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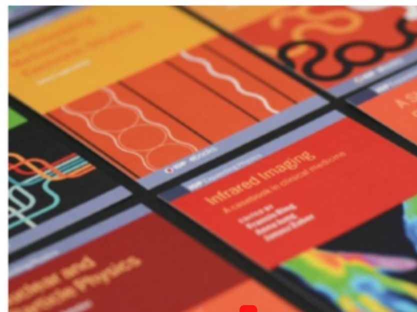

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Cloning of gene-encoded stem bromelain on system coming from *Pichia pastoris* as therapeutic protein candidate

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Abstract. The process of identifying bacterial recombination using PCR, and restriction, and then sequencing process was done after identifying the bacteria. This research aimed to get a yeast cell of *Pichia pastoris* which has an encoder gene of stem bromelain enzyme. The production of recombinant stem bromelain enzymes using yeast cells of *P. pastoris* can produce pure bromelain rod enzymes and have the same conformation with the enzyme's conformation in pineapple plants. This recombinant stem bromelain enzyme can be used as a therapeutic protein in inflammatory, cancer and degenerative diseases. This study was an early stage of a step series to obtain bromelain rod protein derived from pineapple made with genetic engineering techniques. This research was started by isolating the RNA of pineapple stem which was continued with constructing cDNA using reserve transcriptase-PCR technique (RT-PCR), doing the amplification of bromelain enzyme encoder gene with PCR technique using a specific premiere couple which was designed. The process was continued by cloning into bacterium cells of *Escherichia coli*. A vector which brought the encoder gene of stem bromelain enzyme was inserted into the yeast cell of *P. pastoris* and was continued by identifying the yeast cell of *P. pastoris* which brought the encoder gene of stem bromelain enzyme. The research has not found enzyme gene of stem bromelain in yeast cell of *P. pastoris* yet. The next step is repeating the process by buying new reagent; RNase inhibitor, and buying liquid nitrogen.

1. Introduction

The use of protein as the disease therapy has rapidly grown for decades [9].That therapy is well known as therapeutic protein. The protein used as the therapeutic protein must have the high purity level. The enzyme, commonly protease, is a protein group which has the high purity level which will be used in both industry and therapy. More than 60% enzyme used in both industry and therapy is protease enzyme [7].

Bromelain is one of the protease enzymes which can be found on pineapple plant (*Ananas comosus*). Pineapple has been used to cure the digestion problem and reduce inflammatory [5]. In pineapple, this enzyme can be found in all parts. In the 1800s, bromelain enzyme was successful to be isolated from the stem and pollen of pineapple. There are two kinds of bromelain enzymes; stem bromelain with commercial code EC 3.4.22.32 and fruit bromelain with commercial code EC> 3.4.22.33.

In the food industry, bromelain enzyme is mostly used to make meat soft, in the food industry, cosmetics, skin, textile, and health field [10]. In the health field, this enzyme has many benefits as a

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therapeutic protein. In 1957, bromelain enzyme was firstly known as therapeutic compound [5]. In clinic application, this enzyme is used to heal some diseases, but the most effective effect is used to reduce the inflammatory because of infection, sinusitis, osteoarthritis, and cancer [8]. Bromelain enzyme which comes from rough extract is still mixed with protein, enzyme, and carbohydrates. This enzyme must have high purity level so that it can be used as therapeutic protein. This enzyme could be separated from those components with using some stages and methods; centrifugation, precipitation, ultra-filtration, electrophoresis, chromatography method and other procedures [4]. That conventional method caused the production and formulation of bromelain enzymes very difficult, it was almost impossible to be applied. It was often mixed with other compounds and the cost was expensive. Besides that, the process of downstream became one of the main factors the most expensive; more than 50%.

Along with the technological and scientific advancement and the existence of genetic modification technology, the enzyme production could be done in out of its original organism. Recombined enzyme production could be done by isolating that enzyme encoder gene and then being inserted into a plasmid and then being inserted into the host cell which was continually stimulated to produce that enzyme [2]. Some proteolytic enzymes which have been developed with genetic modification technology are papain enzyme using *Pichia pastoris* cell [3]. Genetic modification technology has successfully got recombinant bromelain protein which is fruit bromelain applied on a transgenic plant [6], and stem bromelain coming from *E. Coli* bacterium cell [1].

Currently, host cells that can produce enzymes or proteins have been developed extracellular. One is the *Pichia pastoris* cell. Extracellular proteins will make the process of isolating and purifying proteins easier and it can minimize protein degradation. In addition, *P. pastoris* cells are eukaryotic cells so it is expected that bromelain enzyme confirmation will not change due to the same protein synthesis process with pineapple plants.

There are many benefits to be gained from bromelain enzymes in the industry and health. Therefore, it is necessary to find a cheap and easy method to get this enzyme in a clean condition. Genetic modification technology can produce pure bromelain enzymes with cheap budget and easy methods when on a large scale. Because of the similarity of cell structure and protein synthesis processes that occur in *P. pastoris* and pineapple plants, bromelain enzymes can be produced with genetic modification in *P. pastoris* cells. Production of recombinant stem bromelain enzymes using *P. pastoris* yeast cells can produce pure bromelain rod enzymes and have the same conformation as conformation of enzymes in pineapple plants. This recombinant stem bromelain enzyme can be used as a therapeutic protein in diseases due to inflammation, cancer, and degeneration. This study was an initial step in several steps to obtain bromelain stem protein derived from pineapple made with genetic modification

2. Methods

Genetic modification technology, which is well known as DNA recombination, is a method used to combine some genes to make a component which will be inserted into the host cell which has been modified. This development technology gives the big change in protein and enzyme industry, both in industry and health field. This technology consists of some components; a wanted gene, vector/plasmid, DNA enzyme of polymerase, restriction enzyme, ligase enzyme, the host cell. Briefly, the main work of this technology is isolating the wanted gen from DNA molecule of the root organism, multiplying the wanted gene with in vitro using polymerase chain reaction technique (PCR), the wanted gene is cut and continued by combining those genes into a vector, combined vectors and genes are inserted into the host cell with transformation method [2].

Materials used in this experiment were pineapple, RNA isolation kit from pineapple, *Escherichia coli* enzyme for the first cloning, *Komagataella pastoris* yeast cell for yeast cell transformation, reverse transcriptase enzyme, agarose powder, loading dye, DNA ladder, Tris-Base, glacial acetic acid, EDTA, the primer specific forward and reverse, primer to construct cDNA forward and reverse, DNA enzyme of polymerase, an enzyme of T4 DNA ligase, restriction enzyme of BamH I and Xho I, DNA isolation kit, DNA purification kit from agarose gel, restriction enzyme to make recombined

plasmid linear, ampicillin antibiotic, zeocin antibiotic, tryptone, peptone, yeast extract, yeast nitrogen base, biotin, jelly, glucose, dextrose, methanol.

Equipment used in this research was tools to have separation process of DNA and protein, the process of protein transfer, gene amplification, cloning process, and transformation of yeast *P. Pastoris*, protein extraction, and visualization of DNA fragment. To DNA separation used electrophoresis of agarose gel. To separate protein used Mini Protean (Bio-Rad). To protein transfer process from acrylamide gel to nitrocellulose membrane used semi-dry trans blot (BIO-RAD). To gene amplification used thermocycler PCR machine (Applied Biosystem) and Gradient thermocycler (Bio-Rad). To clone used the water bath and shaker incubator. To transform yeast of *P. pastoris* used electroporator EC100 (Thermo). To visualize DNA used a machine (Bio-Rad) with the software of quantity one. To extract the protein used vortex and water bat. The routine equipment which was used was centrifuged, micropipette, and tips to various volumes.

This research used pineapple, expression vector on *P. pastoris* cell, *Escherichia coli* bacterium, and yeast cell of *Komagataella pastoris*. To isolate pineapple RNA used a kit to isolate RNA. To make cDNA used *reverse transcriptase* enzyme, and to gene amplification was used with polymerase enzyme. To clone an expression vector, a restriction enzyme is used to limit the genes and vectors and ligase enzyme to ligase process.

3. Results and Discussion

3.1 Isolation of pineapple RNA

Isolation of pineapple RNA was used to get pineapple RNA molecule where pineapple RNA molecule kept an informative data of bromelain enzyme gene of pineapple stem. In this experiment, stem (head) of pineapple which was cut and measured for 100mg then was soaked in liquid nitrogen. Liquid nitrogen was kept in the special tube (See Figure 1).

Figure1. A tube to save liquid nitrogen

This submersion was done to make the hard sample in short term so that it could prevent the damage in next process. The final result which was gotten from pineapple RNA extraction which was RNA in the aquadest liquid which was free from RNase and DNase. The result of this step was not pictured because RNA could not be seen visually.

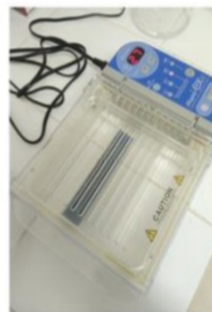
3. 2 Pineapple cDNA Construction

After obtaining the RNA molecules, then it was continued to synthesis process of cDNA and DNA using kit SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, USA). The construction of cDNA and DNA synthesis of specific bromelain enzyme on pineapple stem were done in one step only without stopping when RT-PCR on PCR machine process was running. The PCR machine can be seen in Figure 2. Because of the use of primary molecules (reverse and forward), this process produces DNA molecules of bromelain enzymes which designed by bromelain enzyme gene data residing within the bank genes. The result of this step was specific DNA molecules

of bromelain enzyme whose successful process could be seen from electrophoresis process of agarose gel (see Figure 3).

Figure 2. PCR Machine

Based on the Figure-3B, it was known that DNA synthesis process of bromelain enzyme had not been successful to be synthesized. The occurred molecule on agarose gel was a primer reacting one another which signed that molecule RNA did not exist in RT-PCR reaction. This may have happened because the RNA degradation by RNase enzyme which could destroy RNA molecule happened. Kit, used for DNA synthesis, did not provide RNase inhibitor which could prevent RNase activity when construction process of cDNA and DNA synthesis was running.



A

B

DNA molecule from cDNA construction and DNA synthesis

Figure 3. DNA molecule of cDNA construction and DNA synthesis

4. Conclusion

Until now, the research has not found enzyme gene of stem bromelain yet. The next step is repeating the process by buying new reagent; RNase inhibitor, and buying liquid nitrogen.

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