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Reduced Immunogenic Potential of Membrane Filtered Bovine Thrombin Preparations for Hemostatic Application

He Zhu, MD, Debra Hoppensteadt, PhD, Rakesh Wahi, MD, Josephine Cunanan, MD, Cafer Adiguzel, MD, Rodger L. Bick, MD, PhD, and Jawed Fareed, PhD

There is concern that exposure to bovine thrombin can result in the development of antibodies, usually against factor V/Va, which can lead to hemostatic abnormalities. It is thought that purer preparations of bovine thrombin might be less immunogenic. Utilizing newer methods including a membrane filtration step, bovine crude thrombin is further purified into thrombin 4A and 4B preparations which exhibit a higher specific activity and are devoid of some of the protein contaminants. Bovine crude thrombin and its purified versions were administered intravenously to individual groups of rabbits using standard immunologic protocols.

Antiserum was drawn from each rabbit and the pooled antisera were purified to obtain the IgGs using protein G affinity columns. The results suggest that the reported purification process, including filtration, resulted in the removal of most of the antigens found in crude thrombin, and that none of these preparations generated any detectable antibodies against bovine factor V related antigens.

Keywords: bovine thrombin; Western blot; immunoglobulin G; factor Va; topical hemostatic agents

Although topical bovine thrombin preparations have been widely used for hemostatic purposes in clinical practice, there is concern that exposure to bovine thrombin can result in the development of antibodies, usually against factor V/Va, which can lead to hemostatic abnormalities. It is thought that coagulopathy following exposure to topical thrombin may be attributed to the impurities in bovine thrombin preparations and that purer preparations of bovine thrombin might be less immunogenic. Utilizing newer methods including a membrane filtration step, bovine crude thrombin is further purified into thrombin 4A and 4B preparations which exhibit a higher specific activity and are devoid of some of the protein contaminants. A previous study has reported

on the reduction of trace contaminants such as the bovine factor Va/Va light chain fragments to an undetectable level in thrombin 4B. In order to compare the relative immunogenic potential, bovine crude thrombin and its purified versions, 4A and 4B preparations, were administered intravenously to individual groups of rabbits on day 0 and day 21 using standard immunologic protocols. Antiserum was drawn from each rabbit on day 30 and the pooled antisera were purified to obtain the IgGs using protein G affinity columns. The antibody profile for each of these IgGs was determined by using specific human and bovine coagulation factors such as prothrombin, thrombin, factor Xa, factor VIIa, and factor Va fragment. In addition, bovine crude thrombin as well as 4A and 4B preparations were also profiled in cross immunoblotting studies. Western blotting results suggest thrombin 4B IgG has the most selective binding with thrombin antigen, and thrombin 4B has the least immunogenic potential among the 3 thrombin preparations tested. Furthermore, neither cross-reactivity

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with human coagulation factors nor the recognition of bovine factor Va antigen was observed with any of the IgGs tested. These results suggest that the reported purification process, including filtration, resulted in the removal of most of the antigens found in crude thrombin, and that none of these preparations generated any detectable antibodies against bovine factor V related antigens.

Thrombin is a crucial enzyme that has been widely known for its roles in hemostasis, inflammation, and cell signaling. Thrombin has been purified from numerous sources and used for topical hemostasis for more than 60 years.¹ Topical bovine thrombin preparations are the most widely used hemostatic agents in the United States and may be applied in various forms, including a spray, a paste, or a component of fibrin glue.² As a hemostatic agent, bovine thrombin preparations are commonly used in a variety of surgical procedures including thoracic, cardiac, vascular, neurological, orthopedic, general, transplant, plastic, urological, otolaryngological, maxiofacial, gynecological, and eye surgeries,³⁻⁵ and are also used in endoscopy, interventional radiology, and tooth extraction.^{6,7} It is estimated that at least 500 000 Americans are exposed to a bovine preparation each year.⁵

Although topical bovine thrombin preparations have been used successfully in most patients with isolated reports of serious adverse events, deranged hemostasis has developed in some surgical patients, resulting in severe or refractory bleeding and/or thrombosis.⁸ It is thought that impurities in bovine thrombin preparations might be responsible for the adverse reactions. Patients exposed to topical thrombin preparations may develop antibodies to bovine thrombin, factor V/Va, and various other proteins found in these preparations.⁹⁻¹² In some cases, these antibodies cross-react with the corresponding human coagulation proteins.^{9,13} Ortel et al³ demonstrated, in a prospective series of 151 surgical patients, that more than 95% of patients developed antibodies to bovine thrombin or factor V and that approximately 50% of these patients had cross-reactivity to human coagulation factors. Similarly, Streiff and Ness¹⁴ reviewed the published reports of factor V inhibitors and found that in adults, 40-66% of cardiac surgery patients and 20% of neurosurgery patients developed factor V antibodies after bovine thrombin exposure. Recently, Chapman et al¹⁵ reported that 21.5% of patients receiving bovine

thrombin developed antibodies to the product in a phase 3 trial comparing the efficacy and safety of recombinant human thrombin and bovine thrombin in surgical hemostasis. Furthermore, many case reports suggest that antihuman factor V antibodies elicited in response to exposure to bovine factor V, a contaminant present in bovine thrombin preparations, have played an important role in the development of coagulopathy.^{4,16-18} Factor V is a large, single-chain, plasma glycoprotein. During coagulation, factor V is converted by thrombin to factor Va and 2 additional peptides.¹⁹ If an antibody-mediated depletion of factor V occurs in a patient, that patient may develop a severe and refractory coagulopathy.

Based on the assumption that impurities in bovine thrombin preparations could be responsible for adverse reactions in patients, efforts have been made to reduce or remove contaminants such as factor V from bovine thrombin products to improve the overall safety and efficacy of the products. Recently, Terrab et al²⁰ reported that a membrane-filtration step performed to remove viruses from chromatographically purified bovine thrombin was also highly effective in reducing factor V or its fragments to undetectable levels. This study was designed to determine if the improved purity of bovine thrombin can reduce its overall immunogenic potential and lower the risk of development of factor V antibodies following exposure. Utilizing the Western blotting technique, our study compared acute immunogenic potentials of crude thrombin and its purified forms, namely, thrombin 4A and thrombin 4B, which represent in-process thrombins taken from various stages of the bovine thrombin manufacturing process.

Materials and Methods

Bovine Thrombin Preparations

Bovine crude thrombin was obtained through the activation of bovine prothrombin by thromboplastin. The crude thrombin was initially purified using ion-exchange chromatography to yield an intermediate sample referred to as thrombin 4A. Following elution, the chromatographically purified sample was filtered through the Omega 100K VR membrane filter resulting in thrombin 4B.²⁰ The crude thrombin, thrombin 4A and thrombin 4B preparations used in this study were kindly provided by King Pharma (Middleton, WI).

Bovine Plasma and Specific Human and Bovine Coagulation Factors

Bovine plasma was also provided from King Pharma (Middleton, WI) and diluted with saline to obtain serial dilutions from 25% to 1.6% before loading onto gels. All human and bovine coagulation factors were obtained commercially from Enzyme Research Laboratories (South Bend, IN) and American Diagnostica (Stamford, CT) with the exception of Bovine factor Va light chain which was purchased from Haematologic Technologies Inc. (Essex Junction, VT). All of these proteins were diluted with saline into the following concentrations before loading: human factor Xa (100 µg/ml), human α -thrombin (10 µg/ml), human prothrombin (10 µg/ml), bovine factor Xa (10%), recombinant human factor VIIa (10 µg/ml), bovine prothrombin (0.5%), and bovine factor Va light chain (10 µg/ml).

Generation of Antibovine Thrombin Antisera

To generate specific antisera in rabbits, the thrombin preparations were provided to Lampire Biologics (Pipersville, PA). Bovine crude thrombin, thrombin 4A, and thrombin 4B conjugated with keyhole lymphocyte hemocyanin (KLH) as a carrier were administered intravenously to individual groups of rabbits at a dosage of 100 µg on days 0 and 21 using standard immunologic methods. In brief, pre-immune blood was drawn on day 0 just before antigen injection, and antiserum was harvested on day 30 from each rabbit. The antisera obtained from 3 individual rabbits in each treatment group were pooled and stored at -80°C for further IgG isolation.

Immunoglobulin G (IgG) Isolations

HiTrap Protein G HP columns of 1 ml (GE healthcare Bio-Science Corp, Piscataway, NJ) were employed in this study to isolate IgG from rabbit antibovine antisera. The eluted IgG fractions were carefully collected and the concentration of harvested IgGs from each group was adjusted with saline to 1 mg/ml based on the protein concentration determined using a modified Lowry's assay.²¹ All IgG samples were aliquoted and stored at -80°C .

Gel Electrophoresis and Western Blotting

Samples were denatured and subjected to electrophoresis through 4 to 20% gradient Tris-HEPES-SDS polyacrylamide mini gels (Pierce Biotechnology, Rockford, IL) and then electrotransferred onto nitrocellulose membranes overnight at 4°C . Precision Plus protein standards were included in each gel (Bio-Rad Laboratories, Hercules, CA). After electrotransfer, the nitrocellulose membranes were blocked with TBST/5% milk (Tris buffer saline, pH 7.6, 0.1% Tween-20) for 1 hour with gentle rocking, briefly washed in TBST, and incubated 2 hours with anti-bovine crude thrombin IgG, thrombin 4A IgG, and thrombin 4B IgG (2 µg/ml in TBST/5% milk), respectively, at room temperature. Blots were then washed and incubated for 1 hour with 1:20 000 dilution (in TBST/5% milk) of horseradish peroxidase conjugated donkey anti-rabbit IgG (H+L) (Thermo Scientific, Rockford, IL). The blots were then extensively washed and immunoreactive bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) followed by film exposure for 1 to 5 minutes.

Results

As shown in Figure 1, on blots identically loaded with serial amounts of bovine crude thrombin, rabbit antibovine IgGs at an identical concentration of 2 µg/ml were used to demonstrate their detecting abilities for the antigens in bovine crude thrombin. Evaluated in terms of the lowest amount of crude thrombin sample which was detectable, the rank order of detecting abilities for the antigens in the crude thrombin sample was: crude thrombin IgG > thrombin 4A IgG > thrombin 4B IgG. Crude thrombin IgG could detect as low as 0.125 U of crude thrombin sample showing multiple immunoreactive bands including that of prothrombin at 72 kDa. The lowest amount of crude thrombin sample which thrombin 4A IgG could detect was 0.5 U. From the crude thrombin sample, thrombin 4A IgG mainly revealed 2 clear bands (α -thrombin at 36 kDa and β -thrombin at 22 kDa). Additionally, several low molecular weight bands (≤ 20 kDa) were also detectable in 1 and 2U of crude thrombin samples. With the weakest detecting ability for the antigens in crude

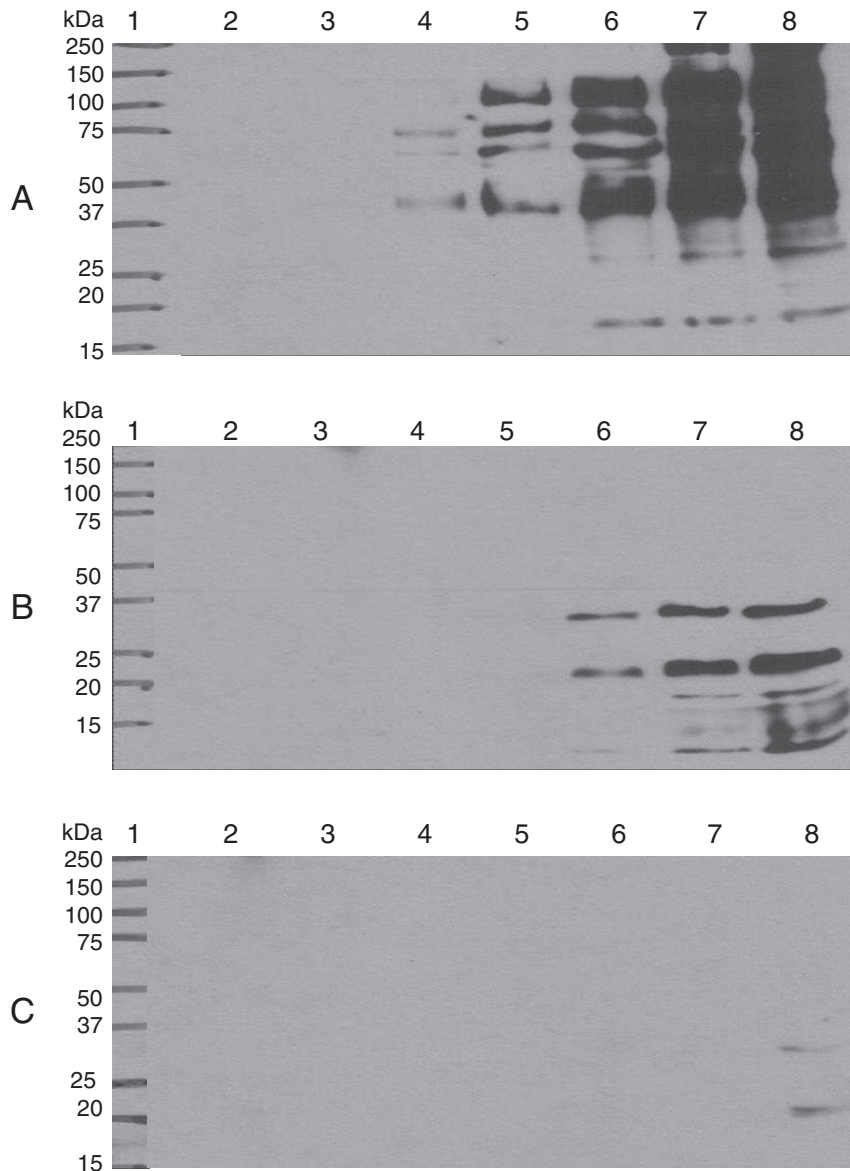


Figure 1. Detection of antigens in bovine crude thrombin sample by Western blot. Serial diluted bovine crude thrombin sample (0.031U, 0.062U, 0.125U, 0.25U, 0.5U, 1.0U, and 2.0U; lanes 2 through 8) were probed with 2 μ g/ml of crude thrombin IgG (panel A), 4A IgG (panel B), and 4B IgG (panel C), respectively.

thrombin samples, thrombin 4B IgG could only detect 2 U of crude thrombin samples showing 2 faint thrombin bands (α -thrombin at 36 kDa and β -thrombin at 22 kDa).

Next, similar blots loaded with serial dilution of bovine thrombin 4A or 4B samples were used to evaluate the detecting ability of each IgG for the antigens in these samples. Employed at the same concentration of 2 μ g/ml, crude thrombin IgG could not detect any visible protein band in thrombin 4A

and 4B samples. The lowest amount of thrombin 4A and 4B samples which thrombin 4A IgG could detect was 1.0 U. From thrombin 4A and 4B samples, thrombin 4A IgG revealed a strong and clear α -thrombin band at 36 kDa. At 2 U of 4A and 4B, thrombin 4A IgG could also detect a weak β -thrombin band at 22 kDa and another weak γ -thrombin band at 17 kDa. With a weaker detecting ability for the antigens in thrombin 4A and 4B samples, thrombin 4B IgG could only detect one

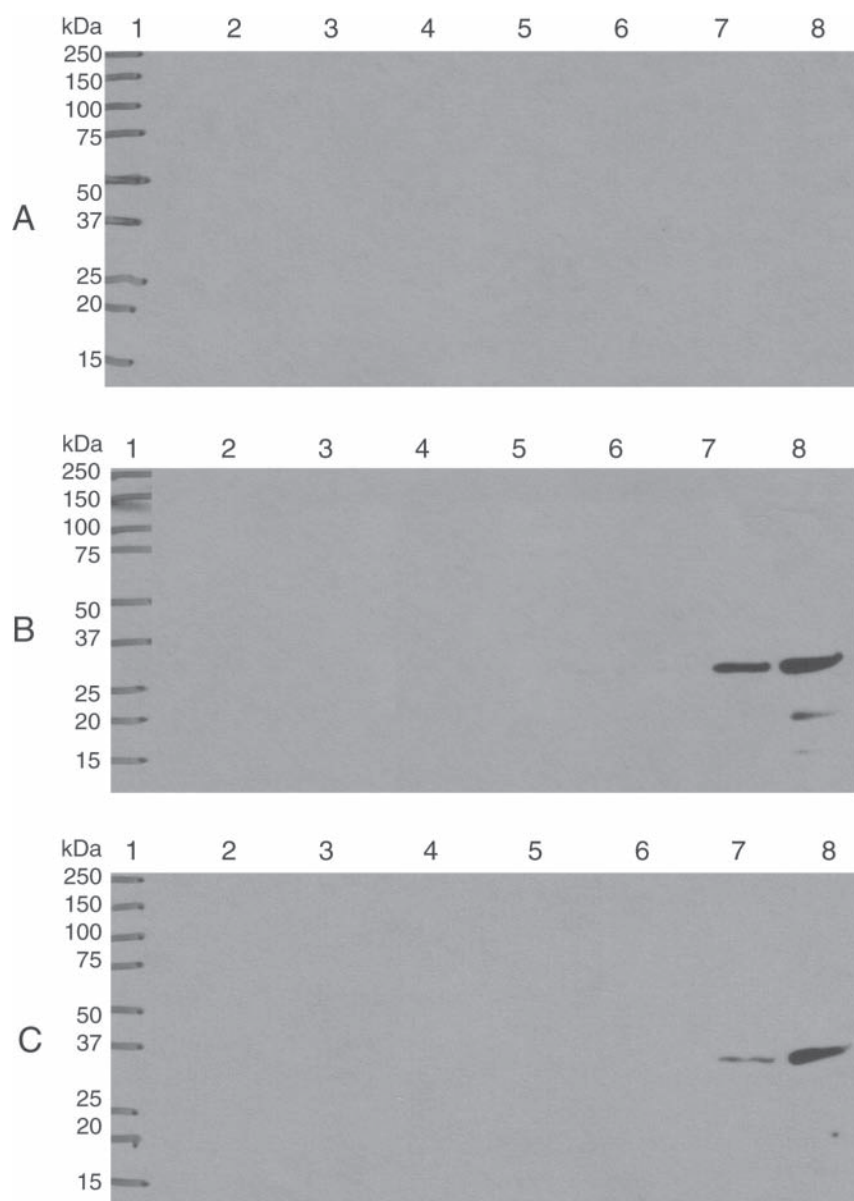


Figure 2. Detection of antigens in bovine thrombin 4A sample by Western blot. Serial diluted bovine thrombin 4A sample (0.031U, 0.062U, 0.125U, 0.25U, 0.5U, 1.0U, and 2.0U; lanes 2 through 8) were probed with 2 μ g/ml of crude thrombin IgG (panel A), 4A IgG (panel B), and 4B IgG (panel C), respectively.

α -thrombin band (36 kDa) in 1 and 2 U of thrombin 4A and 4B samples. This shows much weaker immunodensity of the α -thrombin band when compared with those obtained with thrombin 4A IgG in relevant amounts of thrombin 4A and 4B samples (Figures 2 and 3).

In addition, identical blots loaded with serial dilutions of bovine plasma were also utilized to evaluate further the detecting ability of each IgG. As shown in Figure 4, antbovine crude thrombin IgG detected multiple high molecular weight protein

bands ranging from 80 to 250 kDa with a protein concentration dependent pattern in bovine plasma sample diluted from 25% to 1.6% with saline. However, both thrombin 4A IgG and 4B IgG failed to detect any visible immunoreactive bands from bovine plasma samples.

The specificity of each of these IgGs was determined by using specific human and bovine coagulation factors such as thrombin, prothrombin, factor Xa, factor VIIa, and factor Va. The results demonstrated

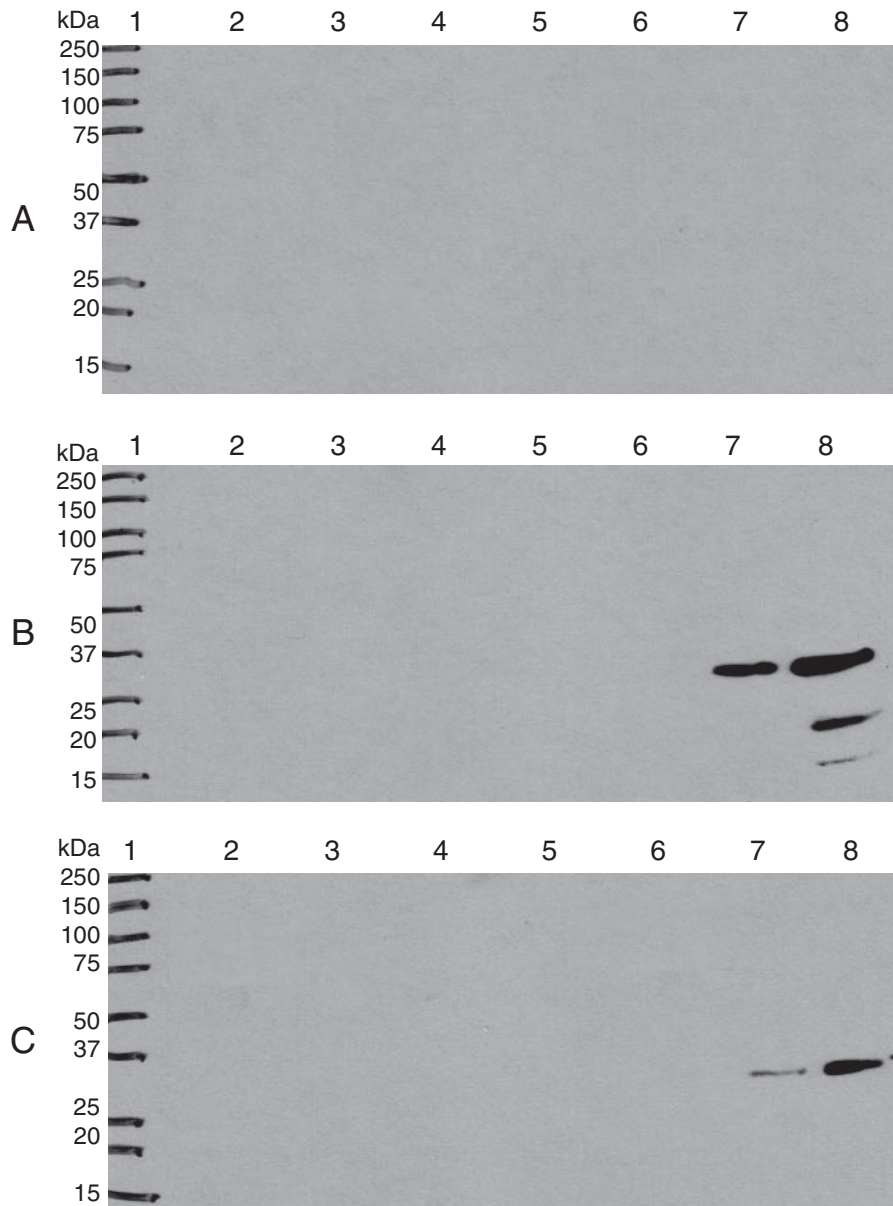


Figure 3. Detection of antigens in bovine thrombin 4B sample by Western blot. Serial diluted bovine thrombin 4B sample (0.031U, 0.062U, 0.125U, 0.25U, 0.5U, 1.0U, and 2.0U, lanes 2 through 8) were probed with 2 μ g/ml of crude thrombin IgG (panel A), 4A IgG (panel B), and 4B IgG (panel C), respectively.

that at a concentration of 2 μ g/ml, crude thrombin IgG could detect multiple high molecular weight protein bands including a faint band at 72 kDa from bovine prothrombin samples. In contrast to crude thrombin IgG, both thrombin 4A IgG and 4B IgG detected only a single immunoreactive band at 72 kDa from bovine prothrombin samples. Neither cross-reactivity with human coagulation factors nor the recognitions of bovine factor Xa and bovine factor Va

antigens were observed with any of the IgGs tested (Figure 5).

Discussion

The IgGs tested in these studies were isolated from the pooled antisera collected on day 30 from rabbits administered with relevant antigens on day 0 and day 21. Initial screening experiments have proved

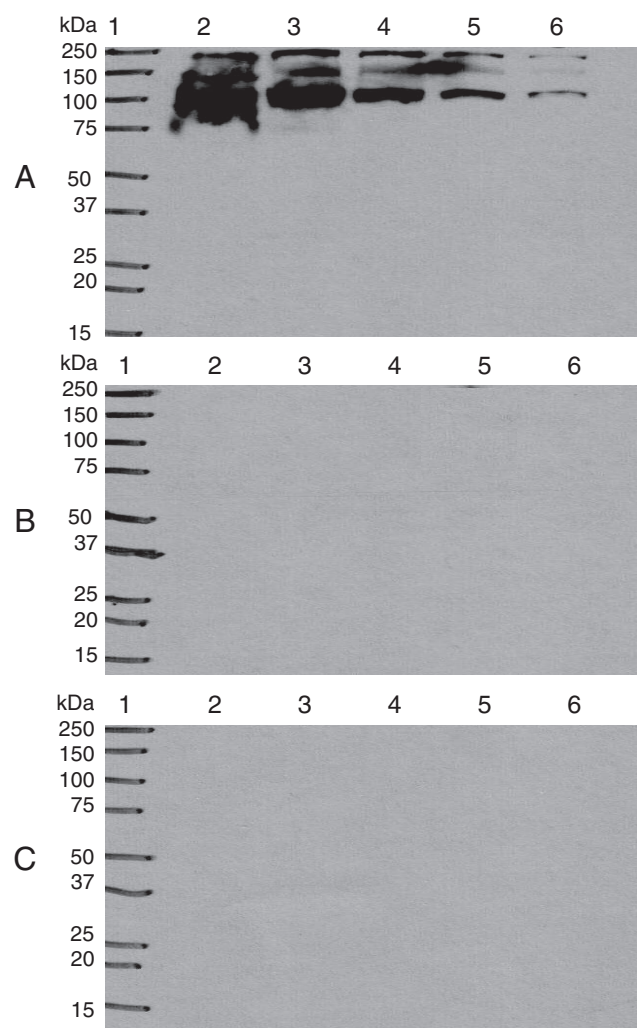


Figure 4. Western blot detection of antigens in bovine plasma. Serial diluted bovine plasma (25%, 12.5%, 6.25%, 3.2%, and 1.6% in dilution with saline; lanes 2 through 6) were probed with 2 μ g/ml of crude thrombin IgG (panel A), 4A IgG (panel B), and 4B IgG (panel C), respectively.

that pre-immune IgG obtained from the rabbits did not demonstrate any recognition of epitopes in bovine crude thrombin, thrombin 4A, 4B, and factor Va light chain (data not included). Therefore, these results suggest that the specific antibodies against bovine crude thrombin, thrombin 4A and 4B, are initially not present in rabbit serum and could be developed in measurable titers by twice repeated immunization of rabbits with those antigens within 30 days. It has been reported that by 4 to 8 weeks after exposure to topical bovine thrombin during surgery, more than 90% of patients had elevated IgG

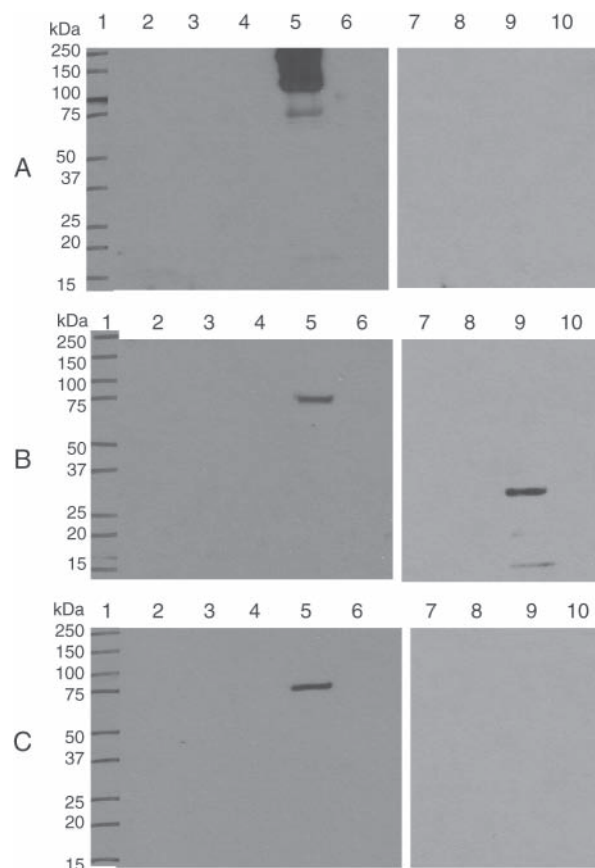


Figure 5. Determination of relative antibody profiles of crude thrombin IgG (panel A), thrombin 4A IgG (panel B), and thrombin 4B IgG (panel C) by Western blot. Specific human and bovine coagulation factors including human factor Xa (2.0 μ g, lane 2), human α -thrombin (0.2 μ g, lane 3), recombinant human factor VIIa (0.2 μ g, lane 4), bovine prothrombin (0.5% in dilution with saline, lane 5), human prothrombin (0.2 μ g, lane 6), bovine factor Xa (undiluted, lane 7), bovine factor Xa (10% in dilution with saline, lane 8), bovine thrombin (2.0 μ g, lane 9), and bovine factor Va light chain (0.2 μ g, lane 10) were probed with 2 μ g/ml of crude thrombin IgG, 4A IgG, and 4B IgG, respectively.

antibody levels to the topical thrombin, independent of type of surgery or thrombin dose.³ Thus, the data obtained in the current study is consistent with that obtained for humans on the time-course of the generation of these antibodies.

The immunogens used to generate the antibodies (IgGs) in this study were bovine crude thrombin, thrombin 4A, and thrombin 4B. Bovine crude thrombin was obtained through the activation of prothrombin by thromboplastin. Purification of crude thrombin by ion-exchange chromatography and filtration yielded thrombin 4A and thrombin 4B,

respectively.²⁰ Compared with crude thrombin, thrombin 4A and 4B preparations represent highly purified and improved thrombins with markedly reduced extraneous protein contaminants with immunogenic potential.²⁰

As to the specificity of each of the IgGs for thrombin-related antigens, these results demonstrate that antbovine crude thrombin IgG detected multiple strong immunoreactive bands from crude thrombin samples, and most of these bands were unrelated to bovine thrombin. On the other hand, antithrombin 4A IgG detected 3 individual bands at 36, 22, and 17 kDa, which correspond to α -thrombin, β -thrombin, and γ -thrombin, respectively. Antithrombin 4B IgG, raised by the immunization of rabbits with thrombin 4B, which represented the most purified preparation of bovine thrombins used in this study, could recognize only one obvious α -thrombin band at 36 kDa. While all 3 individual antibodies (IgGs) profiled were capable of identifying their relevant immunogens, at an optimized concentration of 2 μ g/ml, the cross-reactivity to bovine factor Va light chain was not observed with any of these antibodies at this concentration.

Furthermore, these studies suggest that the thrombin 4B exhibits the weakest immunogenic potential among the 3 thrombin preparations evaluated in this study. In contrast to crude thrombin IgG, thrombin 4B IgG did not show any recognition for nonthrombin related antigens. Also, thrombin 4B IgG failed to detect any measurable protein band from bovine plasma samples when used at the same concentration as that of crude thrombin IgG. In addition, it demonstrated a much weaker detecting ability for antigens in crude thrombin samples in comparison with crude thrombin IgG and thrombin 4A IgG. Finally, at an identical concentration of 2 μ g/ml, thrombin 4B IgG detected less immunoreactive protein bands with much weaker immunodensity from thrombin 4A and 4B samples when compared with thrombin 4A IgG.

In this study rabbit antbovine crude thrombin IgG failed to cross-react with bovine factor Va light chain, which is inconsistent with the data obtained from human patients following exposure to topical bovine thrombin during surgery.⁹⁻¹² It is clear from these results that despite the presence of detectable amounts of bovine factor Va antigens in crude thrombin, this contaminant in crude thrombin failed to elicit the generation of antibodies against factor Va light chain in rabbits. This may be due to a difference in the immune responses among species (ie, unlike

the immune response to bovine thrombin, rabbit and human may have a different immune response to the bovine factor V/Va antigens). Bovine factor V/Va may be less immunogenic in rabbits than in humans. Furthermore, the antibodies against the bovine factor Va may have slower onset as compared to antithrombin IgG in rabbit, and a longer immunization time may be needed to generate antbovine factor Va antibodies in rabbits than in humans. In this study we have only tried probing bovine factor Va light chain with antbovine thrombin IgGs for the evidence of cross-reactivity. Further studies using either the rabbit antbovine crude thrombin IgG obtained from longer/more times of immunization with antigens or bovine factor V as the target protein may help to clarify this issue.

In this study crude thrombin IgG could detect as low as 0.125 U of crude thrombin related signals showing multiple immunoreactive bands. Data shown in Figure 1 demonstrates that in 1 U and 2 U of crude thrombin samples, crude thrombin IgG could also detect a faint α -thrombin band at 36 kDa. However, it did not detect any visible protein band from thrombin 4A and 4B samples. Therefore, there is a possibility that during the process of purification by ion-exchange chromatography from crude thrombin to thrombin 4A, the immune characteristics or the allosteric nature of epitopes on crude thrombin samples have been modified, resulting in the nonrecognition of thrombin 4A and 4B samples by crude thrombin IgG.

It should be noted that humans are constantly exposed to bovine proteins, and beef products constitute a major dietary source. Therefore, the existence of antbovine factor Va related antibodies in humans is not unexpected, as this constant exposure to beef proteins may result in the generation of polyvalent antibodies in some of the individuals. The presence of these antibodies has been previously reported in both healthy individuals and bovine thrombin naive patients.³ On the other hand, the absence of antbovine factor Va antibody from the pre-immune IgG isolated from rabbits clearly demonstrates that the rabbits serum is devoid of this antigen. This is logical since rabbit feed does not include any beef proteins. The results from this study clearly demonstrate that both the crude thrombin and its purified forms did not result in the generation of antbovine factor Va cross-reactive IgGs after a 30-day immunization with double exposure. This observation points to the possibility that the reported presence of antbovine factor Va antibodies in some patients exposed to bovine

crude thrombin may be caused by an immune response to the preexisting bovine immunogens in these patients.

Conclusion

The results reported in this study indicate that the purified bovine thrombin preparations, such as the 4A and 4B, are relatively nonimmunogenic in comparison to the crude thrombin preparation. In particular, the thrombin preparation 4B represents the most purified bovine thrombin exhibiting the least immunogenic potential among the 3 thrombin preparations tested.

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