Journal of Thermal Analysis and Calorimetry, Vol. 95 (2009) 2, 495–499

EFFECT OF BERBERINE ALKALOIDS ON *BIFIDOBACTERIUM ADOLESCENTIS* GROWTH BY MICROCALORIMETRY

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The inhibitory effects of three berberine alkaloids (BAs) from *Coptis chinensis* Franch on *Bifidobacterium adolescentis* growth were investigated by microcalorimetry. The growth rate constant (k) and maximum heat-output power (P_{max}) decreased and peak time of maximum heat-output power (t_p) prolonged with the increase of BAs concentration. Half inhibitory ratios (IC_{50}) BAs were respectively 790.3 (berberine), 339.6 (coptisine) and 229.8 μg mL^{-1} (palmatine), which indicated the sequence of their antimicrobial activity: berberine<coptisine<palmatine. Combined with previous findings, the sequence which could show the bioactivity of *Bacillus shigae* and *Escherichia coli* was: berberine>coptisine>palmatine. The structure-function relationship of BAs indicated that the functional group methylenedioxy or methoxyl at C2 and C3 might be the major group inducing the activities of BAs on *E. coli* and *B. adolescentis*. Meanwhile, the substituent groups at C2, C3, C9 and C10 almost had equal effect on *B. shigae*.

Keywords: *Bacillus shigae, berberine alkaloid, Bifidobacterium adolescentis, Escherichia coli, inhibitory effect, intestinal flora, microcalorimetry*

Introduction

Coptis chinensis Franch (Huanglian in Chinese) is a traditional Chinese medicinal herb, and is officially listed in Chinese Pharmacopoeia [1]. The *C. chinensis* extract has strong antibacterial activity [2]. Major active components are berberine alkaloids (BAs), which are often used as criteria in the quality control of Huanglian products. In this study, the inhibitory effects of BAs, i.e. berberine, coptisine and palmatine, on *Bifidobacterium adolescentis* were investigated by microcalorimetry.

Intestinal flora commonly includes probiotics, harmful bacteria and intermediate flora. Among them, probiotics mainly include *Bacillus bifidus*, *Bacterium lacticum*, etc. The harmful bacteria include *Bacillus aeruginosus*, *Staphylococcus*, etc. The intermediate flora mainly includes enteric bacilli, coliform bacilli, etc. The three floras maintain the relative balance of intestinal microecology. Bifidobacteria, which belongs to probiotics, is a dominant species in intestines, and has a symbiotic bacteria–host relationship with humans [3]. *B. adolescentis* is a type of bifidobacteria with following features: Gram-positive, branched rod-shape and strict anaerobic.

Microcalorimetry is a quantitative, inexpensive and versatile method for measuring various reactions of physics, chemistry and life science [4, 5]. It provides a general analytical tool for the characterization of cell growth processes, and has been extensively used to investigate the interactions between drugs and cultured cells [6–9]. Calorimetry has been used to monitor the whole organism and the cellular metabolism for a long time [10–15].

In this study, we applied this online, kinetic and precise method to investigate the effect of BAs on the *B. adolescentis* growth by microcalorimetry, which focuses on the energy change of microbial growth. Moreover, combined with previous studies [16, 17], we also discussed the action rule of BAs on growth and their structure-function relationship.

Experimental

3115/3238 TAM Air Isothermal Calorimeter (Thermometric AB, Sweden)

B. adolescentis (AS. 1. 2190) was provided by Chinese Center for Type Culture Collections, National Institute for the Control of Pharmaceutical and Biological Products, Beijing 100051, PR China. *B. adolescentis* was grown in a TPY culture medium, which contained 10 g trypticase BBL, 5 g phytone BBL, 2.5 g yeast extract, 0.15 g $CaCl_2$, 5 g glucose, microamount $FeCl_3$, 2 g K_2HPO_4, 1 mL tween-80, 0.5 g $MgCl_2{\cdot}6H_2O$, 0.5 g cysteine hydrochloride, 0.25 g $ZnSO_4{\cdot}7H_2O$ and 15 g agar. All above were dissolved in 1000 mL of distilled water. Medium pH was adjusted to 6.5–6.8 with 1 mol L^{-1} NaOH or

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1388–6150/$20.00

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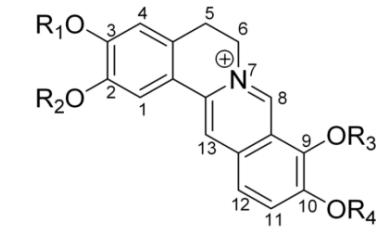

	R1	R2	R3	R4
berberine	—CH2—		CH3	CH3
coptisine	—CH2—		—CH2—	
palmatine	CH3	CH3	CH3	CH3

Fig. 1 Chemical structures of three tested BAs

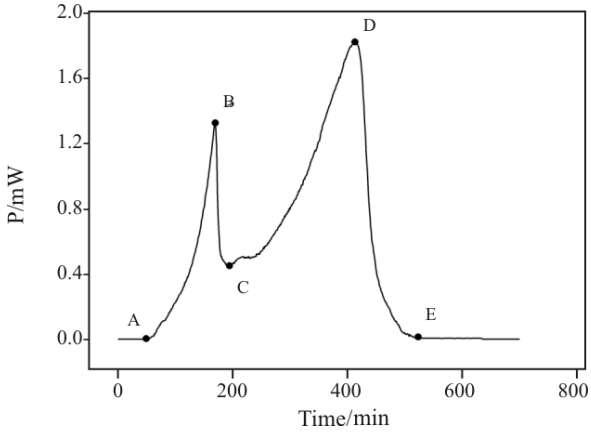

Fig. 2 *P–t* growth curve of *B. adolescentis* cultured in the TPY culture medium and monitored by the microcalorimeter at 37°C

1 mol L^{-1} HCl. Then the medium was sterilized at 121°C for 15 min.

Berberine, coptisine and palmatine were supplied by National Institute for the Control of Pharmaceutical and Biological Products, Beijing 100051, PR China. (purity >95%). The structures of three tested BAs are given in Fig. 1.

B. adolescentis was inoculated into the TPY culture medium ($1.5 \cdot 10^6$ cells per mL) as the first step in the experiments. After that, the fresh prepared BAs solutions (medium as solvent) in different concentrations were added into the cell suspension. Then they were cultivated at 37°C in hypoxia state (GENbox, France).

After that the ampoule method was adopted. These ampoules, filled with BAs and cell suspension, were sealed with wax and put into the 8-channel calorimeter block, the microcalorimeter was thermostated at 37°C. After about 30 min (the temperature of ampoules reached 37°C), the thermogenic curves were recorded until they returned to the baseline. All data were collected continuously by using the dedicated software package. And all procedures were completely sterilized.

Results and discussion

Growth rate constants of B. adolescentis with and without drugs

Figure 2 showed the *B. adolescentis* thermogenic curves without BAs. According to the rule of microorganism growth, the curve can be divided into four phases: exponential phase (A-B), lag phase (B-C), stationary phase (C-D) and decline phase (D-E). The exponential metabolism model may be useful to study [18].

$$\ln(P_t/P_0)=kt \quad (1)$$

In Eq. (1), P_0 represents heat-output power at the beginning and P_t represents that at the time t. The exponential phase of the thermogenic curves corresponds to Eq. (1), while the growth rate constant (k) can be fitted to $\ln P_t$ and t in a linear equation. The values of k were shown in Table 1, and k= 0.02415±0.00041 min^{-1}, indicating a good reproducibility.

The *B. adolescentis* thermogenic curves were monitored. The *P–t* curves showed that the t_p (peak time of maximum heat-output power) prolonged and the k decreased with the increase of concentration (Table 2). All correlation coefficients were larger than 0.9640, indicating a good linear correlationship between k and the corresponding concentration.

The results demonstrated that all the BAs inhibited the growth of *B. adolescentis*, and the inhibitory activity increased with the increase of concentration. Thus, the k decreased gradually, indicating the different antimicrobial activities of BAs. The different inhibitory activities of BAs were demonstrated in Fig. 3.

Peak time and total heat production

With the increase of BAs concentration, the t_p prolonged and the total heat production (Q_t) decreased

Table 1 The values of k of *B. adolescentis* cultured in the TPY culture medium and monitored by the microcalorimeter at 37°C

Experiment No.	1	2	3	4	5	6	RSD[a]/%
k/min^{-1}	0.02401	0.02437	0.2462	0.02443	0.02398	0.02349	1.69

[a]RSD is relative standard deviation

Table 2 The values of k, t_p, P_{max}, I and Q_t of *B. adolescentis* with different concentrations of BAs ($\bar{X}\pm SD$, n=6)

Compound	c/μg mL^{-1}	$k\cdot10^{-4}$/min^{-1}	t_p/min	P_{max}/mW	Q_t/J	I/%
Control	–	241.5±4.1	170.01±1.5	1.34±0.16	19.58±0.21	–
	200	227.5±3.6	192.12±1.39	1.26±0.08	18.37±0.25	5.8±0.81
	600	127.3±2.6	199.23±1.21	0.94±0.11	17.87±0.22	47.3±0.96
Berberine	900	89.5±4.8	237.01±1.07	0.87±0.05	17.41±0.18	62.9±1.05
	1200	56.6±2.2	256.36±1.13	0.64±0.07	17.00±0.08	76.6±1.54
	1500	38.5±2.9	275.19±1.20	0.56±0.04	12.68±0.10	84.1±0.75
Coptisine	50	213.6±6.4	185.97±1.27	1.26±0.08	19.02±0.17	11.6±0.89
	150	171.3±3.4	190.84±2.51	1.12±0.16	18.75±0.10	29.1±1.53
	200	153.8±5.5	210.14±1.98	1.08±0.05	18.12±0.11	36.3±1.25
	300	139.4±3.1	238.09±1.82	1.01±0.04	17.84±0.12	42.3±0.94
	400	103.1±1.1	247.75±1.64	0.90±0.06	17.34±0.14	57.3±1.13
Palmatine	25	220.3±2.4	196.43±2.02	1.27±0.12	18.98±0.23	8.80±.45
	50	209.1±3.7	206.35±2.11	1.09±0.15	18.42±0.12	13.40±.87
	150	146.3±5.7	214.62±1.78	0.86±0.09	18.14±0.09	39.4±1.52
	200	130.2±3.9	218.29±1.43	0.79±0.02	16.37±0.15	46.1±1.16
	300	95.7±2.6	235.51±2.16	0.73±0.05	15.21±0.18	60.4±0.92

gradually. The t_p and Q_t were also listed to show the antibacterial activity of BAs (Table 2).

Inhibitory ratio and the half inhibitory concentration

The inhibitory ratio (I) is defined in Eq. (2), where k_0 is the rate constant of the control in the exponential phase; k is the rate constant in the exponential phase of bacterial growth inhibited by BAs with a c concentration [8].

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

IC_{50} means that ratio I is 50%, which can be regarded as the concentration to cause a 50% decrease of the rate constant. To demonstrate the inhibitory effects of various BAs on *B. adolescentis*, the values of I were also shown in Table 2. Then IC_{50} of BAs were respectively 790.3 (berberine), 339.6 (coptisine) and 229.8 μg mL^{-1} (palmatine). Considering the values of k and IC_{50}, we can conclude easily that coptisine and palmatine had stronger inhibitory effects on *B. adolescentis* than berberine. Thus, the sequence of antimicrobial activity was berberine< coptisine<palmatine.

Comparison of BAs activity on intestinal

The thermogenic curves of intestinal growth indicated that all the tested BAs had inhibitory effects on the tested bacteria. Combined with previous findings [16, 17], IC_{50} of BAs on *B. shigae*, *E. coli* and *B. adolescentis* were analyzed in Fig. 4. As the result, the activity sequences on *B. shigae* and *E. coli* were both berberine>coptisine>palmatine. However, the sequence on *B. adolescentis* was just contrary to it. Berberine could inhibit *B. shigae* and *E. coli* at a low concentration, while it required inhibiting *B. adolescentis* at a comparatively higher concentration. It suggests that when berberine is used to treat diarrhoea in the prescriptive dosage or interval usage, it mainly inhibits harmful bacteria and intermediate flora, while doing little with the probiotics. coptisine and palmatine would not be commonly used to treat diarrhoea due to little of effects on probiotics, harmful bacteria and intermediate flora.

Structure-function relationship of BAs on intestinal flora

All three BAs are berberine alkaloids with different substituents at C2, C3, C9 and C10 of the phenyl ring (Fig. 1). There were great differences among the effects of these BAs on *E. coli* and *B. adolescentis* (Fig. 4). Especially the effects of berberine and palmatine on *E. coli* and *B. adolescentis* were completely different. It suggests that the functional group methylenedioxy at C2 and C3 of the phenyl ring possibly improves antimicrobial activity of BAs on *E. coli*. Meanwhile, the functional group methoxyl at C2 and C3 possibly improves antimicrobial activity of BAs on *B. adolescentis*. Thus, the functional group methylenedioxy or methoxyl at C2 and C3 may be the

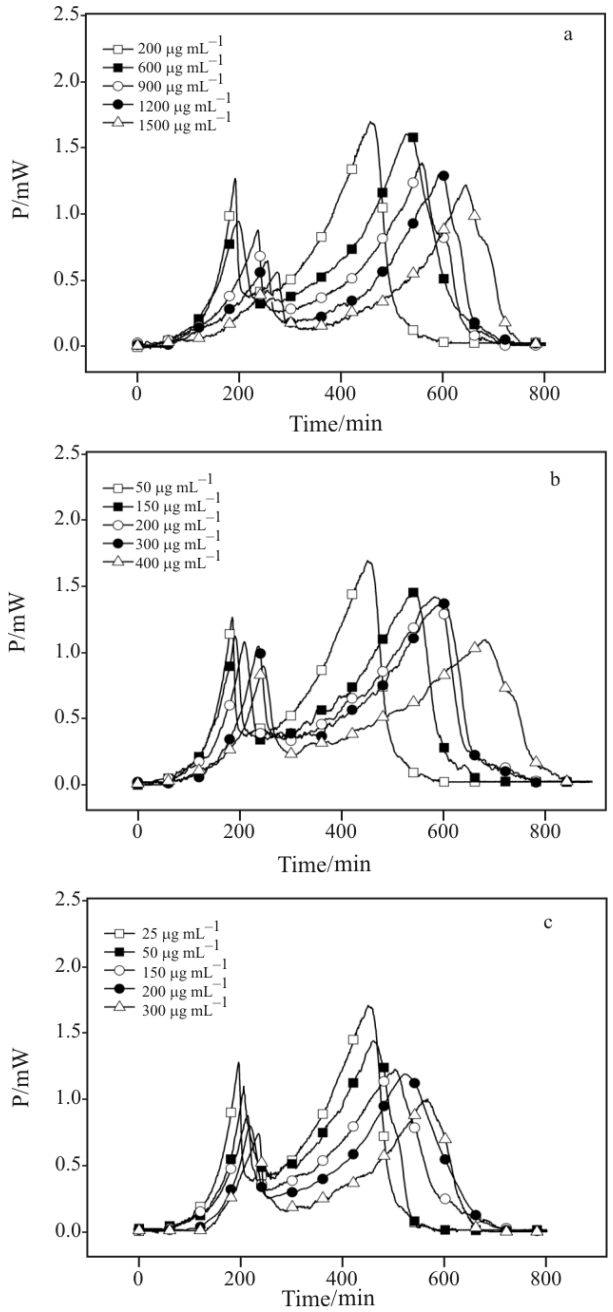

Fig. 3 *P–t* curves of *B. adolescentis* growth with different concentrations of BAs. a – berberine, b – coptisine, c – palmatine. *B. adolescentis* was cultured in the TPY culture medium supplemented with different concentrations of BAs respectively, and monitored by the microcalorimeter at 37°C

major group which could induce the action of BAs on *E. coli* and *B. adolescentis*. However, berberine, coptisine and palmatine have similar effect on *B. shigae*. It also suggests that the substituent groups at C2, C3, C9 and C10 almost have equal effect on *B. shigae*. Besides, the substituent groups all locate at six-membered ring, the spatial structure also shows specificity. It corresponds to the [19] that shows it is

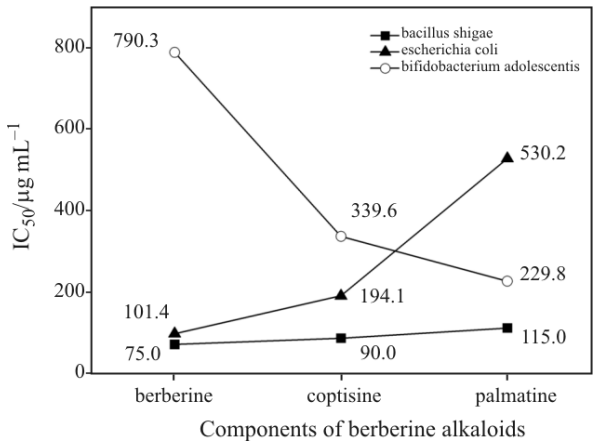

Fig. 4 Effect of BAs on three intestinal floras

the differential of bacterium that could be inserted into the specific molecule spatial structure.

Conclusions and simplified description of the method

In this study, we proposed a microcalorimetric method to assay the bioactivity of BAs on *B. adolescentis*. Compared with the cylinder-plate assay and turbidimetric assay, microcalorimetry not only offers a new way for the evaluation of bioactivity of drugs, but also provides more information about the microbial growth. By using it, the energy changes of four growing periods of *B. adolescentis*, which represent the regularity of microbial population growth, can be distinguished in the heat production curve because the values of P_{max}, t_p, k, as well as AUC (area under curve) determined simultaneously, the heat growing production and the metabolic process of microbes can be described dynamically and precisely.

Regarded as an essential feature of microcalorimetry, it was based on the universal heat exchange involving in all biochemical reactions. Characterized by two-dimension, it can reflect the microbe growth state more completely and directly. It can provide a new method for a wide range of drugs to determine the antibiotic activity. Especially it can supply thermogram as a profile characterized to describe the bioactivity of drug.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 30625042 and 30873385) and National Key Technology R&D Program in the 11th Five Year Plan of China (Grant No. 2007BAI40B05).

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DOI: 10.1007/s10973-008-9273-y