

Screening and partial characterization of inhibitors of insect β -N-acetylglucosaminidase

Hirokazu USUKI, Teruhiko NITODA, Toru OKUDA[†] and Hiroshi KANZAKI*

Laboratory of Bioresources Chemistry, The Graduate School of Natural Science & Technology,
Okayama University, Okayama 700–8530, Japan

[†] Mycology & Metabolic Diversity Research Center, Tamagawa University Research Institute,
Tamagawa University, Tokyo 194–8610, Japan

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Microbial culture broths were screened for novel β -N-acetylglucosaminidase (GlcNAcase) inhibitors specific for the enzyme of insect origin. Four strains of actinomycetes, *Streptomyces griseoloalbus* JCM4480, *S. clauifer* JCM5059, *S. anulatus* NBRC13369 and *S. griseus* subsp. *rhodochrous* NBRC13849, produced unique compounds showing selective inhibition of the insect GlcNAcase. In contrast, 4 fungal strains, *Paecilomyces* sp. F13, F30, *P. carneus* F2281 and *Verticillium* sp. F40, were found to produce GlcNAcase inhibitors showing a broad spectrum of inhibitory activity against GlcNAcases from insects, mammals, plants and fungi. These results indicated obvious differences in GlcNAcases between insects and other organisms. This is the first report of enzyme inhibitors specific for the GlcNAcase of insect origin. © Pesticide Science Society of Japan

Keywords: chitin metabolic turnover, chitinolytic enzyme system, enzyme inhibitor, insect growth regulator (IGR), microbial metabolite, β -N-acetylhexosaminidase (EC 3.2.1.30).

Introduction

Chitin, a linear polysaccharide of β (1,4)-linked N-acetylglucosamine residues, is a structural component of the cuticle and the peritrophic membrane in the mid-gut of insects, and the strict regulation of its metabolism is essential for the normal growth of insects. The chitinolytic enzyme system consisting of chitinase and β -N-acetylglucosaminidase (GlcNAcase) is essential for the metabolic turnover of chitin in insects, and hence, inhibitors of these enzymes are expected to be potential insect growth regulators (IGRs). Over the past few years, chitinases have been regarded as potential target enzymes for IGRs since the hydrolysis of chitin by the enzymes is considered the rate-determining step in the degradation of chitin. Although several compounds such as allosamidin,¹⁾ argifin^{2,3)} and argadin⁴⁾ have already been discovered as chitinase inhibitors, none have been used commercially as IGRs. One reason for this is that these compounds inhibit family 18 chitinases of many different organisms.^{5,6)}

We have previously isolated a novel chitinase inhibitor, FPS-1, from the culture filtrate of *Sphaeropsis* sp. TNPT116-

Cz.⁷⁾ FPS-1 potently inhibited an insect chitinase from *Spodoptera litura* (common cutworm), but did not inhibit an actinomycete chitinase from *Streptomyces griseus*. In contrast, the known chitinase inhibitor allosamidin potently inhibited both chitinases. These results indicated that the insect chitinase was apparently distinct from the chitinases of other organisms.

GlcNAcase forming part of the chitinolytic enzyme system is necessary for the complete degradation of chitin into its monosaccharide, but little attention has been given to it as a target enzyme for new IGRs because of its wide distribution in nature including fungi, actinomycetes, plants and mammals. However, the presence of the insect chitinase inhibitor FPS-1 strongly suggested that the chitinolytic enzyme system in insects consists of a unique chitinase and GlcNAcase. The question then arises, are there enzyme inhibitors specific for the insect GlcNAcase in nature?

This paper reports on the screening and partial characterization of inhibitors specific for the GlcNAcase of insect origin.

Materials and Methods

1. General

Nagstatin was a gift from Microbial Chemistry Research Foundation (Japan). Pupae of *Adoxophyes orana* and *Musca*

* To whom correspondence should be addressed.

E-mail: hkanzaki@cc.okayama-u.ac.jp

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domestica and larvae of *Periplaneta fuliginosa* were kindly provided by Agricultural Chemicals Research Laboratory, Sumitomo Chemical, Japan. β -N-Acetylglucosaminidase from bovine kidney was purchased from SIGMA (USA). All other chemicals were commercially available.

2. Preparation of GlcNAcase solutions

All procedures were carried out at a temperature of between 0 and 4°C. A crude GlcNAcase solution from *S. litura* was prepared using the same method as for the preparation of the chitinase solution described in our previous paper.⁸⁾ Other insect GlcNAcase solutions were prepared with similar procedures but with the extraction buffers changed as follows. (1) Pupae of summer fruit tortrix, *Adoxophyes orana*: 50 mM citrate-phosphate-borate buffer (pH 7.0) containing 0.01% phenylthiourea. (2) Larvae of fruit fly, *Drosophila melanogaster* and larvae of smokybrown cockroach, *Periplaneta fuliginosa*: 50 mM acetate buffer (pH 5.0) containing 0.01% phenylthiourea. (3) Larvae of house fly, *Musca domestica*: 50 mM acetic acid-sodium phosphate buffer (pH 6.5) containing 0.01% phenylthiourea.

Aspergillus fumigatus IAM2046 was cultured in chitin medium (pH 5.0) consisting of colloidal chitin 10, Polypepton (Nacalai Tesque, Japan) 0.5, yeast extract (Nacalai Tesque, Japan) 0.5, glucose 1.0, K₂HPO₄ 0.7, KH₂PO₄ 0.3, and MgSO₄·7H₂O 0.5 (g/l water) for 6 days at 28°C with reciprocal shaking (280 strokes/min, 28°C) and the culture filtrate was used as a crude GlcNAcase solution.

Sphaeropsis sp. TNPT116-Cz was cultured at 28°C for 12 days in YpSs medium (pH 5.6) consisting of potato starch 15.0, yeast extract (Nacalai Tesque, Japan) 4.0, and MgSO₄·7H₂O 0.5 (g/l water) under static conditions and the culture broth was filtered. The mycelia were homogenized in 50 mM citrate-phosphate-borate buffer (pH 4.0) after one cycle of freezing and thawing. The obtained suspension was centrifuged (20,000 g, 20 min) and the supernatant was used as a crude GlcNAcase solution.

A crude GlcNAcase solution from seeds of sweet corn, *Zea mays* L., was prepared as described elsewhere.⁹⁾

3. Methods of assaying GlcNAcase inhibitory activity

In the screening experiment, the following enzyme inhibitory assay was used. Culture filtrates were assayed directly. Culture extracts were evaporated under reduced pressure and the dried materials were suspended in 5% DMSO as test samples. Eighty microliters of the test sample was added to 16 μ l of 5 mM *p*-nitrophenyl-*N*-acetylglucosaminide (*p*NP-GlcNAc) solution. Then, 24 μ l of 232 mM citrate-phosphate-borate buffer, pH 6.0, was added and mixed very well. The enzymatic reaction was initiated by adding 40 μ l of *S. litura* crude GlcNAcase solution in 14.3 mM of the same buffer. This mixture was incubated for 60 min at 37°C. The reaction was quenched by adding 100 μ l of 1.3 M NaOH and the amount of *p*-nitrophenol (*p*NP) liberated was determined at 415 nm. The

amount of enzyme was adjusted so that the increase in A₄₁₅ of the control mixture containing H₂O instead of sample solution was 0.500. Percent inhibition was calculated by the equation.

$$\text{Inhibition (\%)} = 100 - \{(A - B) / (C - D) \times 100\}$$

Where A, B, C and D are the absorbance (A₄₁₅) of the test mixture, substrate blank for the test mixture, control reaction mixture containing no test sample and substrate blank for the control reaction mixture, respectively. One unit of GlcNAcase inhibitory activity is defined as the amount of inhibitor, which caused 50% inhibition of *S. litura* GlcNAcase activity, measured under the above assay conditions.

The inhibitory assay against bovine kidney GlcNAcase was carried out in a similar manner to the inhibitory assay against *S. litura* GlcNAcase, except that 100 mM sodium citrate buffer containing 100 mM NaCl and 0.01% (w/v) bovine serum albumin (pH 5.0) was used instead of 232 mM citrate-phosphate-borate buffer (pH 6.0).

For evaluation of the specificity of the active compounds, GlcNAcases from insects, fungi, plants and mammals were used. The final volume of reaction mixture was decreased to 60 μ l and the concentration of buffer was changed to 50 mM. The types of buffer were selected based on published papers, except for the GlcNAcase of *Sphaeropsis* sp. TNPT116-Cz. In the inhibitory assay of *Sphaeropsis* sp. TNPT116-Cz GlcNAcase, a citrate-phosphate-borate buffer of pH 4.0 was used, which was optimum for the reaction as determined in preliminary experiments.

(1) *S. litura* and *A. orana*: citrate-phosphate-borate buffer (pH 7.0),⁸⁾ (2) *D. melanogaster*: sodium acetate buffer (pH 5.5),¹⁰⁾ (3) *M. domestica*: acetic acid-sodium phosphate buffer (pH 6.5),¹¹⁾ (4) *P. fuliginosa*: sodium acetate buffer (pH 5.0),¹²⁾ (5) *A. fumigatus* IAM2006: citrate-phosphate-borate buffer (pH 5.0),¹³⁾ (6) *Sphaeropsis* sp. TNPT116-Cz: citrate-phosphate-borate buffer (pH 4.0), (7) *Zea mays* L.: citrate-phosphate-borate buffer (pH 5.0),⁹⁾ (8) bovine kidney: sodium citrate buffer containing 100 mM NaCl and 0.01% (w/v) bovine serum albumin (pH 5.0).¹⁴⁾

4. Microbial cultivations and sample preparations for screening

Twenty-five strains of actinomycetes were cultured at 28°C for 4–6 days on a reciprocal shaker at 280 strokes/min in 2 different media, F medium and a modified Bennett's medium containing colloidal chitin as a carbon source. F medium (pH 5.3): glucose 10.0, soybean meal (Mitsubishi Chemical, Japan) 10.0, CaCO₃ 2.0, glycerol 5.0, dry yeast powder (Asahifood & Healthcare, Japan) 5.0, Corn Steep Liquor (Syouno Denpun, Japan) 10.0, and NaCl 5.0 (g/l water). Modified Bennett's medium: colloidal chitin 1.0, NZ Amine Type A (Humko Sheffield Chemical, USA) 2.0, yeast extract (Nacalai Tesque, Japan) 1.0, and beef extract (DIFCO, USA) 1.0 (g/l water). The culture broths were centrifuged (20,000 g, 10 min) to separate mycelia and supernatants. The super-

natants were assayed directly. The mycelia were extracted with acetone/MeOH (1:1, v/v) at room temperature for 4 days.

Two hundred and sixty strains of fungi isolated and collected at Tamagawa University, Tokyo, Japan were cultured at 28°C for 12 days under static conditions in four different media, rolled barley solid medium (RB), buckwheat solid medium (BW), starch soybean liquid medium (SS) and glucose peptone liquid medium (GP). RB medium: rolled barley (Kyowa Seibaku, Japan) 10 g, yeast extract (Aidemar, Spain) 0.02 g, Na-tartrate 0.01 g, KH₂PO₄ 0.01 g, and tap water 10 ml. BW medium: buckwheat grain (Gunma Prefecture, Japan) 10 g, yeast extract (Aidemar, Spain) 1 mg, Na-tartrate 0.5 mg, KH₂PO₄ 0.5 mg, MgSO₄·7H₂O 0.5 mg, FeSO₄·7H₂O 0.05 mg, ZnSO₄·7H₂O 0.05 mg, and tap water 10 ml. SS medium: toast soya (Showa Industry, Japan) 20, glucose 10, potato starch 20, yeast extract (Aidemar, Spain) 2.0, NaCl 2.5, CaCO₃ 3.2, ZnSO₄·7H₂O 0.05, CuSO₄·5H₂O 0.005, and MnCl₄·4H₂O 0.005 (g/l water). GP medium: glucose 20, glycerol 30, Polypepton (Nippon Pharmaceuticals, Japan) 5.0, yeast extract (Aidemar, Spain) 2.0, and NaCl 3.0 (g/l water). The broths were extracted with 20–25 ml of *n*-BuOH to obtain the extracts. These extracts were evaporated to dryness and suspended in 5% DMSO as test samples.

Six hundred and fifty six strains of fungi isolated and collected at Okayama University, Okayama, Japan were cultured at 28°C for 21 days under static conditions in potato dextrose malt extract liquid medium (PDM) as described in a previous paper.¹⁵⁾

5. Characterization of active compounds

The molecular weight of active compounds was estimated using a Centricon ultrafiltration cartridge (10 kDa-cut off for globular protein, Millipore, USA). To evaluate thermal stability, culture filtrates or extracts were heated at 95°C for 10 min. For the evaluation of pH stability, sample solutions were adjusted to pH 3, 7 and 10 with acetic acid or ammonia solution then heated at 60°C for 30 min. Ionic characteristics were determined by ion exchange chromatography using Dowex 50W X8, H⁺ form (DOW Chemical, USA) and AG1 X8, OH⁻ form (Bio-Rad, USA).

Results and Discussion

1. Establishment of the insect GlcNAcase inhibitory assay

We have previously reported the preparation of a *S. litura* chitinase solution.⁸⁾ In the preliminary experiment of this work, GlcNAcase activity was also detected in this crude enzyme solution when *p*NP-GlcNAc was used as a substrate, and hence, the same procedure was used for the preparation of a GlcNAcase solution. The method of inhibitory assay was outlined in “Materials and Methods”.

Nagstatin,^{16,17)} a GlcNAcase inhibitor from the actinomycete *S. amakusaensis* MG846-ff3, was used to evaluate

Table 1. Inhibition of *S. litura* and bovine kidney GlcNAcases by nagstatin

Enzyme sources	IC ₅₀ (nM)	K _i (nM)
<i>S. litura</i>	5.85	1.10
Bovine kidney	1.71	1.64

this assay method (Table 1). The IC₅₀ of nagstatin against *S. litura* GlcNAcase was 5.85 nM. The reported IC₅₀ value against hog kidney GlcNAcase was 4.01 nM.¹⁶⁾ This assay method had been adopted for the screening of GlcNAcase inhibitors from microbial extracts, resulting in the discovery of nagstatin. Therefore, the present *S. litura* GlcNAcase inhibitory assay would possibly detect GlcNAcase inhibitors in crude microbial extracts.

In addition, nagstatin showed inhibitory activity against both *S. litura* and bovine kidney GlcNAcases with similar K_i values. Its mode of inhibition of both enzymes was competitive. These results strongly suggested a broad inhibitory spectrum for this compound.

2. Screening of microbial cultures

Two different media, F medium and modified Bennett's medium were adapted for the cultivation of actinomycetes. The former is one of the best media for the production of antibiotics by actinomycetes and the latter medium contained only colloidal chitin as a carbon source, and therefore, the production of a wide variety of chitin analogs could be expected. Twenty-five strains of actinomycetes were cultured in the above two media and the culture filtrates and cell extracts obtained were screened. Inhibitory activity was found only in the culture filtrates, not in the cell extracts. Out of twenty-five strains screened, four produced GlcNAcase inhibitors only in the presence of colloidal chitin (Table 2), indicating that chitin as a carbon source was directly or indirectly responsible for the production. One other strain, *S. kanamyceticus* NBRC13414, produced inhibitors in the both the F and modified Bennett's medium, suggesting the presence of more than two distinct active compounds.

Twenty-two of nine hundred and sixteen fungal strains showed 50–100% inhibition. Solid phase cultivation was more effective than liquid phase cultivation as shown in Table 3. Four actinomycete strains and four fungal strains were selected for further study because of high levels of the inhibitors (Table 4). The four actinomycete strains showed good productivity on cultivation with CB medium for six days and the culture filtrates possessed potent inhibitory activity with ID₅₀ values in the order of μ l. The four fungal strains were highly productive under solid-static conditions with appropriate media for twelve days and the prepared culture extracts showed potent inhibitory activity with ID₅₀ values in the order of μ g. These crude materials were used for the characterization of the active principles.

Table 2. Results of screening of culture filtrates of actinomycetes^{a)}

Strains	Inhibition(%)	
	F	Modified-Bennett's
<i>Micromonospora carbonacea</i> NBRC14108	21	8
<i>M. chalcea</i> NBRC13503	27	9
<i>M. halophytica</i> NBRC14112	24	15
<i>Saccharomonospora viridis</i> NBRC12207	10	4
<i>Streptomyces albidoflavus</i> NBRC13010	32	7
<i>S. antibioticus</i> NBRC12838	10	8
<i>S. antibioticus</i> NBRC3117	26	6
<i>S. anulatus</i> NBRC13369	14	<u>82</u>
<i>S. atratus</i> NBRC3897	8	16
<i>S. cattleya</i> NBRC14057	14	7
<i>S. canus</i> NBRC12752	17	5
<i>S. chromofuscus</i> NBRC12851	13	10
<i>S. cuspidosporus</i> NBRC12378	30	7
<i>S. violaceoruber</i> NBRC3504	16	-51 ^{b)}
<i>S. diastaticus</i> subsp. <i>diastaticus</i> NBRC13412	12	15
<i>S. filipinensis</i> NBRC12860	24	6
<i>S. fradiae</i> NBRC3360	12	7
<i>S. griseus</i> subsp. <i>rhodochrous</i> NBRC13849	18	<u>80</u>
<i>S. griseoflavus</i> NBRC13044	18	6
<i>S. humidus</i> NBRC12877	10	11
<i>S. kanamyceticus</i> NBRC13414	<u>68</u>	<u>56</u>
<i>S. pactum</i> NBRC13433	6	13
<i>S. rameus</i> NBRC3782	19	<-100 ^{b)}
<i>S. griseoloalbus</i> JCM4480	24	<u>78</u>
<i>S. clauifer</i> JCM5059	11	<u>78</u>

^{a)} Eighty microliters of culture filtrates were assayed against *S. litura* GlcNAcase. Double underline entries; >50% inhibition. Results for double underlined entries are represented as mean values of two independent assays. In these cases, calculated %inhibition was within an experimental error of 0.6%.

^{b)} May be due to the GlcNAcase activity in the culture filtrate.

3. Physicochemical characteristics of active compounds

The physicochemical characteristics of these compounds were examined using the following methods: solvent fractionation, ultrafiltration, heat treatment under an unadjusted pH or acidic, neutral and alkaline pH conditions, and ion exchange chromatography. All active compounds showed similar chemical properties being water soluble, small (<10 kDa as a globular protein), heat stable, stable under acidic to neutral pH conditions, and cationic. However, the active compounds produced by four actinomycete strains were unstable at an alkali

Table 3. Results of screening of fungal metabolites^{a)}

Media	Conditions	Hit numbers (active/total)
RB	solid-static	7/260
BW	solid-static	6/260
GP	liquid-static	4/260
SS	liquid-static	4/260
PDM	liquid-static	1/656

^{a)} Two hundred and sixty strains of fungi were cultured in four different media under static conditions. RB: rolled barley solid medium, BW: buckwheat solid medium, GP: glucose peptone liquid medium, SS: starch soybean liquid medium. The remaining six hundred and fifty-six strains of fungi were cultured in PDM (potato dextrose malt extract) liquid medium under static conditions. One portion of culture extract was assayed against *S. litura* GlcNAcase.

line pH while those from four fungal strains were stable. Therefore, two types of GlcNAcase inhibitors were revealed as judged by their chemical properties. These active compounds, being water soluble, cationic and of low molecular weight, had chemical properties consistent with those of known GlcNAcase inhibitors including nagstatin.¹⁶⁻¹⁸⁾

4. Inhibitory spectra of active compounds

The inhibitors produced by eight active strains could be classified into two groups as judged by their inhibitory spectra, viz., those specific to insect GlcNAcases and non-specific inhibitors (Fig. 1).

The culture filtrates of four actinomycetes showed strong inhibition of a wide variety of insect GlcNAcases, but little or no effect against plant, fungal and mammalian GlcNAcases. In addition, all GlcNAcases used in this study were inhibited by a family 20 GlcNAcase inhibitor nagstatin (data not shown), confirming the novelty of the inhibitors. In contrast, the culture extracts of the four fungal strains showed broad inhibitory spectra similar to known GlcNAcase inhibitors.¹⁸⁾

The presence of inhibitors specific for insect GlcNAcases strongly suggested distinct differences between the GlcNAcases of insects and those of other organisms including plants, fungi and mammals. A similar phenomenon was revealed in our previous study in which a novel insect chitinase inhibitor FPS-1 inhibited the insect chitinase from *S. litura* but not the actinomycete chitinase from *Streptomyces griseus*.⁷⁾ These results strongly suggested that the insect chitinolytic enzyme system is composed of a unique chitinase and GlcNAcase.

This paper described the screening and preliminary characterization of insect GlcNAcase inhibitors. The emphasis of this study is the presence of GlcNAcase inhibitors specific for the enzyme of insect origin. The specificity is quite new, and

Table 4. GlcNAcase inhibitory activity of culture broths from eight active strains

Strains	Media	Days	ID ₅₀ against <i>S. litura</i> GlcNAcase
<i>S. anulatus</i> NBRC13369	CB	6	2.15 ^{a)}
<i>S. griseus</i> subsp. <i>rhodochrous</i> NBRC13849	CB	6	3.98 ^{a)}
<i>S. griseoloalbus</i> JCM4480	CB	6	6.95 ^{a)}
<i>S. clauifer</i> JCM5059	CB	6	5.19 ^{a)}
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<i>Paecilomyces</i> sp. F13	BW	12	14.1 ^{b)}
<i>Paecilomyces</i> sp. F30	RB	12	0.736 ^{b)}
<i>Verticillium</i> sp. F40	RB	12	0.306 ^{b)}
<i>P. carneus</i> F2281	RB	12	12.0 ^{b)}

CB: colloidal chitin-Bennett's medium, RB: rolled barley solid medium, BW: buckwheat solid medium, GP: glucose peptone liquid medium, SS: starch soybean liquid medium, PDM: potato dextrose malt extract liquid medium. ^{a)} As a volume of culture filtrate (μ l).

^{b)} Weight of culture extract (μ g).

therefore, the inhibitors discovered here are expected not only to be potential insect growth regulators but also to become helpful tools to elucidate the physiological role of the chitinolytic enzyme system in insects. In addition, the inhibitors from four fungal strains are also interesting because these compounds have comparatively similar chemical properties to

the inhibitors of four actinomycete strains with distinct inhibitory spectra. Further isolation and structural elucidation of the inhibitors are now in progress.

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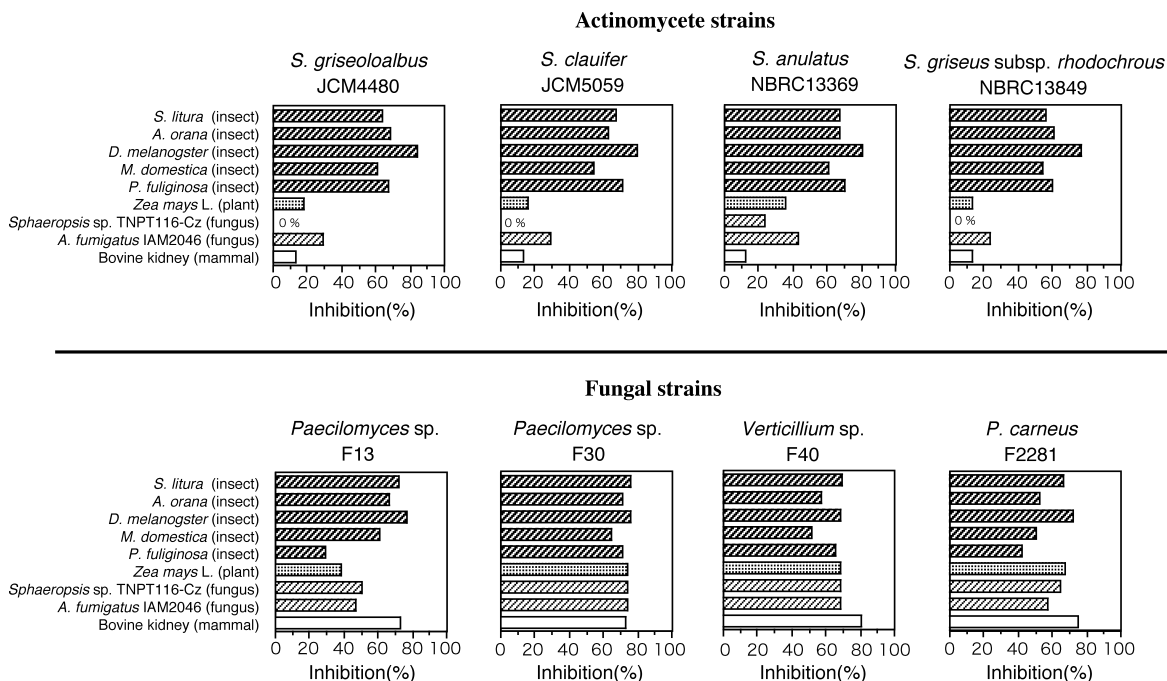


Fig. 1. Inhibitory spectra of active compounds from eight active strains.

Appropriate amounts of culture broth, corresponding to 50–75% inhibition of *S. litura* GlcNAcase, were assayed against various GlcNAcases (JCM4480: 2.27 units, JCM5059: 3.01 units, NBRC13369: 3.18 units, NBRC13849: 1.48 units, F13: 3.02 units, F30: 3.79 units, F40: 2.59 units, F2281: 2.69 units). One unit of GlcNAcase inhibitory activity is defined as the amount of inhibitor which caused 50% inhibition of *S. litura* GlcNAcase activity as measured with the assay used in the screening experiment (see “Materials and Methods”). The amounts of enzymes used in this experiment were so that the increase in A_{415} of the control mixture containing H_2O instead of test materials was 0.500. Assay results are represented as mean values of duplicate determinations. In all cases, the calculated %inhibition was within an experimental error of 5%.

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