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Experimental Design and Statistical Analysis of Protein Buffer to Purify Hydrolases from the Skim Latex of Hevea Brasiliensis.

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ABSTRACT
A lipid acyl hydrolase (LAH) enzyme has been purified from skim latex serum, a waste product of Hevea brasiliensis, using DEAE Sepharose™ Fast Flow ion exchange chromatography. The ~45kDa active LAH was expressed by spectrophotometric study and SDS-PAGE. One-Factor-at-A-Time (OFAT) method and Central Composite Design (CCD) in Response Surface Methodology (RSM) were employed to optimize the protein buffer in order to improve purification. Design Expert® 6.0.8. software was applied using a 2-factor, 5-level to find the optimum pH and concentration of protein buffer to yield the highest lipase acyl hydrolase. In this study, it is concluded that purification at pH 8.0, 20 mM Tris-HCl buffer on ion exchange column chromatography results in the production of the highest total LAH activity (61.152 U). The study has good significance in that it is an initial attempt to purify LAH enzyme, which has known as antifungal agent, from skim latex.

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INTRODUCTION

Skim latex, a byproduct of the highly ammoniated latex concentrate processing technique, is not easily recycled into new rubber products. It is treated in the effluent treatment pond before being released into the stream. Malaysia is one of the biggest producers of natural rubber in the world. However, Malaysia is still repressed with the striving cost of its waste management and environmental problems despite the higher income of natural rubber production. Therefore, finding new uses for this rubber waste is a major concern.

Hydrolases extracted from *Hevea brasiliensis* provides very useful industrial enzyme. Hydrolases play important role in industries such as detergent, food, oil processing, fine chemicals and agrochemicals industries. Skim latex contains a dry rubber content of only 3 to 7 %. The aqueous serum that yields from membrane separation of skim latex comprised of rich source of nitrogen, carbohydrates, proteins, lipids and trace metals. C-serum extracted from fresh *Hevea brasiliensis* latex has been identified to contain lipid acyl hydrolase (LAH), a subtype of lipase which may play an important role in the defense mechanism against plant parasites [1, 2].

Patatin-like-protein from latex of *H. brasiliensis* which shows almost 60 % homology in its amino acid sequence to the patatin from potato tuber also exhibits a nonspecific lipid acyl hydrolase activity [3] that catalyze the cleavage of fatty acids from membrane lipids including phospholipids, lysophospholipids, galactolipids, and mono and diacylglycerols. This property of LAH activity is found to inhibit rubber biosynthesis [3] and also a defense-related protein [4].

In this work, One-Factor-at-A-Time (OFAT) method and Central Composite Design (CCD) under Response Surface Methodology (RSM) were used to improve the purification of LAH enzyme from the skim latex serum of *Hevea brasiliensis*. According to Myers *et al* [5] Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes.

MATERIALS AND METHODS

Sample Collection and Preparation:

The skim latex (effluent) used in this research work was collected from Mardec Industrial Latex Sdn. Bhd. in Tapah, Perak. The preparation of the sample, before being introduced into column chromatography steps, involved coagulation, centrifugation, precipitation, and dialysis. All processes were performed at 4 °C to preserve the stability of the proteins.

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Lipid Acyl Hydrolase Extraction:

After collection, the skim latex (approx. 17 L) was immediately coagulated by adding acetic acid glacial to reduce the pH from 10 to 5. Subsequently, the acidified sample was centrifuged at 10,000 x g and 4 °C for 30 minutes in an Avanti® J-E high-speed centrifuge. This helped to remove the coagulated rubber (skim rubber). The supernatant (skim latex serum) obtained after centrifugation was pooled (approx. 14.5 L, ~85 %). 4M of solid $(NH_4)_2SO_4$ was added to the pooled sample to concentrate the skim latex protein and it was left overnight under slow stirring at 4 °C. The precipitating 100 % saturation was collected by centrifugation at 5,000 x g for 15 min. The supernatant was discarded and the residue was dissolved in minimal phosphate buffered saline at pH 7.0 and dialyzed using 10,000 (MWCO) of Pierce Snake Skin™ Pleated Dialysis Tubing against 0.1 M Tris HCl buffer at pH 7.0 overnight.

Biochemical Analyses:

Several biochemical analyses were conducted to measure the total protein concentration through the dye-binding method of Bradford's assay [6], with bovine serum albumin (BSA) as the standard; protein purity test by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) [7] and several other enzyme activity assays.

Lipid Acyl Hydrolase Activity Assay:

Lipid acyl hydrolase (LAH) activity was estimated according to the method given by Yusof *et al* [3]. *p*-nitrophenyl palmitate stock solution was prepared by dissolving, with stirring, 28 mg in 100 ml of 1% (w/v) Triton 100-X® plus 1.7 ml of 1% sodium dodecyl sulphate in a boiling water-bath. The incubation mixture contains 1.0 ml of 0.1 M Tris-HCl, pH 8.2, 0.5 ml purified sample, and 1.0 ml *p*-nitrophenyl palmitate stock solution. The incubation mixture was incubated in a water-bath for 30 min at 37°C and the reaction was stopped by the addition of 1.0 ml of 1 M NaOH. The product of the incubation, *p*-nitrophenol, was measured by reading the absorbance at 410 nm. One unit of enzyme activity is defined as the amount of enzyme releasing one µmole *p*-nitrophenol/ml in one minute under assay condition. All tests were carried out in triplicate.

The calculation of the total LAH activity was based on the equation:

$$\text{Total LAH activity (Unit)} = \frac{\text{Total reaction volume (ml)}}{\text{Enzyme volume used (ml)}} \times \frac{\Delta \text{ Abs (410 nm)}}{\text{Unit time (min)}} \quad (1)$$

where 1 Unit = 1 µmole / min.

DEAE-Sepharose™ Fast Flow Ion Exchange Column Chromatography:

The enzyme solution was loaded onto a column (1.5 cm diameter, 15.0 cm height) of DEAE–Sepharose™ (Amersham® GE-Healthcare) preequilibrated with protein buffer. The column was washed with 100 ml of the equilibration buffer. The proteins bound to the anion exchange gel were eluted with linear gradient of 1 M NaCl. A 1ml/min flow rate was applied. Fractions (approximately 1.0 ml of each) that exhibited LAH activity in the gradient region of the NaCl concentration were collected. The purification was conducted using AKTA purifier™ (GE Healthcare Bio-Sciences, Sweden).

Experimental Design and Optimization:

The OFAT method was deployed for screening experiments to determine the possible optimum levels of the parameters which may give optimal LAH purity. In OFAT, two set of experiments, with two parameters were conducted. The first, varying the pH of protein buffer while keeping the concentration constant followed by varying the concentration of protein buffer while keeping the pH constant.

In the optimization step, the protein buffer concentrations and pH were varied following the results from OFAT and eluted with linear gradient of 1M NaCl. A flow rate of 1.0 ml/min was applied. To improve the purification of LAH, statistical optimization was conducted by employing Design of Experiment (DOE) method in DESIGN-EXPERT (Version 6.0.8; STAT-EASE Inc., Minneapolis, USA) computer software. The developed regression model was evaluated by analyzing the values of regression coefficient, R^2; analysis of variance (ANOVA; and *p*- and *F*- values). Experimental design was carried out according to Response Surface Methodology (RSM) and using the Central Composite Design (CCD).

RESULTS AND DISCUSSION

Hydrolases Extraction:

When the skim latex rubber was coagulated with acid, the coagulated rubber was about three quarters of the

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total volume. After centrifugation, this coagulated rubber was less than a quarter (~15 %) of the total volume; the rest was contained of the serum. This result agreed with the claim made by Blackley [8] in his book that skim latex contained around 2.5-10 % of rubber while most of it is was contained of the aqueous serum. After centrifugation, a light yellow supernatant was obtained, which became dark brown after precipitation with $(NH_4)_2SO_4$ and dissolved in minimal PBS buffer until it became a clear solution. This solution was dialyzed overnight. However, the $(NH_4)_2SO_4$ present was only partly removed by this dialysis. The next step in the separation procedure, DEAE Sepharose™ Fast Flow ion exchange, removed the remaining $(NH_4)_2SO_4$.

Purification of Lipid Acyl Hydrolase:

IEC was incorporated in this work in order to recover the target LAH bound to the anion exchange gel. This chromatography step has the ability to be used in the capture step due to its high dynamic binding capacity of proteins, unlimited sample loading, and most importantly it is meant for concentration. The previously optimized protein buffer [1] was utilized as the protein buffer for protein attachment to the resin. These attached proteins were eluted using the elution buffer of 1 M NaCl in the same protein buffer.

In this study, DEAE-Sepharose™ (pKa ~11) was used to capture the target LAH. The chromatogram output for this step is shown in Figure 1.

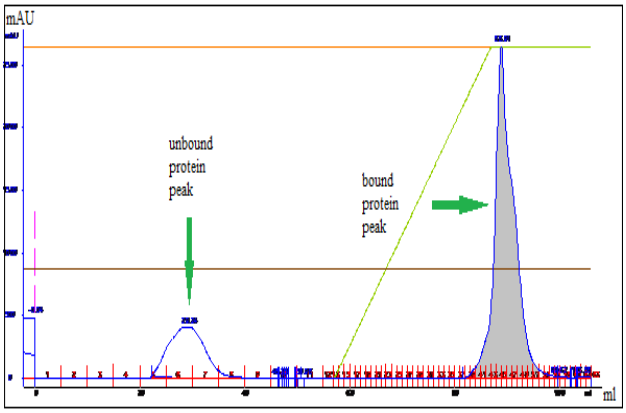

Fig. 1: Example of FPLC chromatogram output from experimental design (Run 4) of DEAE–Sepharose™ IEC for pH 9 and 60 mM Tris-HCl buffer with packed column of 1.5 cm diameter, 15.0 cm height.

In the chromatogram, the big shaded peak represents the bound proteins to the DEAE Sepharose™ and from which the LAH was analyzed through SDS-PAGE while the non-shaded area was the wash-out proteins that unbound to the column resin. SDS-PAGE analysis (Figure 2) revealed the composition of these bound proteins.

All fractions under this shaded peak (bound proteins) were submitted for total LAH activity through LAH activity assay which was taken for statistical analysis.

Biochemical Analysis:

SDS-PAGE Analysis:

The presence of the target protein was detected through SDS-PAGE analysis. SDS-PAGE analysis in Figure 2 revealed the composition of the bound proteins which were represented in the shaded peak in the FPLC chromatogram (Figure 1).

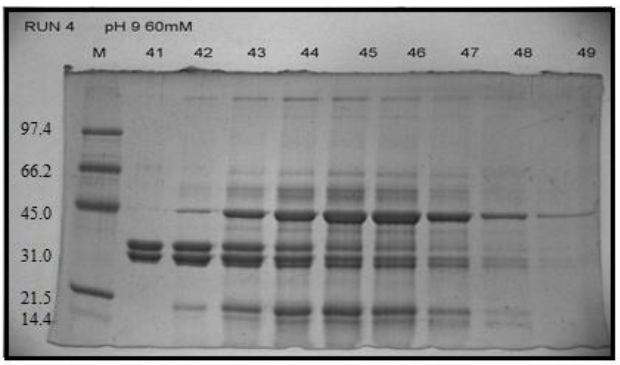

Fig. 2: Analysis of protein separation from experimental design (Run 4) of DEAE–Sepharose™ IEC. The separated protein was electrophoresed on 12% (w/v) of SDS–PAGE for pH 9 and 60 mM Tris-HCl buffer. Lane 1, marker; lane 2 to 10, collected fractions (bound proteins).

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According to the results of the FPLC chromatogram (Figure 1), elution volume between fractions under the bound protein peak were collected and run on SDS-PAGE gel to visualize the separation of proteins in each fraction. From the observation, fractions between 43 and 48 showed higher band intensity at 45 kDa which indicates the presence of LAH enzyme at this fraction compared to the other fractions. It is suggested that these fractions be pooled before being processed in the next chromatography step.

In this study, work done by Yusof *et al* [3] and Abdullah *et al* [1] were referred since the nature of the raw material used was similar. Similar results were obtained by Yusof *et al* [3] and Abdullah *et al* [1] in which patatin-like-protein that exhibits a nonspecific lipid acyl hydrolase activity was identified in the protein profile using Sephadex G-150 gel filtration and DEAE Sepharose™ ion exchange resin, respectively. Yusof *et al* [3] discovered a proteinaceous inhibitor of rubber biosynthesis that has a molecular weight of 43.7 kDa and demonstrates lipid acyl hydrolase activity.

One-factor-at-a-time (OFAT) Analysis:

OFAT is recommended for screening experiments, in which the objective is to reduce the list of candidate variables to a small number so that subsequent experiments can be more efficient before further analysis using statistical method [9].

In the OFAT study, two sets of experiments were conducted. The first, varying the pH of protein buffer between pH 6-10 while keeping the concentration constant at 50 mM. The effect of the variation of pH for this step is shown in Figure 3.

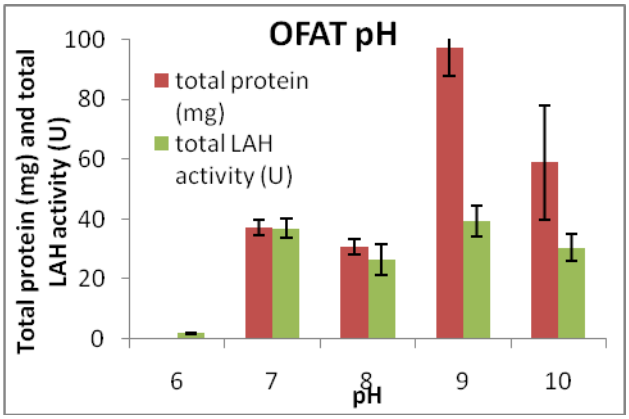

Fig. 3: Effect of varied pH on total protein (mg) and total LAH activity (U) using OFAT method.

Based on the result in Figure 3, it was concluded that, pH 9 results in the production of the highest total LAH activity (39.49 U) and total protein (97.34 mg). Following this result, the second OFAT study was done by varying the concentration of protein buffer between 20-100 mM while keeping the pH constant at pH 9.

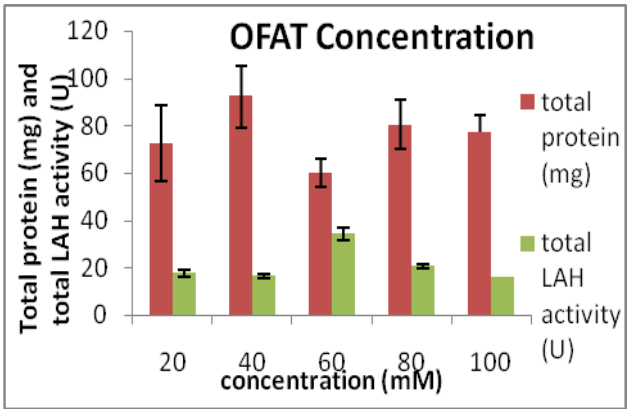

Fig. 4: Effect of different concentrations on total protein (mg) and total LAH activity (U) using OFAT method.

Based on the result in Figure 4, it is observed that the protein buffer concentration of 60 mM results in the production of the highest total LAH activity (34.27 U) while concentration of 40 mM results in the production of the highest total protein (92.38 mg).

Referring on these OFAT results, the protein buffer concentrations and pH were selected between pH 8-10

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and 20-100 mM in the optimization step. A design of experiment using Design Expert® 6.0.8 was applied using a 2-factor, 5-level.

Response Surface Methodology:
Regression Model:

A regression analysis was conducted on the experimental data points obtained to understand the relationship among the pH and concentrations (mM) of protein buffer towards the total LAH activity (U) recovered on DEAE Sepharose Fast Flow ion exchange matrix. Table 1 summarizes the results from the analysis of this design of experiments.

Table 1: Experimental design using CCD of two independent variables with their actual values and three centre points showing the experimental and predicted response.

Run	Std.	Fact. A	Fact. B	Actual	Predicted
		pH	Concen-tration (mM)	Total LAH Activity (U)	Total LAH Activity (U)
1	5	8.5	60	6.94	12.18
2	10	9.0	60	9.31	10.97
3	8	9.0	80	18.37	16.98
4	9	9.0	60	11.28	10.97
5	3	8.0	100	17.59	16.66
6	11	9.0	60	12.64	10.97
7	6	9.5	60	5.52	0.46
8	7	9.0	40	20.41	21.97
9	2	10.0	20	2.29	3.20
10	4	10.0	100	24.42	26.07
11	1	8.0	20	61.15	59.49

The statistical analysis of the data obtained ($p<0.05$, $R^2=0.97$) permitted us to build the response surface (Figure 5) and validate the empirical model presented in Equation 2, for total LAH activity in terms of pH and concentration protein buffer

$$Y = 10.97 - 11.72 * A - 4.99 * B - 18.62 * A^2 + 34.00 * B^2 + 16.42 * A * B \quad (2)$$

where Y is total LAH activity, A and B is pH and concentrations of protein buffer respectively.

The equation was used to predict the output (dependent variables) with planned parameters (independent variables) and compared with observed values.

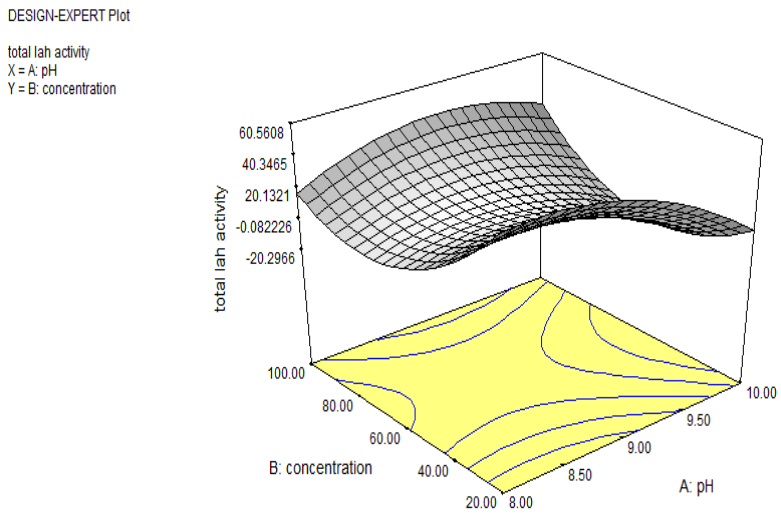

Fig. 5: 3D response surface plot for purification of LAH by IEC, ANOVA analysis-F value= 35.73, p=0.0006, R^2= 0.97

Figure 5: depicted the response surface described by the model equation to estimate the total LAH activity over independent variables; pH and buffer concentration. According to the model graph in Figure 5, it can be interpreted that the maximum response total LAH activity of 60.56 U is obtained by conducting IEC at pH 8 and the buffer concentration at 20 mM. A similar work by Yusof *et al* [3] did purify the patatin-like-protein (exhibits lipid acyl hydrolase activity) from C-serum of fresh latex by using 50 mM Tris-HCl buffer, pH 8 plus 5 mM 2-mercaptoethanol as the protein buffer. Such works were done by Abdullah *et al* [1] in which they use 17 mM

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Tris-HCl buffer, pH 8.7. Although the protein buffer conditions in this work were different with other referred work, it can be deducted that lipid acyl hydrolase (LAH) from *Hevea brasiliensis* has an optimum pH at alkaline condition. Tris-HCl was used as the protein buffer in this work. It was selected due to its cheaply purchased, easy to be made, and most importantly, it is a common buffer for biological sciences.

Scatterplots of the predicted versus observed values of the dependent variable in Figure 6 illustrates a linear trend model that gave the best average fit to all the data. Scatterplot showed that most of the obtained data were scattered at the centered data towards the line Therefore, the scatterplot showed that the predictions were good in a general sense.

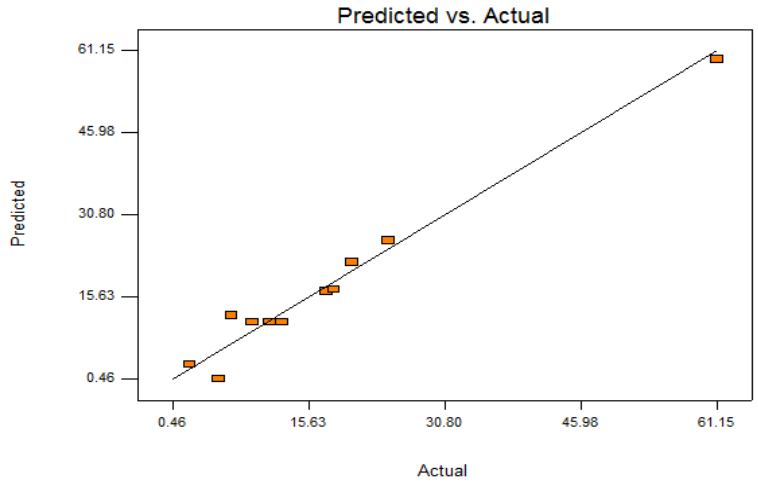

Fig. 6: Plot showing predicted versus actual values.

Analysis of variance (ANOVA) was done and the results are given in Table 2.

Table 2: Anova value of design of experiment

F-value	(Prob > *F*)	Determination coefficient, R^2
35.73	< 0.0001	0.97

The Fisher *F*-test value signifies how greater the mean square of the regressed model as compared to mean square of the residuals (errors). The significance of *F*-value or sometimes referred to as *p*-value is the probability to get larger *F*-value by chance alone. Therefore, *F*-value for the model implies that the model was significant.

The lack-of-fit of the model was checked by the determination coefficient (R^2) which is a measure of the amount of reduction in the variability of Y obtained by using the parameters; coefficients in the models. Based on Table 3, the value of determination coefficient (R^2 = 0.97) indicates that only 3.00 % of the variations are not explained by its regression equation.

Effects of Varied pH and Concentration towards LAH Activity:

For LAH activity, the varied pH and concentration of the protein buffer were analyzed towards the total LAH activity which was measured through absorbance at 410 nm.

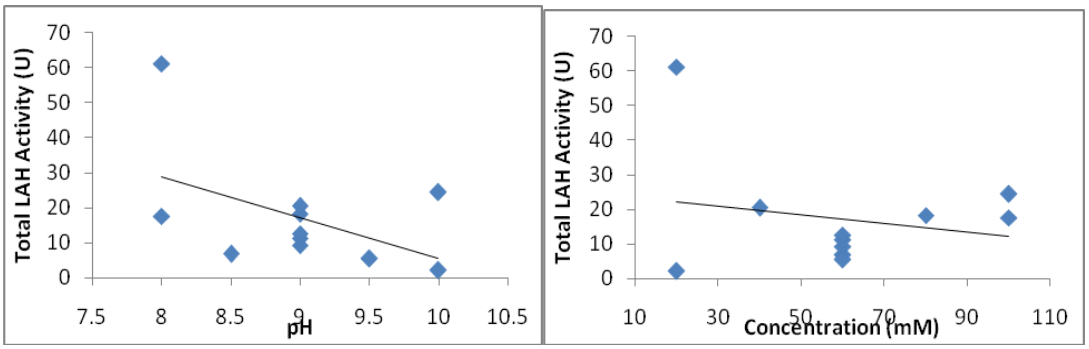

Fig. 7: Correlations between pH and concentration towards the total LAH activity (a) Above: pH (b) Below:

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Concentration:

The analysis recapitulates that a condition of low pH and low concentration of protein buffer was preferred to recover a maximum total protein in ion exchange chromatography step. The contradiction of correlations between these two factors is shown in Figure 7. Changes in pH may not only affect the shape of an enzyme but it may also change the shape or charge properties of the substrate so that, either the substrate cannot bind to the active site or it cannot undergo catalysis.

In this work, the p*I* of LAH protein derived from skim latex is ~5 while the pKa of DEAE Sepharose™ is ~11. By referring to Figure 8, above pH 5, the same protein has a negative net charge and could be bound to an ion exchanger (e.g.: DEAE or QAE). A salt (NaCl) is selected as the source of counter ions in the mobile phase and elution occurs as the salt strength is increased to a higher concentration than the target's binding salt condition.

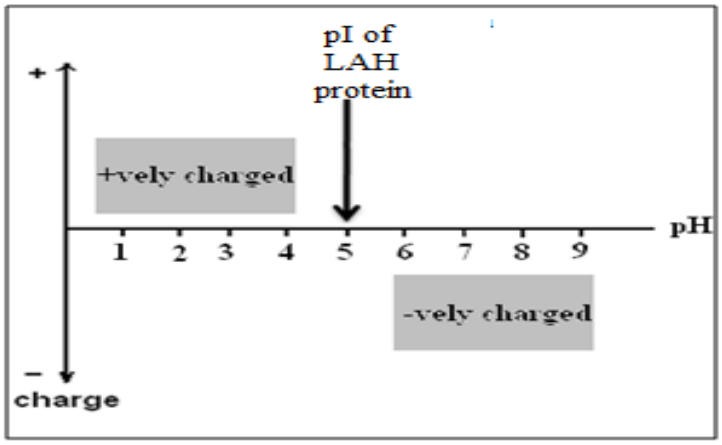

Fig. 8: Effect of pH on protein's charge.

Figure 9 illustrates the pKa of DEAE Sepharose™ resin is 11. The decreased pH of protein buffer below this pKa value was used to equilibrate the resin and thus would make it positively charged while increased pH would make the resin negatively charged. That is why the pH range for buffer (pH 8-10) was chosen below this pKa .

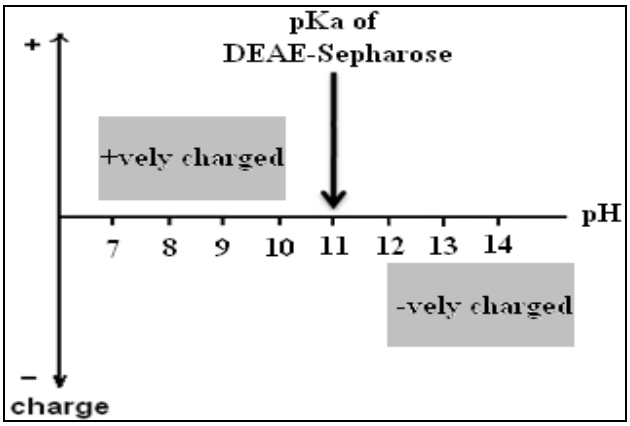

Fig. 9: Effect of pH on DEAE Sepharose.™.

In the column, the lipid acyl hydrolase behaves as an acid protein (or negatively charged ion) while the protein buffer behaves as a salt (protonated form). According to Abdullah *et al* [1] an increase in the concentration of protein buffer will result in increase ionic strength which then increase the buffer capacity to maintain the pH of the buffer at nearly constant. This is because concentration of buffer played a big role in resisting the changes in pH of buffer while pH played the important task give charge on proteins.

Conclusions:

In this study, the optimum pH and concentration of protein buffer towards the maximum recovery of total LAH activity (U) were investigated. It is concluded that the pH 8 and concentration of 20 mM results in the production of the highest total LAH activity (61.152 U).

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