Thermostable Acid Protease Produced by
Penicillium duponti K1014, a True
Thermophilic Fungus Newly Isolated from
Compost

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Received for publication 1 August 1972

A thermophilic fungus, K1014, newly derived from a compost was selected on the basis of protease productivity as the only one of 81 isolates to produce high levels of acid protease. The fungus was named Penicillium duponti K1014 based on taxonomical studies. It grew in the temperature range of 28 to 58 °C, and the optimum was 45 to 50 °C. These temperature characteristics showed that the fungus was the most strongly thermophilic of all the fungi next to Humicola lanuginosa. When P. duponti K1014 was grown on moistened wheat bran, maximal accumulation of acid protease occurred after 2 days at 45 to 50 °C. The addition of ammonium salts, but not nitrate, was effective for the production of the acid protease. The acid protease of P. duponti K1014 was stable at 60 °C for 1 hr and retained more than 65% of original activity after the treatment for 1 hr at 70 °C at pH 4.7. This thermal property was different from those of the ordinary acid proteases, indicating that the enzyme is a thermostable protein.

A number of thermophilic fungi have been isolated by various investigators (4, 6, 9, 14, 15, 18) from self-heating materials and other sources. It has been assumed that thermophilic fungi, as well as other thermophilic microorganisms, play an important role in decomposing plant materials and other organic matter at elevated temperature that usually resulted from microbial thermogenesis (4). Enzymes responsible for the decomposition of plant materials, i.e., cellulase (2, 7, 10; T. Chon et al., Abstr. Papers Annu. Meet. Agr. Chem. Soc. Japan, p. 137, 1970), hemicellulase (M. Matsu et al., Abstr. Papers Annu. Meet. Agr. Chem. Soc. Japan, p. 116, 1969), β-1,3-glucanase (19), lipase (1), and protease (3; S. Hayashida and G. Hongo, Abstr. Papers Annu. Meet. Agr. Chem. Soc. Japan, p. 204, 1968; P. S. Ong and G. M. Gaucher, Abstr. Papers 4th Intern. Ferm. Symp. Japan, p. 47, 1972) were found to be secreted by the thermophilic fungi.

A thermophilic fungus, Zygodesmus sp., produced a thermostable neutral protease (S. Hayashida and G. Hongo, Abstr. Papers Annu. Meet. Agr. Chem. Soc. Japan, p. 204, 1968). Ong and Gaucher found that the alkaline protease produced by a thermophilic fungus, Malbranchae pulchella var. sulphurea, was also thermostable and that its stability was stimulated by the addition of calcium ion (P. S. Ong and G. M. Gaucher, Abstr. Papers 4th Intern. Ferm. Symp. Japan, p. 47, 1972). Thermostable neutral and alkaline proteases of thermophilic bacteria (8, 17) and actinomycetes (5, 16) have been found by several investigators. However, thermostable acid protease of thermophilic fungi has not been reported so far. The present paper (presented at Annu. Meet. Agr. Chem. Soc. Japan, 1970) demonstrates that thermostable acid protease was produced by a true thermophilic fungus identified as Penicillum dupontii.

MATERIALS AND METHODS

Isolation of thermophilic and thermotolerant fungi. A few drops of the suspension of each sample which was collected from soils, hot springs, self-heating composts, etc., in Japan were spread on a Sake-koji extract agar plate (Bally 10, pH 6.0). Sake-koji extract was used after dilution with water to dry matter of 10% determined by a Ballying saccharometer. The plates were incubated at 45 °C, and the
germinating spores were removed by a flamed needle to sterile agar slants. These tubes were incubated at 40°C until growth was complete.

**Cultivation.** Five grams of wheat bran and 3 ml of water were mixed and autoclaved at 120°C for 1 hr and incubated at 40°C for 4 days after inoculation with three hooks of conidia. The culture medium was extracted with 50 ml of tap water at room temperature for 2 hr and filtered through filter paper. The culture filtrates were used as the source of the protease.

**Assay of protease activity.** Protease activity was assayed according to the modified method of Anson (11). Reaction mixture containing 1 ml of enzyme solution and 5 ml of 0.6% Hammarsten milk casein in 0.05 M buffer was incubated at 30°C for 10 min. Except where specified, enzyme reactions were carried out at pH 2.5. The enzyme reaction was stopped by adding 5 ml of 0.11 M trichloroacetic acid containing 0.22 M acetic acid and 0.35 M sodium acetate. The reaction mixture was allowed to stand for 30 min at 30°C and then was filtered. To 2 ml of the filtrate, 5 ml of 0.55 M sodium carbonate was added, followed by the addition of 1 ml of 3-fold-diluted phenol reagent. The blue color was measured at 660 nm by using a spectrophotometer. One unit of activity was defined as the amount of enzyme producing a change of absorbancy equivalent to 1 μg of tyrosine per min, under the above conditions.

**Taxonomical studies.** Morphological characteristics of a newly isolated thermophilic fungus strain K1014 were studied with cover-culture method and identified according to *A Manual of the Penicillia* (Raper and Thom, 1949) and *Thermophilic Fungi* (Cooney and Emerson, 1964).

**Crude enzyme preparation.** *P. duponti* K1014 was grown on 20 g of wheat moistened with 14 ml of water containing 0.2 g of NH₄NO₃ in 500-ml Erlenmeyer flasks at 45°C for 2 days. The culture medium was extracted with five volumes of water at room temperature for 2 hr and filtered through cheesecloth. To the clear filtrate obtained by further filtration with Celite, three volumes of ethanol were added. Resultant precipitates were collected by centrifugation and dried in vacuo.

**RESULTS**

**Isolation of thermophilic and thermotolerant fungi and productivity of protease with the isolates.** Eighty-one fungi capable of growing at 45°C were isolated from 58 samples collected in several widely separated localities in Japan and 29 samples of imported rice and aromatic plants from the tropics. Thermophilic and thermotolerant fungi could be found from various sources except for hot springs, and most of them could be isolated in different types of composting plant materials (Table 1).

The isolates were tested for their ability to hydrolyze protein at acidic and neutral pH values. Forty-two strains in 81 isolates secreted more than 40 units of neutral protease; however, only six strains secreted more than 40 units of acid protease (Table 2). In order of the amount of acid or neutral protease which was secreted in culture medium, three strains were selected from 81 isolates, respectively.

The six strains selected were compared with type cultures of thermophilic or thermotolerant fungi capable of growing at 50°C in protease productivity (Table 3). Table 3 shows that only the fungus K1014 produced more than 100 units of acid protease; however, the three fungi K1012, K1021, and *M. albobioicus* ATCC 16460 produced more than 100 units of neutral protease. It was also found that the fungus K1014 was the most prominent strain.

**Identification of the newly isolated fungus K1014: cultural and physiological characteristics.** The fungus K1014 was isolated at 45°C from the compost in Tochigi-Prefecture, Japan in 1968. This fungus could not grow at temperatures below 27°C, growth

| Source                      | No. of samples | No. of isolated strains |
|-----------------------------|----------------|------------------------|
| Aromatic plants from the tropics | 25             | 4                      |
| Composts                   | 10             | 63                     |
| Hot springs                | 15             | 0                      |
| Imported rice from the tropics | 4              | 2                      |
| Soils                      | 33             | 12                     |

| Protease (units/ml of culture extract) | No. of strains |
|---------------------------------------|----------------|
| pH 3.0                                |                |
| Below 20                              | 67             |
| 20-40                                 | 8              |
| 40-60                                 | 5              |
| 60-80                                 | 0              |
| 80-100                                | 0              |
| Above 100                             | 1              |
| pH 7.0                                |                |
| Below 20                              | 17             |
| 20-40                                 | 22             |
| 40-60                                 | 35             |
| 60-80                                 | 4              |
| 80-100                                | 1              |
| Above 100                             | 2              |

* Culturing was carried out at 40°C for 4 days on wheat bran moistened with water.
This fungus exhibits amylolytic, proteolytic, cellulolytic, and hemicellulolytic properties and assimilates vegetable oils such as rape oil, soybean oil, and rice oil.

**Morphological characteristics.** The present fungus has penicilli varying from monoverticillata to biverticillata. Conidiophores arise almost perpendicularly from the main hyphae, 30 to 60 μm in length by 2 to 4 μm diameter (Fig. 2). Conidia in long, tangled chains from the lanceolate sterigmata (2 to 3 μm by 5 to 11 μm) are elliptical to oval, smooth, 1.5 to 2.0 μm by 3.0 to 4.0 μm (Fig. 3). Perithecia are not observed on Sake-koji extract agar (Ballg 10, pH 6.0) but are produced on Czapek-Dox agar on which it grows less rapidly and very thinly. Ascii consisting of eight ascospores are recognized. The ascospores are nearly spherical (2.5 to 3.0 μm by 2.0 to 3.5 μm).

These characteristics were compared with those of the thermophilic fungi described by Cooney and Emerson (4, 20), and it was found that the present fungus resembled *Penicillium duponti* (Griffon and Maublanc) Emerson. Therefore, the present fungus has been named as *Penicillium duponti* K1014.

**Production of acid protease by P. duponti K1014: effect of cultivation temperature**

![Fig. 1. Effect of temperature on the growth of colonies of newly isolated fungus K1014 cultured on Sake-koji extract agar plates (Ballg. 10, pH 6.0). Symbols: O, 2 days; ●, 3 days.](image-url)

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### Table 3. Production of proteases by thermotolerant and thermophilic fungi grown on moistened wheat bran

| Fungi                        | Protease (units/ml of culture extract) | pH 3.0 | pH 7.0 |
|------------------------------|----------------------------------------|--------|--------|
| Aspergillus fumigatus HUT 2033 | 6.6                                    | 47.3   |        |
| Rhizopus pseudochinensis Yamazaki IAM 6042 | 12.5                                    | 26.6   |        |
| Chaetomium thermophile var. coprophile ATCC 16451 | 0                                      | 0      |        |
| C. thermophile var. dissitum ATCC 16452 | 0                                      | 0      |        |
| Humicola lanuginosa ATCC 16455 | 2.0                                    | 63.7   |        |
| Malbranchea pulchella var. sulfurua ATCC 16456 | 14.7                                    | 65.7   |        |
| Mucor pusillus HUT 1185 5 | 5.1                                    | 17.7   |        |
| Myroccoccus albomyces ATCC 16460 | 10.8                                   | 112.8  |        |
| Penicillium duponti ATCC 16461 | 8.2                                    | 12.8   |        |
| K1009*                        | 47.6                                   | 10.6   |        |
| K1010*                        | 52.7                                   | 45.1   |        |
| K1012*                        | 38.2                                   | 101.7  |        |
| K1014*                        | 193.6                                  | 14.9   |        |
| K1021*                        | 4.0                                    | 127.5  |        |
| K1045*                        | 8.9                                    | 80.9   |        |

* Culturing was carried out at 40 C for 4 days.
** Selected from new isolates capable of growing at 45 C based on protease productivity (Table 2).
on acid protease production. Figure 4 shows the effect of cultivation temperature on protease production. When the thermophilic fungus was grown on moistened wheat bran, maximal protease production occurred after approximately 5 days at 37 C and 3 to 4 days at 40 C. However, at 45 to 50 C, which was the optimal temperature range for growth, maximal accumulation of protease occurred after approximately 2 days and diminished during further incubation.

Effect of nitrogen compounds on acid protease production. The effect of concentration of nitrogen compounds in wheat bran culture medium on protease production is shown in Table 4. The amount of acid protease was increased by addition of ammonium salt, except for ammonium sulfate, but decreased by addition of nitrate. Organic nitrogen compounds had no significant effect.

Effect of moisture in culture medium on acid protease production. The initial real water contents determined by drying in wheat or wheat bran culture medium containing 1% NH₄NO₃ influenced acid protease production (Fig. 5). The maximal protease production occurred at 39 to 43% real water contents in wheat culture medium and at 43 to 47% real water contents in the case of wheat bran.
TABLE 4. Effect of nitrogen compounds on protease production by a thermophilic fungus, P. duponti K1014, grown on moistened wheat bran

| Nitrogen compounds | Protease activity (units/ml of culture extract) |
|--------------------|-----------------------------------------------|
|                    | 0.05 | 0.1 | 0.3 | 0.5 | 1.0 |
| None               | 279  |     |     |     |     |
| NaNO<sub>3</sub>   | 240  | 200 | 155 | 147 | 100 |
| NH<sub>4</sub>Cl    | 300  | 315 | 318 | 290 | 160 |
| NH<sub>4</sub>HPO<sub>4</sub> | 296 | 285 | 295 | 260 | 0   |
| NH<sub>4</sub>NO<sub>3</sub> | 303 | 315 | 348 | 363 | 380 |
| (NH<sub>4</sub>)<sub>2</sub>P<sub>4</sub> | 295 | 305 | 325 | 305 | 65  |
| (NH<sub>4</sub>)SO<sub>4</sub> | 245 | 210 | 205 | 175 | 40  |
| Ammonium citrate   | 205  | 200 | 225 | 190 | 127 |
| Ammonium tartrate  | 250  | 230 | 185 | 155 | 37  |
| Defatted soybean   | 267  | 263 | 247 | 235 | 200 |
| Polypeptone        | 230  | 215 | 215 | 210 | 207 |
| Urea               | 230  | 200 | 155 | 125 | 60  |
| Yeast extract      | 277  | 273 | 253 | 185 | 70  |

* Culturing was carried out at 40°C for 4 days.
  * Percent (w/w) of nitrogen added per wheat bran.

culture medium. It was also found that wheat, as compared with wheat bran, was suitable for the production of acid protease.

Comparison of thermal stabilities of acid proteases. Table 5 shows the thermal stabilities of the acid proteases from P. duponti K1014 as compared with commercially available acid proteases. The acid protease from P. duponti K1014 was stable at 50 and 60°C for 1 hr. However, the other enzymes, except for the acid proteases from Trametes sanguinea and Mucor pusillus, began to lose activity at 50°C and were rapidly inactivated at 60°C. The acid proteases from T. sanguinea and M. pusillus retained more than 50% of original activity for 1 hr at 60°C. After the heat treatment for 1 hr at 70°C at pH 4.9, the present acid protease retained about 45% of original activity, and at pH 4.7 more than 65% of its activity remained. However, all of the other proteases tested were almost completely inactivated after 1 hr at 70°C.

The present acid protease seems to show the marked thermal stability characteristic of proteins from thermophilic organisms although the results presented above were obtained with a crude enzyme preparation.

Some of the other enzymes responsible for the decomposition of plant materials—amylases, cellulases, hemicellulases, and ribonuclease—were also found in the enzyme preparation from P. duponti K1014 used in these studies.

Fig. 5. Effect of moisture of medium on protease production by a thermophilic fungus, P. duponti K1014. Culturing was carried out at 40°C for 4 days on 5 g of wheat (●) or wheat bran (○) moistened with water containing 0.65 g of NH<sub>4</sub>NO<sub>3</sub>. Initial moisture of medium shows real water content of medium determined by drying just after inoculation.

**DISCUSSION**

Thermophilic and thermotolerant fungi capable of growing at 45°C were isolated from various sources except for hot springs. Composting materials were an especially rich source of thermophiles. Fergus (9) isolated a number of the thermophilic and thermotolerant fungi and actinomycetes from mushroom compost during peak heating. Cooney and Emerson (4) demonstrated that certain thermophilic fungi have been associated with the process of microbial thermogenesis.

Maximal temperature for growth of the fungus K1014 newly isolated from compost was found to be 57 to 58°C. Next to Humicola lanuginosa, the fungus K1014 is the most strongly thermophilic of all the fungi. Morphological and cultural characteristics of the thermophilic fungus K1014 were similar to the description (4, 20) of P. duponti (Griffon and Maublanc) Emerson, and it was identified as Penicillium duponti K1014. However, this isolate differed from P. duponti ATCC 16481 originated from Emerson strain in protease productivity (the former produced 23 times as
TABLE 5. Comparison of thermal stability of commercially available acid proteases

| Proteases                              | Manufacturing companies           | Remaining activity (%)a |
|----------------------------------------|-----------------------------------|------------------------|
|                                        |                                   | pH 3.5 | pH 4.9 | pH 3.5 | pH 4.9 | pH 3.5 | pH 4.9 |
| Present acid protease (Penicillium duponti K1014) | Experimental                     | 100    | 100    | 100    | 94.4   | 3.3    | 43.1   |
| Molisin (Aspergillus saitoi)            | Seishin Pharmaceutical Co., Ltd.  | 91.7   | 85.5   | 10.2   | 16.8   | 0      | 0      |
| Panprosin (A. niger)                    | Kinki Yakuruto Co., Ltd.          | 87.8   | 72.6   | 3.3    | 3.1    | 0      | 0      |
| Vernase (A. oryzae)                     | Taisho Pharmaceutical Co., Ltd.   | 75.5   | 92.4   | 0      | 4.2    | 0      | 0      |
| Colonase (Rhizopus delemar)             | Wakamoto Pharmaceutical Co., Ltd. | 64.2   | 64.4   | 2.9    | 2.6    | 0      | 0      |
| Newlase (Rhizopus)                      | Amano Pharmaceutical Co., Ltd.    | 69.4   | 85.3   | 1.5    | 2.7    | 0      | 0      |
| Sanprose F (Rhizopus)                   | Hankyu Kyoei Products Co., Ltd.  | 67.8   | 61.0   | 4.2    | 8.6    | 0      | 0      |
| Rapidase (Trametes sanguinea)           | Takeda Chemical Industries Co., Ltd. | 92.1 | 94.6   | 6.2    | 56.0   | 0      | 0      |
| Meito rennet (Mucor puillus)            | Meito Sangyo Co., Ltd.            | 83.7   | 100    | 4.7    | 50.1   | 0      | 0      |

*a Commercially available enzymes used in these studies were obtained as samples from manufacturing companies in Japan.

*b Enzymes were heated in 0.05 M acetate buffers (pH 3.5, 4.7, and 4.9) at 50 to 70°C for 1 hr. Protease activity was measured at pH 3.0.

much acid protease as the latter). Furthermore, the strain ATCC 16461 seemed to produce more neutral protease than acid protease (Table 3).

It was found that the protease production by P. duponti K1014 was increased by about 50% by adding an adequate amount of ammonium salt on wheat bran culture medium, but decreased by the addition of nitrate. Ichishima and Yoshida (13) reported that the effect of inorganic nitrogen compounds on the acid protease production was closely related to the position of the strain in the classification of Aspergilli.

Maximal production of the acid protease by P. duponti K1014 occurred after short cultivation time at 45 to 50°C which was the optimal temperature range of growth. P. duponti K1014, as well as other thermophilic organisms, may be useful for industrial enzyme production because of the rapid growth rate and reduction in contamination that results from the elevated growth temperature.

The acid protease from P. duponti K1014 was the most thermostable of the acid proteases tested (Table 5).

Some papers (21–23) have been published on the acid proteases from Penicillia, but a thermostable one like the present enzyme has not been reported yet. The acid protease was a thermostable enzyme according to the criterion of thermostable proteins from thermophilic organisms as defined by Howard and Becker (12), since it lost less than 10% activity after treatment at 60°C for 1 hr.

We have already found that the purified acid protease of P. duponti K1014 which was homogeneous on ultracentrifugation and disc gel electrophoresis had a pH optimum of 2.5, and it showed the marked thermal stability characteristic of proteins from thermophilic organisms as well as a crude preparation (H. Hashimoto et al., Abstracts of Papers, P. 131, Ann. Meeting of Agr. Chem. Soc. Japan, Fukuoka, April 1, 1970).

Details of the identification of the enzyme by the nature of the reaction catalyzed and some other properties including the effect of chelating agents, inorganic ions and inhibitors will be presented in a subsequent manuscript.

ACKNOWLEDGMENTS

We thank S. Kitahara for the operation of electron microscope. Thanks are also due to Y. Kaneko and T. Hayakawa for their technical assistance.
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