Effect of culture conditions on the production of an extracellular proteinase by *Thermus* sp. Rt41A

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**Abstract** *Thermus* sp. Rt41A produced a single extracellular proteinase, as determined by fast protein liquid chromatography and isoelectric focusing. Proteinase activity was expressed from very early in the log phase, and halted when the growth substrate was exhausted. There was no continued proteinase production in the stationary phase. Proteinase production was not stimulated by O₂ limitation, not repressed by amino acid growth substrates, and its production could not be correlated to the type or oxidation state of the carbon and energy source or the growth rate on different carbon and energy sources. Growth on certain substrates, e.g. glutamate and glucose, resulted in production of high levels of proteinase, whereas others, such as acetate, resulted in low proteinase levels. Acetate repressed proteinase production in cultures growing on L-glutamate. In continuous culture on L-glutamate, acetate or pyruvate, proteinase production was highest at higher growth (dilution) rates. The kinetics of proteinase production in continuous culture on L-glutamate can be interpreted as evidence for the constitutive nature of proteinase expression by *Thermus* sp. Rt41A. The data obtained show that the control of proteinase production is different to that postulated for *Thermus* sp. Ok6.A1.

**Introduction**

Since the isolation and characterization of *Thermus aquaticus* YT-1, a large number of *Thermus* spp. strains have been isolated. Many of these produce extracellular proteinases, and some of these enzymes have been characterized. The properties of the proteinases produced by different strains vary, suggesting their potential use in a variety of industrial or diagnostic applications (Bergquist and Morgan 1992). Thus a search for different thermophilic proteinases may provide material for diverse final uses. Cloning the appropriate gene into *Escherichia coli* has proved possible (Terada et al. 1990; Bergquist and Morgan 1992; Lee et al. 1992), and production of such proteinases at high levels may prove feasible in the near future. Regulations restricting the end-use of cloned gene products may, however, mean that this approach may not be generally applicable: for example, restrictions on the use of *E. coli*-produced cloned enzymes in the food industry (Cheetham 1985; Quellette and Cheremisinoff 1985). Cloning and expression of aqualysinI from *T. aquaticus* YT-1 has been possible in *T. thermophilus* (Touhara et al. 1991). Some investigations into the production of thermostable proteinases from the native organism have also been done (Jones et al. 1988; Kanasawud et al. 1989; Janssen et al. 1991), but the levels of production are not yet economically viable except perhaps as specialty enzymes.

*Thermus* sp. strain Rt41A is an aerobic thermophilic bacterium obtained from a New Zealand hot spring (Lim 1983), and has been shown to be phenotypically related to *Thermus* spp. strains T351, Rt6.A1 and Ok6.A1 (Hudson et al. 1989). All four strains have been studied to some degree with respect to their extracellular proteinases (Cowan and Daniel 1982; Cowan et al. 1987; Jones et al. 1988; Peek et al. 1992). 16S rRNA gene sequence analysis revealed a close phylogenetic relationship between strains Ok6.A1, T351 and Rt41A (Saul et al. 1993). It is, however, not known whether the growth conditions resulting in maximum proteinase production for one strain will apply to other *Thermus* strains.

The proteinase from *Thermus* sp. strain Rt41A has been characterized (Peek et al. 1992), and stabilization of the proteinase in the growth medium to maximize harvestable enzyme levels (i.e. stabilize the enzyme in the culture medium once it was produced) was the subject of a previously published investigation (Janssen et al. 1991). The present paper describes studies on the
production of the proteinase by Thermus sp. Rt41A under various conditions and on a number of substrates, with the aim of understanding what influences production of the proteinase by this organism.

Materials and methods

Growth of Thermus

Thermus sp. strain Rt41A was maintained by weekly subculture on agar (15 g / l-1) plates of Castenholz’s medium D (Ramaley and Hisson 1970) with 3 g / l-1 each of trypticase peptone and yeast extract, incubated at 65°C.

Inoculum cultures (100 ml) were grown in 500 ml bottles at 70°C and 150 rpm in an orbital incubator, using 162 medium (Janssen et al. 1991) adjusted to pH 7.5 (at room temperature, with NaOH). Then 10 mM NaL-glutamate (autoclaved), 10 µg / g-1 of d-biotin (filter-sterilized) and 0.2 mM CaCl2 (autoclaved) were added just before inoculation.

A CF2000 fermentation plant with a type I fermentor vessel (Chemap, Mannedorf, Switzerland) was used for 51 pH-controlled (to pH 7.0 with 2 M HCl or NaOH) batch cultures at 70°C, stirred at 500 rpm and with dry air delivered at 400 ml·min-1. Low-phosphate medium (LPM) contained (per litre of distilled water): 50 mg MgSO4·7H2O, 160 mg MgCl2·6H2O, 370 mg KCl, 60 mg KH2PO4, 0.28 mg FeCl3, and 1 ml Nitsch’s trace element solution (Ramaley and Hisson 1970). CaCl2 (autoclaved, 2 mM final concentration) and d-biotin (filter-sterilized, 10 µg·g-1 final concentration) were added after autoclaving. Organic acids and L-glutamate were added as sodium salts.

Continuous culture studies were conducted in a 11 glass vessel with a 400 ml working volume controlled to pH 7.0 with 2 M HCl and 2 M NaOH, controlled at 70°C and stirred at 300 rpm. The continuous culture system was assembled from modules of LH Fermentation (Slough, UK) and Gallenkamp (Crawley, UK), fitted with an Ingold (Urdorf-Zürich, Switzerland) autoclavable pH electrode and with a Quickfit water-cooled glass condenser (Bibby, Stone, UK) at the air outlet. Medium was delivered by a Watson Marlow (Falmouth, UK) model 502 pump. The growth rate (dilution rate) was increased stepwise, allowing at least two vessel volume changes to allow the system to stabilize before sampling.

Analytical methods

Glutamate was assayed using a cuprizone-copper-complexing micromethod (Janssen and Barea 1989). Culture growth was followed by measuring the optical density at 650 nm (OD650) in a 10 mm light path. Cell dry weight yields were calculated from a standard curve of OD650 against dry weights determined as described elsewhere (Janssen et al. 1991). An OD650 of 1.00 corresponded to a cell density of 0.64 mg·ml-1. Acetate, pyruvate, and metabolic products were measured by HPLC (Parel et al. 1987).

Crude proteinase preparation

Five litre cultures were rapidly cooled to room temperature, and bacterial cells removed by centrifugation at 13000g for 10 min. Triton X-100 amended (0.01%, v/v) supernatant was filtered through a PM10 ultrafiltration membrane, concentrated to 100 ml with a YM10 membrane in a 2.51 stirred cell (Amicon, Danvers, Mass., USA), then diluted to 1100 ml with 50 mM HEPES/NaOH, pH 7.5, containing 2 mM CaCl2 and 0.01% (v/v) Triton X-100, followed by concentration to 100 ml.

Fast protein liquid chromatography (FPLC) of culture supernatants

Preliminary IEF was performed on IEF 3–9 Phastgels using a Phast System (Pharmacia LKB). Narrow-range Pharmalyte 8.0–10.5 (Pharmacia LKB) 1% (w/v) agarose gels (225 mm × 115 mm) were run on a Pharmacia FBE 3000 flat-bed electrophoresis unit (sealed, and continuously flushed with N2) at 600 V for 2500 V·h-1 at 10°C. High IEF marker proteins (Pharmacia LKB) were run beside the crude proteinase preparation.

After IEF was complete, one half of the gel was fixed in 10% (w/v) sulphosalicylic acid for 30 min. To the other half, containing an identical set of samples, a 1% (w/v) agarose gel containing 1% (w/v) casein and 10 mM HEPES/NaOH, pH 8.0, was overlayed for 15 min. The overlay was incubated for 15 min at 55°C, then fixed in 10% (w/v) trichloroacetic acid/5% sulphosalicylic acid for 15 min. Gels and overlay were washed twice in destain solution (methanol: acetic acid: water: 3:1:6) for 25 min, stained with 0.2% (w/v) Comassie blue R-250 (Sigma, St. Louis, Mo., USA), and destained overnight. Proteolytic activity in the overlay was detected as a zone of clearing against a uniformly stained gel containing undigested casein.

Results

Proteinase production

The proteinase activity produced by Thermus sp. Rt41A, grown in 162 medium or medium LPM with L-glutamate as the carbon and energy source, was expressed linearly with growth from very early in the log phase until the stationary phase began. Proteinase production appeared to occur only during the phase of active growth of the culture concomitant with metabolism of the growth substrate. Production ceased once growth halted, the substrate was exhausted, and the dissolved O2 concentration returned to 100% saturation (Fig. 1). Upon cessation of growth, the proteinase level began to decrease. This phenomenon was the subject of an earlier investigation (Janssen et al. 1991). Addition of chloramphenicol (a potent protein synthesis inhibitor) to batch cultures at the end of the log phase resulted in