Purification, characterization and anticancer potential tests of L-asparaginase enzyme from the leaves siam weed (Chromolaena odorata Linn)

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Abstract. Siam weed (Chromolaena odorata L.) is a plant that interferes with the other plants and it is used as a conventional medicine that has the potential as an anticancer agent. This study aims to determine the optimum activity of the L-asparaginase enzyme purification and its anticancer potential test. The stages of this research include isolation, purification, characterization (pH, temperature, incubation time and metal ion addition) and anticancer potential test by the Brine Shrimp Lethality Test (BSLT) method. The study result showed that the highest specific activity of fractionation was fraction 2 (20-40%) of 19.94 IU/mg. The L-asparaginase enzyme has an optimum activity including pH of 8, the temperature of 37°C, and an incubation time of 30 minutes with metal ions of K⁺ and Na⁺ as activators and with metals of Ca²⁺, Zn²⁺, Mg²⁺, Cu²⁺, Co²⁺ and Mn²⁺ as inhibitors. The result of the toxicity test was an LC50 value of 158.48 μg/mL which was a very toxic level. The purification of the L-asparaginase enzyme from the C. odorata L. leaves has the potential to be developed as an anticancer agent in the future.

1. Introduction
In 2018 there were 18.1 million new cases of cancer with a mortality rate of 9.6 million deaths. In Indonesia, the incidence of cancer is at number 8 [1]. One of the anticancer agents is using the enzyme L-asparaginase. L-asparaginase (EC 3.5.1.1) is a tetramer protein that belongs to the family group of homologous amidohydrolases [2]. L-asparaginase selectively hydrolyzes the free L-asparagine [3] to ammonia (NH₃) and aspartic acid [4]. The enzyme L-asparaginase inhibits the protein synthesis of cancer cells without damaging normal cells [5].

Plants as a source of L-asparaginase enzyme and its potential as an anticancer have been widely studied, such as Curcuma xanthorrhiza that is better known as temulawak, despite less potential as anticancer [6], white turmeric as an anticancer [7], garlic [8], leek [9], and parasitic plants that have the potential as an anticancer agents [10]. The other plant that capable to produce L-asparaginase enzyme which is Chromolaena odorata L.

C. odorata L. is a plant that interferes with the growth of other plants and reduces the soil prolificacy used as wound medicine and antioxidants [11]. C. odorata L. in 100 grams of protein from
the leaves contains essential and non-essential amino acids [12] which can produce the L-asparaginase enzyme. The results of isolation and purification of the L-asparaginase enzyme from *C. odorata* L. have fairly high activity and potential as an anticancer agent.

2. Materials and methods

2.1 Material

The materials of this study were *C. odorata* L. leaves which were collected from the Bone regency, and the other materials were asparagine, ammonium sulphate (NH₄)₂SO₄) cellophane membranes, Nessler reagents, Lowry reagents, Tris-HCl buffers, metal ions K⁺, Na⁺, Ca²⁺, Zn²⁺, Mg²⁺, Cu²⁺, Co²⁺ and Mn²⁺.

2.2 Instruments

The Instruments of this research were glass, blender, micropipette, incubator, centrifuge, and spectronic 20D°.

2.3. Method

2.3.1 Enzyme Isolation. The *C. odorata* L. leaves were obtained at the Pacching village, Patimpang, Bone was added with Tris-HCl buffer pH 8 and then centrifuged at 5,000 rpm.

2.3.2 Enzyme Fractionation. The crude extract of the L-asparaginase enzyme was added by ammonium sulphate based on the saturation level table, left overnight and centrifuged at 13,000 rpm.

2.3.3 Dialysis Enzymes. The highest specific activity of the L-asparaginase enzyme was inserted into the cellophane membrane and immersed into a Tris-HCl buffer solution pH 8.

2.3.4 Characterization of Enzymes. The L-asparaginase enzyme was tested for its activity by a Nessler reagent with the effect characterization of pH 5-10, incubation time of 30-120 minutes, the temperature at 30-45°C and several types of metal ions such as K⁺, Na⁺, Ca²⁺, Zn²⁺, Mg²⁺, Cu²⁺, Co²⁺, and Mn²⁺.

2.3.5 Activity Test of L-asparaginase Enzyme. The activity of the L-asparaginase enzyme was observed by 0.2 ml of Nessler reagents, 1 ml of L-asparagine substrate solution and 0.8 ml of buffer Tris-HCl at a pH of 8. Then, the mix solution was incubated at 37°C for 30 minutes and it was added 1 ml of TCA solution and then left for a moment. Furthermore, 0.5 ml of filtrate was taken and added with 8.5 ml of the distilled water and 1 ml of Nessler reagent, and the absorbance of the solution was measured by spectronic 20D°. Enzyme activity was calculated using formula as follows:

\[
\text{Enzyme Activity (IU/ml)} = \frac{\text{absorbance at 50]}{\text{Intercept}} \times \frac{\text{Total Volume}}{\text{Analysis Volume}} \times \frac{1}{\text{Enzyme Volume}} \times \frac{1}{\text{Incubation Time}} \tag{1}
\]

\[
\text{Specific activity} = \frac{\text{Enzyme Activity (IU/ml)}}{\text{Protein Level (mg/ml)}} \tag{2}
\]

Where one unit (U) of enzyme activity was defined as the amount of enzyme that is catalyzed the liberation of 1 µmole of ammonia at the under of standard assay conditions. Specific activity was stated as IU/mg protein [13].

2.3.6 Toxicity Test Using the Brine Shrimp Lethality Test (BSLT) Method. Toxicity Test using the Brine Shrimp Lethality Test (BSLT) method used the larvae *A. salina* L and enzymes were made in concentrations of 1 ppm, 10 ppm, and 100 ppm, and then the tested samples were stored under lighting for 24 hours. The amount of death and living larvae was observed and calculated. The values of LC₅₀ were determined using probit analysis with the formula as follows:
The LC50 was determined from the data of % mortality based on the probit analysis [6].

3. Result and Discussion

3.1 Isolation and Purification of L-asparaginase Enzymes

Isolation of L-asparaginase was carried out mechanically by extracting C. ordorata L. leaves through the breakdown of leaf tissues with the tris-HCl buffer at pH 8 and then it mashed. The refining process produces a mixture consisting of the component cells, proteins, and non-proteins from the leaves. Separation of proteins from cells and non-protein components was carried out by filtering and centrifuging the filtrate that obtained crude extracts. To obtain the L-asparaginase enzyme with a high level of purity, it is necessary to purify using the method of fractionation and dialysis.

The fractionation stages were conducted by the deposition of proteins using ammonium sulfate with different levels of saturation from low concentrations to high concentrations. This difference in concentration could cause proteins to have high salinity. To separate the salt or molecules that were not desired with the protein, this was carried out the dialysis stage.

The value of the specific activity can be used as a measure of the purity of the enzyme that was produced by isolation [7]. The enzyme activity test used the Nessler method as well as the protein content using the Lowry method. Determination of the enzyme-specific activities of the L-asparaginase enzyme of C. ordorata L. leaves was carried out on crude extracts, fraction 0-20%, fraction 20-40%, fraction 40-60%, and fraction 60-80% in Table 1. The highest result of saturation was obtained at fraction 20-40% with a specific activity of 19.94 IU/mg protein and a purity level of 4.13. This result is different from the L-asparaginase enzyme from ginger which the highest of the specific activity was the fraction 60-80%. [6]. The difference in the level of saturation is caused by the source and the condition of the used plants in the study. The highest fraction result is continued at the dialysis stage and toxicity tests were using the Brine Shrimp Lethality Test (BSLT) method.

| Purification Stage | Total Activity (IU/mL) | Total Protein (mg/mL) | Specific Activity (IU/mg) | Purification Fold |
|--------------------|------------------------|-----------------------|---------------------------|------------------|
| Crude extract      | 13.67                  | 2.83                  | 4.82                      | 1.00             |
| 0-20% Fraction     | 17.38                  | 2.04                  | 6.51                      | 1.35             |
| 20-40% Fraction    | 29.77                  | 1.49                  | 19.94                     | 4.13             |
| 40-60% Fraction    | 7.20                   | 1.34                  | 5.35                      | 1.11             |
| 60-80% Fraction    | 5.61                   | 1.29                  | 4.33                      | 0.89             |

3.2 Characterization of L-asparaginase enzymes

3.2.1 Effect of pH, Temperature and Incubation time on the Enzyme Activity of L-Asparaginase

The L-asparaginase enzyme of C. ordorata L. leaves is optimum at pH of 8, the temperature of 37°C, and an incubation time of 30 minutes which the result of enzyme activity was 0.23 IU/mL in figure 1a, b, and c. Based on the results of studies that have been reported, the L-asparaginase enzymes have different optimum activities. For example, the L-asparaginase enzymes of ginger are optimum at pH 8.6 with a temperature of 37°C with the incubation time of 30 minutes [6] while the L-asparaginase enzymes of white turmeric are optimum at pH 8.5 with a temperature of 37°C and incubation time of 30 minutes [7].
3.2.2 The Effect of Metal Ions on Enzyme Activity L-Asparaginase

Enzyme activity is influenced by metal ion which is an activator that can increase or decrease the activity of an enzyme. Characterization of the metal ion effects on L-asparaginase enzymes using metal is shown in Figure 2. Monovalent (K⁺ and Na⁺) metal ions were an activator with K⁺ as the highest activator compares to divalent metal ions of Ca²⁺, Zn²⁺, Mg²⁺, Cu²⁺, Co²⁺, and Mn²⁺ were the inhibitors with Zn²⁺ as the strongest activator. Based on studies that have been reported which the metal ions of Zn²⁺, Mn²⁺ [13], Ca²⁺, Co²⁺, Cu²⁺, Mg²⁺ and Na⁺ [14] have potentials to inhibit the activity of L-asparaginase enzyme.

![Figure 2. Effect of Metal Ions on Relative Activity of L-Asparaginase Enzyme of the C. Oradorata L. leaves](image)
3.2.3 Toxicity Test Using the Brine Shrimp Lethality Test (BSLT) Method

To determine the toxicity effect of the purification of the L-asparaginase enzyme from C. odorata L. leaves, the LC$_{50}$ value was determined using A. salina or shrimp larvae. The LC$_{50}$ value indicated the amount of sample concentration that able to cause the death of test animals.

| Sample          | Concentration (g/mL) | Axis X (Log [Sample]) | % Larval death - Control | Axis Y (Probit value) | LC$_{50}$ (g/mL) |
|-----------------|----------------------|-----------------------|--------------------------|-----------------------|-------------------|
| Dialysis        | 1                    | 0.00                  | 3                        | 3.12                  |                   |
| Product of Enzyme | 10                  | 1.00                  | 10                       | 3.72                  | 158.48            |
|                 | 100                  | 2.00                  | 47                       | 4.92                  |                   |

The result of observing the death of A. salina showed that the purified enzyme had LC$_{50}$ value of 158.48 µg/mL, as shown in table 2. This LC$_{50}$ value indicated that the purified L-asparaginase enzyme had the potential as an anticancer because of the value of LC$_{50}$ <1000 µg/mL which means the sample was very toxic [15]. The result of toxicity tests from the purification of the L-asparaginase enzyme capable to be developed as anticancer agents.

4. Conclusion

The conclusion of this research is the L-asparaginase enzyme from the purification of C. odorata L. leaves has an optimum activity of 0.23 IU/mL with pH 8 at 37°C and incubation time of 30 minutes with K$^+$ metal ions as the activators. It has potential as an anticancer agent with LC$_{50}$ value of 158.48 µg/mL.

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References

[1] World Health Organization 2018 Latest Global Cancer Data: Cancer Burden Rises to 18.1 Million New Cases and 9.6 Million Cancer Deaths in 2018 http://www.who.int/cancer/en/ (accessed on 17 August 2019)

[2] Abdel F Y R, and Olama Z A 2002 L- asparaginase production by Pseudomonas aeruginosa by solid state culture: evaluation and optimization culture conditions using factorial designs, Process Biochemistry, 38:115-22.

[3] Thakur, Monica. et. al 2014 Extracellular L-Asparagenase from Mucos hiemalis, J. of Biocatalysis & Biotransformation 2 (2): 1-9.

[4] Hendriksen H V. et. al 2009 Evaluating the potential for enzymatic acrylamide mitigation in a range of food products using an asparaginase from Aspergillus oryzae, J. of Agricultural and Food Chemistry, 57:4168-4176.

[5] Shrivastavaa A et al 2015 Recent Developments in L-asparaginase Discovery and its Potential as Anticancer Agent Critical Reviews in Oncology/Hematology 100(2016): 1–10

[6] Puspitasari, Oktarina dan Wuryanti 2010 Isolation of L-asparaginase enzyme from Temulawak (Curcuma xanthorrhiza Roxb.) and Potential Test for Leukemia Cell Culture K562, J. Kimia Sains dan Aplikasi 13 (2): 61 – 65.

[7] Arpintasari, Agustina, Wuryanti, W.H. Rahmanto 2008 Isolation and Potential Test of
L-asparaginase from White Turmeric Rhizome (Curcuma mangga Vall) to Leukemia Type K562. *J. Kimia Sains dan Aplikasi, 11* (3): 57–62.

[8] Kusumaningtias, Nindy. Nies SM dan Purbawatiningrum RS 2016 Calcium Alginate as Supporter of L-asparaginase Immobilization from Garlic (*Allium sativum*), *J. Kimia dan Pendidikan Kimia, 1* (2): 7-15.

[9] Setiawan, Ayu SR, Waryanti, Agustina LNA 2013 Purification of L-asparaginase from Onion (*Allium cepa* L.) using Sephadex G-100 Gel Filtration Chromotography, *Chem Info* 1 (2): 27-34.

[10] Haritini S 2001 Isolation and Purity Level of Ammonium Sulphate Fraction Asparaginase from Keweni Maggo Parasite, *J. Kimia*. 1: 9-18.

[11] Fitrah, Muhammad 2016 Identification of Kopasanda Leaf Extract (*Chromolaena odorata* Linn) Against Antiproliferation of L1210 Leukemia Mice, *Jf Fik Uinam* 4 (3): 99-105.

[12] Ngozi, Igboh M, Jude, Ikewuchi C. and Catherine, Ikewuchi C 2009 Chemical Profile of Chromolaena odorata L. (King and Robinson) Leaves, *Pakistan Journal of Nutrition*, 8 (5) 521-524.

[13] Manna S, Sinha A, Sadhukan R, Chakrabarty SL 1995 Purification, characterization and antitumor activity of L-asparaginase isolated from *Pseudomonas stutzeri* MB-405. *Curr Microbiol*. 30: 291-298.

[14] Kumar, N.S. Mohar HK, Mononmani 2013 Purification, Characterization and Kinetic Properties Extracellular L-Asparaginase Produced by *Cladosporium* sp, *World Journal of Microbiology and Biotechnology*, 29 (4): 577-578.

[15] Carballo JL, Hernandez-Inda ZL, Perez P, Garcia-Gravaloz MD 2002 A Comparison Between Two Brine Shrimp Assays to Detect in Vitro Cytotoxicity in Marine Natural Products. *BMC Biotechnology*, 2 (17): 1472-6570.