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Isolation of *Streptomyces* sp. KK565 as a Producer of β-Amyloid Aggregation Inhibitor

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Abstract β-amyloid (Aβ) peptides from the proteolytic processing of β-amyloid precursor protein (β-APP) aggregates in the brain to form senile plaques, and their aggregation plays a key role in pathogenesis of Alzheimer's disease (AD). To isolate an active compound that has an Aβ aggregation-inhibitory activity, 2,000 microbial metabolite libraries were screened based on their ability to inhibit Aβ aggregation by using both Congo red and thioflavin T assays. As a result, a water-soluble fraction of a soil microorganism, KK565, showed a potent Aβ aggregation-inhibitory activity. The strain was identified as *Streptomyces* species, based on the cultural and morphological characteristics, the presence of diaminopimelic acid in the cell wall, and the sugar patterns for the whole-cell extract. In addition, the purification of active principle resulted in identifying a heat-unstable protein responsible for the Aβ aggregation-inhibitory activity.

Key words: Alzheimer's disease, β-amyloid, *Streptomyces* sp., Aβ aggregation-inhibitor

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder in the elderly, affecting millions of individuals throughout the world. One of the pathological hallmarks of AD is the extracellular protein deposits referred to as senile plaques, that consist predominantly of an aggregated peptide known as β-amyloid (Aβ) [2]. Aβs, 39–43 amino acid peptides, are produced through proteolytic processing of the β-amyloid precursor protein (βAPP) by β-secretase (BACE) and γ-secretase [3, 18]. This peptide is particularly amyloidogenic and appears to form the core of the neuritic plaques. The number of plaques appears to correlate with the degree or severity of the dementia [4, 14]. In addition, fibrillar Aβ, amorphous aggregates of Aβ, was reported to cause neuronal cell death in primary rat hippocampal cultures, whereas soluble monomeric species of Aβ are relatively less toxic than fibrillar Aβ [17]. Other studies suggested that prefibrillar aggregates of Aβ are neurotoxic [20]. Mutations in either APP, the immediate precursor of the Aβ peptides, or presenilins (PS), which seem to have a γ-secretase activity, can elevate the production of Aβ, and are associated with severe and early-onset forms of AD. Taken together, Aβ aggregation is considered as a crucial event in the pathogenesis of AD [19].

Because of the significant role of Aβ aggregation in AD, the search for a compound that inhibits Aβ aggregation and consequently protects against its neurotoxicity is of great interest. Several low molecular weight compounds such as antioxidants, free radical scavangers, cholesterol-lowering drugs, calcium channel blockers, and γ-secretase inhibitors have been investigated to develop them as therapeutic agents for AD [15]. Among these compounds, γ-secretase inhibitors are known to decrease Aβ production by 30–40% [13, 16]. In addition, high molecular weight proteineous compounds have also been highlighted as therapeutic agents for AD. For instance, anti-Aβ antibody, a macromolecular protein, is known to enhance clearing of Aβ deposits in transgenic mice that have already begun to develop plaques, possibly by the recruitment of local microglia [12]. Moreover, neprilysin which was known as enkephalinase as well as the common acute lymphoblastic leukemia antigen (CALLA) has been shown to be involved in degradation of Aβ by its proteolytic activity [5, 6].

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Reduced neprilysin level in the plaque area of AD brain was found, suggesting that reduced degradation of β-amyloid caused by low levels of neprilysin may contribute to AD pathogenesis [18]. Recently, Eckman *et al.* [1] reported that endothelin-converting enzyme-1 (ECE-1) was also able to degrade Aβ *in vitro* at multiple sites. These results demonstrated that the endogenous concentration of Aβ peptides was regulated not only by its production but also by its catabolism with specific proteolytic peptidase, and also suggested that proteolytic degradation of Aβ peptides would be one of the promising means for blocking Aβ aggregation.

Based on these ideas, a screening program for a compound that inhibits Aβ aggregation has been conducted with microbial metabolites, since a variety of bioactive chemicals and proteins have been isolated from the microorganisms [8, 9]. As an assay for the activity, Congo red that binds to β-pleated sheet structure of amyloids was used to measure the degree of Aβ aggregates [10]. Together with this assay, the thioflavin-T (Th-T) assay using a fluorescent dye which binds to fibril structures was used to measure the degree of β-amyloid aggregation [9]. Using these two assay methods, 2,000 microbial metabolite libraries were screened, and a *Streptomyces* sp. KK565 was isolated as a producer of an inhibitor that prevents Aβ aggregation. Herein, the isolation of a soil microorganism, *Streptomyces* sp. KK565, that produced an active protein inhibiting Aβ aggregation is reported.

Aβ peptide (1-40 or 25-35) was purchased from QCB (Hopkinton, MA, U.S.A.). Kieselgel 60 (70-230 mesh) and Kieselgel 60 F254 (Merck, Whitehouse St., NJ, U.S.A.) were used for the silica gel column and thin layer chromatography (TLC), respectively. All solvents and reagents used in this study were the highest grade commercially available. Microbial culture broth libraries were prepared as previously reported [11]. As for the fibrillogenesis assay, Aβ peptide (1-40 or 25-35) was dissolved in dimethyl sulfoxide (DMSO) as concentrated stock solution (1.68 mM) prior to an experiment, and the stock solution was then added to PBS buffer (100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4); the final concentration of Aβ was 100 μM. The mixed Aβ solution was incubated in the presence or absence of library supernatants at 37°C for up to 144 h. The amount of Aβ fibrils remaining intact was measured by the Congo red or thioflavin-T (Th-T) fluorescence assays. First, Congo red assay was used to examine the degree of Aβ aggregates. Aβ peptide and buffer were incubated for 5 days at 37°C to allow fibril formation. Congo red was then added to each sample and the mixture was incubated for 30 min at room temperature. At this point, the optical density (OD) of the samples was measured using a UV spectrophotometer at a wavelength of 540 and 480 nm to assay Congo red binding with Aβ peptide. The Congo red binding (Cb) Aβ quantity was calculated as follows,

$$Cb\ [M]=OD_{540}/25{,}295-OD_{480}/46{,}306$$

Background signals were determined by measuring the OD of a "blank" containing both Aβ and library prior to calculating the total aggregation. Second, the degree of Aβ-aggregation was determined using the fluorescent dye, thioflavin-T (Th-T), which specifically binds to fibrous structures. Aβ peptide stock solution was diluted with 50 mM sodium phosphate buffer, pH 6.0, and Th-T was added to each test sample to a final concentration of 10 μM. The activity was measured at an excitation wavelength of 450 nm and an emission of 482 nm that resulted in the optimum detection of bound Th-T. To account for background fluorescence, the fluorescence intensity measured from each control solution without Aβ peptide was subtracted from that of each solution containing Aβ peptide. The fluorescence spectra of Aβ 40 peptide from different commercial sources and from different lots were in good agreement. Next, the classification and identification of selected strain was carried out on the basis of ISP (International *Streptomyces* Project) method, as described previously [8]. Briefly, to determine the genus of KK565, the type of 2,6-diaminopimelic acid (DAP), one of the cell wall components of *Streptomyces* mycelia, was analyzed by the Beckers method. KK565 was cultured on a tryptic soy broth (17.0 g pancreatic digest of casein, 3.0 g papaic digest of soybean meal, 5.0 g sodium chloride, 2.5 g dipotassium phosphate, 2.5 g dextrose, and 1 l H_2O, adjusted to pH 7.3 before autoclaving) for 7 days at 28°C using a rotary-shaking incubator. The spore chain morphology of KK565 was examined as follows. The strain was incubated for 14 days on a yeast extract-malt extract agar (ISP medium 2) (4.0 g yeast extract, 10.0 g malt extract, 4.0 g dextrose, 20.0 g agar, and 1 l H_2O adjusted to pH 7.3 before autoclaving). The spore chain morphology of the strain was examined using light and scanning electron microscopy (SEM). The anti-amyloidogenic compound producer, KK565, was grown and maintained at 28°C on a YS medium plate (soluble starch 10 g/l, yeast extract 2 g/l, agar 20 g/l) as described previously [8]. For seed cultivation, an agar piece of the stock plate was cut under sterile conditions and inoculated into a 500-ml baffle flask containing 50 ml of the culture medium (G.S.S.): soluble starch 10 g/l, glucose 20 g/l, soy bean meal 25 g/l, yeast extract 4 g/l, NaCl 2 g/l, K_2HPO_4 0.25 g/l, $CaCO_3$ 2 g/l. The pH was adjusted to 7.2 with 1 M NaOH. The flask was cultivated for 48 h at 28°C on the rotary shaking incubator (150 rpm) and then transferred to 2-liter baffle flasks containing the same medium (400 ml/flask) for large-scale cultivation. All flasks were cultured for 7 days at 28°C. Cell viability was assessed by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay [10]. Human neuroblastoma cells, SHSY-5Y (American Type Culture Collection, Rockville, MD, U.S.A.), were plated at the density

of 2.5×10^{4} in 96-well plates and cultured in the growth media, DMEM/F12 (Life Technology, Grand Island, NY, U.S.A.) containing 10% fetal calf serum, 2 mM glutamine, and 1% penicillin/streptomycin, at 37°C, in the 5% CO_2/95% O_2 incubator. The neuronal cells were treated for 24 h with Aβ (1-40 or 25-35) which had been preincubated with a mixture of compounds for 6 days at 37°C. The cells were rinsed with PBS and then 10 µl of the MTT (0.5 mg/ml) was added. After the incubation for 4 h, 100 µl of solution containing SDS (10%) and HCl (0.01 N) were added and incubated overnight. On the next day, absorbance at 550 nm was determined with an automatic microtiter plate reader (Bio-Tek Instruments, Inc., Winooski, VT, U.S.A.). Finally, the active compound in the cultural broth of producing strain was purified as follows. The mycelia were separated from the culture broth by centrifugation (5,000 rpm, 30 min). The broth was extracted with the same volume of n-butanol, and the water-soluble fraction extracted with n-butanol was applied to Diaion HP-20 column (Mitsubishi Chemical Co., Tokyo, Japan). Then, the column was washed with 30% methanol and eluted with 50%, 70%, and 100% methanol in a batch-wise manner. The active 70% methanol fraction was concentrated *in vacuo* and then filtered with a MW 10,000 centricon (VivaScience, Germany). The active fractions were concentrated and applied to mono-Q ion-exchange column chromatography (Pharmacia, Sweden) for further purification of the active principle. Finally, the purity of the active principle was confirmed by 12% SDS-polyacrylamide gel electrophoresis.

To determine the optimum condition for a readily detectable accumulating level of Aβ aggregates, a time course experiment of Aβ aggregation was performed at 37°C.

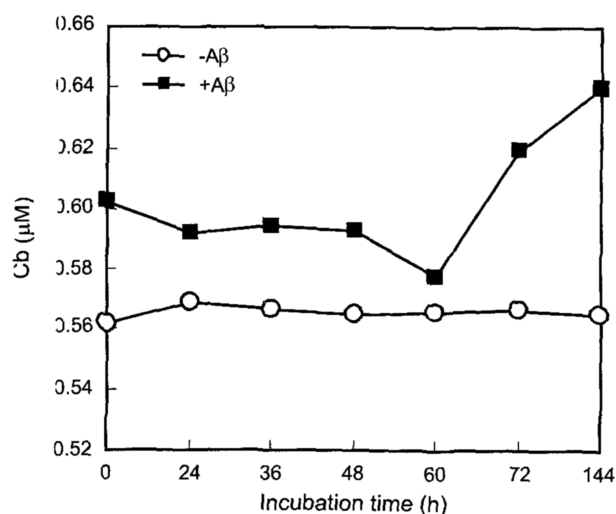

Fig. 1. Time course of Aβ 1-40 peptide aggregation.
Aβ1-40 peptide (100 µM) prepared in 100 mM NaCl and 10 mM NaH_2PO_4 was incubated at 37°C for the indicated time. Aβ aggregation was measured by the Congo red assay as described in the text.

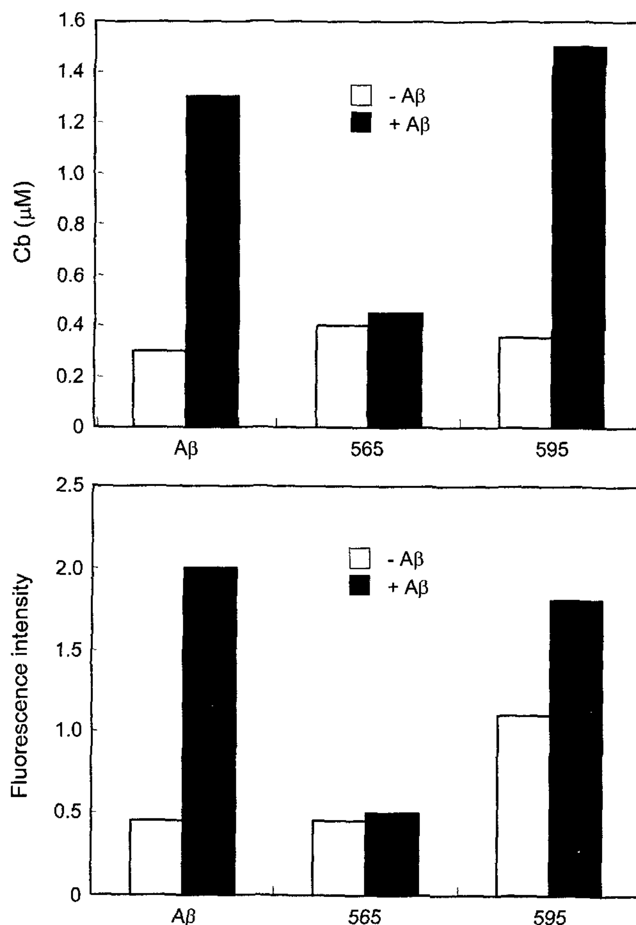

Fig. 2. Effect of microbial metabolites on Aβ1-40 aggregation and fibril formation.
Each microbial library sample was added to Aβ1-40 peptide (100 µM) solutions, and the mixture was incubated at 37°C for 5 days and examined for Aβ fibril formation by the Congo red (A) and Th-T fluorescence (B) assays.

Figure 1 shows the time course of Aβ peptide aggregation at 37°C without any test agent, using the Congo red assay. Aβ aggregates were formed from 4 days and reached maximum at 5 or 6 days. Thus, the aggregation assay was performed by routinely incubating Aβ peptide for 5 days at 37°C in this study. Next, isolation of a microorganism that produced an anti-amyloidogenic compound was conducted using both Congo red and thioflavin-T (Th-T) binding assays, as described above. Thus, Aβ peptide was incubated for 5 days at 37°C to allow fibril formation. The anti-amyloidogenic activity of libraries was measured by adding 5% culture broth of microbial libraries into Aβ aggregation solution in a 96-well plate. Out of the 2,000 microbial metabolite libraries, KK565 showed potent inhibitory activity of β-amyloid aggregation both in Congo red (93.2% inhibition compared to control) and thioflavin-T (97% inhibition) assays (Fig. 2). On the other hand, KK595, another culture broth of microbial libraries, did not inhibit the β-amyloid

Table 1. Morphological characteristics of strain KK565.

Spore surface	Smooth
Spore chain morphology	Spiral
Spore size	0.4-0.7×0.8-1.0 (μm)
Spore mobility	None
Spore number per chain	>30-50

aggregation in both assays. This result demonstrates that a specific microorganism is capable of producing the active principle to inhibit the formation of β-amyloid aggregation, and also rules out the possibility that the substance in the medium may affect the assay. Thus, KK565 was selected as a microorganism to produce an active principle that inhibits the Aβ aggregation.

The classification and identification of KK565 was carried out according to the ISP method. The morphological character of KK565 was investigated by optical and electron microscopy using 14-days-cultured cells (Table 1). A spore chain showed spiral morphology and the superficies of spores were smooth. The shape of the spore was rod with a length of 0.4-0.7×0.8-1.0 mm (Fig. 3). The cultural character of KK565 was investigated using several ISP media conditions with 21-days-cultured cells. The strain grew well in all media tested, except inorganic salt-starch and peptone-yeast extract containing agar medium (Table 2). L-arabinose, D-fructose, raffinose, and D-galactose were utilized by KK565 as a carbon source. Notably, the strain has diaminopimelic acid in cell wall based on the analysis of Beckers method (Table 3). Taken together, it was concluded that KK565 belongs to a *Streptomyces* species and was named *Streptomyces* sp. KK565.

Aβ aggregate is known to induce neuronal cell death. Accordingly, KK565 could protect Aβ-induced neuronal cell death, if the active principle would block the aggregation of Aβ peptide. To verify this possibility, the effect of KK565 on the viability of neuronal cells treated with Aβ peptide was examined. Mixtures containing Aβ peptide (100 μM) and KK565 were incubated at 37°C for 6 days and added into the SHSY-5Y human neuronal cell cultures. KK595, another *Streptomyces* sp. cultural broth having no

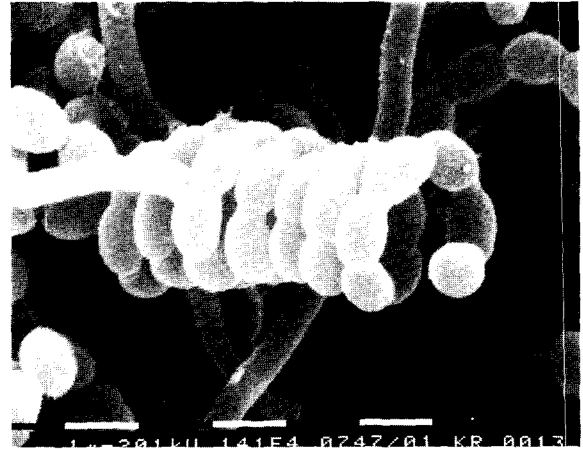

Fig. 3. Electron scanning morphology of *Streptomyces* sp. KK565 in SEM (×2,300).
Bar scale represents 10 μm.

Aβ aggregation-inhibitory activity, was used as a negative control (Fig. 2). After incubation for 24 h, the cells were processed for MTT assay. The viability of neuronal cells was reduced as much as 50% after fibrillar Aβ-peptide treatment (Fig. 4). KK565 significantly blocked Aβ-induced cell death while KK595, a negative control, did not protect the neuronal cells. The viability of neuronal cells treated with KK565 alone was not affected at all, implying that KK565 has no effect on cell proliferation. These data demonstrate that the active principle of KK565 inhibits the aggregation of Aβ peptide, resulting in protection of neuronal cell death by Aβ peptide. This result also suggests a possibility that the active principle of KK565 can be developed as a therapeutic agent for Alzheimer's disease.

It was next attempted to purify the active principle from KK565 culture broth to investigate the biochemical properties of the principle. Using 7 days cultural broth that was prepared as described above, the mycelia was removed from the broth using centrifugation (8,000 ×g, 30 min). A solvent extraction showed that the active principle was not extracted by the treatment of nonpolar solvents such as

Table 2. Cultural characteristics of strain KK565.

Media	Growth	Aerial mycelium	Substrate mycelium
Yeast extract-malt extract agar (ISP No.2)	Good	Gray	Pinkish
Oatmeal agar (ISP No. 3)	Good	Gray	Pale Yellow
Glycerol-asparagine agar (ISP No. 4)	Moderate	Pinkish Gray	Pale Yellow
Inorganic salt-starch agar (ISP No. 5)	Good	White	Pale Yellow
Peptone-yeast extract agar (ISP No. 6)	Moderate	Poor	Pale Yellow
Tyrosine agar (ISP No. 7)	Good	White	Pale Yellow
Glucose-asparagine agar	Good	Gray	Pale Yellow
Bennet's agar	Good	White	Pale Yellow
Nutrient agar	Moderate	White	Pale Brown

Table 3. Physiological characteristics of strain KK565.

		Utilization of C-source	
Starch hydrolysis	+	D-fructose	+
Skim milk hydrolysis	-	Galactose	-
Nitrate reduction	-	Inositol	+
Gelatin liquefaction	-	Mannitol	-
Melanin pigment	-	Raffinose	+
Soluble pigment	-	Rhamnose	-
Milk coagulation	-	Sucrose	-
Diaminopimelic acid	LL	D-xylose	+
		D-glucose	-
		L-arabinose	+
		Cellulose	-

hexane, chloroform, ethylacetate, and n-butanol, suggesting that the active principle would not be low molecular weight compounds (data not shown). Accordingly, after the broth was extracted with an equal volume of n-butanol to remove the nonpolar layer, low molecular weight compounds and a water-soluble fraction were used for

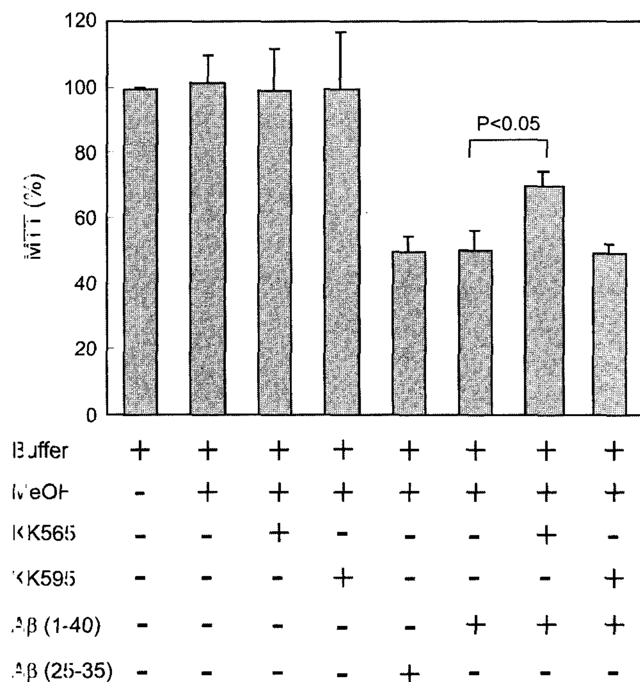

Fig. 4. Protection of Aβ-induced neuronal cell death by KK565.
Aβ1-40 or Aβ25-35 peptide (100 μM each) was prepared in 100 mM NaCl and 10 mM NaH_2PO_4, pH 7.4, and KK565 or KK595 was prepared in MeOH. Mixtures indicated were incubated at 37°C for 6 days and added into the SHSY-5Y human neuronal cell cultures (the final concentration of Aβ was 25 μM). After incubation for 24 h, the cells were processed for MTT assay. The viability of the neuronal cells was reduced as much as 50% after treatment of both fibrillar Aβ1-40 and Aβ25-35. KK565 significantly blocked the cell death (over 20% increased viability than that of control) caused by Aβ1-40, while KK595, which showed no Aβ aggregation-inhibitory activity, did not protect the cells. Each bar represents mean+SEM of three wells.

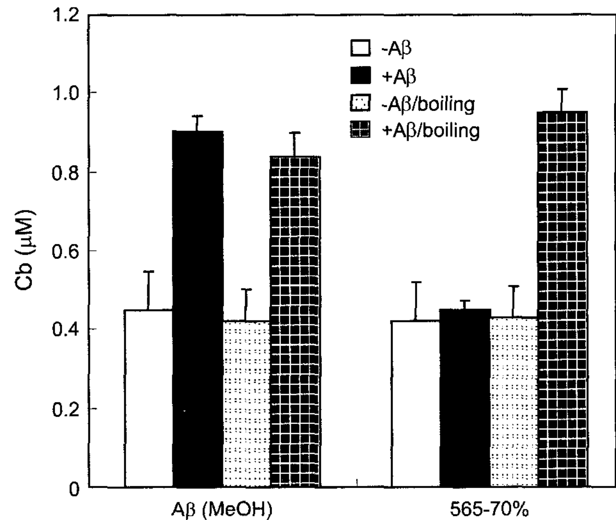

Fig. 5. Effect of heat treatment on the activity of the active compound.
The 70% methanol fraction of a microbial sample (KK565) was purified by HP-20 column, heated for 15 min at 80°C, and incubated with Aβ1-40 (100 μM) in a reaction buffer (100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4) for 5 days at 37°C. Inhibition of Aβ aggregation was determined by Congo red binding value (μM) after measuring O.D. of the samples with a spectrophotometer.

further purification. Among the batch-wise eluted fractions of Diaion HP 20 column chromatography, the 70% methanol fraction showed activity. Interestingly, heat-treatment of the active fraction for 10 min at 80°C completely abolished the activity, suggesting that the active principle would be proteinous materials (Fig. 5). Notably, the preliminary results on the purification of the active principle of *Streptomyces* sp. KK565 indicated that a protein may associate with Aβ peptides to inhibit the aggregation of Aβ peptides or exhibit peptidase activity to hydrolyze Aβ peptides. Detailed study on the characterization and biochemical activity of the active proteinous principle is currently underway to elucidate the mode of inhibitory action of the protein.

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