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Synthesis Optimization of L-Aspartic acid β-hydroxamate by a novel Enzyme, β-Aspartyl-γ-glutamyl transferase

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Abstract. L-Aspartic acid β-hydroxamate or L-β-Aspartyl hydroxamate (BAH), water soluble-chemical compound currently obtains popularity due to its role in several important biochemical processes and to its bioactivities. The information regarding synthesis process of BAH is not available yet. Novel enzyme, *β-aspartyl-γ-glutamyl transferase* from *Pseudomonas syringae* can catalyze the transfer reaction of β-aspartyl moieties from β-aspartyl compounds to water or to hydroxylamine. In this study we describe the synthesis optimization of BAH using this novel enzyme. We prepared the L-β-aspartyl hydroxamate using L-asparagine as a donor substrate and hydroxylammonium chloride as an acceptor substrate. The effects of temperature, pH, concentrations of substrate donor and acceptor were investigated. Spectrophotometry and HPLC analyses were performed to determine the reaction products. The optimum synthesis reaction was observed in 60°C. BAH synthesis was optimum at pH 6. The concentrations of donor and acceptor substrates affected the BAH production and the best concentrations of both substrates were 80 mM and 40 mM, respectively. The BAH production of 0.106 mM has been obtained under the optimized condition and it is approximately two-times higher than 0.047 mM produced under in standard reaction. In conclusion, biosynthesis of L-β-aspartyl hydroxamate using a novel enzyme, *β-aspartyl-γ-glutamyl transferase* from *Pseudomonas syringae* was successfully performed for the first time. Under the optimized conditions, two times higher L-β-aspartyl hydroxamate production was obtained.

Keywords:L-β-Aspartyl hydroxamate, β-Aspartyl-γ-glutamyl transferase, Biosynthesis, *Pseudomonase syringae.*

Introduction

L-Aspartic acid β-hydroxamate or L-β-Aspartyl hydroxamate (BAH), water soluble-chemical has important biochemical such as serine racemase inhibitors. Serine racemase has an important role in the synthesis of neurotransmitter, D-serine and coagonist of N-methyl-D-aspartate (NMDA) receptor. They have an association to the neuron-related diseases. Several studies, suggested that decreasing NMDA through inhibition of serine racemase is a one good option to treat neuron-related diseases [1,2]. An escalation of semicarbazide-sensitive amine oxidase (SSAO), a vascular adhesion protein 1 in human plasma has a positive correlation with diabetes mellitus and hearth failure. A study by Liu and his co-worker indicated that BAH is able to inhibit SSAO production. Hence, it can be used to manage diabetes mellitus and hearth failure symptoms[3]. Furthermore, BAH shows antioxidant, angiotensin converting enzyme (ACE) inhibitory and anticancer activities. The anticancer activity of BAH on the murine leukemia cancer was reported by Thomasset et al. [4]. Moreover, hydroxamic acid derivatives were also

reported to have an antibacterial activity [5]. Hence, due to these bioactivities, further exploration on application of BAH is inevitable.

It is interesting to note that there is no published-report on the production of BAH. only few fine chemical companies have BAH in their catalog products. One of them which currently offering this chemical compound is Sigma Aldrich co. Either, considering BAH bioactivities as our mentioned above or the important of its availability in order to more explore the potency of this compound, the availability of BAH in large quantity throughout effective production is inevitable. The process for production of BAH has barely reported yet. The fact that there is no information on regard of enzyme which is capable of synthesizing BAH, leads us to the prediction that this compound is chemically produced.

A novel BAT-GGT enzyme abel to produce BAH [6], however the optimum condition for the synthesis process was not evaluated yet. Hence, in this study the optimum condition for the reaction of BAH synthesis was reported. Furthermore, preliminary analysis of BAH bioactivities as antimicrobial and antioxidant were investigated.

Material and Methods

Materials

L-Asparaginase, Hydroxylamine Hydrochloride, L-Aspartic acid β-hydroxamate, and Hydrogen Peroxide (H_2O_2) were purchased from Wako Co. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Co. The BAT-GGT enzymes was prepared and obtained based on procedures which is described elsewhere [6].

HPLC Analysis

Analysis of the reaction products was performed by using HPLC analysis. The HPLC analysis followed Prihanto et al. method [6].

Synthesis optimization

The optimization of synthetic reaction of BAH, was performed by investigating the effect of temperature, pH, concentration of substrate donor and acceptors. The temperature effect was carried out in the range of 30 – 70 °C. The effect of pH was investigated under the range of pH 5-11. Substrates donor that is used in this investigation was in the range of 2 – 80 mM. Substrates acceptors for this investigation are 10 – 320 mM.

Bioactivities

The stock of BAH solution was prepared by biosynthesis with 10 mL total volume. The reaction was performed based on their optimum reaction condition. The concentration of the BAH was calculated by both spectrophotometric and HPLC. The peak area compared to the standard of BAH indicated the concentration of synthesized BAH in the reaction. This concentration of BAH, then was further used as stock solutions.

Antibacterial assay

Paper disc diffusion assay was applied for investigation of BAH antibacterial. After bacterial test reach an OD_{600}= 0.2-0.4, they were onto LB agar. The sterile filter paper diameters of 1.5 cm which have impregnated by 50 µl of BAH solution were plated onto the swabbed-LB agar medium. Clear zone (mm) was measured after overnight incubation.

Antioxidant assay

The antioxidant activity of BAH was investigated by DPPH radical scavenging assay. This method was conducted according to the method of Brand-Williams et al. [7]. Control sample was prepared containing the same volume of 99% ethanol without any extract was used as blank. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. All tests were performed in triplicates. Scavenging activity of the DPPH free radical was calculated using the following equation,

$$\text{DPPH radical scavenging activity (\%)} = \frac{(\text{Abs control} - \text{Abs test})}{\text{Abs control}} \times 100 \quad (1)$$

Where, Abs control is the absorbance of the control reaction and Abs test is the absorbance in the presence of the synthetic BAH or standard.

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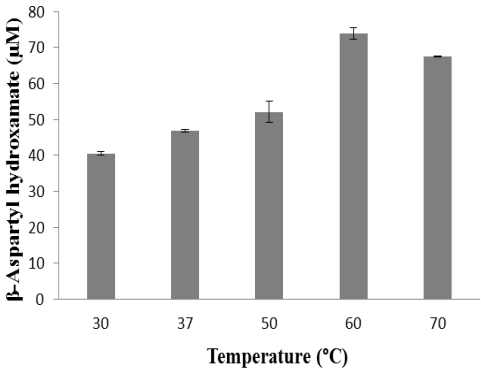
Results

The effect of temperature and pH

The production of BAH was investigated in the range of temperature at 30 - 70 ˚C. A production increased along with an increase of temperature until it reached its peak in temperature of 60 ˚C. The production started to decrease in the 70 ˚C (Fig. 1a). A pH range from 5-11 was used in order to achieved the best pH condition for BAH synthesis. The result indicated that PsGGT was optimally produced BAH in the slightly acid condition (Fig. 1b). The best pH for biosynthesis of BAH was 6.



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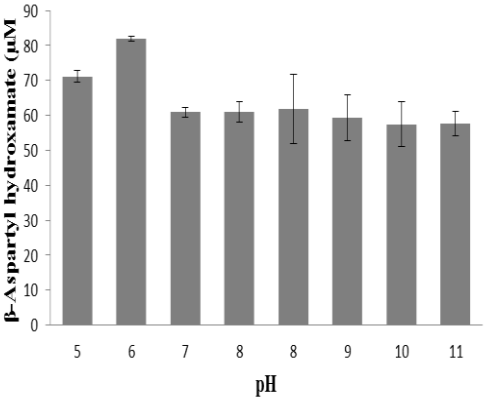
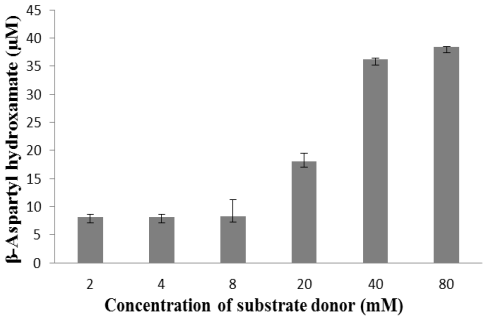


b.

Figure 1. The Effect of temperature (**a**) and pH (**b**) on the BAH synthesis. pH optimum was examined by using temperature of 60 ˚C.

The effect of concentration of substrate donor and acceptor

The substrate donor concentration affected the biosynthesis of BAH. The result revealed that the concentration of donor and acceptor, will affect the production of BAH. Lowest production was showed in the concentration of 2-8 mM L-asparagine. The BAH production increased from 20 mM and continued to increase until 80 mM (Fig. 2a). Different trend production was shown in the effect of substrate acceptor. The optimum BAH production was obtained from 40 mM hydroxylamine. Above 40 mM hydroxylamine, decrease of BAH production occurred.



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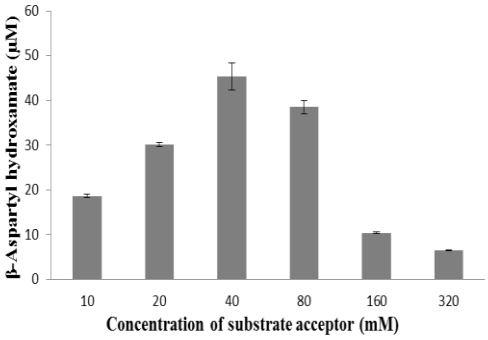

b.

Figure 2. The effect of Substrate donor (**a**) and acceptor (**b**) on the synthesis of BAH. The experiment of substrate donor, the following condition is used: temperature 60 ˚C, pH 6 and 20 mM hydroxylamine. All condition except for substrate acceptor was conducted in the same condition with the experiment as for substrate donor. Substrate donor used is 80 mM L-asparagine.

Optimum condition for BAH synthesis.

The optimum reaction for biosynthesis of BAH was set based on the information of the previous experiment. The analysis was done under the condition of temperature 60 ˚C, pH 6, 80 mM L-Asparagine, and 40 mM hydroxylamine. The reaction was done for 1 h. In order to compare BAH productivity under the optimum condition with standard condition, we run the standard reaction based on the protocol in a section on material

and methods. The formation of β-aspartyl hydroxamate in the reaction mixture was confirmed by the colour change of the mixture to brown or dark brown after an addition of stop mixtures. Unformed β-aspartyl hydroxamate was indicated by the yellow colour. The preliminary investigation of the biosynthesized BAH was carried out by colorimetric analysis at an OD_{540}. HPLC was applied for further in detail analysis.

Different production of BAH was observed. In the optimum reaction condition, the production of BAH was 0.106 mM (Fig. 3). It was about two times higher than that compared with standard reaction which BAH was accounted only 0.047 mM.

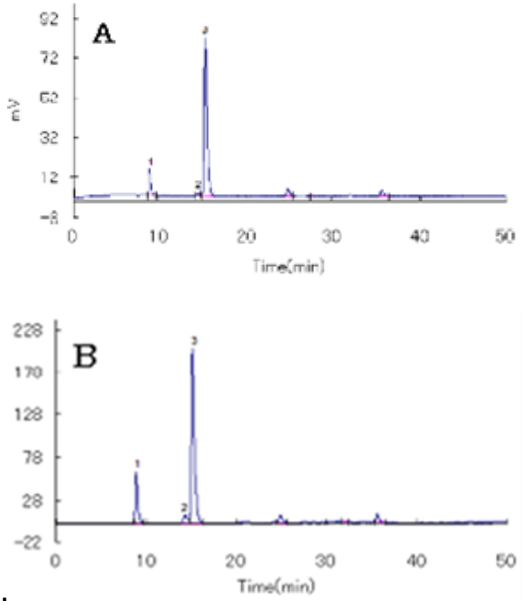

Figure 3. Comparison of standard and optimum reaction. Standard reaction (**a**), Optimum reaction (**b**). Peak No. 1, 2, 3 were L-aspartic acid, BAH, L-Asn, respectively.

Antioxidant activity

At a concentration of 0.2 mM BAT, inhibition for DPPH accounted for 93.58 %. This inhibition was indicated by the discoloration of DPPH from blue-purple to light yellow. This discoloration of the sample is depicted on Fig. 4. Low concentration of BAH gave a nearly same scavenging activity. The lowest inhibition was 75.4 % which was exhibited by 10 µM BAH. Increase of BAH concentration up to 100 µM has a 93.1% of inhibition.

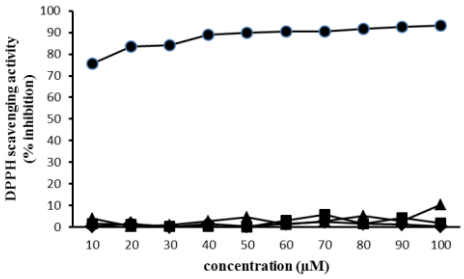

Figure. 4 DPPH scavenging activity of samples. Circle is BAH product, diamond-shape is control reaction, square is L-asparagine, triangle is hydroxylamine.

Antibacterial activity

BAH has antibacterial activity against several gram positive and negative bacteria (Fig. 5). In gram positive bacteria, BAH showed an inhibition toward the growth of *Bacillus cereus* and *B. pumilus*. In *B. cereus* the diameter of clear zone showing inhibition reached 32 mm. This inhibition zone is slightly below chloramphenicol inhibition zone which had a 34 mm. Susceptibility of *B. pumilus* was higher than that *B. cereus*. In *B. pumilus* experiment, BAH showed high inhibition zone. Even it is too large to be measured. An interesting result was exhibited in the inhibition zone of *E. coli*. BAH demonstrated more strong inhibition compared to chloramphenicol with a diameter of 30 mm.

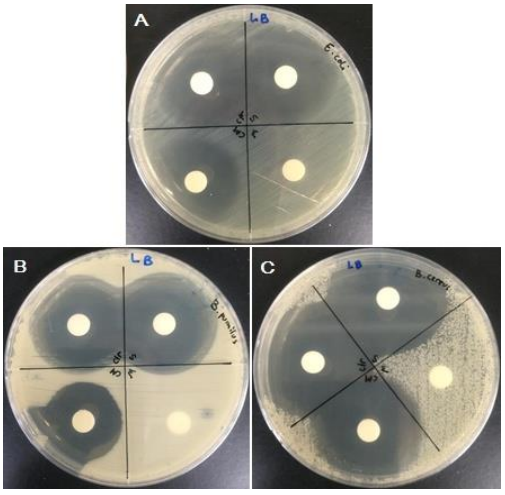

Figure. 5 Antibacterial activity of BAH. The inhibition of BAH against *E. coli* (**a**), *B. pumilus* (**b**), *B. cereus* (**c**). Code `S` is sample, code `W` is control negative of D-water, `CM` is control positive with 30 mg/ml chloramphenicol, `ctr` is control negative control reaction.

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Discussion

BAH is an interesting fine-chemical compound which currently was found its bioactivities. This compound could be synthesized in by using a BAT-GGT enzyme which transfers β-aspartyl moieties to hydroxylamine. Previously, we have been proved that BAT-GGT enzyme catalyzes the synthesis of BAH from L-Asn and hydroxylamine. Optimization of BAH biosynthesis reaction, resulted in two-times increase of BAH production.

An optimum temperature is widely known has a significant effect to the biochemical reaction. We have been demonstrated that relatively high temperature was needed for optimally synthesizing BAH. However, if the reaction was performed in the 70 °C, the yield was decrease. This was most probably due to the inactivation of the enzyme. In our previous finding in chapter III, the presence of hydroxylamine in the reaction mixture will somewhat maintain the enzyme stability.

In slightly acidic environment, the biosynthesis of BAH was recorded high. It was different compared to the theanine synthesis. Most of theanine biosynthesis requires basic environment [8,9,10]. It was interesting to note that the best mol ratio of donor and acceptor substrate was 2:1. It was also different with the biosynthesis of theanine, most optimum theanine biosynthesis occurred in the abundant amount of the substrate acceptor. This was possibly caused by the low hydrolysis activity of the enzyme. If the hydrolysis activity was poorly occurred, the released-β-aspartyl moieties were less. Hence, even small amount of substrate acceptor was enough to catch released-β-aspartyl moiety to form BAH.

Preliminary bioactivities (antioxidant and antibacterial activity) assay of formed BAH in the reaction was performed. The scavenging activity of BAH against radical species DPPH was confirmed. The DPPH, a stable free radical is widely used for assessing the free radical-scavenging activities of deduced antioxidants. Our in vitro assay of antioxidant using DPPH, suggested that BAH has an antioxidant activity. With 92.59% inhibition of DPPH for 30 min reaction, it can be therefore, concluded that BAH is a potent antioxidant. Similar result was also reported by Liu et al.[3]. In their experiment they proved that not only ACE inhibitor but also the scavenging activity of radical DPPH was demonstrated by BAH. Hence, our result was corroborated. Hydroxamate derivates reported to be an antioxidant. Chemical structure in BAH, which responsible for the scavenging activity was functional group of R-CONHOH. The antibacterial activity of control reaction was probably caused by hydroxylamine. However the antibacterial activity of BAH was previously reported. The antibacterial activity of BAH is caused by the peptide deformylase inhibitory effect of hydroxamate [11].

It can be concluded that the optimum condition for the biosynthesis of BAH using BAT-GGT enzyme was determined. The optimized reaction, gave two-times higher production of the BAH. It also confirmed that the BAH has an antioxidant and antibacterial activity.

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