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Biocatalytic peroxy acid formation for disinfection

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Abstract

Novel peroxy acids were prepared by two methods using Novozyme® 435 as a catalyst; either free carboxylic acids were converted with hydrogen peroxide or carboxylic acid esters were perhydrolyzed by hydrogen peroxide. Afterwards, these peroxy acids (as solutions in their respective ethyl or methyl esters) were tested for their sporicidal activity towards spores of *Bacillus subtilis*. The sporicidal activity of peroxy acetic acid made by biocatalytic perhydrolysis was found to be the same as one of commercial peroxy acetic acid. Peroxy methoxy acetic acid, peroxy propionic acid, peroxy phenyl acetic acid, and peroxy citric acid showed comparable or slightly lower activities. Sporicidal activities do not directly relate to oxidative power. Under the conditions applied, long-chain peroxy acids like peroxy dodecanoic acid (perlauric acid) and peroxy octadecanoic acid (perstearic acid) were unsuitable as disinfectants due to their very limited solubility at pH 7.

Keywords: Lipase; Peroxy acids; Disinfectants; Spores; *Bacillus subtilis*

1. Introduction

Peroxy carboxylic acids are important oxidants for chemical processing and syntheses, bleaching and disinfection. The market volume can be estimated to be some 10,000 tonnes worldwide [1]. Industrially, peroxy carboxylic acids are produced by the conversion of carboxylic acids with hydrogen peroxide catalyzed by a strong acid (Scheme 1).

Reaction parameters as temperature and acid catalyst concentration must adapted to more drastic conditions as the size of R increases. Strong carboxylic acids like trifluoro acetic acid or formic acid do auto-catalyze the reaction, but the resulting peroxy acids are unstable and must be used in situ. Due to these limitations, only a few peroxy carboxylic acids are commercially available. Amongst them peroxy acetic acid is the most commonly used one. It is widely applied as a microbicidal agent in agriculture, food industry and hospitals [2]. Some detergent formulations contain precursors of peroxy acetic acid, e.g. TEAD®; their antimicrobial activity was studied recently [3].

The treatment of microorganisms with peroxy acetic acid causes an unspecific oxidation of proteins and other cell components which finally results in cell death. The application of peroxy acetic acid allows a fast inactivation of germs, which can be carried out at low temperature and is effective against a broad range of microorganisms, including bacterial spores. Furthermore, the unspecific nature of the antimicrobial activity reduces the possible selection of resistant microorganisms after prolonged use [4]. Due to these properties, peroxy acetic acid, which decomposes to non-toxic acetic acid, is an attractive reagent to be included in disinfectant formulations. On the other hand, peroxy acetic acid is extremely corrosive and

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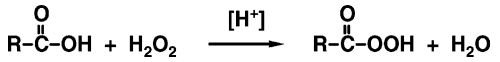
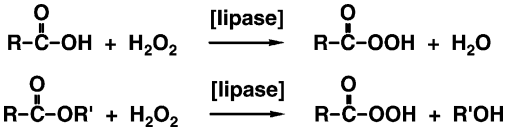


Scheme 1.

can be stored for a limited time only in the presence of stabilizing agents at reduced temperatures. Handling of peroxy acetic acid is hampered by its strong potential to irritate skin, eyes and mucosal tissues [5]. In addition, peroxy acid is suspected to cause cancer in bioassays [6].

Since the early 1990s, an alternative enzyme-catalyzed synthesis of peroxy carboxylic acids has been developed. Some lipases, in particular Novozyme® 435, an immobilized lipase from *Candida antarctica*, are able to catalyze the generation of peroxy acids from either carboxylic acids [7–9] or carboxylic acid esters [10] and hydrogen peroxide (Scheme 2).



Scheme 2.

Whereas the Novozyme® 435-catalyzed reaction of hydrogen peroxide with carboxylic acids is restricted to straight fatty acids, the analogous reaction of carboxylic acid esters (perhydrolysis) has a broader substrate range. It includes the generation of peroxy acetic acid from, e.g. ethyl acetate [11] and the generation of peroxy carbonic acid derivatives from, e.g. diethyl carbonate [12,13]. Thus, the biocatalytic process provides the advantage that a variety of peroxy carboxylic acids can be made, which are not accessible by the conventional method. Furthermore, the products do not contain any mineral acid, which may lower the selectivity of consecutive reactions and may cause corrosion. The mechanism of the lipase-catalyzed perhydrolysis is considered to be very similar to that of hydrolysis—involving the usual triade ser-105/asp-187/his-224—and has been described in detail earlier [14]. Various applications of biocatalytic peroxy acid generation in fine chemical synthesis have been described and reviewed [14,15].

In the present report, the lipase catalyzed synthesis of new peroxy carboxylic acids and their assessment as antimicrobial reactants towards spores of *Bacillus subtilis* are described.

2. Experimental

2.1. Materials

Peroxy acetic acid, phenyl acetic acid ethylester, citric acid triethylester, methoxy acetic acid ethylester, lactic acid ethylester, dodecanoic acid and octadecanoic acid were purchased from Fluka, Seelze, Germany. Propionic acid ethylester was supplied by Sigma–Aldrich, Munich, Germany. Agar-agar, tryptic soy agar and tryptic soy broth were purchased from Sifin, Berlin, Germany. Spores of *B. subtilis* subsp. *spizizenii* (ATCC 6633) were supplied by Difco Laboratories, Augsburg, Germany. Novozyme® 435 was supplied by Novo Nordisk Biotechnology, Mainz, Germany. Hydrogen peroxide 60% (w/v) was supplied by Cognis, Düsseldorf, Germany.

2.2. Preparation of peroxy acids

2.2.1. Peroxy acids from carboxylic acids

Long-chain peroxy carboxylic acids (peroxy dodecanoic acid and peroxy octadecanoic acid) were prepared in the following way: 3 mmol carboxylic acid was dissolved in 30 ml pentane, and 300 mg Novozyme® 435 was added to it. The suspension was stirred and after 15 min 30 μl of hydrogen peroxide (60% w/v in water) was added by a modified Metrohm–Dosimat 775. The addition of H_2O_2 was repeated 24 times after 15 min each, so that after 6 h a total of 15 mmol H_2O_2 had been added. After a further stirring of 24 h, water was removed by the addition of dry sodium sulfate. Inorganic salts and the lipase were removed by filtration and the filtrate was put under the hood in a shallow dish at room temperature for 24 h for the evaporation of the solvent.

Purity of the peroxy acids were determined by a combination of cerimetric and iodometric titration [16]. The resulting crystalline peroxy acids were found to be 67% (peroxy dodecanoic acid) or 83% (peroxy octadecanoic) pure. Yields (Table 1) were calculated from these purity and the weight yields of the products related to the substrate carboxylic acid.

2.2.2. Peroxy acids from carboxylic acid esters

Novozyme® 435 (100 mg) was suspended in 10 ml of carboxylic acid ester. The suspension was stirred, and after 15 min, 10 μl of hydrogen peroxide (60% w/v

Table 1
Lipase-catalyzed preparation of peroxy carboxylic acids

No.	Substrate	Yield (%)	Product	Decomposition rate mmol% per day
1/1	Dodecanoic acid	67	Peroxy dodecanoic acid[a]	None
1/2	Octadecanoic acid	83	Peroxy octadecanoic acid[a]	None
1/3	Acetic acid ethylester	100	Peroxy acetic acid[b]	0.087
1/4	Propionic acid ethylester	87	Peroxy propionic acid[b]	0.038
1/5	Phenyl acetic acid ethylester	50	Peroxy phenyl acetic acid[b]	0.127
1/6	Methoxy acetic acid ethylester	100	Peroxy methoxy acetic acid[b]	0.282
1/7	Citric acid triethylester	34	Peroxy citric acid[b,c]	0.312
1/8	Lactic acid ethyl ester	–		–

For experimental conditions see Section 2.2.
[a] Isolated as crystal solid, weight yield 100%, purity 67% resp. 83%; yield related to carboxylic acid.
[b] Solution in the corresponding ethyl ester; yield related to hydrogen peroxide.
[c] Monoperoxy citric acid diethylester; no effort was made to distinguish between the isomers.

in water) were added by a modified Metrohm Dosimat 775. The addition of H_2O_2 was repeated 24 times after 15 min each, so that after 6 h, a total of 5 mmol H_2O_2 had been added. The lipase was removed by filtration and the resulting solutions were used as such (after dilution and pH-adjustment) for the determination of the sporicidal activity.

Peroxy acid and hydrogen peroxide contents were determined by a combination of cerimetric and iodometric titration [16]. Yields (Table 1) were calculated from these peroxy acid contents and the weight of the resulting solution related to the substrate hydrogen peroxide.

It should be noted, that for both methods (a) and (b) of peroxy acid preparation, an excess of one educt is necessary to drive the equilibrium to the product side. Either a carboxylic acid reacts with a five-fold excess of hydrogen peroxide (a) or an excess of an ester reacts with hydrogen peroxide (b).

The enzyme is not deactivated by hydrogen peroxide but by peroxy carboxylic acids. Detailed studies have shown the feasibility of a continuous fixed-bed reactor. The catalyst worked over 4 months and a turnover of 800,000 was obtained [19].

2.3. Treatment of bacterial spores with peroxy carboxylic acids

If not stated otherwise all solutions and media were sterilized for 15 min at 121 °C. Spores from *B. subtilis* were treated with peroxy carboxylic acids according to Sagripanti and Bonifacino [17] with minor modifications. A spore suspension (ampoule, ca. 1 ml, as supplied by the manufacturer Difco) was centrifuged at 16,000 × *g* for 10 min and re-suspended in 1 ml 50 mM KH_2PO_4, pH 7.0. The final spore concentrations were 5×10^7 to 5×10^8 cfu/ml. For each assay 50 μl of the suspension were added to the bottom of a 1.5-ml plastic conical reaction vial. A volume of 50 μl of peroxy carboxylic acid solution, dissolved in 50 mM KH_2PO_4, pH 7.0 at twice the final concentration of the assay, were added and thoroughly mixed. The following incubation (at 20 °C for 30 min) was terminated by adding 32 μl sodium thiosulfate solution (1 M) and 1 ml cold (4 °C) tryptic soy broth. After centrifugation at 16,000 × *g* the precipitate was re-suspended in 1 ml tryptic soy broth and the assay was heated for 10 min at 80 °C. Ten-fold serial dilutions with 0.85% NaCl were prepared from each assay. A volume of 0.1 ml of the dilution was set on 100-mm tryptic soy agar plates enriched with 0.3% agar-agar to suppress swarming of *B. subtilis* colonies. Colonies were counted after incubation for 3 days at 30 °C. For blanks, spores were incubated in phosphate buffer only.

3. Results and discussion

3.1. Preparation of percarboxylic acids

Table 1 shows the results of the Novozyme® 435-catalyzed preparations of peroxy acids. Since standard procedures were used and no optimization of reaction conditions for each substrate was carried out,

the yields vary between 34% (citric acid) and 100% (peroxy acetic acid; peroxy methoxy acetic acid).

We utterly failed to prepare peroxy lactic acid by perhydrolyis of lactic acid ethyl ester. An increase in viscosity was noticed during the experiments; blind experiments with water instead of hydrogen peroxide resulted in oligomerization.

However, concentrations of all other resulting peroxy acid solutions were more than sufficient to carry out tests of their sporicidal activities. It has been shown earlier that peroxy acids made by biocatalytic perhydrolysis can be isolated by chromatography [15]; since disinfectants are applied as dilute solutions, anyway, such techniques are considered superfluous for the purpose described here.

We also examined the deterioration of the peroxy acids over a period of 1–3 months at −18 °C, because the stability of the oxidants is important for all potential applications. Without the addition of any stabilizers, all peroxy acids were reasonably stable with decomposition rates ranging from 0.038 mmol% (peroxy propionic acid) to 0.312 mmol% (peroxy citric acid).

Peroxy dodecanoic acid and peroxy octadecanoic acid were found to be insufficiently soluble in all solvents appropriate for disinfectant formulations at pH 7. Further work with these peroxy acids at higher pH is in progress.

3.2. Sporicidal activity of peroxy acetic acid

So far, peroxy carboxylic acids, generated by lipase-catalyzed methods, were not tested for their antimicrobial activity. To evaluate this property, the inactivation of spores from *B. subtilis* by a commercially available peroxy acetic acid (Fluka) was considered a reference test. These bacteriospores were chosen because they represent the most resistant microbial organisms to disinfectants [18]. In the first experiment, the sporicidal activity of peroxy acetic acid, made by perhydrolysis of acetic acid ethylester, was analyzed. As shown in Fig. 1, the inactivation of *B. subtilis* spores were similar for both peroxy acetic acids over a concentration range from 1.3 to 26 mM.

The biocatalytic generation of peroxy carboxylic acids is highly selective; therefore, the resulting solutions of peroxy acids in carboxylic acid esters contain no by-products and further purification is not necessary. Typical impurities in commercial peroxy acetic acid (e.g. sulfuric acid), apparently do not contribute significantly to the sporicidal activity.

Each data point in Fig. 1 (as well as in Fig. 2) represents a mean from at least five independent experiments. Numerical data for mean and SD are summarized in Table 2.

From the experiments shown in Fig. 1, it can be concluded, that peroxy acetic acid, made by perhydro-

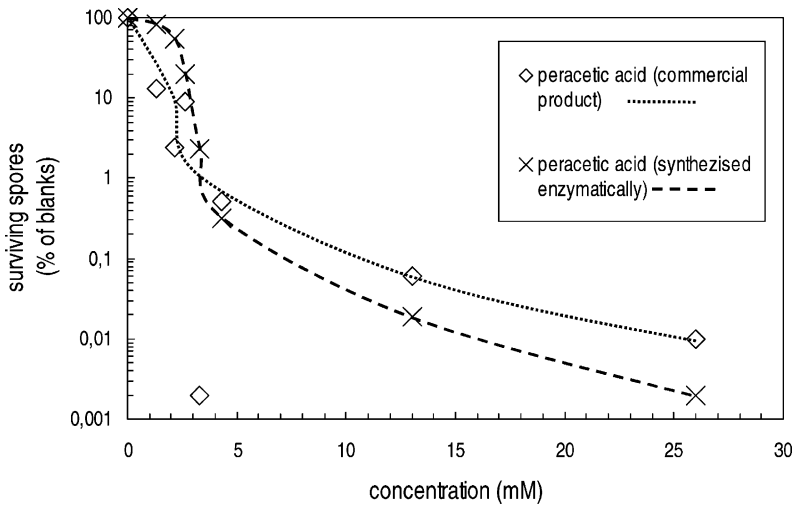


Fig. 1. Inactivation of *B. subtilis* spores with peroxy acetic acid. Each data point represents a mean of five experiments. Blanks (100%) were between 2.8×10^6 and 1×10^7 cfu/ml.

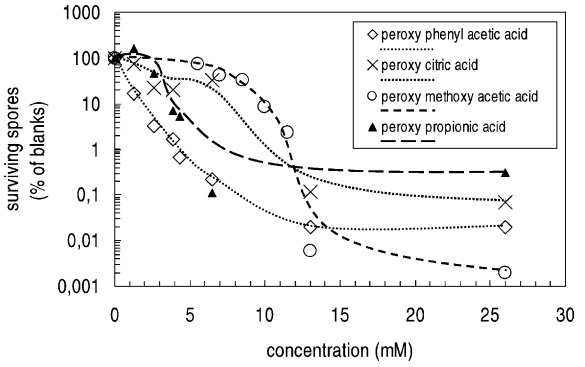


Fig. 2. Inactivation of *B. subtilis* spores with different peroxy carboxylic acids. Each data point represents a mean of five experiments. Blanks (100%) were between 2.7×10^6 and 1.8×10^7 cfu/ml.

lysis of acetic acid ethylester, was equivalent to a commercial product with respect to sporicidal activity.

The concentration of peroxy acetic acid stock solution prepared by enzyme catalysis was at 4.3%, w/v (0.58 M), whereas the commercial product was at 40% (w/v). Thus, the dilution rate to adjust the experimental concentration to 26 mM was only 22-fold for the prepared peroxy acetic acid. The first control experiment with pure ester diluted 22-fold showed no significant sporicidal activity. A further control experiment was carried out treating the spores with both precursor reagents hydrogen peroxide and acetic acid ethylester. Spores were treated with a 22-fold dilution of this non-reacted mixture of precursors, which corresponds to the experiments performed at 26 mM final concentration of peroxy acetic acid. All other experimental conditions were as described for peroxy acetic acid assays (see Section 2). Under these conditions, a 60% inactivation of spores compared to blanks was observed (data not shown). This effect is probably caused by traces of hydrogen peroxide and can be considered of minor importance, since the peroxy acetic acid showed an inactivation power of over four orders of magnitude higher at the corresponding concentration (Fig. 1).

3.3. Sporicidal activity of other peroxy acids

Peroxy methoxy acetic acid, peroxy citric acid, peroxy propionic acid and peroxy phenyl acetic acid made by lipase-catalyzed peroxy acid generation have so far not been tested for their antimicrobial potential. Therefore, these derivatives were tested in the same way as described for peroxy acetic acid to evaluate their sporicidal activity. In Fig. 2, the results from several experiments are summarized.

Peroxy phenyl acetic acid and peroxy methoxy acetic acid exhibited sporicidal activities comparable to peroxy acetic acid. An inactivation of five and four orders of magnitude could be observed for peroxy methoxy acetic acid and peroxy phenyl acetic acid, respectively. Peroxy propionic acid and peroxy citric acid were less effective with respect to the final degree of inactivation. The maximum sporicidal effect, which was at three orders of magnitude, was observed at 7 mM for peroxy propionic acid and 13 mM for peroxy citric acid. An increase of concentrations up to 26 mM did not result in a further inactivation of spores. The reason for this resistance is unclear. At concentrations >6.5 mM, a phase separation could be observed during incubation of spores with peroxy phenyl acetic acid. Therefore, in these cases the apparent concentration may not reflect the active concentration towards spores, and correspondingly the data may not represent the optimal sporicidal activity at elevated concentrations of this peroxy carboxylic acid. Preliminary experiments with the addition of a non-ionic detergent pave the way to avoid phase separations.

Interestingly, the sporicidal activities do not relate directly to their oxidative power. Out of the peroxy acids examined peroxy methoxy acetic acid is, by far the strongest oxidant; peroxy citric acid on the other hand is by far the weakest, as can be shown by competitive epoxidation experiments [14]. If sporicidal activity were based on unspecific oxidation of S–H- and S–S-groups [4], peroxy methoxy acetic acid would be a superior sporicide and consequently the sporicidal effect of peroxy citric acid should be much lower. Understanding influences of the peroxy acid's structure on the efficiency of disinfectants is a long-term goal of our work.

In conclusion, all these four peroxy carboxylic acids deserve further investigation. Experiments to evaluate the sporicidal activity at different pH, temperature, fat and protein concentration are under way. In future studies microorganisms which are known to spoil food or cause food-borne infections will be included. The antimicrobial activity on surface contaminations

Table 2
Treatment of *B. subtilis* spores with various peracids

No.	Peroxy carboxylic acid	Concentration (mM)	Surviving spores (%)[a]	Surviving spores (σ)[b]
2/1	Peroxy acetic acid (commercial)	1.3	13.12	12.25
2/2		2.17	2.38	2.51
2/3		2.6	9.07	9.1
2/4		3.26	0.002	<0.01
2/5		4.33	0.51	0.6
2/6		13	0.06	0.04
2/7		26	0.01	0.02
2/8	Peroxy acetic acid (enzyme)	1.3	83.46	16.54
2/9		2.17	55.408	29.15
2/10		2.6	19.52	15.69
2/11		3.26	2.319	4.03
2/12		4.33	0.317	0.42
2/13		13	0.019	0.01
2/14		26	0.002	<0.01
2/15	Peroxy phenyl acetic acid	1.3	17.01	11.03
2/16		2.6	3.27	3.37
2/17		3.9	1.66	1.85
2/18		4.33	0.68	0.57
2/19		6.5	0.22	0.26
2/20		13	0.02	0.01
2/21		26	0.02	0.03
2/22	Peroxy citric acid	1.3	73.49	5.83
2/23		2.6	22.32	2.23
2/24		3.9	20.58	4.84
2/25		6.5	33.15	21.49
2/26		13	0.12	0.07
2/27		26	0.07	0.05
2/28	Peroxy methoxy acetic acid	5.5	72.836	7.42
2/29		7	41.666	21.46
2/30		8.5	32.37	20.17
2/31		10	8.554	5.34
2/32		11.5	2.279	1.5
2/33		13	0.006	0.01
2/34		26	0.002	<0.01
2/35	Peroxy propionic acid	1.3	162.63	91.53
2/36		2.6	46.55	43.39
2/37		3.9	6.9	4.97
2/38		4.33	5.3	5.07
2/39		6.5	0.11	0.14
2/40		26	0.31	0.36

[a] Related to untreated controls; mean of at least five independent experiments.
[b] Standard deviation.

and biofilms will also be investigated. Peroxy phenyl acetic acid and other hydrophobic peroxy acids may be useful in areas with fatty contaminations, where due to their hydrophobic character they can reach microorganisms otherwise protected in apolar niches. For other applications, peroxy citric acid is of particular interest; it will decompose to citric acid, which is a non-toxic ingredient of many food items. Nevertheless, the toxicity of the peroxy acid itself has to be studied separately.

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References

[1] C.W. Jones, Application of Hydrogen Peroxide and Derivatives, Royal Society of Chemistry, Cambridge, 1999, p. 61ff.
[2] S.S. Block, in: S.S. Block (Ed.), Disinfection, Sterilisation, and Preservation, Lippincott, Williams & Wilkins, Philadelphia, PA, USA, 2001, pp. 185–204.
[3] M.M. Betz, Dissertation, München University of Technology, 2001.
[4] G. McDonnell, A.D. Russell, Clin. Microbiol. Rev. 12 (1999) 147.
[5] Material Data Sheet, Peracetic Acid Solution, EC Patent No. 201-186-8 (2001).
[6] Senatskomission zur Prüfung gesundheitsschädlicher Arbeitsstoffe der deutschen Forschungsgemeinschaft, MAK-Listing, Chapter III, Category 3B, Germany, 2000.
[7] F. Björkling, S.E. Godtfredsen, O. Kirk, J. Chem. Soc. Chem. Commun. (1990) 1301.
[8] O. Kirk. Björkling, S.E. Godtfredsen, A Process for Preparing Peroxy Carboxylic Acids Using an Enzyme Catalyst, WO 91/04333 (12.9.1990/4.4.1991).
[9] S. Warwel, M. Rüsch gen. Klaas, J. Mol. Catal. B: Enzym. 1 (1995) 29.
[10] M. Rüsch gen. Klaas, M.S. Warwel, J. Mol. Catal. A: Chem. 117 (1997) 311.
[11] M. Rüsch gen. Klaas, S. Warwel, in: G. Emig, C. Kohlpaintner, B. Lücke (Eds.), DGMK Tagungsbericht 9803, Proceedings of the DGMK Conference Selective Oxidations in Petrochemistry, Hamburg, 8–9 September 1998, p. 233.
[12] M. Rüsch gen. Klaas, S. Warwel, Organic Lett. 1 (1999) 1025–1026.
[13] S. Warwel, M. Rüsch gen. Klaas, Perkohlensäurehalbester DOS 19738442 (3.9.97/4.3.99).
[14] S. Warwel, M. Rüsch gen. Klaas, in: W. Adam (Ed.), Peroxide Chemistry—Mechanistic and Preparative Aspects of Oxygen Transfer, Wiley–VCH, Weinheim, 2000, pp. 209–231.
[15] M. Rüsch gen. Klaas, S. Warwel, in: U.T. Bornscheuer (Ed.), Enzymes in Lipid Modifikation, Wiley–VCH, Weinheim, 2000, pp. 116–127.
[16] I. Hilker, Masters Thesis, University of Paderborn, 1997.
[17] J.-L. Sagripanti, A. Bonifacino, Appl. Environ. Microbiol. 62 (1996) 545.
[18] P. Setlow, in: M.P. Doyle, L.R. Beuchat, T.J. Montville (Eds.), Food Microbiology–Fundamentals and Frontiers, ASM Press, Washington, DC, 1997, p. 30.
[19] M. Rüsch gen. Klaas, S. Warwel, in: F.N. Rohstoffe (Ed.), Schriftenreihe Nachwachsende Rohstoffe Band 18: Nachwachsende Rohstoffe für die Chemie, Landwirtschaftsverlag, Münster, 2001, p. 651.