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Characterization β-glycosidase of Tempeh from Rejected Edamame Soybean and Determination Method of Extracted Genistein by Conventional and Compared Using of Modern Method

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Abstract. Background: The exploration of natural active material became special attention in recent research dimensions, such as genistein. As we know, one of the genistein resources which never researched yet is edamame, especially tempeh from rejected edamame soybean. Tempeh made from rejected edamame soybean is known to have a β-glycosidase enzyme and can be used to increase the content of genistein. The result of early research by Wibisono gave the conclusion that identification by 16S rRNA for IS.5 as dominant bacterial and β-glycosidase enzyme producer on soymilk from tempeh was *L. plantarum* IMAU: 10246 (formerly called *L. plantarum* Polije: 15420). **Methods:** The research was divided into 3 (three) stages, such: stage I was the determination of *L. plantarum* IMAU: 10246 induced nisin in producing of the β-glycosidase enzyme on tempeh fermentation; stage II was a purification of the β-glycosidase enzyme and stage III was the final stage which determination method of extracted genistein by conventional method and compared using of the modern method. **Results and conclusions:** The resulting research on early-stage gave the conclusion that GC content of *L. plantarum* IMAU: 10246 ratio was 56,37 mol%, had log phase of the 25 – 30th, stationary phase in 35th with death log phase in 45 - 50th from optimum temperature levels. On the other result, this bacterial had optimum growth on 35 – 38^{o}C, pH 5,0 – 5,5 with having μ_m value as 0,276/hour, saturation constantan (K_8) was 0,051 g/l and growth yield (Y) with 0,311 g biomass/g substrate. The result of stage II showed that using ammonium sulfate 40% was resulting specific activity of the highest enzyme approximately 1,856 x 10^{-2} unit.mg^{-1}. Using Sephadex C-50 had yielded the increasing of specific activity becoming 0.218 unit.mg^{-1}. The result of the molecular of the enzyme showed 11.51 kDa and has an optimum temperature of 40^{o}C (by activity 0,0550 unit), optimum pH 6,5 (0,0923 unit), and had K_m and V_{max} values are 2,572 mM and 4,276 U.mg^{-1}. β-glycosidase can be inhabiting by CaCl2 > EDTA > NaCl > KCl and allegedly belong to family 1. The final result showed that the extraction method using ultrasound yielded 1,526 mg.g^{-1}, increasing 15,51% higher than conventional extraction. Hydrolysis optimally needs duration for about 120 minutes on 50 ml water containing 5 g powder of tempeh from rejected edamame soybean.

Keywords: β-glycosidase, Genistein, Rejected Edamame Soybean, Tempeh

1. Introduction

Lifestyle changes (diet), stress, radiation exposure, and pandemics/epidemics cause degenerative diseases. On the other hand, the increase in food commodity prices as a result of inflation and restrictions on incoming goods, including imported soybeans, has made it difficult for people to consume affordable and healthy foods. This can reduce the quality of human resources in an era of competition with other countries.

The exploration of active ingredients to reduce the risk of degenerative diseases has received special attention, including genistein. Since 1978, genistein therapy has been carried out in developed countries (especially in America) [7]. Genistein is reported to have inhibition against cancer cells in general, including breast cancer cells [13]. Genistein is also reported to be able to reduce prostate, antimicrobial, decrease LDL, and even prevent DNA mutation [5]. The use and therapy of genistein led to the selling price in the international market reaching $ 265.32 per 10 mg and is predicted to experience a price hike in the next five years [14]. The demand for genistein will provide commercial genistein export opportunities.

The potential source of genistein is soybeans, however, 60 - 70% (0.414 mg / g) is still bound to sugar and has no functional activity, so it needs to be hydrolyzed with β-glucosidase enzymes. [14] . The results of early research by Wibisono gave the conclusion that bacteria called *L. plantarum* IMAU: 10246 can produce the highest β-glucosidase, but further identification of these bacteria is still needed. [14] .

In Indonesia, PT Mitratani Dua Tujuh Jember is the largest exporter of edamame soybeans. From the export amount, as much as 12.8 tons per year is a rejected edamame. The use of *L. plantarum* IMAU:10246 bacteria in the fermentation process of tempeh from raw edamame and extracted genistein using modern equipment (such as ultrasound) compared to Sephadex C-50, has also never been carried out in-depth research and has never been published in an international journal.

2. Methods

The research was divided into 3 (three) stages, such as stage I was the determination of *L. plantarum* IMAU: 10246 induced nisin in producing of the β-glycosidase enzyme on tempeh fermentation; stage II was the purification of the β-glycosidase enzyme, and stage III was the final stage which determination method of extracted genistein by conventional method and compared using of the modern method.

2.1 Procedure in phase 1 research

The research in phase I was to study the β-glycosidase enzyme produced by *L. plantarum* IMAU: 10246 induced by nisin from the fermentation process of tempeh with a rejected edamame as a raw material. The bacteria were isolated and grown in MRSA media with additional 2% glucose to study the log phase, the stationary phase of fermented edamame tempeh according to its optimum conditions. Making standard curves and determining the above values using the modified Zotta method [16]. The bacteria *L. plantarum* IMAU: 10246 induced with nisin were also studied for optimum pH and growth temperature of the media, μm and K_s values, and growth yield. [1]

In stage 1, the profile of bacterial isolates was also determined by using native gel electrophoresis [3]. The bacterial culture comes from the optimal phase and uses the above procedure to obtain the supernatant. The results of the cell-free extract were then visualized and negative gel electrophoresis was carried out.

Measurement of β-glycosidase enzyme activity used a modified procedure [5], by measuring the hydrolysis rate of the pNPG substrate. One enzyme unit is defined as the amount of β-glycosidase enzyme which liberates 1 micromol of p-nitrophenol from the pNPG substrate per ml under the above measurement conditions.

2.2 Purification of the β-glycosidase enzyme

Extraction and purification were carried out by homogenization and centrifuge using glass beads that produced crude enzymes. [4] . The enzyme solution was then precipitated with ammonium sulfate with saturation levels of 30, 45, 60, and 75%. The next step is a preparation with a dialysis bag and then drying it using freeze-drying for 24 hours. Observations were made by testing protein levels and specific activity according to a modified method [6]

The β-glycosidase enzyme purification then used Sephadex G-25 and continued with Sephadex C-50 which was eluted with a NaCl gradient at a concentration of up to 1 M. Eluent was collected using a fraction collector (per 5 minutes for setting 5 ml tubes) and the highest fraction was freeze. drying for 24 hours (until frozen) and prepared and again freeze-drying for 2 days until dry enzymes are obtained. [2]

2.3 Extraction of the β-glycosidase enzyme by conventional methods and by the ultrasound method

This extraction was carried out by mixing 40 ml of crude enzyme extract solution with 160 ml of ethyl acetate, added with 20 ml of 4N HCl, and ultrasound at 40 kHz for 30 minutes. This mixture is stored in a special cupboard for 18 hours to allow the release of aglycone. The waste is then dissolved with a mixture of the previous chemical and the filtrate is added with anhydrous $Na_2SO_4$to bind the water, filtered, and evaporated again in vacuum at a temperature of 50 ◦C. The last step is the quantitative determination of genistein levels using HPLC [11]

For the conventional method, the procedure is the same as above, only without using ultrasound. HPLC has been prepared with a standard solution containing genistein and genistin at final concentrations of 4, 10, 20, 30, and 40 μg / ml and injected

3. Result and Discussion

The results of the initial research have obtained 11 bacterial isolates (IS) from the results of natural fermentation using edamame rejected soybean as raw material. Further test results obtained isolate *L. plantarum* IMAU: 10246 which can produce β-glucosidase enzymes for further screening. Screened-selected bacteria were identified and further confirmed using 16S rRNA. A total of 719 16S rRNA sequences were successfully read using Primer 283F 5 '- GAG AGT TTG ATC CTG GCT CAG GAC - 3' and Primer 261R 5 '- AAA GGA GGT GAT CCA GCC GC - 3'. The primer used is based on the results of previous studies to identify lactic acid bacteria that can produce β-glucosidase enzymes [10] [12]

The order of bases obtained is as follows.

AGAGACACCTAAGCTGGAGGCGTGCCTAAACAAGCAAGTCGAACGAACTCTGGTATTGATTGGT
GCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCC
CAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGG
TCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATG
GTGAGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATT
GGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACG
AAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTA
AAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTA
AAGCGAGCGCAGCGGTTTTTTAAGTCTGATGTGAAAGCCTTTCGCTCACCCGAGAGTGCATCGG
AAAACTGGGGTAACTGGGTGCAGAAGAGGACAGTGTACTCATGTGTAGCGGTGAATGCGTAGT
TATTTATGGTAGAAAACCCCCAGTTGGCCGA

The optimal pH condition of *L. plantarum* IMAU: 10246 to produce β-glucosidase enzyme is at pH 6 and if it is conditioned below pH 5 there will be a sub-lethal injury. The adaptability of *Lactobacillus* bacteria to acidic conditions is due to the presence of the enzyme F_oF_1-ATPase subunit which can remove excess H^+ protons from its cell membrane using ATP.

Nisin induced in the fermentation process has been reported to bind lipid II to the precursors of cell wall synthesis of positive Gram bacteria [15] but it is suspected that *L. plantarum* IMAU: 10246 has a protective barrier against bacteriocins and the inability of nisin to pass through the cytoplasmic membrane and peptidoglycan layer in negative Gram bacteria.

Native gel electrophoresis was carried out to see the profile of *L. plantarum* IMAU: 10246 and the results are presented below.

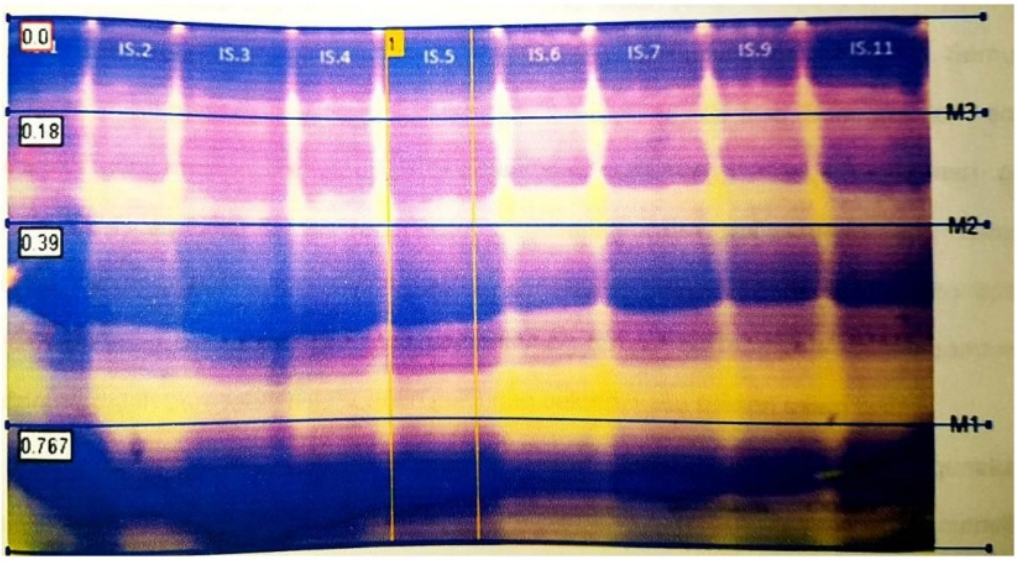

Figure 1. **Protein profile of *L. plantarum* IMAU: 10246 isolates by native gel electrophoresis.** M1 description: aprotinin bovine lunge, 6,500 Da (Rf 0.767); M2: ovalbumin, 42,700 Da (Rf 0.39); and M3: bovine serum albumin, 66,000 Da (Rf 0.18). Calculation of Rf using a Gel Analyzer.

The GC content of *L. plantarum* IMAU: 10246 was 56.37 mol%, as shown in the following figure. The basic composition of the amino acid content of the IS.5 isolate, respectively, was glycine (29.69 mol%), alanine (27.36 mol%), threonine (22.30 mol%), and cysteine (20.66 mol%).

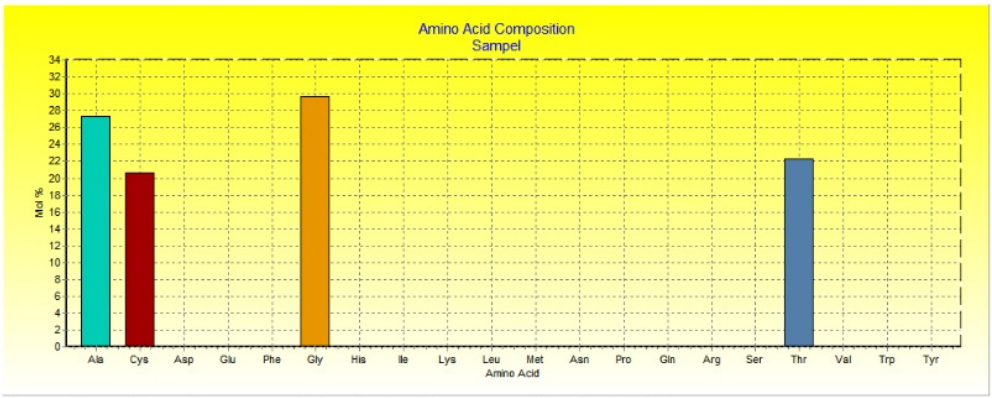

Figure 2. **Amino acid composition in *L. plantarum* IMAU: 10246**

L. plantarum IMAU: 10246 has been further tested, has a log phase of the 25 - 30th, stationary phase in 35th with death log phase in 45 – 50th from optimum temperature levels. On the other result, this bacterial had optimum growth on 35 - 38 ^{o}C, pH 5,0 - 5,5 with having μ_m value as 0,276 / hour, saturation constantan (K_m) was 0,051 g / l and growth yield (Y) with 0.311 g biomass / g substrate

Table 1. The specific activity of the crude β-glucosidase enzyme at ammonium sulfate level

Ammonium Sulfate Saturation%	Specific Activity (Unit/mg)	
	Supernatant	Sediment
30	1,486 x 10^{-2} ± 0,0036	1,686 x 10^{-2} ± 0,0036
45	1,500 x 10^{-2} ± 0,0010	1,856 x 10^{-2} ± 0,0015
60	1,433 x 10^{-2} ± 0,0035	0,095 x 10^{-2} ± 0,0037
75	0,766 x 10^{-2} ± 0,0067	0,085 x 10^{-2} ± 0,0044

The precipitating results with 45% ammonium sulfate were selected for further use with selopan dialysis and using Sephadex C-50 showed that after the 55th tube there was no further enzyme activity. This is shown in the following figure

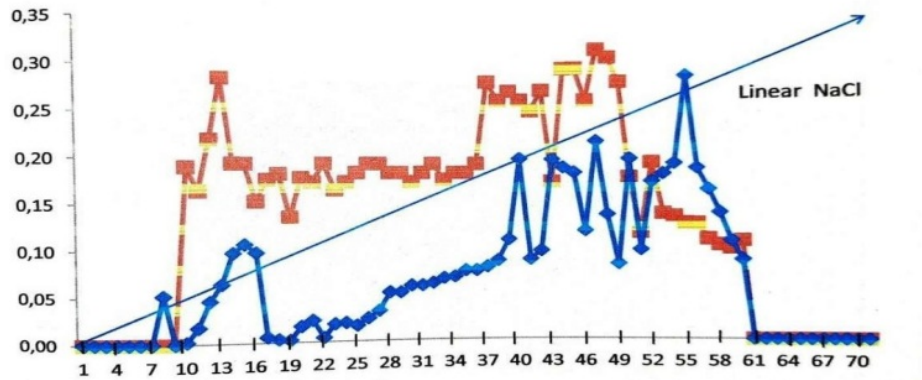

Figure 3. **Profile of β-glucosidase enzyme elution with Sephadex C50**. On the x-axis is the specific activity (Unit/mg) and the y-axis is the fraction number (5 ml/tube)

Purification results with Sephadex C-50 provide a purity increase of 7.17 times compared to Sephadex G-25. Meanwhile, the use of Sephadex G-25 gave an increase of 6.15 times compared to the initial conditions (only with 45% ammonium sulfate). The highest specific activity result was shown with a value of 1.856 x 10^{-2} unit/mg.

The molecular weight of the β-glucosidase enzyme is 11.51 kDa with Rf = 0.88 as shown in the following figure

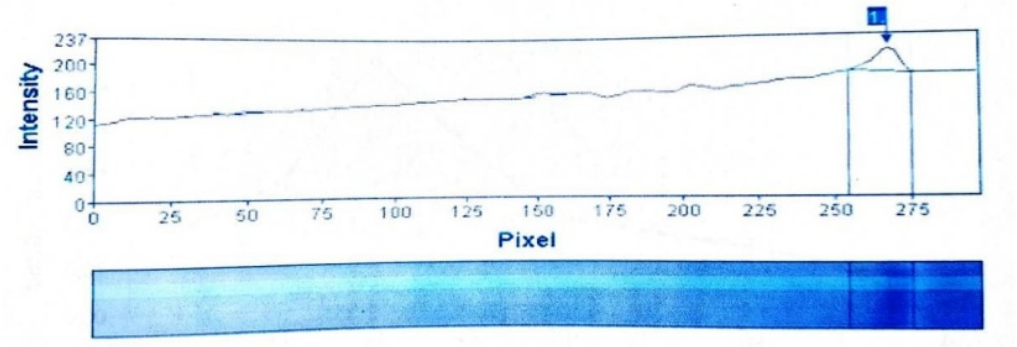

Figure 3. **The profile of the purification results of Sephadex G-25 and continued with Sephadex C-50 and calculated with a gel analyzer.** Figure 1 shows the single band in column C.

The β-glucosidase enzyme is known to have different temperatures and pH, depending on the source of isolation. The β-glucosidase enzyme from *L. plantarum* IMAU: 10246 has an optimum temperature of 40 °C (by activity 0.0550 units), an optimum pH of 6.5 (0.0923 units), and had K_m and V_{max} values are 2.572 mM and 4,276 Umg^{-1}. β-glycosidase can be inhabiting by $CaCl_2$ > EDTA > NaCl > KCl and allegedly belong to family 1. Further research has shown that the β-glucosidase enzyme is thought to be not only applicable for genistin hydrolysis but also can be used for hydrolysis of agricultural waste. This is indicated by the data above that the enzyme can survive in relatively high NaCl conditions at a concentration of 6.5%.

The results of the third stage of research, namely extraction and purification using ultrasound, gave genistein 15.51% higher results than conventional methods. This increase is due to the ability of ultrasound to increase solubility, increase the emulsion activity index, and the efficiency of the chemicals used concerning the physical, chemical, and functional intervention of the cavitation produced by the device [8] [9]. The results of this study showed that there was no reduction in genistein extracted by ultrasound, although genistein, like isoflavones, has OH groups and radical properties that cause changes in the active site of the hydroxyl group. The inducer frequency of 40 kHz has been known to have the highest cavitation bubble formation and a greater hydromechanical force

5. Conclusion

The resulting research on early-stage gave the conclusion that GC content of *L. plantarum* IMAU: 10246 ratio was 56,37 mol%, had log phase of the 25 – 30^{th}, stationary phase in 35^{th} with death log phase in 45 - 50^{th} from optimum temperature levels. On the other result, this bacterial had optimum

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The final result showed that the extraction method using ultrasound yielded 1,526 mg.g^{-1}, increasing 15,51% higher than conventional extraction. Hydrolysis optimally needs duration for about 120 minutes on 50 ml water containing 5 g powder of tempeh from rejected edamame soybean.

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