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IN VITRO ACTIVITY OF CYP2J RECOMBINANT PROTEASE FROM BACTRIAN CAMEL

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ABSTRACT

The purpose of this experiment was to study the *in vitro* activity of recombinant protease CYP2J of Bactrian camel. The activity of CYP2J enzyme was determined by fluorescence method according to the change of fluorescence peak after the conversion of ethoxycoumarin to hydroxycoumarin in NADPH generation system and ethoxycoumarin deethylase reaction system. Then, the affinity of the recombinant protease with its specific substrates arachidonic acid (AA) and astemizole was detected by localised surface plasmon resonance (LSPR) technique, and its activity was further determined. The results showed that the standard curve is y=17 533x+190.73 and R^2=0.998; The fluorescence detection result of the repeated experiments was 0.1350±0.0251 nM/min/mg, and the significant value between the repeated experiments was greater than 0.05, indicating that the difference was not statistically significant. The affinity of the recombinant protease for arachidonic acid was 3.53×10^{-5} M and that for astemizole was 4.07×10^{-5} M, respectively. Therefore, the Bactrian camel CYP2J recombinant protease has sufficient stable activities *in vitro* and can meet the basic requirements of further research.

Key words: Bactrian camel, CYP2J, *in vitro* activity, recombinant protease

CYP2J enzyme is the most abundant in cytochrome P450s, and its content accounts for 1/3 of the total enzyme content of CYP family. Mainly involved in the metabolism of most exogenous and endogenous substances including drugs and environmental compounds. CYP2J is widely distributed in different tissues of Bactrian camels, and mainly existed in digestive and metabolic organs such as small intestine, pancreas and liver (Maayah *et al*, 2019; Lu *et al*, 2020). Therefore, it is speculated that these unique biological characteristics of Bactrian camels are related to the specific CYP2J enzyme. The activity of the recombinant protease can be effectively detected by using the change of fluorescence peak after ethoxycoumarin is converted into hydroxycoumarin and ethoxycoumarin deethylase in NADPH generation system (Messina *et al*, 2010).

LSPR is a surface plasmon resonance technology that has been widely used in such fields as biomolecular interaction, proteomics, drug screening and real-time monitoring of related pharmacokinetics (Park *et al*, 2021). This method can obtain important information such as biomolecular interaction, interaction between different drugs or drug modified structures and biomolecules, the speed of molecular interaction and separation (Acimovic *et al*, 2014), when molecular interaction reaches equilibrium, and magnitude of interaction force in real time without labeling.

Materials and Methods

Recombinant protease

Bactrian camel CYP2J gene was expressed by *E. coli* prokaryotic expression system, purified and stored in the refrigerator at -80°C in laboratory (Jia, 2018).

Main reagents and instruments

In vitro fluorescence quantitative detection kit for cytochrome P450 sub-enzyme CYP2J (ECOD) activity: GENMED Scientifics Inc. USA; Tris-HCl buffer and PBS buffer: Solarbio company; Multifunctional microplate reader: Biotek Synergy H4 Hybrid Reader, USA; Open SPR biomolecular interaction analyser: Restar Communications Corporation, Japan; Arachidonic acid standard (SA9940-20mg): Solarbio company; Astemizole standard (YZ100301-50mg): National Institutes for Food and Drug Control.

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Determination of standard curve

The reaction conditions were set as follows: temperature 37°C, excitation wavelength of 370 nm and emission wavelength of 450 nm. According to table 1, prepared 20μL standard solutions with different concentrations, then 155μL buffer solution, 20μL reaction solution and 5μL substrate solution were sequentially added and mixed, incubated them at 37°C in the dark for 30 min, added stop solution and incubated for another 5 min for detection.

Determination of total activity of CYP2J recombinant protease

Added 20μL reaction solution, 5μL substrate solution and 20μL sample to 155μL buffer successively and mixed thoroughly. Incubated at 37°C for 30 min, and then added 75μL stop solution, and incubated for another 5 min in the dark. The relative fluorescence unit of the total activity of the sample was determined. The concentration of hydroxylcoumarin in the enzyme was obtained according to the standard curve.

Determination of nonspecific activity of CYP2J recombinant protease

20μL colourimetric solution, 20μL reaction solution, 5μL substrate solution and 20μL sample were added sequentially to the 135μL of buffer solution, and mixed thoroughly. Incubated at 37°C for 30 min, added 75μL of stop solution, then incubated again for 5 min. The relative fluorescence unit of nonspecific activity of the sample was detected. The concentration of hydroxylcoumarin in the enzyme was obtained according to the standard curve.

Detection of interaction between CYP2J recombinant protease and its specific substrates by LSPR

Sample pretreatment

The CYP2J recombinant protease was 0.6mg/mL and its molecular weight was 57.98 kDa. The specific substrates were arachidonic acid (AA) with molecular weight of 304.47Da and astemizole with molecular weight of 458.57Da. Standards were diluted with anhydrous ethanol to 10, 20, 40 and 100 μM.

Experimental process

The NTA chip was installed according to the standard operating procedure of OpenSPR instrument, and the PBS buffer (pH7.4) was run at the 150 μL/min. After reaching the signal baseline, 200μL 80% IPA(isopropanol) was added and the reaction temperature was 25°C. After running for 10s, the sample loop was flushed with buffer, then the flow rate of buffer was adjusted to 20 μL/min. The NTA chip was charged by Ni^{2+} ions by adding 200 μL of 40 mM $NiCl_2$ solution. 200 μL of CYP2J recombinant protein was injected and run for 4min. Then the samples were loaded at different concentrations of specific substrates to observe the binding and dissociation time of the enzyme and substrates.

Table 1. Preparation of standard tube concentration.

Centrifuge tube	buffer solution	Standard solution	Determination system standard hydroxycoumarin concentration
1	25 μL	25 μL	1 μmol/L
2	25 μL	25 μL Centrifugal tube No. 1	0.5 μmol/L
3	25 μL	25 μL Centrifugal tube No. 2	0.25 μmol/L
4	25 μL	25 μL Centrifugal tube No. 3	0.125 μmol/L
5	25 μL	Blank control	0

Result

Specific activity of CYP2J recombinant protease

According to the standard curve, the total activity and non-specific activity values of the recombinant protease were obtained by the above detection, and the specific activity of the recombinant protease was further obtained by calculation (Messina, 2010).

Sample activity = [corresponding hydroxycoumarin concentration × 0.2 × sample dilution times] ÷ [0.02 × reaction time] ÷ concentration of recombinant protein

Specific activity of sample = total activity of sample - nonspecific activity of sample

Table 2. Standard curve parameters.

Concentration of standard hydroxycoumarin	1	0.5	0.25	0.125	0
RFU (actual relative fluorescence unit)	17739	8683	5099	2307	0

Construct a standard curve: the vertical coordinate (y axis) is the actual relative fluorescence unit (RFU); The abscissa (x axis) is the standard hydroxycoumarin concentration (μmol/L) (Table 2). The standard curve is y = 17 533x+190.73, R^2 = 0.998

(Fig 1). The correlation coefficient is close to 1, and the agreement between theory and practice is high.

Three repeated experiments were conducted to determine and compare the specific activities of protease. The results are shown in table 3. The specific activities of the three groups were as follows: 0.1333± 0.0175, 0.1450±0.0214 and 0.1267±0.0342, respectively. After the significance test, P=0.467, which was much higher than 0.05, indicated that there was no significant difference between groups. The total average value of the three groups was 0.1350±0.0251. Therefore, the Bactrian camel CYP2J recombinant protease obtained by *E. coli* prokaryotic expression system had certain *in vitro* activity.

Detection of interaction between CYP2J recombinant protease and its specific substrates by LSPR

It could be seen from the Fig 2 that the recombinant protease of bactrian camel CYP2J could be well combined with AA and astemizole, with smooth curve and high coincidence. Local module and one-to-one analysis model were used to determine the binding rate constant of AA and CYP2J was Ka=6.55×10^{-1} $Lmol^{-1}s^{-1}$, dissociation rate constant was Kd= 2.31×$10^{-3}$$Ls^{-1}$, and affinity constant

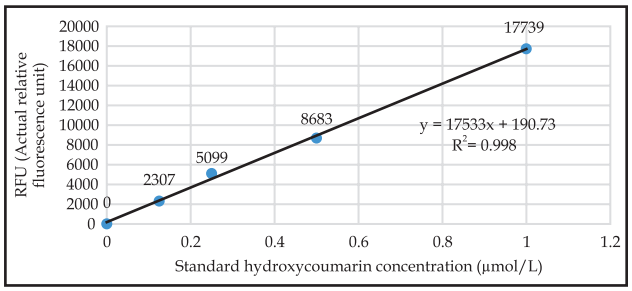

Fig 1. Standard curve.

was KD=3.53×10^{-5}M. After Trace Drawer analysis, the binding curve of AA and CYP2J recombinant protease had good waveform and the kinetic fitting accuracy was high. The results showed that AA had a good affinity with CYP2J recombinant protease, and the activity of CYP2J recombinant protease was stable.

Table 3. Comparison of specific activity and significance of repeated experiments in three groups.

Repeat group	1	2	3
Specific activity	0.1 333± 0.017 5	0.1 450± 0.021 4	0.1 267± 0.034 2
Significance test	0.467		
Total average	0.1 350±0.025 1		

Table 4. Interaction of CYP2J recombinant protease with arachidonic acid and astemizole.

Substrate	Ka(1/m*s)	Kd(1/s)	KD (M)
AA	6.55×10^{-1}	2.31×10^{-3}	3.53×10^{-5}
Astemizole	4.04×10^{-2}	1.64×10^{-2}	4.07×10^{-5}

Ka: Binding rate constant: Kd: Dissociation rate constant: KD: Dissociation equilibrium constant, also called affinity constant.v

As an exogenous specific substrate of CYP2J, astemizole could combine well with the recombinant protease of CYP2J in Bactrian camel. The binding rate constant was Ka=4.04×10^{-2} Lmol-$1s^{-1}$, the dissociation rate constant was Kd=1.64×10^{-2} Ls^{-1} and the affinity constant was KD=4.07×10^{-5} M.These parameters were obtained by fitting with the global module and one-to-one analysis model. Through the analysis of Trace Drawer, The smooth curve and good fitting directly indicate that astemizole had good affinity with recombinant protease. The activity characteristics of the recombinant protease *in vitro* of bactrian camel CYP2J are well represented.

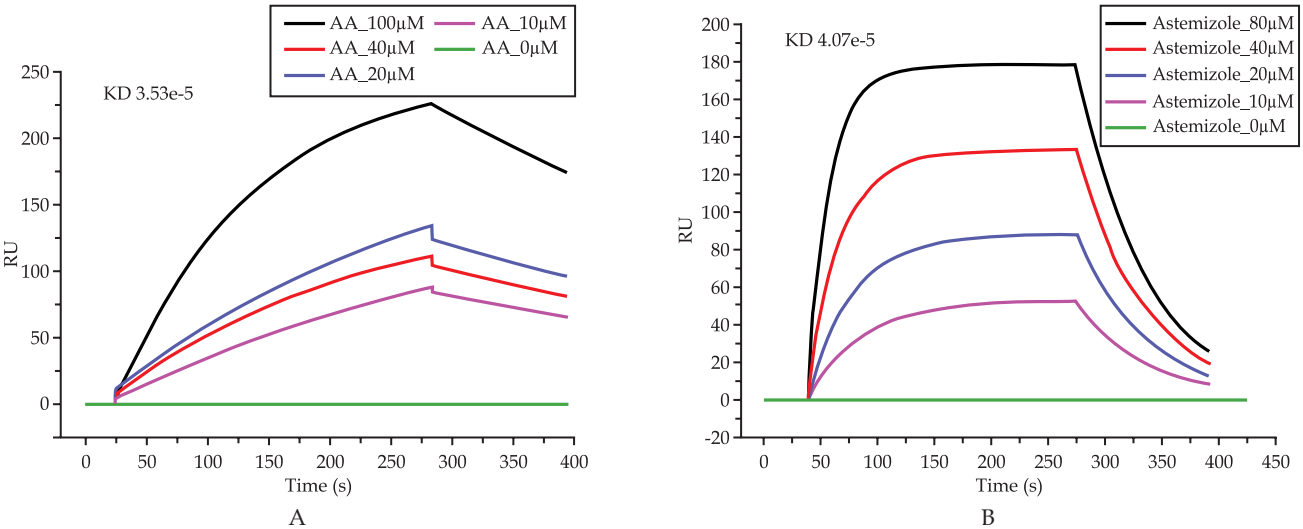

Fig 2. The interaction of CYP2J recombinant protease with AA (A) and astemizole (B) was detected by LSPR.

Discussion

Current studies had shown that the CYP2J subfamily genotypes were detected in different animals included, i.e. rabbit CYP2J1, rat CYP2J3/ 4, Mouse CYP2J8/ 11/ 12/ 13 (Graves *et al*, 2013), Human, ape, and cattle CYP2J2 (Lu *et al*, 2020). The substrates and reactions of CYP2J mainly included aniline hydroxylation, aminopyrine demethylation, ethoxycoumarin deethylation, testosterone hydroxylation, all-trans retinoic acid oxidation, astemizole-O-demethylation, diclofenac hydroxylation, arachidonic acid epoxidation and so on. The activity of ethoxycoumarin deethylase was a diagnostic marker of CYP2J (Hanif *et al*, 2017). The activity of cytochrome P450 subenzyme 2J was quantitatively determined by the change of fluorescence peak after the conversion of cytochrome P450 subenzyme to hydroxylcoumarin catalysed by ethoxycoumarin deethylase in the presence or absence of danazole, the sensitive inhibitor, and to screen inducers and inhibitors (Messina *et al*, 2010).

Bactrian camel CYP2J protein was mainly distributed in the small intestine, pancreas, liver, heart and other important organs, with anti-inflammatory, vasodilation, relaxation of smooth muscle, promote angiogenesis and other functions (Hasi *et al*, 2018; Peng, 2019). It mainly acts on aminopyrine, all trans retinoic acid, arachidonic acid and its derivatives prostaglandins, leukotrienes and epoxy eicosatrienes, among others. It could also oxidised and metabolised other exogenous compounds included astemizole, ebastine, terfenadine, albendazole, amiodarone, phentermine and diclofenac, etc. Thereby regulated hormone secretion, vascular tension, renal microtubule secretion, etc (Kang *et al*, 2011). CYP2J was also highly expressed in tumour tissues, which promoted tumour growth and reproduction. Abnormal CYP2J might lead to pathological conditions such as vascular diseases, tumour manifestations, inflammation, hormone secretion variation and renal microtubule filtration failure (Yu *et al*, 2000). Therefore, in this experiment, the activity of recombinant protease CYP2J of Bactrian camel was detected *in vitro*, so as to provide convenience for the later research of disease experiment or clinical medication.

In order to better confirm the activity of the recombinant protease, the specific activity of the recombinant protease was detected by conventional fluorescence method. According to the repeated tests, it was confirmed that the recombinant protease was relatively completely expressed and had good activity. Since CYP2J is widely distributed and abundantly expressed in the body, participating in endogenous and exogenous multiple metabolism, the specific substrates unique to CYP2J are selected to further use from both endogenous and exogenous substrates (Messina *et al*, 2010; Arnold *et al*, 2017), and the activity of recombinant protease can well be expressed by the sensitive reaction and intuitive effect of LSPR.

Arachidonic acid was one of the essential polyunsaturated fatty acids, which had many biological functions and participates in multiple metabolic reactions in the animal body, and was a specific substrate of CYP2J enzyme. Therefore, detecting the affinity of CYP2J recombinant protease and arachidonic acid could well show that the constructed recombinant protease could be used for later research and had a certain effect (Kamel *et al*, 2018; Olivares-Rubio and Espinosa-Aguirre, 2020). As a representative of exogenous CYP2J metabolism, astemizole affinity test not only could prove the activity of the recombinant protease, but also showed that the recombinant protease constructed could play a highly similar role with the protease actually existing *in vivo* (Lu *et al*, 2020; Matsumoto *et al*, 2002).

Localised surface plasmon resonance was generated by metal nanoparticles, typically gold and silver, as continuous thin films of gold. LSPR produced a strong resonance absorption peak in the visible light range, and the location of which was highly sensitive to the local refractive index around the particle (Chen *et al*, 2020). This technique only needs a small number of samples, which greatly saves the cost (Faridfar *et al*, 2020). The experiment could be easily repeated by reproducible sample injection and accurate results were obtained from reproducible measurements.

In conclusion, the recombinant protease was transformed into hydroxycoumarin under the catalysis of ethoxycoumarin deacetylase in NADPH production system. According to the change of fluorescence peak, the activity of recombinant protease was confirmed to be good, and the specific endogenous and exogenous active substances were further detected by LSPR technology. Therefore, the recombinant protein expressed in *E. coli* showed good activity, which could provide the basis for further research or clinical application.

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