

*Full Length Research Paper*

# Simple and efficient methods for isolation and activity measurement of the recombinant hirudin variant 3 from *Bacillus subtilis*

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**A simple purification approach of the recombinant hirudin variant 3 from the *Bacillus subtilis* was established, by which the hirudin could be purified to the purity of 95% through one-step chromatography with the total recovery rate of 83.9%. A modified Markwardt thrombin titration method for measuring hirudin activity was also set up. Briefly, a series of concentrations of thrombin was prepared and titrated to hirudin sample, respectively and the anti-thrombin activity-range of hirudin was narrowed down by several thrombin solutions at high or low concentration and the optimum group of thrombin concentrations was determined for titration of the hirudin sample. In this modified method, the hirudin activity was determined more accurately, concisely and promptly than the classic Markwardt method.**

**Key words:** Hirudin, thrombin titration method, chromatography, purification.

## INTRODUCTION

Since recombinant hirudin variant was expressed from *Escherichia coli* in 1986, intensive research had been performed on large scale of production and clinic application of hirudin (Harvey et al., 1986; Greinacher and Warkentin, 2008; Fischer et al., 2004). Purification is the most expensive step in the product process of hirudin. In the previous reports, at least three steps of chromatography were always employed, including reversed-phase chromatography in which organic solvent with side effect to patients was used as eluent (Tan et al., 2002; Rosenfeld et al., 1996; Scacheri et al., 1993; Zhou and Zhang, 2004; Bi et al., 2001). Therefore, these processes might not be suitable for scale-up purification

process (Shi et al., 2006). Recently, a low cost two-step chromatography process for hirudin purification was described by Chen et al. (2009). In this study, we established a more simple purification approach of the recombinant hirudin variant 3 (rHV3) which consist of an efficient pretreatment of the fermentation broth and a one-step chromatography.

The importance of recombinant hirudin as an hirudin also necessitate the development of efficient and antithrombotic agent and large-scale production of the economical method for the quantification of its biological activity. Colorimetric methods described by Chang et al. (1983) and Iyer and Fareed (1995) needed expensive chromogenic substrates and the linear range of the assay was too narrow. Rayleigh scattering method described by Zhou et al. (1997) needed expensive fluorescence spectrophotometer and the standard curve of hirudin should be set up. Thrombin titration method established by Markwardt (1970) was the classic method to determine the activity of hirudin. This method was convenient and economical which was employed widely, but repeatability and accuracy were limited. In this study, a modified

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**Abbreviations:** rHV3, The recombinant hirudin variant 3; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Markwardt method was set up for measuring hirudin activity with better accuracy and the new method was more concise.

## MATERIALS AND METHODS

### Reagents and instruments

Fibrinogen and thrombin were from Shanghai Institute of Biological Products. Dry powders of *Hirudo medicinalis* Linnaeus and *Whitmania pigra* were from Shanghai Center of Research and Development of New Drugs. Normative hirudin was from Sigma Company. Chromatography media preassemble column, liquid chromatography AKTA Purifier 10 and 100 were all bought from Swedish Amersham Company.

### Determination of hirudin purity

Hirudin purity was determined by high performance liquid chromatography (HPLC) with Sephasil Peptide C18 preassemble column and purity was expressed as "area of hirudin absorption peak/combined area of all peak", as described previously by Chen et al. (2009).

### Pretreatment of fermentation broth

Cultured medium of the rHV3 was separated from the *Bacillus subtilis* cells by continuous flow centrifugation and 50% trichloroacetic acid was added until pH value reaching 2.5, then the unwanted proteins were precipitated by heating at 100°C for 30 min. The supernatant was kept after centrifugation and the pH value of the supernatant was adjusted to 4.0 by the saturated NaOH solution, then the proteins were precipitated by  $(\text{NH}_4)_2\text{SO}_4$  with 90% saturation and the precipitated proteins were washed by solution of  $(\text{NH}_4)_2\text{SO}_4$  with 90% saturation twice. Finally, the hirudin activities of the samples with pre-and post-treatments were determined and recovery rates were calculated.

### Gel filtration

Sephacryl S-100 was used to purify recombinant hirudin. Equilibrium and elution buffer was: 50 mM phosphate buffer (pH 7.0) + 0.15 M NaCl. The flow rate was set at 1.1 ml/min and the volume of applied sample which came from dissolution of the proteins precipitated by  $(\text{NH}_4)_2\text{SO}_4$  was 10 ml. Absorption peaks were collected and identified, furthermore, specific activity, purification multiple and activity recovery rate were calculated. The purity of collected sample was determined by HPLC.

### Samples and reagents preparation for thrombin titration

0.5% fibrinogen buffer was prepared in 0.05 M Tris-HCl (pH7.4, containing 0.05 M NaCl). Thrombin concentration gradients were prepared with 20 NIH/ml as benchmark concentration (1 times), 40 NIH/ml as 2 times, 100 NIH/ml as 5 times, 200 NIH/ml as 10 times, 400 NIH/ml as 20 times and so on. 5  $\mu\text{l}$ (20 NIH/ml, 1 times) was set as a 1 titration volume (1 V), 5  $\mu\text{l}$ (40 NIH/ml, 2 times) was set as a 2 titration volume (2 V), 5  $\mu\text{l}$  (100 NIH/ml, 5 times) was set as a 5 titration volume (5 V) and so on.

Two kinds of dry leech powder were soaked by physiological saline for 30 min, then after centrifugation, the supernatants of *H. medicinalis* Linnaeus and *W. pigra* were named as titration sample A and B, respectively. The rHV3 supernatant from zymotic fluid of

the *B. subtilis* was named as titration sample C. Normative hirudin from Sigma Company was named as titration sample D.

### Thrombin titration

According to the method described by Markwardt (1970), one test tube (0.75 × 10 cm) containing 200  $\mu\text{l}$  of 0.5% fibrinogen solution mixed with 50  $\mu\text{l}$  sample was incubated in 37°C water for 5 min, then 5  $\mu\text{l}$  of 5 times thrombin solution (5 V) was added to the mixture in the test tube, if coagulation of the fibrinogen in the test tube did not happen in 1 min, another 5  $\mu\text{l}$  of 5 times thrombin solution was added, till coagulation happened in 1 min and the total titration volume and activity of the thrombin employed in the titration were calculated.

In the modified thrombin titration method, the mixtures all containing 200  $\mu\text{l}$  of 0.5% fibrinogen solution and 50  $\mu\text{l}$  sample in several little test tubes (0.75 × 10 cm) were incubated at 37°C water for 5 min, then one tube was added with 5  $\mu\text{l}$  of 5 times thrombin solution, if the mixture coagulated in 1 min after rapidly shaken up, the activity should be below 10 ATU/ml (for example, <5 V of titration volume), so another tube would be added with 5  $\mu\text{l}$  of 1 times thrombin solution, if the mixture did not coagulate in 1 min, another 5  $\mu\text{l}$  of 1 times thrombin solution will be added, until the mixture coagulated in 1 min, the total titration volume and activity was calculated. After adding 5  $\mu\text{l}$  of 5 times thrombin solution, if no coagulation happened in 1 min, another 5  $\mu\text{l}$  of 20 times thrombin solution was added. if no coagulation happened in 1 min, another 5  $\mu\text{l}$  of 20 times thrombin solution was added subsequently, if coagulation happened in 1 min after the rapid mixture, the thrombin titration volume should be between 25 and 45 V. Therefore, another test tube would be titrated with 5  $\mu\text{l}$  of 20 times thrombin solution and 5  $\mu\text{l}$  of 10 times thrombin solution, if coagulation happened in 1 min after the rapid mixture, the thrombin titration volume should be between 25 and 30 V. At last steps, 5  $\mu\text{l}$  of 20 times thrombin solution and 5  $\mu\text{l}$  of 5 times thrombin solution were added to another test tube, then, 5  $\mu\text{l}$  of 1 times thrombin solution was added until coagulation happened. In this way, the anti-thrombin activity-range of the hirudin sample was narrowed down by several thrombin solutions at proper concentration and the optimum group of thrombin concentration was determined for titration of the hirudin sample at last. Anticoagulation activity of the sample (ATU/ml) was the 20 times of the total activity of the thrombin employed in the titration. Titration of every sample was repeated seven times and the standard deviation (SD) of every sample was calculated.

## RESULTS

### Pretreatment of fermentation broth

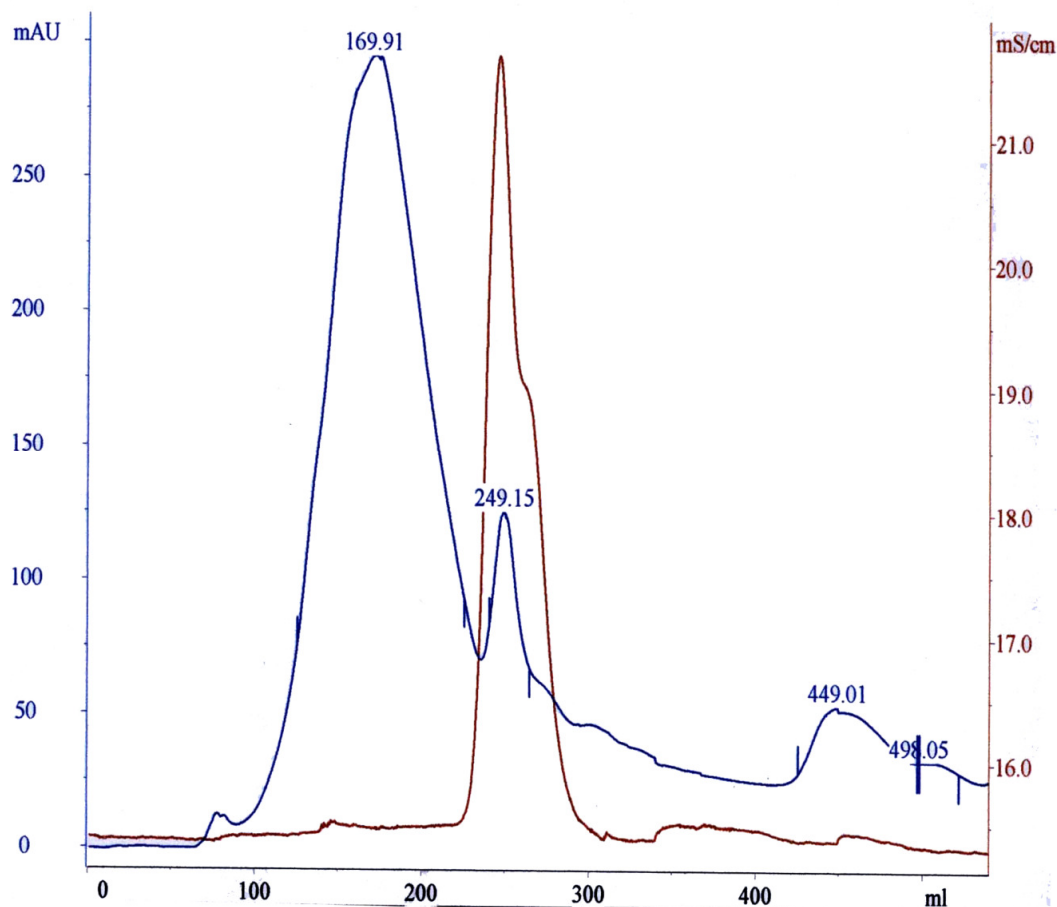
Almost 98.8% of the impurity protein of the fermentation broth was removed by pretreatment of heating at both 100°C and pH 2.5 for 30 min and the purification multiple and recovery rate of the rHV3 protein was 22.9 and 95%, respectively (Table 1). Then, pH was adjusted to 4 (isoelectric point), after precipitation and subsequent wash by  $(\text{NH}_4)_2\text{SO}_4$  with 90% saturation, the total purification multiple and recovery rate were 23.9 and 90.3%, respectively (Table 1).

### Gel filtration

After gel filtration chromatography (Figure 1), the purity of

**Table 1.** Purification results of the rHV3 from the *B. subtilis*.

Purification procedure	Protein amount (mg)	Total activity (ATU)	Specific activity (ATU /mg)	Recovery (%)	Purification multiple	Purity (%)
Fermentation supernatant	1425.6	65581.2	46	100	1	—
Treatment at 100 °C and pH 2.5	59.1	62302.2	1053.4	95	22.9	—
Precipitation by ammonium sulfate	53.8	59187.2	1099.4	90.3	23.9	—
Gel filtration by Sephacryl S-100	44.0	55043.9	1251.1	83.9	27.2	95

**Figure 1.** Gel filtration chromatography by Hiprep 26/60 S-100 preassemble column.

final product could reach 95% assayed by HPLC (Figure 2), with 93% recovery rate and 1.14 purification multiple for this chromatography step (Table 1). Seeing from the chromatography, the impure peaks that appeared in salt peaks or after them mainly were pigments or degraded small-peptide fragments. The SDS-PAGE analysis of the final purified rHV3 revealed a single band (Figure 3).

### Thrombin titration

Titrate was done following the same samples by

Markwardt method and by the improved method to compare the process and merit of these two methods. According to the method described by Markwardt, the mean values of the activities of sample A, B, C and D were  $50 \pm 9.1$ ,  $10 \pm 0$ ,  $136 \pm 15.2$ ,  $105 \pm 5.6$ , respectively as shown in Figure 4.

With the modified thrombin titration method, one tube was added with 5  $\mu$ l of 5 times thrombin solution, but the mixture did not coagulated in 1 min after rapidly shaken up, then 5  $\mu$ l of 20 times thrombin solution was added, no coagulation happened in 1 min, so another 5  $\mu$ l of 20 times thrombin solution was added in this mixture, also,

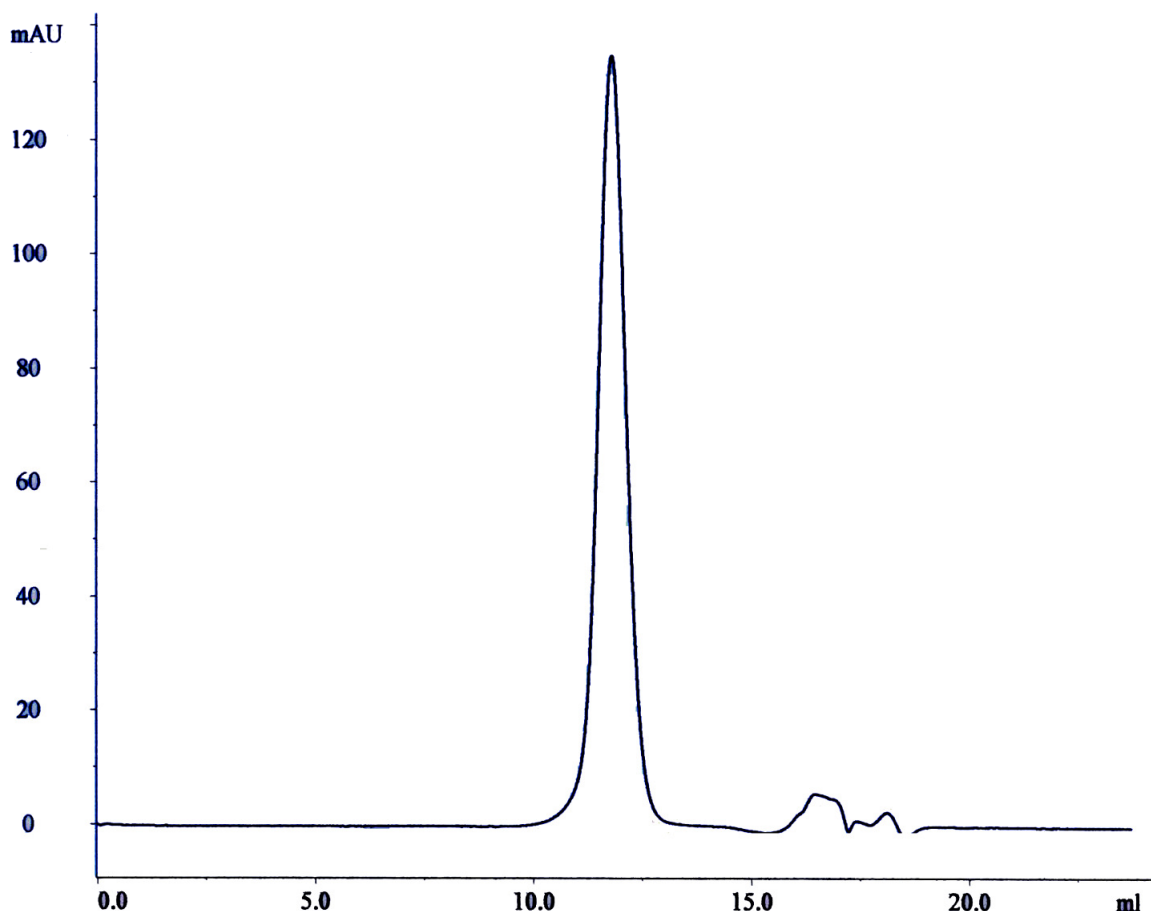


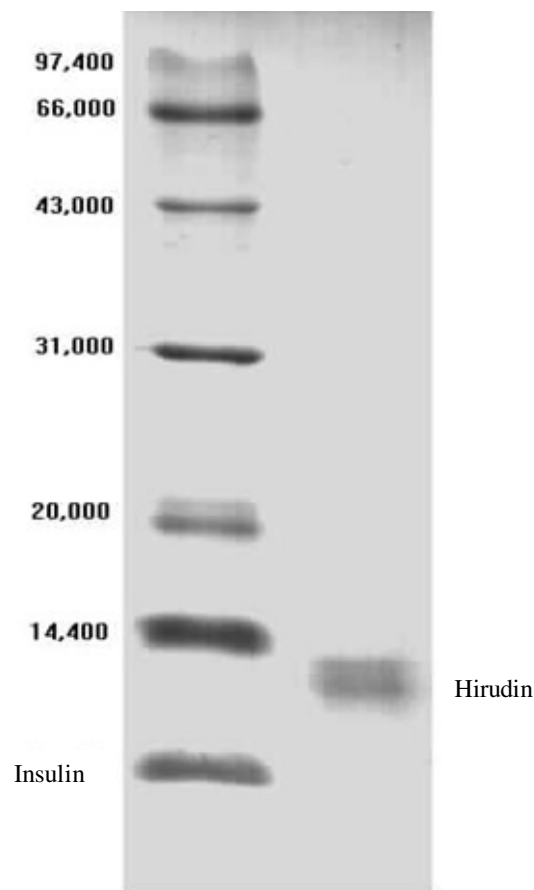
Figure 2. HPLC of the product after the gel filtration chromatography.

no coagulation happened in 1 min, then another 5  $\mu$ l of 20 times thrombin solution was added and the mixture coagulated in 1 min after rapid shake, that is to say, thrombin volume was between 45 and 65 V. Thereafter, another test tube was added with 5  $\mu$ l of 40 times thrombin solution and 5  $\mu$ l of 10 times thrombin solution, but no coagulation happened in 1 min after rapid shake and 5  $\mu$ l of 2 times thrombin solution, no coagulation and 5  $\mu$ l of 1 times thrombin solution, also no coagulation, after 6 times with 5  $\mu$ l of 1 times thrombin solution, little floccule happened, this could be regarded as coagulation, because no big agglomerate happened after continuative titration, so the total titration volume was regarded as 59 V. But the accuracy was low with too many times of titration, so another test tube was added with 5  $\mu$ l of 40 times thrombin solution and 5  $\mu$ l of 10 times thrombin solution, no coagulation and 5  $\mu$ l of 5 times thrombin solution, also no coagulation and 5  $\mu$ l of 1 times thrombin solution, then coagulation happened in 1 min after shake, as a result, the total titration volume was 56 V, for example, 5.6 ATU, therefore, the activity of the sample C was 112 ATU/ml. This result was repeated 7 times, with the mean value and the standard deviation (SD) being 112.3 and 2.3, respectively, as shown in

Figure 4. With the same improved thrombin titration method, the mean values of sample A, B and D were  $44 \pm 1.6$ ,  $2 \pm 0$  and  $98 \pm 1.8$ , respectively, as shown in Figure 4. When the estimated hirudin activity was high, the initiative titration concentration of thrombin was high also.

## DISCUSSION

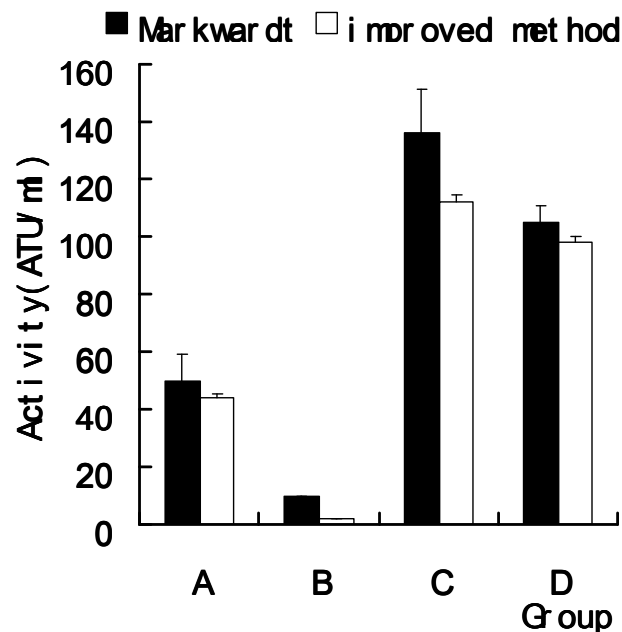
Pretreatment of fermentation broth was very important step in protein purification process; unfortunately, many researchers often hasten to employ chromatography method without careful consideration of protein characteristic and preliminary isolation method (Marshak et al., 1996). In the previous report, little attention was paid to the pretreatment of fermentation broth (Tan et al., 2002; Rosenfeld et al., 1996; Scacheri et al., 1993; Bi et al., 2001; Shi et al., 2006; Kochanowski et al., 2006). Here, the rHV3 was very stable under the condition of both 100°C and pH 2.5 for 30 min, with little hirudin inactivated, so the supernate of the fermentation broth was treated under this condition and almost 98.8% of the impurity protein was removed. Then by precipitation and



**Figure 3.** SDS-PAGE of hirudin product from the gel filtration chromatography. Left lane, Protein mark; right lane, hirudin dimmer.

wash with ammonium sulfate of 90% saturation, main pigments or small fragments were removed. These pre-treatment methods gave the possibility of conciseness and efficiency to the total isolation process.

Exact and brief activity measurement method was very important to the total isolation process (Marshak et al., 1996). By the improved concentration gradient titration method, the standard deviation of the Sample C was 2.3 ATU/ml, which was much lower than that of 15.2 ATU/ml by Markwardt method, so the degree of variation of the determined value by the improved method was much lower than that by the Markwardt method. The activity of the normative hirudin (Sample D) was determined as  $98 \pm 1.8$  ATU/ml, which was near the known value 96 ATU/ml from Sigma and the standard deviation of 1.8 ATU/ml was much lower than that of 5.6 ATU/ml by Markwardt method. The degrees of variation of the determined value of other samples by the improved method were all also much lower. The precision by the improved method was 2ATU/ml, because 5  $\mu$ l of 1 times thrombin solution could make the mixture coagulate in 1 min. However, the lowest concentration of the thrombin solution was 5 times (100 NIH/ml), therefore, the pre-



**Figure 4.** Sample activities by Markwardt's titration method and by the improved titration method. The values were the means  $\pm$  standard deviations, obtained from at least seven experiments. A, The supernatant of *Hirudo medicinalis* Linnaeus; B, the supernatant of *W. pigra*; C, The rHV3 supernatant from zymotic fluid of the *B. subtilis*; D, normative hirudin from Sigma.

cision measured by Markwardt method were relatively coarse (with  $\pm 10$  ATU/ml precision), for example, Sample B activity measured by Markwardt method was 10 ATU/ml, but was only 2 ATU/ml by the improved method, obviously, the latter was more fine and accurate.

There was only one kind of titration concentration in Markwardt method (100 NIH/ml), so when hirudin concentration was high, much more times of titration should be needed for coagulation and often in little floccule, not in big agglomerate. Sometimes several dozens of times of titration made the mixture not coagulate. One reason was that the fibrinogen solution became too thin to coagulate easily. Another reason was that too many times of titration made the feeble initial coagulation invisible and destroyed by shake and sometimes coagulation did not happen forever. Therefore, the activity value determined by Markwardt method was of higher variability, lower repeatability and accuracy.

The result changed evidently when temperature differed. If it is below 37°C, the activity became on the high side. All the thrombin or fibrinogen solution should be prepared just before titration assay. In conclusion, the concise and efficient isolation line of the rHV3 from the *B. subtilis* was as follows: Fermentation supernate  $\rightarrow$  heating at both 100°C and pH 2.5 for 30 min  $\rightarrow$  precipitation by ammonium sulfate with 90% saturation  $\rightarrow$  gel filtration  $\rightarrow$  pure products (Total recovery rate =  $95\% \times 95\% \times 93\% = 83.9\%$  and the purity was 95%).

By successively titrating several test tubes of the same sample with a series of thrombin concentration and gradually reducing the activity range of the sample, the optimum group of thrombin concentration was selected for the last steps titration, therefore, with this improved thrombin titration method and the hirudin activity was determined more accurately, concisely and promptly.

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