococcus aureus* and spores of *Bacillus subtilis* more rapidly than betadine. The effects of normal saline and these enzyme-based iodine compositions on the rate of epidermal regeneration in superficial swine wounds were comparable.

These results suggest that an effective germicide containing a high level of molecular iodine need not be irritating or toxic.

Iodine's broad spectrum of activity (Hoehn 1976), speed of kill (Gershenfeld 1977) and lack of tendency to induce resistance (Houang et al 1976) has resulted in its acceptance as an active agent in topical disinfectants and hard-surface sanitizers. Commercial iodine compositions are based upon four different approaches to iodine formulation: iodide as a complexing agent (US Pharmacopeia National Formulary 1990); organic complexing agents (Shelanski 1956); solid compositions that release elemental iodine (Marchin & Fina 1989) and oxidation reactions to produce iodine in-situ (Kinman & Layton 1976). Each approach has inherent constraints and potential benefits because of the intrinsic properties of iodine. The overwhelming majority of iodine products sold in commerce is based upon the first two approaches. In health care the two most widely recognized classes of iodine composition are iodophors (Schenck et al 1979) and preparations that contain only elemental iodine and iodide. Povidone-iodine solution (10% polyvinylpyrrolidone iodine) is an example of an iodophor; tincture of iodine or Lugol's solution contain elemental iodine and iodide only.

Although broadly accepted as disinfectants in medicine, iodophoric preparations are not ideal economically or ecologically because they contain excess iodine that does not contribute to biocidal efficacy. For instance, in 10% povidone-iodine approximately 60% of the iodine species exists as triiodide (and iodine) bound to polyvinylpyrrolidone (PVP) and about 40% as iodide; only approximately 0.1% is free molecular iodine, the species found to be responsible for biocidal activity (Gottardi & Koller 1986). It is likely that some of the toxic properties associated with existing iodine compositions result from iodine species that do not contribute to biocidal efficacy.

For the in-situ preparation of iodine-based disinfecting formulations from iodide, the most common oxidants are active chlorine compounds and hydrogen peroxide. However, a catalyst is necessary to increase the rate of iodine formation from hydrogen peroxide; the enzyme peroxidase can be used as biological catalyst for this purpose. Peroxidase was associated with biocidal activity as early as 1924 (Hanssen 1924) and it has subsequently been determined that mammalian cells, e.g. leucocytes, associated with biocidal activity are exceptionally rich in peroxidase (Anger 1941). Several peroxidases including horseradish peroxidase are known to catalyse the oxidation of iodide to iodine (Association of Official Analytical Chemists 1990) and the subsequent iodination of bacteria (Klebanoff 1967). Horseradish peroxidase catalyses this reaction as depicted in equation 1 across a broad pH range and in the presence of diverse additives.



The possibility of using a peroxidase enzyme to generate only enough free molecular iodine to achieve the bactericidal activity desired is intriguing. This study was performed to establish the main aspects of iodine chemistry, bactericidal efficacy and toxicity of enzyme-based iodine germicides using horseradish peroxidase.

Materials and Methods

Materials

Sodium iodide, hydrogen peroxide, sodium thiosulphate, sodium chloride, foetal bovine serum, pig mucin, Lugol's solution and citric acid were purchased from Sigma (St Louis, MO). Horseradish peroxidase was from Seravac USA (San Diego, CA), starch indicator solution from Fisher Scientific (Pittsburgh, PA), manganese chloride from Aldrich (Mil-

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waukee, WI), betadine from Purdue-Fredrick (Norwalk, CT), and Dey-Engley broth, nutrient agar and trypticase soy broth from Difco (Detroit, MI). Membrane filters (0.45 μm) were purchased from Whatman International (Maidstone, UK).

Streptococcus agalactiae, *Staphylococcus aureus* and *Bacillus subtilis* were purchased from the American Type Culture Society (Rockville, MD).

Measurement of iodine species

Iodide levels were measured using an iodide-selective electrode (Corning, NY; Catalogue No. 476127) calibrated against concentration using standards prepared from analytical-grade sodium iodide. Total titratable iodine was determined by titrating 10-mL volumes from a sample with 0.010 N thiosulphate solution in the presence of starch indicator. Free molecular iodine was measured by a potentiometric method (Gottardi 1983) which relies upon measurement of the iodide activity and the redox potential. Iodide activity was measured using an iodide-ion selective electrode calibrated against standards with known activity, and the redox potential was measured with a platinum electrode (Fisher Cat. No. 13-620-115) calibrated with an iodine-iodide redox buffer. A Fisher reference electrode (Cat. No. 13-620-51) and Corning Model 345 pH meter were used for potentiometric measurements. In one experiment the absorbance at 460 nm ($\epsilon I_2 = 742$, $\epsilon I_3 = 1030 \text{ mol}^{-1} \text{ cm}^{-1}$), measured with a Shimadzu UV-1601 spectrometer (Columbia, MD) was used to establish qualitative changes in the concentration of iodine.

Conditions for enzymatic iodine generation

Hydrogen peroxide (30%, 0.91 mL), sodium iodide (0.6 g) and horseradish peroxidase (1 mg) were added to citrate buffer solution (0.10 M, pH 4.5; 1 L) at 25°C. The concentrations of iodide, free molecular iodine and total titratable iodine were measured as a function of time according to the methods described above.

Time course of enzymatic iodine regeneration

Hydrogen peroxide solution (3%, 0.06 mL) was added to citrate buffer solution (0.10 M, pH 4.0; 3.0 mL) containing horseradish peroxidase (0.01 mg mL⁻¹) and sodium iodide (1 mg mL⁻¹). The reaction was monitored photometrically at 460 nm and reached a visible plateau in approximately 20 min. At the reaction plateau several 0.01-mL volumes of 0.10 N sodium thiosulphate solution were added and the absorbance was observed continuously.

Bactericidal activity of enzyme-generated iodine and betadine in the presence of a bioburden

A series of five enzyme-generated iodine solutions was prepared in phosphate buffer (0.10 M, pH 6.0) containing between 45 and 280 ppm thiosulphate-titratable iodine. Horseradish peroxidase (5 mg), hydrogen peroxide solution (3%, 5 mL) and sodium chloride (7.9 g) were added to citrate buffer solution (0.10 M, pH 6.0; 1.0 L) and sodium iodide (0.5, 0.4, 0.3, 0.2 or 0.10 g) was then also added. The reaction mixtures were stirred and analysed for titratable and free molecular iodine immediately before efficacy testing. Betadine was used for comparison; this proved to contain 10 000 ppm titratable iodine and 5.6 ppm free molecular iodine.

The biocidal activity of these solutions was tested at 20°C

against *S. aureus* in the presence of 10% porcine serum. The method of the Association of Official Analytical Chemists (1990) for germicidal and detergent sanitizing action was used, with modification. A sample (9.9 mL) was removed from the 99-mL test solution and replaced with porcine serum (9.9 mL). This was left to incubate at 20°C for 30 s before addition of culture suspension (1.0 mL). Samples were removed and iodine was neutralized with 0.1 N sodium thiosulphate at times ranging from 10–300 s. The initial concentration of *S. aureus* in all assays was determined to be at least 10⁷ colony-forming units mL⁻¹.

Inactivation of *Bacillus subtilis* spores

B. subtilis was grown in trypticase soy broth containing 0.08% MnCl₂. The growth suspension (1.0 mL) was placed on trypticase soy agar plates and the plates were tilted to spread the broth. After incubation for 70 h at 37°C, the plates were scraped and the cells suspended in sterile deionized water, washed three times in deionized water and concentrated to approximately 108 colony-forming units mL⁻¹. *B. subtilis* suspension (1 mL) was added to the germicide or to sterile water (49 mL) at 20°C. At various time intervals, 1.0 mL was withdrawn and added to sodium thiosulphate solution (0.50 N, 9.0 mL). Ten-fold serial dilutions (1.0 + 9.0 mL) in Dey-Engley medium were filtered through a 0.45- μm membrane filter, rinsed with sterile water (approximately 50 mL), placed on nutrient agar in a Petri plate, and incubated for 48 h at 37°C to enumerate surviving colony-forming units. An enzyme formulation containing 175 ppm free molecular iodine and betadine were used as germicides.

Swine epidermal skin regeneration

The rate of wound healing was measured in an animal model (Mertz et al 1986) using 30 Yorkshire pigs. Ten each were designated for two different concentrations of free molecular iodine and one for saline. Two incisions were made in the animals of the iodine groups and two or three in the control group. The shallow wounds (7 mm wide, 10 mm long and 0.3 mm deep) were made in the paravertebral and thoracic areas with an electric dermatome. Wounds were treated daily for the duration of the study with normal saline or an iodine formulation prepared from citrate buffer solution (0.10 M, pH 6.0; 1.0 L), horseradish peroxidase (5 mg), hydrogen peroxide solution (3%, 5 mL), sodium chloride (7.9 g) and sodium iodide (either 0.2 or 0.5 g). Three times daily 1.0 mL was applied to wound sites and held there by means of an occlusive bandage. Beginning on day 4 after wounding and each day thereafter, 5 to 6 wounds and the surrounding normal skin were excised. Harvested specimens were incubated with 0.5 M sodium bromide at 37°C for 24 h and the epidermis was then separated from the dermis. The epidermal sheet was examined by an evaluator (working blind) for defects. Wounds were considered healed if there were no defects in the epidermis and not healed if there were one or more defects.

Results

Development of iodine by enzymatic generation

The change in the concentrations of iodide, free molecular iodine and titratable iodine under the reaction conditions established is shown in Fig. 1. The concentration both of

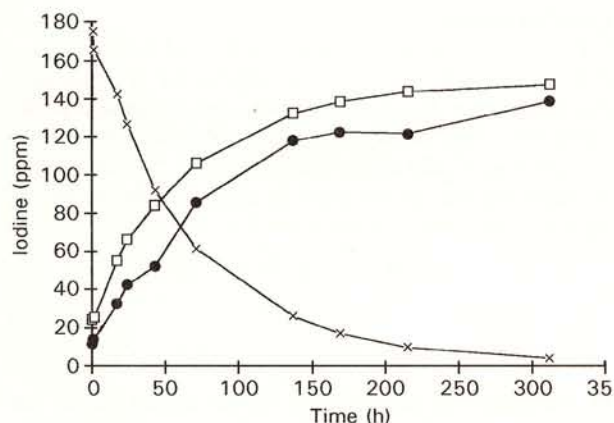


FIG. 1. Time-course of the horseradish peroxidase-catalysed formation of iodine. The reaction was run in citrate buffer solution (0.10 M, pH 4.5) at 25°C. The concentration of hydrogen peroxide was 8.03×10^{-3} M, the initial concentration of sodium iodide was 3.77×10^{-3} M and the concentration of horseradish peroxidase was $1 \mu\text{g mL}^{-1}$. □ Titratable iodine, ● free molecular iodine, × sodium iodide.

titratable iodine and of free molecular iodine increased, with a corresponding decrease in iodide concentration, as the reaction progressed. At 50 min approximately 41% of the total iodine comprised free molecular iodine; this value increased to 75% at 300 min.

Time-course for enzymatic iodine regeneration

The peroxidase-catalysed reaction between hydrogen peroxide and iodide regenerated titratable iodine that was chemically reduced to iodide. Fig. 2 illustrates the regeneration of iodine after repeated reduction by reaction with thiosulphate. Under the initial reaction conditions the absorbance (which gives an estimate of the developed iodine) reaches a level plateau at about 20 min. The addition of sodium thiosulphate reduces both free molecular iodine and triiodide to iodide and results in an immediate decrease in the absorbance. When the equilibrium concentration of titratable iodine was displaced, the enzyme reaction regenerated iodine as shown by the increase in absorbance at 460 nm. The loss of absorbance owing to thiosulphate reduction of iodine and subsequent formation of colour owing to enzyme-generated iodine results in the saw-tooth pattern observed in Fig. 2.

Bactericidal activity of enzyme-generated iodine and betadine in the presence of a bioburden

The effect of a varying concentration of sodium iodide (6.29×10^{-4} to 3.14×10^{-3} M) on a fixed concentration of horseradish peroxidase and hydrogen peroxide is shown in Table 1. Titratable iodine increased from 45 to 280 ppm and free molecular iodine increased from 20 to 175 ppm as the concentration of sodium iodide was increased. As expected, the time required for inactivation of *S. aureus* is remarkably reduced by increasing the concentration of free molecular iodine. The time required for the enzymatic formulations to achieve a six-log reduction of *S. aureus* in 10% porcine serum ranged from 15 to 245 s; with betadine a contact time of 240 s was necessary to achieve a six-log reduction.

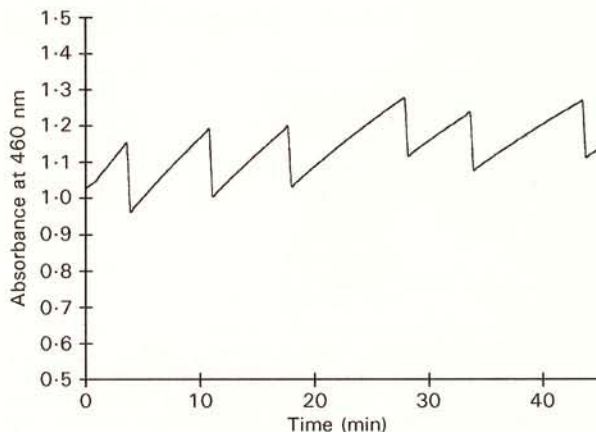


FIG. 2. Regeneration of titratable iodine by horseradish peroxidase-catalysed oxidation of iodide. The reaction was run in citrate buffer (0.10 M, pH 5.5) at 25°C. The concentration of hydrogen peroxide was 8.03×10^{-2} M, the initial concentration of sodium iodide was 5.75×10^{-3} M and the concentration of horseradish peroxidase was $1 \mu\text{g mL}^{-1}$.

Table 1. Inactivation of *S. aureus* suspended in 10% porcine serum at 20°C*

Enzyme-iodine (molarity NaI)†	Titratable iodine (ppm)‡	Free molecular iodine (ppm)‡	Time to achieve 6-log reduction of <i>S. aureus</i> (s)§
3.14×10^{-3}	280 ± 6.5	175 ± 3.9	15
2.51×10^{-3}	216 ± 4.3	128 ± 3.8	60
1.88×10^{-3}	152 ± 3.4	85 ± 2.7	60
1.26×10^{-3}	102 ± 2.6	47 ± 1.9	90
6.29×10^{-4}	45 ± 2.0	20 ± 1.0	240
Betadine	$10\ 000 \pm 7.3$	5.6**	240

*The assay used was the Association of Official Analytical Chemists (1990) Germicidal and Detergent Sanitizing Action of Disinfectants, Method 960.09. †Enzyme reactions were conducted in 1.8×10^{-3} M citrate buffer at pH 5.0. The concentration of horseradish peroxidase was $5 \mu\text{g mL}^{-1}$, the concentration of sodium chloride was 0.146 M and the concentration of hydrogen peroxide was 4.41×10^{-3} M. ‡Five replicates \pm s.d. §The experiment was performed in duplicate. **One measurement.

Inactivation of *Bacillus subtilis*

As shown in Table 2, betadine had little or no effect over a 24-h period whereas the enzyme formulation inactivated more than $1 \log \text{h}^{-1}$ of the *B. subtilis* spores and complete kill was achieved after 4 h.

Swine epidermal skin regeneration

Table 3 shows that the rate of epidermal regeneration in superficial wounds after treatment with enzyme-generated iodine compositions containing either 47 or 175 ppm of free molecular iodine, with a corresponding concentration of titratable iodine of 102 and 280 ppm, was equivalent to that after treatment with normal saline. By day 8 post-wounding, 100% of the wounds were healed in all three treatment groups. There were no significant differences among the rates of wound healing for the three treatment groups at day 7.

Table 2. Inactivation of *Bacillus subtilis* spores by enzyme-based iodine and betadine.

Time (h)	Enzyme formulation* (colony-forming units mL ⁻¹)	Betadine (colony-forming units mL ⁻¹)
0	2.1 × 10 ⁵ †	2.1 × 10 ⁵ †
1	5.9 × 10 ³ †	>10 ⁵
2	1.4 × 10 ² †	>10 ⁵
4	Not detected	>10 ⁵
6	Not detected	>10 ⁵
24	Not detected	>10 ⁵

*The enzyme formulation contained 280 ppm titratable iodine and 175 ppm free molecular iodine. The concentration of horseradish peroxidase was 5 µg mL⁻¹, the concentration of sodium chloride was 0.146 M and the concentration of hydrogen peroxide was 4.41 × 10⁻³ M. †Two replicates, counts were within 20% of each other.

Table 3. Swine epidermal skin regeneration: percentage of wounds healed after different times.

Enzyme-iodine (molarity NaI)*	Titratable iodine (ppm)	Free molecular iodine (ppm)	Number of wounds healed day after wounding)†			
			5	6	7	8
3.14 × 10 ⁻³	280 ± 6.5	175 ± 3.9	0	0	50	100
1.26 × 10 ⁻³	102 ± 2.6	47 ± 1.9	0	0	50	100
Saline	0	0	0	0	71	100

*Wounds were treated with the test mixture (1.0 mL) three times daily during the course of this experiment. Each treatment group comprised ten animals; two incisions were made in each animal. †Enzyme reactions were conducted in 1.8 × 10⁻³ M citrate buffer at pH 5.0. The concentration of horseradish peroxidase was 5 µg mL⁻¹, the concentration of sodium chloride was 0.146 M and the concentration of hydrogen peroxide was 4.41 × 10⁻³ M.

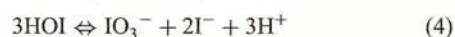
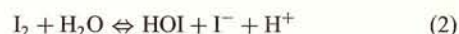
Discussion

Enzymatic generation of iodine

Horseradish peroxidase proved to be a very effective catalyst for the in-situ preparation of iodine-based formulations from hydrogen peroxide and iodide. At constant hydrogen peroxide concentration (c(H₂O₂)) and pH the titratable iodine and the relative distribution of the iodine species iodide, triiodide and free molecular iodine were a function of the initial concentration of iodide. It is possible to formulate enzyme-driven iodine systems which generate any required concentration of free molecular iodine up to its solubility limits of approximately 330 ppm at room temperature. Under the reaction conditions examined, the ratio of free molecular iodine to titratable iodine ranged from 0.44 to 0.63. These ratios are substantially higher than the corresponding values for commonly used iodophors. For instance, the corresponding ratio for betadine is ≈ 0.001. Because iodine is generated in-situ in this system, only a fraction of the iodide typically found in iodophor preparations is required to provide an equivalent concentration of free molecular iodine. Because the pH range suitable for the generation of iodine by peroxidase extends from acidic pH values as low as 2.5 to pH values as high as 7.5, the whole pH range generally used for disinfection is encompassed.

Chemical aspects

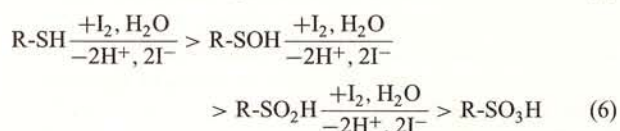
It has been shown that under the usual conditions of iodine-based disinfection only five iodine species are of importance: molecular iodine (I₂), triiodide (I₃⁻), hypoiodous acid (HOI), iodate (IO₃⁻) and iodide anion (I⁻), with I₂ and HOI being the effective bactericidal agents (Gottardi 1991). The interactions of these species are governed in aqueous solution by the equilibria represented by equations 2-4.



In evaluating the properties of enzyme-driven iodine formulations the question arises of the relevance of HOI because of its tendency to disproportionate into non-bactericidal iodate (equation 4). Using the values shown in Table 1, an estimate from calculated equilibrium concentrations (Gottardi 1978) shows that the proportion of HOI ranges from 0.01 to 0.04% of the measured molecular iodine. Because of this very low concentration of HOI, equations 2 and 4 can be ignored with the consequence that the iodine chemistry in such a system is essentially controlled only by the triiodide equilibrium (equation 3) and that at pH < 6 enzyme-driven formulations can be considered to be very stable (no iodate formation).

Regeneration of consumed iodine

The enzyme's ability to catalyse the regeneration of iodine is a very important feature because it ensures the persistent activity of the formulation. One drawback of halogen-based disinfectants is consumption of oxidative capacity by oxidizable components in the material to be disinfected (bacteria, tissue surfaces, body fluids). For iodine the main reducing agents are thiol groups, which are immediately oxidized either to disulphides or to sulphur-oxygen acids (Gottardi & Koller 1986):



It is apparent from Fig. 2 that an enzyme-driven iodine system can compensate very quickly for such unwanted side-reactions. The reactions depicted in equations 5 and 6 not only consume titratable iodine but also, by releasing an appreciable amount of iodide, reduce the concentration of free molecular iodine in the partly reduced germicide in favour of triiodide (Eqn 3). This results in an above-average reduction of bactericidal efficacy by the quoted consumption effects. Because the regeneration of iodine proceeds via re-oxidation of the iodide formed, reduction of titratable iodine and shift of the triiodide equilibrium (Eqn 3) towards I₃⁻ are both counteracted.

Biocidal activity and toxicity

Biocidal tests with enzyme-generated iodine solutions (45-280 ppm total iodine) and betadine against *Staphylococcus aureus* and against *Bacillus subtilis* spores confirm that the concentration of free molecular iodine is responsible for microbiocidal efficacy and not total (i.e. titratable) iodine.

Another important observation is that inactivation of *B. subtilis* is possible only with high doses of iodine. In this regard the enzyme-based iodine formulation has favourable properties compared with povidone-iodine preparations.

The biocidal efficacy of the enzyme-based iodine composition is not surprising because of the high concentration of free molecular iodine. However, they invite the question of the toxicity of such formulations. The instinctive reaction of most scientists is to assume that the toxicity of a composition is proportional to the concentration of free molecular iodine.

The experiments with the swine epidermal skin regeneration model, however, clearly indicated that high levels of free molecular iodine (e.g. 175 ppm) are not necessarily toxic because, compared with saline, they do not inhibit wound healing. It can reasonably be concluded from these results that free molecular iodine might be substantially less irritating and toxic than other forms of iodine, depending upon the specifics of administration. Clues to the correctness of this assertion are provided by Thrall (1990) and Thrall & Bull (1990), who demonstrated that the pharmacokinetic behaviour of iodine is different from that of iodide, and by Shelanski (1956), who showed that iodine is substantially less toxic orally than is iodide. The different behaviour of I_2 and I_3^- can also be inferred from the skin-staining properties of Lugol's solution and of saturated aqueous iodine solution after the same contact time (20 s). Lugol's solution ($[I_2] = 170$ ppm, $[IO_3^-] = 0.196$ M) results in deep brown coloration of the skin which lasts several hours whereas with saturated iodine solution ($[I_2] = 334$ ppm, $[IO_3^-] \approx 6 \times 10^{-6}$ M), which contains much more molecular iodine, the colour formed is only very slight and soon vanishes. This result clearly indicates that triiodide and not molecular iodine is primarily responsible for the staining of the skin.

These observations doubtless suggest re-assessment of the use and toxicity of topical iodine and also suggest the need for exact clarification of the individual contributions of the iodine species relevant to disinfection (i.e. I_2 , IO_3^- and I^-) to toxic side-effects in topical applications. According to current knowledge all three species contribute to toxicity but only molecular iodine is responsible for the microbiocidal action required. Therefore, a preparation such as the enzyme-based formulation presented in this paper, which contains less iodide and triiodide, might be favoured because it results in a higher concentration of free molecular iodine with the same or even less toxicity than that of a traditional preparation.

The new approach introduced for the efficient generation of free molecular iodine from hydrogen peroxide and iodide using peroxidase as a biological catalyst seems to be an attractive

approach to the intrinsic goal of any disinfection, which is to achieve an optimum compromise between maximizing efficacy and minimizing toxicity.

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