EGYPTIAN JOURNAL OF BOTANY (EJBO)

Print ISSN: 0375-9237
Online ISSN: 2357-0350

Improving the chromatographic purification protocol for recombinant hirudin produced by *Hansenula polymorpha*

Khaled S. Ewies, Jan Rohde, Ahmed Ibrahim, Ashraf El-Baz

PUBLISHED BY
THE EGYPTIAN
BOTANICAL SOCIETY

Improving the chromatographic purification protocol for recombinant hirudin produced by *Hansenula polymorpha*

Khaled S. Ewies[1], Jan Rohde[1], Ahmed Ibrahim[2], Ashraf El-Baz[2]
[1]*Rhein-Minapharm Biogenetics for biopharmaceutical production (RM-BG),44916, Egypt*
[2]*Industrial Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt*

REVIEW ARTICLE

This study modified a factory-integrated recombinant Hirudin downstream process of successive chromatographic purification steps to improve their yield. The effect of different ion exchanges chromatographic resins for protein capture and altering process conditions was investigated on the yield of the process. The obtained results showed that tuning the pH of the protein solution while keeping the same conventional chromatographic matrix could improve the capture step yield by 5.7% compared to the conventional yield. Further, the particle sizes and internal surface structures of the chromatographic matrices significantly impacted the yield of the intermediate and polishing downstream steps by 21.2% and 24%, respectively. Accordingly, these minor modifications significantly impacted the yield of the production process by a 58% increase compared to the conventional process without affecting any of the product's critical quality attributes. This change has a significant economic impact, saving 35% of the production cost.

Keywords: Chromatography; Fermentation; Improvement; Ion Exchange; Reversed phase

ARTICLE HISTORY
Submitted: May 11, 2024
Accepted: October 01, 2024

CORRESPONDANCE TO
Khaled S. Ewies,
Rhein-Minapharm Biogenetics for Biopharmaceutical Production (RM-BG), Egypt
Email: khaledewies@yahoo.com
DOI: 10.21608/ejbo.2024.288599.2842

EIDTED BY: R. Gaafar

INTRODUCTION

Hirudin is an acidic polypeptide with anti-coagulant activity, secreted by the salivary gland of the medicinal leech, *Hirudo medicinalis*. Unlike heparin, it has a specific and potent anti-thrombin activity. Hirudin is a single-chain polypeptide with 65 amino acids and a molecular weight of 7.0 kDa (Mo et al., 2009). The natural hirudin molecule is characterized by its high content of acidic amino acids, sulfated tyrosine 63, and three disulfide bridges (Markwardt 1992). Hirudin activity is measured in anti-thrombin units (ATUs), reflecting the amount of hirudin complexes with one IU of thrombin complexes and one thrombin IU (Markwardt 1992).

Historically hirudin was extracted from medicinal leeches for therapeutic approaches. However, yields were very low (maximum 20 micrograms per leach) (Lee 1997). There were many attempts to use genetically engineered microorganisms, including bacteria (*Escherichia coli*) and yeast (*Saccharomyces cerevisiae*), to produce a higher amount of hirudin for therapeutic use (Lee 1997). Minapharm Pharmaceuticals uses the yeast *Hansenula polymorpha* for the recombinant expression of hirudin. Recombinant hirudin has the same configuration as the natural hirudin obtained from leeches, with the absence of a sulfate group on Tyrosine-63. The lack of this sulfate group increased the inhibition coefficient ki value of the Hirudin molecule tenfold (Markwardt 1992). The lower the Ki for a specific drug at a specific receptor, the stronger its affinity for that receptor. *H. polymorpha* is a methylotrophic yeast that has been expanded as an expression system to produce heterogeneous proteins. It is a facultative yeast that uses glycerol as the sole energy and carbon source.

The need for highly productive bioprocesses justifies optimizing fermentation strategies to maximize yields and/or productivity. Unlike exponential substrate feeding intervals, specific protein production rates significantly increased during short carbon-starvation periods (Jaishankar and Srivastava 2017). As a result, using carbon-free periods as an innovative operational strategy was proposed, resulting in up to 50% increases in yields and total production (Garcia-Ortega et al. 2016). Liquid chromatography utilizes intra-molecular differences between molecules, such as charge, hydrophobicity, and molecular size, to purify the target product (Cummins, Dowling, and O'Connor 2011).

Ion exchange chromatography (IEC) was explicitly used to separate ionizable and charged molecules such as polypeptides and proteins (Cummins, Dowling, and O'Connor 2011). Many factors may affect protein adsorption in ion exchange chromatography, such as operation parameters (pH and conductivity) and physical properties of the matrix (Urmann et al. 2010). The slight change in the pH values could affect the protein conformation (S. G. Kim, Bae, and Kim 2009). Knowing the isoelectric

point (pI) of the target protein could help initiate a purification scheme that relies on changing the protein charge with different pH values. Dynamic binding capacity decreases with increasing ionic strength and approaching the pH of the solution near the isoelectric point (Ming-Ju Chen, Kreuter 1996). Concerning the physical properties of the IEC matrix, tentacle resin is a promising technology that allows ionic groups to be more accessible to the highly flexible tentacles, avoiding steric hindrance with increasing binding (Supplementary 1A) more than other conventional chromatographic matrices (Supplementary 1B).

Reversed-phase chromatography is widely used to achieve better resolution of closely related compounds. Increasing the efficiency (N) could continuously improve the resolution to be directly proportional to the square root of N (Fornstedt, Forssén, and Westerlund 2015). According to Van Deemter curves, upon using the optimum flow rate, decreasing particle size will reduce the plate height and increase both efficiency (N) and resolution (R) (Everley and Croley 2008). The resolution is inverse to the particle size square root–(Mazzeo et al. 2005). Maximum product recovery could be achieved using a stationary phase with a smaller particle diameter (Peskin and Rudge 1992). It was observed in batch chromatography that using a shorter column with a smaller particle diameter is better than a long column with a larger particle diameter (Samuelsson et al. 2018).

Liquid reversed-phase chromatography separation of proteins could also be improved by tuning gradient slope, pH, and organic modifier concentration and using a physically competent stationary phase (Lavine, Ritter, and Peterson 2002). Ion pairing agents such as trifluoroacetic acid react with ionized protein groups, which could be either the protonated amino groups or the ionized free carboxylic groups (Rotkaja, Golushko, and Mekss 2014). The more promising method for better resolution is a selectivity-related challenge through changing the stationary phase. One more forward step is to use a spherical uniform monosized matrix (Supplementary 1C)to decrease the irregular paths inside the column (Fornstedt, Forssén, and Westerlund 2015) while using the conventional chromatographic matrices (Supplementary 1B).

In this work, we aim to improve the conventional downstream process outcome without affecting the product quality using different beads particle sizes, matrices (after measuring the plate number N), binding techniques, and process parameters like pH and conductivity that may improve the binding capacity.

EXPERIMENTAL

Materials

Trifluoroacetic acid (High-Performance Liquid Chromatography grade), citrate buffer, ortho-phosphoric acid, sodium chloride, acetone (HPLC grade), 2-propanol, and ammonium bicarbonate were purchased from Sigma-Aldrich. XK 16/20 columns and Source 30 rpc were purchased from Cytiva. Toyopearl SP 550C resin came from Tosoh Bioscience. Eshmuno S resin was purchased from Merck. Amberchrom CG 300M and Amberchrom CG 300S resins were delivered by Dow.

Protein expression in the fermentation phase

H. polymorpha was propagated in a growth medium of 1% glycerol as a carbon source at 30° C, pH of 3.8 for 48 hours. Then, the protein expression phase was started to produce the target Hirudin molecule for the next 48 hrs. The cultivation was carried out as a fed-batch culture with a pO_2-controlled mode wherein glycerol was intermittently added to maintain its concentration at 0.05-0.4% (Weydemann et al. 1995). This trial was carried out on a large-scale fermenter of 300L with different additions of shots of glycerol during the protein expression phase.

Hirudin purification steps

Buffers used in the downstream process (DSP) were prepared and filtered before usage using 0.2-µm sterilizing filters. The downstream process of r-HIR consists of three main chromatographic steps. These steps were downscaled to a lab-scale for ease of parameter modification (Table 1). The chromatographic resins used in this work are listed with their properties in Table 2.

Capture step (IEC)

In this step, Toyopearl SP 550C and Eshmuno S resins were packed in XK 16/20 columns (Cytiva) with a bed height of 10 cm. The harvested cell-free culture supernatant was loaded onto the capture cation exchange chromatography column pre-equilibrated with a citrate buffer of low ionic strength (1.5 mS/cm) at a 73.7 cm/hr flow rate (Table 1). Hirudin was eluted

Table 1. Columns properties of both the conventional (large scale) and the lab scale processes for Hirudin processes.

Parameters	Large-scale (Conventional process)	Lab scale (Experimental changes)
Operating System	MPLC Bioprocess	AKTA explorer 100
Capture (Cation Exchange Chromatography)		
Column Type	Bioprocess 450 x 600	XK 16/20
Matrix	Toyppearl SP 550C	Toyppearl SP 550C
		Eshmuno S
Bed Height	19 cm	10 cm
Linear flow rate	140 cm/hr	73.7 cm/hr
Residence time	8.1 min	8.1 min
Intermediate and Polishing (RPC)		
Column Type (ID x H)	Bioprocess 400 x 800	XK 16/20
Matrix	Amberchrom CG 300M	Amberchrom CG 300M
		Amberchrom CG 300S
		Source 30rpc
Bed Height	35 cm	10 cm
Linear flow rate (acid/alkaline)	150 cm/hr 168 cm/hr	43 cm/hr 48 cm/hr
Residence time (acid/alkaline)	14 min 12.5 min	14 min 12.5 min

Table 2. Specifications of the used chromatographic resins

Resin	Type	matrix	Particle size (µm)
Toyopearl SP 550C	Cation exchange resin	Methacrylic polymer	100
Eshmuno S		Hydrophilic polyvinyl ether	85
Amberchrom CG 300M	Reversed-phase resin	Polystyrene divinyl benzene polymer	75
Amberchrom CG 300S			35
Source 30rpc			30

isocratically with a citrate buffer of high ionic strength of 11 mS/cm (Figure 1). The pH of the culture supernatant was adjusted with ortho-phosphoric acid to 2.0 while keeping conductivity at 5.0±0.5 mS/cm (Table 3) and loaded on Toyopearl SP resin. (Table 3). The conductivity of the culture supernatant was adjusted to 1.5 mS/cm by adding water for injection (WFI). The adjusted sample is loaded on both Toyopearl and Eshmuno S resins.

The intermediate purification step (Acidic RPC)

In this step, Amberchrom CG 300M (Dow), Amberchrom CG 300S (Dow), and Source 30 rpc (Cytiva) resins were packed in XK 16/20 columns (Cytiva) with a bed height of 10 cm. The protein sample was adjusted with the conventional percent of TFA and 2-propanol, then loaded on the three different packed columns (conventional 2.0, proposal 2.2, and proposal 2.3) as shown in (Figure 2, Table 3). The protein sample was prepared with 0.2% TFA instead of 0.1%, and Amberchrom CG 300M was used to improve the protein's resolution (proposal 2.1) (Figure 2, Table 3).

The polishing purification step (Alkaline RPC)

The chromatographic matrices used for the polishing step are the same ones used in the intermediate phase (Figure 6). The protein sample was adjusted with the conventional percent of ammonium bicarbonate and 2-propanol, then loaded on the three different packed matrices (conventional 3.0, proposal 3.2, and proposal 3.3) as shown in (Figure 3, Table 3). The protein sample was prepared with 40mM instead of conventional 20mM Ammonium bicarbonate while using Amberchrom CG 300M (proposal 3.1) to improve the resolution of the protein (Figure 3, Table 3). (The results should be written in a descriptive form in detail). Finally, the ultrafiltration process was done for the purest protein fractions for concentration and buffer exchange purposes using cassette membranes with a cut-off of 3KDa.

Quantitative analysis of Hirudin

Samples obtained from the runs were immediately analyzed by quantitative RP-HPLC to calculate the process yields. The validated analytical method of Hirudin is based on RP- HPLC (Shimadzu), using a C8, nucleosil, 3 microns, 120A, 150 x 4.5mm column (Phenomenex), applying a gradient of 15 – 30% of acetonitrile in water (Lee 1997).

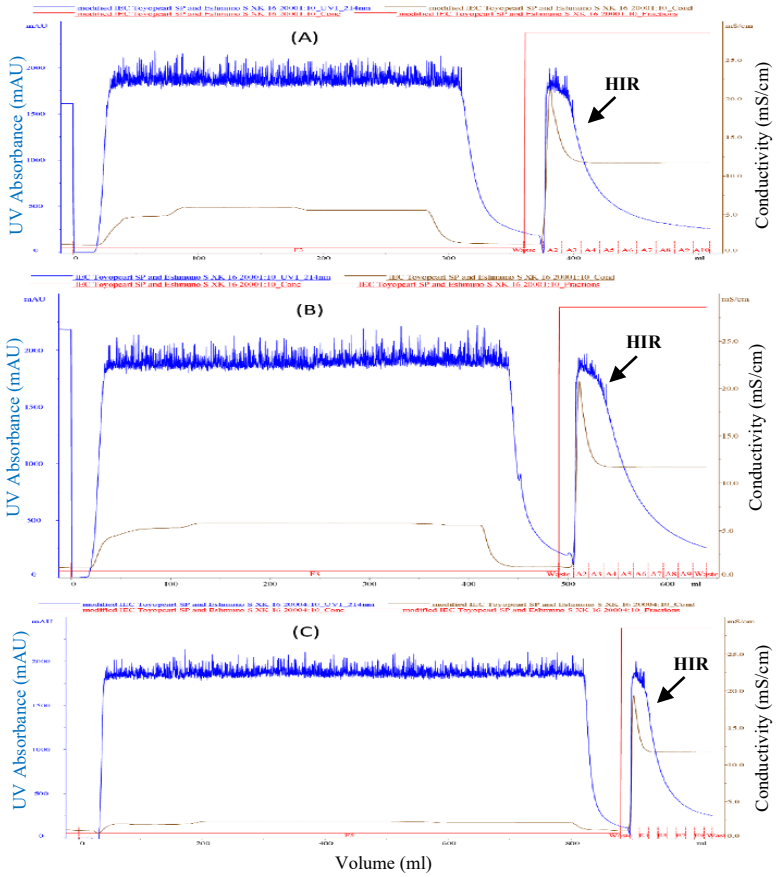


Figure 1. Purification of recombinant Hirudin by cation exchange chromatography. (A) Conventional 1.0: Toyopearl SP 550C column: Process pH 3.0±0.2 / conductivity 5.0 mS/cm; (B) Proposal 1.1: Toyopearl SP 550C column: Process pH 2.0±0.2 / conductivity 5.0 mS/cm; (C) Proposal 1.4: Eshmuno S column: Process pH 3.0±0.2 / conductivity 1.0 mS/cm. Process conditions: Column equilibration = 3 CV, Hirudin concentration = 0.13 mg/mL in A & B and 0.044 mg/ml in C, temperature = 25◦C, Residence time = 8.1 min. Purification procedures: Sample volume (260 mL in A & B and 780 ml in C) of the harvest supernatant was injected to a 20 mL XK 16/20 (Toyopearl SP 550C / Eshmuno S) column pre-equilibrated with 3CV of buffer A (20 mM sodium citrate, pH 3.0, conductivity 1.5 mS/cm) using an AKTA explorer 100 system at room temperature. After the column was washed by 5-fold column volumes of buffer A, the IEC fractions were eluted isocratically by 7-fold column volumes with buffer B (20 mM sodium citrate, 0.1 M NaCl, pH 3.0, conductivity 11 mS/cm) over55 min. The flow rate was 2.5 mL/min, and the detection UV wavelength was 214 nm.

Table 3. Different proposal parameters of the Hirudin downstream processes on the lab scale

Capture (Cation Exchange Chromatography)			
Matrix Name	**pH**	**Cond. (mS/cm)**	**Type**
Toyopearl SP 550C	3.0 ± 0.2	5.0 ± 0.5	Convent. 1.0
	2.0 ± 0.2		Proposal 1.1
	3.0 ± 0.2	1.0 ± 0.5	Proposal 1.2
Eshmuno S	3.0 ± 0.2	5.0 ± 0.5	Proposal 1.3
		1.0 ± 0.5	Proposal 1.4
Intermediate (Acidic RPC)			
Matrix Name	**% TFA**	**% IPA**	**Type**
Amberchrom CG 300M	0.1	5%	Convent. 2.0
	0.2		Proposal 2.1
Amberchrom CG 300S	0.1		Proposal 2.2
Source 30rpc	0.1		Proposal 2.3
Polishing (Alkaline RPC)			
Matrix Name	**Amm. Bicarbonate**	**% IPA**	**Type**
Amberchrom CG 300M	20mM	5%	Convent. 3.0
	40mM		Proposal 3.1
Amberchrom CG 300S	20mM		Proposal 3.2
Source 30rpc	20mM		Proposal 3.3

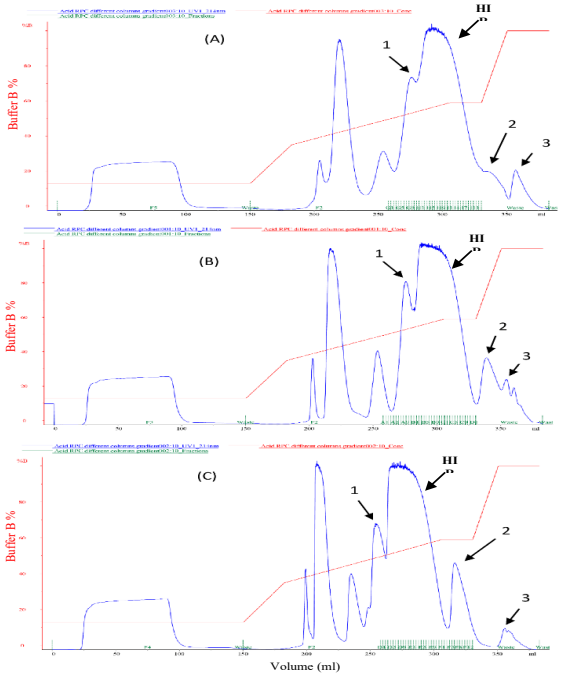


Figure 2. Purification of recombinant Hirudin by acidic reversed phase chromatography. (A) Conventional 2.0: Amberchrom CG 300M column; (B) Proposal 2.2: Amberchrom CG 300S column; (C) Proposal 2.3: Source 30rpc column. Process conditions: Hirudin concentration = 0.88 mg/mL, temperature = 25◦C, Buffer A (0.1%TFA in WFI, pH 2.2), Buffer B (0.1% TFA + 40% Isopropanol in WFI, pH 2.2), Residence time = 14 min. Purification procedures: Sample volume (70 mL) of the selected IEC fractions adjusted with 0.1% TFA and 5% Isopropanol was injected to the 20 mL XK 16/20 column pre-equilibrated with 4-fold column volumes of 13% buffer B using an AKTA explorer 100 system at room temperature. After the column was washed by 4-fold column volumes of 13% buffer B, the fractions were eluted via segmented gradient technique by 11.2-fold column volumes over 160 min. The flow rate was 1.4 mL/min, and the detection UV wavelength was 214 nm. The target protein was eluted at 50% Buffer B (Weydemann et al. 1995).

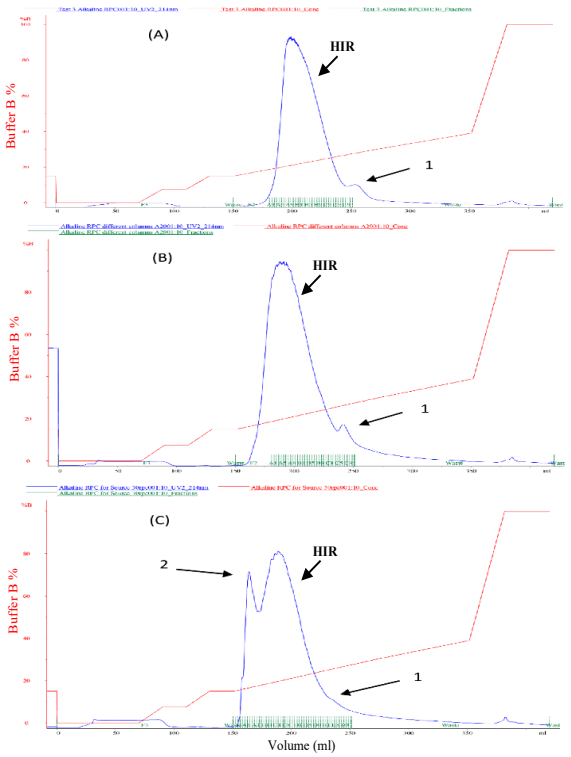


Figure 3. Purification of recombinant Hirudin by alkaline reversed phase chromatography. (A) Conventional 3.0: Amberchrom CG 300M column; (B) Proposal 3.2: Amberchrom CG 300S column; (C) Proposal 3.3: Source 30rpc column. Process conditions: Hirudin concentration = 2.8 mg/mL, temperature = 25◦C, Buffer A (20mM sodium bicarbonate in WFI, pH 7.5), Buffer B (20mM sodium bicarbonate + 40% Isopropanol in WFI, pH 7.5), Residence time = 12.5 min. Purification procedures: Sample volume (70 mL) of the selected acid RPC fractions adjusted with 20mM sodium bicarbonate and 3-fold of WFI was injected to the 20 mL XK 16/20 column pre-equilibrated with 4-fold column volumes of 15% buffer B using an AKTA explorer 100 system at room temperature. After the column was washed by 4-fold column volumes of 15% buffer B, the fractions were eluted via segmented gradient technique by 14-fold column volumes over 186 min. The flow rate was 1.5 mL/min, and the detection UV wavelength was 214 nm. The target protein is eluted at 30% Buffer B (Weydemann et al. 1995).

RESULTS AND DISCUSSION

Fermentation and protein expression

The growth of *H. polymorpha* in the different bioreactor sizes relied on glycerol concentration (carbon source). The last 48 hours in the commercial batch (represents the stationary phase in the cell's growth curve) is the growth-protein expression phase. The shots of glycerol were continuous over the 48 hours. The impact of using different glycerol addition shots on the oxygen level (PO_2) in the culture medium is shown in (Figure 4), and its impact on the hirudin level is shown in (Table 4).

The hirudin content in the final fermentation sample was positively impacted by the optimum dose of 600 gm glycerol per shot (Table 4), as it showed a long starvation period (Figure 4). The increase in protein production during carbon starvation is a complex process regulated by several factors, including the type and level of nutrients that are being starved, the length of the starvation period, and the environmental conditions (Jaishankar and Srivastava 2017). During the *H. polymorpha* growth-protein expression phase (last 48 hrs.), the duration of the growth phase is directly proportional to the duration of the starvation phase (Jaishankar and Srivastava 2017) (Figure 4).

In this context, we were optimizing the glycerol addition during the expression phase to increase the hirudin yield in the fermentation process. Adding 600 gm glycerol per shot extends the growth phase and

the starving period to express more protein. Starvation was monitored by following the increase of dissolved oxygen level, which means low consumption of glycerol (starvation), and accordingly, more protein expression is expected (Figure 4). On the other hand, adding 500 gm glycerol per shot produced short-growth and starvation periods, and, accordingly, less protein was expressed.

Hirudin purification

The most common purification strategy is based on capture, intermediate, and polishing steps, targeting a final

The Capture step (IEC)

The Hirudin molecule contains 12 free carboxylic groups (Aspartic acid (D) and Glutamic acid (E)) and three free amino groups (Lysine amino acid (K)) (Supplementary 2).

Lowering pH value in the cation exchange chromatography (Proposal 1.1)

In the CEX capture step, adjusting the pH of the sample load to a pH value away from the isoelectric point of hirudin showed increasing protein yield (%) through decreasing protein loss in chromatography flow-through. Lowering the pH value to 2.0 increased the hirudin IEC yield by 5.7% (n=5, $p<0.05$) (Figure 1B, Table 5). Although cation exchange chromatography is a capture step aiming to catch the most considerable amount of protein, part of the hirudin was detected in the collected flow-through fraction during the sample loading step during the conventional process, leading to protein losses.

Changing the sample pH will affect the molecule ionization, and the strength of binding could be affected. The structural changes of bovine serum albumin (BSA) in albumin-gold nanoparticle bioconjugates were consistent with previous findings. These studies showed that albumin in the bioconjugates underwent significant conformational changes at the secondary and tertiary structural levels at pH 3.8, 7.0, and 9.0. These changes were consistent with the formation of various albumin isomeric forms. (Shang et al. 2007). Also, it mimics the modifications to the SP Sepharose FF (cation exchange resin) binding mechanism, in which the protein orientations change from one binding site at low pH to a second binding site at high pH (Dismer, Petzold and Hubbuch 2008).

Proteins are neutral at their isoelectric point (pI), having a positive charge if the solution pH is lower than the pI and having a negative charge if the solution pH is larger than the pI (Shaw et al. 2001). The isoelectric point of the hirudin molecule is 4.0, and upon adjusting the pH of the sample load to 3.0±0.2, it acquires the molecule positive charge (NH_4^+) through protonation of the three amino groups. This charge does not seem strong enough, leading to weak binding of the protein molecule on the IEC matrix and then to the loss of some protein amount in the flow-through. Lowering the sample pH away from the isoelectric point (pH = 2) produces a more substantial positive charge (NH_4^+) that might strengthen the protein binding on the IEC matrix. The protein loss in the flow-through during the loading step is diminished. Accordingly, the yield % of the capture step is increased. Similarly, a one-unit shift in the isoelectric point of plasma (pI) altered antibody clearance. Antibodies with improved neonatal Fc receptor (FcRn) affinity at pH 6.0 had longer serum half-lives and clearance rates than wild-type antibodies. (Bumbaca et al. 2012).

Table 4. Results of different shoots of glycerol during the expression phase

Amount of glycerol per shoot	Result
500 gm	100%*
600 gm	110%
700 gm	100%

The yield of the conventional large-scale process was considered 100%.

Table 5. Results of the proposals of the downstream steps

Capture (Cation Exchange Chromatography)		
Matrix Name	**Test**	**Yield%**
Toyopearl SP 550C	Conventional 1.0	100%*
	Proposal 1.1	105.7%
	Proposal 1.2	100%
	Proposal 1.3	85%
Eshmuno S	Proposal 1.4	103.5%
Intermediate (Acidic RPC)		
Matrix Name	**Test**	**Yield%**
Amberchrom CG 300M	Conventional 2.0	100%*
	Proposal 2.1	100%
Amberchrom CG 300S	Proposal 2.2	121.1%
Source 30rpc	Proposal 2.3	105.1%
Polishing (Alkaline RPC)		
Matrix Name	**Test**	**Yield%**
Amberchrom CG 300M	Conventional 3.0	100%*
	Proposal 3.1	100%
Amberchrom CG 300S	Proposal 3.2	124%
Source 30rpc	Proposal 3.3	85.1%

The yield of the conventional large-scale process was considered 100%.

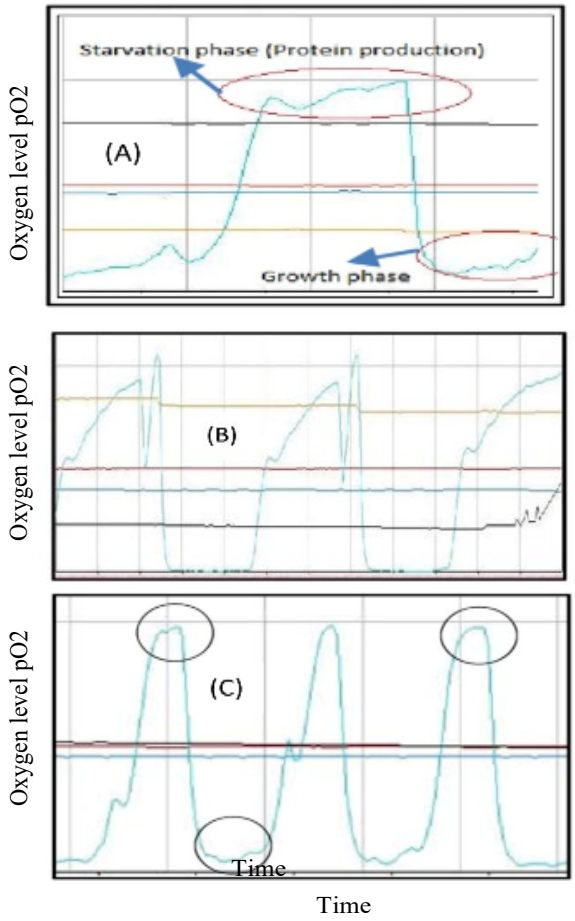


Figure 4. Impact of different addition shots of glycerol, during H. polymorpha growth-protein expression phase, on po2 level vs time: (A) 600 gm/shot showed long starvation period, (B) 700 gm/shot, (C) 500gm/shot showed short starvation period.

Replacing the conventional cationic exchange matrix with another with a tentacle working technique (Proposal 1.3 and 1.4)

Proposal 1.4: Applying a low conductivity sample on tentacle working resin (Eshmuno S) positively impacted the yield (Figure 1C, Table 5) but showed no impact while using Toyopearl SP 550C. Eshmuno S showed higher dynamic binding capacity than Fractogel SO_3 and Toyopearl GigaCap S over a range of different pH and conductivity values during the purification of monoclonal antibodies (Urmann et al. 2010). Eshmuno S resin is a cation-exchange resin that has tentacle-like structures. These tentacles are made of a polymer grafted onto the surface of resin beads. These tentacles provide a large surface area for the mobile phase to interact with the stationary phase, increasing separation efficiency. The positive charge of the Hirudin will interact with the negatively charged tentacles in this interaction. Eshmuno S resin showed that the tentacle working technique increased the yield of the capture step by 3.5% just in case of lowering the sample conductivity.

Theoretical plate test for the reversed-phase matrices

The Source 30rpc resin showed the highest theoretical plates (13022 plate/m), followed by Amberchrom CG 300S (6063 plate/m) and Amberchrom CG 300M (2345 plate/m). The results of the theoretical plate tests confirmed the higher column efficiency of the media with smaller particle sizes (Source 30rpc followed by Amberchrom CG 300S, and then Amberchrom CG 300M), but curves are not shown.

The intermediate and polishing purification steps (Acidic and Alkaline RPC)

The intermediate purification step aims to get the target protein with higher purity. The polishing purification step aims to remove the remaining trace impurities, resulting in a final purity of NLT of 98%.

Increasing % of TFA in Acidic RPC (Proposal 2.1)

Applying the sample load with a higher % of TFA (0.2%) on Amberchrom CG 300M showed peak elution at a higher percent of buffer B, but no improvement in the yield of this chromatography step was observed (data are not shown). Altering the sample pH could affect the molecule ionization, thereby affecting the molecule hydrophobicity and retention time. The acidic conditions achieved by the TFA hinder the ionization of carboxylic groups and activate the ionization of amino groups in the hirudin molecule. Ionization of NH_3 will occur by producing the acid form, NH_4^+, affecting the total hydrophobicity of the molecule. Nevertheless, increasing the concentration of TFA didn't improve the separation, although the Hirudin peak has been eluted lately.

Replacing the RPC matrix with one of lower particle size (Proposal 2.2 and 3.2)

Both acidic RPC (intermediate step) and alkaline RPC (polishing step) work with the same resin.

Proposal 2.2 (acidic RPC): Applying the sample load with the conventional conditions (0.1% TFA and 5%IPA) on Amberchrom CG 300S (particle size of 20-50µm) instead of Amberchrom CG 300M (particle size of 50-100µm) showed an increase in yield % through improving peak resolution (Table 5). The peak numbers 1 and 2 were better separated (Figure 2B) than the conventional large particle size (Figure 2A).

The yield of the intermediate step increased by 21.1% (n= 3, p<0.05). (Figure 2B, Table 5).

Proposal 3.2 (alkaline RPC): Applying the sample load with the conventional conditions (20mM ammonium bicarbonate and 5% IPA) on Amberchrom CG 300S (particle size of 20-50µm) instead of Amberchrom CG 300M (particle size of 50-100µm) showed an increase in yield % through improving peak resolution (Table 5). The impurity peak number 1 (Figure 3B) was better separated than the conventional large particle size (Figure 3A). The yield of the polishing step increased by 24% (n= 4, p<0.05). (Figure 3B, Table 5). According to Van Deemter curves, decreasing the particle size might decrease Eddy diffusion (multiple paths) and molecular diffusion due to reducing the irregular pathway.

Accordingly, a better resolution was achieved by decreasing plate height (N). So, using the polystyrene divinyl benzene matrix with a smaller particle size showed an improvement in both the intermediate and polishing steps compared to the conventional particle size used in both steps (Figure 2B, 3B, indicated by arrows). As mentioned in the literature, many trials of epigallocatechin gallate (EGCG) at greater particle sizes and have been made before separating epigallocatechin gallate (EGCG) at greater particle sizes. They were demonstrated by comparing the isolation of EGCG with particle size at a preparative scale. At particle sizes of 15 and 40-63 microns in the isocratic mode, separation of EGCG was experimentally obtained, but at 150 microns, EGCG was not completely separated (J. Il Kim, Hong, and Row 2002)

Replacing the RPC matrix with one of smaller particle size and monosized beads (Proposal 2.3 and 3.3)

Proposal 2.3 (acidic RPC): Applying the conventional load (0.1% TFA and 5% IPA) on Source 30rpc showed an improvement in the yield % of the intermediate purification step (Table 5). The peak numbers 1, 2, and 3 (Figure 2C), which represent the closely related impurities, were better separated than the conventional large particle size (Figure 2A)

Proposal 3.3 (Alkaline RPC): Applying conventional load (20mM ammonium bicarbonate and 5% IPA) on Source 30rpc negatively impacted the yield % of the polishing purification step (Table 5). Although the impurity peak number 2 was perfectly separated (Figure 3C), the yield decreased compared to the conventional large particle size (Figure 3A). In chromatography, monosized beads are used to improve separation efficiency. When the beads are all the same size, they pack more tightly together, giving the mobile phase more surface area to interact with the stationary phase. This increased surface area results in a more efficient separation of the mixture's components. The use of monosized beads reduces band broadening and improves Hirudin resolution.

Source 30 rpc resin (HETP=13022 N/m) was the monobead candidate to be tried. It has the same ligand, unsubstituted polystyrene divinyl benzene, and smaller particle-size monosized beads. These features proposed better separation and, accordingly, higher yield (Figures 2C, 3C, indicated by arrows for the impurity peaks). Nevertheless, Source 30 rpc showed promising results in the yield of the intermediate step (5.1% increment) but negatively impacted the yield of the polishing step purity of hirudin of not less than 99%. Both intermediate and polishing steps were conducted using Amberchrom CG 300M as a conventional process, with a medium particle size of 75 µm. Replacing the matrix with (Amberchrom CG 300S), the smaller particle size of the same matrix showed increased protein recovery through improving protein separation. This modification has a dual benefit as it increased the yield % of both intermediate and polishing steps (Figure 5).

Effect of increasing pH in the alkaline RPC (Proposal 3.1)

Applying the sample load with a higher % of ammonium bicarbonate (40 mM) on Amberchrom CG 300M showed no improvement in the yield. The alkaline conditions hinder the ionization of amino groups and activate the ionization of many carboxylic groups in the Hirudin molecule, decreasing its total hydrophobicity. Therefore, in acidic conditions, it was eluted at 30% buffer B instead of 50% buffer B.

CONCLUSION

Minor modifications in the parameters of the purification steps of r-HIR, produced from transformed methylotrophic yeast *H. polymorpha* culture, could increase the overall production yield by 58.7% higher than the conventional process (Figures 5, 6). Thus, any increase in market demand could be easily fulfilled by increasing the process's economy.

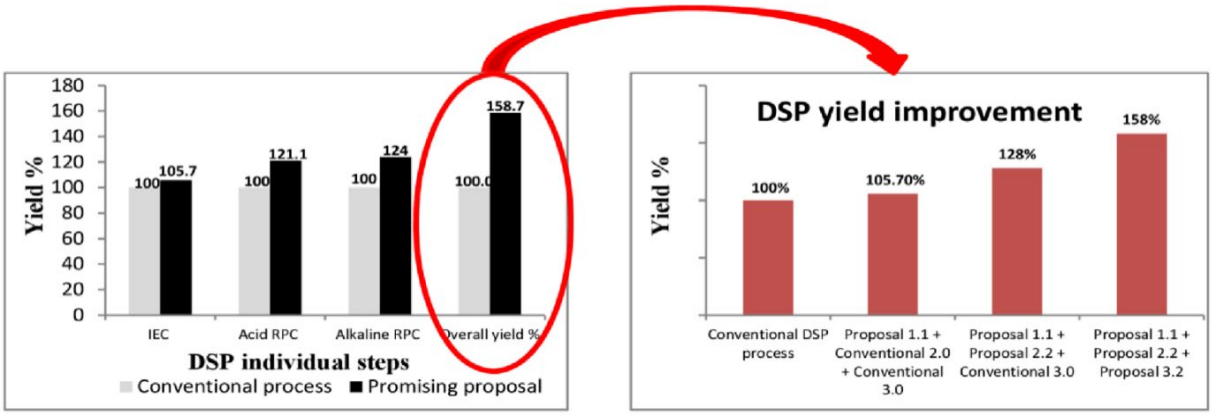


Figure 5. Yield of each downstream step and overall yield % while applying both Conventional and the promising proposals (The yield of the conventional large scale process was considered as 100%).

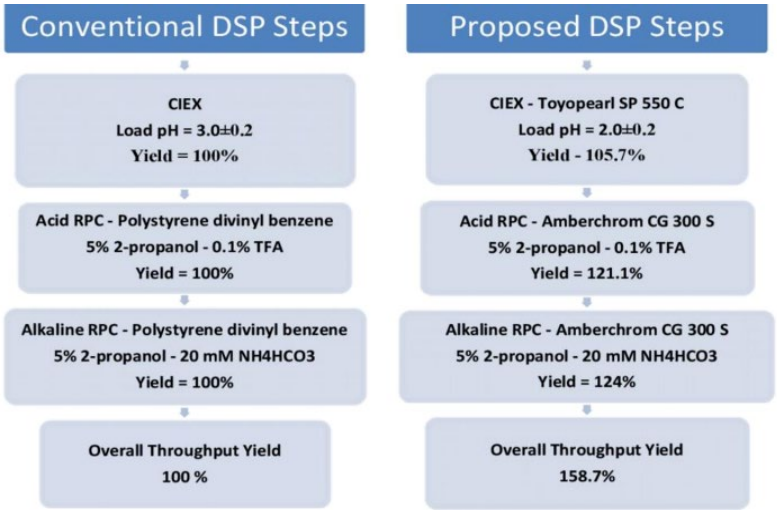


Figure 6. Downstream process of r-HIR on a lab-scale, illustrating both conventional and promised modified DSP steps with applying different parameters

The protein loss in the capture step has been minimized by decreasing the pH of the protein solution. The intermediate and polishing steps yield was improved using the same chromatographic matrix with lower particle size. With this Hirudin molecule, Toyopearl SP resin showed an optimum binding capacity at a slightly decreased pH, whereas Eshmuno S resin showed maximum capacity with a lowering conductivity value.

Concerning the capture step, Toyopearl has an advantage over Eshmuno S as using Eshmuno S is considered a major change unacceptable for the commercial process.

More studies might be required to exploit the numerous carboxylic groups (12 groups). This could be done by adjusting the pH to more than 4.0 and using an anionic exchange matrix to look for better separation between the closely related compounds.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

ACKNOWLEDGEMENTS

Many thanks to the upstream processing team for conducting the fermentation trials needed for this study and to the analytics team of MiGenTra Egypt for their help in analyzing all samples whenever possible.

REFERENCES

Bumbaca, Daniela, C Andrew Boswell, Paul J Fielder, and Leslie A Khawli. 2012. "Physiochemical and Biochemical Factors Influencing the Pharmacokinetics of Antibody Therapeutics." *The AAPS Journal* 14 (3): 554–58. https://doi.org/10.1208/s12248-012-9369-y.

Cummins, Philip M., Oonagh Dowling, and Brendan F. O'Connor. 2011. "Ion-Exchange Chromatography: Basic Principles and Application to the Partial Purification of Soluble Mammalian Prolyl Oligopeptidase." *Methods in Molecular Biology (Clifton, N.J.)* 681: 215–28. https://doi.org/10.1007/978-1-60761-913-0_12.

Dismer, Florian, Martin Petzold, and Jürgen Hubbuch. 2008. "Effects of Ionic Strength and Mobile Phase PH on the Binding Orientation of Lysozyme on Different Ion-Exchange Adsorbents." *Journal of Chromatography. A* 1194 (1): 11–21. https://doi.org/10.1016/j.chroma.2007.12.085.

Everley, Robert A., and Timothy R. Croley. 2008. "Ultra-Performance Liquid Chromatography/Mass Spectrometry of Intact Proteins." *Journal of Chromatography A* 1192 (2): 239–47. https://doi.org/10.1016/j.chroma.2008.03.058.

Fornstedt, Torgny, Patrik Forssén, and Douglas Westerlund. 2015. "Basic HPLC Theory and Definitions: Retention, Thermodynamics, Selectivity, Zone Spreading, Kinetics, and Resolution." *Analytical Separation Science*, 1–24. https://doi.org/10.1002/9783527678129.assep001.

Garcia-Ortega, Xavier, Núria Adelantado, Pau Ferrer, José Luis Montesinos, and Francisco Valero. 2016. "A Step Forward to Improve Recombinant Protein Production in Pichia Pastoris: From Specific Growth Rate Effect on Protein Secretion to Carbon-Starving Conditions as Advanced Strategy." *Process Biochemistry* 51 (6): 681–91. https://doi.org/10.1016/j.procbio.2016.02.018.

Jaishankar, Jananee, and Preeti Srivastava. 2017. "Molecular Basis of Stationary Phase Survival and Applications." *Frontiers in Microbiology* 8 (OCT): 1–12. https://doi.org/10.3389/fmicb.2017.02000.

Kim, Jung Il, Seung Bum Hong, and Kyung Ho Row. 2002. "Effect of Particle Size in Preparative Reversed-Phase High-Performance Liquid Chromatography on the Isolation of Epigallocatechin Gallate from Korean Green Tea." *Journal of Chromatography. A* 949 (1–2): 275–80. https://doi.org/10.1016/s0021-9673(01)01273-0.

Kim, Sang Gon, Young Chan Bae, and Jae Jun Kim. 2009. "Effect of PH on Phase Separation of Globular Protein." *Korean Journal of Chemical Engineering* 26 (3): 742–47. https://doi.org/10.1007/s11814-009-0124-8.

Lavine, Barry K, Jason P Ritter, and Sean Peterson. 2002. "Enhancement of Selectivity in Reversed-Phase Liquid Chromatography." *Journal of Chromatography. A* 946 (1–2): 83–90. https://doi.org/10.1016/s0021-9673(01)01520-5.

Lee, Hae Don. 1997. "United States Patent [191," 1–4.

Markwardt, Fritz. 1992. "Hirudin: The Promising Antithrombotic." *Cardiovascular Drug Reviews* 10 (2): 211–32. https://doi.org/10.1111/j.1527-3466.1992.tb00247.x.

Mazzeo, Jeffrey R, Uwe D Neue, Marianna Kele, Robert S Plumb, and Waters Corp. 2005. "A New Separation Technique Takes Advantage of Sub-2-Mm HPLC." *Journal of the American Chemical Society*, 460–67.

Ming-Ju Chen, Kreuter, J Yi-Tzu Kuo. 1996. "Nanoparticles and Microparticles for Drug and Vaccine Delivery." *Journal of Anatomy* 189 (Pt 3 (Ii): 503–5. https://doi.org/10.1002/bit.

Mo, Wei, Yan Ling Zhang, Hong Shan Chen, Long Sheng Wang, and Hou Yan Song. 2009. "A Novel Hirudin Derivative Characterized with Anti-Platelet Aggregations and Thrombin Inhibition." *Journal of Thrombosis and Thrombolysis* 28 (2): 230–37. https://doi.org/10.1007/s11239-008-0251-9.

Peskin, Adele P., and Scott R. Rudge. 1992. "Optimization of Large-Scale Chromatography for Biotechnological Applications." *Applied Biochemistry and Biotechnology* 34–35 (1): 49–59. https://doi.org/10.1007/BF02920533.

Rotkaja, O., J. Golushko, and P. Mekss. 2014. "The Behavior of Proteins on Reversed-Phase Supports During High-Performance Liquid Chromatography and Prediction of Protein Retention Time on Ymc Columns." *Latvian Journal of Chemistry* 52 (1–2): 71–76. https://doi.org/10.2478/ljc-2013-0008.

Samuelsson, Jörgen, Marek Leśko, Martin Enmark, Joakim Högblom, Anders Karlsson, and Krzysztof Kaczmarski. 2018. "Optimizing Column Length and Particle Size in Preparative Batch Chromatography Using Enantiomeric Separations of Omeprazole and Etiracetam as Models: Feasibility of Taguchi Empirical Optimization." *Chromatographia* 81 (6): 851–60. https://doi.org/10.1007/s10337-018-3519-z.

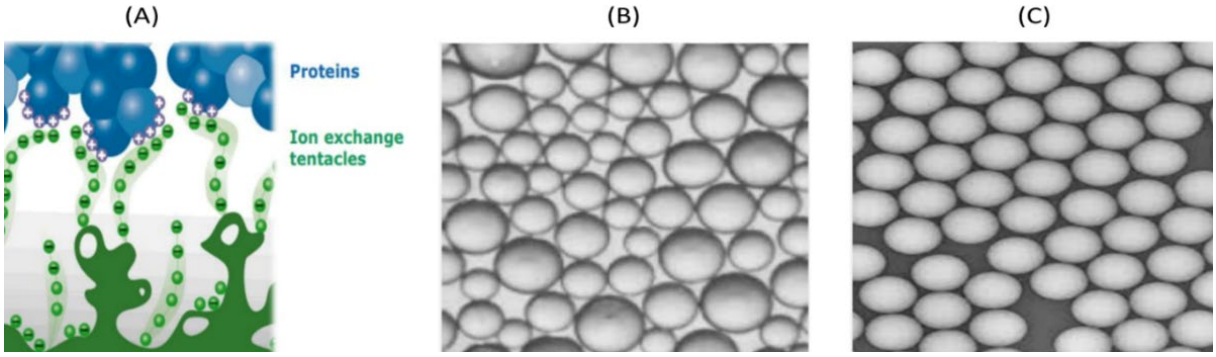
Shang, Li, Yizhe Wang, Junguang Jiang, and Shaojun Dong. 2007. "PH-Dependent Protein Conformational Changes in Albumin:Gold Nanoparticle Bioconjugates: A Spectroscopic Study." *Langmuir : The ACS Journal of Surfaces and Colloids* 23 (5): 2714–21. https://doi.org/10.1021/la062064e.

Shaw, Kevin L., Gerald R. Grimsley, Gennady I. Yakovlev, Alexander A. Makarov, and C. Nick Pace. 2001. "The Effect of Net Charge on the Solubility, Activity, and Stability of Ribonuclease Sa." *Protein Science* 10 (6): 1206–15. https://doi.org/10.1110/ps.440101.

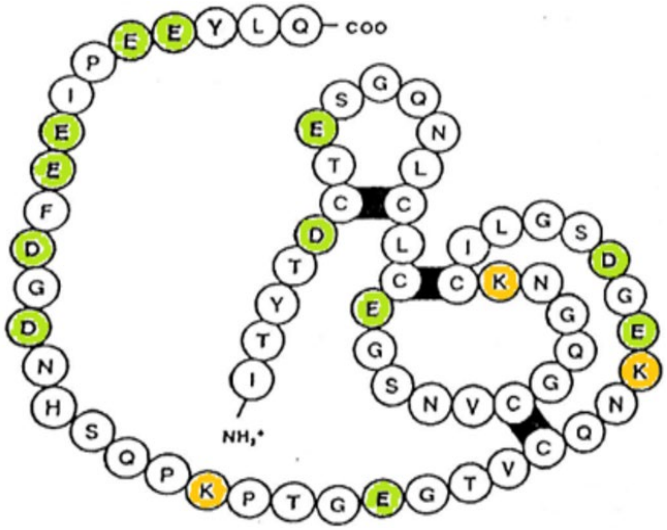
Urmann, Marina, Heiner Graalfs, Matthias Joehnck, Lothar R. Jacob, and Christian Frech. 2010. "Cation-Exchange Chromatography of Monoclonal Antibodies: Characterization of a Novel Stationary Phase Designed for Production-Scale Purification." *MAbs* 2 (4): 395–404. https://doi.org/10.4161/mabs.12303.

Weydemann, U., P. Keup, M. Piontek, A. W.M. Strasser, J. Schweden, G. Gellissen, and Z. A. Janowicz. 1995. "High-Level Secretion of Hirudin by Hansenula Polymorpha -Authentic Processing of Three Different Preprohirudins." *Applied Microbiology and Biotechnology* 44 (3–4): 377–85. https://doi.org/10.1007/BF00169932.

SUPPLEMENTARY FILES



Supplementary 1. Physical features of some chromatographic matrices: (A) The working principle of the tentacle resin, (B) Conventional variability in the matrix particle size, and (C) Monosized beads of the chromatographic matrix.



Supplementary 2. Structure of recombinant Hirudin, depicting both acidic and basic amino acid residues. (D: Aspartic acid, E: Glutamic acid, K: Lysine amino acid)