Fractional Factorial Analysis Of Immunoglobulin G Anti-Diphtheria Serum Fragmentation By Pepsin

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Abstract. The number of diphtheria outbreaks was observed to be quite high in the last 5 years. One way to treat diphtheria at the time of an outbreak is to use an anti-diphtheria serum. The availability of anti-diphtheria serum products worldwide has been reduced for years because manufacturers in several countries have stopped producing. The cessation of production is partly due to a decrease in demand, production failures and regulatory requirements in the manufacture of increasingly stringent biological products from the blood. In addition, the use of certain types of pepsin as a component of production becomes problematic with the issuance of Law No. 34 of 2014 concerning Guarantees of Halal Products. This study aims to analyze the use of pepsin A as a substitute for pepsin B, get parameters that have a significant effect on IgG fragmentation using fractional factorial design, get the optimum parameter value of IgG fragmentation (highest titer value, shortest Kf time and absence of IgG bands in SDS PAGE) and increase anti-diphtheria serum product antibody titers. Risk analysis was carried out to find out several factors that influence the process of pepsin A fragmentation against IgG anti-diphtheria serum products using fishbone and FMEA methods. Factors belonging to the unacceptable and intolerable classification are purification methods (RPN 60 value), pH termination of the fragmentation process (RPN 100 value), fragmentation temperature (RPN 60 value), number of pepsin (RPN 125 value), and fragmentation time (RPN 125 value) Changes in the method carried out by replacing the ammonium sulfate fractionation process to diafiltration using a 100kDa filter membrane on the initial plasma showed insignificant results. Fractional factorial analysis was carried out on 4 factors, namely: pH; temperature; amount of pepsin and time. Based on pareto analysis and main effect, the four factors showed a very significant effect on the IgG fragmentation process by pepsin A (p-value <0.05) by using antibody titers as the main response. Characterization of the results of fragmentation is done using the BCA test and SDS PAGE. The conclusion of this study is that pepsin A can replace pepsin B, a factor that has a significant effect is the amount of pepsin, time, temperature, and pH of the incubation termination; optimum value (highest titer, shortest Kf time and no IgG band) IgG fragmentation process by pepsin A is the amount of pepsin 1163mg / L, temperature 37ºC, time 135 minutes, and pH 5.95; and increasing antibody titer at IgG fragmentation stage to 2.67 times than the previous method using pepsin B.

Keywords: Anti-diphtheria serum, horse plasma, pepsin, IgG fragmentation, halal.

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1. Introduction

Diphtheria is an infection caused by the Corynebacterium diphteriae. Signs and symptoms begin 2-5 days after exposure. Corynebacterium diphteriae produce a toxin that causes thick gray or white patches on the back of the throat (Unicef, 2017). In 2017, Indonesia experienced an Extraordinary Incidence (KLB) of diphteria with a report up to November 2017 outbreaks totalling 593 cases and 32 deaths spread across 20 provinces. 66% of the total prevalence of non-immunization, 31% ogf immunization, but the immunization status is incomplete (Health Ministry, 2017). The appropriate handling of diphteria outbreaks in addition to increasing the coverage of diphteria immunization is by administering anti-diphteria serum, intravenously or intramuscular injection (Unicef, 2017)

Anti diphtheria serum is a preparation containing specific immunoglobulin fragments obtained from natural serum (plasma) through purification that is influenced by enzyme treatment, fractionation and other chemical or physical procedures (WHO, 1969). Anti diphtheria serum has been included in WHO's Essential Medicines List (EML), which consists of a list of important medicines that must be available in all health facilities. The availability of anti-diphtheria serum has decreased over the years because producers in several countries have stopped producing due to several factors including decreased demand, as well as increasingly stringent regulatory requirements in the manufacture of products that originate from blood (Unicef, 2017). In the 2016-2017 period there were only three pharmaceutical companies in the world capable of producing and supplying the world's needs for diphtheria anti-toxin, namely Haffkine Bio Pharmaceutical Corporation (India), Microgen (Russia) and Vins Bioproducts Limited (India).

One of the stages of anti diphtheria serum production that is suspected to be problematic is the fragmentation of immunoglobulin G (IgG) to obtain divalent immunoglobulin fragments, F(ab')2. The divalent immunoglobulin fragment F(ab')2 produced so far has been considered not optimal so as to produce anti-diphtheria horse plasma antibody titer values that do not meet the requirements set by WHO which is > 350Lf / mL. The process that has been carried out using the protease enzyme pepsin B. The use of pepsin B also in the future will have problems with the Law of the Republic of Indonesia No. 33 of 2014 concerning Halal Product Guarantee.

This study aims to analyze the use of pepsin A as a substitute for pepsin B, obtain parameters that have a significant effect on fragmentation of IgG by using fractional factorial design, obtain optimum parameters for IgG fragmentation (highest titer value, shortest Kf time and absence of IgG bands in SDS PAGE) and increase the antibody titer of anti diphtheria serum products. Optimized parameters in the digestion process are the amount of pepsin A, incubation temperature, pH of digestion process termination, and digestion time. The response used in assessing a significant factor (P-value <0.05) is a quantitative parameter of the IgG fragmentation process, which is the antibody titer value. Characterization of IgG fragmentation results used bichinconic acid (BCA) test to determine the total protein content produced and SDS-PAGE profile to see the character of IgG fragmentation into antibody fragment F (ab) 2.

2. Digestated enzymes on immunoglobulin

Immunoglobulin fragments are produced through proteolytic breakdown which has been proven to be able to elucidate structures / functions related to immunoglobulin. Several different enzymes produce different immunoglobulin fragments. The papain enzyme breaks down immunoglobulin molecules in the joint region before disulfide bonds between H chains. This process results in the formation of 2 identical fragments containing L chains and VH, CH1 regions in the H. chain. These fragments are called Fab fragments which contain antigen-binding sides to antibodies that are able to bind to certain antigens because they have special combinations of VH and VL. The Fab fragment produced by the papain enzyme is monovalent. The use of the papain enzyme also produces fragments containing the remaining 2 H chains, each containing CH2 and CH3, which are named as fragments of Fc that are easily crystallized (Mayer, 2017).

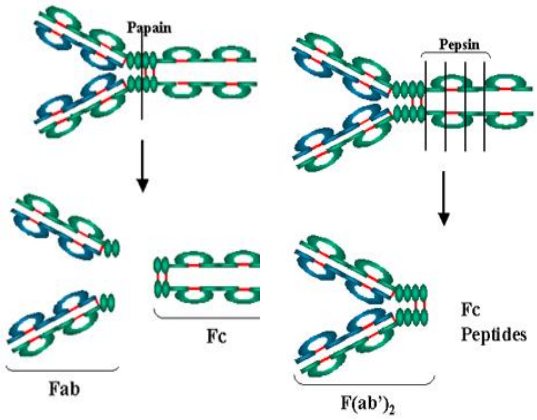


Figure 1.1 Side cutting of immunoglobulins and by protease enzymes. (a) Papain enzyme; (b) Pepsin enzyme (Mayer, 2017)

The addition of the pepsin enzyme aims to obtain divalent immunoglobulin fragments namely F(ab')2. Pepsin cuts the structure of immunoglobulins in the H chain after disulfide bonds between the H-H chains to produce divalent fragments that contain both sides of the binding of antigens. The Fc region of the protein molecule is broken down into small peptides (Figure 1.1). The resulting fragment F(ab')2 is capable of binding to antigens, but is unable to mediate the effector function of antibodies (Mayer, 2017). This fragmentation method using pepsin also causes degradation of non-IgG proteins such as albumin (WHO TRS 1004, 2017).

There are different specifications of the use of pepsin from type B and A. These differences determine the fragmentation process that occurs in the production process of Anti Diphtheria Serum. Fab fragments are very sensitive to temperature changes, while Fc fragments are very sensitive to pH reduction. The difference in specifications between the 2 types of pepsin can be seen in table 1.1. The difference in molecular weight of each fragment and immunoglobulin causes significant differences in pharmacokinetic and pharmacodynamic parameters. Monovalent antibody fragment (Fab) which has a molecular weight of ± 50kDa has the largest

volume of distribution and can reach the extravascular part quickly, but Fab fragments are also very quickly eliminated mainly through renal excretion so that it has a short elimination half-life (4-24 hours). Divalent fragment Fab (F (ab′) 2 with molecular weight ± 120kDa) and IgG molecules (molecular weight ± 180kDa) are not eliminated by the kidney route (elimination through phagocytosis through the reticuloendothelial system) so that it has a longer half-life (2-4 days) (WHO TRS 1004, 2017).

Table 1.1 The difference in the specifications of pepsin B and pepsin A

Parameter	Pepsin B	Pepsin A
pH	pH 2	pH 2-3
Temperature	37°C – 42°C	52°C
pH inaktif	pH 6.5 – 8	N/A
Enzym Activity	1:10.000	1:10.000

3. Critical factor screening for igg fragmentation

The identification of critical factors is done by using a fishbone diagram and the Failure Mode and Effect Analysis (FMEA) method. The method is used as an initial selection of factors affecting the IgG fragmentation process quantitatively assessed from the titer value of anti diphtheria serum products. Fishbone diagrams use several categories used in the manufacturing industry, namely machinery; method; material; human; measurement; and the environment. Each of these categories is determined by factors that have a possible influence on the response. FMEA is one method of determining the risk generated by several factors towards the response in the form of a Risk Priority Number (RPN) score. The FMEA method uses parameters of severity (S), level of likelihood of occurrence of a risk (P) and level of detectability in capturing a risk (D) (Rathore, 2013). These parameters are ranked in five levels as shown in Figure IV.1. The RPN score is the result of multiplication of the severity, the likelihood of a risk event and the ability to detect several factors towards the risk. Risk classification is determined based on the risk severity matrix (Figure IV.1) which is a description of the RPN value obtained. The risk classification specified includes; negligeble (RPN score 1-12); acceptable (score 13-36); unacceptable (score 37-64); and intolerable (score 65-125). RPN scores that are in the unacceptable and intolerable matrices are analyzed using factorial fractional designs to determine the influence of these factors and the interactions between them.

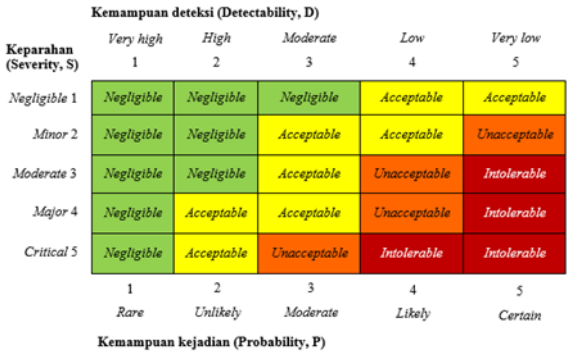


Figure 1.2 Risk severity matrix of antibody titer values IgG fragmentation by pepsin A (Ganzevoort W, 2017)

4. Material and method

This research uses anti diphtheria horse plasma which is carried out by the diafiltration process using 100kDa membrane filter. Centrifugation was carried out on the early plasma of the diphtheria horse to separate the sediment and the supernatant. The diafiltration process uses a 100kDa membrane filter with 0.85% NaCl buffer exchange and 0.25% phenol. The results of the separation were characterized by SDS PAGE to analyze the results of purification of immunoglobulins from diphtheria horse plasma, a BCA test was performed to determine the total protein content and determine the initial plasma titer value of the anti-diphtheria horse using the flocculation test method.

5. Statistical analysis

Pepsin fragmentation analysis was carried out using a factorial fractional ½ fraction design at the IgG fragmentation stage with the addition of pepsin. The response that will be observed from the factorial ½ fraction design is the titer of the flocculation test results. The antibody titer value is expressed as a response from several predetermined factors, namely the amount of pepsin; time; pH; and fragmentation temperature. Factorial design uses 3 midpoints with the aim to see the most significant factors that influence the process of fragmentation of anti-diphtheria horse IgG plasma by pepsin A. The number of experiments as many as 11 was obtained using Minitab software 17. The assessment of significant factors was seen from the results of ANOVA, pareto diagrams, main effects and response optimizer results of antibody titers among several of these factors.

6. Result and discussion

The use of fishbone diagrams as initial screening of the risk of several factors on the results of antibacterial titer of anti diphtheria products can be seen in Figure V.1. Several factors that are described to influence the response in the form of antibacterial titer values of anti diphtheria

serum products are categorized into 6 categories, namely humans; machine; measurement; ingredients; method and environment.

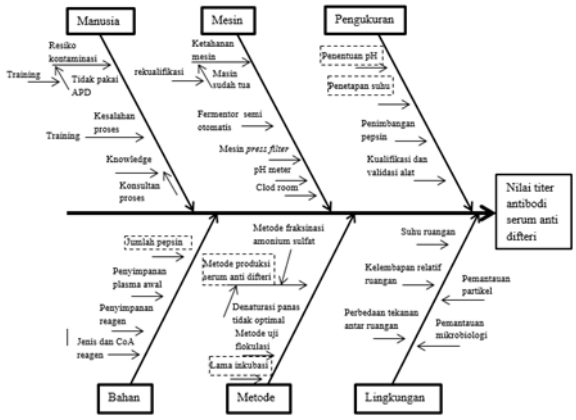


Figure 1.3 Fishbone diagram of factors and risks to anti diphtheria serum antibody titer values.

The fishbone diagram provides an overview of the causative factors that influence the value of antibody titers resulting from IgG fragmentation by the use of pepsin A. Fragmentation and serum antibody titer values are influenced by several factors including; amount of pepsin; pH; temperature; time; production methods (fractionation and heat denaturation) (Pope et al, 1951 and Harms et al, 1948). Other factors that influence the production process also contribute to the antibody titer value such as the risk of contamination resulting from the operator's working procedures in classy rooms, operator qualifications in carrying out the production process, the life of the fermenter and press filter produced in 1961 greatly contributed to the results product titers due to decreased ability of the machine in the product purification process; qualification and validation of tools used in the production process; initial storage of plasma and reagents used in the production process influences the initial diphtheria horse plasma titer so it needs to be considered and maintained according to its specifications; and some environmental conditions that must be determined according to the room qualifications used in the production process such as room temperature; relative humidity; pressure difference between spaces; microbiology; and the number of particles. These factors are grouped into six categories according to type to calculate the RPN score using the FMEA method.

The next risk management analysis uses the FMEA method to determine the RPN score of several factors that influence the titer value. RPN score calculations can be seen in Table V.3. Factors that are in the unacceptable and intolerable matrix include pH stopping the incubation process (RPN value 60); fragmentation temperature (RPN value 60); fragmentation process time (RPN value 125); the number of pepsin used in the production process (RPN 125); and the ammonium sulfate fractionation purification method (RPN value 60) can be seen in Figure V.2. Some of these factors are included in the classification of unacceptable and intolerable because optimization has never been done to get the optimal value of each factor. These factors become critical parameters because at the time of starting the production process, initial adjustments are made to the time, temperature and pH, so that these three factors

become parameters that must be considered during the production process because it determines the value of titer and yield. The amount of pepsin is known to affect how many antibody fragments are produced (Pope, 1939). Pepsin works as a protease enzyme that breaks down IgG into fragments F (ab) 2 and F (c) in anti diphtheria horse plasma. The purification method is classified as unacceptable because the fractionation process using ammonium sulfate directly impacts the damage to some antibodies in the anti-diphtheria horse plasma, so it is necessary to modify the purification method of anti diphtheria serum products.

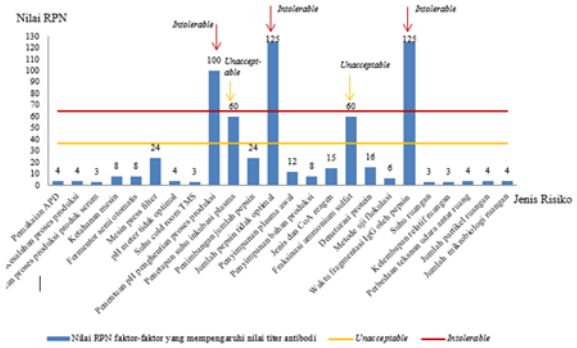


Figure 1.4 The RPN histogram of the factors that influence the antibody titer value

7. Analisa fragmentasi igg terhadap rancangan eksperimen faktorial fraksional ½ fraksi

The flocculation test results obtained through experiments and calculations using equation of each sample show different titer (Lf) and Kf values. Titer and KF values can be seen in Table 1.2. This difference in results is influenced by the different parameter values for each experiment, namely the amount of pepsin, temperature, pH and incubation time. Fractional factorial design of the ½ fraction uses replication 3 times at the mean value, from the flocculation test it was found that the results of the three obtained variations that were not much different from one experiment to another. This relatively small variation proves that the error rate of the experiments carried out is relatively small.

In the factorial fractional design ANOVA results showed that all four factors and their interactions had a significant influence on the value of anti diphtheria serum titers. The Pareto graph and the main effect plot also show that the four factors and their interactions have a significant effect on the resulting titer value. ANOVA shows a p-value <0.05 for all four factors and interactions that occur among several of these factors (Table 1.3).

These values indicate that the four factors have a significant influence on the value of titer as a parameter of the success of IgG fragmentation by pepsin A on anti-diphtheria serum products. The interaction between the two factors and the four factors shows a significant value (p-value <0.05), this indicates that the four factors interact strongly with each other in influencing the anti diphtherin antibody titer. The influence of these factors can also be seen from the Pareto diagram (Figure 1.5) which shows how much influence these factors have on responses. The

main effect plot (Figure 1.6) of each factor shows that the effect of some of these factors is significant to the response of anti diphtheria serum antibody titer values (Montgomery, 2013).

No	Sampel	Pepsin (mg/L)	pH	Suhu (°C)	Waktu (menit)	Nilai titer (Lf)
E0	Plasma Kuda Difteri	-	-	-	-	150
E1	Eksperimen 1	2000	8	22	30	120
E2	Eksperimen 2	326	8	52	30	0
E3	Eksperimen 3	2000	3,9	52	30	0
E4	Eksperimen 4	1163	5,95	37	135	380
E5	Eksperimen 5	326	3,9	22	30	0
E6	Eksperimen 6	1163	5,95	37	135	400
E7	Eksperimen 7	326	8	22	240	96
E8	Eksperimen 8	2000	3,9	22	240	1200
E9	Eksperimen 9	1163	5,95	37	135	400
E10	Eksperimen 10	2000	8	52	240	0
E11	Eksperimen 11	326	3,9	52	240	0

Table 1.2 Titer value of each experiment

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	8	1315093	164387	1232,90	0,001
Linear	4	731808	182952	1372,14	0,001
Pepsin	1	187272	187272	1404,54	0,001
pH	1	121032	121032	907,74	0,001
Suhu	1	250632	250632	1879,74	0,001
Waktu	1	172872	172872	1296,54	0,001
2-Way Interactions	3	481176	160392	1202,94	0,001
Pepsin*pH	1	172872	172872	1296,54	0,001
Pepsin*Suhu	1	187272	187272	1404,54	0,001
Pepsin*Waktu	1	121032	121032	907,74	0,001
4-Way Interactions	1	102109	102109	765,82	0,001
Pepsin*pH*Suhu*Waktu	1	102109	102109	765,82	0,001
Error	2	267	133		
Total	10				

Table 1.3 Results Factorial fractional ANOVA antibody titer values

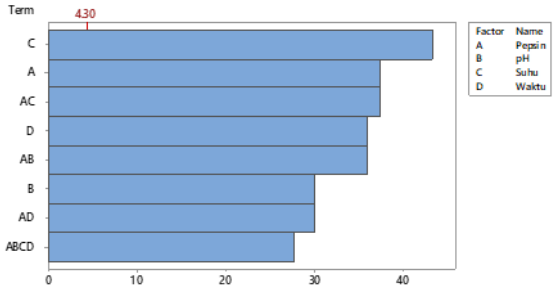


Figure 1.5 Pareto diagram of the influence of factors on the titer value (α = 0.05)

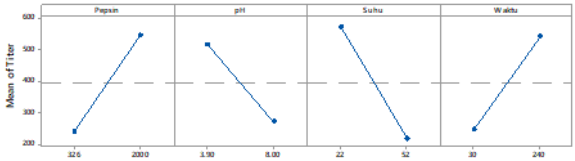
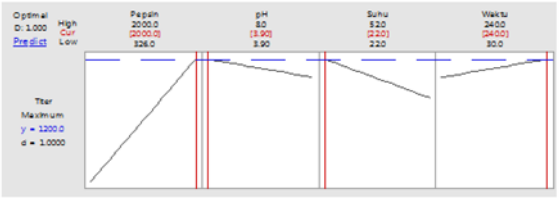


Figure 1.6 Main effect of factor influence on titer value

Fractional factorial is also used to predict the optimum response value of the factors that affect the response. By using regression analysis, obtained by the equation model for the response are as follows:

$$\begin{aligned}\text{Titer} = {} & 393.33 + 153.00\ \text{Pepsin} - 123.00\ \text{pH} - 177.00\ \text{Suhu} \\ & + 147.00\ \text{Waktu} - 147.00\ \text{Pepsin*pH} - 153.00\ \text{Pepsin*Suhu} \\ & + 123.00\ \text{Pepsin*Waktu} - 216.33\ \text{Pepsin*pH*Suhu*Waktu}\end{aligned}$$

The model equation can then be used to identify the optimal factor values, namely the number of pepsin 2000mg / L; pH 3.9; Temperature of 22° C; and 240 minutes (Figure 1.7). The value generated from the response model equation does not differ significantly from the factual titer value that has been done that is equal to 1160Lf. This value is still included in the value of confident interval (CI) which is 1150.3-1249.7 Lf / mL (Figure 1.7)



Variable	Setting
Pepsin	2000
pH	3.9
Suhu	22
Waktu	240

Response	Fit	SE Fit	95% CI	95% PI
Titer	1200.0	11.5	(1150.3, 1249.7)	(1129.7, 1270.3)

Figure 1.7 The maximum parameter value of anti diphtheria serum antibody titers

The optimal parameter values need further analysis. The success rate of pepsin A fragmentation process against IgG in addition to the resulting titer value> 350Lf / mL, also needs to be seen from the SDS-PAGE profile where there are no more IgG bands and Kf values which indicate the speed of reaction between antigens and antibodies in forming flocculation in flocculation tests. The IgG fragmentation process is then seen from the characterization of the experimental sample. Characterization included BCA test to see the total protein content and SDS-PAGE test to see the fragmentation profile with protein amount of 10 µg / well.

IgG fragmentation experiments by pepsin A can be seen from the results of the SDS PAGE, pepsin A with different treatments namely the amount of pepsin, temperature, pH and time, giving different profiles between treatments. Center points (ex 4, 6 and 9), which are the middle values of the experimental range, show profiles that are close to each other that there are no more IgG bands in the lane. It can be said that the optimization process at the center point parameter values (pepsin 2000mg / L, temperature 37 ° C, 135 minutes and pH 5.95) provides an optimum fragmentation profile. The optimum value parameter from this center point can also be seen from the titer value in table V.1 which shows that the antibody titer between the three center points is in the adjacent range (380-400 Lf).

No	Sampel	Pepsin (mg/L)	pH	Suhu (°C)	Waktu (menit)	Jumlah Protein (ug/mL)
E0	Plasma Kuda Difteri	-	-	-	-	13634,08
E1	Eksperimen 1	2000	8	22	30	10695,48
E2	Eksperimen 2	326	8	52	30	10954,16
E3	Eksperimen 3	2000	3,9	52	30	10344,04
E4	Eksperimen 4	1163	5,95	37	135	10412,04
E5	Eksperimen 5	326	3,9	22	30	11828,84
E6	Eksperimen 6	1163	5,95	37	135	9515,36
E7	Eksperimen 7	326	8	22	240	10488,8
E8	Eksperimen 8	2000	3,9	22	240	11999,08
E9	Eksperimen 9	1163	5,95	37	135	10108.8
E10	Eksperimen 10	2000	8	52	240	8515,62
E11	Eksperimen 11	326	3,9	52	240	11065

Table 1.4 Results of total protein levels from several experiments

Every protein in the blood plasma has a certain charge, the protein becomes uncharged at a certain pH and tends to interact and form aggregates. The pH at which the protein becomes uncharged is called pI (isoelectric point), IgG and F (ab) 2 horse plasma has pI above pH 6.5; albumin A has a pI of 4.9; and PI Fc 5.0-5.5 (Cheung et al, 2003). The pI value can be utilized in the next step of purifying antibody fragments by using the ion exchange chromatography method. Flocculation test is also influenced by pH, in general flocculation is not formed if the pH is outside the range 5-9 (Dennison, 2002). This can be seen in experiments 3,5 and 11, besides due to the influence of temperature, the pH of experiment 3.9 did not form flocculates which are visual parameters for the formation of interactions between antigens and antibodies. Experiment 8 showed an anomaly because the experiment used the largest amount of pepsin and time compared to Experiment 5, so the enzymes work were still active but the flocules formed were very small when compared to other experiments that showed titer values.

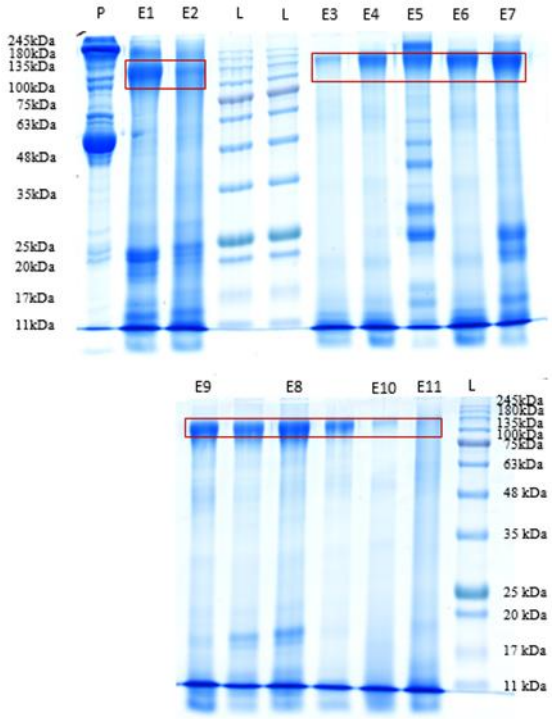


Figure 1.8 SDS PAGE electrophorogram in the design of the experiment. P = Initial plasma; E1-E11 = Experiments 1-11; L = Ladder protein.

Electroporegram profiles were analyzed using densitometry with Totallab software. The molecular intensity of the proteins separated during electrophoresis is marked by the height of the peak and the area of the area produced during densitometry. Densitometry results on electropheregrams produce peak heights that vary between experiments. This shows the differences in the profile of IgG fragmentation by pepsin enzyme due to different treatments between experiments. The results of densitometry analysis of the middle value can be seen in Figure 1.9. In experiments 4,6 and 9 no peaks formed on the band above> 135kDa, this is because in all three experiments IgG was fragmented into smaller fragments, especially F(ab)2 by the protease enzyme, namely pepsin A. Experimental densitogram 5 (Figure 1.9) shows that there is still a peak in the IgG band which shows that the fragmentation process is not optimal, this is because in experiment 5 using the smallest parameters of the range of factor levels in Table 1.5. Lf, Kf and SDS-PAGE profile data for some of the experimental samples shows that the parameter values at the center point provide optimal values in the IgG fragmentation process by pepsin A (Table 1.5).

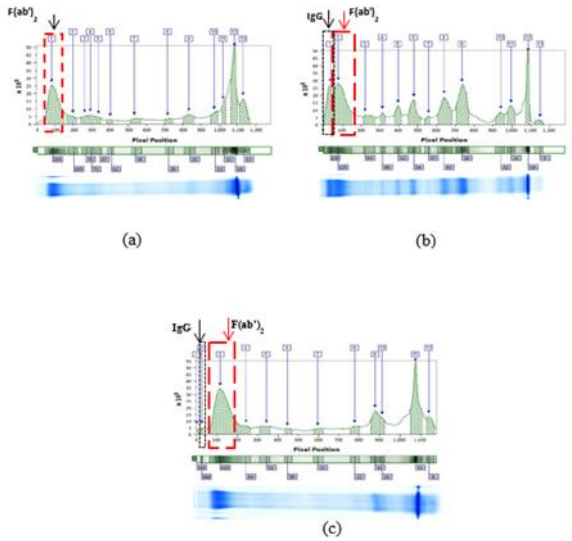


Figure 1.9 Comparison of densitogram results from SDS PAGE. (a): experiment 6 (middle value); (b): experiment 5; (c): experiment 8

No	Sampel	Pepsin (mg/L)	pH	Suhu (°C)	Waktu (menit)	Jumlah Protein (ng/mL)	Luas Area		Nilai titer (Lf)	Kf (Menit)
							F(ab')2	IgG		
E0	Plasma Kuda Difteri	-	-	-	-	13634,08	-	10584	150	55
E1	Eksperimen 1	2000	8	22	30	10695,48	8232	3528	120	120
E2	Eksperimen 2	326	8	52	30	10954,16	6384	0	0	-
E3	Eksperimen 3	2000	3,9	52	30	10344,04	10584	0	0	-
E4	Eksperimen 4	1163	5,95	37	135	10412,04	16128	0	380	3
E5	Eksperimen 5	326	3,9	22	30	11828,84	12348	4914	0	-
E6	Eksperimen 6	1163	5,95	37	135	9515,36	18144	0	400	3
E7	Eksperimen 7	326	8	22	240	10488,8	20664	4704	96	120
E8	Eksperimen 8	2000	3,9	22	240	11999,08	14750	1408	1200	240
E9	Eksperimen 9	1163	5,95	37	135	10108.8	18048	0	400	3
E10	Eksperimen 10	2000	8	52	240	8515,62	6254	0	0	-
E11	Eksperimen 11	326	3,9	52	240	11065	6498	0	0	-

Table 1.5 The results of the characterization of IgG fragmentation by pepsin A

8. Conclusion

Pepsin A can be used as a substitute for pepsin B in the anti-diphtheria serum production process as one of the commitments to deliver halal products. Factors that have a significant influence on the antibody titer value of anti-diphtheria serum products are the amount of pepsin, time, temperature, and pH of incubation termination. The optimum value (highest titer, shortest Kf time and no IgG band) IgG fragmentation process by pepsin A is the amount of pepsin 1163 mg / L, temperature 37ºC, time 135 minutes, and pH 5.95. Using the optimum parameters of pepsin A can increase the antibody titer in the IgG fragmentation stage to 2.67 times from the previous method using pepsin B.

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