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Original Article

Degradation of dexamethasone by acclimated strain of Pseudomonas Alcaligenes

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Abstract: This study is to investigate the use of microbial remediation technology for degradation of dexamethasone in polluted water. A strain of Pseudomonas Alcaligenes with the ability of dexamethasone degradation was isolated from hospital polluted water. This strain was further acclimated into a bacterial strain that could highly degrade dexamethasone. Domesticated bacterial proteins were separated by osmotic shock method and were analyzed using SDS-PAGE. Enzyme activity of dexamethasone degradation was detected by high performance liquid chromatography. Protein bands with different molecular weight were found in all regions of the bacteria and a band with molecular weight of about 100 kDa was most obvious. In intracellular and periplasmic liquid, there was a band with molecular weight of about 41 kDa. Enzyme activity mainly existed in intracellular liquid. The 41 kDa protease was purified using ammonium sulfate precipitation, DEAE-52 ion exchange column and Sephadex G-100 column. Dexamethasone and dexamethasone sodium phosphate degrading rates of the purified enzyme were 36% and 95%, respectively. The 100 kDa protein had a 19% coverage rate to TonB receptor dependent protein, with 11 peptides matching. The 41 kDa protein had a 56% coverage rate to isovaleryl coenzyme A dehydrogenase, with 5 peptides matching. The 41 kDa protein had good degradation between the temperature of 25-40°C and PH value of 6.5-8.5. The enzyme kinetics equation was $Ct = C_0 e^{-0.1769t}$, in accordance with the first-order kinetic equation. This study laid the foundation for further preparation of bioremediation agents for clearance of dexamethasone pollution in water.

Keywords: Pseudomonas Alcaligenes, dexamethasone, degrading enzymes, extraction, purification, properties

Introduction

Dexamethasone is a synthetic long term glucocorticoid using natural hydrocortisone as synthetic material, belongs to steroidal compounds. Its chemical composition is $C_{22}H_{29}FO_5$ and it is structural stability, almost insoluble in water. Clinical commonly used dexamethasone preparation is the soluble dexamethasone sodium phosphate. Dexamethasone is a kind of hormone drugs widely used in the world scope in clinical for treatment of multiple diseases such as autoimmune diseases, allergies, inflammation, asthma, etc. Generally, dexamethasone medicine has no obvious adverse reactions while used within physiological dose. However, long-term or high-dose use of dexamethasone medicine can induce adverse reactions such as iatrogenic Cushing syndrome, immune suppression [1], aggravated status epilepticus [2], inhibited children's growth, glaucoma, cataract and so on.

In the application of dexamethasone drugs, the residual liquid drug can pollute the environment through various pathways, especially by hospital waste water. It also can be discharged from the body through secretion or urine [3], thus pollute extensive water environment. At present, hospital waste water disposal mainly adopts the methods of physics, biochemistry and disinfection, which, can remove wastewater pathogens, radioactive substances and heavy metals but could not effectively deal with hormone pollutants. Different levels of dexamethasone and other glucocorticoids pollut-

ants exist in hospital waste water, city sewage and river water [4]. Hormone in the environment can cause serious impact on biological and human health through food chain and bioaccumulation effect [5, 6].

In this study, based on a Pseudomonas Alcaligenes strain that can degrade dexamethasone sodium phosphate and dexamethasone isolated from hospital waste water [7] (namely degrading bacteria in this study), degradation relevant enzymes were extracted and purified from the bacteria. The characteristics were investigated for the preparation of biological purification agent of dexamethasone clearance and to provide experimental basis for eliminating drug adverse effects caused by clinical application of dexamethasone.

Materials and methods

Domestication of degrading bacteria

A Pseudomonas Alcaligenes strain (2012YZB) with the ability of dexamethasone sodium phosphate and dexamethasone degradation isolated by our laboratory and preserved in China Center for Type Culture Collection (CCTCCNO: M2014231) was used in this study. Dexamethasone sodium phosphate and dexamethasone degrading rates of this strain were 50.89% and 23.63% [7] respectively. In order to obtain a strain with higher dexamethasone degrading rate, the degrading bacteria was further acclimated according to Wang et al. [7]. Briefly, the preserved bacterium was cultured on LB agar at 37°C for 3 days and was then prepared into 4.2×10^6 cfu/ml bacterium fluid with dexamethasone sodium phosphate solution. After 1 ml bacterium fluid was added to 9 ml freshly made dexamethasone sodium phosphate, the bacterium was cultured at 37°C, 200 rpm/min for 10 days. Then, 1 ml bacterium fluid was added to 9 ml freshly made dexamethasone sodium phosphate and was cultured with the same condition. By cyclic training of this method for 10 times, the bacterium liquid was transferred to LB plate and was cultured at 37°C for 3 days. The bacterium was made into 4.2×10^6 cfu/ml bacterium fluid with dexamethasone sodium phosphate and 1 ml bacterium fluid was added to 9 ml freshly made dexamethasone sodium phosphate. Meanwhile, 10 ml dexamethasone sodium phosphate solution without bacteria was taken as control. Parallel experiments of 200 rpm/min and conventional incubator were taken at 37°C for 10 days. With newly prepared 280 g/ml dexamethasone sodium phosphate as standard solution, dexamethasone sodium phosphate solution contents in culture medium and control medium were determined by high performance liquid chromatography (HPLC).

Localization of acclimated bacteria enzyme activity

The acclimated bacteria was inoculated into LB agar, cultured at 37°C for 18 h, then an individual colony from the plate was inoculated in dexamethasone sodium phosphate culture medium and cultured at 37°C for 50 h to achieve best growth period for dexamethasone sodium phosphate degradation [7]. Each part of the domesticated bacteria protein fluid was extracted using osmotic shock method [8, 9]. Extracted proteins were detected by SDS-PAGE electrophoresis analysis and gel image analyzer. BCA kit (Beijing Dingguochangsheng Biotechnology Co.LTD, Beijing, China) was used to determine protein concentration in each part of the separated fluid.

The aforementioned protein separation was used for evaluation of enzyme activity in various parts of the domesticated bacteria. Dexamethasone was firstly dissolved in methanol and then prepared into 500 g/ml dexamethasone solution using pH7.5 Tris-Hcl buffer. A volume of 400 μl protein liquid from each part was taken and mixed with equal volume of dexamethasone solution. Then, the mixtures were incubated at 37°C for 24 h and the reaction was terminated at 100°C water bath. With double distilled water as control, dexamethasone contents in the control and protein solutions were detected by HPLC. Enzyme activity (i.e, at the condition of pH7.5 and 37°C, degradation of microgram amounts of dexamethasone in 1 min is 1 unit enzyme activity) was calculated. Specific activity (U/mg) namely enzyme activity unit per mg protein contains was calculated.

Extraction and purification of dexamethasone degrading enzyme

The above-mentioned experiment showed that dexamethasone degrading enzyme activity mainly existed in intracellular fluid, hence, only intracellular protein was extracted. HPLC meth-

od was used to determine the ammonium sulfate salting out optimum concentration and intracellular protein was salted out using the optimum concentration. After centrifugation, the protein was re-suspended with pH7.5 Tris-Hcl buffer and this was taken as crude enzyme. The crude enzyme was further purified using DEAE-52 and Sephadex G-100 chromatography and was analyzed by SDS-PAGE electrophoresis.

After purification, protein concentration was measured and dexamethasone degrading amount was analyzed with the same method above mentioned. Enzymatic activity and specific activity were calculated, enzyme purification factor, recovery rate and the total activity were compared. Enzyme purification factor was the ratio of specific activity after salting out and chromatography to protein activity of the extracted whole protein. Recovery rate was the ratio of total activity after salting out and chromatography to that of the extracted whole protein. Evaluation of degrading effect of dexamethasone sodium phosphate and dexamethasone of the purified enzyme was carried out using the same method as above mentioned.

Mass spectrometric analysis

In order to understand the main properties of domesticated bacterial proteins, the 100 kDa and 41 kDa bands showed by SDS-PAGE electrophoresis were tested by mass spectrometry. The mass spectrometric analysis results were blasted using MSCOT search engine. Matching rate score more than 61 points was considered credible, whereas matching rate score less than 61 points was not reliable [10].

Effects of temperature and pH value on the degrading reaction

Generally, temperature and pH value are the influence factors of enzyme activity, and in this study, these two elements were tested. PH4 to PH9 Tris-HCL buffer containing 200 g/ml dexamethasone with the interval pH value of 0.5 was prepared and 200 μL purified protein solution was added to equal amount of buffer. The mixtures were incubated at 37°C for 24 hours. Meanwhile, 200 μL distilled water was used as blank control and 3 parallel experiments were carried out at the same time. HPLC was used to detect dexamethasone degrading rates at different pH conditions.

PH7.5 Tris-HCL buffer containing 200 g/ml dexamethasone was added to equal amount of 200 μL purified protein solution and the mixture was incubated at 10 to 50°C with the interval of 5°C for 24 h. At the same time, 200 μL distilled water was used as blank control and 3 parallel experiments were performed. HPLC was used to detect dexamethasone degrading rates at different temperatures.

Effect of substrate concentration on the degrading reaction

To evaluate the influence of dexamethasone concentration on degradation of the purified protein, 400 μL different concentrations of dexamethasone solution were added to equal amount of 20 mg/ml purified protein solution respectively. Dexamethasone solutions were 10 to 80 μg/ml with the interval of 10 μg/ml. The mixtures were incubated at 37°C for 5 d and dexamethasone contents were measured each day. Degrading curve of degrading enzyme to dexamethasone was drawn, the curve was analyzed using SPSS statistical software, the dynamics equation was established, and then dynamics equation was analyzed by the SPSS statistics software.

Statistical analysis

All the statistical analyses were performed using SPSS version (SPSS Inc, Chicago, IL, USA) for windows. Linear correlation analysis was used for analyzing the curve and variance analysis method was carried for analyzing data of the experimental and control groups. $P < 0.05$ was considered as statistically significant.

Results

Domestication of degrading bacteria

To obtain degrading bacteria strain that with high degrading rate, multiple cycles of domestication was performed and HPLC was used to determine dexamethasone degrading rate. The degrading rate of domesticated bacteria to dexamethasone sodium phosphate increased from 50.89% [7] to 98.7% and the degrading rate to dexamethasone increased from 23.63% to 74.99%. As shown in **Table 1**, there was statistical significant difference in the degradation of dexamethasone sodium phosphate and dexamethasone before and after domestication ($P < 0.05$). Together, after acclimation,

Table 1. Comparison of degradation rates of dexamethasone sodium phosphate and dexamethasone of degrading bacteria before and after acclimation

Bacteria strain	Culture method	Degradation rates		
		Dexamethasone sodium phosphate (%)	Decompose into dexamethasone (%)	Decompose into other substrate (%)
2012YZB*	Shake culture	50.86	64.63	23.63
2012YZB	Routine culture	20.60	75.90	24.10
2013YZB*	Shake culture	98.7	25.01	74.99
2013YZB	Routine culture	73.8	57.33	42.67

Note: *There were statistically significant differences in 2012YZB and 2013YZB by shake culture and routineculture.

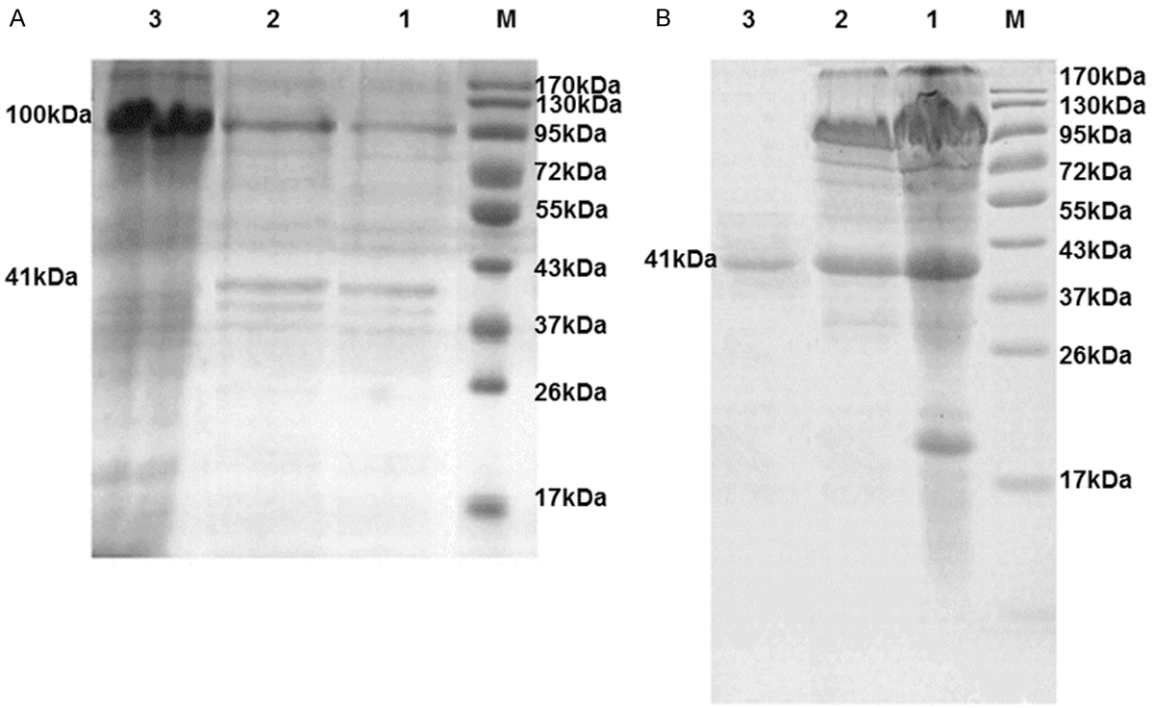

Figure 1. SDS-PAGE electrophoresis analysis of domesticated bacterial proteins in each part (A) and purified proteins of domesticated bacteria (B). (A) M: protein maker; 1: domesticated bacterial extracellular protein liquid; 2: domesticated bacterial periplastic protein liquid; 3: domesticated bacterial intracellular protein liquid. (B) M: protein maker; 1: ammonium sulfate salting out crude elution; 2: enzyme liquid after DEAR-52 chromatography; 3: enzyme liquid after Sephadex-100 chromatography.

degrading bacteria that with high degrading rate was successfully obtained.

Location of domesticated bacteria degrading enzymes

To determine the location of degrading enzymes in domesticated bacteria, extracellular, intracellular and periplasmic proteins were isolated by osmotic shock method. SDS-PAGE electrophoresis analysis showed that there were huge amount of different molecular weight of proteins in all the lanes and the band with molecular weight of approximately 100 kDa was most obvious. Additionally, there was an obvious band with molecular weight of about 41 kDa in intracellular and periplasmic protein liquid but was not seen in extracellular protein solution (**Figure 1A**). Dexamethasone degrading enzyme activity detection results showed that enzyme activity of intracellular protein liquid was the high, enzyme activity of periplasmic protein liquid enzyme activity was low and no enzyme activity was detected in extracellular protein liquid. These observations indicated that enzyme activity of domesticated bacteria existed mainly in intracellular protein fluid (**Table 2**).

Table 2. Enzyme activity in domesticated bacterial

Group	Degrading rate (%)	Enzyme activity (U)	Specific activity (U/mg)	Enzyme activity percentage (%)
Intracellular liquid	80.96	0.78	0.101	70.90
Periplasm liquid	45.61	0.320	0.004	29.09
Extracellular liquid	0	0	0	0

Extraction and purification of degrading enzyme

To understand the degrading rate of purified enzyme, degrading enzyme was extracted and purified from intracellular protein solution and enzyme activity related coefficients were measured. As stepwise chromatography was carried out, protein contents, total protein and enzyme activity gradually decreased, whereas specific activity increased significantly (**Table 3**). SDS-PAGE electrophoresis analysis showed that there was a large amount of proteins after ammonium sulfate salting out and only one protein band after Sephadex-100 chromatography. The molecular weight was about 41 KDa and the purity was about 97% (**Figure 1B**). HPLC showed that the degrading effects of the purified proteinase to dexamethasone sodium phosphate and dexamethasone were 37.0% and 96.5% respectively. Dexamethasone could be degraded by this enzyme directly and dexamethasone sodium phosphate could be degraded into dexamethasone at first and then be further degraded. In all, this suggested that we had obtained high purity proteinase with molecular of about 41 KDa and the enzyme activity increased significantly.

Identification of the main protein produced by domesticated bacteria

In order to exactly know the enzymes these played roles in enzymatic hydrolysis, the two main protein bands of 100 kDa and 41 kDa from acclimated bacteria were digested and analyzed by mass spectrometry. MSCOT search engine retrieval result showed a 19% coverage rate of the 100 kDa protein to TonB receptor dependent protein (EVT73323), with 11 peptides matching. The score was 792, $P < 0.05$, and the result was credible. The amino acid sequence of matching peptides was as follows: 1, TNTETPSPVQVVTR; 2, TFGNGFAGGGAGISLR; 3, VFTDLSTIPLDAVER; 4, DGASAIYGSDAIAGVVNIILR; 5, ATVLGPNHPDNPIPGQASR; 6, YSAWDVGPR; 7, VTNNTNEFNR; 8,IGDNANLNSQALYDYISPTISAR; 9, GGPLGLAIGTEWR; 10, APNPAENGDGGLAAFSNASDPVR; 11, WNITEAFELNASVANVFDR. The 41 kDa protein had a 56% coverage rate to isovaleryl coenzyme A dehydrogenase (WP-010487056), with 5 peptides matching. The result scored 273 with $P < 0.05$, and was identified as trusted. The amino acid sequence of matching peptides was as follows: 1, MHVPSLNFDLGEDIDLLR; 2, RHQFGEAIGSFQLIQGKI; 3, RAYVYAVARA; 4, KATWLTGQAIQILGGNGYINEYPTGRL; 5, KLYEIGAGTSEIRR. To sum up, the result showed that the two main proteins of 100 kDa and 41 kDa responsible for dexamethasone degrading reaction were TonB receptor dependent protein and isovaleryl coenzyme A dehydrogenase.

Influence of temperature and PH value on the degrading reaction

To identify the influence of temperature and PH value on degrading reaction, degrading enzyme was mixed with dexamethasone and incubated at different temperatures and PH values. At 37°C, degrading enzyme had degrading activity in Tris-HCL buffer with different pH values and the enzyme had high degrading rate to dexamethasonme between pH 6.5-pH 8.5 (**Figure 2A**). Under the condition of pH7.5, the degrading enzyme had a higher degrading rate to dexamethasone at 25-40°C (**Figure 2B**). The result showed that temperature and PH value had effect on the degrading enzyme.

Effect of substrate concentration on the degrading reaction

To test whether substrate concentration can affect degrading reaction of degrading enzyme, different concentrations of dexamethasone were prepared and incubated with degrading enzyme. Degrading curves were drawn according to dexamethasone contents at different times in dexamethasone solution of different concentrations (**Figure 3**). The results showed that the degrading activity was in accordance with exponential model of $C_t = C_0 e^{-kt}$ (**Table 4**), in which, C_t was the concentration of dexamethasone, C_0 was the initial concentration, K was kinetic parameters of dexamethasone degrading rate, half-life $t_{1/2}$ was $\ln^{2/k}$. There was significant statistical difference in dynamical

Table 3. Comparison of enzyme activity in degrading enzyme during purification process

Purification process	Protein content (mg)	Total enzyme activity (U)	Specific activity (U/mg)	Purification factor	Recovery rate (%)
Intracellular fluid	405.54	22.80	0.05	1.00	100.00
Ammonium ulfate salting out solution	124.50	14	0.11	2.2	61.4
DEAE-52 chromatography liquid	26.63	13.00	0.49	9.8	57.01
Sephadex-100 chromatography liquid	9.00	9.50	1.05	21.0	41.67

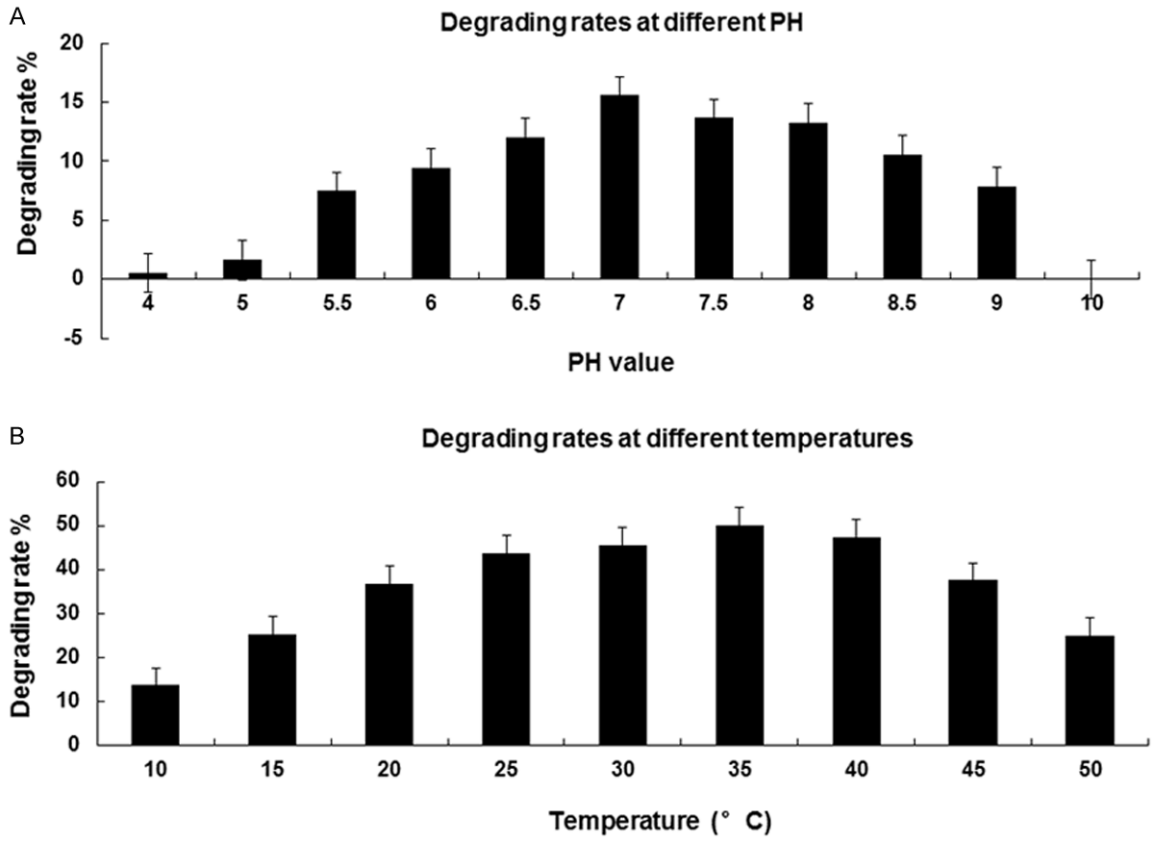

Figure 2. Effects of pH and temperature on enzymatic reaction.

equation while the initial dexamethasone concentration was more than 50.00 μg/ml ($P < 0.05$). However there was no significant statistical difference in dynamical equation with the initial dexamethasone concentration of less than 50.00 μg/ml ($P < 0.05$). Dynamics equation of degrading enzyme to dexamethasone under concentration of 50.00 μg/ml was got by the curvet fitting method. The dynamics equation was $Ct = C_0 e^{-0.1769t}$, and the half-life were 1.70 days.

Discussion

In this study, based on isolated dexamethasone degrading bacteria, domesticated strain with high dexamethasone sodium phosphate and dexamethasone degrading effect was obtained after long-term acclimation. Meanwhile, degrading enzyme was extracted and purified for further experiments. In early stage of this study, it was demonstrated that bacteria growth was affected after dexamethasone was dissolved in methanol and added to the culture medium. Therefore, only dexamethasone sodium phosphate was added to the bacterial culture medium. In studying the activity of degrading enzyme, both dexamethasone sodium phosphate and dexamethasone were used for degrading evaluation. The results showed that degrading enzyme could degrade dexamethasone directly and dexamethasone sodi-

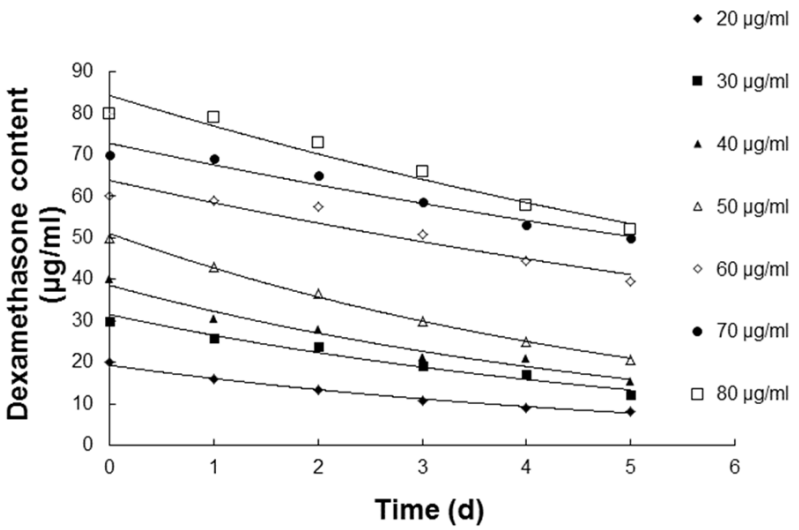

Figure 3. Degradation of degrading enzyme to different initial concentrations of dexamethasone.

Table 4. Degradation kinetics of degrading enzyme to different initial concentrations of dexamethasone

Initial concentration (μg /ml)	First-order kinetic equation	Correlation coefficient R^2	Parameter K	Half-life (d)
80	$C = 88.062\ e^{-0.1005t}$	0.9453	0.1005	2.99
70	$C = 74.441\ e^{-0.0797t}$	0.9812	0.0797	3.77
60	$C = 66.494\ e^{-0.0983t}$	0.9594	0.0983	3.70
50	$C = 50.739\ e^{-0.1765t}$	0.9967	0.1765	1.70
40	$C = 38.588\ e^{-0.1776t}$	0.9629	0.1776	1.69
30	$C = 31.863\ e^{-0.1749t}$	0.9617	0.1749	1.72
20	$C = 19.03\ e^{-0.1786t}$	0.9883	0.1786	1.65

Note: There were no statistically significant differences with concentrations of substrate under 50 μg/ml.

um phosphate was degraded into dexamethasone at first and then into other substances. The purified enzyme had the same degrading effect to the domesticated bacteria, confirming that the degrading enzyme extracted was the protease responsible for dexamethasone sodium phosphate and dexamethasone degradation.

In this study, to identify the location of degrading enzymes, osmotic shock method was used for extraction of intracellular, periplasmic and extracellular proteins [9, 10]. The result showed high enzyme activity in intracellular protein solution, less enzyme activity in periplasmic protein solution and no enzyme activity in extracellular protein solution. It is possible to assume that dexamethasone degrading enzyme was a kind of endoenzyme, therefore, intracellular fluid was used for bacteria degrading enzyme extraction.

By SDS-PAGE electrophoresis analysis, it was found that in addition to an apparent molecular weight of about 41 kDa protein band in intracellular and periplasmic fluid but not in extracellular protein fluid, the remaining protein bands had no obvious difference. There was a distinct protein band with the molecular weight of about 100 kDa in all parts of protein fluid. Mass spectrometry identification results showed that the amino acid sequences of 100 kDa protein and TonB had 11 peptides matching, suggesting that 100 kDa protein might have a similar function to TonB dependent receptor protein. TonB dependent receptor is the key protein in gram negative bacteria responsible for nutrients transportation. A large amount of 100 kDa protein was found in all regions of the bacteria, indicating that dexamethasone and dexamethasone sodium phosphate were transported to intracellular part via 100 kDa protein in domestication bacteria. Dexamethasone and dexamethasone sodium phosphate are then degraded by degrading enzyme [11]. The 41 kDa protein which existed mainly in intracellular fluid and isovaleryl coenzyme A dehydrogenase had 5 peptides matching. It is possible to assume that the 41 kDa protein might have a function similar to isovaleryl coenzyme A dehydrogenase. Tanaka K et al. [12] found that with flavin adenine dinucleotide (FAD) as an auxiliary factor, isovaleryl coenzyme A dehydrogenase can dehydrogenize isovaleryl coenzyme A into 3-methylcrotonoyl coenzyme A. Förster et al. [13, 14] also found isovaleryl coenzyme A dehydrogenase in Pseudomonas aeruginosa plays a key role in organic matter use. It is possible that the purified dexa-

methasone degrading enzyme might degrade dexamethasone and dexamethasone sodium phosphate by dehydrogenation. Common steroidal compounds degrading enzyme like 3-α hydroxy steroid dehydrogenase is steroid dehydrogenase [15]. In this study, no amino acid sequence matching was found, hence, the purified enzyme might be another kind of steroidal compounds degrading enzyme.

Degrading kinetics research result showed that the degrading kinetic equation of degrading enzyme to dexamethasone was exponential model. Under dexamethasone initial concentration below 50 μg/ml, the enzymatic reaction was $Ct = C_0 e^{-0.1769t}$, the half-life was 1.70 days, with no significant difference between the dynamics equation. This enzyme had good degrading effect between pH 6.5-pH 8.5 and 25°C-40°C, therefore, had application value in clearance of dexamethasone polluted water.

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Disclosure of conflict of interest

None.

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