Inhibition of Model Compound of Purple Acid Phosphatases on Growth of *Aerobacter aerogenes* Investigated by Microcalorimetry

YAO, Jun[a,c](姚俊) LIU, Yi[b](刘义) LIU, Jian-Ben[c](刘建本) ZHOU, Qin[a](周琴)
Qin, Xia[b](秦霞) LIU, Peng[b](刘鹏) DONG, Jia-Xin[b](董家新) QU, Song-Sheng[b](屈松生)
YU, Zi-Niu*,[a](喻子牛)

[a] *Key Laboratory of Agricultural Microbiology of Chinese Agriculture Ministry, Huazhong Agricultural University, Wuhan, Hubei 430070, China*

[b] *Department of Chemistry, College of Chemistry and Molecular Sciences, Wuhan University, Wuhan, Hubei 430072, China*

[c] *College of Life Sciences and Chemistry, Jishou University, Jishou, Hunan 416000, China*

Microcalorimetry was used to study the inhibitory or antibiotic action of six kinds of the model compounds of purple acid phosphatases on a strain of *Aerobacter aerogenes*. Difference in their capacities to inhibit the metabolism of this bacterium was observed. The extent and duration of the inhibitory effect on the metabolism as judged from the growth rate constant, k, and the half-inhibitory concentration, IC_{50}, varied with the different drugs. The rate constant k of *A. aerogenes* (in the log phase) in the presence of the compounds decreased with the increasing of concentrations. The experimental results reveal that the order of the antibiotic activity of the compounds is: LD-1 > LD-2 > LD-3 > XF-1 > LD-4 ~ LD-5.

Keywords model compound of purple acid phosphatases, *Aerobacter aerogenes*, inhibition, metabolism, microcalorimetry

Introduction

Iron is one of the fourteen types of essentially biological trace elements. Iron plays an important role in living body and it was regarded as the required trace element for life. Many studies indicate that deficiency or excess of iron may be the cause of many diseases. Furthermore, iron is active center of pigment oxidase, and succinic dehydrogenase, *etc*. Consequently, the study of the influence of iron and its compounds on microorganism is significant for understanding life phenomenon.[1]

Inhibitory or antibiotic action of purple acid phosphatases (PAPs) has not been studied previously. PAPs are iron-containing proteins, and can be separated from plant and animal. In pH = 5—6, it can catalyze the hydrolization of phosphate,

$$ROPO_3^{2-} + H_2O \longrightarrow ROH + HPO_4^{2-}$$

In the way, Utreroferrin (Uf) and Bovine spleen purple acid phosphatases (BSPAP) are studied clearly. They all have double nucleus iron central active site. Every protein molecule has two iron atoms, and the atoms have activity when they exist in the form of $Fe^{II}Fe^{III}$. Their sequence of amino acid is same above 90%, their activities are identical too. Researchers are interested in PAPs recently, because this study is related to hydrolization of DNA and RNA.[2] The studies of catalytic mechanism cause researchers to pay more attention to the model compounds of purple acid phosphatases.[3] Their model compounds can recognize and cut DNA and RNA differently as a "molecular scissors", and their unique physiological function and potential value in medicine draw people's more attention. The study will promote progress in genetic engineering technique.

Microcalorimetry provides a general analytical tool for the characterization of the microbial growth process. It has been used extensively to investigate drug and the microbial cell interaction and has furnished much useful information.[4-8] One of the most prominent features of the microbial growth process is the production of heat. If the heat is monitored by microcalorimeter, much useful information, both qualitative and quantitative, may be obtained. Each type of microbe has a unique power-time trace, as recorded by the microcalorimeter, under a defined set of growth conditions. Any substance that can modify the metabolic growth processes involved in cell will change the power-time curve obtained from the microcalorimetry. From the power-time curves, not only thermodynamic but also kinetic information can be obtained.

In this paper, the power-time curves, produced by *A. aerogenes* alone and *A. aerogenes* under the action of

* E-mail: liuyi@chem.whu.edu.cn & yz41@public.wh.hb.cn; Tel.: +86-27-87218284, 87218304
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six kinds of model compound of purple acid phosphatases of different concentrations, were determined with an LKB-2277 Bioactivity monitor. From these power-time curves (log phase), the growth rate constant k, and the generation time G, classic parameters of microbiology were calculated.

Experimental

Instruments

LKB-2277 Bioactivity Monitor was used to determine the growth metabolic power-time curves of *A. aerogenes*. The microcalorimeter was thermostated at 37 ℃. The voltage signal was recorded by means of an LKB-2210 recorder (1000 mV range). The baseline stability (over a period of 24 h) is 0.2 μW. The performance of this instrument and the details of its structure have been described previously.[9-12]

Materials

Aerobacter aerogenes strain (Chester CMCC (B) 45102) was provided by Department of Biology, Huazhong Normal University, Wuhan 430079, China.

The peptone culture medium contained per 1000 mL (pH = 7.2): proteose peptone (Oxoid, 5.0 g), beef extract (Oxoid, 3.0 g) and NaCl (5.0 g). It was sterilized in high pressure steam at 120 ℃ for 30 min.

The model compound of purple acid phosphatases were synthesized and characterized by the Bioinorginac Group, Department of Chemistry, Hazhong Normal University, China.[3] The following are chemical formula of them.

LD-1: $[Fe_2(\mu\text{-}O)(\mu\text{-}OAC)DTPB]Cl\cdot(BF_4)_2\cdot 3CH_3OH$
LD-2: $[Fe_2(\mu\text{-}OH)(\mu\text{-}OAC)DTPB\cdot Cl_2]Cl_2\cdot 5H_2O$
LD-3: $Fe_2DTPB\cdot(BF_4)_2\cdot Cl_4\cdot 4CH_3OH$
LD-4: $Na_3[Fe_2(\mu\text{-}O)(\mu\text{-}OAC)(CA)_2]\cdot 6H_2O$
LD-5: $Na_2[Fe_2(\mu\text{-}OH)(\mu\text{-}OAC)(CA)_2]\cdot 8H_2O$
XF-1: $[Fe_2(\mu\text{-}O)(\mu\text{-}OAC)_2\cdot(IDB)_2]\cdot Cl_2\cdot CH_3OH$
DTPB: 1,1,4,7,7-pent(2′-benzimidazolymethy)-diethyltriamine
IDB: *N*,*N*-di(2′-benzimidazolymethy)mine
CA: citrate
OAC: acetate

Methods

In the calorimetric experiments, the flow cell was completely cleaned and sterilized. The procedure was as follows: sterilized distilled water, NaOH (0.1 mol·L^{-1}), alcohol solution (75%), HCl (0.1 mol·L^{-1}), and sterilized distilled water were pumped in sequence by an LKB-2132 microperpex peristaltic pump through the cell, each for 15 min at a flow rate of 50 mL·h^{-1}. Once the system was cleaned and sterilized and the baseline was stabilized, the bacterial suspension, initially containing 1×10^6 bacteria/mL and the model compound of purple acid phosphatases, was pumped through the calorimetric cell with an LKB-2132 perplex peristaltic pump at a flow rate of 50 mL·h^{-1}. When the flow cell (volume 0.6 mL) was full, the pump was stopped and the monitor was used to record the power-time curves of the bacterial growth.

In this type of experiment, the bacteria used were suspended in the peptone culture medium. The model compounds of purple acid phosphatases were added at the beginning of the experiment, *i.e.*, they were introduced as soon as the bacteria were inoculated in the peptone culture medium. These solutions were prepared in sterilized distilled water and were prepared freshly every time.

Results

Growth thermogenic curve of Aerobacter aerogenes at 37 ℃

The power-time curves reflect the metabolic activities of *A. aerogenes* under the action of PAPs. From Figs. 1 and 2 and Table 1, it can be seen that diversity was the shape of the power-time curves of *A. aerogenes* under the action of six kinds of the model compounds of purple acid phosphatases. It indicated different inhibiting mode on *A. aerogenes* by different compounds. But, with the increase of the concentration of the compounds, log phase, *i.e.*, the period between the start of the experiment and the ascending phase of the curves, became longer, the whole metabolic time also became longer, the metabolic peak moved backward, the maximum heat production rate P_{max} decreased, and the generation times G increased. In the same experimental condition, the curves have very good reproducibility and correlation.

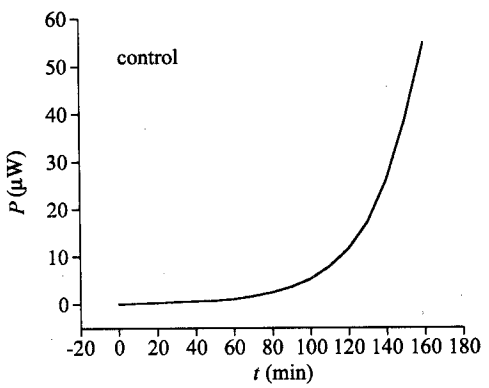

Fig. 1 Growth thermogenic curve of *Aerobacter aerogenes* in log phase at 37 ℃.

Calculation of the growth rate constant and generation time of Aerobacter aerogenes

The growth curves of *A. aerogenes* have shown that the log phase of growth obeys the equation,[13]

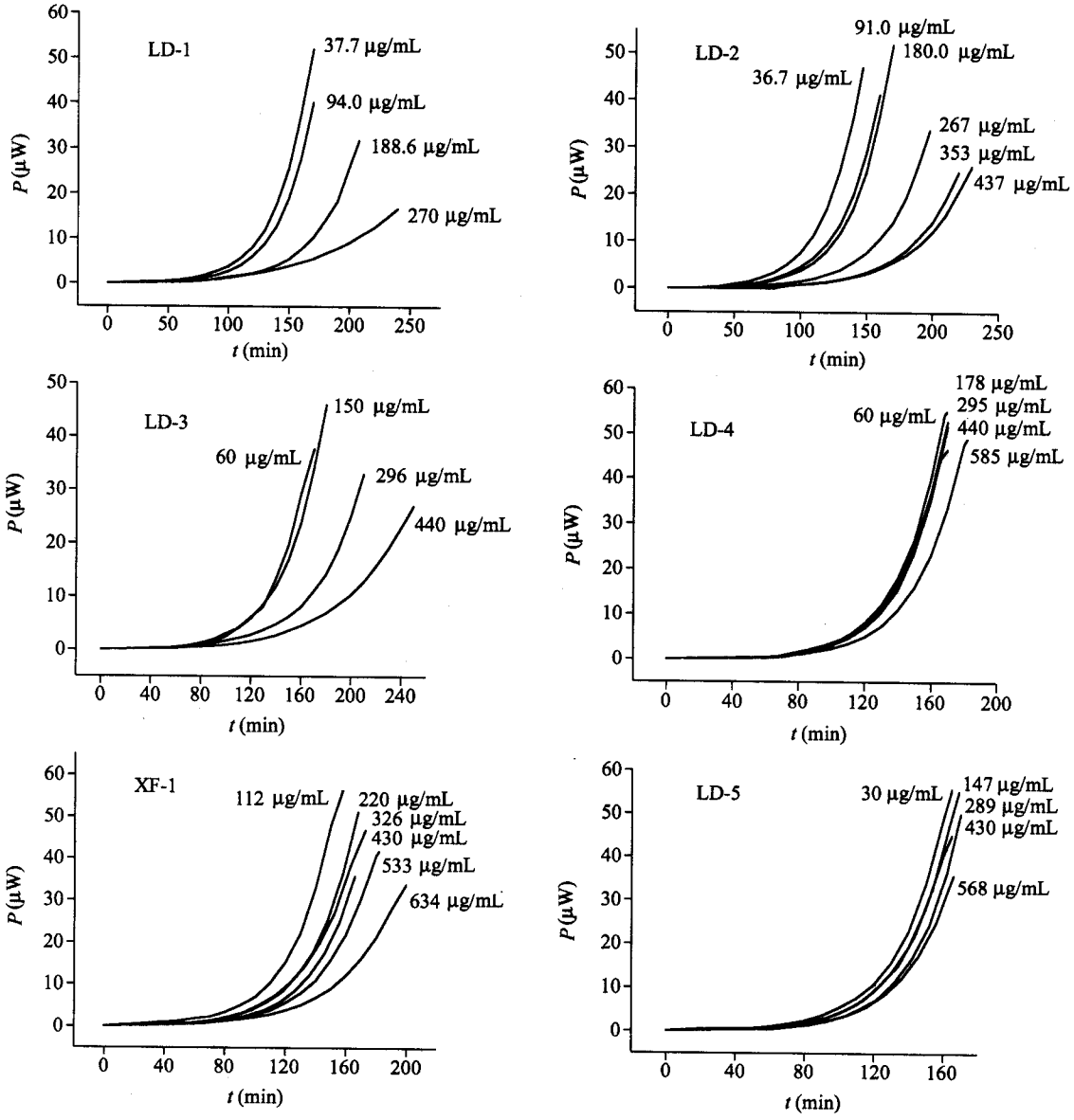

Fig. 2 Growth power-time curves of *Aerobacter aerogenes* under the action of six kinds of the model compounds of purple acid phosphatases in log phase at 37 ℃.

$$P_t = P_0 \exp(kt) \text{ or } \ln P_t = \ln P_0 + kt \quad (1)$$

where t is the time, P the calorimetric power at time t, P_0 the power at time $t = 0$ and k the growth rate constant. Using this equation, the growth rate constant of *A. aerogenes* was calculated. The generation times (G), which are ($\ln 2/k$), were also obtained. Corresponding k and G are shown in Table 1. in Table 1, all of the correlation coefficients, R, are larger than 0.9950, indicating a good reproducibility and correlation.

Inhibitory ratios and half inhibitory concentrations

The inhibitory ratio of the process of growth metabolism can be defined as:

$$I = [(k_0 - k_c)/k_0] \times 100\% \quad (2)$$

Where k_0 is the rate constant of the control, and k_c is the rate constant for growth inhibited by an inhibitor with a concentration of C. The values of I are shown in Table 1. When the inhibitory ratio I is 50%, the corresponding concentration of inhibitor is called the half inhibitory concentration IC_{50}. IC_{50} can be regarded as the inhibiting concentration of causing a 50% decrease of the growth rate constant. According to above definition, we calculate the inhibitory ratio I of *A. aerogenes* at different concentration of different compound, and the results are shown in Table 1. Fig. 3 shows the relationship of I and C, from the relationship of I and C, we can obtain directly the values of IC_{50}.

Discussion

The time of the log-phase of bacteria growth was longer with increasing concentrations of the model compound

Table 1 Parameters of *A. areogenes* inhibited by different compounds

System	c ($\mu g \cdot mL^{-1}$)	k (min^{-1})	G (min)	I (%)	IC_{50}($\mu g \cdot mL^{-1}$)	R
Control	0	0.0398	17.4	—	—	0.9996
LD-1	37.7	0.0386	17.9	3.0	266	0.9996
	94.0	0.0385	18.0	3.2		0.9998
	188.6	0.0306	22.7	23.2		0.9998
	270.0	0.0195	28.1	51.2		0.9923
	370.0	0.0000		100		—
LD-2	36.7	0.0378	18.3	5.1	524	0.9997
	91.0	0.0410	16.9	-3.0		0.9994
	180.0	0.0398	17.4	0.1		0.9987
	267.0	0.0344	20.1	13.5		0.9995
	353.0	0.0288	24.1	27.7		0.9999
	437.0	0.0255	27.2	36.0		0.9996
LD-3	60.0	0.0422	16.4	-6.1	544	0.9978
	150.0	0.0037	16.4	6.8		0.9984
	296.0	0.0274	18.7	31.3		0.9997
	440.0	0.0023	25.3	42.2		0.9973
LD-4	60.0	0.0412	16.8	-3.4	$k = 0.03801$	0.9993
	178.0	0.0420	16.5	5.6		0.9993
	295.0	0.0422	16.4	6.1		0.9988
	440.0	0.0379	18.3	4.7		0.9966
	585.0	0.0381	18.2	4.4		0.9998
LD-5	30.0	0.0374	18.5	6.1	$k = 0.03778$	0.9997
	147.0	0.0379	18.3	4.8		0.9997
	289.0	0.0403	17.2	-1.2		0.9990
	430.0	0.0387	17.9	2.9		0.9992
	568.0	0.0372	18.6	6.6		0.9993
XF-1	112.0	0.0407	17.0	2.1	730	0.9992
	220.0	0.0384	18.1	3.7		0.9993
	326.0	0.0376	18.4	5.5		0.9996
	430.0	0.0361	19.2	9.3		0.9984
	533.0	0.0336	20.6	15.6		0.9969
	634.0	0.0286	24.2	28.2		0.9988

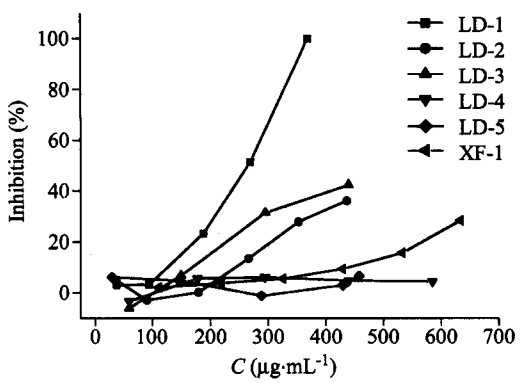

Fig. 3 Relationship between the inhibitory ratios I and C.

of purple acid phosphatases. This indicated that the bacteria took longer time to generate a detectable signal. This result, probably was due to excess iron inhibited the growth of *A. aerogenes* or killed the bacteria. The experiment indicate that these six compounds all have the capacity to inhibit the growth of *A. aerogenes* to different extents, and the inhibitory extent and mode varied with different compounds. Considering the rate constant, half inhibitory concentration and the molecular mass, it could be concluded that LD-1 gave the best inhibitory effect on *A. aerogenes* growth. The inhibition increased with the increase of the concentration of LD-1, k decreased, I and G all increased, it can not grow when the concentration is up to 370 $\mu g/mL$, the half inhibitory concentration IC_{50} is 266 $\mu g/mL$. LD-4 and LD-5 have inhibition on the growth of *A. aerogenes*, but their effect is less than that of LD-1. Low concentration (60—295 $\mu g/mL$) of LD-4 can promote the growth of *A. aerogenes*, high concentration can inhibit its growth, but the relationship between inhibition and concentration is not obvious. LD-5 has no relation in whole concentrations. LD-3 and XF-1 have similar inhibition on *A. aerogenes*. Low concentration can promote the growth, the inhibitions increased with the increase of the concen-

tration of the compounds. But the inhibition of LD-3 is bigger than that of XF-1. Their IC_{50} are 544 μg/mL and 730 μg/mL, respectively. For LD-2, the inhibition decreased with the increase of the concentration of the compound; in the concentration range of 36.7—91 μg/mL, and in the concentration range of 91—437 μg/mL, the inhibition increased with the increase of the concentration.

From the power-time curves of *A. aerogenes* effected by the model compound of purple acid phosphatases, it can be seen that at the low concentrations the model compound of purple acid phosphatases has a promoting action on *A. aerogenes* growth, but at high concentrations of them inhibits the growth of *A. aerogenes*. The factors of determining the characteristics of a dose-response curve are the drug's mode of action in cells, its number of target sites and its affinity for those target sites. Iron is active center of purple acid phosphatases (PAPs), it can catalyze hydrolyzation of phosphate.[3] In this study, the growth was inhibited by excess of iron probably through the catalysis of oxidation reactions of SH groups to S—S bonds.[1] During this process more active free radicals may be produced, which further damage the membrane structure and functions of cells. According to IC_{50}, it is concluded that the sequence of antibiotic activity of the model compound of purple acid phosphatases is: LD-1 > LD-2 > LD-3 > XF-1 > LD-4—LD-5.

In conclusion, microcalorimetry offers a means for studying the kinetics of the antibacterial action of antibiotics and for estimation of the relative bioactivity of antibiotics. It provides kinetic and thermodynamic information that can not be obtained by conventional bacteriological techniques, and all of this information is very significant for the further studies of antibiotics, such as the studies of toxicology and pharmacology.

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