Vol. 120, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1984 Pages 45-51

PROLINE HYDROXYLATION BY CELL FREE EXTRACT OF A STREPTOMYCETE

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Received February 23, 1984

SUMMARY: Free L-proline was hydroxylated to free L-hydroxyproline by cell free extract of Streptomyces griseoviridus P8648. The hydroxylation reaction required ferrous ion, 2-ketoglutarate and ascorbate. Zinc ion, ethylenediaminetetraacetic acid and α,α'-dipyridyl inhibited the reaction. Optimum temperature and pH were 25.0 °C and 7.5, respectively.

Hydroxyproline is found in a certain protein such as collagen and in some peptide antibiotics such as actinomycin[1] and viridogrisein(= etamycin)[2]. In animal system, the biosynthetic mechanism of hydroxyproline is well established[3]. In the system, only peptidyl proline is hydroxylated to peptidyl hydroxyproline by an enzyme named proline, 2-oxoglutarate, dioxygenase. The enzyme requires 2-ketoglutarate, ferrous ion, ascorbate and atmospheric oxygen as co-factors. On the other hand, the formation mechanism of hydroxyproline in microorganism has not been clarified.

We reported previously that S. griseoviridus P8648 produced neoviridogrisein II, one of viridogrisein(=etamycin) analogs together with viridogrisein[4]. The former contains D-proline instead of D-hydroxyproline(allo) in the latter. It was also revealed that both imino acids were originated from L-proline, and that the direct precursor of the hydroxyproline was L-hydroxyproline. Furthermore, the production ratio of neoviridogrisein II to viridogrisein was affected by the addition

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ABBREVIATIONS: TES, N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TLC, thin layer chromatography; SDS, sodium dodecyl sulfate.

0006-291X/84 $1.50

of ferrous ion, zinc ion and α,α'-dipyridyl, which are known as effectors for prolyl hydroxylase in animal system[5),6)]. Additionally, Katz et al. reported that when S. griseoviridus P-D04955, one of viridogrisein producers, was cultured in the presence of L-[^{14}C-(U)]proline, free form of L-[^{14}C-(U)]hydroxyproline was formed in the medium[7)]. These phenomena suggested that these streptomycetes had proline hydroxylating ability.

For the sake of clarification of the hydroxyproline formation from proline in these streptomycetes, we attempted to isolate the proline hydroxylating enzyme. This paper deals with the cell free extract containing the hydroxylation activity from S. griseoviridus P8648, and some properties of the hydroxylation reaction.

Materials and Methods

Materials: L-[^{14}C-(U)]Proline(250 mCi/mmole) was purchased from New England Nuclear(Boston, MA). Amino acids used as standards were obtained from Sigma Chemical(St. Louis, MO). DE-81 Cellulose papers were the products of Whatman Chemical Separation(Clifton, NJ). Cellulose thin layers were purchased from Funakoshi Pharmaceutical(Tokyo, Japan). Other chemicals were of the highest quality commercially available.
Microorganism and Cultivation: S. griseoviridus P8648 was used in this study. Cultivation methods were described previouly[5)].
Preparation of cell free extract: Unless stated otherwise, all the procedures were carried out at 0°C. Mycelia were harvested from 1 liter broth by filtration and washed two times with 0.9% saline. The washed mycelia(62 g wet weight) were suspended in 80 ml of TES buffer(25 mM, pH 7.5) and subjected to sonic oscillation, which was carried out at 5 kc for total 10 min(1 min oscillation at 4 min intervals). The sonicates were centrifuged at 10,000 x g for 30 min. Streptomycin sulfate solution(20%) was slowly added to the supernatant to the final concentration of 1.4%. After stirring for 30 min, the centrifugation was carried out at 10,000 x g for 30 min. The supernatant was further centrifuged in the 55.2 Ti rotor of a Beckman ultracentrifuge. Centrifugation was at 130,000 x g for 90 min at 4°C. The obtaining supernatant was subjected to ammonium sulfate fractionation. Precipitate of 30-60% saturation was collected by centrifugation and dissolved in 20 ml of the buffer described above. Until stated otherwise, this fraction was used as the cell free extract.
Assays: Proline hydroxylation was performed at 25°C in a 100 µl reaction mixture containing 20 mM TES buffer(pH 7.5), 1 mM sodium ascorbate, 0.5 mM 2-ketoglutarate, 0.5 mM ferrous ammonium sulfate, 10 µg catalase, 1 mM L-proline containing 0.1 µCi of labeled L-proline and 20 µl of the cell free extract. The reaction was initiated by the addition of the extract to the reaction mixture. The reaction was terminated by spotting 20 µl of the reaction mixture on a DE-81 paper within 10 seconds. After development with 1 mM NaCl, the paper was airdried and colored with 0.2% ninhydrin. Proline and hydroxyproline spots were cut off and transferred into counting vials, respectively. After solubilization with 2 ml of 5% SDS solution at 30°C for 16 hours, 10 ml of scintillation fluid were added. Radioactivity of each spot was measured by a liquid scintillation spectrometer. One unit of the proline hydroxylating activity

was defined as the amount of the activity which produced 1 nmole of hydroxyproline per min at 25°C. The distribution of radioactivity in the reaction mixture was determined by the following methods: a) Two dimensional TLC was carried out on a cellulose plate as described previously[4]. The ninhydrin positive and radioactive spots were detected by coloration with ninhydrin followed by radioautography using a X-ray film. After 2 days' exposure, the film was developed. Ninhydrin positive spots corresponding to radioactive spots on the plate were scraped off and the radioactivities were determined by a liquid scintillation spectrometer. b) Identification of radioactive amino acid in the reaction mixture was carried out with an automatic amino acid analyzer. The analytical conditions were as follows: apparatus, Hitachi high speed amino acid analyzer Model 835-50; resin, Hitachi custom ion exchange resin #2619; column, 4 mm x 150 mm; eluent, sodium citrate buffer(pH 3.2); temperature, 55°C; flow rate, 0.225 ml/min. Each amino acid peak was collected to a counting vial to which 10 ml of scintillation fluid were added and the radioactivity was measured. Protein contents in various samples were determined by the method of Bensadoun and Weinstein using bovine serum albumin as a standard[8].

Results

Preparation of cell free extract and time course of the reaction: Table 1 gives a summary of the extraction procedures. Since there was the hydroxylation activity in the supernatant of the ultracentrifugation(130,000 x g for 90 min), the activity was probably solubilized. Time course of the hydroxylation is presented in Fig. 1. The reaction proceeded linearly at least to 10 min. The extract inactivated by heat treatment (100°C for 10 min) did not form hydroxyproline from proline. Hydroxyproline formation was slightly jacked up because the labeled proline used in this study containing a small amount of labeled hydroxyproline (below 0.25%).

Table 1. Extraction of proline hydroxylation activity from Streptomyces griseoviridus

Step	Total protein (mg)	Total units*	Recovery (%)	Specific** activity
Sonication supernatant	2158	755.3	100	0.35
Streptomycin treatment	1821	564.5	75	0.31
Ultracentrifugation Supernatant	1375	605.0	80	0.44
Ammonium sulfate fractionation	454	404.1	54	0.89

* 1 unit : 1 nmole hydroxyproline formed/min at 25°C.
**Specific activity : unit/mg protein.

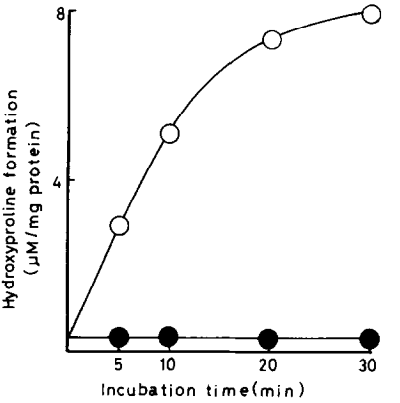


Fig. 1. Time course of the hydroxyproline formation by the cell free extract. ○, control; ●, after inactivation by heat treatment.

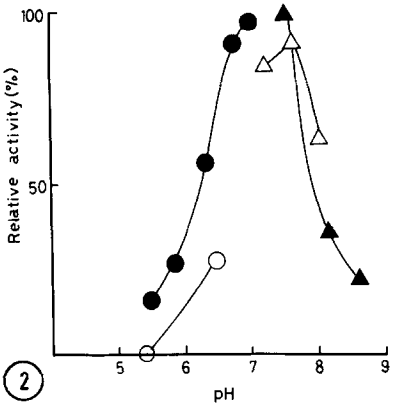
Identification of the reaction products: In order to identify hydroxyproline in the reaction mixture, two chromatographic procedures were carried out as described in Materials and Methods. The results are shown in Table 2. In both analyses, no radioactive compound except proline(substrate) and hydroxyproline(product) was detected. Based on the results, we concluded that hydroxyproline was formed from proline by the cell free extract of S. griseoviridus P8648.

Some properties of the hydroxylation reaction: Optimum pH of the reaction at 25°C was determined in the following buffer: pH 5.5-6.5, citrate buffer; pH 6.5-7.0, malate buffer; pH 7.0-8.0, TES buffer;

Table 2. Identification of hydroxyproline formed in the reaction mixture

Fraction	Before inactivation		After inactivation	
	TLC	Amino acid analyzer	TLC	Amino acid analyzer
	dpm/ml	dpm/ml	dpm/ml	dpm/ml
Proline	1.11×10^5	1.12×10^5	1.11×10^5	1.11×10^5
Hydroxyproline	1.29×10^4	1.34×10^4	0.35×10^3	0.46×10^3

Before and after inactivation, the hydroxylation reaction was carried out for 30 min at 25°C and pH 7.5. After the reaction, each reaction mixture containing 10 mM of cold hydroxyproline was subjected to cellulose TLC and to an automatic amino acid analyzer. Proline and hydroxyproline parts on the thin layer were scraped off and the radioactivity of each part was measured by a liquid scintillation spectrometer. Proline and hydroxyproline peaks on the analyzer were collected to counting vials and each radioactivity was measured by a liquid scintillation spectrometer.



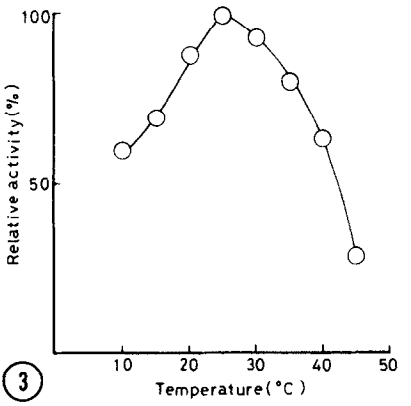


Fig. 2. Effect of pH on the hydroxyproline formation by the cell free extract. The reaction mixture contained 20 mM of the indicated buffer instead of TES buffer in the standard conditions at various pH. The incubation was carried out at 25°C for 10 min. The activity was indicated as relative values against the highest activity. ○, sodium citrate; ●, sodium malate; △, TES; ▲, borate.

Fig. 3. Effect of reaction temperature on the hydroxyproline formation by the cell free extract. The reaction was carried out under the standard conditions except the reaction temperature for 10 min. The activity was indicated as relative values against the highest activity.

pH 7.5-8.6, borate buffer. From the results(Fig. 2), the optimum pH was decided to be 7.5. The optimum temperature of the reaction in TES buffer(pH 7.5) was decided to be 25.0°C(Fig. 3). Effects of co-factors and inhibitors are summarized in Table 3. From the results, hydroxyproline formation from proline by the cell free extract required ferrous ion and 2-ketoglutarate, while the reaction was inhibited by zinc ion, EDTA and α,α'-dipyridyl.

Discussion

Hydroxyproline formation from proline by cell free extracts or by enzymes of microbial origin has never been observed before. Our cell free system obtained from S. griseoviridus P8648 proceeded the hydroxylation reaction(Fig. 1). The system essentially required ferrous ion and 2-ketoglutarate. Ferric ion instead of ferrous ion was not effective(data not shown). Ascorbate also contributed to the reaction but not essentially. Additionally, the reaction was inhibited by zinc ion, EDTA and α, α'-dipyridyl which are known as inhibitors of prolyl hydroxylase

Table 3. Effects of co-factors and inhibitors on the hydroxylation

Factor deleted or Inhibitor added	Concn.(mM)	Relative activity(%)
Complete	-	100
Fe^{2+}	-	0
2-ketoglutarate	-	0
Ascorbate	-	78
Catalase	-	100
Zn^{2+}	0.03	39
	0.25	31
	0.50	0
α,α'-Dipyridyl	0.01	82
	0.05	57
	1.00	29
	5.00	22
	10.0	0
EDTA	0.25	0

The effects of co-factors were determined by deletion method. After each factor was omitted from the complete reaction mixture, the reaction was carried out as described in Materials and Methods. The inhibitors were added to the indicated concentration at the initial time and then the reaction was performed.

of animal system. Since these co-factors and inhibitors were same as those of the animal system(Table 3 and Ref. 3), the reaction mechanism of the microbial system might be similar to that of animal system. But, about the substrate, the two systems were different from each other. In animal system, only peptidyl proline can be hydroxylated to hydroxyproline, while in our microbial system free proline could be hydroxylated to free hydroxyproline. It was not elucidated that in the microbial system peptidyl proline was the possible substrate or not. The precise properties of the hydroxylation by the cell free extract of the streptomycete remains to be clarified because the purification of the system has not been accomplished.

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