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THE EFFECT OF CHLORHEXIDINE ON THE ANAEROBIC FERMENTATION OF *SACCHAROMYCES CEREVISIAE* RELATED TO THE RELEASE OF PROTEIN

JØRN ERIK JENSEN

Department of Biochemistry, The Royal Dental College, Vennelyst Boulevard, DK-8000 Århus C, Denmark

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Abstract—The relationship between the inhibitory effect of chlorhexidine (CHX) on the anaerobic fermentation of baker's yeast and the release of protein from the cells has been studied. The experiments were based on the ability of Ca^{2+} to protect the cells against the effect of CHX. Maximal depression of the anaerobic fermentation was produced by a concentration of 21 nmoles per mg of wet cells (29 μM) without the addition of Ca^{2+}. This amount of CHX had no effect when 41 mM Ca^{2+} was added prior to or concomitantly with CHX. The inhibitory effect of CHX increased proportionally to the time of incubation up to about 75 sec. Fifty per cent inhibition of the CO_2 production was achieved after 41 sec of incubation without Ca^{2+}, while maximal inhibition (90 per cent) was obtained after 100 sec of incubation. The yeast could lose up to 5 μg of protein per mg of cells corresponding to 4 per cent of their total content without any impairment of CO_2 production. The release of protein at the maximal inhibition of the CO_2 production amounted to 14 μg per mg of cells corresponding to 11 per cent of the total content. CHX exerted a concentration dependent protein releasing effect on the yeast in total concentrations up to 836 nmoles per mg of cells (1153 μM). The maximal release of protein was 50 μg per mg of cells corresponding to 38 per cent of the total content. Higher concentrations of CHX exerted a precipitating effect on the released proteins.

In a previous study it was shown that chlorhexidine (Hibitane®), 1,1'-hexamethylene bis(5-(4-chlorophenyl))biguanide exerts a concentration dependent inhibitory effect on the glycolysis of yeast [1]. The depressive effect was among other things ascribed to an increase of the permeability of the cytoplasmic membrane. Experiments with yeast and various bacteria have shown that treatment with chlorhexidine is followed by extensive leakage of intracellular material and loss of viability [2–12]. As the antimicrobial effect of chlorhexidine is suggested to be due to the loss of intracellular material it was found of interest to study the relationship between the inhibition of the glycolysis and the release of proteins from yeast cells. The effect of chlorhexidine can be counteracted by cations added before or concomitantly with the antimicrobial [1]. As no information about the effect of cations added later than the chlorhexidine is available, the significance of the addition-time of cations was studied. Finally the present paper describes a precipitating effect of chlorhexidine on the proteins released.

MATERIALS AND METHODS

Yeast. All experiments were performed on non-growing suspensions of a strain of baker's yeast type RN 102 (De Danske Spritfabrikker, Copenhagen). The yeast cells were washed two times in distilled water and finally centrifuged at 35,000 *g* for 30 min. The pellet was subsequently suspended in deaerated distilled water 0.5 hr prior to the experiments. The weight of yeast used in the experiments is given as wet weight.

Determination of protein. The content of protein of whole cells was determined according to Stickland [13]. The protein released from the cells was determined by the method described by Lowry *et al.* [14].

Determination of CO_2 production. The production of CO_2 was determined by Warburg constant volume respirometers by the conventional technique [15]. The deaerated incubation medium consisted of 3.3 mM KH_2PO_4, 95.8 mM glucose, 29 μM chlorhexidine, 41 mM $CaCl_2$ and 17.2 mM sodium acetate buffer pH 5.0. The amount of yeast used was 8.0 mg in 5.8 ml medium. The respirometers were flushed for 10 min and filled with oxygen-free nitrogen. The experiments were performed at 30°. The reaction was initiated by the addition of the glucose. Chlorhexidine was added concomitantly with the glucose, while the $CaCl_2$ was added from 0 to 240 sec after the addition of chlorhexidine. All experiments were performed in duplicate.

Determination of the release of protein. The release of protein in relation to the concentration of chlorhexidine was determined by incubating 8 mg of cells in 5.8 ml calcium-free medium containing 0–9.6 mM chlorhexidine. The mixtures were incubated at 30° for 15 min and then centrifuged at 1,500 *g* for 10 min. The supernatants were removed and their content of released proteins was determined.

The release of protein as a function of the time of incubation was determined by incubating 8 mg of cells in 5.8 ml medium containing selected amounts of chlorhexidine. Samples were taken and centrifuged at 1,500 *g* for 10 min followed by a determination of the protein content of the supernatants.

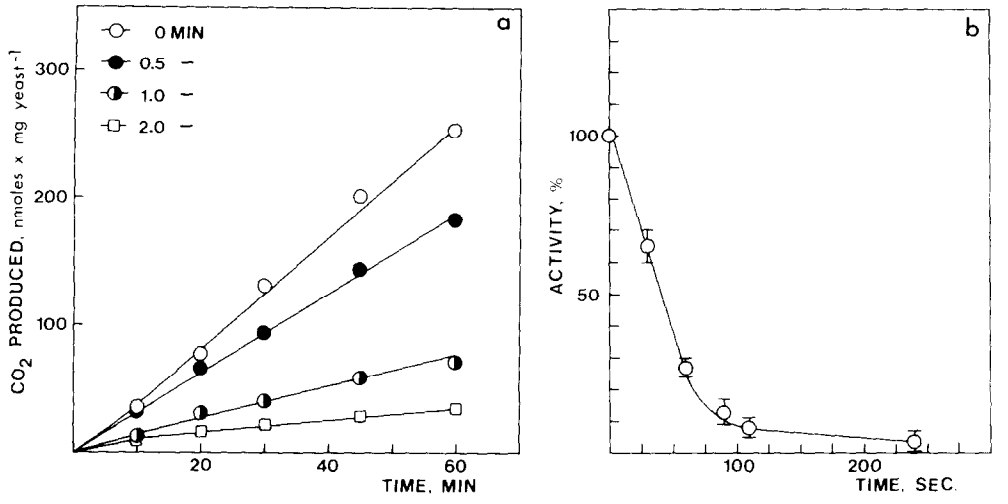

Fig. 1. The interference of Ca^{2+} with the inhibitory effect of chlorhexidine on fermentation. (a) CO_2 production related to the difference between the time of addition of chlorhexidine and Ca^{2+}. ○: control and Ca^{2+} added after 0 sec; ●: Ca^{2+} added after 30 sec; ◑: Ca^{2+} added after 60 sec; □: Ca^{2+} added after 120 sec. (b) Relationship between fermentation activity and the time interval between chlorhexidine and Ca^{2+} addition. The vertical bars show the standard deviation. The CO_2 production of the controls amounted to 4.5 ± 0.2 (mean ± S.E.M.) (n = 4) nmoles per mg of yeast.

Chemicals. Chlorhexidine digluconate was obtained from I.C.I., Macclesfield, England. Other chemicals were of analytical grade.

RESULTS

The influence of the time of addition of Ca^{2+} on the effect of chlorhexidine. The addition of chlorhexidine to a final concentration of 21 nmoles per mg of yeast (29 μM) had no inhibitory effect on the anaerobic fermentation of yeast in the presence of 41 mM Ca^{2+}, when the Ca^{2+} was added concomitantly with the chlorhexidine. Without Ca^{2+} this amount of chlorhexidine depressed the CO_2 production to a basic level of 5–10 per cent of that of the controls. From Fig. 1a it is evident that the later the Ca^{2+} is added the greater is the inhibitory effect of chlorhexidine. The graphs show furthermore, that the CO_2 production preserved remains at a constant level after addition of Ca^{2+}. From Fig. 1b it is seen that the activity of the fermentation decreases proportionally to the difference of time between the addition of chlorhexidine and Ca^{2+} up to about 75 sec. At a difference of time greater than 100 sec was the Ca^{2+} without any protective effect on the cells as the CO_2 production proceeded at the basic level corresponding to maximal depression.

The protein releasing effect of chlorhexidine. Chlorhexidine exerts a concentration dependent protein releasing effect on yeast at concentrations up to about 836 nmoles per mg of cells (1153 μM) as seen from Fig. 2. The maximal release of protein at this concentration amounts to 50 μg per mg of yeast corresponding to 38 per cent of the total cell content. Higher concentrations of chlorhexidine provoke a concentration dependent decrease of the protein content of the supernatants. A minimal protein content of about 33 μg per mg of yeast (25 per cent of the total content) is obtained with 2 μmoles chlorhexidine per mg of cells (2880 μM). Incubation with more than 2880 μM chlorhexidine causes a concentration dependent increase of the protein content of the supernatants to a maximal level of about 45 μg protein per mg of yeast at 7 μmoles per mg of cells (9600 μM).

The effect of the time of addition of Ca^{2+} on the release of protein. The addition of Ca^{2+} inhibits the ability of chlorhexidine to release proteins from the

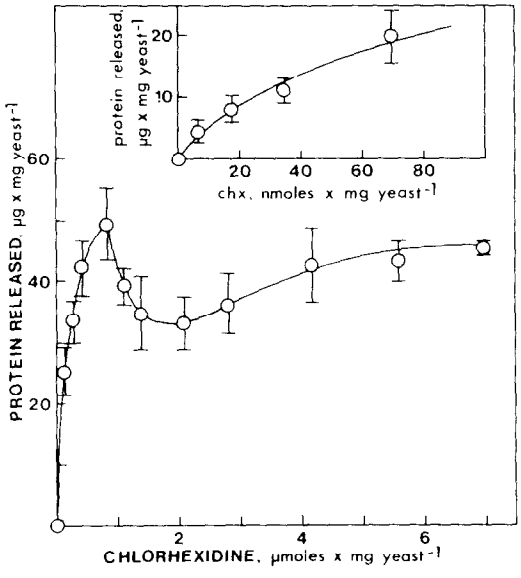

Fig. 2. The release of protein from yeast related to the total concentration of chlorhexidine. The insert shows the same relationship at low concentrations of chlorhexidine. Each point on the curves represents the mean of four determinations. The vertical bars show the standard deviation.

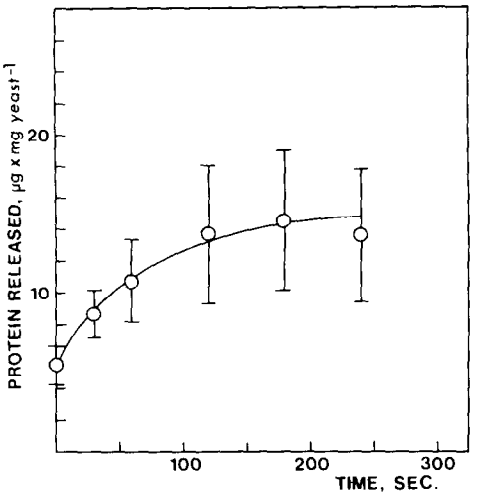

Fig. 3. The release of protein from yeast cells by chlorhexidine. The abscissa gives the time of addition of Ca^{2+}. The experimental points represent the means of four determinations. The vertical bars show the standard deviation. Controls with Ca^{2+} and without chlorhexidine showed no release of protein.

yeast cells as shown in Fig. 3. The controls without chlorhexidine showed no release of protein. Addition of 29 μM chlorhexidine and 41 mM Ca^{2+} at the same time caused a release of about 5 μg protein per mg of cells corresponding to 4 per cent of the total content of cell-protein. Addition of Ca^{+} subsequent to the addition of chlorhexidine showed a time-dependent increase of the release of protein. The maximal release of protein was obtained after about 120 sec of incubation without Ca^{2+}. Addition of Ca^{2+} later than 120 sec subsequent to the addition of chlorhexidine gave a constant release of protein about 14 μg per mg yeast equivalent to 11 per cent of the total cell content.

The precipitating effect of chlorhexidine on the released proteins. The precipitating effect of chlorhexidine on the released proteins was investigated by incubating 8 mg of cells in 5.8 ml medium without $CaCl_2$ in the presence of 836 nmoles chlorhexidine per mg of cells (1153 μM). After incubation at 30° for 15 min the samples were centrifuged at 1,500 *g* for 10 min. To the supernatants were added increasing amounts of chlorhexidine and they were again incubated at 30° for 15 min. Finally the mixtures were centrifuged at 1,500 *g* for 10 min and the content of protein in these supernatants was determined.

From Fig. 4 it is evident that an increase of the total amounts of chlorhexidine from 836 nmoles per mg of cells to 1,000 causes a decrease of the concentration of released protein from 50 μg per mg of cells to 35 μg. The addition of more chlorhexidine up to a total amount of 2.5 μmoles per mg of cells does not provoke any further precipitation of protein. At total concentrations of chlorhexidine above 2.5 μmoles per mg of cells the precipitating ability of chlorhexidine decreases as the protein concentration increases up to a final level of 51 μg per mg of cells at 6.2 μmoles per mg of yeast.

The kinetics of the release of protein. The release of protein from yeast provoked by low concentrations of chlorhexidine exhibits a biphasic pattern characterized by an initial phase of rapid release followed by a secondary phase of slow release. From Fig. 5a it is seen that about 40 μg of protein per mg of yeast is released after 90 min of incubation with 279 nmoles of chlorhexidine per mg of cells (384 μM) and that 70 per cent of this amount of protein is released during the first 5 min of incubation. Incubation with 836 nmoles of chlorhexidine per mg of cells (1153 μM) caused a maximal release of about 50 μg protein per mg of cells after 15 min of incubation as seen from Fig. 5b. Prolongation of the incubation beyond 30 min caused a progressive precipitation of the released proteins. After 90 min of incubation a final level of solubilized protein of about 35 μg per mg of cells was obtained.

DISCUSSION

The present study shows that the protective effect of Ca^{2+} on the anaerobic fermentation of yeast cells decreases parallel to the difference of time between the addition of chlorhexidine and cation. It is noteworthy that if Ca^{2+} is added before total inhibition of the fermentation is achieved, then the residual activity remains constant during the following hour. This protective effect of Ca^{2+} has been ascribed to the ability of Ca^{2+} to interfere with the binding of chlorhexidine to the cells [1].

The release of protein was not directly proportional to the depression of the fermentation, as the cells could lose up to 4 per cent of the total content of protein without any detectable decrease of the CO_2 production. The proteins released initially are thus without significance to the fermentation and must be

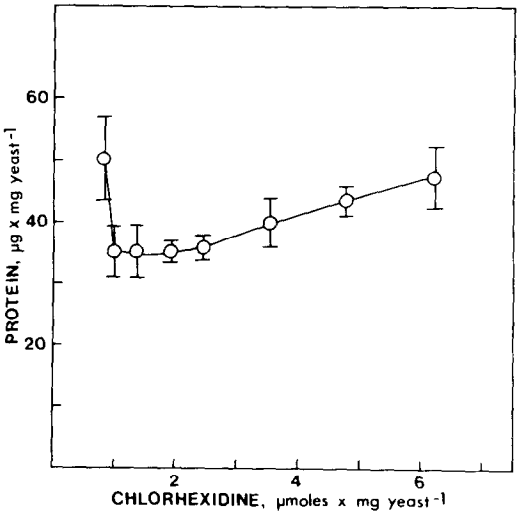

Fig. 4. The precipitating effect of chlorhexidine on the proteins released by 836 nmoles chlorhexidine per mg of yeast after 15 min of incubation. The abscissa gives the total concentration of chlorhexidine i.e. the 836 nmoles per mg of cells added to the whole cells suspension plus the amount added to the supernatants. The points on the curve represent the means of 6 determinations. The vertical bars show the standard deviation.

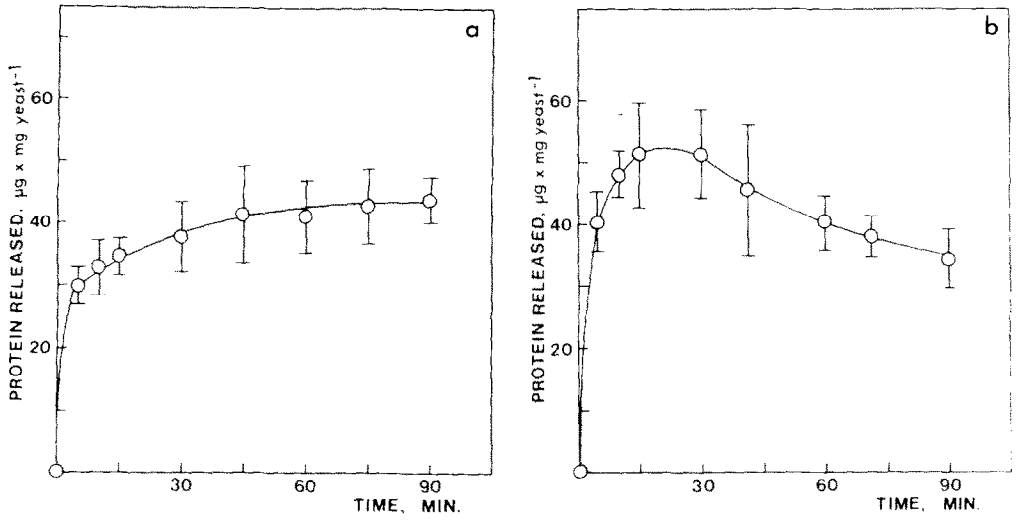

Fig. 5. The release of protein from yeast related to the incubation time. Each point on the curves represent the mean of 6 determinations. The vertical bars give the standard deviation. (a) The release of protein caused by 279 nmoles chlorhexidine per mg of cells (384 μM). (b) The release of protein caused by 836 nmoles chlorhexidine per mg of cells (1153 μM).

different from rate limiting glycolytic enzymes as a loss of some of the rate limiting glycolytic enzymes would lead to a decrease of the fermentation. The proteins released initially are presumably elements with a loose binding to structures external to the cell membrane i.e. the cell wall and the membrane surface. It cannot be ruled out, however, that some cytoplasmic proteins are released as it has been shown that treatment of yeast with basic proteins cause a release of ultraviolet-absorbing components while the glycolytic enzymes remain inside the leaky cells [16, 17]. The release of protein above the initial loss of 4 per cent of the total content is nearly proportional to the depression of the fermentation with a release of about 11 per cent of the total content at total inhibition. Half maximal inhibition of the fermentation and release of half part of their protein (between 4 per cent and 11 per cent i.e. 7.5 per cent of the total content) is thus obtained concomitantly after 40 sec of incubation. This parallelism between a certain part of the protein release and the depression of the fermentation is different from the relationship between cell death and the release of phosphorus-32 from labelled cells of *E. coli* as it was found that the percentage of phosphorus-32 released was significantly higher than the percentage of cells killed [9].

The ability of chlorhexidine to release intracellular constituents from a variety of cells is widely recognized [2–12]. In general, low concentrations of chlorhexidine induce a concentration dependent release of such material whereas higher concentrations lead to a decrease. This decrease has been explained by a sealing of the cytoplasmic membrane and a precipitation *in situ* [3–6, 12]. The release of protein from yeast as a function of the chlorhexidine concentration follows the classical course as shown in the present study. The detected decrease of the amounts of protein released at higher concentrations of chlorhexidine may be false, however. Thus a decrease of the concentration of released protein into the supernatants from the samples with whole cells can be provoked by the addition of small amounts of chlorhexidine. When such small amounts of chlorhexidine were added to the amounts already added to the whole cells the new graph was nearly an exact reproduction of the graph concerning the whole cells (cf. Figs 4 and 5). These experiments, however, give no information about the site of precipitation of protein i.e. inside or outside the cells. To get further insight into this problem, the release of protein as a function of the time of incubation was studied. It was found that higher concentrations of chlorhexidine caused a progressive precipitation of the initially released protein whereas low concentrations of chlorhexidine caused a secondary slow release. The reduced concentration of released protein obtained with higher concentrations of chlorhexidine must therefore be ascribed to a precipitation outside the cells with a subsequent removal during the centrifugation. As a consequence the theory of the membrane sealing should be modified.

In conclusion the inhibitory effect of chlorhexidine on the anaerobic fermentation of yeast proceeds proportionally to the incubation time. The provoked inhibition is irreversible but may within a certain time be blocked by Ca^{2+}. The effect of chlorhexidine on the fermentation is directly proportional to certain parts of the release of protein. The seeming decrease of the release of protein at higher concentrations of chlorhexidine is due to a precipitation outside the cells.

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