Culture Conditions for an α -Amylase Inhibitor-Producing Marine Actinomycete and Production of the Inhibitor "Amylostreptin"

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(Received June 18, 1992)

Streptomyces corchorusii subsp. *rhodomarinus* subsp. nov. which was isolated from marine sediment produced an extracellular α -amylase inhibitor named "amylostreptin". The optimal medium of the strain for the production of amylostreptin was investigated by using shake flask and an improved basal medium. Soluble starch as a carbon source and Polypepton as a nitrogen source were excellent for amylostreptin production as well as the growth of the strain. The maximum production of amylostreptin (62.6×10^3 units/0.1 ml of broth) was observed in a medium consisting of 1.0% soluble starch, 1.2% Polypepton, and 0.1% Bacto-yeast extract at pH 7.5 in 25% (v/v) seawater. Under the optimal cultivation conditions mentioned above, the production of amylostreptin increased about 3 times compared to the initial level.

The crude amylostreptin was very stable up to 100°C for 30 min as well as in a wide range of pH stability from 1 to 13.

Recently, amylase inhibitors have received increasing attention because they are not only a useful tool for the study of the active site of amylases,¹⁾ but also potential drugs for carbohydrate-dependent diseases such as diabetes, obesity, and hyperlipaemia.²⁻⁴⁾

As reported previously, the authors examined a large number of marine bacteria and actinomycetes for amylase inhibitor-producing activity, and encountered an actinomycete strain from a sediment sample of a marine environment.⁵⁾ We named this amylase inhibitor "amylostreptin". It was necessary to investigate the improvement in production of the inhibitor because of the potential applications of the inhibitor in industry, medicine, and biochemistry. Culture conditions such as the composition of medium, pH, and various physiological factors affect the production of secondary metabolites like antibiotics^{6,7)} and enzyme inhibitors.⁸⁻¹⁰⁾

This paper describes the results of an investigation for establishing the optimal cultivation conditions as well as some properties of the inhibitor.

Materials and Methods

Microorganism

Streptomyces corchorusii subsp. *rhodomarinus* T-178 isolated from a marine sediment (5 m depth) of Aburatsubo Inlet, Kanagawa, Japan was used throughout this experiment.⁵⁾ The strain was maintained on an inorganic salt-starch agar medium (ISP No. 4)¹¹⁾ at 10°C.

Cultivation of Microorganism

A liquid medium composed of 0.6% Polypepton (Daigo Eiyo Kagaku), 0.1% Bacto-yeast extract (Difco), and 1.0% soluble starch in full strength seawater, pH 7.0, was used for the initial culture of the microorganism. A hundred milliliters of the medium was put into a 500 ml Erlenmeyer flask and inoculated from a slant culture. Incubation was carried out on a rotary shaker (speed, 200 rpm; eccentricity, 7 cm) at 20°C until the logarithmic growth phase. Then, 2 ml of the preculture was inoculated in 100 ml of the same medium and incubated for 2 days at 20°C. The growth of the strain was assessed by the weight of mycelium from 5 ml of the broth, and after drying at 60°C for 2 h. The mycelium was

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removed by centrifugation (10,000 rpm, 10 min) and the inhibitory activity in the supernatant was assayed.

Assay System for Amylase Inhibitory Activity

Inhibitory activity of amylostreptin was assayed by a modified blue value method.¹²⁾ The enzyme used for the assay was α -amylase from human saliva type IX-A (Sigma) and was dissolved in 50 mM Tris-HCl buffer (pH 7.0) at the concentration of 5 μ g/ml. The substrate used was 0.5% soluble starch (Merck) solution in the same buffer. A reaction mixture containing 0.1 ml of suitably diluted culture supernatant and 0.2 ml of enzyme solution was preincubated at 30°C for 10 min. Then, 0.2 ml of substrate was added to the preincubated mixture. The mixture was incubated for 12 min at 30°C, then 0.2 ml of 1 M acetic acid was added to terminate the enzyme reaction. Thereafter, 0.2 ml of iodine reagent (0.2% iodine and 2% potassium iodide) was added. The volume was made up to 6 ml with deionized water, and the optical density of the resulting blue colour was measured at 690 nm. One unit of inhibitory activity of amylostreptin was defined as the amount of inhibitor required to decrease the enzyme activity by 50% under the above conditions.

The Effect of Various Factors on the Growth of the Strain and Production of Amylostreptin

The seawater used in the present study was collected from the open ocean off the Japanese coast and was stored for several months in the dark. The salinity of the seawater thus obtained was 3.5% (w/v). To investigate the effect of the concentration of seawater, it was diluted with deionized water. To study the effect of initial pH, the medium was adjusted to various pHs (3.5–10.5) with 1 N NaOH or HCl. Various nitrogen and carbon compounds were added to the medium in order to find the most suitable nitrogen and carbon sources.

The time course of the growth of the strain, the fluctuation of pH, and the production of the inhibitor during cultivation were examined. An aliquot of 5 ml of the broth was sampled every day.

Thermostability of Amylostreptin

The inhibitor was kept at 100°C for 10 to 60 min and the residual inhibitory activity was assayed.

The pH Stability of Amylostreptin

The inhibitor solution was incubated at various pHs for 10 min at 30°C. After incubation, the pH was adjusted to 7.0, and the residual inhibitory activity was measured.

Results

The Effect of Preincubation Time on Degree of Inhibition

Inhibitory activity increased depending on increasing preincubation time and reached a maximum after 12 min, indicating that the time required for enzyme-inhibitor (E-I) complex formation was 12 min (Fig. 1).

Fig. 1. The effect of preincubation time on the degree of inhibition.

Fig. 2. Percentage of inhibition versus arbitrary concentration of amylostreptin.

Table 1. Effect of the concentration of seawater on the growth and inhibitor production

Seawater conc. (%, v/v)	Final pH	Growth (Mycelium dry wt., mg/0.1 ml)	Inhibitory activity ($\times 10^3$ units/0.1 ml)
0	7.9	0.21	28.6
25	7.5	0.49	40.0
50	7.4	0.56	32.4
75	7.4	0.53	29.7
100	7.3	0.49	22.0

Basal medium: Polypepton 0.6%, Bacto-yeast extract 0.1%, soluble starch 1.0%, pH 7.0.

Table 2. Effect of pH on the growth and inhibitor production

pH		Growth (Mycelium dry wt., mg/0.1 ml)	Inhibitory activity ($\times 10^3$ units/0.1 ml)
Initial	Final		
3.5	3.7	0.25	0.0
4.5	5.9	0.58	26.9
5.5	7.6	0.59	51.7
6.5	7.9	0.51	55.2
7.5	8.1	0.51	58.3
8.5	8.4	0.43	21.0
9.5	8.3	0.36	1.0
10.5	8.8	0.17	0.0

Basal medium: Polypepton 0.6% (w/v), Bacto-yeast extract 0.1% (w/v), soluble starch 1.0% (w/v), seawater 25% (v/v).

Percentage of Inhibition versus Arbitrary Concentration of Amylostreptin

The correlation between the amount of amylostreptin and inhibition against α -amylase was not linear above a concentration which displayed 68% inhibition, as shown in Fig. 2. It was necessary to determine the inhibitory activity within the linear range (0–68% inhibition).

The Effect of Various Factors on the Growth of the Strain and Production of Amylostreptin

Table 1 shows the effect of the concentration of seawater on the growth of the strain and the production of amylostreptin. Fifty percent (v/v) of seawater was the most suitable for growth of the strain, whereas 25% (v/v) of seawater was the best for the production of amylostreptin. The growth as well as the production of the inhibitor was less effective in the absence of seawater.

From the result shown in Table 2, an initial pH of 7.5 was found to be the most favorable for production. Consequently, the initial pH of the basal medium in 25% (v/v) seawater was set at 7.5 for further investigations. When amylostreptin was produced, the final pH of the broth changed to about 8.

Various mono-, di-, and polysaccharides, and sugar alcohols were tested as carbon sources in

terms of production and growth of the strain. As shown in Table 3, soluble starch was the most effective and the optimal concentration for production was 1.0% (w/v). Higher concentrations of soluble starch caused an adverse effect on the production. Several carbohydrates such as lactose, arabinose, sucrose, and maltose were less effective either for production or growth. From these results, soluble starch was added at a concentration of 1.0% (w/v) to 25% (v/v) seawater basal medium at pH 7.5.

From the results shown in Table 4, it is apparent that inorganic nitrogen compounds such as NH_4NO_3 and NH_4Cl were not effective for growth or inhibitor production. Complex organic nitrogen sources such as Bacto-gelatin, Bacto-casamino acids, and Pharmamedia were also not effective for production or growth. Among the various nitrogen sources, Polypepton was found to be excellent for growth as well as production and the optimal concentration was 1.2 or 1.8% (w/v). The final medium for the study of the time course of growth and the production of inhibitor was composed of 1.0% soluble starch, 1.2% Polypepton, and 0.1% Bacto-yeast extract in 25% (v/v) seawater at pH 7.5.

Figure 3 illustrates the time course of growth of the strain, the fluctuation of pH, and the production of amylostreptin. After inoculation

Table 3. Effect of carbon source on the growth and inhibitor production

Carbon source	Conc. (%, w/v)	Final pH	Growth (Mycelium dry wt., mg/0.1 ml)	Inhibitory activity ($\times 10^3$ units/0.1 ml)
None	0.0	8.4	0.28	1.9
Soluble starch	0.5	7.6	0.38	28.6
Soluble starch	1.0	8.0	0.43	58.3
Soluble starch	2.0	7.0	0.78	30.2
Glucose	1.0	6.6	0.53	2.3
Lactose	1.0	8.4	0.27	2.0
Levulose	1.0	7.8	0.44	2.6
Arabinose	1.0	7.9	0.31	2.7
Galactose	1.0	8.3	0.31	2.6
Xylose	1.0	8.2	0.29	2.1
Sucrose	1.0	8.4	0.28	2.2
Maltose	1.0	8.4	0.26	1.4
Starch, wheat	1.0	7.6	0.55	58.1
Starch, corn	1.0	7.7	0.60	52.8
Starch, sweet potato	1.0	7.6	0.51	50.6
Starch, potato	1.0	7.6	0.32	28.6
Laminarin	1.0	8.2	0.42	9.3
Glycerol	1.0	5.6	0.53	10.3
Inuline	1.0	8.3	0.36	2.5
Amylose	1.0	8.0	1.21	6.9
Dextrin	1.0	7.6	0.51	37.6
Mannitol	1.0	7.9	0.55	12.1
Inositol	1.0	8.2	0.30	8.8
Dulcitol	1.0	8.3	0.35	2.6

Basal medium: Polypepton 0.6% (w/v), Bacto-yeast extract 0.1% (w/v), seawater 25% (v/v), pH 7.5.

Table 4. Effect of nitrogen source on the growth and inhibitor production

Nitrogen source	Conc. (%, w/v)	Final pH	Growth (Mycelium dry wt., mg/0.1 ml)	Inhibitory activity ($\times 10^3$ units/0.1 ml)
None	0.0	5.6	0.20	0.6
Polypepton	0.3	6.3	0.49	44.1
Polypepton	0.6	7.4	0.64	62.6
Polypepton	1.2	8.1	0.95	65.8
Polypepton	1.8	8.2	1.03	65.8
Proteose peptone No. 3 (Difco)	0.6	8.3	0.32	4.5
Bacto-soytone	0.6	7.6	0.36	32.1
Phytone (BBL)	0.6	7.9	0.30	18.0
Bacto-casitone	0.6	7.4	0.40	33.2
Bacto-tryptose	0.6	5.4	0.65	19.1
Meat extract	0.6	8.2	0.40	16.9
Bacto-gelatin	0.6	7.9	0.22	8.4
Bacto-casamino acids	0.6	8.3	0.37	0.5
Corn steep liquor	0.6	5.9	0.15	5.3
Pharmamedia*	0.6	5.3	0.29	1.5
Bacto-tryptone	0.6	7.7	0.35	18.0
Trypticase peptone	0.6	7.8	0.38	28.9
Casein	0.6	6.0	0.47	12.5
NaNO ₃	0.6	6.1	0.13	0.2
NH ₄ Cl	0.6	4.7	0.12	0.3
NH ₄ NO ₃	0.6	5.2	0.13	0.3

* Traders Oil Mill Co., USA.

Basal medium: Bacto-yeast extract 0.1% (w/v), soluble starch 1.0% (w/v), seawater 25% (v/v), pH 7.5.

Fig. 3. Time course of the growth of the strain, fluctuation of pH, and production of amylostreptin. ○—○, Growth; ■—■, pH; ●—●, inhibitory activity.

of the strain, it grew steadily within 3 days, followed by a plateau afterwards. The production of amylostreptin was almost parallel to the growth. The maximum production was reached after 4 days and this level was maintained thereafter. However, there was not much fluctuation of pH in the broth and the pH remained between 7 and 8.5 throughout the experiment.

Thermostability and pH Stability of Amylostreptin

Amylostreptin was stable at 100°C with no loss of inhibitory activity up to 60 min.

It was also quite stable in a pH range from 1 to 13 with a loss of no more than 20% of inhibitory activity.

Discussion

A number of amylase inhibitors were isolated and characterized from various plants.¹³⁾ However, inhibitors of microbial origin are limited in number. In terms of the production of useful substrates, microorganisms have advantages over plants: they grow fast, are easily and economically cultivated, and many experiments can be undertaken within a short period of time. In the present study, amylostreptin production increased more than three times under the modified condition compared to that under the initial culture conditions. In the case of protease inhibitor producing bacterium isolated from seawater, the production of the inhibitor also increased about three times by improving the culture conditions.¹⁴⁾ The production of amylostreptin

was less effective in the presence of full-strength seawater, suggesting that this actinomycete was of a terrestrial origin. It may be transported to rivers through rain or irrigation water, finally brought to the marine environment and adapted to the environment.

Murao *et al.*¹⁵⁾ reported that an actinomycete strain isolated from soil required corn starch for the production of amylase inhibitors because chemical structures of these inhibitors were oligosaccharides. The same result was obtained in the present study, that is, the production of amylostreptin increased considerably in the presence of starch, suggesting that the chemical structure of amylostreptin was a saccharide. The crude amylostreptin was very thermostable and pH stable. It should be taken into consideration, however, that the amylostreptin used in the present study was in a crude form. Further studies of the purification of amylostreptin are underway.

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