Efficient Selection for Thermostable Protease in *Thermus thermophilus*

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An efficient procedure was established to select for thermostable proteases in an extreme thermophile, *Thermus thermophilus*. A non-protease-secreting mutant derived from *T. thermophilus* TH125 was used as host and the expression plasmid for aqalysin I from *T. aquaticus* YT-1 was constructed as a source of thermostable protease. *T. thermophilus* cells harboring the recombinant plasmid produced active aqalysin I into the medium and were able to grow on a minimal medium containing milk casein as the sole source of carbon and nitrogen.

Key words: aqalysin I; screening; thermostable protease; *Thermus thermophilus*

One important aim of protein engineering is to increase the thermostability of enzymes. Recently, a new strategy was tried in the selection of thermostable proteins using an extreme thermophile, *Thermus thermophilus*.1,2) *T. thermophilus* is a Gram-negative aerobic microorganism that can grow at temperatures from 50 to 80°C,3) and is the only therophilic bacterium for which a host-vector system is now available.4) Maseda and Hoshino5) recently constructed a new expression vector for *T. thermophilus*, in which a *T. thermophilus* promoter was added upstream of the kanamycin resistance gene. Thus, this efficient host-vector system would be useful for selecting heat-resistant mutated proteins in *T. thermophilus* after the introduction of random mutagenesis into the genes. Arnold et al.6,7) have developed a directed evolution approach to screen laboratory-evolved thermostable enzymes. However, no study has been reported on a mutated protease with increased thermostability using the *T. thermophilus* expression system.

Recently, we cloned an alkaline serine protease subtilisin E gene from the mesophilic *Bacillus subtilis* into an expression vector of *T. thermophilus*.8) The gene product was produced as an inactive pro-form, because autoprocessing to the active form did not occur at 60°C. After the introduction of random muta-

![Protease Assay with *T. thermophilus* Cells.](image)

After overnight growth on TM liquid medium, two μl of each *T. thermophilus* culture were spotted on TM agar plates containing 1% skim milk. Halo formation was detected on the plate after 4 days at 60°C.

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mutants that did not secrete proteases into the medium. *T. thermophilus* TH125 cells harboring pTT8 in the late exponential phase in TM medium were mutagenized at 37°C for 45 min with N-methyl-N'-nitro-N-nitrosoguanidine (100 μg/ml in 0.1 M phosphate buffer, pH 7.0; Sigma Chemicals Co., St. Louis, MO). Mutagenized cells were spread onto TM agar plates containing 1.0% skim milk (Wako Pure Chemical Industries, Osaka, Japan) and incubated at 60°C for 20 h. Several mutants that failed to form halos on TM agar plates were selected as possible mutants. As shown in Fig. 1, TH125-DP, a mutant with a tryptophan auxotrophy and harboring pTT8, was used as the host, as described below.

Aqalysin I was used as a thermostable protease for the establishment of selection system. Aqalysin I is a heat-stable subtilisin-type serine protease secreted by *T. aquaticus* YT-1. When *T. thermophilus* cells carrying pNK006, the expression plasmid for the aqalysin I gene carrying the trpB5 gene as a selection marker, were cultured on a MM agar plate containing skim milk, some of the transformants did not form halos around the colonies. It is possible that plasmid pNK006 gradually disappeared from the cells during cultivation due to the homologous recombination between trpB5 genes on the *T. thermophilus* chromosome and pNK006. Therefore, the aqalysin I gene containing the signal sequence and the putative terminator region was cloned into pT8-131, an expression vector carrying the heat-stable kanamycin resistance (Km') gene, as described in the legend for Fig. 2. The resultant plasmid, pAQ-131, was then introduced into *T. thermophilus* TH125-DP with marker rescue transformation. As shown in Fig. 1, a large halo was formed by the recombinant strain harboring pAQ-131. The transformant carrying the pT8-131 vector did not form a halo. This finding indicates the production and extracellular secretion of aqalysin I in *T. thermophilus*.

The growth conditions under which *T. thermophilus* cells produce thermostable aqalysin I were established. Venkeki et al. developed a method for rapid screening of active trypsin revertants among many random mutants in *Escherichia coli* using protease-selective plates containing 0.2% glucose, 0.125% bovine serum albumin, and other constituents on the plates. In the *T. thermophilus* expression system, bovine serum albumin was used as the sole source of carbon and nitrogen. However, the colony growth of each transformant remained insensitive, and the bovine serum albumin was easily precipitated due to heat denaturation (data not shown). Heat-denatured milk casein (Hammarsten; Merck, Darmstadt, Germany) was tested as the sole source of carbon and nitrogen (Table 1). *T. thermophilus* cells harboring the recombinant plasmid pAQ-131 were able to grow on plates containing 0.5% and 1.0% casein after 4 days at 60°C, but the cells carrying pTOS, a *T. thermophilus* expression plasmid for the mesophilic *B. subtilis* subtilisin E gene, did not form colonies even after cultivation for 5 days. At high temperature (60°C), prosubtilisin E could not be processed autocatalytically and was then not folded to the active form. A clear halo was only observed around cells producing aqalysin I (data not shown). On the other hand, both strains harboring pTOS and pAQ-131 failed to grow on MM medium without any carbon
and nitrogen sources. These findings indicate that only cells secreting thermostable protease will be selected on the minimal medium containing casein as the sole source of carbon and nitrogen. Similar results were obtained by cultivation in liquid medium. The growth was monitored by measuring absorbance at 660 nm. As shown in Fig. 3, growth of cells harboring pAQ-131 was observed in the presence of 0.5% and 1.0% casein as the sole source of carbon and nitrogen after cultivation at 60°C for 2 days. This finding indicates that the production of aqulasin I in the recombinant strain causes cell growth. It is worth noting that the optical density of the cells harboring pAQ-131 containing casein was somewhat higher than that of the culture in normal MM medium (Fig. 3). This is perhaps due to the degradation of casein by newly secreted aqulasin I in the medium, which caused small peptide fragments to form. This process led to the increase in optical density as the transformants grew on the medium. In the case of liquid culture, the cells that did not produce thermostable protease would have been able to grow slowly by using the peptides and amino acids. It would be necessary to isolate a single colony capable of growing on a selection plate from the culture.

In conclusion, this study indicated that efficient selection conditions for thermostable protease are established by the use of a T. thermophilus expression system. The use of such a system will select for only the transformants capable of growing on a particular medium. This method would be useful for isolating thermostable mutant proteases such as subtilisin after the introduction of random mutagenesis into wild-type genes; such a study is currently in progress.

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References


