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Production of Trypsin Inhibitor by a *Cephalosporium* sp.

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Conditions for the production of the trypsin inhibitor from *Cephalosporium* sp. KM 388 were investigated. Polypeptone-meat extract-glucose medium supported excellent production of the trypsin inhibitor. In this medium, polypeptone and meat extract were utilized both as carbon and nitrogen sources and as limiting substrates for the cell growth. Glucose was consumed during the stationary growth phase and prevented the disappearance of inhibitor activity. *Cephalosporium* sp. KM 388 grew at a rate of a first-order reaction for the cell concentrations. Trypsin inhibitor production paralleled cell growth. At 27°C, the maximum specific rates of growth and inhibitor production were 0.14 h^{-1} and 2.1 U of inhibitor/h per mg of cell, respectively. The production rate and the maximum yield of the inhibitor were increased 1.5- and 1.2-fold, respectively, when the initial pH 6.3 was maintained throughout the fermentation.

Inhibitors of proteolytic enzymes have been found to be produced by animals and plants (8) and also to occur in microorganisms (7, 13). Since some of them are useful in the diagnosis and therapy of inflammation, pancreatitis, etc., caused by proteases (14), production of the protease inhibitors has been investigated. Protease inhibitors have been reported to be produced by actinomycetes (13) and some fungi (12). Recently, *Cephalosporium* sp. KM 388, which formed the trypsin inhibitor, was screened by S. Ohmura, Faculty of Pharmaceutical Sciences, Kitasato University.

Fermentation studies of antibiotics have been reported (4, 9, 11), but little is known about fermentation of protease inhibitors from microorganisms. To investigate the kinetics of their formation, conditions for the production of the trypsin inhibitor from *Cephalosporium* sp. KM 388 were studied.

MATERIALS AND METHODS

Microorganism. The microorganism used in this work was *Cephalosporium* sp. KM 388, which was supplied by S. Ohmura and was maintained on potato dextrose agar.

Cultural medium and fermentation. The basal medium was composed of: glucose, 10.0 g; polypeptone, 5.0 g; meat extract, 1.0 g; KH_2PO_4, 1.0 g; NaCl, 2.0 g; and tap water, 1 liter (pH 6.3). The inocula were prepared by culturing the mycelial mats, which had been taken from the stock culture, in 100 ml of this liquid medium in a 500-ml flask for 48 h at 27°C on a reciprocal shaker at 110 rpm. This culture broth (4 ml) was inoculated and cultured under the same conditions as the preculture. The compositions of the other fermentation media are specified below.

Determinations of cell and substrate concentrations. The mycelial weight of the fungus was determined by pipetting 10 ml of the culture broth into a graduated tube, centrifuging at 1,500 × *g* for 5 min, and measuring the packed volume. One milliliter of packed volume was equivalent to 3.6 mg of cell dry weight per ml. The concentrations of glucose and nitrogen sources in the culture broth were measured by the phenol-sulfonic acid (5) and micro-Kjeldahl methods, respectively.

Determination of trypsin inhibitor activity. The activity of the trypsin inhibitor was determined by the method of Murao et al. (10), with modifications as shown in Fig. 1. Since the trypsin inhibitor was secreted into the culture medium, the culture broth was centrifuged at 1,500 × *g* for 5 min, and the supernatant was used for the determination of its activity. A 31.25-μg/ml amount of trypsin solution was reacted with 0.6% denatured hemoglobin in 0.1 M phosphate buffer, pH 7.5 (standard [*S*]). A 0.1-ml portion of sample was added to the standard solution, and the inhibition of trypsin activity was measured (test [*T*]). As a control (*C*), the activity of protease, which was produced by *Cephalosporium* sp., in the sample was measured. These reaction mixtures were incubated at 37°C for 30 min and treated with 1.7 M perchloric acid at 37°C for 30 min, and the optical density of the perchloric acid-soluble fraction at 280 nm was measured with a spectrophotometer (model 101, Hitachi Ltd.). The rate of inhibition (*R*) was calculated by using the following equation: R (percent) = $[(S - B) - (T - C)]/(S - B) \times 100$, where B = blank. The activity of the trypsin inhibitor was obtained from the standard curve shown in Fig. 2. One unit of its activity is equivalent to 50% inhibition of the trypsin used.

Chemicals. Lyophilized trypsin (1:12,700) and hemoglobin were purchased from Sigma Chemical Co. Ltd.; polypeptone, meat extract, and yeast extract were from Daigo Eiyo Chemical Co. Ltd.; and Casamino Acids (vitamin free) were from Difco Laboratories. Other chemicals were obtained from commercial sources.

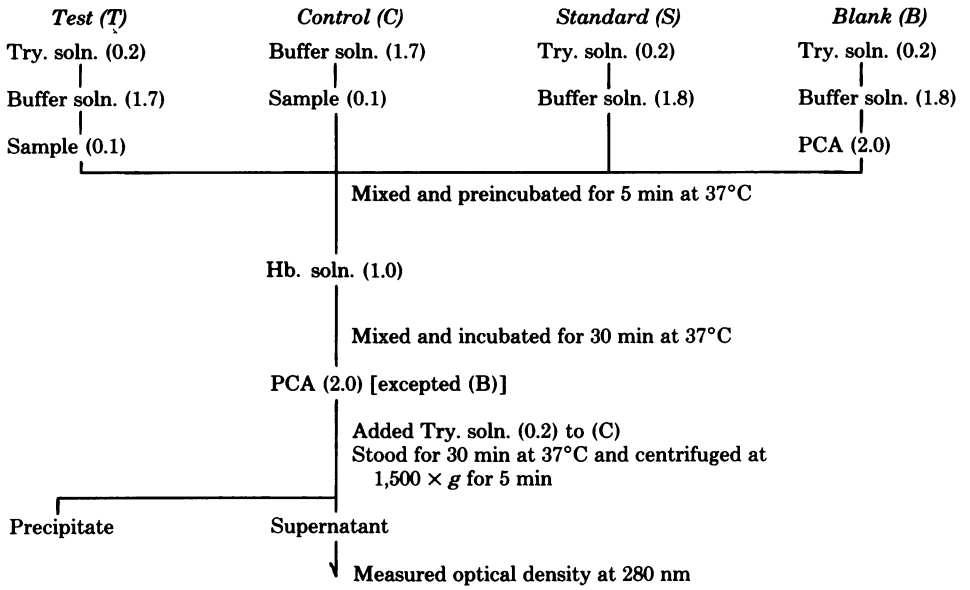

FIG. 1. *Schematic representation of assay procedure for trypsin inhibitor. Values in parentheses indicate volume (milliliters) of added solution. Try. soln., Trypsin (31.25 μg/ml); Hb. soln., 0.6% denatured hemoglobin; Buffer soln., 0.1 M phosphate; PCA soln., 1.7 M perchloric acid.*

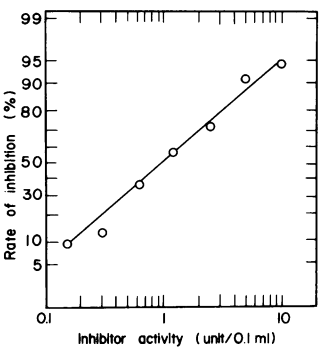

FIG. 2. *Determination of trypsin inhibitor activity. Coordinates are logarithmic-normal distribution.*

TABLE 1. *Effect of carbon sources on production of trypsin inhibitor*[a]

Carbon source (10.0 mg/ml)	Specific productivity (U of inhibitor/mg of cell)	Cell yield (mg/ml)
Glucose	7.7	2.6
Maltose	8.3	3.0
Starch	6.2	2.6
Mannose	3.2	2.9
Lactose	0	0.4
Fructose	0	0.3
Galactose	0	0.3
Xylose	0	0.3
Raffinose	0	0.5
Sucrose	0	0.6
Cellobiose	0	0.4

[a] Other compositions tested: $NaNO_3$, 6 mg/ml; KH_2PO_4, 1 mg/ml; NaCl, 2 mg/ml. Length of incubation: 48 h.

RESULTS AND DISCUSSION

Effects of carbon and nitrogen sources on the growth and production of trypsin inhibitor. Table 1 shows the effects of various carbon sources on the growth and production of the trypsin inhibitor. *Cephalosporium* sp. KM 388 utilized glucose, maltose, starch, and mannose as carbon sources and also for the production of the trypsin inhibitor. Other sugars were ineffective.

Table 2 shows the effects of various nitrogen sources on the growth and production of the trypsin inhibitor. Among the tested inorganic nitrogen sources, sodium nitrate gave the highest yields of cells and inhibitor, whereas ammonium salts gave the lowest yields. On the other hand, the cells grew well on all organic nitrogen sources. Yeast extract, polypeptone, and polypeptone with added meat extract were extremely beneficial to the production (average, 9.7 U of inhibitor/mg of cell) of the trypsin inhibitor, whereas about 27% of that value was obtained when urea and Casamino Acids (vitamin free) were used. The medium consisting of polypeptone and meat extract in basal medium, as de-

scribed in Materials and Methods, was investigated further.

The correlation between the cell yields and the concentration of polypeptone at 1 mg of meat extract per ml is shown in Fig. 3. The maximum cell yield was obtained at a concentration of polypeptone >12 mg/ml in the medium. This result indicates that the cell growth is limited by polypeptone and meat extract in the basal medium.

Fermentation of *Cephalosporium* sp. KM 388. A typical time course of growth, trypsin inhibitor production, and pH variation at 27°C is shown in Fig. 4. The exponential growth was observed up to 20 h after a lag time of 4 h, after which time the cell concentration remained constant. The fungus formed pellets (2 to 3 mm in diameter) during the fermentation. This growth rate of a first-order reaction for the cell concentration seems to be unusual for growth of the pellet-forming fungi, which have been reported to grow at a rate proportional to two-thirds the power of the cell concentration (1, 3).

Polypeptone and meat extract were completely consumed at the end of the cell growth phase, whereas glucose was not utilized during the exponential growth phase, but rather began to be consumed during the stationary growth phase. This indicates that polypeptone and meat extract are utilized not only as nitrogen sources, but also as carbon sources. A role of glucose that has not been consumed during cell growth will be considered below.

The production of the trypsin inhibitor paralleled the cell growth at a constant rate up to the end of the growth phase. This is unusual in the production of secondary metabolites, which are observed during the idiophase (2), and may be closer to the so-called growth-associated type (6). The trypsin inhibitor activity gradually decreased with the consumption of glucose in the stationary growth phase. The pH level decreased slightly during the formation of the inhibitor and rose after formation stopped.

TABLE 2. *Effect of nitrogen sources on production of trypsin inhibitor*[a]

Nitrogen source (6.0 mg/ml)	Specific productivity (U of inhibitor/mg of cell)	Cell yield (mg/ml)
Inorganic		
$NaNO_3$	7.7	2.6
$Ca(NO_3)_2$	0	1.8
KNO_3	0	1.9
NH_4NO_3	0	0.9
NH_4Cl	0	1.0
$(NH_4)_2SO_4$	0	0.6
Organic		
Urea	2.7	3.5
Casamino Acids (vitamin free)	2.5	2.9
Yeast extract	9.4	3.9
Polypeptone	9.2	3.6
Polypeptone-meat extract	10.5	5.5

[a] Other compositions tested: glucose, 10 mg/ml; KH_2PO_4, 1 mg/ml; NaCl, 2 mg/ml. Length of incubation: 48 h.

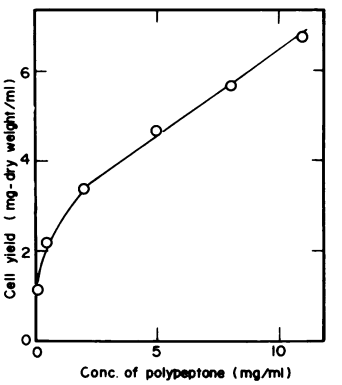

FIG. 3. *Cell yield as a function of the concentration of polypeptone. Meat extract was added at 1 mg/ml.*

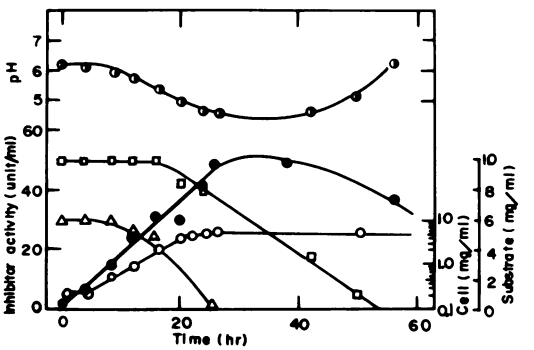

FIG. 4. *Fermentation pattern of Cephalosporium sp. KM 388 at 27°C. Symbols:* (○) *cell concentration;* (●) *trypsin inhibitor activity;* (△) *polypeptone-meat extract concentration;* (□) *glucose concentration;* (◑) *pH.*

Effect of glucose on inhibitor production. Glucose was not consumed during cell growth, whereas polypeptone and meat extract were utilized during that period (Fig. 4); however, glucose prevented the disappearance of the inhibitor activity in the stationary growth phase (Fig. 5). Glucose affected neither cell growth nor inhibitor production. In the absence of glucose, however, inhibitor activity rapidly decreased after the termination of cell growth. This can be explained by the following hypothesis. The in-

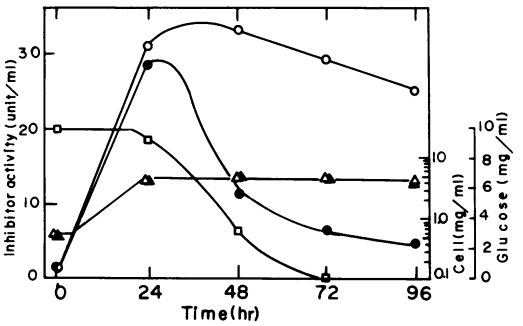

FIG. 5. *Effect of glucose on the production of trypsin inhibitor. Symbols: (△) cell concentration in medium containing 1% glucose; (▲) cell concentration in medium without glucose; (○) inhibitor activity in medium containing 1% glucose; (●) inhibitor activity in medium without glucose; (□) glucose concentration.*

hibitor is utilized as a substrate for the maintenance of cellular metabolism after the polypeptone and meat extract is consumed, since this trypsin inhibitor is a polypeptide of low molecular weight, i.e., ca. 1,000 (S. Ohmura, personal communication). When glucose is present, it prevents the disappearance of the inhibitor activity in that phase, since it serves as a carbon source.

Effect of pH control on inhibitor production. As mentioned above, the initial pH 6.3 decreased to pH 4.3 as cell growth progressed and rose gradually during the stationary phase (Fig. 4). When the initial pH 6.3 was maintained with 0.1 N NaOH or 0.1 N HCl throughout, the growth was not affected, but the production rate and the maximum yield of the trypsin inhibitor were increased 1.5- and 1.2-fold, respectively (Fig. 6).

Effect of fermentation temperature. The effect of fermentation temperature on the cell growth is shown in Fig. 7. A 4-h lag phase was observed at each temperature tested. Specific growth rates at various temperatures were determined from slopes of semilogarithmic plots of the cell concentration versus fermentation time. The variation in the specific growth rate with fermentation temperature is shown in Fig. 9. The maximum specific growth rate, 0.14 h^{-1}, was obtained at 27°C, but cell yield was independent of temperature. There was no growth at 35°C.

Inhibitor production proceeded linearly at each temperature tested (Fig. 8), and its specific rate was affected by the fermentation temperature (Fig. 9). The maximum specific rate, 2.1 U of inhibitor/h per mg of cell, was also obtained at 27°C.

Arrhenius plots for the maximum specific rates of growth and inhibitor production are shown in Fig. 9. Both rates were exponential

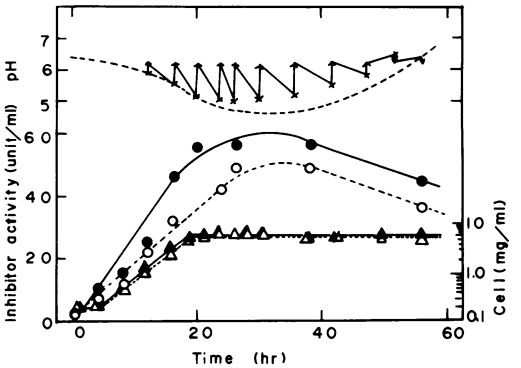

FIG. 6. *Effect of pH control on the production of trypsin inhibitor. Dotted line shows change in pH value without pH control. At the points of the symbol (×), pH was adjusted to 6.3 of the initial value with 0.1 N NaOH or 0.1 N HCl. Symbols: (△) cell concentration in medium without pH control; (▲) cell concentration in medium with pH control; (○) inhibitor activity in medium without pH control; (●) inhibitor activity in medium with pH control.*

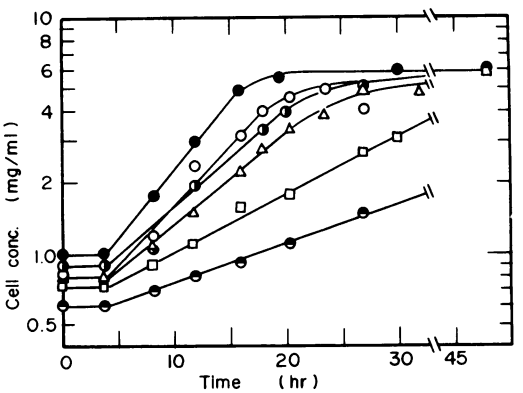

FIG. 7. *Effect of fermentation temperature on cell growth. Temperatures were: (◓) 33°C; (◑) 30°C; (●) 27°C; (○) 24°C; (△) 20°C; (□) 15°C.*

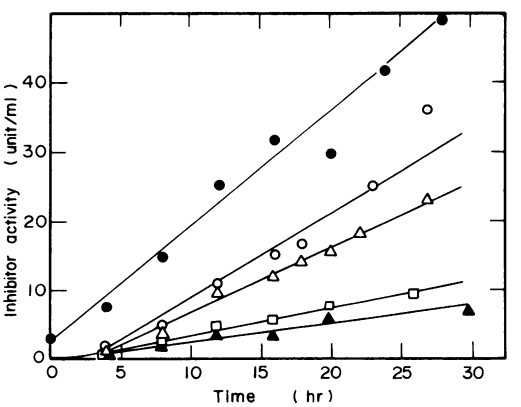

FIG. 8. *Effect of fermentation temperature on the production of trypsin inhibitor. Temperatures were: (▲) 33°C; (●) 27°C; (○) 24°C; (△) 20°C; (□) 15°C.*

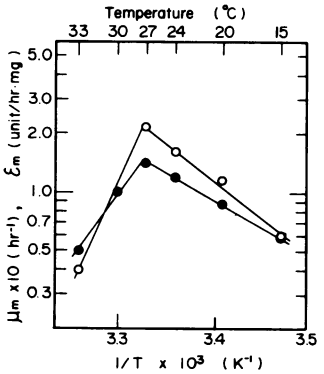

FIG. 9. *Arrhenius plots for maximum specific rates of growth and inhibitor production. Coordinates are semilogarithmic. T, Absolute temperature. Symbols:* (●) *maximum specific growth rate* (μ_m); (○) *maximum specific rate of inhibitor production* (ϵ_m).

functions of the temperature. The activation energies for the specific rates of growth and inhibitor production were calculated to be 54.4 and 95.3 kJ/mol, respectively.

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