Study on the Discovery and Properties of Anticancer Fibrinolytic Active Protein from *Sympiezomias velatus Chevrolat*

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Abstract: *Sympiezomias velatus Chevrolat*, commonly known as the cereal leaf beetle, is an insect pest that mainly affects tobacco crops. Feeding on tobacco leaves, it causes significant economic losses and impacts tobacco production. Due to its wide distribution and high adaptability, *S. velatus* has become an important model organism for studying croppest control and ecological adaptability. In this experiment, *S. velatus* was used as the research subject. The crude protein of *S. velatus* was isolated and extracted using the ammonium sulfate partial salting-out method. The protein activity was assessed using the fiber active plate method. The results demonstrated that three different concentration gradients of crude protein exhibited strong fibrinolytic activity. The optimum temperature for protein activity was found to be 35℃, while the optimal pH was approximately 7.0. Fe^{3+} significantly inhibited the activity of the fibrinolytic active protein in *S. velatus*, whereas Ba^{2+} showed a slight inhibitory effect on βME. Na^{+} and EDTA did not exhibit any impact. MTT experiments revealed that the fibrinolytic active protein displayed a certain inhibitory effect on cancer cell growth. This study provides valuable references for other research on *S. velatus*.

Keywords: *Sympiezomias velatus Chevrolat*; Fibrinolytic Activity; Purification; Anticancer

1. Introduction

Thrombotic diseases and tumor diseases pose severe threats to human health, with high rates of disability and mortality. The incidence of both diseases is significant and tends to occur at younger ages. The pathogenesis of thrombus formation is highly complex, involving a cascade reaction of numerous factors that result in the formation of insoluble fibrin-based blood clots within blood vessels and the subsequent dissolution of these clots[1]. When the fibrinolytic capacity is compromised due to either a hypercoagulable or hypocoagulable state, it can lead to thromboembolic diseases[2]. Fibrinolytic active proteins possess significant ability to hydrolyze fibrinogen, which can be utilized for the prevention and treatment of thrombotic diseases[3]. In recent years, fibrinolytic active proteins have been discovered from various medicinal insects, and these proteins exhibit anti-thrombotic and anti-tumor effects, offering potential as novel sources for thrombolytic and anticancer agents. Among the various methods currently used for treating thrombosis, thrombolytic therapy has been widely employed. Thus far, thrombolytic agents have evolved from first to third generation, showing good efficacy in the prevention and treatment of thromboembolic diseases. However, there are still certain limitations associated with these agents, such as a high risk of bleeding, high cost, significant toxic side effects, difficulties in administration, and limited availability[2]. Therefore, the search for natural thrombolytic agents with advantages and promising prospects becomes particularly important. Extracting natural fibrinolytic active compounds from traditional Chinese medicinal herbs has attracted widespread attention due to their economic, safe, and efficient characteristics, as well as their dual therapeutic effects against both thrombosis and tumors. Medicinal insects refer to insects or their products that can be directly used for disease treatment or health maintenance, and they are

an important component of traditional Chinese medicinal herbs. Due to their diverse species, abundant quantity, and significant medicinal value, they have gained favor among researchers.

Sympiezomias velatus Chevrolat, commonly known as the cereal leaf beetle, is a common agricultural pest that primarily affects tobacco crops[4]. In addition to causing damage to tobacco leaves, research has indicated that *S. velatus* may have potential medicinal value in other aspects. It has been reported that *S. velatus* is rich in nutrients such as proteins, carbohydrates, and lipids, and possesses properties such as promoting blood circulation, detoxification, reducing swelling, diuresis, and facilitating bowel movements. Recent studies have shown significant therapeutic effects of *S. velatus* extracts in certain fields. For instance, in cancer research, *S. velatus* extracts have been found to inhibit the proliferation of tumor cells, arrest their cell cycle, and induce apoptosis. Furthermore, *S. velatus* extracts have also been shown to enhance the phagocytic activity of macrophages, thereby aiding the immune system in combating inflammation and pathogens. However, the specific mechanisms underlying the bioactive compounds of *S. velatus* are still poorly understood. To shed light on its therapeutic potential and provide a scientific basis for drug development and treatment strategies, further investigation is warranted into the chemical composition and bioactive components of *S. velatus*, as well as its potential applications in the field of medicine[5].

In this experiment, *S. velatus* fibrinolytic crude proteins were isolated using ammonium sulfate fractionation. The fibrin plate method was employed to assess their fibrinolytic activity, while the MTT assay was utilized to determine whether *S. velatus* fibrinolytic active proteins exhibit anticancer effects on colon cancer cells.

2. Methods and Materials

2.1 Extraction of Crude Proteins

Cut the dialysis bag into small sections, approximately 10cm in length. Boil the dialysis bag in a mixture solution containing 2% (w/v) $NaHCO_3$ (4.0g/200mL) and 1mmol/L EDTA (pH 8.0, 0.0749g/200mL) for 10 minutes. Rinse the dialysis bag thoroughly both inside and outside using distilled water. Soak the prepared dialysis bag in distilled water and store it at 4℃ for future use.

Freeze the *S. velatus* to death, wash it, drain it, and weigh 162g. Place it in a beaker and add phosphate buffer solution (pH 7.4) to submerge the *S. velatus*, approximately 500mL. Soak for 2 hours. Pour the soaked *S. velatus* and buffer solution into a high-speed blender and mash them together. During the mashing process, start the blender for 3-5 seconds, then stop and wait for 5 seconds before starting again. Repeat this process 7-8 times to fully mash and obtain a slurry. Collect the slurry in the beaker. Wash the residue on the inner wall of the blender with 100mL of PBS buffer solution and add it to the beaker. Soak at 4℃ for 24 hours. Pre-cool a high-speed refrigerated centrifuge and set it to a temperature of 4℃, a speed of 10000r/min, and a time of 15 minutes. Centrifuge the soaking solution under these conditions to remove the precipitate and collect the supernatant. Using the method of ammonium sulfate saturation, as shown in Table 1[6], perform stepwise salt precipitation of the crude extract obtained from the *S. velatus* at 0℃ to obtain target proteins in the ranges of 0~35%, 35~70%, and 70~100%. Collect the precipitates. Pack each fraction of the salt-precipitated precipitates into pre-treated dialysis bags. Dialyze for 4 hours using distilled water, followed by dialysis with Tris-HCl buffer solution (pH 7.4). Change the buffer solution every 2 hours in the initial stage. Before changing the buffer solution, take out the dialysis bag and check for the presence of white precipitate in the dialysate using 1mol/L $BaCl_2$ solution. If present, replace the buffer solution with a new one for dialysis. Change the buffer solution every 4 hours in the later stage, using the same method of detection, until no white precipitate is detected in the buffer solution using barium chloride solution. Place the dialyzed protein crude extract at different concentrations into culture dishes and pour polyethylene glycol 10000 powder onto the surface to concentrate it. After half an hour, observe if the PEG 10000 powder has become wet throughout. If so, add more powder to the surface until the protein in

the dialysis bag is concentrated to a sticky state without water. Take out the test liquid from the dialysis bag, divide it into portions, and store at 4℃ for future use.

Table 1. Adjust the Ammonium Sulphate Solution Saturation Calculation Table (0℃)

Ammonium sulfate initial concentration, % saturation	Ammonium sulfate final concentration at 0℃, % saturation																	
		20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Grams of solid ammonium sulfate added per 100mL of solution																	
	0	10.6	13.4	16.4	19.4	22.6	25.8	29.1	32.6	36.1	39.8	43.6	47.6	51.6	55.9	60.3	65.0	69.7
	5	7.9	10.8	13.7	16.6	19.7	22.9	26.2	29.6	33.1	36.8	40.5	44.4	48.4	52.6	57.0	61.5	66.2
	10	5.3	8.1	10.9	13.9	16.9	20.0	23.3	26.6	30.1	33.7	37.4	41.2	45.2	49.3	53.6	58.1	62.7
	15	2.6	5.4	8.2	11.1	14.1	17.2	20.4	23.7	27.1	30.6	34.3	38.1	42.0	46.0	50.3	54.7	59.2
	20	0	2.7	5.5	8.3	11.3	14.3	17.5	20.7	24.1	27.6	31.2	34.9	38.7	42.7	46.9	51.2	55.7
	25	0		2.7	5.6	8.4	11.5	14.6	17.9	21.1	24.5	28.0	31.7	35.5	39.5	43.6	47.8	52.2
	30	0			2.8	5.6	8.6	11.7	14.8	18.1	21.4	24.9	28.5	32.3	36.2	40.2	44.5	48.8
	35	0				2.8	5.7	8.7	11.8	15.1	18.4	21.8	25.4	29.1	32.9	36.9	41.0	45.3
	40	0					2.9	5.8	8.9	12.0	15.3	18.7	22.2	25.8	29.6	33.5	37.6	41.8
	45	0						2.9	5.9	9.0	12.3	15.6	19.0	22.6	26.3	30.2	34.2	38.3
	50	0							3.0	6.0	9.2	12.5	15.9	19.4	23.0	26.8	30.8	34.8
	55	0								3.0	6.1	9.3	12.7	16.1	19.7	23.5	27.3	31.3
	60	0									3.1	6.2	9.5	12.9	16.4	20.1	23.1	27.9
	65	0										3.1	6.3	9.7	13.2	16.8	20.5	24.4
	70	0											3.2	6.5	9.9	13.4	17.1	20.9
	75	0												3/2	6.6	10.1	13.7	17.4
	80	0													3.3	6.7	10.3	13.9
	85	0														3.4	6.8	10.5
	90	0															3.4	7.0
	95	0																3.5
	100	0																

2.2 Determination of Fibrinolytic Activity and Preparation of Urinary Plasminogen Activator Standard Curve

Use the fibrin plate method to determine if the protein crude extract has fibrinolytic activity[7]. Open the laminar flow hood and place pre-sterilized petri dishes, tip boxes, pipettes, and sterile PBS in it. Turn on the UV lamp for approximately 30 minutes to sterilize. Melt the pre-sterilized culture medium (microwave heating) and let it cool to 45℃. Add 3mL of pre-prepared bovine blood fibrinogen (10mg/mL) and 120μl of thrombin (100U/mL) to the cooled culture medium, mix quickly, and pour onto the plates. Insert an inverted 200μl pipette tip into the prepared plate and make evenly spaced holes, then label them.

Add 20μl of test samples, UK (100U/mL) standard, and PBS control into each well, with concentration gradients ranging from 0% to 35%, 35% to 70%, and 70% to 100%. Label the wells accordingly and incubate them in a constant temperature incubator at 37°C for 24 hours. After 24 hours, remove the plates and add staining solution for a duration of 10 minutes. Then pour out the staining solution, rinse with distilled water, and add decolorizing solution. Change the decolorizing solution every ten minutes. If the crude protein exhibits fibrinolytic activity, transparent zones will be observed against the blue background. Take photographs to document the experimental results.

In each well, add 20μL of urinary plasminogen activator solution (prepared using PBS) at different concentrations: 10U/mL, 20U/mL, 40U/mL, 60U/mL, 80U/mL, 100U/mL, 120U/mL, and 200U/mL, in the order indicated by the labels. Use PBS as a negative control and perform three parallel reactions for each concentration. Incubate the plate at 37°C for 24 hours. After incubation, stain the plate and decolorize until the transparent zones are clearly visible. Measure the two perpendicular diameters of the transparent zones using a ruler, calculate the average,

and determine the area. Plot a standard curve with the area of the transparent zone as the x-axis and the activity of urinary plasminogen activator as the y-axis.

2.3 Determination of Protein Concentration

Dilute bovine serum albumin (BSA) standard solution with PBS according to Table 2[8]. Prepare the BCA working solution by mixing BCA-A and BCA-B in a volume ratio of 50:1, and ensure thorough mixing. Add 25μL of the diluted BSA standard solution into each well of a 96-well plate, with three parallel reactions for each sample. Label the wells accordingly. Then add 200μL of the BCA working solution into each well and mix well. Incubate at 37°C for 30 minutes, cool to room temperature, and immediately measure the absorbance of the BSA standard solutions at 570nm. Record the values and calculate the average. Plot a standard curve with the concentration of BSA standards as the x-axis and the absorbance as the y-axis.

Table 2. BCA Standard Sample Loading Table

Tube number	PBS (uL)	Amount of BSA standard solution(uL)	Final concentration of BSA standard solution(ug/uL)
A	0	100	2
B	200	200	1
C	200	200(Take from tube B)	0.5
D	200	200(Take from tube C)	0.25
E	200	200(Take from tube D)	0.125
F	200	200(Take from tube E)	0.0625
G	200	0	0(Blank)

Add 25μL of gradient *S. velatus* protein samples, with concentrations ranging from 0%-35%, 35%-70%, and 70%-100%, respectively, to each well of a 96-well plate. Label the wells accordingly. Then add 200μL of BCA working solution to each well and mix thoroughly. Perform 3 parallel reactions. Incubate at 37℃ for 30 minutes, then cool to room temperature. Immediately measure the absorbance of each sample at 570nm and record the values. Calculate the average and refer to the standard curve to determine the protein concentration in the samples.

2.4 Effects of Different Physicochemical Factors on Protein Activity

Prepare a mixture of equal amounts of the sample and PBS buffer to form a 0.1 M solution of NaCl, $BaCl_2$, $FeCl_3$, EDTA, and β-mercaptoethanol (βME). After incubating at 37℃ for 2 hours, measure the enzyme activity using the aforementioned fibrin plate method.

Pre-set the temperatures of various constant temperature water baths. Place equal amounts of the sample in each of the 7 groups (4℃, 25℃, 35℃, 45℃, 55℃, 65℃, 75℃) and incubate for 0.5 hours, then test their activity.

Dissolve equal amounts of the sample in buffers with different pH values (pH 5, 6, 7, 8 prepared with sodium phosphate-citric acid buffer; pH 9, 10 prepared with glycine-sodium hydroxide buffer). After standing at room temperature for 2 hours, measure the activity.

2.5 MTT Assay for Measuring *S. velatus* Fibrinolytic Activity and Crude Protein Anticancer Activity

Resuscitate colon cancer cells and perform media exchange and subculture during the process to cultivate them to the logarithmic phase. Prepare MTT solution and add the samples according to Table 3 and Table 4[9-11]. Place them in a 37°C, 5% CO_2 incubator for 36 hours. After cultivation, add 20μL of MTT solution to each well (taking care to avoid light exposure). Continue cultivation for 4 hours, then centrifuge and carefully remove the supernatant (avoiding the removal of crystals). Add 150μL of dimethyl sulfoxide (DMSO) to each well and gently tap the 96-well plate until the blue-purple crystals are completely dissolved. Shake the plate on a microplate reader for 10 seconds, then measure the absorbance at 492nm and record the experimental results.

Table 3. The Table about Protein Concentration

Serial Number	Drug Concentration (ug/mL)	Sample Concentration (ug/mL)
Protein 1	1	87.8

Protein 2	125	131.7
Protein 3	250	439
Protein 4	500	1317

Table 4. Anti-cancer Experiment Gradient Setting and Sample Loading Table

Group	Reagent (uL)					
	1640 Medium	Protein 1	Protein 2	Protein 3	Protein 4	PBS
1	200		15.2			
2	200	14.0				
3	200			15.6		
4	200				12.2	
5	200					15.0

3. Results

3.1 Crude Protein Fibrinolytic Activity Assay

Based on Figure 1, it can be observed that well number 4 is the positive control - urokinase, showing a larger clear zone and stronger fibrinolytic activity. Well number 5 is the negative control - PBS, showing no clear zone and no fibrinolytic activity. Wells 1, 2, and 3 all exhibit fibrinolytic activity. Among them, well 2 with 35%-70% sample concentration shows the strongest fibrinolytic activity, followed by well 1 with 0%-35% sample concentration. Well 3 with 70%-100% sample concentration shows the weakest fibrinolytic activity. There is also a dark blue ring inside the clear zone, which is tentatively identified as impurities. However, in subsequent experiments, after crude protein chromatography, when measuring the clear zone using a fiber plate method, there is no dark blue ring in the center.

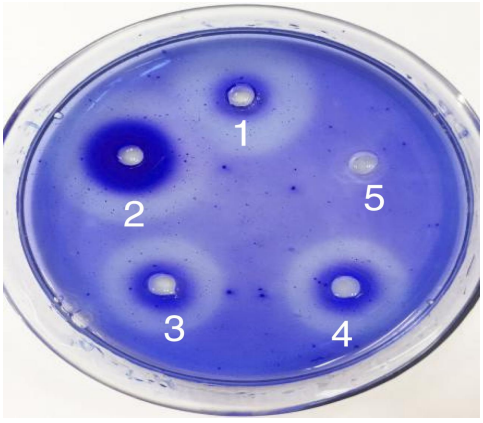

Figure 1. *S. velatus* Active Protein Fibrinolytic Plate. 1: 0%-35% concentration of *S. velatus* protein; 2: 35%-70% concentration of *S. velatus* protein; 3: 70%-100%concentration of *S. velatus* protein; 4: UK(100U/mL); 5: PBS

3.2 Preparation of the Standard Curve for Urokinase

The activity of urokinase is shown in Table 5. The standard curve for urokinase activity is presented in Figure 2.

Table 5. Urokinase Activity Table

Well Number	Urokinase Activity (U)	Sample Volume (uL)	Clear Zone Diameter (cm)
1	20	20	1.20
2	40	20	1.35
3	80	20	1.58
4	160	20	1.90
6	240	20	2.20
7	320	20	2.50
8	400	20	2.70
9	500	20	3.00

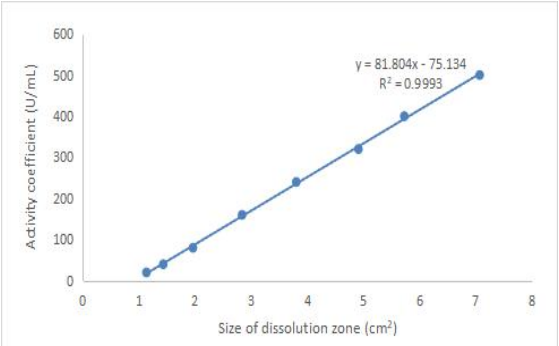

Figure 2 Urokinase Viability Standard Curve

3.3 Protein Concentration Determination

The protein concentration standard curve is shown in Figure 3. Table 6 displays the crude concentrations of three different gradient *S. velatus* proteins.

3.4 Effect of Physicochemical Factors on Protein Activity

The impact of various inhibitors, including metal ions and denaturants, on the fibrinolytic activity of fibrous protein is shown in Table 7. The results indicate that Fe^{3+} inhibits the activity of *S. velatus* fibrinolytic protein close to 100%, almost completely suppressing its activity. Ba^{2+} and βME have a mild inhibitory effect, while Na^{+} and EDTA have little impact on its fibrinolytic activity.

Table 6. Concentration of Large Concentration of S. velatus Protein

Concentration Gradient	Absorbance			Mean	Rough Concentration (mg/mL)
0%-35%	0.990	0.942	0.963	0.965	1.285
35%-70%	1.048	0.942	0.963	0.984	1.317
70%-100%	0.430	0.428	0.402	0.420	0.402

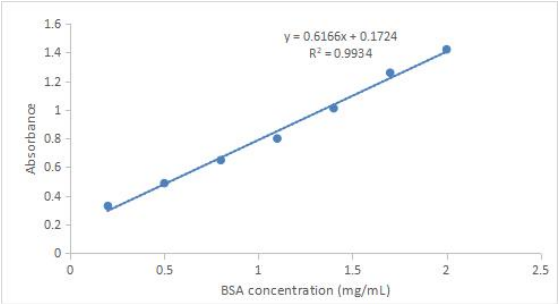

Figure 3. Protein Concentration Standard Curve

Table 7 Effect of Some Inhibitors on Fibrinolytic Activity

Metal salt/Inhibitors	Concertration	Residual activity(U/mL)	Inhibition/%
Na^{+}	0.1mol/L	513.171	1.79
Ba^{2+}	0.1mol/L	424.984	18.67
Fe^{3+}	0.1mol/L	2.607	99.50
EDTA	0.1mol/L	477.942	8.53
βME	0.1mol/L	445.262	14.79
Control	—	522.540	0

Figure 4 demonstrates the effect of temperature on *S. velatus* fibrinolytic protein. Significant changes in crude protein activity are observed at different temperatures. The enzyme activity steadily increases from 4°C to 35°C. However, beyond 35°C, if the temperature continues to rise, the protein's fibrinolytic activity significantly decreases. In summary, the optimal temperature for *S. velatus* fibrinolytic protein is 35°C.

Figure 5 illustrates the effect of pH on the activity of *S. velatus* fibrinolytic protein. Between pH 6 and 10, the activity of *S. velatus* fibrinolytic protein remains relatively stable. However, when the pH is below 6, its activity sharply declines. The protein exhibits stable activity under alkaline conditions, indicating it might be an alkaline protein. According to the graph, the optimal pH for *S. velatus* fibrinolytic protein is 7.0.

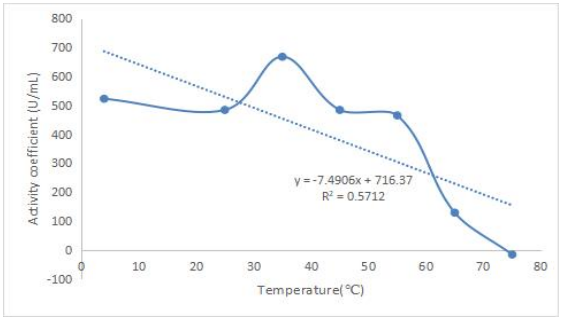

Figure 4. The Effect of Temperature on the Fibrinolytic Protein from *S. velatus*

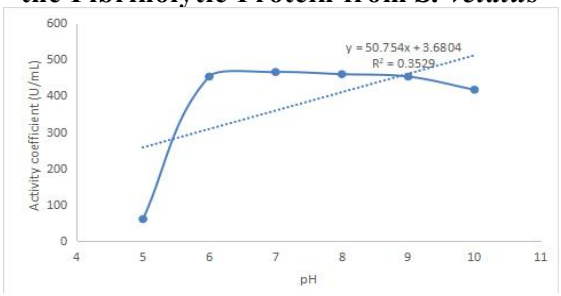

Figure 5. The Effects of pH on the Fibrinolytic Protein from *S. velatus*

3.5 Effect of the Target Protein on Cancer Cell Growth

The results of the impact of *S. velatus* fibrinolytic crude protein on the growth of colon cancer cells at different concentration gradients are shown in Table 8. The cell inhibition rate can be calculated from the absorbance values using the formula:

$$Inhibition(\%) = \frac{A_{control} - A_{test}}{A_{control}} \times 100\% \quad (1)$$

As shown in Table 9. In this formula, $A_{control}$ represents the absorbance value of the control group, and A_{test} represents the absorbance value of the test group.

Table 8. OVCAR-3 Absorbance Change Table after Crude Protein

Group	Absorbance(A)			Mean
	A1	A2	A3	
1	1.121	0.992	0.105	1.073
2	0.822	0.820	0.818	0.820
3	0.668	0.643	0.677	0.663
4	0.258	0.221	0.245	0.241
5	1.464	1.453	1.478	1.465

Table 9. Survival Rate of Cancer Cells under Different Concentrations of Protein

Protein Concentration (ug/mL)	Inhibition Rate (%)
1	26.78
125	44.03

250	54.77
500	83.53

4. Discussion

All experimental procedures in this study were repeated at least three times, with consistent results obtained each time. However, there are some limitations in the experiment. Firstly, the purpose protein used in the experiment was crude extract of *S. velatus* protein, without purification, which contains impurities. As observed from the fibrin plate assay for crude protein fibrinolytic activity, there is a deep blue ring within the dissolution zone, indicating the interference of impurities in the results. Therefore, the impurities in crude protein may have certain interference on the experimental results. Secondly, in the physicochemical property experiments, the variety of metal ions and denaturing agents was limited, which cannot fully reflect the influence of various physicochemical factors on the target protein. The number of temperature and pH groups was also limited, and it is necessary to expand the temperature and pH range to improve the accuracy of the experiment. Thirdly, due to limitations in equipment and conditions during the experiment, it was not possible to accurately control factors such as temperature and time, which also had an impact on the experimental results. Due to these limitations, the experiment used crude protein with fibrinolytic activity, and the influence of other factors on the experimental results cannot be completely ruled out. However, this experiment has laid the groundwork for the subsequent purification of individual proteins and is more conducive to in-depth research on the anticancer activity and mechanism of *S. velatus* fibrinolytic proteins.

5. Discussion

This experiment utilized the method of ammonium sulfate fractionation to isolate proteins of different concentrations from living *S. velatus*, and their fibrinolytic activity was determined through a fibrin plate assay. The results from the fibrin plate assay indicated that proteins with concentration gradients of 0-35%, 35-70%, and 70-100% all exhibited fibrinolytic activity. The optimal temperature was found to be 35°C, while the optimal pH value was 7.0. Fe^{3+} showed an inhibitory effect on the fibrinolytic activity of *S. velatus* proteins, with inhibition approaching 100%. Different protein concentrations were set to investigate the anticancer activity against colon cancer cells. The results showed that as the concentration gradient increased, the growth of cancer cells was inhibited to a certain extent, indicating the anticancer effect of *S. velatus* active proteins.

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