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Medium Optimization and Application of Affinity Column Chromatography for Trypsin Production from Recombinant *Streptomyces griseus*

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The production of *Streptomyces griseus* trypsin (SGT) by *S. griseus* IFO13350 transformed with the expression vector pWHM3-TR1R2, containing *sprT* encoding SGT and the two positive regulatory genes *sgtR1* and *sgtR2*, was investigated in various media. Cultivation in Ferm-0 gave 1.4 times more trypsin activity than in C5/L medium. In addition, replacement of 2% glucose and 1% skim milk in Ferm-0 with 2% dextrin and 1% tryptone (designated Ferm-II) enhanced trypsin activity 4.1-fold. To simplify the purification process, the supernatant from the *S. griseus* transformant cultured in Ferm-II medium was fractionated with ammonium sulfate (25–55%), then subjected to Hitrap Benzamidine FF affinity column chromatography. The specific activity of SGT purified by one-step chromatography was 69,550 unit/mg protein and the overall purification yield was above 8%, indicating that this method is more effective than those previously reported. Purified SGT was most active at pH 8.0 and 50°C, and it maintained activity between pH 7.0 and 9.0 and at temperatures up to 70°C. These enzymatic properties are very similar to those of authentic eukaryotic trypsin purified from bovine pancreas.

Keywords: *Streptomyces griseus* trypsin (SGT), medium optimization, affinity column chromatography

The soil-dwelling filamentous bacterial genus *Streptomyces* is comprised of gram-positive bacteria with a characteristic complex life cycle referred to as morphological differentiation.

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S. griseus produces not only about 180 secondary metabolites, including streptomycin, but also several types of proteases, sold under the commercial name Pronase.

S. griseus trypsin (SGT) is one of the major extracellular proteases of this organism. SGT is a serine protease that hydrolyzes amides and esters, and a triad of serine, histidine, and aspartic acid residues is located in its catalytic center [15, 19]. SGT encoded by *sprT* represents most of the trypsin protease produced by *S. griseus* [7]. The genes for two trypsins, SGT and its homologous protein, SprU [11, 20], and three chymotrypsins, SGPA, SGPB, and SGPD, have been cloned [3–5, 12]. All of these genes were found to be members of the AdpA regulon [7, 18], which is actively controlled by AdpA, the key transcriptional activator in the A-factor regulatory cascade that modulates the initiation of morphological and physiological differentiation in *S. griseus* [6, 8].

Overexpression of *sprT* induced significant morphological changes in *S. lividans*, but none were apparent in *S. griseus* strains overexpressing *sprT* or with disrupted *sprT* [2, 7]. Besides being directly controlled by AdpA, SGT production is also enhanced by two regulators, SgtR1 and SgtR2, whose genes are located downstream of *sprT* on the chromosome [16]. Interestingly, the transcription of *sgtR1* and *sgtR2* is not dependent on AdpA; therefore, they are not members of the AdpA regulon [16].

Although SGT is industrially important because it can be used in medications as an antiphlogistic and a digestive, in the food industry in bakery and cheese applications, and in detergent formulations, little is known about its biochemistry and conditions of overproduction in bacteria. Recently, we constructed the expression vector pWHM3-TR1R2 for *sprT* followed by the two regulatory genes

sgtR1 and *sgtR2*, and compared SGT production in *S. lividans* TK24 and *S. griseus* IFO13350 transformed with this construct [10]. Various media of different composition were used to maximize the production of SGT in the recombinant strains. SGT production was best when *S. griseus* IFO13350/pWHM3-TR1R2 was cultivated in C5/L medium [10].

In the present study, we chose *S. griseus* IFO 13350/pWHM3-TR1R2 as the SGT producer, developed a better medium than C5/L for SGT production, and evaluated a very simplified SGT purification process.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Media

The wild-type strain *S. griseus* IFO13350 was obtained from Prof. S. Horinouchi (The University of Tokyo, Japan) and *S. lividans* TK24 [9] was acquired from the John Innes Institute, United Kingdom. R2YE medium [9] was used for maintaining *Streptomyces* strains, and R2YE plates containing 2% agar were used for regenerating protoplasts and, after addition of thiostrepton (25 µg/ml), for selecting transformants. The high-copy-number plasmid pWHM3, a *Streptomyces–E. coli* shuttle vector, was used for overexpressing SGT in *Streptomyces* [16].

Enzymes and Chemicals

The artificial chromogenic substrate *N*-benzoylarginine *p*-nitroanilide (BAPNA) for the trypsin protease assay was obtained from Boehringer Mannheim GmbH, Germany. Other fine chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Fermentation and Sample Preparation

Stocks of *Streptomyces* transformant spore suspensions were used to inoculate 100 ml of R2YE liquid medium in 500 ml baffled flasks. After incubation at 28°C with vigorous shaking at 200 rpm for 48 h, 1 ml of the culture broth was transferred to 100 ml of various fresh liquid media in 500 ml baffled flasks. To investigate the effects of different carbon and nitrogen sources on SGT production, 1 ml of *S. griseus* IFO13350/pWHM3-TR1R2 preculture was used to inoculate 100 ml of modified media. After 24 h of cultivation under the same conditions, a 10 ml sample of the culture broth was taken every 48 h and centrifuged at 6,000 rpm for 10 min. The supernatant was used to measure protein concentration and trypsin protease activity, and the cell pellet was disrupted by sonication and used to measure protein concentration.

Trypsin Activity Assay

The release of *p*-nitroanilides by the enzymatic hydrolysis of an artificial chromogenic substrate was estimated spectrophotometrically [17, 19]. Briefly, 890 µl of a reaction buffer (50 mM Tris-Cl, pH 8.0, and 20 mM $CaCl_2$) and 10 µl of 50 mM BANPA in DMSO were prewarmed for 5 min at 37°C, then rapidly added to 100 µl of the enzyme. The reaction mixture was incubated for 30 min at 37°C, then the reaction was stopped by the addition of 400 µl of 30% acetic acid in dioxane. The absorbance at 405 nm was recorded. One unit of trypsin was defined as the amount of enzyme corresponding to an increase in absorbance of 0.1 under the above conditions.

SDS–PAGE Protein Analysis

SDS–PAGE was performed according to the method of Laemmli [14] using a 15% acrylamide gel on a Mini-Protean II Cell (Bio-Rad, U.S.A.). After electrophoresis at 40 mA, gels were stained with Coomassie Brilliant Blue R-250. Protein concentrations were measured according to Bradford method [1] using bovine serum albumin (BSA) as the standard.

Purification of SGT from the *S. griseus* IFO13350 Transformant

S. griseus/pWHM3-TR1R2 was grown in 100 ml of Ferm-II medium in a 500-ml baffled flask at 28°C with vigorous shaking for 7 days. After centrifugation at 6,000 rpm for 10 min, the supernatant was concentrated by precipitation with 25–55% ammonium sulfate. The precipitate was resuspended in 5 ml of buffer A (10 mM Tris-Cl, pH 8.0, 10% glycerol, and 1 mM EDTA) and dialyzed overnight against 100 ml of the same buffer. The sample was loaded onto a Hitrap benzamidine FF column (Φ1.6×2.5 cm, GE Healthcare, catalog number 17-5144-01) previously equilibrated with buffer A, and the column was washed with buffer B (10 mM NaOAc, pH 5.0, 10% glycerol, 1 mM EDTA, and 0.5 M NaCl). Proteins were eluted with 6 M guanidine chloride solution at a flow rate of 2.5 ml/min, and fractions containing trypsin activity were collected and dialyzed against buffer A.

Analysis of SGT Enzymatic Properties

To study the enzymatic properties of purified SGT, the activity toward BAPNA was compared with that of bovine pancreatic trypsin (T1426, Sigma, U.S.A.) at various pHs ranging from 3 to 9 at 37°C. Trypsin activity was also measured at various temperatures ranging from 4 to 90°C at pH 8.0.

RESULTS

Selection of Medium for SGT Production

Previously, we reported that *S. griseus* IFO13350/pWHM3-TR1R2 could produce more SGT than *S. lividans* TK24/pWHM3-TR1R2 in all of the media tested [10]. Therefore, *S. griseus* IFO13350/pWHM3-TR1R2 was chosen as the host strain for SGT production, and further investigation of optimal medium composition was performed. The culture media that we tested are listed in Table 1. In Ferm-0 medium, trypsin activity increased sharply until 3 days of cultivation, then rose more gradually to reach the highest activity (A_{405}=2.048) at 7 days of cultivation (Fig. 1). Protease activity in C5/L and R2YE culture broth at 7 days of cultivation was 1.518 and 1.284, respectively. In Livid and NDSK media, trypsin activity was very low compared to that in Ferm-0 medium as previously described [10]. This result indicates that Ferm-0 medium is the best among those tested for overproduction of SGT by recombinant *S. griseus*. In contrast, R2YE has been reported to be the best medium for overexpression of *sprA*, *sprB*, *sprD*, and *sprT* in *S. lividans* [2, 5, 12]. In the case of *S. griseus* IFO13350/pWHM3-TR1R2, C5/L has been known to be the best medium for SGT production [10]. However, our data clearly

Table 1. Culture media used in this study.

Medium	Ingredient	Amount (g/l)	Medium	Ingredient	Amount (g/l)
NDSK	$NaNO_3$	4.28	Livid	MOPS	1.49
	$K_2HPO_4 \cdot 3H_2O$	0.23		Nutrient broth	1.0
	HEPES	4.77		$CaCl_2 \cdot 2H_2O$	0.735
	$MgSO_4 \cdot 7H_2O$	0.12		KH_2PO_4	0.5
	Trace elements	2 ml		K_2HPO_4	0.5
	Skim milk	10		Fructose	0.25
	Maltose	45.04		Skim milk	20
				pH 7.2	
C5L	Glucose	12.5	R2YE	Sucrose	103
	Yeast extract	5.0		K_2SO_4	0.25
	$CaCO_3$	8.0		$MgCl_2 \cdot 6H_2O$	10.12
	NaCl	5.3		Glucose	10
	pH 7.2			Casamino acid	0.1
				Yeast extract	5
Ferm-0	Glucose	20		**After autoclaving, add**	
	Skim milk	10		0.5% K_2HPO_4	10 ml
	KH_2PO_4	1.0		3.68% $CaCl_2 \cdot 2H_2O$	80 ml
	K_2HPO_4	3.4		20% L-proline	15 ml
	$MgSO_4 \cdot 7H_2O$	0.3		5.73% TES (pH 7.2)	100 ml
	$FeSO_4 \cdot 7H_2O$	0.01		Trace elements	2 ml
	$ZnCl_2$	0.1		***Trace element solution**	**(mg/l)**
	$CuSO_4 \cdot 7H_2O$	0.01		$ZnCl_2$	40
	$MgCl_2 \cdot 4H_2O$	0.003		$FeCl_3 \cdot 6H_2O$	200
	$CaCl_2$	0.01		$CuCl_2 \cdot 2H_2O$	10
	NaCl	0.03		$MnCl_2 \cdot 4H_2O$	10
	pH 7.0			$Na_2B_4O_7 \cdot 10H_2O$	10
				$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	10

demonstrate that cultivation of *S. griseus* IFO13350/pWHM3-TR1R2 in Ferm-0 could give 1.35 times more trypsin activity than in C5/L medium.

Optimization of Medium Composition for SGT Production

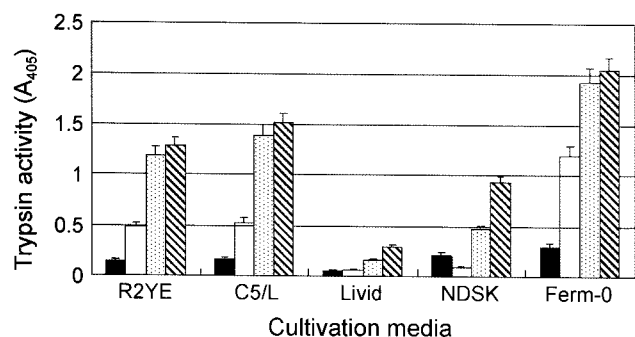Once Ferm-0 was chosen as the best medium for SGT production by *S. griseus* IFO13350/pWHM3-TR1R2, the

Fig. 1. Comparison of *S. griseus* IFO13350/pWHM3-TR1R2 trypsin activity in various media.
Protease activity is expressed as the absorbance at 405 nm following the enzyme reaction described in Materials and Methods. Transformants were cultured in the indicated media and sampled on day 1 (black bars), 3 (white bars), 5 (dotted bars), and 7 (striped bars) of cultivation. Each value is an average of three parallel replicates.

effect of various carbon and nitrogen sources was investigated. First, to determine which carbon source can best enhance SGT production, 2% maltose, sucrose, starch, lactose, dextrin, or fructose was added to Ferm-0 medium instead of 2% glucose. When dextrin or starch was used, trypsin activity was highest (4.03) at 7 days of cultivation (Fig. 2A), 2.2-fold higher than that obtained in the control containing glucose. SGT activity in modified Ferm-0 medium containing glucose, maltose, sucrose, lactose, or fructose was 1.8, 0.087, 1.19, 1.01, and 0.32, respectively. Thus, dextrin was chosen as the best carbon source among those tested for SGT production.

Next, the effect of various nitrogen sources on SGT production was tested. Ferm-I broth containing 2% dextrin instead of 2% glucose was used as the basal medium. Then, the 1% skim milk in Ferm-I was replaced by 1% yeast extract, peptone, tryptone, casein, or soytone. When *S. griseus* IFO13350/pWHM3-TR1R2 was cultured in each medium for 7 days, the highest SGT activity (7.29) was found in the culture medium containing 1% tryptone (Fig. 2B). Trypsin activity in culture medium containing skim milk, yeast extract, peptone, casein, or soytone was 6.0, 5.27, 6.86, 4.63, and 6.33, respectively. Based on these results, tryptone was selected as the best nitrogen source for SGT production by *S. griseus* IFO13350/pWHM3-TR1R2.
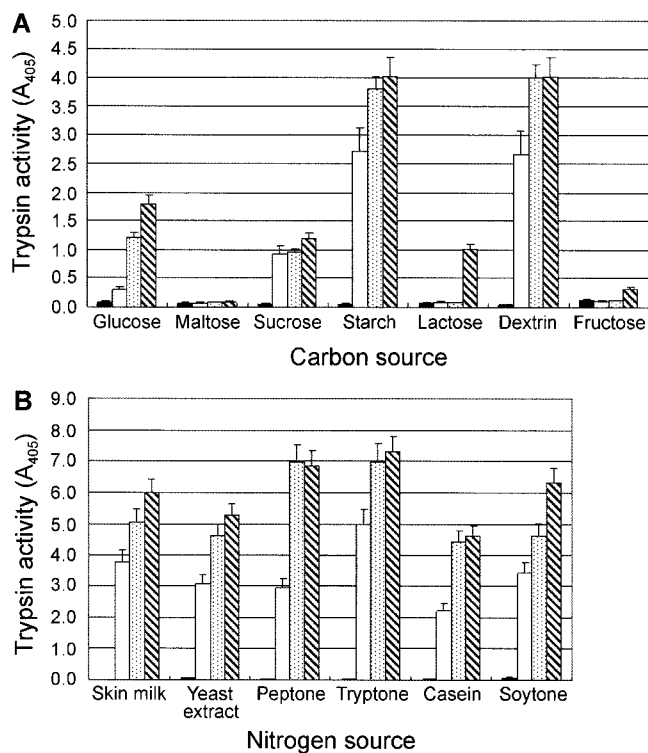
Fig. 2. Effects of carbon (**A**) and nitrogen (**B**) sources on trypsin production by *S. griseus* IFO13350/pWHM3-TR1R2.
A. Ferm-0 medium containing 2% glucose was used as the basal medium and the carbon source (glucose) was replaced by 2% maltose, sucrose, starch, lactose, dextrin, or fructose. **B.** Ferm-1 medium containing 2% dextrose and 1% skim milk was used as the basal medium and the nitrogen source (skim milk) was substituted with 1% yeast extract, peptone, tryptone, casein, or soytone. Protease activity is expressed as absorbance at 405 nm following the enzyme reaction described in Materials and Methods. Transformants were cultured in the indicated media and sampled on day 1 (black bars), 3 (white bars), 5 (dotted bars), and 7 (striped bars) of cultivation. Data are the average of three experiments.

Fig. 3. Trypsin production by *S. griseus* IFO13350/pWHM3-TR1R2 and concentration of total cellular protein depending on cultivation time in R2YE and optimized Ferm-II media.
Ferm-II is Ferm-0 medium modified containing 2% dextrin and 1% tryptone as the carbon and nitrogen sources, respectively. Trypsin production is expressed as units per milligram of cellular protein following the enzyme reaction. The concentration of cellular protein was measured by Bradford method after cell disruption by sonication and is expressed in microgram of cellular protein per milliliter. Each value is the average of three parallel replicates. Filled and open circles: trypsin production and total cellular protein in R2YE medium, respectively. Filled and open squares: trypsin production and total cellular protein in Ferm-II medium, respectively.

Thus, Ferm-II broth containing 2% dextrin and 1% tryptone as carbon and nitrogen sources was used for fermentation of *S. griseus* IFO13350/pWHM3-TR1R2 (Fig. 3). The highest level of trypsin production in this medium was 8,863 unit/mg of cellular protein, observed at 7 days of cultivation, whereas that in R2YE was 3,576 unit/mg of cellular protein. The synthesis of cellular protein (*i.e.*, cell growth) reached the maximum level in both media at 3 days of cultivation, whereas R2YE gave a higher concentration of cellular protein (140 μg/ml) than did Ferm-II (97 μg/ml). However, a more rapid cultivation time-dependent decrease in cellular protein level was observed in R2YE.

Purification of SGT by Affinity Column Chromatography
Several reports have described the purification of SGT from the culture broth of *S. griseus* [10, 13]. However, most methods employed several steps of ion-exchange chromatography, using either CM-Sepharose and SP-Sepharose, or CM-Sepharose, Sephadex G-200, Mono-S,
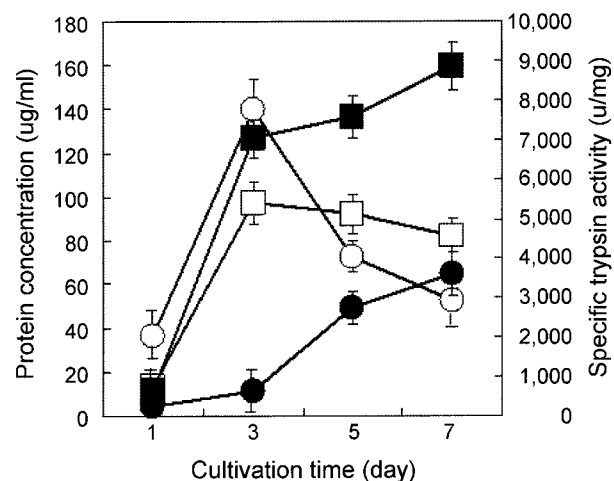
and Superose-12, both of which resulted in very poor yield (1.4% and 2.6%, respectively).

To improve the yield, a simple new purification method was evaluated. The supernatant from *S. griseus* transformant cultured in Ferm-II medium was fractionated with ammonium sulfate (25–55%), then subjected to Hitrap benzamidine FF affinity column chromatography. The highly purified SGT obtained by this method was confirmed by SDS–PAGE (Fig. 4). The specific activity of the purified SGT was 69,550 unit/mg protein and the overall purification yield was above 8%, indicating that this method was more effective than those in previous reports.

Effects of pH and Temperature on SGT Activity
We tested the effects of pH and temperature on the protease activity of purified SGT using the artificial substrate, BAPNA, and bovine pancreatic trypsin as the control. Purified SGT and the control showed the highest level of trypsin activity at pH 8.0 and maintained their activity at pH ranging 7.0 to 9.0 (Fig. 5A). Although sharp decreases in enzyme activitiy were observed under extreme acidic conditions, the degree of the decrease was greater for bovine pancreatic trypsin than for SGT, implying that SGT is more stable under acidic conditions.

SGT showed the highest activity at 50°C, and it was maintained up to 70°C. Activity decreased slowly in proportion to the decrease in temperature below 50°C, but decreased
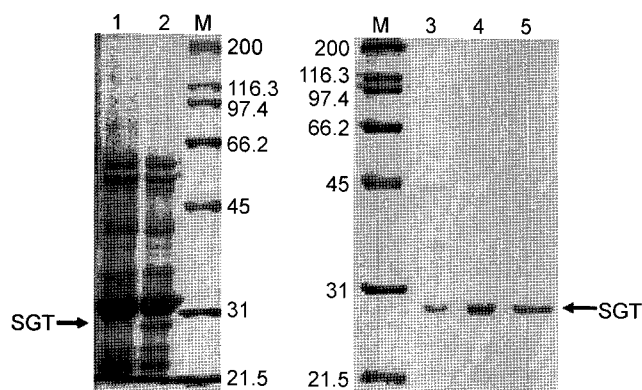
Fig. 4. Purification of SGT from *S. griseus* IFO13350/pWHM3-TR1R2.

Extracellular protein in *S. griseus* culture broth was precipitated with ammonium sulfate and analyzed by SDS–polyacrylamide gel electrophoresis. Lane M, molecular weight standards; lane 1, total extracellular protein from *S. griseus*/pWHM3 as the control; lane 2, total extracellular protein from *S. griseus*/pWHM3-TR1R2; lanes 3–5, fractions containing SGT purified by affinity column chromatography. SGT protein with a molecular mass of 28 kDa is indicated by an arrow.

sharply above 70°C, and only 19.76% of the maximum protease activity remained at 80°C (Fig. 5B). Although bovine pancreatic trypsin activity was highest at 60°C, its relative activity at various temperatures was very similar to that of SGT. These data clearly show that the enzymatic properties of prokaryotic SGT are almost the same as those of eukaryotic trypsin.

DISCUSSION

Pronase is a commercially available mixture of proteinases isolated from the culture broth of *S. griseus*, and SGT is one of these proteinases. The *sprT* gene encoding SGT has been cloned, and a molecular study revealed that two regulatory genes, *sgtR1* and *sgtR2*, located downstream of *sprT*, could stimulate its expression at the transcriptional level [16]. Based on this finding, the overexpression vector pWHM3-TR1R2 containing *sprT*, *sgtR1*, and *sgtR2* was previously constructed and the production of SGT by the transformant *S. griseus* IFO13350 was investigated in various media (Fig. 1). Among the media tested, Ferm-0 supported the highest SGT production, and optimization of this medium by replacing 2% glucose and 1% skim milk with 2% dextrin and 1% tryptone as the carbon and nitrogen sources, respectively (designated Ferm-II; see Table 1) enhanced trypsin production by 4.1-fold (Fig. 3).

The results of our previous study indicate that R2YE supported the highest production of SGT in *S. lividans* TK24/pWHM3-T [13]. However, Ferm-II, C5/L, and R2YE supported production of decreasing amounts of SGT in *S. griseus*/pWHM3-TR1R2 in that order. These results strongly imply that the two strains are quite different in
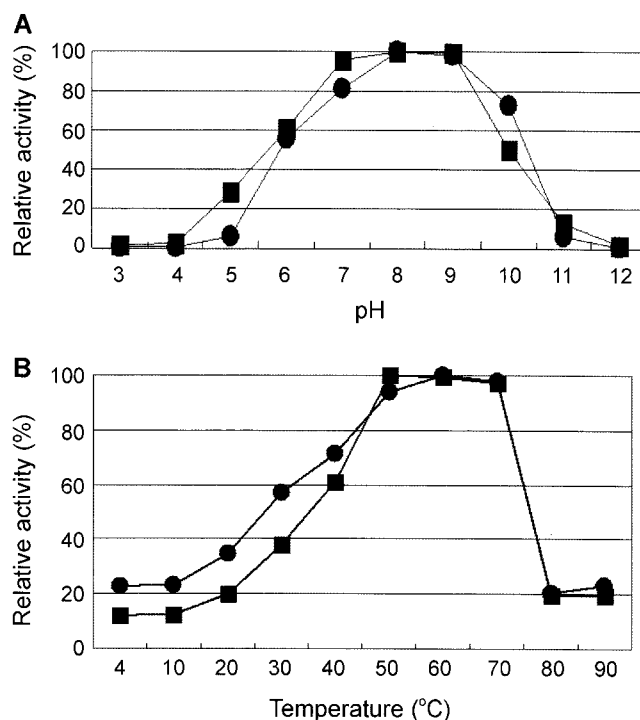

Fig. 5. Enzymatic properties of SGT at various pHs and temperatures. The enzyme activity of SGT purified from *S. griseus*/pWHM3-TR1R2 was measured with the artificial substrate BAPNA in Tris-universal buffer at pH ranging from 3 to 9 at 37°C (**A**) and at temperatures ranging from 4 to 90°C at pH 8.0 (**B**). The highest protease activity was considered to be 100% when calculating relative activities. Authentic trypsin purified from bovine pancreas was used as the standard for comparison. Squares, SGT purified from *S. griseus*/pWHM3-TR1R2; Circles, trypsin purified from bovine pancreas.

their preferences for carbon and nitrogen sources. R2YE contains 11.3% carbon (10.3% sucrose+1% glucose) and 0.81% nitrogen (0.01% casamino acid+0.5% yeast extract +0.3% proline), which may more adequately support microbial growth. In fact, cultivation in R2YE gave a higher concentration of cellular protein (140 µg/ml) than in Ferm-II (97 µg/ml), but resulted in a more rapid decrease in cellular protein concentration over time. The specific activity of trypsin produced by *S. griseus*/pWHM3-TR1R2 was nearly 2.5 times higher in Ferm-II (8,863 unit/mg of cellular protein) than in R2YE (3,576 unit/mg of cellular protein). The biased proportion of carbon in R2YE seems to stimulate cell lysis and decrease production of metabolites. This result provides an interesting example of how better growth does not guarantee higher cellular productivity.

Hitrap benzamidine FF affinity column chromatography has been used to purify many serine proteases. We also succeeded in purifying SGT by this method. The specific activity of the purified SGT was 69,550 unit/mg protein, which is five times higher than that of authentic eukaryotic trypsin from bovine pancreas. The purified SGT was most active at pH 8.0 and 50°C, and maintained its activity

between pH 7.0 and pH 9.0 and at temperatures up to 70°C. These enzymatic properties are very similar to those of the eukaryotic trypsin, but SGT appears to be a little more stable under acidic and high temperature conditions.

Acknowledgment

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