Improvement of Desferrioxamine B Production of *Streptomyces pilosus* ATCC 19797 With Use of Protease Inhibitor and Minerals Related to Its Activity

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Received: 12 November 2011 / Accepted: 22 February 2012 / Published online: 24 March 2012
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Abstract The relationship between protease and Desferal production was assayed. Experiments were performed using a cultivation of *Streptomyces pilosus* ATCC 19797 in soybean broth medium containing 2% soybean flour and 2% mannitol. The metabolism of the trihydroxamic acid siderophore desferrioxamine B and protease production by a *S. pilosus* in nine continues days after culture were investigated as well as the effect of protease inhibitors was examined. It is found that the Desferal formation decreased with increased protease production. Also the effect of protease inhibitors and minerals in determined day of protease production in the culture medium by *S. pilosus* has been investigated.

Keywords Desferrioxamine B production · Protease · *Streptomyces pilosus*

Introduction

Desferrioxamine B (DFB) is produced by *Streptomyces pilosus* and act as siderophore. Desferal® is the only siderophore substance which is currently commercially available (Novartis Pharma AG, Basel, Switzerland). It is produced by the fermentation of *S. pilosus* and is used in the therapy of patients with iron and aluminium overload.

Also desferrioxamine has antiproliferative activity against leukemia and neuroblastoma cells in vitro and in vivo and has shown anti tumor activity in clinical trails. Desferal, the methane sulfonate salt of DFB, has been used with some success in the treatment of acute accidental iron poisoning and iron overload disease.

In this investigation the effect of protease on Desferal® is investigated for nine continues days of culture. The protease activity and DFB production were assayed in each day. Additionally, we investigated the effect of minerals that play a role in DFB secretion to external environment and protease activity.

Proteolytic enzymes that depend on serine residue for their catalytic activity are commonly spread in nature. The role played by protease in degradation of DFO has already proved. How siderophores are degraded and returned to the carbon and nitrogen cycles is not well understood. The catalytic activity of an enzyme from a bacterium that degrades the siderophore DFB has been examined. The data presented is most consistent with the hypothesis that the enzyme uses a hydroxyl moiety (serine peptidase) to catalyze the degradation of DFB. Analysis of the inhibitor experiments conducted led to the conclusion that the DFB degrading enzyme is a serine-peptidase-like enzyme [1, 2].

Materials and Methods

Materials

Casein for protease assay was purchased from Gibco and Desferal (desferrioxamine mesylate) was provided by CIBA Pharmaceuticals; all other components and chemicals were used of high purity grade available commercially from Merck.

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Microorganism

Streptomyces pilosus ATCC 19797 was obtained lyophilized.

Culture Condition

Protease production in batch culture of *S. pilosus* was studied. The bacterial strain was cultured in 250 ml of Erlenmeyer flasks containing 100 ml soybean broth medium consisting (g/l): soybean flour, 20; D-mannitol 20; the initial pH of the medium was adjusted to about 7.5 with sterile sodium hydroxide solution. Erlenmeyer flask experiments were carried out at 29°C and 150 rpm for 9 days, ten Erlenmeyer flask for each days and control separately. The cells at each day were removed from the culture medium by gentle filtration with 0.22 µm filters and were dried in incubator and the its pH were determined.

Each of the ten flasks was removed at the determined days and analyzed the protease production and residual DFO was estimated. The contents of each flask were centrifuged at 4°C and this supernatant was used as the source of Desferal or crude enzyme. The culture was subjected to centrifugation (4000×g for 10 min to remove cells).

Determination of Produced Desferrioxamine B

The amount of DFO was estimated calorimetrically at 430 nm [3, 4]. The standard curve was provided for Desferal. The assay was applied an iron (III) chloride assay consisted in adding 0.9 ml of filtered medium or supernatant medium to 0.1 ml of FeCl₃ reagent (0.1% v/v iron chloride), then measuring the absorbance (430 nm) using a 1 cm quartz cell was used.

Protease Activity by Agar Plate Assay

The casein plate assay was used as test for the presence of alkaline protease. An agar casein medium was used for the protease activity assay. The samples of cultured medium cell free were applied onto a Luria–Bertani (LB)-agar medium containing 1% casein and incubated overnight [5].

Protease Assay

The alkaline serine protease of *S. pilosus* was characterized spectrophotometrically by the casein-Folin phenol method [6, 7]. The protease was assayed at 37°C in 100 mM tris-base buffer. One milliliter of appropriately diluted enzyme was incubated with 1 ml of 0.5% casein solution (prepared in tris base buffer) for 10 min at 37°C. The reaction was arrested by the addition of 200 µl of 5% trichloroacetic acid (TCA). The contents were centrifuged after 15 min at

13,000 rpm for 15 min and total protease yield was determined by Lowry's method. One unit of alkaline protease activity was equivalent to the amount of enzyme required to release 1 µg of tyrosine per min per ml under the standard assay conditions.

Effect of Various Metal Ions and Inhibitor on Desferal Production

The effect of protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), on DFB content of cultured medium was assayed. The medium was inoculated with inhibitor and metal ions and incubated at 37°C for 5 days at a given concentration [8, 9]. For determining the influence of metal ions, the following salts were used: CaCl₂, ZnSO₄, MgSO₄, MnCl₂, FeSO₄, and Na₂HPO₄. The produced Desferal was then measured as described above. The activity without inhibitors or metal ions was considered to be 100% (Table 1).

Results and Discussion

pH Variation During Protease Production

The medium pH declined from 7.5 to 6.7 during 5 days fermentation to be the end of exponential growth phase and then the pH started to rise and at 9 days old cultures pH reached to 8.2. This decline of medium pH might be due of acid production from glucose utilization during growth phase and rate reduced, pH started to rise. This pH variation during fermentation is a good indicator of the state (start or end) of protease production. A rise in culture pH due to production of free amino protease fermentation has been reported before [10].

Table 1 Effect of metals and inhibitor on protease activity

Inhibitor	Concentration (mM)	Residual activity (%)
None	–	100
PMSF	0.5	70
	1	40
	2	20
Minerals		
CaCl ₂	2	125
ZnSO ₄	2	131
MgSO ₄	2	121
MnCl ₂	2	112
FeSO ₄	2	60
Na ₂ HPO ₄	2	108

Protease Plate Assay

Assaying for alkaline protease production of the *S. pilosus* was done by casein plate assay. For casein plate assay, only five samples out of 9 days of culture of bacteria were positive for alkaline protease activity. The samples of 3–7 days old cultures digested the casein in agar medium and showed clear zone around the wells contain them.

Comparison of Desferal and Protease Production

The result of protease production during bath culture in compare with DFB of *S. pilosus* has been shown in Fig. 1. The protease begins to produce at third days. When the carbon source, glucose, was completely utilized by bacterial cells, the protease production lightly increased. As the maximum alkaline protease was produced during the stationary and death phase, it is considered as a partially growth associated product. The result showed that the maximum protease activity were 900 IU/ml in days fifth. Then protease activity was decreased to 580 IU/ml in day seventh.

We assay the DFB production in 9 days and it was defined that its concentration in the medium is highest in fifth day of culture, when protease also is at highest concentration. With regard this, it is the best point for adding protease inhibitor or decries the pH for inhibit alkaline protease activity to extend the culture duration for DFB purification.

Effect of Some Chemicals and Inhibitor on Desferal Production

It seems that the metal ions concentration affects the protease by *S. pilosus*. Also it possibly affects the release of Desferal directly from bacteria in culture medium [11].

Fig. 1 Desferal produced by *S. pilosus* cultured in soybean medium and protease activity

Minerals or inhibitor were added in determined day of protease production and assayed DFO production in such medium. Table 1 shows the effect of various minerals and protease inhibitor on the DFB production of *S. pilosus* (Table 1). These result indicated that DFB was enhanced in the presence of most metal ions such as Ca^{2+} , Zn^{2+} , Mn^{2+} , Mg^{2+} and Na^{+} , whereas the DFB production was decreased in the presence of Fe^{2+} . The cystine protease inhibitor, PMSF, has significant effect on enzyme activity. PMSF were tested at the following concentrations: 0.5, 1 and 2 mM. The protease inhibition profile showed that the enzyme activity was 60 and 80% inhibited by 1 and 2 mM PMSF respectively.

Conclusion

Our aim of this study was to increase the yield of DFO production by *S. pilosus* in soybean medium with inhibition of protease activity or mineralization of the culture medium. In this study we attempted to increase the DFO production with use of protease inhibitor, PMSF, and minerals that effects on protease activity.

Protease production at first was characteristics in plate and quantitatively assays were performed at nine continues days. Desferal production *S. pilosus* was determined using 9 day-old cultures. It is shown that production of Desferal is elevated (induced) in minerals addition. The effect of different minerals was studied in mannitol-soybean media (CaCl_2 , ZnSO_4 , MgSO_4 , MnCl_2 , FeSO_4 and Na_2HPO_4). Supplementation with Ca^{2+} , Zn^{2+} , Mn^{2+} , Mg^{2+} and Na^{+} improved the production of Desferal in media. However, the DFO production increased by Ca^{2+} , Zn^{2+} , Mn^{2+} , Mg^{2+} and Na^{+} , indicated that the enzyme might inhibited by metal ions for its activity. FeSO_4 reduced the DFO production by 40%. It was shown previously that iron has negative effect of DFO production because its effect on iron-regulated gene expression that exists in *S. pilosus* [12].

The protease was strongly inhibited by PMSF. The medium containing protease inhibitor provides more Desferal B production. This study indicates that dissimilation of DFO by *S. pilosus* is correlated with protease production and it is maybe for protease activity. Deferrioxamine B was rapidly (degrade) by protease. The rise in culture pH is due to production of free amino protease fermentation.

Acknowledgment This work was supported by Pasteur institute of Iran.

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